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AWI Alfred-Wegener-Institut für Polar- und Meeresforschung in der Helmholtz-Gemeinschaft

Adaptation and stress defence in intertidal and subtidal Antarctic limpets (*Nacella concinna*): A study of the plasticity of molecular and biochemical stress response in Antarctic invertebrates

Anpassung und Stressabwehr bei der Antarktischen Napfschnecke *Nacella concinna* aus dem Sublitoral und Eulitoral: Eine Studie über die Plastizität molekularer und biochemischer Stressreaktionen in Antarktischen Wirbellosen

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Terms, Definitions, and Abbreviations

•HO	hydroxyl radical
ADP	adenosine diphosphate
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
Asn	asparagine
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix motif
CAT	catalase
CBP	cyclic AMP response element-binding protein (CREB)-binding protein
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CoQ	coenzyme Q, ubiquinone
C-TAD	C-terminal transactivation domain
DNA	deoxyribonucleic acid
EC	energy charge
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FAD	oxidized flavin adenine dinucleotide
$FADH_2$	reduced flavin adenine dinucleotide
FIH	factor inhibiting HIF
GF-AAS	graphite furnace atomic absorption spectrometry
GPX	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
H_2O	water
H_2O_2	hydrogen peroxide

HIF	hypoxia inducible factor (transcription factor)
HO-2	haemoxygenase-2
HPLC	high performance liquid chromatography
HRE	hypoxia responsive element
HSP	heat shock protein
ICP/OES	inductively coupled plasma optical emission spectrometry
K_m	Michaelis constant
Ma BP	million years before present
n	number of individuals
$NaClO_4$	sodium perchlorate
$NAD(P)^+$	oxidized nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced nicotinamide adenine dinucleotide (phosphate)
NaOH	sodium hydroxide
NLS	nuclear localization signal
NO	nitric oxide
N-TAD	N-terminal transactivation domain
O_2	oxygen
O_2^-	superoxide anion
ODD	oxygen dependent degradation domain
^-OH	hydroxyl ion
PAGE	polyacrylamid gel electrophorese
PAS	Per-Arnt-SIM
PCA	perchloric acid
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
PGC-1α	peroxisome proliferators-activated receptor-γ coactivator-1α
PHD	prolyl hydroxylase domain

PK	pyruvate kinase
PO_2	partial pressure of oxygen
Pro	proline
psu	practical salinity unit
Q_i	inner quinone pool
Q_o	outer quinone pool
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
Tris	tris(hydroxymethyl)aminomethane
VHL	von Hippel-Lindau protein
V_{max}	maximal velocity

Zusammenfassung

Die Antarktische Napfschnecke *Nacella concinna* kommt in zwei morphologisch unterschiedlichen Unterpopulationen vor. Während die eulitorale Unterpopulation jedes Frühjahr für die Sommermonate in den Gezeitenbereich (das Eulitoral) wandert und dort zweimal am Tag bei Ebbe trocken fällt, verbleibt die andere Unterpopulation das ganze Jahr im Sublitoral, wo sie permanent von Wasser bedeckt ist. Die globale Klimaerwärmung führt zu einem Rückgang der Gletscher, wodurch das Antarktische Eulitoral zunehmend eisfrei wird, und möglicherweise neuer Lebensraum für die Napfschnecken entsteht.

Um zu untersuchen, ob die eulitorale *N. concinna* physiologische Anpassungen an das Trockenfallen im Eulitoral entwickelt hat, wurden Tiere beider Unterpopulationen für mehrere Stunden sowohl der Luft, als auch einer Hypoxie ausgesetzt, und die biochemische und molekulare Stressantwort in beiden Unterpopulationen untersucht. Hypoxie kann als Folge von Trockenfallen auftreten, wenn die Tiere ihre Schalen schließen, um sich vor zu großem Wasserverlust zu schützen.

Sauerstoffmessungen in dem Schalenwasser der eulitoralen Napfschnecken zeigen eine Abnahme der Sauerstoffkonzentration während des Trockenfallens, was auf eine reduzierte Ventilation des Schalenwassers hindeutet. Dieses Verhalten könnte ein Grund für den reduzierten Wasserverlust sein. Gleichzeitig scheinen die Napfschnecken aus dem Gezeitenbereich Sauerstoff aus der Luft über ihren Fuß aufzunehmen, da der Stoffwechsel des Fußmuskels während der gesamten Untersuchungszeit aerob bleibt; weder Succinate noch Lactate akkumulieren. Erhöhte Malondialdehydekonzentrationen, ein Marker für Lipidperoxidation, während des Trockenfallens im Fußmuskel von eulitoralen *N. concinna* im Vergleich zu sublitoralen *N. concinna* deuten ebenfalls auf eine Sauerstoffaufnahme über den Fuß hin. Zusätzlich ist nach 12 Stunden Trockenfallen das Verhältnis von oxidiertem zu reduziertem Glutathion (GSSG/GSH) in eulitoralen *N. concinna* höher als in Napfschnecken aus dem Sublitoral. Im Gegensatz zu den eulitoralen Napfschnecken, erscheinen die sublitoralen *N. concinna* durch Trockenfallen stark gestresst. Sie können ihren Wasserverlust nicht reduzieren und verlieren dadurch viel Wasser. Hohe Sauerstoffkonzentrationen im Schalenwasser der sublitoralen Napfschnecken während des Trockenfallens deuten zudem auf eine fortwährende Ventilation des Schalenwassers hin. Zusätzlich reichern sie nach 12 Stunden Trockenfallen sowohl Succinat, als auch Lactat im Fußmuskel an, was auf einen anaeroben Stoffwechsel hindeutet. Vermutlich kontrahieren die Napfschnecken ihren Fußmuskel stark, um ihre Schale fest auf das Substrat zu pressen und dadurch einen weiteren Wasserverlust zu verhindern, was dann möglicherweise zu einer funktionellen Anaerobiose führt.

King George Island ist eine Insel vulkanischen Ursprungs, deren Küstengewässer hohe Eisen- und andere Metallkonzentrationen aufweisen. Besonders im Sommer nehmen diese Konzentrationen zu, wenn durch die Gletscherschmelze große Sedimentmengen in das Meer transportiert werden. *Nacella concinna* nimmt diese Metalle über die Nahrung auf und lagert sie hauptsächlich in ihrer Mitteldarmdrüse ein. Die Napfschnecken aus dem Sublitoral weisen dabei 10fach höhere Eisenkonzentrationen in ihren Mitteldarmdrüsen auf, als die Napfschnecken aus dem Gezeitenbereich. Die hohen Eisenkonzentrationen bedingen vermutlich die erhöhten Superoxiddismutase- (SOD) aktivitäten in den Mitteldarmdrüsen sublitoraler *N. concinna*. Im Gegensatz dazu weisen eulitorale *N. concinna* trotz niedrigerer Eisenkonzentrationen, höhere SOD- und Katalase- (CAT) aktivitäten in ihren Kiemen auf, als die Napfschnecken aus dem Sublitoral. Schwankende Sauerstoffkonzentrationen, verursacht durch den ständig variierenden Wasserstand im Gezeitenbereich, führen vermutlich zu verstärktem oxidativen Stress für die Napfschnecken und somit zu erhöhten Enzymaktivitäten in ihren Respirationsorganen. Das oxidiertere Redoxmillieu in den Kiemen der eulitoral *N. concinna* spricht ebenfalls für erhöhten oxidativen Stress. Eulitorale Napfschnecken zeigen unter Trockenfallen und Hypoxie unterschiedliche Stressantworten, für SOD und CAT überwiegt jedoch eine gewebeabhängige Antwort.

Im Rahmen dieser Arbeit habe ich das HIF-α (α Untereinheit des hypoxia-inducible factor) Gen aus *N. concinna* isoliert und damit das erste Gastropoden HIF-α sequenziert. Der Vergleich von Evertebraten und Vertebraten HIF-α Sequenzen zeigt, dass Evertebraten einen auffälligen Aminosäure-Ausstausch in der sauerstoffabhängigen Abbau Domäne (oxygen dependent degradation domain; ODD) aufweisen. Dieser Austausch könnte Auswirkungen auf den Mechanismus der HIF-α-Stabilisierung haben und somit ein möglicher Grund für die nachweisbaren HIF-α Proteinkonzentrationen unter Normoxie sein. Darüber hinaus zeigt die phylogenetische Analyse des Proteins, dass Evertebraten ein „frühes“ HIF-α zu besitzen scheinen. Während Vertebraten mehrere HIF-α Isoformen besitzen (HIF-1α, HIF-2α, HIF-3α), konnte in Evertebraten bisher nur eine HIF-α Isoform nachgewiesen werden. Die Untersuchung der mRNA Expression in der Mitteldarmdrüse mittels real time PCR zeigt keinen signifikanten Einfluss von Hypoxie. HIF-α Proteinlevel wurden anhand von Immunoblots bestimmt, wozu ein zuvor gegen die *N. concinna* HIF-α Sequenz konstruierter Antikörpers verwendet wurde. Nach 12 Stunden Hypoxie akkumuliert das HIF-α Protein in den Mitteldarmdrüsen beider *N. concinna* Unterpopulationen. Während des Trockenfallens wird HIF-α dagegen nur in den Mitteldarmdrüsen der Napfschnecken aus dem Gezeitenbereich stabilisiert. Nach weiteren 12 Stunden beider Stressinkubationen wird das HIF-α Protein wieder herunterreguliert. Diese Ergebnisse zeigen eine stressinduzierte HIF-α Anreicherung in der Antarktischen Napfschnecke. Da die HIF-α Anreicherung während des Trockenfallens in den beiden *N. concinna* Unterpopulationen eine Übereinstimmung mit den

jeweiligen Sauerstoffkonzentrationen im Schalenwasser der Napfschnecken aufweist und deutet dies auf eine sauerstoff-anhängige Regulation des HIF-α hin.

Die Ergebnisse meiner Arbeiten zeigen sowohl eine deutliche physiologische wie auch molekulare Anpassung der Napfschnecken aus dem Gezeitenbereich an Trockenfallen, wodurch die Individuen dieser Unterpopulation in ihrem stressreichen Lebensraum überleben können. Möglicherweise erlauben ihnen diese Anpassungen auch eine Neubesiedlung, der durch den Rückgang der Gletscher neu entstehenden Flächen. Die sublitoralen *N. concinna* dagegen weisen diese Anpassungen nicht auf.

Summary

In the summer months, a subpopulation of the Antarctic limpet *Nacella concinna* migrates into the intertidal zone, which becomes increasingly ice-free and expands by glacier retreat due to global warming. Animals inhabiting intertidal zones are air exposed twice a day during low tide while sub-littoral animals are always covered by water. Intertidal and permanently sub-littoral *N. concinna* can easily be distinguished by their shell morphology. To test whether these morphological differences are accompanied by a physiological adaptation to the intertidal environment, I investigated the biochemical and molecular response of both limpet subpopulations to air exposure and hypoxia, a possible result of desiccation, if the limpets clamp down their shell to the substratum to avoid water loss.

The comparison of the desiccation stress response in intertidal and sub-littoral limpets show a clear adaptation of intertidal limpets: PO_2-measurements in the extrapallial water of air exposed and submerged limpets reveal decreased oxygen concentrations in air exposed intertidal limpets, presumably due to reduced ventilation, which enables the limpets to minimize water loss. Simultaneously, they seem to take up oxygen via the lateral margin of their foot indicated by the lack of succinate and lactate accumulation. Moreover, during air exposure higher malondialdehyde concentrations are found in the foot tissue of intertidal limpets compared to sub-littoral *N. concinna*. Malondialdehyde is a marker for lipid peroxidation and together with a more oxidized GSSG/GSH (oxidizied/redused glutathione) ratio after 12 h of air exposure these findings support the hypothesis of oxygen up-take via the foot margin. In contrast, sub-littoral limpets suffer from desiccation. They lose high amounts of water already within the first hours of air exposure. Furthermore, they accumulate succinate and lactate after 12 h, indicating that sub-littoral limpets potentially perform functional anaerobiosis caused by clamping down the shell against the substratum to minimize further water loss.

King George Island is a volcanic island with extremely high iron and other metal concentrations in coastal waters, especially in the summer season when melt water carries sediment loads into the sea. *N. concinna* incorporates these metals into its tissues, predominantly into the digestive gland. Sub-littoral limpets have 10-fold higher iron concentrations in their digestive gland than their intertidal congeners, probably causing the elevated superoxide dismutase (SOD) activities. Despite elevated iron concentrations in the gills of sub-littoral *N. concinna,* activities of the antioxidant enzymes SOD and catalase (CAT) are higher in intertidal limpets. Changing oxygen concentrations due to oscillating water levels may cause oxidative stress and account for the more active antioxidant enzymes. Elevated oxidative stress in the gills of intertidal limpets is additionally mirrored by the more

oxidized redox state. Regarding the antioxidative stress response, intertidal limpets seem to differentiate between air exposure and hypoxia while sub-littoral limpets do not show these characteristics. But even during either form of stress, SOD and CAT activities are regulated predominantly tissue-specific.

I sequenced the first gastropodean HIF-α (hypoxia-inducible factor), isolated from *N. concinna*. The comparison of the HIF-α peptide sequences of invertebrates with vertebrate sequences reveal a prominent amino acid exchange in the oxygen-dependent degradation domain. This process potentially modifies the HIF-α stabilization mechanism leading to detectable HIF-α levels even in normoxic limpets. In addition, the phylogenetic analysis of the invertebrate HIF-α indicates the occurrence of an ancestral HIF-α in only one isoform whereas vertebrates developed up to three isoforms (HIF-1α, HIF-2α, HIF-3α). The investigation of HIF-α mRNA expression by real time PCR does not show any significant changes under hypoxia. HIF-α protein levels in the digestive gland were determined by immuno blotting using an antibody constructed against the deduced peptide sequence of *N. concinna*. HIF-α protein levels increase significantly after 12 h of hypoxia in both subpopulations, but only in intertidal limpets after 12 h of air exposure. These results indicate a stress-dependent HIF-α accumulation in the Antarctic limpet. As HIF-α stabilization coincide with oxygen concentrations in the extrapallial water during air exposure, the posttranslational degradation of the α-subunit seems to be regulated in an oxygen-dependent manner.

The results obtained within my thesis clearly indicate a physiological and molecular adaptation of the intertidal *N. concinna* to desiccation stress which the sub-littoral limpets do not show. These adaptations enable the limpets to survive in this extreme environment and will potentially allow the repopulation of emerging areas due to glacier retreat.

1 Introduction

Antarctic regions, especially the Antarctic Peninsula and the offshore islands are the most affected areas due to global climate change (Turner et al., 2005). Environmental conditions, which were stable for millions of years, are changing; ice masses are retreating and the intertidal, where these changes are most perceptible, may become completely ice-free. *N. concinna* inhabits both the very variable and stressful intertidal zone, where it is the most abundant macroorganism, and the stable sub-littoral zone. A spring migration of *N. concinna* from the sub-littoral to the intertidal zone followed by a return to the sub-littoral in autumn and winter has previously been documented (Walker, 1972). More recent observations found limpets to remain in the intertidal zone covered by ice even in the winter months. The aim of my thesis was to investigate whether the two subpopulations differ in their response to desiccation and hypoxia stress, possible consequencess of aerial exposure.

The transcription factor HIF is a key regulator of the hypoxia-stress response and as well other stress factors (Rissanen et al., 2006). The goal of my work was to characterize the gene and investigate the expression pattern of HIF-α in this marine invertebrate. I sequenced the *N. concinna* HIF gene and investigated its mRNA expression under hypoxic conditions as well as the protein expression levels under air exposure and hypoxia.

1.1 Living in the Antarctic intertidal

The Antarctic intertidal zone is one of the harshest environments on earth. Organisms living there have to deal with extreme conditions like high UV radiation, variable seasonal food availability, ice scouring in summer and encasement in winter (Peck et al., 2006). In addition to typically variable factors that occur in intertidal zones all over the world like wave action and predatory pressure, changing salinities, 17 - 40 PSU (Waller et al., 2006b), and temperatures, they must also withstand additional factors particular to Polar Regions. Air temperatures (daily average) at King George Island, Jubany station varied in the summers 2004 – 2007 (October - April) between -12°C and 7°C and decreased in winter months even down to -22.5°C (Irene Schloss, pers. communication). Abele et al. (1999) measured water temperatures between 0°C and 8°C in intertidal rock pools at King George Island in summer. Clark et al. (2008) measured an average foot temperature of 3.8°C for air exposed intertidal *Nacella concinna* from Adelaide Island, Rothera Station.

Additionally, water levels decrease twice in 24 hours and expose intertidal animals that do not burrow in the sediment or retreat to rock pools, to air. In the rock pools oxygen concentrations can range from hyperoxic values during daytime, when algae have high photosynthetic activities, to hypoxic values, when respiration rates exceed O_2-production,

(Abele et al., 1998). For exposed animals, the changing water level is also associated with desiccation stress and separation from the respiratory medium. Intertidal molluscs have two options: They can withdraw into the shell to avoid water loss which excludes gas exchange and leads to anaerobiosis. The other option is to allow gas exchange by air gaping, or shell lifting in the case of the limpets. Oxygen can diffuse from the air into the extrapallial or inner shell water wherefrom the limpets can take up the O_2 via the skin. The limpets can also extend their pallial gills to take up O_2 (McMahon, 1988). Brinckhoff et al. (1983) and McMahon (1988) showed that intertidal invertebrates, like the limpet *Patella vulgata* and the bivalve *Cerastoderma edule*, take-up oxygen from air when emerged during low tide. Temperate intertidal gastropods, living on high shore levels respired even more when emerged than submerged (McMahon, 1988).

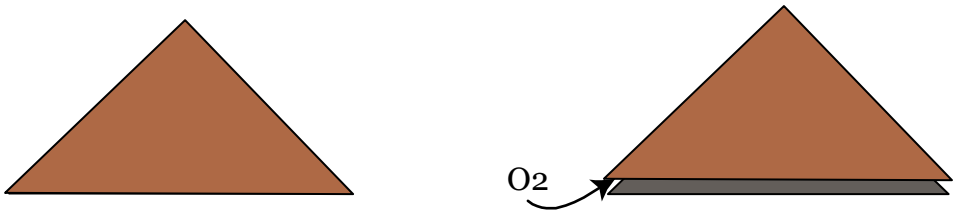

Figure 1-1: Limpets during aerial exposure.
Left limpet clampes down it shell to the rock. Right limpet lifts its shell to perform air gaping, oxygen can diffuse in the inner shell water.

By contrast, the Antarctic sub-littoral is a very stable environment. Temperatures vary only between -1.9°C and 1.4°C (1997-2004) in 15 m depth (Rothera Station, Peck et al., 2004) and salinity is usually between 33 PSU and 34 PSU. Antarctic invertebrates survive only in temperature windows of 6-12°C, whereas temperate and tropical bivalves experience a 2-6 fold temperature range (Peck & Conway, 2000). The completely loss of critical biological functions (righting of *N. concinna*) occurs at 5°C, while 50% of the investigated limpets lose their righting ability already at 2 - 2.5°C (Peck et al., 2004). Sub-littoral animals are therefore considered to be stenothermal (Peck & Conway, 2000) and stenohaline (Davenport, 2001). Not only temperature and salinity are much more stable in the sub-littoral, oxygen concentrations are also constant due to the high oxygen solubility in cold water.

Over the past 50 years the annual mean air temperature has risen by about 2.5°C on the Antarctic Peninsula (Turner et al., 2005). At King George Island the average annual air temperature increased by 1.2°C, 1.6°C during the last 14 years (1991-2005), when only winter months are calculated. Water temperature (upper 10 m) increased from 1992 to 2003 by approx. 1.2°C in summer and approx. 2°C in winter (Schloss et al., 2007). These changes are not only restricted to elevated temperatures but are accompanied by reduced salinity due to fresh water intake from melting ice and increased sedimentation by retreating glaciers (see

Schloss et al. 2007 for data). For intertidal areas this warming also means a shortened ice-cover period in winter as well as many new areas, which were formerly permanently ice-covered are now ice-free. Recent studies of the intertidal fauna detected more species in this area than previously (Barnes & Brockington, 2003). The Antarctic limpet, described as migrating to the intertidal only in summer, has recently been found in the intertidal zone covered by ice at Adelaide Island during winter months (Waller et al., 2006a).

Clear morphological differences exist between intertidal and sub-littoral *Nacella concinna* (Beaumont & Wei, 1991). They are induced by a combination of physical, biological and behavioural influences (Walker, 1972), for example, sub-littoral limpets have a flattened shell. Besides this, physiological differences have also been described, indicating an adaptation to the stressful environment. Limpets from 6m water depth were found to have a temperature-independent metabolic rate over a temperature range of -1.5°C to +0.5°C, which is close to their environmental range (Jan. 1979 – March 1980). Other gastropods from the sub-littoral showed a significant increase of metabolic rate with rising temperatures (Houlihan & Allan, 1982). Comparing limpets from both habitats revealed that intertidal *N. concinna* had a lower super-cooling point, accompanied by a higher survival rate in down-cooling experiments to –6°C (Waller et al., 2006a), and slightly higher tenacity (Davenport, 1988) than sub-littoral *N. concinna*. Furthermore, both subpopulations were found to differ with respect to heat shock protein (HSP70) induction (Clark & Peck, 2009). Not only Antarctic Nacellidae show shore-dependent differences. Malanga et al. (2004) also described different oxidative stress levels in *Nacella deaurata* from the sub-littoral zone and *Nacella magellanica* from the intertidal zone, both close relatives of the Antarctic limpet from the Beagle Channel, supporting the hypothesis of adaptations to different tidal zones. Despite these physiological differences, no genetic differentiation between the two *N. concinna* subpopulations could be clearly detected (Beaumont & Wei, 1991). A study by de Aranzamendi et al. (2008) used ISSR markers and found first hints for a starting separation of the two subpopulations, however this work still needs verification by other markers.

1.2 Reactive oxygen species and low oxygen concentrations

Oxygen (O_2) is the basis of aerobic life. During aerobic respiration, 90% of the consumed O_2 is used as the ultimate electron acceptor at the end of the electron transport chain (ETC) in the inner membrane of the mitochondria. This ETC and the oxidative phospohorylation units are a series of oxidation-reduction reactions that provide the cell with the high energy compound ATP (Storey, 1996; Winston & Di Giulio, 1991). Upon incomplete reduction of oxygen to water, reactive oxygen species (ROS) are formed. ROS is a phrase used to describe a variety of molecules and free radicals, chemical species with one unpaired electron, derived from molecular oxygen (Turrens, 2003). Four successive transfers of single electrons reduce

the oxygen molecule to water. The intermediates include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^\bullet HO$) (Storey, 1996). H_2O_2 indeed a non-radical species but usually the term ROS is used for radicals and H_2O_2.

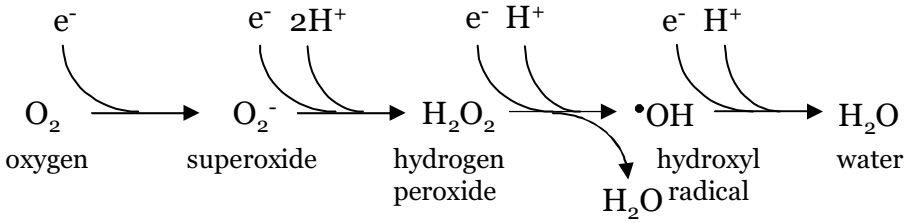

Figure 1-2: The univalent reduction of the oxygen molecule.
Taken from Heise (2005).

Although the mitochondria is the main ROS production site due to the leaky electron transport system, various other enzymatic and non-enzymatic reactions result in partial reduction of O_2, generating ROS like O_2^- and H_2O_2 (Hermes-Lima, 2004). Superoxide is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of O_2^- by the enzyme superoxide dismutase (SOD) produces H_2O_2, which in turn may be fully reduced to water by catalase (CAT) or glutathione peroxidase (GPX), which requires reduced glutathione. These reactions prevent the reduction of H_2O_2 to hydroxyl radicals ($^\bullet OH$), one of the strongest oxidants (Turrens, 2003). This formation of $^\bullet OH$ is catalysed by reduced transition metals (Fe^{2+} and Cu^+) and can be described by the Fenton reaction (see Figure 1-3)

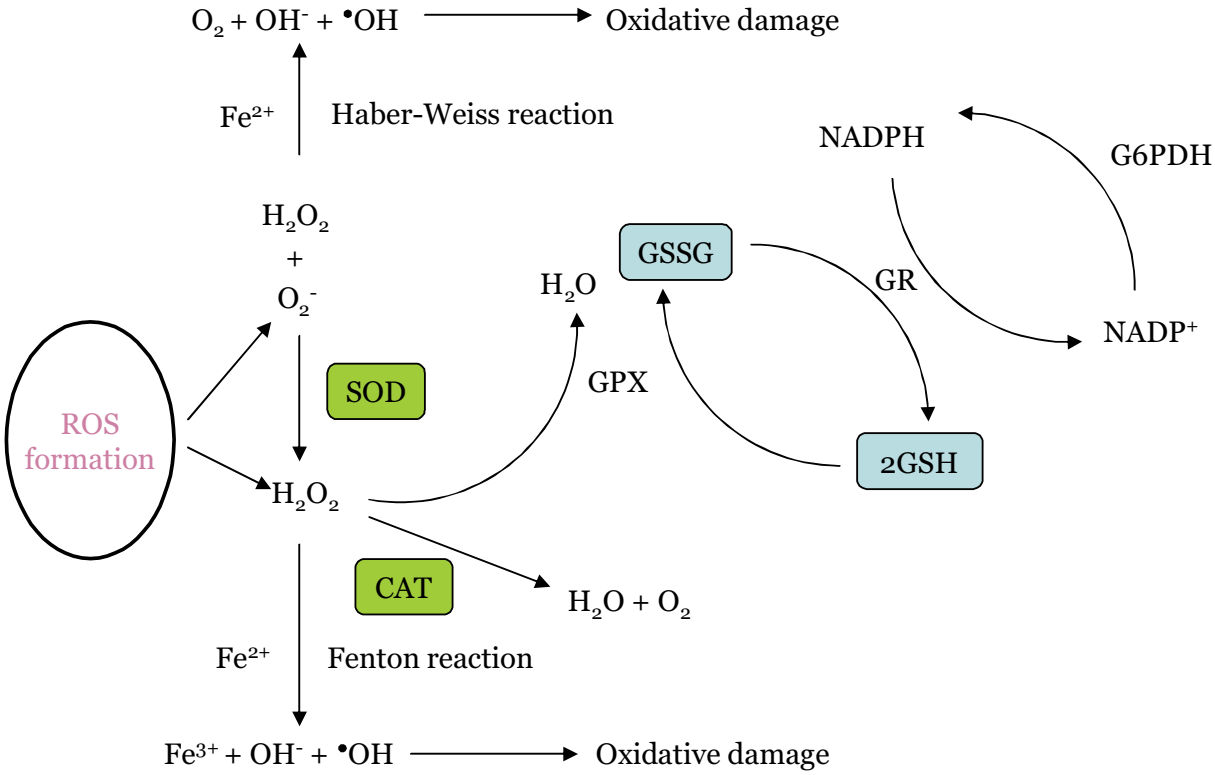

Figure 1-3: ROS formation and antioxidant defence.
Adapted from Hermes-Lima (2004). Coloured boxes indicate measured antioxidants.

Although O_2^- can only pass through membranes via anion channels (Chandel et al., 2000), H_2O_2 is uncharged and can easily diffuse across biological membranes. Therefore it can cause damage to DNA directly even though it is only mildly reactive (Lesser, 2006). As such, both are not highly reactive, but their danger lies in the fact that both are transformed into the highly reactive hydroxyl radicals ($^\bullet OH$) and hydroxyl ions (^-OH), which can do extensive damage to almost all biological macromolecules, including membrane lipids, proteins and DNA (Hermes-Lima et al., 1998).

Consequently, aerobic organisms have evolved very effective defence mechanisms to protect against oxidative damage. These include enzymatic and non-enzymatic antioxidants. The

main enzymatic defences include SOD, CAT and selenium-dependent GPX (see Figure 1-3). Non-enzymatic, low molecular weight, antioxidants include glutathione, ascorbate, vitamin E and uric acids, to name just a few. Reduced glutathione (GSH) can act as an antioxidant on its own, in addition to its role as a co-substrate of GPX and glutathione S-transferase (GST). In both cases GSH is oxidized to GSSG. The ratio of GSH/GSSG can be used as an indicator of change in the cellular redox state. Ascorbate can act as ROS scavenger and it can recycle vitamin E (Hermes-Lima, 2004). When the ROS formation exceeds the antioxidant capacity of the cells, oxidative stress occurs. This is reflected in changes of the cellular redox state and goes in line with oxidative damage of cell compounds (Sies, 1997).

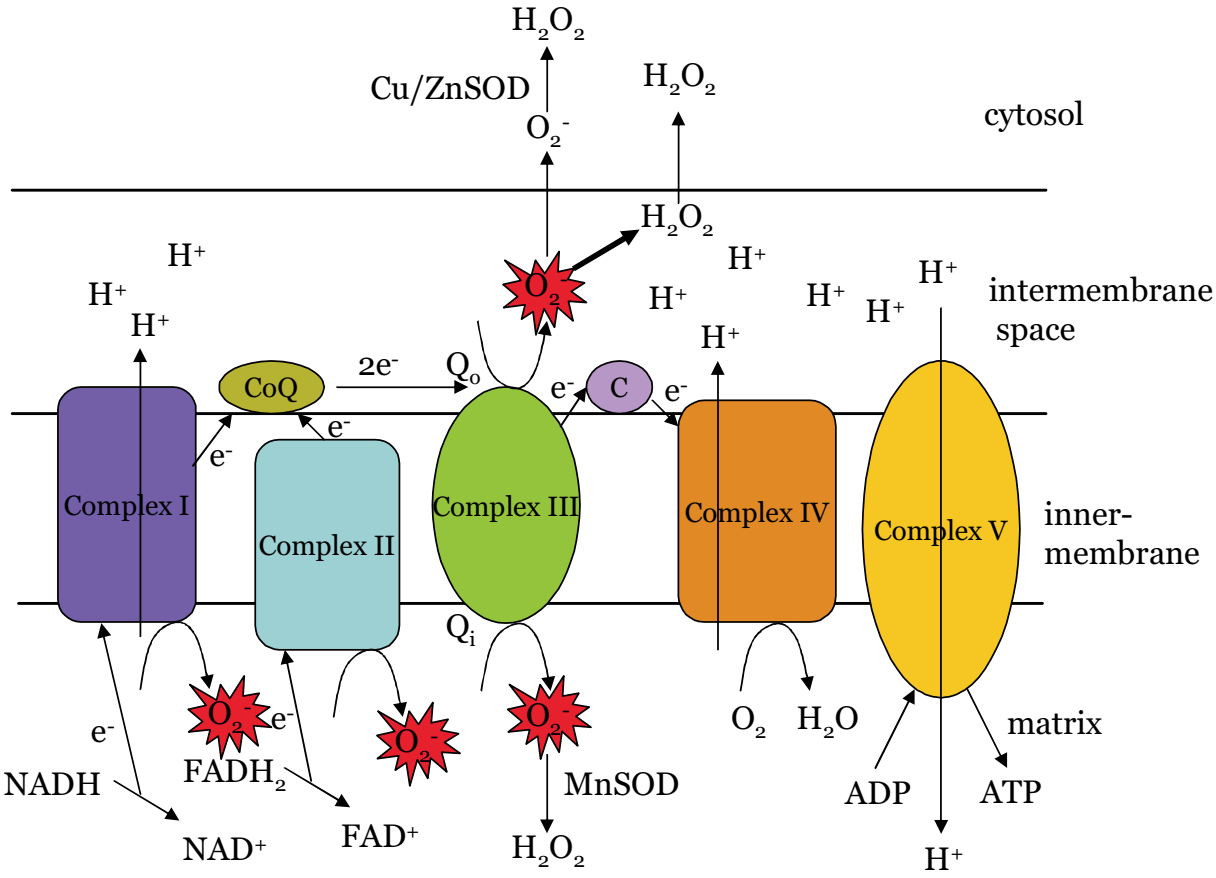

Figure 1-4: Electron transport chain in the mitochondrial membrane.
Adapted from Turrens, 2003. C = cytochrome C; CoQ = coenzyme Q, ubiquinone; Q_o = outer quinone pool; Q_i = inner quinone pool.

Under normoxic conditions the mitochondrial ETC generates O_2^- at complex I, II and III which is mainly released to the mitochondrial matrix. The anionic superoxide is highly membrane-impermeable and remains in the matrix, where it can be dismutated by Mn-SOD (superoxide dismutase) to H_2O_2, which can diffuse into the cytosol. Superoxide produced at the Q_o site of complex III ubisemiquinone can be released into the intermembrane space where it is again converted to H_2O_2, which can pass the membrane and move into the cytosol,

but the $O_2^{-\cdot}$ can also enter the cytosol via anion channels in the mitochondrial membrane (Chandel et al., 2000; Muller et al., 2004).

The rate of O_2^- formation by the respiratory chain is controlled primarily by mass action, increasing both when electron flow slows down, leading to increased concentrations of electron donors, and when the concentration of oxygen increases (Turrens, 2003). This suggests that under hypoxic conditions, when O_2 concentrations are low, the formation of O_2^- also declines. Fandrey and colleagues (1994) could show an oxygen-dependent slow down of H_2O_2 production in hypoxic HepG2 cells (human liver carcinoma cell line). On the contrary, recent studies have shown a paradoxic increase in O_2^- formation under hypoxic conditions (Schumacker, 2002). Several hypotheses exist to explain this. Under low O_2 concentrations cytochrome oxidase may be inhibited by NO (nitric oxide), resulting in an increase in its K_m for oxygen and an increased reduction of electron carriers located upstream from the terminal oxidase, favouring O_2^- formation at low O_2 concentartions (Figure 1-4) (Turrens, 2003). Chandel et al. (1998) suggested that increased ROS formation at the mitochondrial complex III may be a result of changes in redox state, caused by the reduced V_{max} of cytochrome oxidase due to hypoxia. O_2 -dependent structural changes at complex III are also under discussion (Guzy et al., 2005). ROS formation by membrane NADPH oxidase or cytochrome P_{450} is in contrast still expected to decrease at low O_2 concentrations (Chandel et al., 1998).

During re-oxygenation a burst of mitochondrial ROS production can occur due to the interaction of oxygen with accumulated electrons (Turrens, 1991) as well as due to xanthine oxidase using the reintroduced oxygen and ATP (Dykens & Shick, 1988).

1.3 Cellular oxygen sensing and response

ROS are not only injurious by-products of cellular metabolism, they are also essential participants in intracellular signalling and regulatory processes (D´Autréaux & Toledano, 2007). When oxygen concentrations decrease and energy production becomes insufficient cells have the ability to react and sustain metabolic demands for limited time. The ability to sense the level of oxygen, and to sound the alarm when it falls, is therefore a fundamental requirement for the survival of aerobic organisms (Aragonés et al., 2009; Guzy & Schumacker, 2006). Mitochondria, in particular the ETC is very likely to act as bioenergetic oxygen sensors in the cells, dependent on perturbation of mitochondrial function and energy state. The information is transmitted via three signalling pathways 1) ROS 2) cytosolic redox state and 3) energy state (Ward, 2008).

When oxygen concentrations decrease in the cells, elevated amounts of O_2^- are released from the Q_o site of complex III of the ETC to the innermembrane space of the mitochondrion (see

1.2). These ROS function as signal agents in the cell and trigger diverse functional responses, including the stabilisation of the transcription factor HIF-1 (hypoxia-inducible factor-1, see 1.6).

This signalling is mainly done by H_2O_2 due to its stability and mobility. It can lead to oxidative modification of proteins resulting in altered protein structure and modified critical amino acid residues, such as protein dimerization, and interactions with metal complexes. The best described modification involves cystein residues. The sulfhydryl group of a single cystein residue may be oxidized and then alter the activity of an enzyme or the ability of a transcription factor to bind to DNA (Thannickal & Fanburg, 2000).

Beside the modification effect on proteins, ROS can also influence the intracellular redox state of cells. ROS, namley H_2O_2 can react with reduced glutathione (GSH) to produce water and oxidized glutathione (GSSG). Cellular depletion of GSH has been found to be associated with decreased cell proliferation in vascular endothelial cells (Thannickal & Fanburg, 2000). A reduced redox state in pulmonary artery smooth muscle cells was proposed to lead to inhibition of redox-sensitive K^+ channels, depolarisation and Ca^{2+} entry via voltage-dependent Ca^{2+} channels (Ward, 2008).

The energy state of the cells plays a decisive role in metabolic regulation. The AMP/ATP ratio in cells is a sensitive indicator of cellular energy status. Increasing AMP/ATP ratios result in activation of the AMPK (AMP-activated protein kinase) system. It modulates many cellular functions but in particular anaerobic ATP production and consumption, including up-regulation of glucose uptake and glycolysis in hypoxia (Hardie, 2003).

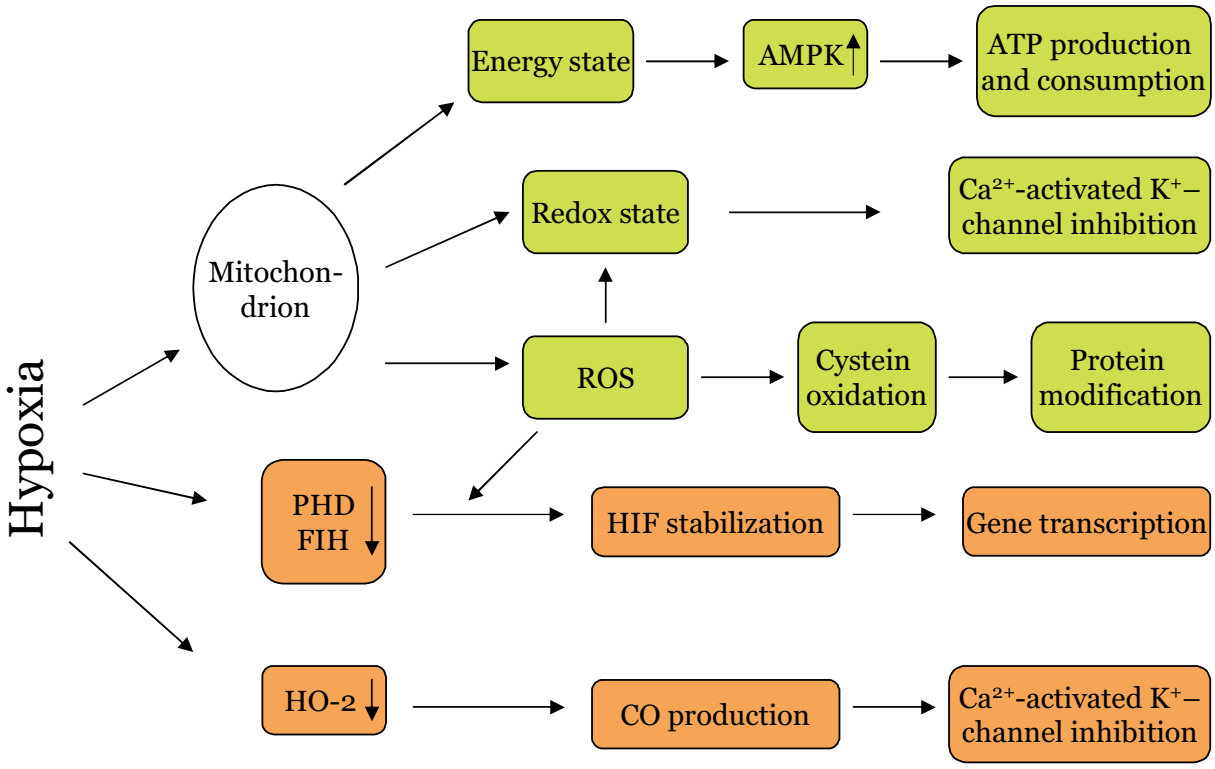

Figure 1-5: Summary of bioenergetic and biosynthetic O_2 sensors and signalling mechanisms.
Adapted from Ward 2008. Bioenergetic sensors are marked green and biosynthetic sensors are marked orange.

Next to mitochondria (bioenergetic mechanisms) oxygen consuming enzymes like hydroxylases can function as O_2 sensors (biosynthetic mechanisms). The prolyl- and aspraginyl-hydroxylases, which regulate HIF-1α stability and transciption activity, were recently shown to act as O_2 sensors. They require O_2 for the hydroxylation of the HIF-α protein together with other co-substrates and co-factors, discussed below (1.6). Without O_2 the HIF-α protein cannot be hydroxylated. It is stabilizes and activates the transcription of target genes in the nucleus. This means that oxygen concentrations are measured in the cytosol and modulate gene expression in the nucleus.

The haemoxygenases-2 (HO-2) is another O_2-sensing enzyme. In the presence of O_2 and NADPH HO-2 degrades haem to biliverdin and iron and forms CO that keeps Ca^{2+}-sensitive K^+ channels open. Under hypoxia, when the HO-2 substrate oxygen is low, CO formation slows down and K^+ channels closure forces an increase in respiration rates (ventilation) (Williams et al., 2004).

All oxygen related processes are linked with each other and cannot be easily separated. Several signal circuits regulate ROS induced oxidative stress-protective responses, but the upstream ROS receptors are still unknown. One example is the peroxisome proliferators-

activated receptor-γ coactivator-1α (PGC1α), which stimulates mitochondrial biogenesis in response to increased energy demand and also regulates the H_2O_2-inducible antioxidant transcriptional programme that includes superoxide dismutase, catalase and glutathione peroxidase. PGC1α thus forms an important link between the control of mitochondrial respiration that generates ROS and a ROS defence programme (D´Autréaux & Toledano, 2007).

1.4 Organismal response to changing oxygen (marine)

Whereas air-breathers control oxygen up-take by monitoring CO_2 concentrations in the blood, in water-breathers O_2 levels are the prime factor that control ventilation rates, and thus the oxygen availability to tissues and cells (Schmidt-Nielsen, 1999). This is because of the higher variability and the lower solubility of oxygen, compared to CO_2, in water (Randall et al., 1997).

Carotid bodies, internal oxygen sensing organs, are only found in mammals and birds (Randall et al., 1997). They measure oxygen concentrations and are associated with the regulation of ventilation to ensure oxygen supply to the gas exchange surface. Fish and amphibia have oxygen sensor cells, ancestors of the carotid bodies, in their gills (Milsom & Burleson, 2007). Nacellidae exhibit a pair of osphradia and associated wart organs, which have sensory structures, located on their mantle and on either side of the neck near the shell attachment muscles (Lindberg & Ponder, 2001b). There the osphradia lie in the path of the respiratory currents and are therefore ideally placed to sample the environment (Croll, 1983).

Independent of ambient water PO_2 changes, which routinely occur in aquatic ecosystems, water-breathers were found to maintain their arterial PO_2 within a low and narrow range between 1 and 3 kPa (10 – 14 kPa in air-breathers). Massabuau (2001; 2003) described this as the “low blood PO_2 strategy” or “low tissue oxygenation strategy”. Preventing high PO_2 is the simplest and most efficient tool to limit the production of reactive oxygen species (ROS) (Abele, 2002). On the other hand, hypoxic conditions often mean insufficient O_2 supply for energy production. Therefore two different respiratory strategies have been developed in the animal kingdom to cope with changing O_2 concentrations. Most vertebrates and active marine organisms like squid, scallops and active fish are oxyregulators. They maintain their respiration and metabolic performance independent of the dissolved oxygen concentration by increased water ventilation and blood pumping rates until the lower critical PO_2 is reached. Below this critical PO_2 oxygen demand starts to decline and becomes oxygen dependent (anaerobic oxyconformity). That is the point where anaerobiosis starts (Pörtner & Grieshaber, 1993). By way of contrast, anoxia- and hypoxia-tolerant marine invertebrates (*Arctica islandica, Nereis pelagica*) are (aerobic and anaerobic) oxyconformers (Tschischka

et al., 2000). They reduce their respiration rate when the water PO_2 declines. This strategy is crucial for survival as it delays early onset of tissue hypoxia and allows them to survive longer periods of environmental hypoxia (Abele, 2002) and at the same time it causes the reduction of mass driven mitochondrial radical formation (Abele et al., 2007). When the lower critical PO_2 is reached and the oxygen demand of the cells cannot be met any longer, the metabolism is changed to anaerobiosis (see 1.5).

1.5 Anaerobic metabolism

Facultative anaerobe organisms, like intertidal invertebrates for example, have the ability to gain high amounts of ATP from oxidative phosphorylation when oxygen is available, but have equally the ability to rely on fermentation to produce ATP when oxygen is lacking (Schmidt-Nielsen, 1999). When oxygen concentrations are reduced and insufficient to meet the oxygen requirements of the mitochondria for continuous NADH reoxygenation, the ETC comes to a halt, and NADH and $FADH_2$ are no longer re-oxidized to NAD^+ and FAD (Figure 1-4), the essential electron acceptors for glycolysis (Grieshaber et al., 1994). Under anaerobic conditions the reduction of pyruvate to lactate, oxaloacetate to malate, and fumarate to succinate are then the essential NADH-consuming reactions to maintain redox balance (light green balls in Figure 1-6) (Tielens & Van Hellemond, 1998).

Functional and environmental hypoxia (i.e. anaerobiosis) need to be differentiated in marine invertebrates (Urich, 1990). Functional hypoxia can occur in air-breathing as well as water-breathing animals when the oxygen supply to the tissue is not sufficient to meet the oxygen demand (Gäde, 1983). This can be the case when ATP turnover rates are dramatically increased due to stress like temperature or when animals perform intense muscle activity, e.g. during the flight response (De Zwaan & Putzer, 1985). Furthermore the inhibition of the mitochondrial oxidative respiration, for example by H_2O_2, leads to functional hypoxia. Functional hypoxia is usually a short-term event and requires and alternate and speedy form of energy production. High amounts of glycogen are catabolised to lactate, the main end-product, yielding 2 moles of ATP per glucosyl unit (Zammit & Newsholm, 1978). Pörtner et al. (1999) measured only elevated octopine concentrations in the foot muscle of the Antarctic limpet *Nacella concinna* after short-term temperature stress, whereas neither succinate nor acetate accumulated. Lactate was not measured in their study. Functional anaerobic metabolism has a low efficiency, but high yield in terms of energy (ATP) production through the Pasteur effect.

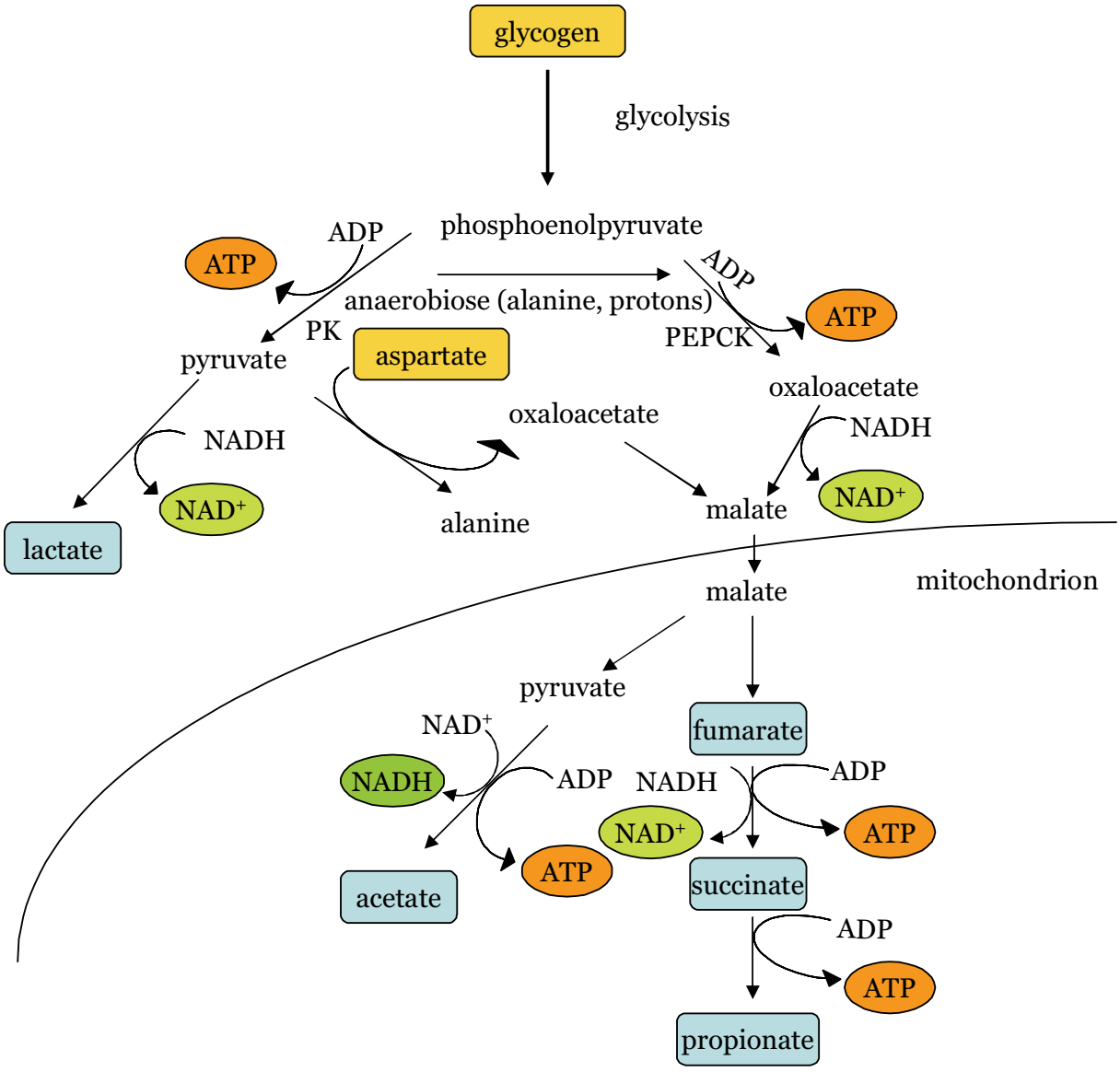

Figure 1-6: Anaerobic metabolic pathways.
Adapted from Grieshaber et al. (1994). Blue boxes indicate metabolites measured by HPLC adapted from Eertman et al. (1996).

Environmental hypoxia is defined as exposure of the whole organism to hypoxic or anoxic conditions in the natural environment. This kind of hypoxia mainly happens to water-breathing organisms due to oxygen deprivation in the water and can last for weeks, e.g. in frozen lakes in winter (Gäde, 1983). In hypoxia tolerant invertebrates energy expenditure can drop to an extent, 1 – 10% of the corresponding aerobic rate under hypoxic conditions (Larade & Storey, 2006). Consequently there may even be no need for a compensatory increase in anaerobic glycolysis to generate ATP for survival ("reverse Pasteur effect"). As a result of the hypometabolic state, O_2 consumption is reduced, often by more than 10-fold (Aragonés et al., 2009). This extreme metabolic reduction allows the animals to stay aerobic also at low PO_2 and survive long-term hypoxia, because glycogen stores are preserved.

Glycogen is the main fuel during long-term anaerobic metabolism, while aspartate is used as an energy source during the initial phase. During the first hours of anaerobic metabolism the accumulating NADH is reoxidized in the cytosol by lactate dehydrogenase (De Zwaan & Putzer, 1985). Additional substrate-level phosphorylation reactions are coupled to the glycolysis to increase the ATP output per glucosyl-unit (Larade & Storey, 2009). First by using the aspartate-succinate pathway and later when aspartate pools are depleted by the glycogen-succinate-propionate pathway cells can gain up to the double amount of ATP, 4 (succinate) or rather 6 (propionate) moles ATP per mole glucose compared to the classical glycolysis (Larade & Storey, 2002). When succinate is mainly formed, a small part of the malate is oxidized via pyruvate and acetate to generate NADH (Grieshaber et al., 1994). This leads to acetate formation in addition to succinate and propionate (De Zwaan & Putzer, 1985; Tielens & Van Hellemond, 1998). Decreasing pH and increasing alanine concentrations are the consequences of prolonged hypoxia. This favours the catabolism of phosphoenolpyruvate (PEP) via the phosphoenolpyruvate carboxykinase (PEPCK) pathway, resulting in acetate and succinate respectively propionate, rather than via the pyruvate kinase (PK) pathway resulting in lactate. Despite these alternative pathways, anaerobic metabolism remains much less efficient than aerobic metabolism in utilizing energy stores for ATP production (McMahon & Russel-Hunter, 1978). A further advantage of these pathways, beside increased ATP production, is the reduced acidification of the tissue from accumulating succinate, propionate and acetate, which are highly soluble and can be excreted into the surrounding water. This kind of anaerobiosis can be described as relatively efficient, but still has a low rate of energy production.

1.6 The hypoxia inducible factor

The hypoxia-inducible factor (HIF) is a master regulator of cellular and systemic O_2 homeostasis in animals which facilitates both O_2 delivery and adaptation to O_2 deprivation (Semenza, 2001). The transcription factor HIF plays a central role in the transcriptional response to oxygen flux by being degraded in an oxygen-dependent manner. When stabilized under hypoxic and anoxic conditions HIF initiates the expression of genes that control cellular processes including a switch from oxidative to anaerobic metabolism, inhibition of cellular proliferation, and stimulation of oxygen delivery (Benita et al., 2009).

HIF is a heterodimer of two constantly expressed subunits (α and β). The oxygen sensitivity of HIF is caused by posttranslational hydroxylations of the α-subunit. The oxygen-dependent hydroxylation of two functional proline residues by prolyl hydroxylases (PHD) is the initial step for the proteasomal degradation of the α-subunit. It prepares a binding-site for the von Hippel-Lindau protein (VHL) that leads to ubiquitylation of HIF-α, which targets the protein for proteasomal degradation (Ivan et al., 2001; Jaakkola et al., 2001). Under hypoxia the α-

subunit is stabilized and accumulates. After translocation into the nucleus it dimerizes with the β-subunit and binds to the hypoxia-responsive element (HRE) on more than 100 target genes (Figure 1-8e)(Benita et al., 2009), including genes coding for glycolytic enzymes (Kaelin Jr, 2005). The transcriptional activity is modulated by hydroxylation of one functional asparagine residue, located within the C-terminal part of the α-subunit, by the factor inhibiting HIF (FIH). When the asparagine is hydroxylated, the p300/CBP co-activators, which are essential for the transcriptional activity of HIF, cannot bind and target genes are not expressed (Figure 1-8b).

Both hydroxylation steps are independent from each other. Koivunen and colleagues (2004) found FIH to have a higher affinity for O_2 than the PHDs. In that way FIH remains active at reduced O_2 tensions when PHDs have already lost their HIF degrading activity. This led to the speculation of a "fine-tuning" of HIF-α regulation by the FIH mechanism.

Both subunits are Per-Arnt-SIM (PAS) -proteins and contain the characteristic basic helix-loop-helix domain, which is responsible for DNA binding and dimerization with the β-subunit. The two PAS domains are important for target specification, dimerization and transactivation. Furthermore, the α-subunit contains the oxygen dependent degradation domain (ODD), where both functional proline residues (Pro 402, Pro 564) are located, and the C-teminal transactivation domain with the asparagines residue (Asn 803) (see Figure 1-7).

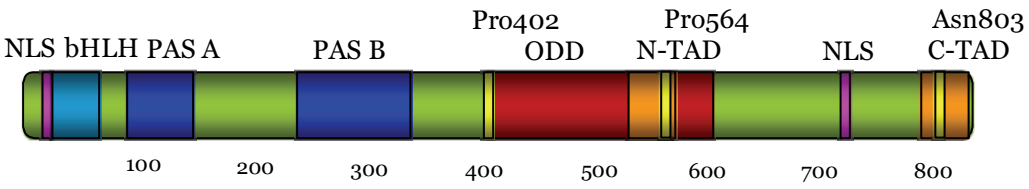

Figure 1-7: HIF-α peptide structure.
Domains and functional sites (yellow), adapted from Dery et al. (2005). NLS = nuclear localisation signal (pink); bHLH = basic helix-loop-helix motif (light blue); PAS = PER-ARNT-SIM (dark blue); ODD = oxygen dependent degradation domain (red); N/C--TAD = N-/C- terminale transactivation domain (orange).

Both hydroxylase enzmymes (PHDs and FIH) require oxygen, 2-oxoglutarate which is converted to succinate during the hydroxylation, Fe^{2+} as co-factor which is oxidised to Fe^{3+}, and ascorbate to reduce the Fe^{3+} to Fe^{2+} again. One oxygen atom of the O_2 molecule is bound to the hydroxylated residue and the other one is used for the oxidative decarboxylation of 2-oxoglutartate to succinate and CO_2 (Figure 1-8 a).

Recent studies have shown that the hydroxylases are inhibited by two different mechanisms depending on the PO_2 in the cells. Under anoxic conditions the hydroxylases are inhibited because of the lack of O_2 (Figure 1-8d). Guzy et al. (2005), Brunelle et al. (2005), and

Mansfield et al. (2005), could prove that under hypoxia elevated H_2O_2 concentrations in the cytosol, originating from the increased mitochondrial $O_2^{-\cdot}$ output (see 1.2), have an inhibiting effect on the hydroxylases also leading to the accumulation of HIF-α (Figure 1-8c). Furthermore, anaerobic metabolites like succinate were found to inhibit the PHDs resulting in elevated HIF-protein levels.

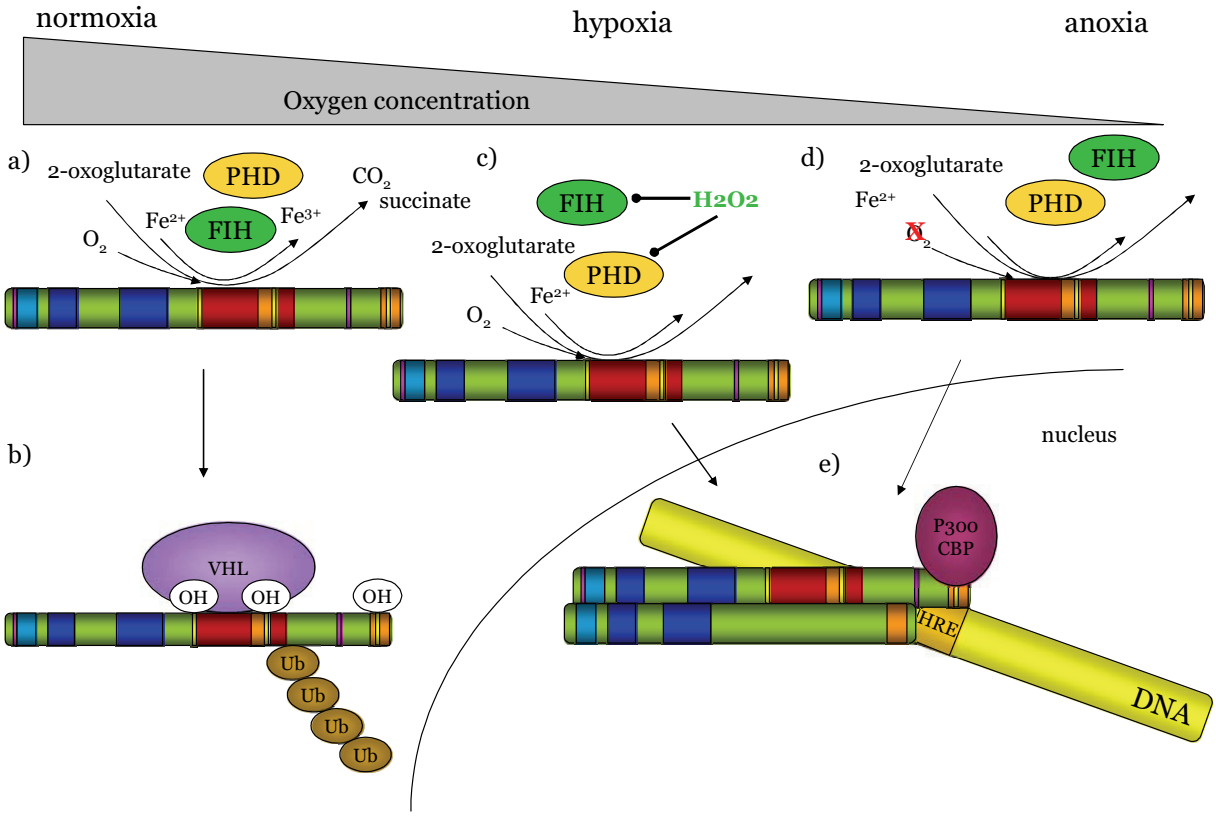

Figure 1-8: HIF-α regulation scheme.
Adapted from Kaelin, 2005.

Degradation of the HIF protein not only takes place under normoxic conditions, but also after prolonged hypoxia. PHDs were found to be target HIF genes (Benita et al., 2009). This implicates a direct negative feedback mechanism: HIF stabilisation and activity initiates its own degradation by enhancing the transcription of PHDs (Marxsen et al., 2004). The early hypoxic regulation allows the cell to adapt to hypoxia, without becoming completely deprived of energy and challenged with life-threatening exhaustion of vital ATP (Aragonés et al., 2009).

Beside further anaerobic metabolites such as lactate, fumarate and pyruvate (Kaelin Jr & Ratcliffe, 2008) the redox state is thought to have a regulatory effect on HIF activity (Lando et al., 2000). Tajima et al. (2009) found GSSG had an enhancing effect on HIF binding activity while GSH had a negative effect. How this regulation functions in detail is not clear, but the GSH/GSSG ratio may determine binding of thiol groups of regulatory cellular

proteins. Nikinmaa et al. (2004) has previously discussed the impact of the redox state on the phosphorylation of HIF, in particular in the cystein residues.

So far most of the work on HIF has been conducted in mammalian systems, cell lines and invertebrate model organisms like *Caenorhabditis elegans* and *Drosophila melanogaster*. Recent investigations of lower vertebrates (fish and frogs) and invertebrates (shrimp and insect larvae) could detect HIF-protein levels under normoxic conditions (Heise et al., 2006; Li & Brouwer, 2007; Rissanen et al., 2006) and found variable mRNA levels (Morin et al., 2005; Soñanez-Organis et al., 2009; Terova et al., 2008). Milloning and colleagues (2009) found that it is not the final oxygen concentration to be crucial for HIF-α stabilization, but the intensity of the PO_2 decline in the tissue. These results indicate that the HIF-regulation is more complex than presently known.

Currently nothing is known about the characteristics of HIF-α and its regulation in molluscs. One of the central questions in the current project was, whether or not this cellular oxygen sensing system, which translates the oxygen signal into metabolic regulation within the tissues, is employed in early evolved and oxyconforming patellid gastropods. Furthermore we wanted to know how and when HIF-α is regulated in hypoxia tolerant invertebrates such as the limpets.

1.7 Objectives of the study

The aim of the present work was to investigate whether the morphological differentiation between the intertidal and the permanently sub-littoral *Nacella concinna* subpopulation is going along with a physiological and molecular adaptation to desiccation. Biochemical parameters for oxidative stress, cellular redox state, and onset of anaerobiosis were studied during air exposure as well as hypoxia which can be a result of air exposure. For the adaptation on molecular level, the α-subunit of transcriptionfactor HIF was investigated.

More specifically, the following questions were asked:

- What characterizes the metabolic stress response during desiccation and hypoxia exposure in intertidal *N. concinna* and in their sub-littoral congeners, especially with respect to onset of anaerobic metabolism?
- What effects have desiccation and hypoxia on the oxidative stress parameter, in particular, the cellular redox potential? And do in-situ tissue iron levels have an influence on the oxidative stress parameters in sub-littoral and intertidal *N. concinna*?
- Does the hypoxia-tolerant Antarctic limpet express the hypoxia inducible factor (HIF-α) and how is HIF-α expression regulated during desiccation and hypoxia stress?

2 Materials and Methods

2.1 The Antarctic limpet *Nacella concinna*

Nacella concinna, first described as *Patella polaris* by Hombron and Jaquinot (1841), is the only Antarctic representative of the widespread subclass Patellogastropoda or Docoglossa. Strebel (1908) already distinguished between two morphotypes, the shallow water form *Patinella polaris* and the deep water form *P. polaris* var. *concinna*. Powell described two types as well in 1951, *Patinigera polaris and P. polaris concinna*, but classified in 1973 both forms as *Nacella concinna* within the subgenus *Patinigera*. This is the current valid nomenclature (Beaumont & Wei, 1991). Both subpopulations can be clearly distinguished by the morphology of their shells. The intertidal animals have smaller and denser shells with a greater height/length ratio than the limpets from the sub-littoral (Figure 2-8).

N. concinna is abundant around the Antarctic Peninsula and the islands of the Scotia arc (Stanwell-Smith & Clarke, 1998), (Figure 2-1). In the intertidal zone (Figure 2-2), *N. concinna* is the most conspicuous and usually the only macro-invertebrate south of lat. 60°C S. In the subtidal zone, it usually inhabits depths of up to 15 m (Figure 2-3), though it has also been found as deep as 110m (Picken, 1980; Powell, 1973; Stanwell-Smith & Clarke, 1998).

Currently it is not clear whether the *N. concinna* spread from the north to the south into the Antarctic when the Drake Passage opened 25 – 30 Ma BP or vice versa (Clarke and Crame 1992, cited by De Aranzamendi et al., 2009). Recent studies indicate a migration from South America into the Antarctic regions (Kevin Pöhlmann, AWI, pers. communication).

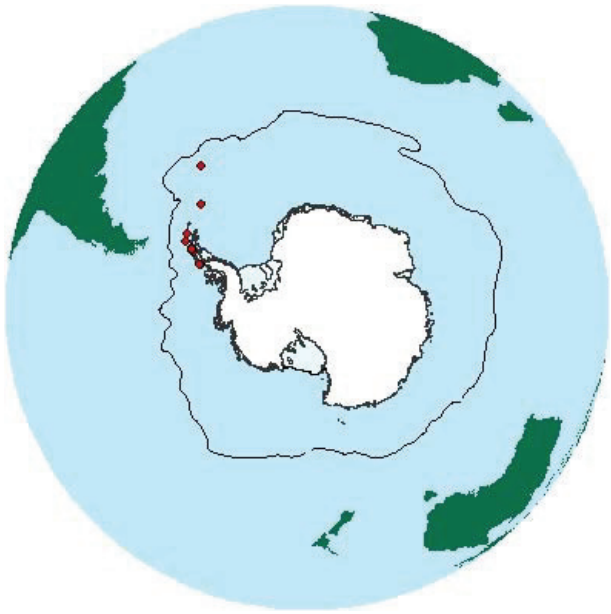

Figure 2-1: Distribution of *Nacella concinna*.
Red dots indicate distribution. Map is taken from Southern Ocean Mollusc Database (SOMBASE 2009). http://www.antarctica.ac.uk/bas_research/data/access/sombase/gastropod_species_map.php?family=Nacellidae&genus=Nacella&species=concinna (link verified 05.12.2009)

Walker (1972) reported that intertidal *N. concinna* migrate to the subtidal zone for wintering and reproduction in austral spring. Reproduction, where up to 35 individuals of both sexes aggregate in stacks, occurs in a very narrow timeframe triggered by the spring phytoplankton bloom in early spring (Brêthes et al., 1994; Stanwell-Smith & Clarke, 1998). After spawning the animals disperse and the intertidal limpets return back to the intertidal (Walker, 1972). Hargens and Shabica (1973) was the first to report that intertidal limpets remain in higher tidal zones underneath the ice, even during winter months. Newer studies confirmed these observations, with individual *N. concinna* able to survive the winter in the intertidal zone (Waller et al., 2006a). The mucus, produced in the foot muscle of the limpets, protects the tissue from inoculative freezing and lowers the interstitial fluid content, which avoids extracellular ice propagation that can only occur below –8°C (Hargens & Shabica, 1973).

Nacella concinna is a non-homing grazer that feeds on microphytobenthos, microalgae and diatoms all over the year (Davenport, 1997; Fraser et al., 2002). It roams over a variety of hard and soft substrata (Stanwell-Smith & Clarke, 1998). Contrary to many temperate, homing limpets, *N. concinna* does not fit its shell perfectly to the contours of the substratum, in order to minimizing water loss during low tide when the animals are exposed to the air (Davenport, 2001). *Nacella concinna* is an important member of the Antarctic food chain as

it links primary producers (algae) with higher trophic levels. Many species prey on *N. concinna*, for example: the Dominican gull *Larus dominicanus*; Sheathbill *Chionis alba*; fish (*Notothenia coriiceps*); pycnogonids (*Colossendeis robusta*, *C. megalonyx*, *Pentanymphon sp.*); echinoids (*Sterechinus neumayeri*); the sea star (*Neosmilaster georgianus*); nemertine worms and prosobranch gastropods (Kidawa et al., 2008; Nolan, 1991; Shabica, 1971).

The Antarctic limpet is very eurythermal compared to many other polar animals. Most cold-water invertebrates die in experiments at temperatures of 5-10°C (Peck et al., 2004). *N. concinna* can tolerate in extremes temperatures between -12.9°C (median lower lethal temperature) and +15.6°C for up to 12h (median upper lethal temperature) (Davenport, 1997) whereas the long-term upper lethal temperature was reported to be at 9°C (Peck, 2002).

Figure 2-2: *Nacella concinna* in the intertidal on King George Island during low tide.
The picture was taken at Peñon 1 in 2007

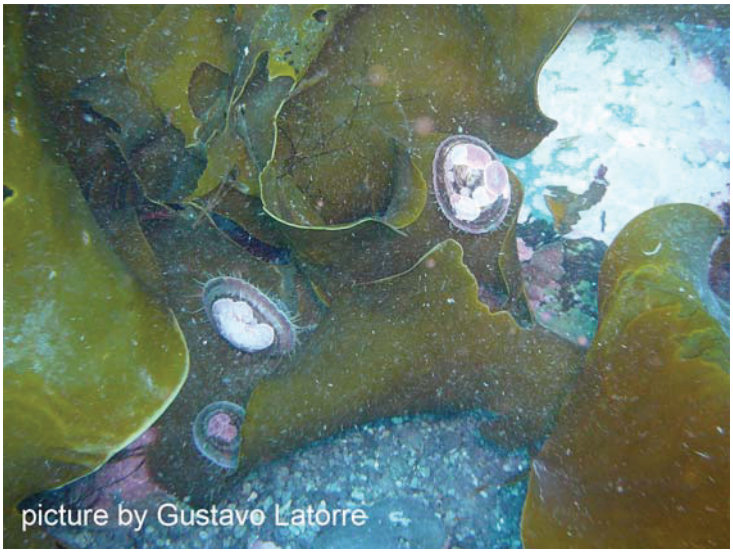

Figure 2-3: *Nacella concinna* in the sub-littoral in 13m depth at King George Island.
The picture was taken by Gustavo Latorre in 2007.

2.1.1 Animal collection

All *N. concinna* used in this thesis were collected during two summer expeditions to King George Island (South Shetland Island, Antarctica) in 2005 and 2007. Intertidal limpets were collected by hand during low tide at Peñon 1 in Potter Cove (62° 14' S - 58° 40' W) (Figure 2-5). Sub-littoral animals were collected by SCUBA divers in 13 – 15 m depth in Potter Cove about 1 km from the intertidal sampling location at Peñon de Pesca (62° 14' S - 58° 43' W) (Figure 2-5). The animals were gently removed from their rocky substratum using a rounded spittle and immediately transferred to the aquarium at Dallmann Laboratory at Jubany

Station, and held in aerated, 0°C cold seawater which had been obtained from the cove. The limpets were maintained in the aquaria for a minimum of 10 days and up to 3 weeks before air exposure experiments were initiated. Both subpopulations were permanently submersed in flow through aquaria so that any differences in control parameters were not caused by acute exposure to different *in-situ* conditions.

Shell lengths ranged from 2.7 to 4 cm (mean 3.3 cm) for intertidal and from 3.04 to 5.78 cm (mean 4.3 cm) for sub-littoral specimens. Sexes were not distinguished.

Figure 2-4: Map of King George Island.
Red box indicates Potter Cove. Darker colour indicates ice free areas (source: http://www.kgis.scar.org/mapviewer/kgis.phtml, 2009).

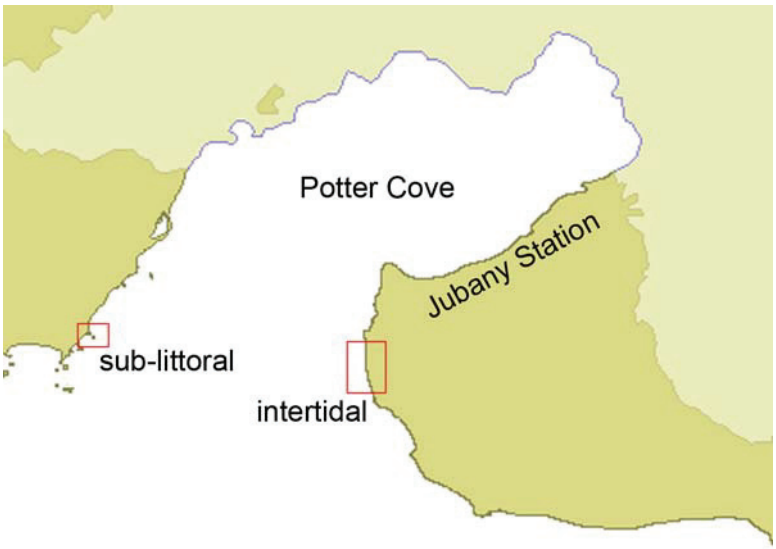

Figure 2-5: Map of Potter cove.
Red boxes mark limpet collection sites Peñon 1 (intertidal) and Peñon de Pesca (sub-littoral). (Source: http://www.kgis.scar.org , 2009).

2.2 Methods

Within this thesis two expeditions were conducted to the Dallmann Laboratory, King George Island, in winter 2005 and 2007. *Nacella concinna* of both subpopulations were exposed to two different experimental incubations, air exposure and hypoxia. Frozen tissues were used for measurements of several biochemical and molecular parameters (Table 2-1), wherefore different tissues were used (Table 2-2). In addition water loss and extrapallial O_2 concentrations were investigated of air exposed animals and beyond shell parameters were measured.

Table 2-1: Overview of expeditions, conducted experiments, measured parameters and where the results are published.

Expedition	Exposure experiment	Measured parameters	Publication
Expedition 2005	Air exposure	Antioxidant enzymes Glutathione Anaerobic metabolites HIF protein	Publication I, II and III
	Hypoxia	Antioxidant enzymes Anaerobic metabolites HIF mRNA and protein	Publication II and III
Expedition 2007	Air exposure	Water loss PO_2 in extrapallial water Shell morphometrics Adenylates pH	Publication I
	Hypoxia	Glutathione Adenylates pH	Publication II and III Additional results

Table 2-2: Overview of measured parameters and tissues

Parameters	Whole animal	Digestive gland	Gills	Foot muscle
Shell morphology	X			
Extrapallial PO_2	X			
Water loss	X			
Heavy metal		X	X	
SOD and CAT		X	X	
Glutathione			X	X
pH			X	X
Ascorbate				X
Malondialdehyde				X
Adenylates				X
Organic acids				X
HIF sequence		X		
HIF mRNA expression		X		
HIF protein levels		X		

2.2.1 Experimental set-ups and tissue sampling

To investigate the response of intertidal and sub-littoral limpets to air exposure individuals of both subpopulations were placed on small plastic weighing scales over silica drying pearls in a desiccator for up to 24 hours (Figure 2-6). The desiccator was placed into the snow and covered with a dark cloth (not shown) to reduce light impact and keep temperatures close to 0°C. Incubations were started in the evening, as the animals were more actively crawling during day time.

Figure 2-6: Set up for air exposure experiment.
Limpets are placed on plastic dishes in the desiccator.

Figure 2-7: Hypoxia incubation of *Nacella concinna*.
Limpets were placed in a 2L glass jar containing 2% oxygen.

For the hypoxia incubation, animals were placed in 2L glass jars for up to 48 hours. The jars were placed in a thermostated water bath at 0°C and the water was permanently bubbled with an air mixture containing 2% oxygen (Figure 2-7). The oxygen concentration was controlled using a fiber optical respirometer (Precision Sensing GmbH, Regensburg, Germany). Hypoxia incubations were conducted to have environmental hypoxic conditions and to compare animals´ response to air exposure with hypoxia. No animal died during either the air exposure or hypoxia incubations.

After the incubations animals were killed quickly by decapitating. Tissues (foot, gills and digestive gland) were dissected and immediately frozen in liquid nitrogen using freeze clamps for biochemical and molecular biological analysis

2.2.2 Whole animal analyses

2.2.2.1 Shell morphology

For comparison of shell morphology for both *N. concinna* subpopulations, shell length (L), height (H), width (W), and weight of every animal were measured (Figure 2-8). From these parameters shell volume (V), circumference (C), steepness (S), and roundness (R) were calculated using the following equations:

$$C = 2(L^2/8 + W^2/8)^{0.5}$$

$$V = LWH/12$$

according to Lowell (1984) and

$$S = 2H/(L+W)$$

$$R = W/L$$

according to Orton (1932) cf. Wallace (1972). For more details see publication I.

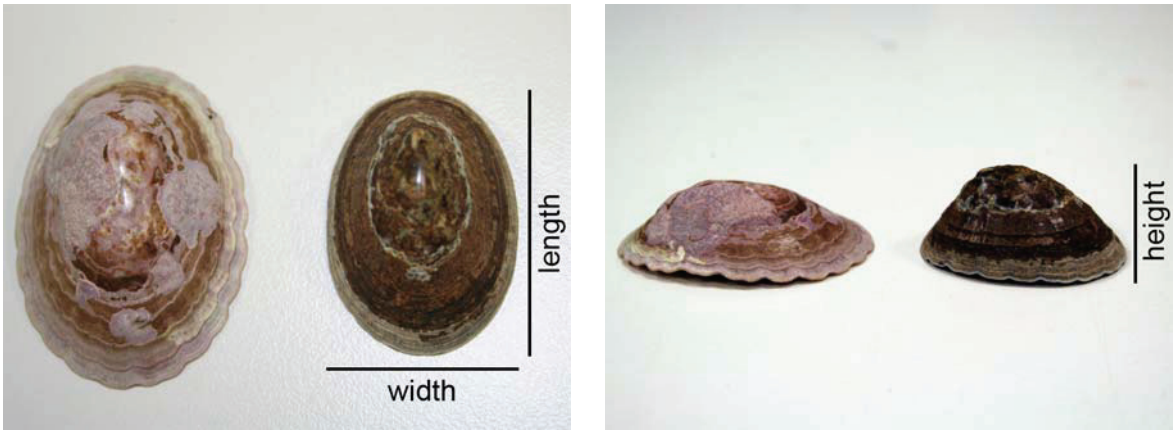

Figure 2-8: Shells of sub-littoral and intertidal *Nacella concinna*.
Sub-littoral shell: left light shell; intertidal shell: right dark shell

2.2.2.2 Oxygen measurement in extrapallial water

In patellids, the gills are very rudimentary, only a pallial gill fringe exists. However, the entire epidermis, especially the roof of the pallial cavity and the epidermis of head and foot, are capable of cutaneous oxygen uptake. Small oxygen chemoreceptors, the osphradia, are located in the anterior pallial roof of the patellogastropods (Lindberg & Ponder, 2001a). These sensors in the innermost part of the open circulatory system of limpets provide a signal for the adjustment of the ventilation activity in the mantle cavity.

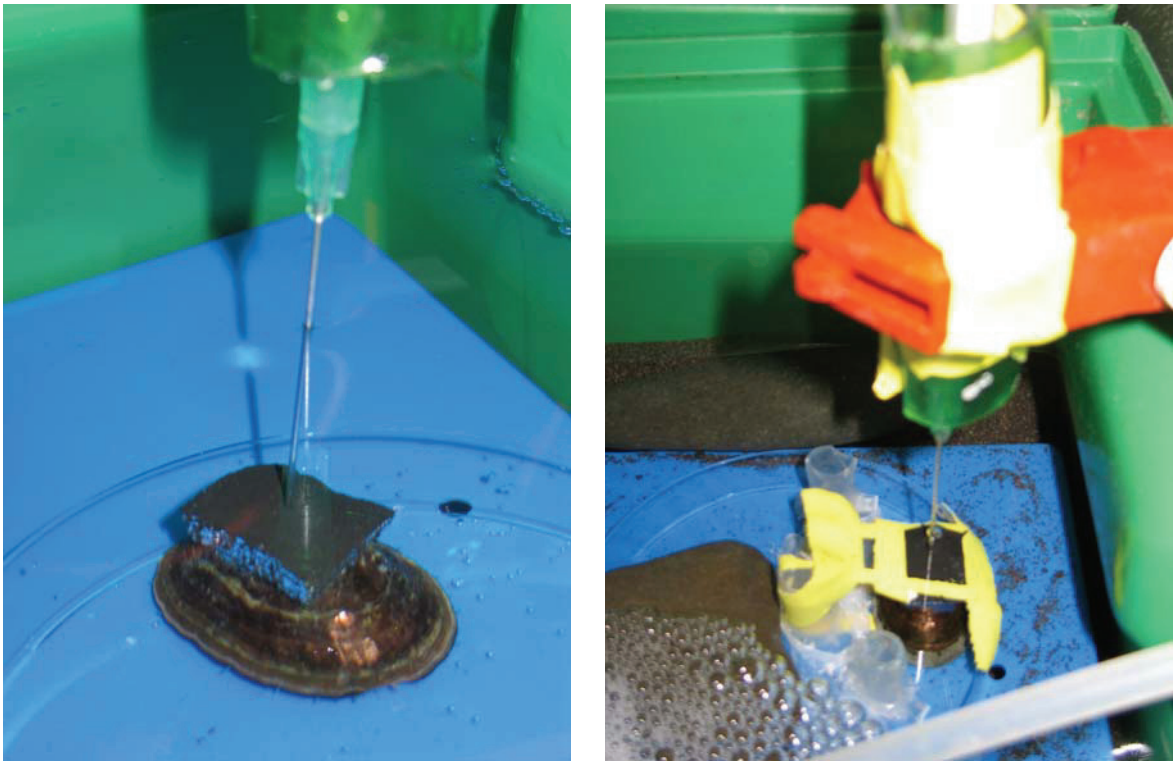

Figure 2-9: Experimental set-up of PO_2 measurement in the inner shell water.
Left: limpet with implanted optode and insulating material. Right: fixed limpet with implanted optode and isolation material.

Oxygen concentrations were measured in the inner shell water of submerged as well as of air exposed limpets. For this purpose, a hole was drilled in the shell and covered with a thin elastic latex foil and isolation material (Armaflex) to avoid exchange with the ambient medium water or respectively air. The fiber oxygen optodes for O_2 measurements were implanted through a hole in the insulating material into a small, sea-water filled space between the mantle and shell (Figure 2-9 left). The animals were fixed to stop them crawling around and breaking the optode (Figure 2-9 right). Nonetheless, the limpets could still lift their shells. The animals were exposed to cycles of submersion and emersion. During the whole experiment the temperature was maintained at 0°C.

2.2.2.3 Water loss

Water loss was calculated by the difference in shell-free wet mass before and after aerial exposure and expressed as a percentage of the initial shell-free wet mass.

For information about statistical analysis see publication I.

2.2.3 Biochemistry

2.2.3.1 Heavy metal measurements

The heavy metals iron (Fe), cadmium (Cd), zinc (Zn) and aluminium (Al) were determined in digestive glands and gill tissues of limpets collected in the intertidal and the sub-littoral. The analyses were conducted by Ilse Stölting (AWI). Fe, Zn and Al were measured by inductively coupled plasma optical emission spectrometry (ICP/OES) and Cd by graphite furnace atomic absorption spectrometry (GF-AAS) and are described in detail in publication II.

Figure 2-10: Picture of the sub-littoral in Potter Cove showing plumes of sedimentation.

High sediment loads are carried into the bay (Figure 2-10) due to glacier melting and surface erosion in austral summer. The volcanic rock sediments contain high concentrations of heavy metals which are taken up by sedimentary grazers and benthic filter feeders leading to accumulation in the tissues. Metals like the Fe^{2+} can lead to elevated formation of ROS.

2.2.3.2 Antioxidants

The antioxidant enzymes SOD and CAT were measured photometrically according to Livingstone et al. (1992) and Aebi et al. (1984), respectively. Both enzyme activities were determined in the same extracts of digestive gland tissue and gills at 20°C. For a more detailed description see publication II.

Concentrations of the two antioxidants glutathione and ascorbate were determined using high performance liquid chromatography (HPLC). Glutathione was measured in gill and foot tissues according to Farris and Reed (1987) whereas ascorbate was determined only in foot

tissue as per Lazzarino et al. (2003). GSSG is formed by the oxidation of two molecules of GSH, thus, total glutathione was calculated as 2*GSSG + GSH.

The enzyme activities of the two antioxidant enzymes as well as the concentrations of glutathione and ascorbate shed light on the antioxidant capacities of the two investigated tissues. Beyond that glutathione, or more precisely the ratio of oxidized (GSSG) to reduced (GSH) glutathione (GSSG / GSH), gives information about the redox status of the tissue. The redox potential of the foot tissue was calculated using the Nernst equation (Schafer & Buettner, 2001):

$$\Delta E = E^{o}{}_{pH} - (RT * 2.303 / nF) \log ([GSH]^2 / [GSSG]) \text{ mV}$$

2.2.3.3 Adenylates

The adenylates ATP, ADP and AMP were measured as per Lazzarino et al. (2003), as described in publication I. ATP is primarily used as energy carrier within a cell. The total adenylate pool was calculated using the following equation:

$$\text{Adenylate pool or total adenylates} = [ATP] + [ADP] + [AMP]$$

The balance between energy production and expenditure determines the energy charge of the cell and the ratio of [ATP] to the adenylate pool. Energy charge was calculated using the following equation (Atkinson, 1968).

$$EC = ([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP])$$

Energy charge is typically between 0.85 – 0.95 in skeletal muscle (Ataullakhanov & Vitvitsky, 2002). Increasing ratio of AMP/ATP indicates energy depletion in the cells and activates enzymes like the AMPK, which in turn modulates anaerobic ATP production and consumption (Hardie, 2003).

2.2.3.4 Organic acids

The organic acids acetate, lactate, succinate, propionate, and fumarate are typical anaerobic metabolites in marine invertebrates. They were investigated to gain information about the onset and degree of anaerobic metabolism in the limpets.

The amount of organic acids in the foot tissues was determined using HPLC as described by Eertman et al. (1996) with some modifications. Frozen samples were not lyophilized and so only the 3-fold volume of 7% PCA was used for homogenation instead of the 30-fold volume. pH was adjusted to 2-3 with NaOH instead of K_2CO_3 to minimize salt precipitation. To precipitate the NaClO4 formed during pH adjustment, supernatants were frozen. For detailed information about sample preparation and measurement see publication I or III.

2.2.4 Molecular biology

2.2.4.1 RNA Isolation

Total RNA was isolated from digestive gland tissues of *N. concinna* and *P. vulgata* as described in publication I. This method, the combination of TRIZol, DNA digestion and clean up was developed as part of my diploma thesis working with the ocean quahog *Arctica islandica*. RNA concentrations and quality were determined spectrophotometrically (Nanodrop ND-1000, PecLab). Measurement of RNA quality resulted always in $A_{260/280}$ ratios > 2. Integrity of the RNA was checked using a bioanalyser (Agilent Technologies, Waldbronn, Germany).

Total RNA was reverse transcribed into cDNA, which was used as a template for PCR (for more detailed description see publication III). Total RNA was isolated from digestive gland tissues of *N. concinna* and *P. vulgata* as described in publication I. This method, the combination of TRIZol, DNA digestion and clean up was developed as part of my diploma thesis working with the ocean quahog *Arctica islandica*. RNA concentrations and quality were determined spectrophotometrically (Nanodrop ND-1000, PecLab). Measurement of RNA quality resulted always in $A_{260/280}$ ratios > 2. Integrity of the RNA was checked using a bioanalyser (Agilent Technologies, Waldbronn, Germany).

Total RNA was reverse transcribed into cDNA, which was used as a template for PCR (for more detailed description see publication III).

2.2.4.2 Sequencing and cloning of the transcription factor HIF-α

The first HIF-α gene fragment was sequenced from the common limpet *Patella vulgata*. This fragment was then used as a template to construct primers for the *Nacella concinna* gene. Total RNA was transcribed into cDNA, which was used as template for gene sequencing as described in publication III. MacVector 9.5 (Accelrys Software Inc., San Diego) was used for primer design, assemblage and sequence analysis. First primers for *Patella vulgata* were designed by aligning published fish and invertebrate HIF-1α nucleotide sequences (*Danio rerio* AY326951; *Palaemonetes pugio* AY655698; *Caenorhabditis elegans* NM075607; *Daphnia magna* AB425958). The first fragment was obtained by performing nested PCR with touch down program (see publ. III).

Therefore, a primer matrix of three outer and two inner primer pairs (named HIF-DF and HIF-DB, see Table 2-3) was used to perform nested PCR. Every possible combination was tested. Only one inner primer pair (DF2; DB1) yielded a fragment of the gene (orange arrows). Using this first fragment as a template, further primers were constructed to enlarge

the sequence by PCR (blue arrows) and rapid amplification of cDNA ends (RACE; green arrows) (Figure 2-11).

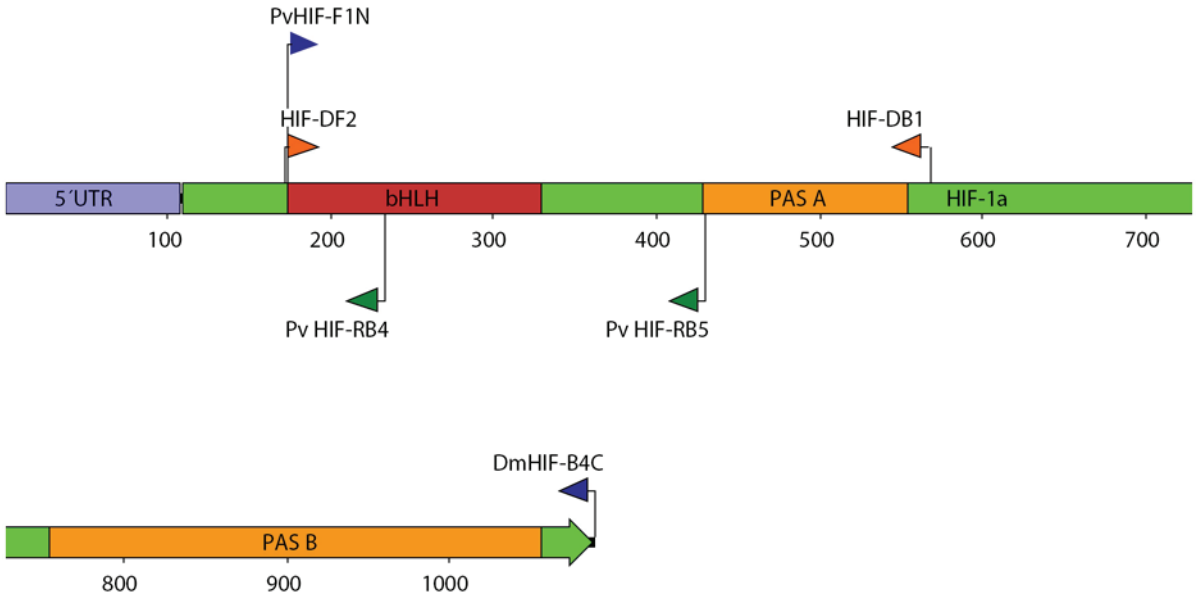

Figure 2-11: Schematic overview of the *P. vulgata* HIF-α cDNA fragment, its functional domains, and the used primers.
The 5´ untranslated region (UTR) is indicated by a light blue bar, the open reading frame by the green bar. The basic-helix-loop-helix domain is indicated by a red bar and the two PER-ARNT-SIM (PAS) domains by orange bars. The locations of the RT-PCR primers are indicated by arrows above the sequence and locations of 5´RACE primers by arrows below the sequence. Identical colours of the arrows indicate primer pairs.

Using the previously obtained HIF-α fragment of *P. vulgata* cDNA as a template, primers (PvHIF-F3; PvHIF-B1; yellow arrows) for the *Nacella concinna* gene were constructed. Subsequent PCRs were performed for completing the sequence, using "primer walking" with one *N. concinna* specific primer (NcHIF-F23 orange arrow/HIF-CT-F2/F4 blue arrows) and a degenerated primer (NcHIF-B41 orange arrow/HIF-CT-B1 blue arrow;Figure 2-12) designed on the basis of sequence segments conserved between published vertebrate and invertebrate HIF-1α sequences. To complete the gene, 5´RACE (NcHIF5´-RB5/NcHIF5´-RB10 green arrows) and 3´RACE (NcHIF3´-R1/NcHIF3´-R8/NcHIF3´-R12 purple arrows) were carried out using First Choice RLM-RACE Kit following the manufactorer´s instructions (Applied Biosystems). For further description and cloning see publication III.

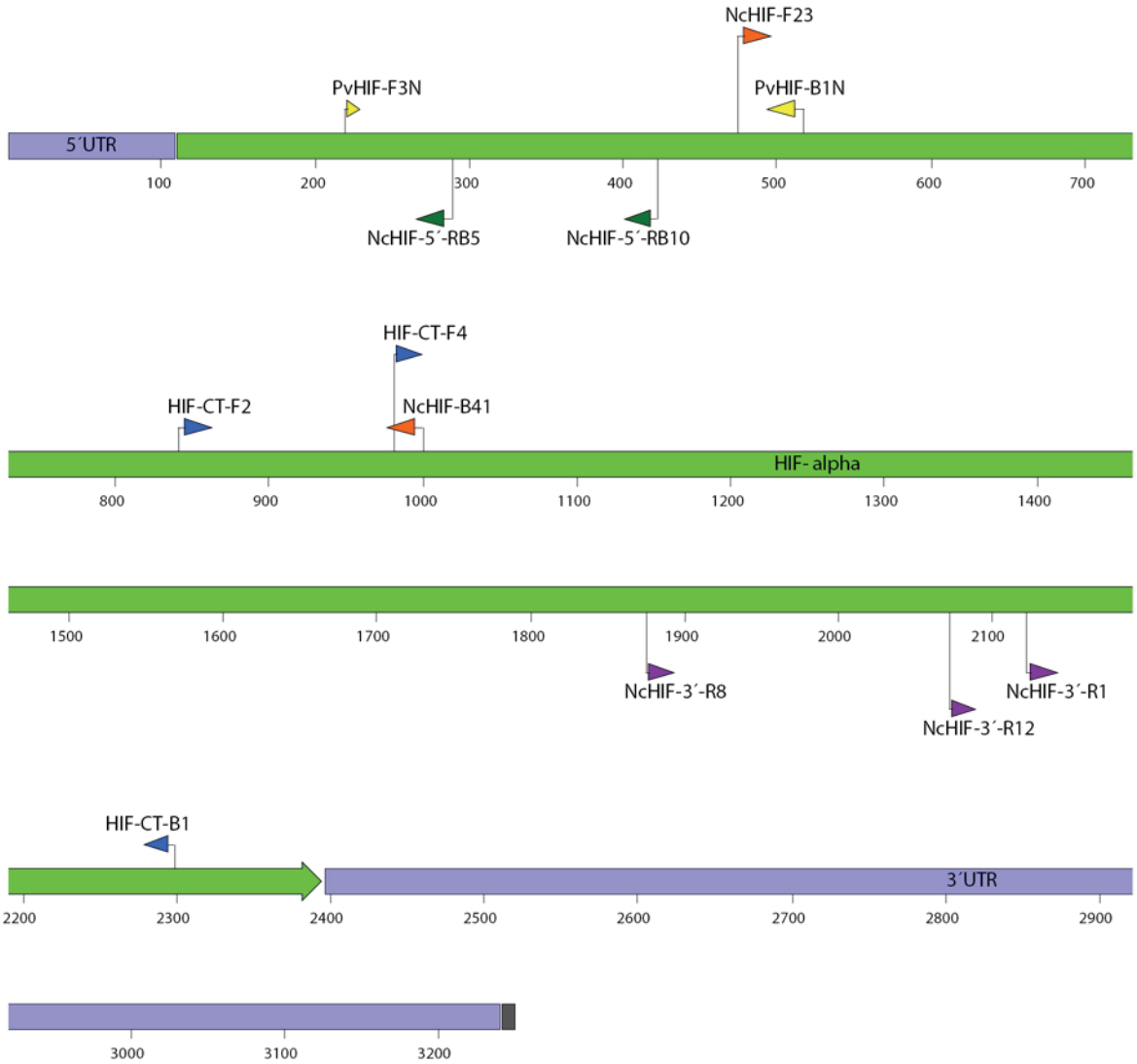

Figure 2-12: Schematic overview of the *N. concinna* HIF-α cDNA and the used primers.
The 5´and 3´untranslated regions (UTR) are indicated by light blue bars, the open reading frame by the green bar, the poly-A-tail by the grey bar. The locations of the RT-PCR primers are indicated by arrows above the sequence and locations of RACE primers by arrows below the sequence. Identical colours of the arrows indicate primer pairs.

Table 2-3: List of all primers used in this study for sequencing.
Pv indicate primers constructed using the *Patella vulgata* sequence as template, Dm indicate primers constructed using the *Daphnia magna* sequence as template. F indicate forward primers, B indicate backward primers.

Primer	Sequence	Purpose
	Patella vulgata	
HIF-DF1	SMAAAGRAAARGAGRSGGAAYTCGG	Outer RT-PCR
HIF-DB2	CCARCGGMRTYTCRATGTTSGARGG	Outer RT-PCR
HIF-DB3	TCTCVACRTAGGTGWACTTCATRTCC	Outer RT-PCR
HIF-DF2	TCGGAMARGCGSAAGGAGAAVTCCCG	Inner RT-PCR
HIF-DB1	AGYTCVTCRTGRTCGCASGGGTG	Inner RT-PCR
PvHIF-F4	TCTGTGTTCAAAGGCTCTGGAAG	RT-PCR
PvHIF-F1	AAAACGGAAGGAGAAATCCCG	RT-PCR
DmHIF-B4C	GTCTGGAMCCAMRCAWAACCWCC	RT-PCR
Pv HIF RB4	CCTCTGTTTCTTTACTCCGACGAC	5´RACE
Pv HIF RB5	CCTTCCAGAGCCTTTGAACACAG	5´RACE
	Nacella concinna	
PvHIF-F3N	CGGTGTCGTCGGAGTAAAGAAAC	RT-PCR
PvHIF-B1N	TAGTTCAATCTGCTGTATGCCCAG	RT-PCR
NcHIF-F23	AGAAACTGTTGCCAAGTATCTGGG	RT-PCR
NcHIF-B41	TTCTCAACAACATGGCTGTCCAG	RT-PCR
HIF-CT-F2	TCTTGACAGCAACACCTTTCTTACC	Outer RT-PCR
HIF-CT-F4	CAGCCATGTTGTTGAGAACGC	Inner RT-PCR
HIF-CT-B1	TGAACRGGRGCRTTBACTTC	RT-PCR
NcHIF5´-RB5	TGGCTTGTAATACTGGTTGGCAG	5´-RACE
NcHIF5´-RB10	TGCTTTTGAACACAGGGCGTC	5´-RACE
NcHIF3´-R8	TATTTGAAGTGCCTCCCCAGC	3´-RACE
NcHIF3´-R12	CCACCTCTAATAGGGATGAGC	3´-RACE
NcHIF3´-R1	CAATCGCTATCATACCTACTTCCG	3´-RACE
	Actin	
ACT-F1	GWGTGATGGTTGGTATGGGWCAG	RT-PCR
ACT-B2	GCKGTGGTTGTGAAWGARTARCC	RT-PCR

2.2.4.3 HIF-α mRNA quantification by real-time PCR

Total RNA, which was DNA-free because of digestion and clean-up steps, was transcribed into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems, Germany). This served as a DNA template for quantitative real-time PCR performed on an ABI 7500

Sequence Detection System (Applied Biosystems, Germany) using its universal PCR protocol. Primers and reporters used for the Real-Time PCR (Table 2-4) were designed using the customer design service by Applied Biosystems. The mRNA expression values were evaluated relative to β-actin. For the calculation of the relative expression levels under hypoxia conditions (x) compared to control (0), the comparative Ct method was used according to the following formula, where Ct correspond to the threshold cycle number:

$$2^{[(Ct_{HIF\text{-}1\alpha,x} - Ct_{actin,x}) - (Ct_{HIF\text{-}1\alpha,0} - Ct_{actin,0})]}$$

For more detailed description see publication III.

Table 2-4: Primer and reporter used for Real-Time PCR.

Name	Sequence	Purpose
NcHIF-α-ANYF	GATTATCTATCAGTTATTTGAACTTGTGTAATATTTTGGATT	Forward Primer
NcHIF-α-ANYR	CAGGGCGTCCATTTTCTTTTCTT	Backward Primer
NcHIF-α-ANYM1	CTGCTGCTGAAACTGA	Reporter
NcActin-ANYF	CCAGAAGAACATCCCGTCCTT	Forward Primer
NcActin-ANYR	GGTCATCTTTTCTCGGTTGGCTTTA	Backward Primer
NcActin-ANYM1	AAGCCCCACTCAACCC	Reporter

2.2.4.4 Immunoprecipitation and HIF-α protein quantification by Immunoblotting

To increase the amount of HIF-α protein to clearly detectable amounts, the protein was concentrated by immunoprecipitation before immunoblotting.

Primary antibodies were produced in two rabbits by Biotrend Chemikalien GmbH (Germany) based on the deduced *N. concinna* HIF-1α amino acid sequence CTLTSKGRNVNLKSA (position 179 – 193) located between the two PAS domains. For detailed description of the extraction see publication III. Crude extracts were incubated with one of the two antibodies (animal # 2097) over night at 2°C. On the following day, incubated samples were added to 30µl pre-washed Sepharose CL-4B beads (Sigma, P3391), having protein A immobilized, and incubated for 3 h at 2°C. The immunoprecipitate bound to the beads was collected by 5 min centrifugation at 1000 g, and 2°C, and washed three times with ice-cold buffer containing 100 mM KCl, 0.2 mM EDTA and 20 mM Tris/HCl pH 7.8. The interacting proteins were detached from the beads by suspending the pellet in 60µl of the sample loading buffer, containing 62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol and 5% β-mercaptoethanol and incubating for 10 min at 75°C under slight agitation.

The proteins were fractionated by polyacrylamid gel electrophoresis (PAGE) as per Laemmli (1970) and transferred to PVDF (Polyvinylidene difluorid)- membranes (Bio-Rad, Germany). Membranes were blocked and blots were incubated with the other primary antibody (animal #2096) overnight at 2 °C. After thorough washing, blots were incubated with the secondary antibody (mouse anti-rabbit antibody conjugated to horseradish peroxidase). The resulting signal was detected using ECL detection reagent (GE Bioscience). Chemiluminescence was detected and quantified with a cooled CCD-camera system (LAS-1000, Fuji, Japan) and AIDA software package (raytest, Germany). Protein levels in each sample were expressed relative to the signal intensity of the antibody used for immunoprecipitation.

3 Publications

List of publications and declaration of my contribution towards them

Publication I

Ellen Weihe, Doris Abele

Differences in the physiological response of inter- and subtidal Antarctic limpets *Nacella concinna* to aerial exposure

Aquatic Biology 2008: Vol. 4, 155 – 166

I conducted all the practical work and data analyses, with the exception of the statistical analyses of the water loss and PO_2 measurements including the full factorial interaction models, which were performed by the second author. I wrote the first draft of the manuscript that was revised by the second author.

Publication II

Ellen Weihe, Michael Kriews, Doris Abele

Differences in heavy metal concentrations and in the response of the antioxidant system to hypoxia and air exposure in the Antarctic limpet *Nacella concinna*

Marine Environmental Research 2009: doi:10.1016/j.marenvres.2009.09.003

The practical work and data analyses were performed by myself with the exceptions of the metal concentration measurements, which were conducted by Ilse Stölting, the technician in AG Kriews. I wrote the first manuscript that was improved in co-work with the third author.

Publication III

Ellen Weihe, Magnus Lucassen, Christoph Held, Doris Abele

Characterisation and expression patterns of hypoxia inducible transcription factor HIF-α in the Antarctic limpet *Nacella concinna*

Molecular Ecology, submitted

The laboratory work was carried out all by myself under the supervision of the second author. The phylogentical analyses were conducted by the third author. I analysed and discussed the data with the second and fourth author and wrote the first manuscript, which was improved together with all of the co-authors.

Publication I

Differences in the physiological response of inter- and subtidal Antarctic limpets *Nacella concinna* to aerial exposure

Ellen Weihe, Doris Abele

2008

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Differences in the physiological response of inter- and subtidal Antarctic limpets *Nacella concinna* to aerial exposure

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ABSTRACT: Antarctic intertidal zones are extremely harsh environments, and the Antarctic limpet *Nacella concinna* is one of the most conspicuous components of the macrofauna, colonizing such areas at King George Island, South Shetlands. The limpet subpopulation encompasses an intertidal and a subtidal ecomorph; these differ with respect to shell height and inner volume. We studied morphological, behavioural and physiological traits that distinguish both *N. concinna* subpopulations. We found a higher volume to circumference ratio of intertidal versus subtidal limpets to go along with a drastically reduced loss of body water during air exposure in intertidal specimens. Smaller intertidal limpets were less susceptible to desiccation than larger individuals. Further, the intertidal snails avoided accumulation of anaerobic metabolites (succinate, lactate and propionate), whereas subtidal limpets switched to anaerobic fermentation when exposed to air for >12 h. Only acetate accumulated in both types of snails after 12 h. Mean pO_2 in shell water was higher in intertidal (2.6 kPa) than subtidal individuals (1.3 kPa) when submersed under water, and only the intertidal snails appeared able to respire air on emersion. These differences document strong physiological diversification between shore levels and shell morphs in the Antarctic limpet subpopulation at King George Island.

KEY WORDS: *Nacella concinna* · Antarctic limpet · Air exposure · Water loss · Morphometry · Onset of anaerobic metabolism · pO_2-regulation

INTRODUCTION

The Antarctic limpet *Nacella concinna* (Strebel, 1908) is the main macrobenthic invertebrate colonizing maritime Antarctic and sub-Antarctic rocky intertidal shores south of 60° S (Davenport 1988). These high-latitude intertidal zones are extremely harsh environments, in which survival of sessile macro-invertebrates is severely challenged by freezing and icefoot in winter, and melt water runoff from thawing ice and snowfields, among other factors, in the summer season. Walker (1972) was the first to distinguish 2 different *N. concinna* shell morphs with different shore level distributions at Signy Island (South Orkneys), a phenomenon that has now been accepted as a common feature of all populations on the shores of the Antarctic Peninsula and the nearby South Shetland and South Orkney archipelagos. The intertidal and shallow subtidal shell morph is found in the upper littoral, including the splash zone, and down to approximately 4 m water depth, and has a high, heavy and dense shell, whereas the deep subtidal morph has lighter and flatter shells and is found at depths >4 m and down to 110 m (Davenport 1988).

These 2 shell and ecomorphs are considered as only 1 limpet species, which reproduces in the early spring in the subtidal (Stanwell-Smith & Clarke 1998). The onset of the spring phytoplankton bloom seems to be a spawning cue (Stanwell-Smith & Clarke 1998), and recruitment of the pelagic larvae occurs in subtidal areas, from where individuals migrate towards the intertidal during later life. Young limpets of <20 mm

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shell length were described as being sensitive to desiccation (Walker 1972) and, therefore, confined to the subtidal (Kim 2001). Of the adult limpets >20 mm, those with heavier shells are described as a migratory ecomorph, moving seasonally between intertidal and subtidal shore levels (upshore in spring, downshore in autumn), whereas the other ecomorph forms a non-migratory and permanently subtidal subpopulation (Davenport 1988, Brêthes et al. 1994). Recently, observations were made around Rothera Station (Adelaide Island) which indicate that intertidal *Nacella concinna* specimens are able to survive the Antarctic winter in rock crevices under the ice (Waller et al. 2006). The authors identified mean super cooling survival temperatures of around −5°C for *N. concinna* and found significantly higher tolerance of intertidal compared to sublittoral limpets to experimental deep freezing.

Nacella concinna is a non-homing limpet of high mobility, which can crawl over extended distances including sandy spaces, reaching a maximum speed of 10 mm s^{-1} at 0°C (Davenport 1997). In line with the enhanced migratory mobility, *N. concinna* is of low tenacity, i.e. low adhesive forces to clamp to the substratum, if compared to temperate limpet species. Davenport (1988) compared tenacity in both shore level subpopulations and found higher adhesive force and closer contraction of the shell to the substratum during tidal emersion in the intertidal ecomorph, although differences did not reach significance. Based on his own ecophysiological investigations and an earlier paper on genetics by Beaumont & Wei (1991), in which 5 polymorphic gene loci showed no genetic differentiation between both *N. concinna* subpopulations, Davenport (2001) concluded that 'there is no evidence of genetic or physiological differences between the 2 limpet morphotypes'.

In the light of the rapid climate warming that is happening in the maritime Antarctic, where air temperature means have risen by 2.5 K over the past 50 yr (Turner et al. 2005), the question arises as to how Antarctic limpets will be able to cope with the rather dramatic environmental change in their intertidal habitat. Increased air temperature is not the only changing factor; however, increased temperature drives glacier melting, leading to intensified meltwater formation, surface freshening and an increase of inshore water temperatures in the upper 10 to 15 m of coastal surface water (Schloss et al. 2008). Moreover, increasing aerial warming will directly affect intertidal fauna during low tides. Warming of the shell will happen more rapidly in air-exposed limpets, and especially in the non-homing species, which cannot tightly contract its shell to the rocky substratum, leaving it prone to desiccation. Rapid environmental change may accelerate specific adaptation in the intertidal limpet subpopulation and thereby may add to a physiological diversification of both *Nacella concinna* subpopulations and may, eventually, cause genetic splitting of the subpopulation. Indeed, in a recent paper, Aranzamendi et al. (2008) used ISSR-PCR (Inter-Simple Sequence Repeats-polymerase chain reaction) markers for analysis of the genetic differentiation between both limpet morphotypes in Potter Cove, King George Island. For 1 out of 3 sites tested in Potter Cove, the authors came to the conclusion that the 2 morphotypes can be considered genetically distinct subpopulations that maintain only low levels of gene flow. The limpets can therefore be regarded as model species for the adaptive response of a stenothermal (narrow thermal tolerance window) and stenosmotic (low tolerance of osmotic variability) subtidal species, which, in colonizing shallow intertidal habitats, is increasingly challenged by ongoing rapid local climate change.

We conducted a multilevel study of the possible adaptive response of both *Nacella concinna* subpopulations to environmental challenge, testing their tolerance to air exposure and hypoxia. Intertidal limpets with their higher shell might better ventilate their inner shell environment than subtidal conspecifics. We hypothesized that this would lead to enhanced tolerance to air exposure. We present data from air exposure experiments with intertidal and subtidal *N. concinna* from King George Island. We investigated the extent of water loss upon prolonged aerial exposure and recorded the pO_2 values maintained in the extrapallial water in the top of the shell in both ecomorphs during sub- and emersion. We further investigated tissue adenylate levels, as well as the accumulation of anaerobic metabolites, and recorded the changes of gill tissue pH over the duration of a 24 h experimental air exposure, to unravel possible differences in the metabolic response between both subpopulations.

MATERIALS AND METHODS

Limpet collection. Intertidal *Nacella concinna* were collected during low tide at Peñon 1, Potter Cove, King George Island, South Shetlands, on 2 occasions in November 2005 and 2007 during 2 subsequent expeditions. Parallel to the intertidal samplings, divers collected subtidal individuals in 13 to 15 m water depth in Potter Cove at about 1 km from the intertidal sampling location. All snails were immediately transferred to the aquarium of Dallmann Laboratory, Jubany Station, and held in aerated, 0°C cold seawater from the cove for between 10 d and 3 wk before air exposure experiments were started. In the aquaria both groups of limpets were maintained

permanently submersed, so that any differences between intertidal and subtidal control groups were not due to acute exposure to different *in situ* conditions.

Morphometric differences between subpopulations. Shell morphometric parameters were recorded for 181 intertidal and 136 subtidal experimental individuals. Length (L), width (W) and height (H) of the shells were measured to the nearest 0.01 mm using digital dial callipers. L was determined as the maximum distance from the anterior to the posterior shell margin, W as the maximum distance between the lateral shell margins, and H as the vertical distance from the ventral margin to the apex of the shell.

From these data, circumference (C) and inner volume (V) were calculated as:

$$C = 2(L^2/8 + W^2/8)^{0.5} \quad (1)$$

and

$$V = LWH/12 \quad (2)$$

according to Lowell (1984). Steepness (S) and roundness (R) were calculated using:

$$S = 2H/(L + W) \quad (3)$$

and

$$R = W/L \quad (4)$$

according to Orton (1932) cf. Wallace (1972).

As statistical analyses, differences between shells of the 2 subpopulations were tested by t-test (GraphPad Prism 4) and an ANCOVA (GraphPad InStat 3) model for comparing shell volume to circumference or shell volume to wet mass relationships in the 2 subpopulations.

Experimental air exposure and determination of water loss. Air exposure experiments for analysis of water loss, anaerobic metabolites and tissue adenylate levels in both subpopulations were carried out in 2005 and 2007 following identical protocols. In 2007, an additional sampling point after 2 h air exposure was included, whereas in 2005, samples were taken after 0, 6, 12, and 24 h of air exposure only. Prior to air exposure, limpets were blotted dry with tissue paper, weighed and placed individually on small pre-weighed plastic dishes over silica drying pearls in a desiccator. For each experimental time group 10 intertidal or 8 subtidal individuals were exposed to air. The desiccator was placed in snow and covered with dark cloth to minimize the impact of light and wind and to keep temperatures at 0°C. Experiments were started in the evening, as the limpets were observed to be more active during the day, when they left the dishes to crawl around in the desiccator.

Whole-animal wet mass was determined again after the incubations. Subsequently, the limpets were killed, and shell and shell-free soft body wet mass (SFWM) were determined separately for calculations of water loss during air exposure in each group. Water loss from the limpets' tissue was calculated as the percentage of shell-free wet mass (% SFWM). Tissues (gills, foot, digestive gland) were snap frozen in liquid nitrogen for biochemical analysis. No individual died during air exposure.

As statistical analyses, effects of subpopulation (subtidal, intertidal), air exposure time (h) and individual body mass (SFWM) on water loss (arcsine-transformed percentage data) were examined by analysis of covariance (ANCOVA, SAS software JMP 5.0.1a). The Box-Cox routine was applied to the data to achieve homogeneity of variance (Sokal & Rohlf 1995). Multivariate outliers in the sample space (water loss, exposure time, body mass) were identified by Mahalanobis jackknife distances (Barnett & Lewis 1994) and excluded from further analysis.

Measurements of oxygenation (pO_2) in shell water. Measurements of the pO_2 in the limpets' shell water were carried out in a thermostated aquarium at 0°C and 34‰ salinity natural seawater, using a fibre optical system by PreSens (Precision Sensing). Optodes were calibrated to 100% air saturation with aerated seawater and to 0% using a saturated solution of ascorbic acid at 0°C. Calibration was confirmed by measuring the O_2 concentration by the Winkler method. Air saturation was recorded in shell water of experimental individuals at 5 min intervals, using TX-3 oxymeters and TX3_v520 software by PreSens. The digital data of oxygen saturation were saved as a text file and copied into Excel for data calculation. Percent data were converted to oxygen partial pressure (kPa) before performing statistical analysis.

To insert the fibre optical oxygen sensor, a hole was drilled into the top of the shell of experimental individuals. The hole was covered with thin elastic latex foil (Rubber Dam, Heraeus Kulzer). This foil was covered with isolation material (Armaflex, Armacell) to avoid exchange with the outside water. To insert the fibre optodes, a hole was punched through the isolation material using an injection needle, and the optodes were implanted through the hole into a small, medium-filled space in the upper shell. Individuals were exposed to cycles of submersion and emersion, starting with submersion for up to 10 h. After 8 to 10 h, the water was drained from the thermostated aquarium and the limpets were exposed to air for 6 to 8 h, followed by a second period of submergence. Only 5 intertidal and 3 subtidal limpets received 2 cycles of air exposure. During air exposure temperature was maintained at 0°C. In total, 8 intertidal (L: 33.5 ± 2.2; C: 91.3 ± 6.1) and 10 subtidal specimens (L: 46.5 ± 5.1; C: 126.1 ± 15.3) were analysed.

Analysis of the pO_2 data. Effects of subpopulation (intertidal vs. subtidal) and exposure state (air vs. water) on pO_2 were analysed using ANCOVA (SAS software JMP 5.0.1a), applying the full interaction model: pO_2 = subpopulation × exposure state × exposure time.

Exposure time was set to zero at each change of exposure state (air–water–air). pO_2 data were Box-Cox-transformed (Sokal & Rohlf 1995) to achieve normality and homogeneity of variances. The Tukey post hoc test ($\alpha = 0.05$) was applied to detect differences between means. Data are shown in the figures as shell water pO_2 frequency distributions during water and air exposure, calculated from the original data using Excel.

Tissue pH (pHi). Tissue pH was determined in both years in gill tissue of limpets from both subpopulations, using the homogenate technique (Pörtner et al. 1990). The system was thermostated to 4°C, the lowest operable temperature, and the pH electrode (SenTix Mic, WTW) was calibrated with precise calibration solutions (AppliChem; pH 6.865-A1259; pH 7.413-A1260). Readings of pH were recorded on a Kipp & Zonen chart recorder. Gill tissue (100 to 200 mg) was ground in liquid nitrogen, and the powder was added to a 0.5 ml Eppendorf cup containing 0.15 ml of medium composed of 160 mM potassium fluoride and 2 mM nitrilotriacetic acid. The cup was closed after layering with air-bubble-free medium, and the tissue was homogenised by ultrasound (Brandson sonifier 450, duty cycle 40%, output control 8) at 0°C and centrifuged at 20 000 × *g* at 4°C for 30 s.

Organic acids modified after Eertman et al. (1996). The foot tissue was ground in liquid nitrogen and homogenised in the 3-fold volume (w:v) of 7% perchloric acid (PCA). After centrifugation (25 min, 25 000 × *g*, 4°C for all 3 centrifugation steps), the pH in the supernatant was adjusted to pH 2–3, with 3 M NaOH. Samples were centrifuged again, and the supernatant was collected and frozen at –20°C to precipitate residual NaCl. The thawed sample was centrifuged again, and the supernatant was injected into the HPLC (high-performance liquid chromatography).

Organic acids were separated on a BioRad Aminex HPX-87H column (300 × 7.8 mm) by isocratic elution with 2.25 mM H_2SO_4, flow rate 0.6 ml min^{-1}, at 60°C column temperature and UV detection at 210 nm. Standards were succinate (S2378; Sigma), D(–)lactate (L1000; Sigma), fumarate (F1506; Sigma), acetate (24,285-3; Aldrich) and propionate (P1880; Sigma).

ATP/ADP/AMP. Adenylate concentrations were measured after Lazzarino et al. (2003) using HPLC. Frozen foot tissue was ground in liquid nitrogen and homogenised with a micropistill in a 1.5 ml reaction vial with ice-cold, nitrogen-saturated precipitation solution (CH_3CN [Acetonitril] + 10 mM KH_2PO_4, at a ratio of 3:1, pH 7.4) at a 1:10 (w:v) tissue to medium ratio. The precipitation solution was prepared weekly, and the pH was checked daily. The homogenate was centrifuged at 20 690 × *g* for 10 min at 4°C, and the clear supernatants were stored on ice. Pellets were supplemented with 1 ml of the precipitation solution and resuspended for several seconds using an ultraturrax, centrifuged again as above, and the supernatants combined. This extract was washed with the double volume of chloroform (10 s vortexed with HPLC grade CH_3Cl) and centrifuged as above. The upper aqueous phase, containing the water-soluble low molecular weight compounds, was collected and washed again twice with chloroform. Supernatants were then stored at –80°C until measurement.

Samples were separated by HPLC using a Kromasil 250 × 4.6 mm, 5 µm particle size column (Eka Chemicals, AB) and its own guard column. The injection volume was 50 µl of undiluted extract. HPLC conditions (solvents, gradient, flow rate, detection) were applied as described in Lazzarino et al. (2003). AMP, ADP and ATP standards were purchased from Sigma. Adenylate concentrations in the samples were calculated using Karat Software 7.0. Energy charge (EC) after Atkinson (1968) (cf. Ataullakhanov & Vitvitsky 2002):

$$EC = [ATP + (ADP/2)]/(ATP + ADP + AMP) \quad (5)$$

The total amount of adenylates was calculated after Ataullakhanov & Vitvitsky (2002):

$$\text{Total adenylate} = ATP + ADP + AMP \quad (6)$$

Citrate synthase activity. The enzyme activity of the mitochondrial marker citrate synthase (CS: EC 4.1.3.7, key enzyme of the citric acid cycle) was measured in foot tissue of intertidal and subtidal control specimens following Sidell et al. (1987). Extraction was carried out as described by Abele et al. (2008).

As statistical analyses, influence of the factors time of air exposure and subpopulation on the biochemical parameters was analysed using multiple regression with the Tukey post hoc test ($\alpha = 0.05$) to detect differences between group means. A Gaussian distribution of the data was tested using the Kolmogorov-Smirnov test (GraphPad InStat 3).

RESULTS

Differences in shell morphology between intertidal and subtidal *Nacella concinna*

Mean length, width, height and weight of intertidal limpets were significantly smaller than those of the subtidal limpets (Table 1). The calculated parameters shell volume, circumference, steepness and roundness

Table 1. *Nacella concinna.* Shell morphometrics of intertidal (n = 181) and subtidal (n = 136) limpets from Potter Cove, King George Island, South Shetlands. All values are means ± SD

	Length (mm)	Width (mm)	Height (mm)	Weight (g)	Volume (mm^3)	Circumference (mm)	Steepness	Roundness
Intertidal	32.1 ± 2.66	23.18 ± 2.03	12.04 ± 1.63	1.58 ± 0.52	2467 ± 691	89.6 ± 7.32	0.43 ± 0.04	0.70 ± 0.02
Subtidal	43.17 ± 6.16	29.85 ± 4.53	12.89 ± 2.99	1.79 ± 0.83	4680 ± 2210	116.6 ± 16.9	0.35 ± 0.05	0.69 ± 0.03
t-test	p < 0.0001	p < 0.0001	p = 0.0013	p = 0.008	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001

also differed significantly between intertidal and subtidal shells (Table 1). Fig. 1A indicates intertidal shells to be steeper than subtidal shells, with a higher volume to circumference relationship in intertidal limpets (ANCOVA, F = 2821.42, p < 0.001, n = 314). Fig. 1B indicates subtidal limpets to have larger inner shell volumes than intertidal specimens of the same wet mass (ANCOVA, F = 68.298, p = 0.0001, n = 72). The amount of shell water was calculated by subtracting shell weight and individual SFWM from the weight of the intact/live individual. Statistical analysis confirmed that subtidal limpets have significantly more shell water (subtidal 2.16 ± 1.4 g of shell water, n = 28; intertidal 0.76 ± 0.8 g, n = 45; Kruskal-Wallis, p < 0.001) than intertidal limpets. However, SFWM was also higher in the subtidal limpets so that the ratio of shell water to SFWM remained the same in both subpopulations.

Water loss in air-exposed *Nacella concinna*: differences between subpopulation and dependence on body mass

Fig. 2 shows the time course of the percent water loss in intertidal and subtidal limpets exposed to air at 0°C recorded in 2007. The percent water loss in intertidal limpets was lower than that in subtidal specimens, but increased significantly over time in air-exposed individuals (ANCOVA, F = 5.96, p = 0.0051, n = 47). On the contrary, in subtidal limpets the percentage of water loss was higher from the beginning of the air exposure on and remained constant over the full 24 h (ANCOVA, F = 2.52, p = 0.10, n = 28).

ANCOVA indicated significant effects of subpopulation (inter- vs. subtidal), exposure time and body mass on water loss (percentage data were arcsine transformed), as well as a significant interaction between the parameters subpopulation and body mass (Table 2). Subsequent separate ANCOVAs for both subpopulations revealed distinct differences of the effect of SFWM in intertidal and subtidal limpets. In the intertidal subpopulation, larger individuals lost more water than smaller snails. On the contrary, in the subtidal subpopulation, the larger individuals appeared less susceptible to desiccation and lost comparably less water than smaller individuals (negative correlation between water loss and body mass are shown in the model in Table 2).

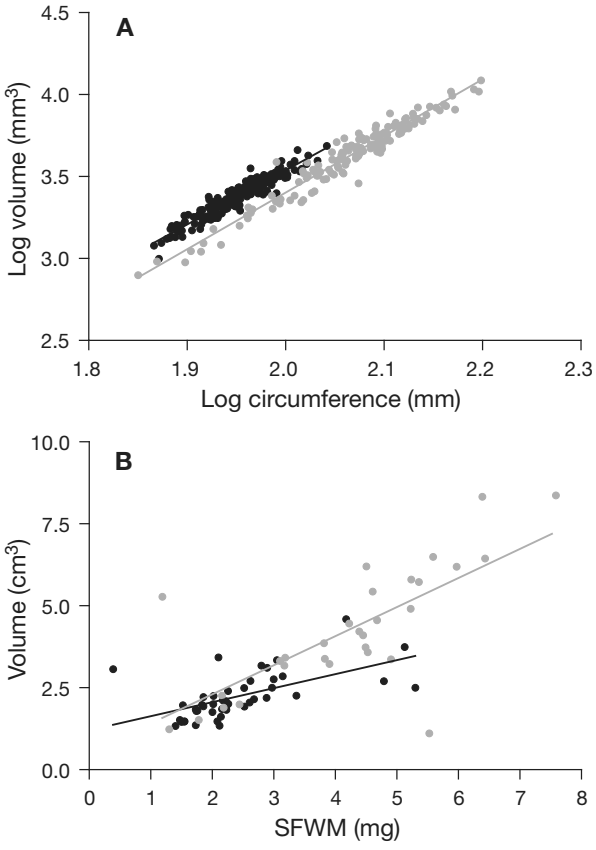

Fig. 1. *Nacella concinna.* Inner shell volume plotted against (A) circumference and (B) shell-free wet mass (SFWM) for the intertidal (●) and subtidal (●) limpets from Potter Cove, King George Island, South Shetlands. (A) Intertidal: log (volume) = 3.3 log (circumference) – 3.139, r^2 = 0.9035, n = 136; subtidal: log (volume) = 3.46 log (circumference) – 3.508, r^2 = 0.9436, n = 136. (B) Intertidal: volume = 0.427 SFWM + 1.206, r^2 = 0.346, p < 0.05, n = 45; subtidal: volume = 0.887 SFWM + 0.527, r^2 = 0.529, p < 0.05, n = 30. Volume–wet mass relationship differed between subpopulations (ANCOVA, p = 0.0125)

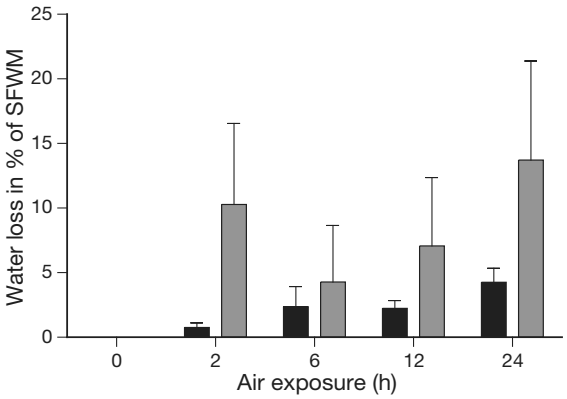

Fig. 2. *Nacella concinna.* Water loss in percent of whole individual shell-free wet mass (SFWM) during air exposure in intertidal (black bars) and subtidal (grey bars) limpets. Mean + SD, n = 6 to 10 limpets per group

Gill tissue pH in intertidal and subtidal limpets during air exposure

Tissue pH was analysed in gills of subtidal and intertidal control specimens in 2005 and 2007. After confirming that pH did not differ significantly between years (ANOVA, $p > 0.05$), values were pooled and analysed for general differences between subpopulations. Intertidal *Nacella concinna* gill pH (7.391 ± 0.064, n = 10) was significantly more acidic ($p = 0.0019$) than subtidal gill pH (7.707 ± 0.188, n = 8) under submersed control conditions.

Table 2. *Nacella concinna.* Full factorial ANCOVA interaction model of effects of subpopulation (subtidal, intertidal), air exposure time (h) and individual shell-free wet mass (SFWM) on water loss (percentage data, arcsine transformed) in limpets from King George Island. Full interaction model: $F = 5.9283$, $p = 0.0004$, n = 75. The effect of body mass was tested in 2 separate models for both subpopulations—intertidal: $F = 5.9587$, $p = 0.0051$, n = 47; subtidal $F = 2.5188$, $p = 0.1008$, n = 28

	Estimate	*t*-ratio	p
Full interaction model:			
Intercept	20.437	8.15	<0.0001
Subpopulation	–2.12	–2.14	0.0362
Exposure time	0.205	2.10	0.0393
SFWM	0.7467	1.25	0.2160
SFWM × Subpopulation	1.585	2.65	0.0099
Exposure time × Subpopulation	0.1381	1.40	0.1673
Intertidal limpets:			
Intercept	13.55193	4.19	0.0001
Exposure time	0.30535	2.75	0.0086
SFWM	2.0017	2.08	0.0435
Subtidal limpets:			
Intercept	24.7129	6.13	<0.0001
Exposure time	0.0407	0.24	0.8152
SFWM	–1.20024	–2.19	0.0378

Fig. 3 shows changes in pHi in the gill tissue from the 2007 air exposure experiment. The gill pHi rose significantly within the first 6 h of air exposure in intertidal limpets and was back to the control level in 12 and 24 h air-exposed snails (ANOVA and Tukey, $p < 0.05$). The pHi in subtidal limpet gills (Fig. 3) decreased slightly during air exposure from 7.7 to 7.5, but the change did not reach significance ($p > 0.05$).

Adjustment of shell water pO_2 in submersed and air-exposed limpets from both shore levels

pO_2 measurements were carried out in 2007 only. Although the experimental snails were submersed in fully oxygenated seawater and exposed to air on emersion, pO_2 in the shell never reached >15 kPa (70% air saturation) in either subpopulation. pO_2 frequency distribution under water was more variable (Fig. 4A) than during air exposure (Fig. 4B) in both subpopulations. Especially intertidal limpets under water had very variable pO_2 in their shell tip, with values ranging between 0 and 13 kPa. In subtidal limpets, 75% of all pO_2 values recorded for 5 min measuring intervals under water were between 0 and 1 kPa, and the highest recorded pO_2 was 12 kPa. During air exposure the overall pO_2 range in both limpet subpopulations was significantly narrowed to between 0 and 3 kPa in intertidal (Fig. 4A) and 0 to 4 kPa in subtidal limpets (Fig. 4B); 90% of intertidal and 85% of subtidal limpet pO_2 data were between 0 and 0.5 kPa, indicating that the inner shell space becomes hypoxic in both groups during air exposure.

The full factorial interaction model with the parameters exposure (air vs. water), shore level (intertidal vs. subtidal) and exposure time indicated that all parameters have significant effects on limpet shell water pO_2. Moreover, cross effects were visible for the interaction between shore level and exposure, exposure and expo-

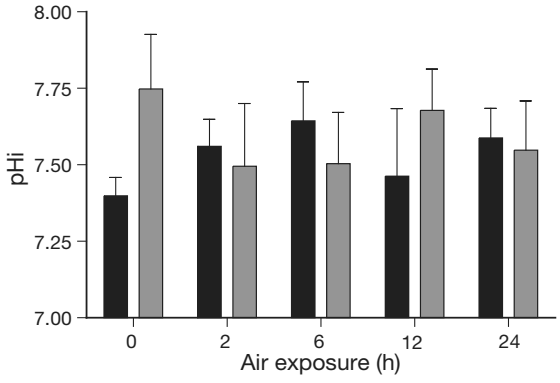

Fig. 3. *Nacella concinna.* Gill tissue pH (pHi) of intertidal (black bars) and subtidal (grey bars) limpets during air exposure in 2007. Mean + SD, n = 5 to 7 limpets per group

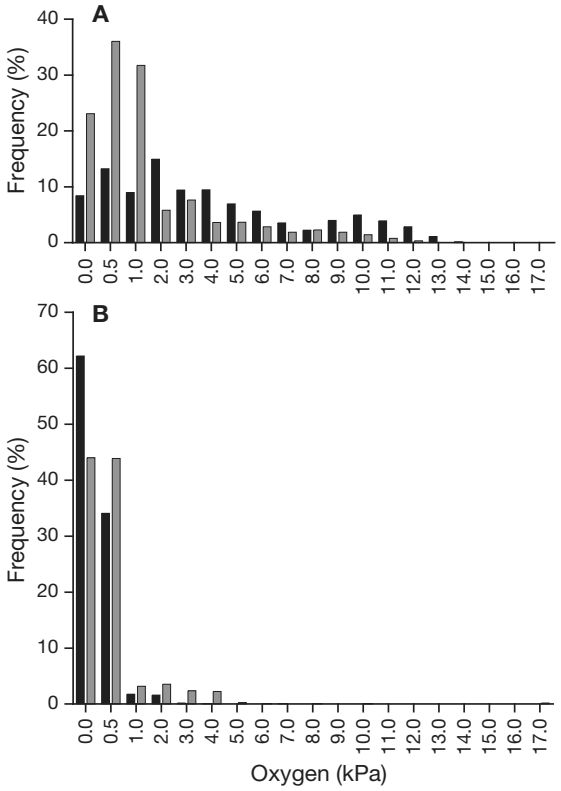

Fig. 4. *Nacella concinna.* Mean pO_2 frequency of 5 min measuring intervals in the shell water of intertidal (black bars, n = 9) and subtidal (grey bars, n = 5) limpets (A) under water and (B) during experimental air exposure

sure time, and shore level and exposure time (Table 3). Hence, inter- and subtidal limpets differ significantly in the adjustment of shell water pO_2 during experimental air exposure and submergence; moreover, the changes in shell water pO_2 differed in both subpopulations over the time of air exposure. The Tukey honestly significant difference (HSD) test performed with Box-Cox-transformed data for the 4 groups of inter- and subtidal limpets, each submerged and during aerial exposure, indicated each group to be significantly different from all others with $p < 0.05$ (Table 4). The alphabetical order of the letters in Table 4 indicates the distance from the value of the 'intertidal water' group. Hence, the statistical analysis of the pO_2 data confirmed the impression from the frequency distribution graphs (Fig. 4): intertidal and subtidal limpets differ significantly with respect to the change of shell water oxygenation during air exposure, as well as when submerged under water. Whereas the pO_2 in intertidal limpet shell water changes significantly upon air exposure, no such clear response could be distinguished in the 'reaction' of subtidal specimens during experimental emersion.

Table 3. *Nacella concinna.* Factor impact estimates obtained from the full factorial interaction model for the effects of the factors subpopulation (intertidal vs. subtidal), exposure (air vs. submerged in water) and time on the pO_2 in the extrapallial water of the shell top of the limpet *N. concinna.* All factors and interactions had significant effects with p (prob > *t*) < 0.001

Term	Estimate
Exposure (air)	−0.198945
Subpopulation (intertidal)	−0.08611
Exposure time	0.0006065
Subpopulation (intertidal) × Exposure (air)	−0.374168
Exposure (air) × Exposure time	0.0005211
Shore level (intertidal) × Exposure time	−0.000175

Table 4. *Nacella concinna.* Differences between shell water pO_2 in intertidal and subtidal limpet under water and during air exposure. Analysis of Box-Cox-transformed data in different groups with Tukey post hoc. Different letters indicate significant differences ($p < 0.05$) between pO_2 levels recorded in different groups during the entire time of air exposure (air) or submergence (water). The alphabetical order of the group indicates the distance of each group from the intertidal water group

Level	pO_2 means (kPa)	Estimate	Grouping with 0
Intertidal, water	2.577	1.4837245	A
Intertidal, air	0.0933	0.3374979	D
Subtidal, water	1.339	0.9076090	C
Subtidal, air	1.482	1.2580552	B

Onset of anaerobic metabolism in intertidal and subtidal limpets during air exposure

Fig. 5 shows changes in organic acids, intermediates of anaerobic metabolism in hypoxia-tolerant marine invertebrates (Tielens et al. 2002) in foot tissue of intertidal and subtidal limpets during up to 24 h of air exposure (data from 2005). ANCOVA detected significant differences in the concentrations of lactate ($F = 10.48$, $p < 0.001$, n = 53), propionate (effect of subpopulation only, $F = 4.25$, $p = 0.014$, n = 53) over time and between subpopulations, with higher concentrations in subtidal than intertidal limpets. After 12 h of air exposure, acetate, lactate and succinate levels were significantly increased over non-air-exposed controls (Tukey HSD) in subtidal specimens. Propionate values increased 5-fold only after 24 h of air exposure in subtidal limpets, but the increase did not reach significance due to the high inter-individual variability. The only anaerobic metabolite that increased to the same extent in air-exposed limpets from both subpopulations was acetate (ANCOVA, $F = 10.12$, $p = 0.001$, n = 55 for all data; subtidal only $p = 0.0014$; Fig. 5A). The acetate concentration seems even higher in intertidal than in

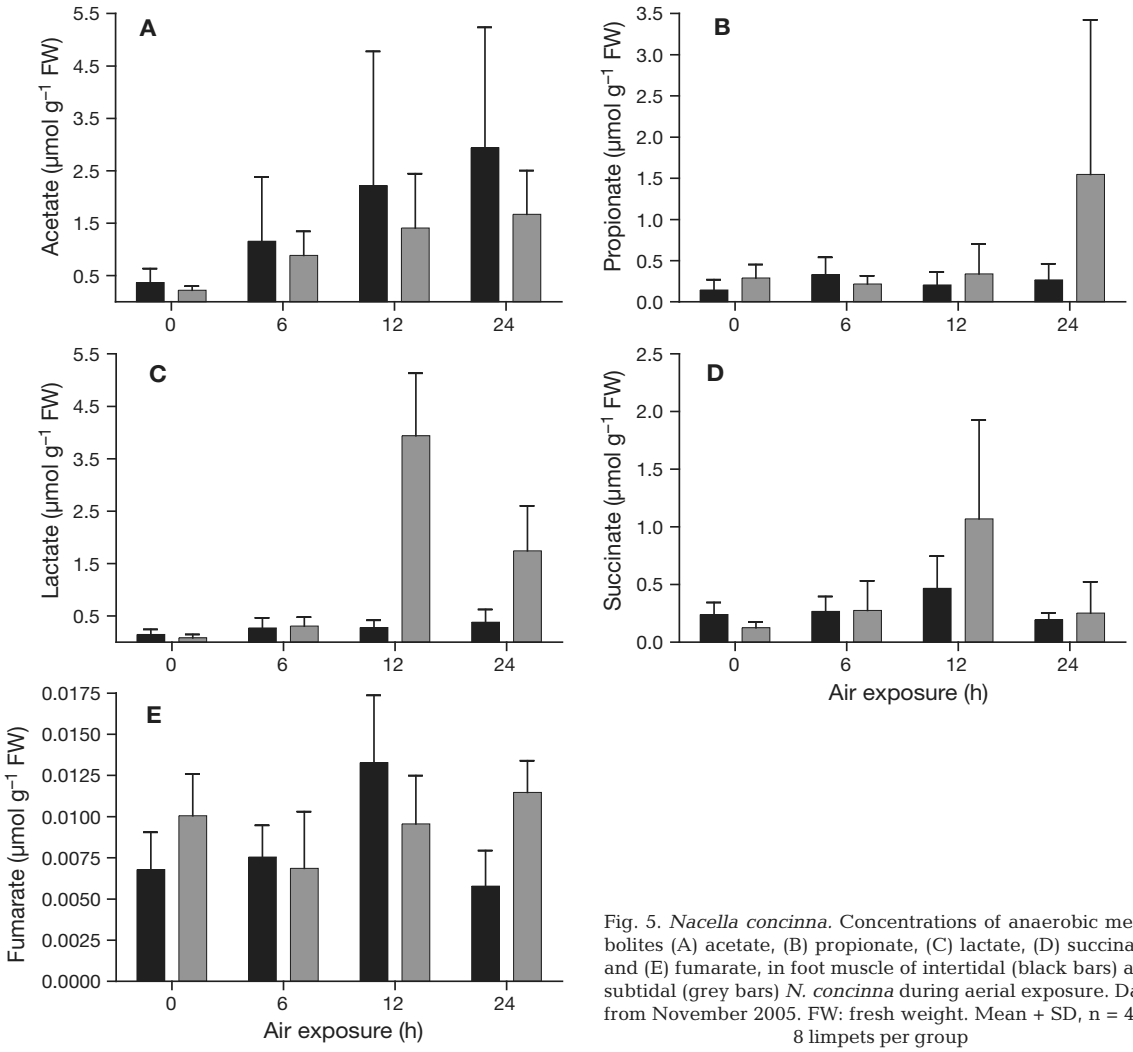

Fig. 5. *Nacella concinna*. Concentrations of anaerobic metabolites (A) acetate, (B) propionate, (C) lactate, (D) succinate, and (E) fumarate, in foot muscle of intertidal (black bars) and subtidal (grey bars) *N. concinna* during aerial exposure. Data from November 2005. FW: fresh weight. Mean + SD, n = 4 to 8 limpets per group

subtidal limpets, but statistically there was no difference between subpopulations (p = 0.8).

Adenylate concentrations and CS activity in intertidal and subtidal limpets during air exposure

Intertidal limpets had higher overall adenylate concentrations (ATP + ADP + AMP) in foot tissue than subtidal snails (F = 25.34, p < 0.0001, n = 60; Table 5), mainly due to higher ATP concentration (F = 28.1, p < 0.0001, n = 60) in control and air-exposed individuals. If only submerged control specimens were considered, the difference between subpopulations was still significant with p < 0.05. The amount of ADP was also higher in intertidal than subtidal limpets, although the difference was less clear (F = 5.23, p = 0.0356, n = 60), whereas AMP concentrations were similar in both subpopulations (F = 0.27, p = 0.602, n = 60). EC increased in both subpopulations over the time course of air exposure (intertidal: F = 4.8, p = 0.046, n = 29; subtidal: F = 8.07, p = 0.008, n = 31; Table 5). Total adenylate concentration increased in both subpopulations during air exposure (ANCOVA, p = 0.0242), with a major increase after 12 h.

CS activity was 1.2 ± 0.16 U g^{-1} wet mass (0.013 ± 0.002 U mg^{-1} protein) in intertidal (n = 7) and 1.4 ± 0.33 U g^{-1} wet mass (0.016 ± 0.003 U mg^{-1} protein) in subtidal (n = 7) control limpet foot muscle, with no significant difference between subpopulations (p = 0.13). Only a small number of snails yielded sufficient foot tissue to determine the individual ATP:CS ratio, which was significantly higher in the intertidal specimens (intertidal: 0.4 ± 0.1 µmol ATP U^{-1} CS, n = 3; subtidal: 0.16 ± 0.06 µmol ATP U^{-1} CS, n = 6; Mann-Whitney test p = 0.024).

Table 5. *Nacella concinna*. Adenylate concentrations in the foot muscle of limpets during air exposure; p-values indicate the influence of factors exposure time and population on foot muscle adenylate concentrations. Data in µmol g^{-1} fresh weight, mean ± SD, n = 6. EC: energy charge

	ATP	ADP	AMP	EC	ATP + ADP + AMP	AMP/ATP
Intertidal						
0 h	0.351 ± 0.12	0.067 ± 0.02	0.016 ± 0.01	0.879 ± 0.05	0.443 ± 0.13	0.038 ± 0.02
2 h	0.405 ± 0.05	0.072 ± 0.02	0.012 ± 0.01	0.9 ± 0.03	0.491 ± 0.04	0.036 ± 0.03
6 h	0.384 ± 0.06	0.076 ± 0.03	0.022 ± 0.01	0.876 ± 0.04	0.482 ± 0.08	0.059 ± 0.03
12 h	0.453 ± 0.10	0.07 ± 0.015	0.01 ± 0.005	0.915 ± 0.015	0.534 ± 0.12	0.022 ± 0.01
24 h	0.44 ± 0.10	0.069 ± 0.03	0.013 ± 0.01	0.909 ± 0.036	0.523 ± 0.12	0.032 ± 0.03
Time	p = 0.321	p = 0.291	p = 0.186	p = 0.046	p = 0.615	p = 0.109
n = 29	*F* = 0.846	*F* = 1.458	*F* = 2.2	*F* = 4.8	*F* = 0.159	*F* = 3.107
Subtidal						
0 h	0.22 ± 0.08	0.052 ± 0.01	0.014 ± 0.007	0.846 ± 0.06	0.286 ± 0.08	0.084 ± 0.076
2 h	0.269 ± 0.048	0.055 ± 0.01	0.015 ± 0.01	0.876 ± 0.04	0.338 ± 0.05	0.060 ± 0.05
6 h	0.245 ± 0.08	0.06 ± 0.016	0.024 ± 0.026	0.838 ± 0.06	0.329 ± 0.11	0.09 ± 0.1
12 h	0.294 ± 0.11	0.066 ± 0.02	0.016 ± 0.01	0.862 ± 0.045	0.376 ± 0.12	0.065 ± 0.04
24 h	0.346 ± 0.096	0.058 ± 0.018	0.011 ± 0.005	0.905 ± 0.02	0.414 ± 0.11	0.030 ± 0.01
Time	p = 0.016	p = 0.997	p = 0.263	p = 0.008	p = 0.052	p = 0.044
n = 31	*F* = 6.603	*F* = 1.14 e^{-5}	*F* = 1.304	*F* = 8.075	*F* = 4.103	*F* = 4.423
Population	p < 0.001	p = 0.026	p = 0.602	p = 0.006	p < 0.0001	p = 0.028
n = 60	*F* = 28.103	*F* = 5.234	*F* = 0.275	*F* = 8.119	*F* = 25.341	*F* = 5.1053

DISCUSSION

In the Antarctic, intertidal limpets *Nacella concinna* are, on the one hand, confronted with extreme environmental hazards, whereas, on the other hand, they can exploit the microalgal biofilm and macroalgal propagules (Zacher et al. 2007) that support high densities of consumers in spring and summer. The average subpopulation density at King George Island is 32 ind. m^{-2}, with maximum densities of >400 ind. m^{-2} (Silva et al. 1999). Split into 2 morphologically distinguishable subpopulations, exploiting food resources on different shore levels, presents an advantage for the productivity of herbivorous grazers; Wolcott (1973) described this strategy as the 'expansionist exploitation theory'. However, the conundrum remains that obviously only one part of the subpopulation can make it to the higher shore and survive there.

Different response to air exposure in subtidal and intertidal Antarctic limpets

The primary aim of our work was to understand the behavioural strategies and biochemical patterns that distinguish migratory intertidal from stationary subtidal limpet morphotypes, their behavioural differences and their metabolic adaptation to life on different shore levels. This paper focuses on the capacity to balance water loss and to regulate the shell water pO_2, 2 features under behavioural control of the individuals (Truchot 1990, see Massabuau 2001 for pO_2 balance). We found some clear-cut differences between the intertidal and subtidal *Nacella concinna* morphotypes, which are summarized for overview in Table 6. First, intertidal limpets have a smaller shell circumference than subtidal limpets of identical shell volume. This minimizes the contact zone over which water can evaporate during air exposure on tidal emersion. Under *in situ* conditions Antarctic limpets avoid prolonged air exposure. Fewer individuals crawl upwards and most limpets stay in moist cracks and crevices or at the bottom of the rocks (authors' pers. obs.). The maximum emersion time of intertidal limpets was between 4 and 6 h. Bigger intertidal specimens were less able to control water loss, possibly due to their longer shell circumference and the higher amount of water in their shell (higher volume to wet mass ratio). The inverse effect of size on water loss in subtidal and intertidal limpets highlights the physiological differentiation between both subpopulations. Higher offset of water loss in all subtidal specimens underlines their generally reduced capability to avoid desiccation once exposed to air and is in line with lower contractive power in general as reported earlier by Davenport (1988), who stated that subtidal individuals contract less tightly to the rocks and are easier removed during collection by divers. Subtidal limpets have more shell water and possibly lose most of it in an early phase of aerial exposure, before contracting their shells and becoming hypoxic in an attempt to avoid further desiccation.

The second behaviourally regulated parameter we analysed was the pO_2 in the extrapallial water in the top of the shell. Capability to regulate tissue and body water oxygenation and pHi in response to changeable environmental conditions in macro-invertebrates is an important prerequisite to stabilize metabolic home-

Table 6. *Nacella concinna*. Summary of morphological differences and parameter changes in subtidal and intertidal limpets during aerial exposure. Arrows indicate direction of change during aerial exposure; →: no change

Trait	Subtidal	Intertidal
Morphology		
Height	Short	Tall
Circumference to volume ratio	High	Low
Volume to wet mass ratio	High	Low
Change during air exposure		
Water loss	High ↑	Low →
Effect of size on water loss	Large sizes less	Large sizes more
Shell water pO_2	↑	↓
Organic acids	↑	↓
Tissue pH	→	↑
Adenylates	High	Less
Adenylates × time	↑	↑

ostasis and survive extreme and rapidly occurring environmental change. The water in the shell top is representative of the level of oxygenation the limpets maintain next to their tissues, often against variable oxygen levels in the outside medium (Massabuau 2003). It is not necessarily a reflection of an individual's oxygen uptake, but rather of its ventilation activity. However, shell water is also the last oxygen reserve under hypoxic conditions, and a decrease in shell water oxygenation indicates that oxygen is consumed by the limpet without ventilatory compensation.

Our measurements of shell water pO_2 indicate more active ventilation of the inner shell space in intertidal than subtidal limpets under water. pO_2 values between 2 and 13 kPa were recorded with much higher frequency than in sublittoral specimens, where pO_2 was mainly between 0 and 1 kPa even in a fully oxygenated environment. Better ventilation of the inner shell volume is presumably indicative of higher oxygen demand of intertidal limpets compared to non-migratory subtidal specimens under water, but so far nobody has investigated the difference. We observed intertidal limpets in our aquaria to be more active crawlers than their subtidal conspecifics; moreover, their higher ATP:CS ratio speaks for more active aerobic metabolism in intertidal limpet foot muscle during routine activity.

During experimental emersion the picture changed completely. Shell ventilation was stopped altogether in intertidal specimens, and >90% of all measurements were <1 kPa. Together, low pO_2 in the shell top, absence of lactate accumulation, as well as stable ATP levels and energy charge in the foot muscle of air-exposed intertidal *Nacella concinna* indicate that the limpets switch to breathing air during low tides. This is a characteristic behaviour for high intertidal molluscs, including several species of patellids from temperate waters (for review see McMahon 1988). These animals perform short uplifting movements (limpets) or openings of the shells (cockles), so-called gaping behaviour, in an attempt to maintain an aerobic metabolism and avoid desiccation over several hours without water (Truchot 1990). A 3-fold increase in the mean acetate concentration observed in foot tissue, caused by elevated acetate levels in 3 out of 6 intertidal *N. concinna* in the 24 h exposure group, is not huge. Brinkhoff et al. (1983) exposed the common limpet *Patella vulgata* to complete anoxia and found a 5-fold increase in acetate levels after 12 and 24 h. However, it indicates that additional input from mitochondrial anaerobic energy production during prolonged air exposure is possible in Antarctic intertidal limpets. Air-exposed subtidal specimens had significantly lower foot ATP content and EC; they were generally less tolerant of air exposure and switched to anaerobic glycolysis, which was visible in an 8-fold increase in tissue lactate levels after 12 h of desiccation stress. Although they clamped shells to the bottom and obviously were not able to gape air during emersion, they lost more shell water then intertidal individuals, presumably due to their lower tenacity and perhaps also due to their higher shell water content.

Exposed to air in the cold: comparing the response in Antarctic limpets and temperate molluscs

Our data represent a first analysis of energy metabolism of limpets collected from the South Shetland Islands, distinguishing inter- and subtidal limpets. The adenylate concentrations we measured are strikingly lower than those reported by Pörtner et al. (1999) for *Nacella concinna* foot muscle from Signey Island of the South Orkneys. The sum of ATP + ADP + AMP (*Ade* in Pörtner et al. 1999; Table 1) was around 5 times higher in South Orkney subtidal limpets, which were collected in summer. This may be due to prolonged maintenance under laboratory conditions (>6 mo) by Pörtner et al. (1999) rather than to a higher temperature regime at the lower latitude sub-Antarctic South Orkney sampling site. In contrast, the anaerobic capacity of the limpets, sampled *in situ* at King George Island, was higher than in the South Orkneys study and more comparable to values for temperate intertidal bivalves and patellides (Brinkhoff et al. 1983, Nicchitta & Ellington 1983).

Nicchitta & Ellington (1983) investigated adenylate energy charge and metabolite levels in intertidal (*Geukensia demissa*) and subtidal (*Modiolus squamosus*) bivalves over 12 h of aerial exposure and 12 h of subsequent recovery. The adenylate EC in both individuals was congruent with the values in the Antarctic

limpets, remained stable throughout air exposure in the intertidal *G. demissa*, and was significantly lower during air exposure of *M. squamosus* (adductor muscle in all 3 individuals). In contrast, *Nacella concinna* EC values and even the ATP concentrations (only of the subtidal individuals) increased significantly in both intertidal and subtidal limpets during air exposure, indicating that Antarctic snails are able to respire air (intertidal) or induce anaerobic fermentation and reduce ATP consumption (subtidal) to maintain EC when emersed. Moreover, this is in keeping with the observations of Brinkhoff et al. (1983) for 6 h *in situ* air exposure of the limpet *Patella vulgata* in Roscoff, Britanny, France. The authors attributed the maintenance of EC and the increase in ATP levels in foot muscle to metabolic rate depression (quiescence) of the limpet and to the possibility of air gaping in the cockle *Cardium* (*Cerastoderma*) *edule*. They found no indication for the onset of anaerobic metabolism in the common limpet *P. vulgata*, which may be due to the shorter exposure time (6 h) and the fact that the limpets were collected from the natural environment, whereas we exposed the Antarctic limpets to desiccation in a longer lasting laboratory experiment. It is interesting to note that also in our study only acetate fermentation was already switched on after 6 h air exposure in *N. concinna*, in keeping with the intertidal bivalves investigated by Brinkhoff et al. (1983), but contrasting to the common limpet *P. vulgata*, where acetate remained below the detection limit. Only after 12 h of air exposure, did lactate, succinate and propionate accumulate exclusively in the subtidal limpets. This is in keeping with the concept of Kluytmans et al. (1977), who found propionate fermentation commencing only after 12 h of hypoxia/anoxia in bivalves. The subtidal specimens were obviously and logically more stressed by our treatment than their intertidal companions, and the initiation of anaerobic energy production, and especially the accumulation of lactate, indicates higher sensitivity to air exposure than in common limpets from Brittany.

Cold temperatures (and the low light regime), as maintained in our experimental set-up, supported maintenance of energetic and tissue homeostasis in the Antarctic limpet. Sokolova & Pörtner (2001) investigated periwinkles *Littorina saxatilis* from the White and North Seas, in high- and low-shore environments during 60 h of aerial exposure in a desiccator (dried air), but additionally warmed the periwinkles to 30°C. This caused rapid loss of body water by up to 50%, and, also in their study, snails from low-shore habitats were much more affected than high-shore periwinkles from the White Sea. The difference in water loss and in most biochemical/energetic parameters between high- and low-shore snails was always more pronounced in White Sea than in North Sea specimens, leading Sokolova & Pörtner (2001) to conclude that habitat conditions, and consequently adaptation to different shore levels, were more pronounced in the White Sea population. Both studies have much in common, including the finding that an increased ability to maintain body water was associated with a reduced accumulation of organic metabolites in the high-shore periwinkles and in intertidal compared to subtidal Antarctic limpets in our study. Thus, body water might also be a temperature buffer and confer some cooling of the heated individual during evaporation. However, there are also important differences: high-shore periwinkles had 20% lower aerobic metabolic rates during aerial exposure than under water and the authors concluded that, in contrast to their low-shore conspecifics, they are metabolically depressed in air. In contrast, the intertidal *Nacella concinna* were more mobile during air exposure than the subtidal specimens and also seemed to be more active when covered by water.

We conclude that there appears to be a pronounced physiological differentiation between inter- and subtidal *Nacella concinna* with respect to physiological capacities and metabolic regulation, both under water and during air exposure. Even if the intertidal Antarctic limpets are physiologically sensitive to freshwater and high temperatures, they have evolved behavioural strategies that help them to survive, remain active under water, maintain body water balance and energetic homeostasis during air exposure presumably by respiring air, and, during all this, clamp their feet to the rocks on emersion in order to, as much as possible, avoid water loss and predation. In contrast, subtidal limpets, when exposed to air, appear metabolically depressed and switch to anaerobic fermentation for survival. This indicates that fundamental physiological diversification is the basis for the ability of one of the King George Island limpet subpopulations to colonize intertidal areas, and this diversification may have a genetic background (see Aranzamendi et al. 2008). If there is a trade-off for this extraordinary physiological adaptation in this stenothermal Antarctic invertebrate, it may consist of slower growth within the migrating intertidal subpopulation.

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Differences in heavy metal concentrations and in the response of the antioxidant system to hypoxia and air exposure in the Antarctic limpet *Nacella concinna*

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Differences in heavy metal concentrations and in the response of the antioxidant system to hypoxia and air exposure in the Antarctic limpet *Nacella concinna*

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ABSTRACT

During the austral spring and summer months, the Antarctic limpet *Nacella concinna* colonizes intertidal environments in the Western Antarctica Peninsula region. The species is divided into a permanently sub-littoral and a seasonally intertidal, migratory subpopulation. We investigate the physiological differentiation between the two limpet groups to identify cellular and molecular changes that accompany adaptation of stenothermal Antarctic invertebrates to life under more stressful intertidal habitat conditions. A major difference between the two groups is the significantly higher concentrations of heavy metals (Fe, Al, Zn) from ingested sediments in sub-littoral limpet digestive glands (DG), associated with higher rates of reactive oxygen species (ROS) formation in this organ. ROS formation is accompanied by significantly higher SOD activity in sub-littoral limpet DG. These high SOD activities are, however, not conserved during either air exposure or hypoxic stress exposure of the sub-littoral limpets, when ROS production is slowed due to the absence of oxygen. The intertidal animals maintain higher levels of SOD and also conserve catalase activity at higher levels during hypoxia or air exposure compared to sub-littoral individuals under the same exposure conditions. More oxidized redox potential in gills and foot muscle and higher antioxidant enzyme activities in gills indicate that intertidal limpets maintain more oxygenated tissues during air exposure, in keeping with shell-lifting for oxygen up-take by the gills of intertidal limpets which migrate up the shore in the spring and down in the autumn. An increase of the redox ratio (GSSG/GSH) and accumulation of the lipid oxidation derived malonedialdehyde in intertidal limpet foot muscle during 12 h of exposure to air shows that indeed this tissue becomes more oxidized before the limpets eventually contract their shells tightly to minimize water loss and eventually become anaerobic. Intertidal limpets obviously avoid early onset of anaerobic energy production seen in their sub-littoral congeners when exposed to air and are still able to maintain tissue redox ratio balance when exposed to air.

1. Introduction

The Antarctic limpet *Nacella concinna*, key species on the shores of the Western Antarctic Peninsula (AP) and nearby South Shetland and South Georgia archipelagos, colonizes intertidal and shallow sub-littoral habitats down to 130 m. A subgroup of the limpet population with distinguishable shell morphology (Nolan, 1991; Walker, 1972) migrates upwards during the Austral spring, presumably to exploit the newly sprouting algal propagules in the intertidal (Zacher et al., 2007). This "intertidal subpopulation" tolerates fluctuations of environmental factors such as temperature and salinity on the high rocky shores, and limpets are therefore regarded as comparatively stress resistant among Antarctic stenotherms (Peck, 2005). In the context of the current massive climatic change happening in the AP region (Vaughan, 2006), species like *N. concinna* are studied as models for the response of Antarctic stenotherms to environmental change, a response which encompasses behavioral, ecological and physiological traits within a single species. We want to clarify, how important the split is into two morphologically distinguishable subpopulations, one remaining sub-littoral and the other one "going intertidal". In addition to the conspicuous differences in shell morphology between the two subpopulations, behavioral traits differ such as the capacity to regulate the PO_2 in shell water (Weihe and Abele, 2008).

Many Antarctic shelf species are extremely eurybathic with wide distribution on different shore levels or seasonal migrations between depth horizons (Brey et al., 1996). Thus, intertidal *N. concinna* were reported to retreat to sub-littoral areas for the winter to mingle with the sub-littoral subpopulation before reproduction

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and recruitment take place in early spring (Stanwell-Smith and Clarke, 1998). Indeed, new observations of scientists overwintering at the British Rothera station on Adelaide Island, West Antarctic Peninsula, report that a few *N. concinna* individuals survive the winter in intertidal rock crevices (Waller et al., 2006), and at King-George Island (KGI) some very small individuals of <12 mm shell length are found in the intertidal during early November (D. Abele and G. Husmann, pers. observation). These observations raise the question whether seasonal migration is indeed a species phenomenon that works similarly at all *N. concinna* residence sites along the AP. It is possible that there may be two differently adapted limpet populations drifting further apart under the impact of the ongoing climatic change. Very subtle evidence has been put forward documenting the existence of two genetically separating groups locally at King-George Island. These investigations of ISSR markers (inter simple sequence repeats) saw differences only during spring and early summer when migration is at a seasonal standstill and intertidal and sub-littoral limpets are most clearly separated (Aranzamendi et al., 2008).

Both subpopulations would be differing not only with respect to their physiological status-quo, especially when sampled in spring and summer on different shore levels, but moreover would presumably differ with respect to their general stress response capacities, resulting from their different environmental adaptation. Limpets colonizing the high intertidal and surf zone are daily exposed to air or caught in confined water bodies of the tide pools in one of the most demanding rocky shore environments in the world. Temperature can fluctuate up to 10 °C during daytime low tides, whereas during the night new ice may form on the rocks. Snow and ice melt waters were shown to be extremely stressful for the limpets, which clamp their shells down to the rock in order to avoid fresh water exposure (Davenport, 2001). Compared to this, the sub-littoral habitats, even under the influence of climate change are comparably stable with temperature fluctuating between −1.8 and +1.5 °C and salinity between 34.1–34.2‰ over the year (O. Gonzalez, Argentine Antarctic Institute, unpubl. data of temperature and salinity in Potter Cove, KGI between September 2007 and December 2008 in 20 m water depth). However, other factors such as increased input of lithogenic particles from underneath melting glaciers and from eroding land surfaces during the austral summer (Ahn et al., 1997; Klöser et al., 1994; Yoo et al., 1999) are bound to primarily affect the sub-littoral limpet fauna in the coastal environment of this volcanic archipelago (Ahn et al., 2002). Plumes of volcanic rock sediments carry high concentrations of heavy metals including Fe, Al, Cu and Zn into the nearshore waters of the South Shetland Archipelago (Abele et al., 2008; Dick et al., 2007), and similar phenomena are described from areas along the maritime AP (Dierssen et al., 2002). Once taken up by sedimentary grazers or benthic filter feeders, these metals can exacerbate oxidative stress in animal tissues. Fe^{2+} is a well known Fenton reactant which, if not tightly bound to ferritin Fe-storage protein, catalyzes the reduction of H_2O_2 to the detrimental hydroxyl radical ($OH^{\cdot}$) and hydroxyl anion (OH^-) (Gonzalez et al., 2008). The resulting Fe^{3+} can be reoxidized mainly by interaction with $O_2^{\cdot-}$ released in small amounts by the mitochondria, or in large quantities during oxidative burst reactions. Aluminum (Al^{3+}) is the most abundant metal in the earth crust and although not a transition metal and Fenton reactant, exacerbates Fe^{2+} catalyzed lipid peroxidation in animal tissues (Quinlan et al., 1988). Likewise, Zn is not in itself a ROS producing metal, but when highly concentrated it can damage the mitochondria and induce higher rates of $O_2^{\cdot-}$ release (Halliwell and Gutteridge, 2007). The pro-oxidant effect of the non-essential element Cd is mainly via depletion of glutathione and protein-bound sulfhydryl groups, resulting in enhanced production of reactive oxygen species (ROS) (Stohs and Bagchi, 1995).

To test for differences in physiological regulatory capabilities, we have conducted air and (aquatic) hypoxia exposure experiments with both limpet subpopulations at King-George Island (KGI, South Shetland Islands). As a first set of results we described how intertidal limpets minimize water loss, how they manage to remain active, maintain energetic homeostasis and avoid tissue hypoxia and accumulation of anaerobic metabolites during exposure to dry air, in comparison to their sub-littoral conspecifics (Weihe and Abele, 2008).

The present paper deals with the possibility of oxidative stress occurring during aerial exposure in both limpet subpopulations at KGI, which could arise when limpets start air gaping, or during hypoxia re-oxygenation. Basically, one would expect antioxidant enzyme activity of superoxide dismutase (SOD) and catalase (CAT) to become reduced during hypoxia or to possibly increase as animals gape air. We also analyzed the reduction status of the redox buffer glutathione and the concentrations of the antioxidant ascorbate over time in two different tissues of submerged controls and air exposed intertidal and sub-littoral specimens. Moreover, experimental hypoxia exposure (2 kPa PO_2) was conducted to demonstrate whether the limpets merely close their shells and become hypoxic, or whether the intertidal subpopulation is capable of aerial respiration. Because oxidative stress is exacerbated by heavy metals possibly ingested during grazing of the sediment surface, we compared Fe, Cd, Zn and Al concentrations in digestive gland and gills of sub-littoral and intertidal specimens.

2. Materials and methods

2.1. Sample collection

Intertidal *N. concinna* were collected during low tide at Peñon 1, Potter Cove, King-George Island on two occasions in November 2005 and November 2007. SCUBA divers collected sub-littoral animals in 13–15 m depth in Potter Cove at about 1 km from the intertidal sampling location. All animals were immediately transferred to the aquarium of Dallmann Laboratory, Jubany Station, and held in aerated, 0 °C cold seawater from the cove for between 10 days and 3 weeks before air and hypoxia exposure experiments were started. Both groups of animals were maintained permanently submersed in flow through aquaria, so that any differences in control parameters were not caused by acute exposure to different *in-situ* conditions. Ascorbate and MDA concentrations and glutathione concentration under hypoxia were measured in samples collected in 2007. Samples from 2005 were used for the antioxidant enzyme measurements and glutathione concentrations in air exposed limpets.

2.2. Experimental set-up and tissue sampling

To test the response to dry air exposure, animals were individually placed on small plastic dishes over silica drying pearls in a desiccator for 2 h (only in 2007), 6, 12 and 24 h. Each time-group consisted of either 10 intertidal or eight sub-littoral animals. The desiccator was placed in snow and covered with dark cloth, to reduce light and wind impact and keep temperatures close to 0 °C. With this experimental routine we obtained reproducible results with respect to water loss and physiological parameters (see also Weihe and Abele, 2008). Contrary to other works (Kensler, 1967), we did not attempt to measure natural survival times or LT50 values of air exposed limpets. Experiments were started in the evening, as the animals were observed to be more active during the day, when they left the dishes to crawl around in the desiccator. No animal died during air exposure.

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For the hypoxia incubations animals were placed in 2 L glass jars for 2, 6, 12, 24 and 48 h. Each time-group consisted of either 15 intertidal or 10 sub-littoral animals. The jars were cooled to 0 °C in a thermostated water bath, and the water in the jars was bubbled with a gas mixture containing 2% oxygen (= 2 kPa). Oxygen concentration was controlled using a fiber optical respirometer (Precision Sensing GmbH, Regensburg, Germany).

Tissue samples for biochemical analyses were snap frozen and stored in liquid N_2 at Jubany–Dallmann. Samples were transported to Bremerhaven, Germany and stored in liquid N_2 until analysis.

2.3. Heavy metal measurement

The Fe and Cd, Zn and Al concentrations were measured in digestive gland and gill tissues of animals collected in 2005. The lyophilised samples were digested in 5 ml subboiled HNO_3 (65%) and 1 ml subboiled HF (40%) for 2 h at 60 °C, 3 h at 100 °C and 5 h at 160° in closed teflon beakers. Afterwards the samples were heated in 5 ml ultrapure H_2O for 5 h at 160 °C with the lid not firmly closed to evaporate acid and water. Subsequently, sample volume was adjusted to 10 ml with 1 M subboiled HNO_3. Fe analysis was carried out by inductively coupled plasma optical emission spectrometry (ICP/OES, IRIS Intrepid Typ Duo, Thermo Nicolet GmbH) at 259.9 nm, Al at 396.1 nm, and Zn at 206.2 nm (Nölte, 2003). Cd was measured in the same samples by GF-AAS (graphite furnace atomic absorption spectrometry) (Zeeman 4100-Perkin-Elmer) (Schlemmer and Radziuk, 1999).

Calibrations were performed for ICP–OES measurements with multi-element solutions (Merck IV) in a range from 100 $\mu g\,L^{-1}$ to 10 $mg\,L^{-1}$. Cd calibration was in a range from 0.1 $\mu g\,L^{-1}$ to 2 $\mu g\,L^{-1}$.

2.4. Measurements of antioxidant enzyme

Superoxide dismutase (SOD) activity was measured in aliquots of 30–180 mg of frozen gill and digestive gland tissue that were ground in liquid nitrogen and homogenised with a micropistill in Tris buffer (20 mM Tris–HCl, 1 mM EDTA, pH 7.6) at 1:4 (gill) and 1:6 (digestive gland) (w/v). Samples were centrifuged for 3 min at 18,000*g* and at 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 according to (Livingstone et al., 1992). One Unit SOD causes a 50% inhibition under the assay conditions. Mitochondrial and cytosolic SOD isoforms were not distinguished. Catalase (CAT) activities were determined in the same extracts. The activity was determined by recording the time of H_2O_2 decomposition, resulting in a decrease of absorbance from 0.45 to 0.4 at 240 nm (1 U) after Aebi (1984).

2.5. Determination of reduced (GSH) and oxidized (GSSG) glutathione by HPLC

The glutathione status represents the most important determinant for the cellular redox environment. The content of GSH and GSSG was determined according to Fariss and Reed (1987). Frozen tissue was ground in liquid nitrogen and homogenised in 1:10 (w/v) pre-cooled PCA (10% containing 2 mM bathophenanthroline-disulfonic acid) bubbled with nitrogen. After centrifugation at 15,000*g* for 5 min at 4 °C, 500 µl of the supernatant were mixed with 10 µl pH-indicator (1 mM m-cresol purple sodium salt containing 0.5 M iodoacetic acid (IAA)). Fifty microliter of 1 mM γ-glutamyl-glutamate (in 0.3% PCA) was added as internal standard. The pH was adjusted to 8.5 with 5 M KOH (containing 0.3 M *N*-morpholine-propanesulfonic acid). The mixture was incubated at room temperature for 45 min, to allow IAA to bind GSH. Subsequently samples were centrifuged for 5 min at 15,000*g* and 4 °C. 300 µl of the supernatant were added to the double amount of 1% 1-fluor-2,4-dinitrobenzene (diluted in 100% ethanol, HPLC-grade) and derivatized in dark vials at room temperature over 24 h. Samples were stored in dark HPLC vials at −20 °C. Prior to measurement, thawed samples were centrifuged at 7500*g* for 1 min at 4 °C and filtered through 0.2 µm nylon membrane filters. HPLC sample separation was carried out on a Beckmann Coulter HPLC System using a NH_2-spherisorp column, 5 µm 240 × 4 mm (Waters, Germany). Solvent A: 80% methanol and solvent B: 20% sodium acetate stock and 80% solvent A. Sodium acetate stock was prepared by dissolving 272 g sodium acetate trihydrate in 122 ml Milli-Q water and 378 ml of concentrated HPLC-grade acetic acid. The gradient program was as follows: 3 min hold at 92% A followed by a 28 min linear gradient to 40% A and 15 min re-equilibration phase. Flow rate was 1.2 $ml\,min^{-1}$ at 2.3–2.8 psi backpressure. Peaks were recorded with a photodiode array detector at 365 nm.

Tissue redox potential (E_{mV}) can be calculated based on Nernst's equation as a function of the total glutathione concentration [GSH], the glutathione redox ratio (GSSG/GSH), and the tissue specific pH (Schafer and Buettner, 2001).

2.6. Tissue pH

We measured the pH in the gills of control and air exposure groups and in the foot tissue of control animals. *N. concinna* foot tissue is buffered by $CaCO_2$ and, therefore, pH-changes cannot be reliably measured in this tissue (Pörtner et al., 1999) for comparison between groups.

Tissue pH was determined using the homogenate technique in a system thermostated at 4 °C, the lowest operable temperature. Prior to measurements the pH electrode (SenTix Mic, WTW, Germany) was calibrated at 4 °C with precise calibration solutions (AppliChem Darmstadt; pH 6.865-A1259; pH 7.413-A1260). Readings of pH were recorded on a Kipp and Zonen chart recorder. For tissue measurements, foot and gill tissue (100–200 mg) was ground in liquid nitrogen and the powder added to a 0.5 ml eppendorf cup containing 0.15 ml of medium composed of 160 mM potassium fluoride, 2 mM nitrilotriacetic acid. The cup was closed after layering with air bubble free medium, and the tissue homogenised by ultrasound (Brandson sonifier 450, duty cycle 40%, output control 8) at 0 °C and centrifuged at 20,000*g* at 4 °C for 30 s. For most samples, the pH values were used to calculate the tissue redox potential. In cases, where sample size was too small, to measure the glutathione concentration and tissue pH, a mean pH of the corresponding experimental group was used to calculate the tissue redox potential.

2.7. Ascorbate and malondialdehyde

Ascorbate and malondialdehyde (MDA) concentrations were measured after Lazzarino et al. (2003) using HPLC. Samples were prepared as described in Weihe and Abele (2008) and separated using a Kromasil column (250 × 4.6 mm, 5 µm, Eka Chemicals, AB, Bohus, Sweden) and its own guard column. Injection volume was 50 µl of undiluted extract. HPLC conditions (solvents, gradient, flow rate, detection) were applied as described in Lazzarino et al. (2003). Calibration was carried out using an ascorbate (Applichem A1052) and a MDA (Merck 805797) standard. Ascorbate was diluted in water and MDA dissolved in 1% sulfuric acid and then diluted with water. Calculations of sample concentrations were done using 32 Karat Software 7.0 (Beckmann Coulter, Krefeld, Germany).

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2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software. Previously data were tested for normality (Gaussian distribution) by Kolmogorov–Smirnov test. Parameter changes within one subpopulation were analyzed by one-way ANOVA (Kruskal–Wallis test for not Gaussian distributed data) and Tukey post-hoc tests for normally distributed data, whereas Dunn's test was used if normality was not given. Two-way ANOVA with Bonferroni post-hoc test was used to analyze differences between the two subpopulations. t-Test was used to compare two single data groups, depending on normality unpaired t-test or Mann–Whitney test.

3. Results

3.1. Differences in heavy metal concentrations and tissue antioxidant levels between intertidal and sub-littoral limpet populations

Heavy metal concentrations in gill (a) and digestive gland (DG, b) of sub-littoral and intertidal limpets are shown in Table 1. Metal concentrations in gills did not differ statistically between subpopulations except iron ($p = 0.0076$). However, all metals were more concentrated in sub-littoral than intertidal DG and, with the exception of Cd, this difference was highly significant. Especially iron and aluminum were much more concentrated in sub-littoral than intertidal limpet digestive glands.

Under control conditions (submerged) both antioxidant enzymes were more active in gills of intertidal specimens. Especially the SOD activity was twice as high compared to gills of sub-littoral limpets (Table 2). By contrast, SOD activity in DG of intertidal limpets was 40% lower than in sub-littoral specimen DG, whereas DG catalase activity was the same in both limpet subpopulations (Table 2). Glutathione concentration, the ratio between oxidized (GSSG) and reduced (GSH) glutathione, and tissue ascorbate (vitamin C) concentrations were the same range in intertidal and sub-littoral limpet gills and also in foot tissue (Table 3). Glutathione levels in DG were not analyzed.

3.2. Effects of hypoxic exposure on tissue antioxidant parameters

Hypoxic exposure of limpets over 2 h and longer caused a significant decrease in intertidal limpet gill SOD activities (Fig. 1a). Sub-littoral animals had low gill SOD activity in the 0 h hypoxia group (= submerged and normoxic), which increased significantly until 12 h of hypoxia, but returned to control levels after 24 and 48 h of hypoxia. Gill catalase activity remained stable over the entire time of hypoxia exposure in both groups (Fig. 1b). Values were always slightly higher in intertidal animal gills, a difference which became significant only in the 24 h-group. Gill glutathione concentrations (GSH + 2GSSG) were not influenced by hypoxic exposure in either of the two subpopulations, and furthermore no difference was observed between intertidal and sub-littoral gill glutathione content during hypoxia (data not shown). Gill pH values (data not shown) were very variable in both subpopulations throughout the hypoxic exposure. In neither subpopulation did gill tissues become clearly acidified, as we had, indeed, expected. Tissue pH and glutathione values were used to calculate changes of gill redox potential according to Nernst's equation and results are shown in Fig. 1c. Gill redox potential was significantly more oxidized (−333 ± 6.97 mV) in the intertidal than the sub-littoral (−359 ± 4.03 mV) control group (0 h) and was rapidly reduced during hypoxia with significant effects after 2 and 12 h at 2% oxygen. Gill tissues of sub-littoral limpets were generally more reduced throughout the hypoxic exposure without significant change over time.

Table 1
Metal content [μg × g^{-1} dwt] in gill and digestive gland tissue of the Antarctic limpet *Nacella concinna*. Data as means ± SD, $n = 4$–6. Unpaired *t*-test (Mann–Whitney if not normally distributed).

	Intertidal	Sub-littoral	*p*-Value
(A) Gill			
Fe	335.2 ± 59.78	731.7 ± 189.9	0.0079
Cd	6.2 ± 2.7	3.7 ± 0.6	0.2000
Al	96.54 ± 37.65	79.46 ± 65.30	0.5476
Zn	43.04 ± 14.69	44.70 ± 5.3	0.6905
(B) Digestive gland			
Fe	271.5 ± 65.99	2669 ± 1595	0.0159
Cd	25.3 ± 7.0	30.7 ± 17.0	1.0000
Al	162.2 ± 85.53	2365.14 ± 2188	0.0159
Zn	74.95 ± 7.76	119.9 ± 14.25	0.0286

Table 2
Superoxide dismutase (SOD) and Catalase (CAT) activities [U × mg^{-1} fwt] in gill and digestive gland tissue of the Antarctic limpet *Nacella concinna*. Values were normally distributed and significant differences tested by unpaired *t*-test. $n = 7$–10, means ± SD.

	Intertidal	Sub-littoral	*p*-Value
SOD (gill)	1.018 ± 0.1	0.554 ± 0.1	0.0001
CAT (gill)	0.526 ± 0.15	0.348 ± 0.16	0.0190
SOD (DG)	2.228 ± 0.69	3.623 ± 1.21	0.0110
CAT (DG)	9.541 ± 2.89	9.693 ± 1.95	>0.05

Table 3
Glutathione [μmol × g^{-1} fwt] and ascorbate was measured in animals from 2007.

	Intertidal	Sub-littoral	*p*-Value
(A) Gill			
GSH	345.5 ± 61.04	390.2 ± 56.29	>0.05
GSSG	61.57 ± 51.36	64.42 ± 45.47	>0.05
GSSG/GSH	0.141 ± 0.06	0.136 ± 0.07	>0.05
(B) Foot			
GSH	450.8 ± 61.58	480.8 ± 144.4	>0.05
GSSG	34.7 ± 16.9	47.89 ± 26.22	>0.05
GSSG/GSH	0.068 ± 0.023	0.078 ± 0.023	>0.05
Ascorbate	0.10 ± 0.06	0.08 ± 0.01	>0.05

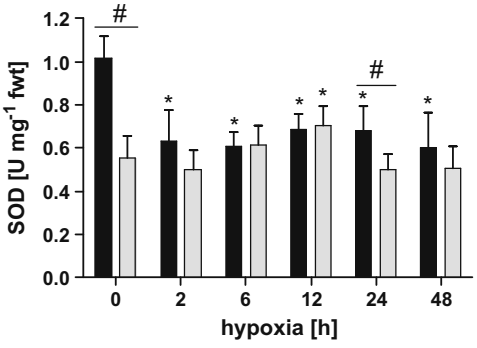

Fig. 1a. SOD activity in *gills* of *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Means + SD, $n = 5$–10. * Significant difference from 0 h-value; one-way ANOVA. Intertidal $p < 0.0001$; $F = 12.55$; $n = 35$; subtidal $p = 0.0014$; $F = 5.102$; $n = 40$. Subpopulations ($p < 0.0001$; $F = 33.26$) time of hypoxia incubation ($p < 0.0001$; $F = 10.70$) interaction ($p < 0.0001$; $F = 10.97$). # Significant differences between subpopulations: significance level $p < 0.001$ between controls and $p < 0.05$ between 24-values.

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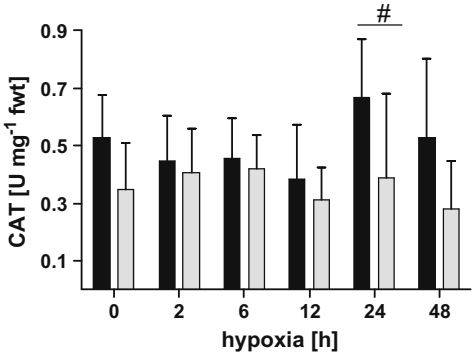

Fig. 1b. CAT activity in *gills* of *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Means + SD, $n = 5–11$. Subpopulation $p = 0.001$; $F = 11.76$, $n = 81$ two-way ANOVA. # Significant difference between the subpopulations (two-way ANOVA, Bonferroni, $p < 0.05$).

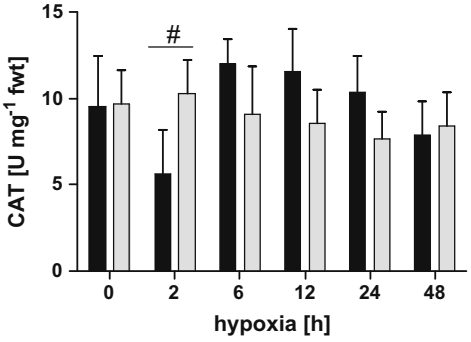

Fig. 2b. Catalase activity in *digestive glands* of intertidal (black) and subtidal (grey) *Nacella concinna* during hypoxia incubation for several hours. Means + SD. $n = 5–10$. Intertidal $p = 0.0011$; $F = 5.363$ (one-way ANOVA). Hypoxia $p = 0.0376$; $F = 2.529$; interaction $p = 0.0005$; $F = 5.171$ two-way ANOVA. # Indicates significantly different values $p < 0.01$ Bonferroni.

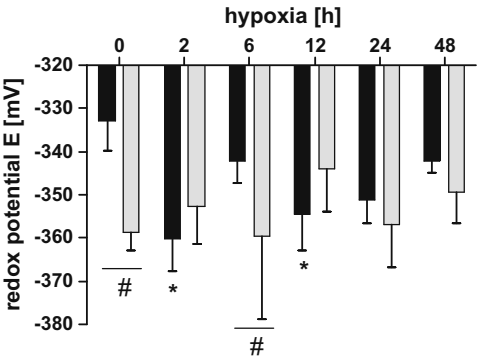

Fig. 1c. *Gill tissue* redox potential of *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Means + SD, $n = 3–7$. * Significantly more reduced than controls ($p < 0.05$ one-way ANOVA). # Significant differences between subpopulations ($p < 0.05$ two-way ANOVA). Interaction: $p = 0.0004$; $F = 5.576$; population: $p = 0.0125$; 6.775. $n = 57$.

Hypoxic exposure caused significant decrease in digestive gland SOD activities of both subpopulations to significantly lower levels after 2 h (sub-littoral) and 6 h (intertidal) (one-way ANOVA, intertidal: $p = 0.0002$; $F = 6.076$, $n = 37$; sub-littoral: $p < 0.0001$, $F = 13{,}87$, $n = 36$, Fig. 2a). Hypoxia effected CAT activity only in intertidal limpet DG ($p = 0.0011$; $F = 5363$; one-way ANOVA) (Fig. 2b). CAT activity in sub-littoral DGs remained unchanged during hypoxic exposure, which led to a highly significant interaction term of hypoxia and subpopulation ($p = 0.0005$; $F = 5.171$).

Hypoxia exposure of limpets over 48 h caused no change in total glutathione (GSH + 2GSSG, data not shown) or ascorbate concentrations in limpet foot muscle. Ascorbate values in intertidal and sub-littoral limpets (only intertidal shown in Fig. 3) were very variable and, if anything, declined over the 2 days at 2 kPa PO_2, but the trend was not significant. The glutathione redox ratio GSSG/GSH was more variable and significantly influenced by hypoxia in sub-littoral ($p = 0.0383$; KW = 11.75; one-way ANOVA) than intertidal foot muscle (Fig. 4) with no significant change over time of hypoxia exposure. Both subpopulations differed significantly concerning their redox ratio (subpopulation $p = 0.0263$; $F = 5.32$; interaction $p = 0.0055$; $F = 3.918$; $n = 52$; two-way ANOVA). We did not measure antioxidant enzyme levels in limpet foot muscles. Concentrations of malondialdehyde (MDA) were a little higher in intertidal (0.01 ± 0.07 μmol g^{-1} fwt) than sub-littoral animal foot muscles (0.007 ± 0.003 μmol g^{-1} fwt), but the difference was not statistically significant (Mann–Whitney test, $p = 0.081$, $U = 142$, $n = 39$).

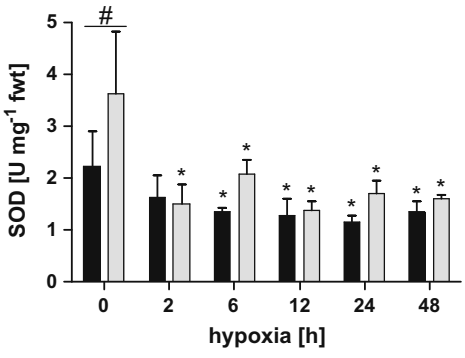

Fig. 2a. Superoxide dismutase activity in *digestive glands* of intertidal (black) and subtidal (grey) *Nacella concinna* during hypoxia incubation for several hours. Means + SD. $n = 5–9$. * Indicates significantly lower activities than in the controls (one-way ANOVA). Intertidal $p = 0.0002$; $F = 6.976$; $n = 37$; subtidal $p < 0.0001$; $F = 13.87$; $n = 36$ (one-way ANOVA). # Significant difference between the subpopulations (two-way ANOVA, Bonferroni, $p < 0.001$). Time $p < 0.0001$; $F = 20.25$. Subpopulations $p = 0.0001$; $F = 16.58$. Interaction $p = 0.0031$; $F = 4.045$; $n = 73$; two-way ANOVA.

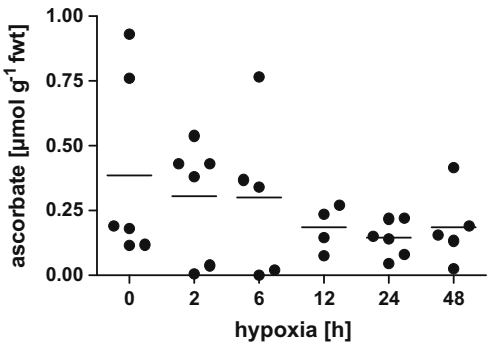

Fig. 3. Ascorbate concentration in *foot muscle* of intertidal *N. concinna* exposed to 2%-oxygen.

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3.3. Effects of air exposure on tissue antioxidant parameters

High SOD values in gills of the intertidal control animals were significantly reduced during 6 h air exposure and equalled the values in sub-littoral 6 h-group ($p > 0.05$, Fig. 5a). Between 6 and 12 h of air exposure, gill SOD activity increased in both groups (significant only in sub-littoral $p < 0.05$). In 24 h air exposed limpets, gill SOD values were all back to low levels, resembling the controls in the sub-littoral group, significantly lower with respect to the high 12 h-value, and also much lower than the control values of the intertidal limpets ($p < 0.001$, Tukey HSD). Thus, gill SOD activities decline in intertidal and, following a short phase of mild induction, also in sub-littoral limpets during air exposure. After 12 h CAT activity became significantly lower in gills of sub-littoral limpets than intertidal specimens, which kept CAT activity constant (two-way ANOVA, subpopulation $p < 0.0001$, $F = 23.87$, $n = 54$, Fig. 5b).

Alterations within the concentration of glutathione (GSH + 2GSSG, Fig. 6a) in limpet gills during air exposure were significantly different between subpopulations in the overall two-way ANOVA ($p = 0.0078$; $F = 7.838$; $n = 46$). Air exposure had a significant effect on total glutathione concentration ($p = 0.0103$; $F = 4.289$, two-way ANOVA). Pair wise comparisons did not yield significant differences between both subpopulations at any time period. Gill glutathione in intertidal animals remained stable and even increased slightly throughout the exposure. In contrast, sub-littoral animal gill glutathione declined from 511.9 ± 86.02 nmol g^{-1} fwt ($n = 8$) in controls to 335.2 ± 118.2 nmol g^{-1} fwt during the first 6 h of air exposure, but the difference remained insignificant because of the low number of samples (6 h-value, $n = 3$). Subsequently glutathione concentration increased also in the sub-littoral limpet gills (significant between 6 and 24 h) but sub-littoral values remained below the values in the intertidal specimens.

Tissue pH was measured in gills and foot tissue of sub-littoral and intertidal control animals in 2005 and 2007. After confirming that pH did not differ between control animals in both years, the values were pooled for each tissue and analysed for the general differences between subpopulations. In submersed control groups, gill tissue of intertidal *N. concinna* had a significantly lower pH (7.391 ± 0.064, $n = 10$) than sub-littoral limpet gills

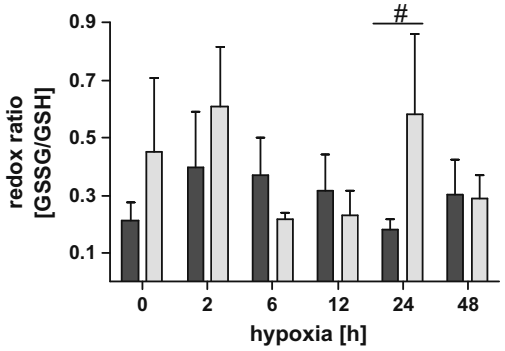

Fig. 4. Ratio of oxidized (GSSG) and reduced (GSH) glutathione (GSSG/GSH) in *foot tissue* of the Antarctic limpet *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Subtidal $p = 0.0383$; KW = 11.75; $n = 25$ (one-way ANOVA). Means + SD. $n = 3–5$. Time $p = 0.0414$; $F = 2.573$; subpopulation $p = 0.0263$; $F = 5.32$ # Indicates significant differences $p < 0.01$ (Bonferroni); interaction $p = 0.0055$; $F = 3.918$. $n = 52$; two-way ANOVA.

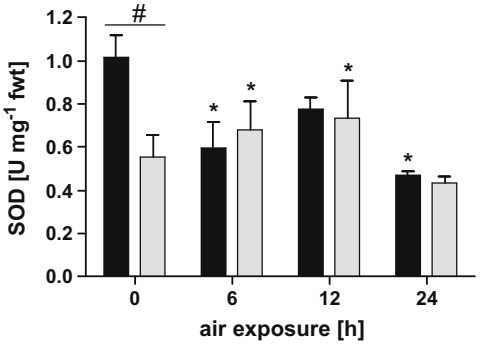

Fig. 5a. Superoxide dismutase activity in *gills* of intertidal (black) and subtidal (grey) Antarctic limpets *Nacella concinna* during several hours of air exposure. Mean values + SD. $n = 6–10$. * Significantly different SOD activity to control animals. (ANOVA, Tukey if normally distributed and Dunn's Multiple Comparison Test if not, $p < 0.05$) intertidal $p = 0.0001$; KW = 20.87; subtidal $p = 0.0059$; KW = 12.5. # Significant difference between the subpopulations (two-way ANOVA, Bonferroni, $p < 0.001$) Subpopulation $p = 0.0002$; $F = 15.97$; interaction $p < 0.0001$; $F = 20.79$; time $p < 0.0001$; $F = 27.9$; $n = 54$ (two-way ANOVA).

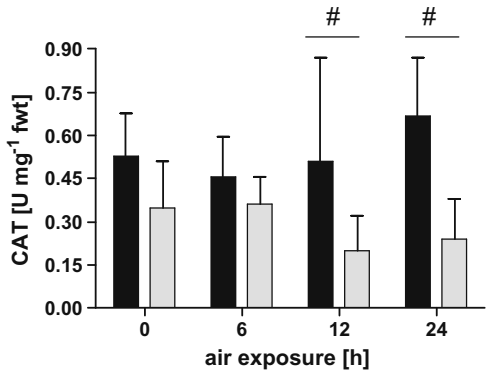

Fig. 5b. Catalase activity in *gill* of the Antarctic limpet *Nacella concinna* from the intertidal (black) and the subtidal (grey) during several hours of air exposure. Mean + SD. $n = 3–10$. # Significant difference between the subpopulations (two-way ANOVA, Bonferroni, $p < 0.05$). Subpopulation $p < 0.0001$; $F = 23.87$; $n = 54$ (two-way ANOVA).

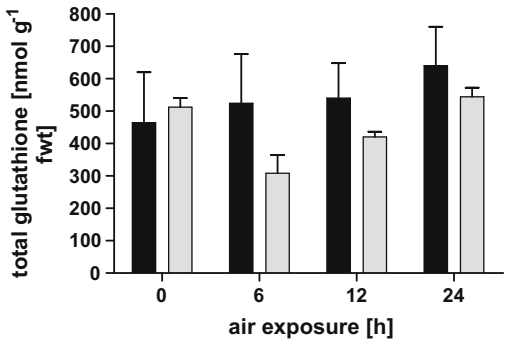

Fig. 6a. Glutathione concentration (GSH + 2GSSG) in *gills* of intertidal (black) and (grey) sub-littoral *Nacella concinna* during air exposure. Subtidal $p = 0.0099$; KW = 11.36. Subpopulation $p = 0.0078$; $F = 7.838$; time $p = 0.0103$; $F = 4.289$; $n = 46$. Means + SD. $n = 3–8$.

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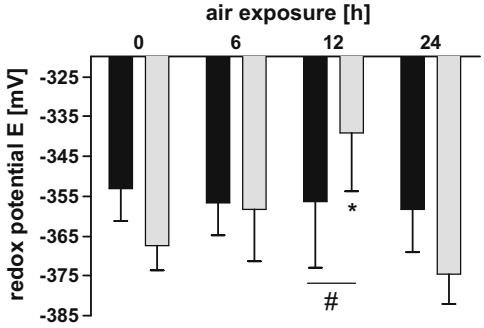

Fig. 6b. Redox potential in *gills* of intertidal (black) and (grey) subtidal *Nacella concinna* during air exposure. ∗ Significantly lower than in control group ($p < 0.05$, ANOVA, Dunn's Multiple Comparison Test). # Significant difference between subpopulations (two-way ANOVA, Bonferroni, $p < 0.05$) interaction $p = 0.001$; $F = 6.524$; time $p = 0.0013$; $F = 6.275$; $n = 50$. Means + SD. $n = 3–8$.

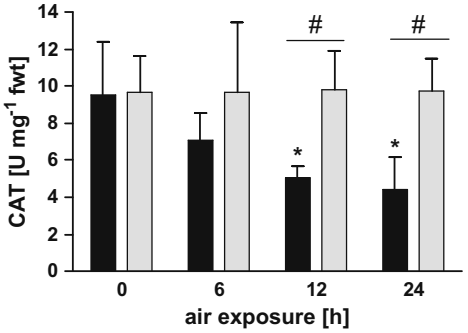

Fig. 7b. Catalase activity in *digestive gland* of the Antarctic limpet *Nacella concinna* from the intertidal (black) and the subtidal (grey) during several hours of air exposure. Mean + SD. $n = 3–10$. ∗ Significantly lower CAT activity than in control animals (ANOVA, Dunn's Multiple Comparison Test, $p < 0.05$) intertidal $p = 0.034$; $F = 13.65$, $n = 23$. # Significant difference between the subpopulations. Subpopulation $p < 0.0001$; $F = 19.91$; interaction $p = 0.024$; $F = 3.491$, time $p = 0.0295$; $F = 3.306$; $n = 49$ (two-way ANOVA, Bonferroni, $p < 0.05$).

(7.707 ± 0.188, $n = 8$, t-test $p = 0.0019$). Changes of gill pH over time differed significantly between intertidal and sub-littoral limpets when exposed to air (interaction $p = 0.001$; $F = 7.236$; aerial exposure (time) $p < 0.0001$; $F = 10.53$; $n = 16–19$). This was mainly due to a dramatic decrease from pH 7.7 to 7.1 in sub-littoral limpet gills between controls and the 12 h air exposure group ($p < 0.05$; ANOVA; Dunn's Multiple Comparison Test, *data not shown*). Fig 6b depicts the resulting redox potential calculated according to Nernst's equation. The low number of samples in some groups is due to limitation of gill tissue for both analyses. Low pH and higher glutathione redox ratio (GSSG/GSH) in gills of intertidal specimens resulted in a redox potential of −350 mV in control animals, which was maintained throughout the time of air exposure (ANOVA, $p = 0.8668$; $F = 0.241$). Sub-littoral limpet gills were initially (0 h) more reduced than intertidal gills at 0 h and became transiently oxidized after 12 h (one-way ANOVA, $p < 0.05$), before going back to reduced control values at 24 h of air exposure.

High SOD activities in digestive gland of sub-littoral control limpets dropped significantly within 6 h of experimental air exposure (Fig. 7a) and continued on low levels. No significant change of SOD activity occurred in DG of either subpopulation between 6 and 24 h of air exposure. DG CAT activity (Fig. 7b) decreased significantly in the intertidal subpopulation during air exposure and remained constant in sub-littoral animals (subpopulation $p < 0.0001$, $F = 19.91$, $n = 49$; interaction $p = 0.024$; $F = 3.491$; two-way ANOVA).

The effect of air exposure time and tidal level (= subpopulation) on glutathione concentrations in the foot were significant (time: $p = 0.0006$; $F = 7.155$; tidal level: $p = 0.0021$; $F = 10.92$; $n = 46$) with an insignificant interaction between factors time and tidal level (interaction term: $p = 0.2461$; $F = 1.441$), indicating similar changes of glutathione concentration to occur in the foot muscle in both subpopulations during aerial exposure. After 24 h, sub-littoral foot tissue had significantly more glutathione than the intertidal 24 h-group ($p < 0.001$, ANOVA, Bonferroni PostHoc test). Fluctuation of the redox ratio (GSSG/GSH) differed significantly between intertidal and sub-littoral foot tissues during air exposure. After 12 h the foot tissue of intertidal limpets had accumulated proportionally more GSSG per GSH and the redox balance was twice as oxidized (0.1252 ± 0.07) compared to sub-littoral limpet foot muscles (0.0602 ± 0.02) ($p < 0.05$; population $p = 0.0446$; $F = 4.36$; interaction $p = 0.035$; $F = 3.225$; $n = 17$ sub-littoral, $n = 18$ intertidal, two-way ANOVA, Bonferroni) (see Figs 8a and 8b).

The ascorbate concentration in limpet foot muscle did not change during air exposure (two-way ANOVA $p > 0.05$) and did

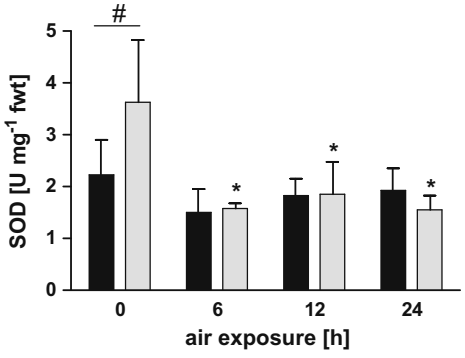

Fig. 7a. Superoxide dismutase activity in *digestive gland* of intertidal (black) and subtidal (grey) Antarctic limpets *Nacella concinna* during several hours of air exposure. Mean values + SD. $n = 6–10$. ∗ Significantly different SOD activity to control animals. (ANOVA, Tukey if normally distributed and Dunn's Multiple Comparison Test if not, $p < 0.05$) subtidal $p < 0.0001$; $F = 11.51$; $n = 24$. # Significant difference between the subpopulations (two-way ANOVA, Bonferroni, $p < 0.001$) interaction $p = 0.0036$; $F = 5.235$; time $p < 0.0001$; $F = 13.58$; $n = 51$ (two-way ANOVA).

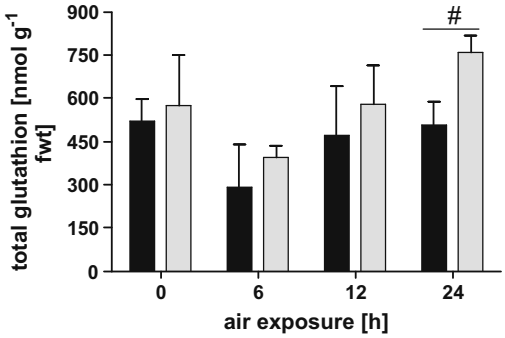

Fig. 8a. Total glutathione concentration in *foot muscle* of intertidal (black) and (grey) subtidal *Nacella concinna* during air exposure. # Significant difference between the subpopulations (two-way ANOVA, Bonferroni, $p < 0.05$). Subpopulation $p = 0.0021$; $F = 10.92$; time $p = 0.0006$; $F = 7.155$; $n = 45$. Means + SD. $n = 3–8$.

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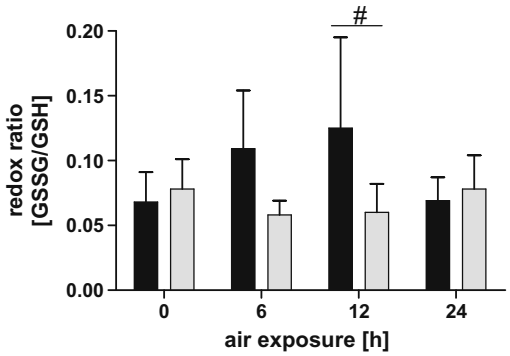

Fig. 8b. GSSG/GSH ratio in *foot muscle* of intertidal (black) and (grey) subtidal *Nacella concinna* during air exposure. Means + SD. $n = 3$–8. # Indicates significant differences $p < 0.05$ (Bonferroni). Subpopulation $p = 0.0446$; $F = 3.225$; interaction $p = 0.035$; $F = 4.36$; $n = 41$ (two-way ANOVA).

not differ between subpopulations (intertidal 0.104 ± 0.09 μmol ascorbate g^{-1} fwt; $n = 27$; sub-littoral 0.093 ± 0.06 μmol ascorbate g^{-1} fwt; $n = 27$; unpaired t-test $p = 0.5949$). Malondialdehyde (mean ± SD) was more concentrated in foot muscle of intertidal limpets (0.00869 ± 0.002 μmol g^{-1} fwt, $n = 29$) than of their sub-littoral congeners (0.00679 ± 0.003 μmol g^{-1} fwt, $n = 29$; $p = 0.0066$; $F = 8.066$, two-way ANOVA, $n = 58$ samples, DF = 1).

4. Discussion

4.1. Heavy metal concentrations and oxidative stress in N. concinna

Absorbed metals in *N. concinna* are at least transiently stored and concentrated in the digestive gland (Ahn et al., 2002, 2004; Moreno et al., 1997), and sub-littoral limpets had 10-times as much Fe and 15-times as much Al in digestive gland compared to the intertidal specimens. This is due to surface grazing of near-shore sub-littoral sediments and ingestion of freshly deposited particulate matter, including sediment particles highly charged with terrigenous trace elements. In contrast, intertidal limpets graze on rock surfaces and presumably ingest proportionally less inorganic matter. An overload of the three analyzed elements (and presumably other metals that we did not analyze) is bound to accelerate oxidative stress and lipid peroxidation in DG, and is antagonized by 40% more SOD background activity in sub-littoral limpet DG. As additional protection, high CAT activities in DG tissue of both subpopulations control the concentration of H_2O_2 and, in so doing, presumably minimize activation of radical chain reactions and Fenton catalyzed hydroxyl radical ($OH^{\cdot}$) production. Contrary to the elements Fe, Al and Zn contained in particulate lithogenic run-off, Cd is mostly dissolved in water and sediment pore water and evenly distributed throughout KGI coves (Ahn et al., 2004), resulting in similar concentrations in sub-littoral and intertidal limpets (gills and DG). Dissolved metals can be absorbed from the water column directly into molluscan gills (P. Gonzalez unpublished experimental data with dissolved Fe) and our present data document this form of uptake also for Fe and Al, the two most abundant metals in the KGI environment. However, primary enrichment of metals in sub-littoral limpets clearly occurs through deposit feeding. Migration of limpets to the intertidal at the beginning of the melting season in spring may thus help to reduce the risks related to heavy metal enrichment. However, the different heavy metal loads in both subpopulations persisted also after more than 2 weeks of maintenance in the same holding system without sediment, and consequently de-enrichment will take much longer, if at all it happens during summer.

4.2. Response of the antioxidant system of intertidal and sub-littoral N. concinna to hypoxia and aerial exposure

Intertidal limpets are more mobile and active with respect to shell water ventilation when submerged (Weihe and Abele, 2008). Oxygen consumption is possibly higher and additional oxidative stress arises from the changing oxygen availability during tidal cycles. Especially during low tide aerial exposure, gills are a main target for oxidative injury (Malanga et al., 2005), and consequently gills of intertidal limpets must be better protected from oxidative stress by antioxidant enzymes. The oxidative stress during low tides arises either from breathing (gaping) air in intertidal *N. concinna*, or from hypoxia during shell contraction and subsequent re-oxygenation. It is typical that the antioxidant response under such variable conditions is catalyzed through the enzymes SOD and CAT for a rapid, ad-hoc response to change, whereas the glutathione status, a basal buffer of tissue redox potential does not differ between intertidal and sub-littoral limpets.

Response to aerial exposure and hypoxia in the oxidative stress parameters differed in intertidal *N. concinna*, whereas the antioxidant enzyme activities in sub-littoral specimens responded in the same way to both forms of stress (Table 4). This fits with our observation that intertidal limpets respond more rigorously to air exposure than sub-littoral limpets, which maintained shell water PO_2 at low levels independently of submergence state. Whereas sub-littoral limpets responded equally to air exposure and hypoxia, by inducing anaerobic metabolism, intertidal limpets differentiate between air exposure, during which they remain aerobic and hypoxia during which anaerobic metabolites accumulate (Weihe and Abele, 2008; Weihe, unpublished data). The most obvious difference with respect to the antioxidants occurs in the gills. Intertidal limpets reduce (SOD) activities, whereas in sub-littoral limpets gill SOD activity increases under both forms of stress.

Our data support a concept formulated earlier (Weihe and Abele, 2008) that the first strategy of intertidal limpets is to down regulate metabolism and save energy, including the synthesis of antioxidants, during air exposure/hypoxia. This is in keeping with stable glutathione concentration and maintenance of tissue reducing capacity (low redox potential at −350 to −355 mV), as well as stable pH in the gills of the intertidal animals throughout the whole time of air exposure. Up-regulation of gill SOD activities in sub-littoral animals exposed to air and hypoxia would then indicate a response to stress by induction of enzymatic antioxidant protection to maintain tissue redox potential low (E_{mV} sub-littoral gill in Table 4). Glutathione levels diminished (6 h) and the gills became transiently oxidized (12 h) and acidified during air exposure, indicating that the sub-littoral specimens had more problems to

Table 4
Changes of oxidative stress parameters in intertidal and sub-littoral *Nacella concinna* upon exposure to air and hypoxia (2 kPa). Arrows indicate increase (↑) or decrease (↓) of enzyme activity. In case of pHa and redox potential (E_{mV}) "↓" indicates acidification/reduction. No change observed is indicated as "↔" and an initial decrease with subsequent slight recovery of a parameter by "$\downarrow_{\uparrow}$" and "$_{\downarrow}\uparrow$" stands for early transient decrease followed by recovery.

	Intertidal		Sub-littoral	
	Air	Hypoxia	Air	Hypoxia
SOD (gill)	↓	↓	↑	↑
CAT (gill)	↔	↔	↔	↔
E_{mV} (gill)	↔	↓	$\downarrow_{\uparrow}$	↔
pH (gill)	↔	↔	$\downarrow_{\uparrow}$	↔
SOD (DG)	↔	↓	↓	↓
CAT (DG)	↓	$_{\downarrow}\uparrow$	↔	↔

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maintain redox and pH homeostasis when exposed to desiccation, a previously not experienced state for them.

Metabolic down regulation during air exposure in intertidal animals included down regulation of CAT activity in DG, whereas DG CAT activity during hypoxia was maintained high after a short drop in the beginning. The risk of oxidative stress upon re-oxygenation is presumably too high in this trace metal loaded tissue.

Foot muscle tissue appears well buffered by glutathione, with more oxidized GSSG/GSH ratio during the first 12 h of exposure in air exposed intertidal compared to sub-littoral limpets. Especially in combination with significantly higher MDA levels in foot muscle of intertidal limpets, this indicates higher ROS production and lipid peroxidation in foot muscle of intertidal limpets during air exposure and may be suggestive of oxygen uptake happening directly over the surface of the foot during air exposure. Indeed, in our first paper we showed that intertidal limpet foot muscle is maintained aerobic during air exposure, whereas sub-littoral limpets switch to anaerobic metabolism (Weihe and Abele, 2008), again documenting insufficient adaptation for life under intertidal exposure conditions.

The most important conclusion from our study is therefore that physiological adaptation for survival in the Antarctic intertidal shapes limpet behavior including their biochemical and metabolic response to stress exposure. One important strategy seems to be metabolic down regulation of unnecessary enzyme activity and presumably a general metabolic reduction during stress exposure in intertidal limpets (gill and digestive gland) for the purpose of energy saving. Aerial respiration during low tide may further prevent the limpet foot muscle from becoming anaerobic and accumulating major amounts of lactate and short chained organic acids. The differences in stress response are not immediately lost upon maintenance of both limpet sub-groups under standardized conditions, and thus are not purely adaptive, but indicate fundamental differences in the metabolic strategies between the two limpet populations, which may perhaps have a genetic background (Aranzamendi et al., 2008). However, it clearly indicates that some Antarctic marine invertebrates have the capacity to adjust their response and survive in changing Antarctic coastal environments. Only shared genetic and physiological research on biogeographical gradients can show whether genetic limitations exist to physiological flexibility and adaptive capacities in these species.

Acknowledgements

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Publication III

Characterisation of hypoxia inducible transcription factor HIF-α in the Antarctic limpet *Nacella concinna*

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2009

Molecular Ecology

(submitted)

Characterisation of hypoxia inducible transcription factor HIF-α in the Antarctic limpet *Nacella concinna*

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Running title: HIF-α in the Antarctic limpet

Abstract

Hypoxia inducible factor (HIF) activates genes under hypoxia. Together with factors and enzymes regulating the amount of the HIF-α protein subunit in a PO_2-dependent manner, the transcription factor constitutes the cellular security system for hypoxic survival in metazoans. We sequenced the HIF-α subunit of the Antarctic limpet *Nacella concinna* and measured its transcription under hypoxia with real-time PCR. Using a newly synthezised antibody against a protein motif deduced from the *N. concinna* HIF sequence, we measured the protein expression in hypoxia exposed and air exposed intertidal and sub-littoral limpets. Whereas HIF-α mRNA levels did not change under hypoxia, protein expression was induced following 12h of hypoxic exposure in both populations. Air exposure caused a HIF-α signal only in intertidal limpets. In all cases, HIF-α protein expression returned to control level at 24h of stress exposure. HIF-α up-regulation in limpets exposed to hypoxia occurs later than in fish or mammals which may be characteristic of hypoxia tolerant aquatic invertebrates. Phylogenetic analyses indicate invertebrate HIF-α to be an ancestral form of the transcription factor, and the observed regulatory pattern may relate to structural divergence, especially in the transactivation and the Pro^{564} oxygen-dependent degradation motif. The capacity of intertidal limpets to induce HIF-α in the body core during air exposure relates to their survival strategy during tidal emergence, which involves tight contraction of the shell over the body resulting in severely hypoxic mantle cavity PO_2. Sub-littoral limpets were unable to react in the same way and, consequently, they were less tolerant of air exposure.

Keywords: HIF-1α, hypoxia, aerial exposure, gastropod, polar, phylogeny

Introduction

Aquatic molluscs are famous for their pronounced endurance of hypoxic and anoxic conditions occurring when the animals close their shells in order to avoid predator attack, when intertidal species prevent desiccation on air exposure, or when bivalves burrow into the sediment for shelter. Many of these hypoxia/anoxia tolerant species are oxyconforming, meaning that metabolic rates decrease at declining outside oxygen tension, and that tissue energy (e.g. ATP) turnover is correspondingly depressed. Metabolic rate depression is not a passive downfall of organismal and cellular metabolism, but rather implicates a strategic and highly organized suppression of energy consuming rate processes, such as protein synthesis and active ion pumping (for summary see Storey & Storey 2004). Hypometabolic down-regulation also requires some genetic re-organization, including synthesis of specific regulators and effectors of hypoxic/anoxic survival. Hypometabolism is a fundamental and evolutionary early strategy in marine invertebrates, adopted to survive unfavourable

environmental conditions e.g. limited food supply during winter. It also helps to survive shorter periods of extreme conditions, especially under highly fluctuant intertidal conditions, extremely demanding with respect to maintenance of the energetic balance in an organism. Among such highly demanding conditions are summer and winter thermal extremes, or the exposure to desiccation during low tides.

Contrasting the situation in air breathers, respiration rates in water breathers are controlled by the PO_2. Small osphradia oxygen chemoreceptors are located in the anterior pallial roof of the patellogastropods (Lindberg & Ponder, 2001). These sensors in the innermost part of the open circulatory system of limpets provide a signal for the adjustment of the ventilation activity in the mantle cavity. As only very rudimentary gills are conserved in patellids, the entire epidermis, especially the roof of the pallial cavity and the epidermis of head and foot are capable of cutaneous oxygen uptake (Lindberg & Ponder, 2001). The question therefore arises, whether or not a cellular oxygen sensing system, which translates the oxygen signal into metabolic regulation within the tissues, is employed in these evolutionary early, oxyconforming gastropods. It is well known that a multileveled controlling system for metabolic rate processes exists in molluscs that functions under anaerobic conditions and mainly involves chemical modification of metabolic enzymes. We were, however, interested to see whether oxygen dependent gene regulation is induced in hypoxia tolerant invertebrates. Such a cellular oxygen sensing system has been characterized mostly in hypoxia sensitive mammals, and involves the hypoxia inducible transcription factor-1 (HIF-1). HIF-1 is a heterodimer composed of the oxygen regulated subunit HIF-1α and the constitutively expressed HIF-ß, the aryl hydrocarbon nuclear translocater (ARNT), which interacts with a diverse set of specific transcription factors. In mammals, which maintain clearly defined tissue PO_2, the role of HIF in the organization of short-term survival of tissues in hypoxia has received major interest with respect to cardiac and brain stroke pathologies (Hoogewijs et al., 2007; Jaakkola et al., 2001). However, HIF plays more fundamental roles in ontogenesis and more generally supports blood vessel growth and dilatation, as well as erythropoiesis. Three HIF-α homologues are known from mammals (HIF-1α, HIF-2α and HIF-3α). HIF-1α and HIF-2α have 48% overall amino acid identity (Hu et al., 2003). All three proteins accumulate under hypoxia, dimerize with HIF-β and bind to hypoxia response elements (HRE) in the promoter region of hypoxia-regulated genes (Wang et al. 2005).

Marine invertebrates and especially molluscs are evolutionary early organisms, and studies of the ecophysiological role of HIF, its oxygen-dependent regulation, or the identity of target genes in these organisms are only just beginning. In fish, HIF-1 has been shown to play a role in the adaptation to seasonal cold climate and also to be induced upon short-term (< 24h) experimental exposure to low temperature. This makes sense as marine animal ectotherms experience hypoxaemia as blood flow slows in the cold (Heise et al., 2007).

Therefore, we questioned the immediate role of HIF (erythropoiesis) in low aerobic performers and wanted to understand the dynamics and response to hypoxia inducing stress (air exposure) on the hypoxic gene transcription system in the Antarctic limpets. The species *Nacella concinna* at King George Island comprises two subpopulations, one remaining permanently sub-littoral, whereas the other one is migratory, with a greater part of this subpopulation moving between intertidal and sub-littoral shore areas in spring and autumn. Several marked differences between both subpopulations with respect to shell morphometrics as well as in survival strategies and physiological response to hypoxia and desiccation have already been reported (Weihe & Abele, 2008; Weihe et al., 2009).

In this paper we report the first characterisation of the HIF-α protein in gastropod molluscs and its involvement in the response to hypoxia and to stressful air exposure under experimental conditions. We have sequenced a full length HIF-α gene to be used for assessment of gene transcription, and for constructing specific antibodies against the deduced limpet HIF-α protein sequence. These antibodies were used for protein detection in experimental timelines of up to 48h of limpet stress exposure.

Material and Methods

Animal collection and tissue sampling

Intertidal *Nacella concinna* were collected during low tide at Potter Cove, King George Island on two occasions in November 2005 and November 2007. SCUBA divers collected sub-littoral animals in 13 – 15 m depth at about 1 km from the intertidal sampling location. All animals were immediately transferred to the aquarium of Dallmann laboratory, Jubany Station, and held in aerated, 0°C cold seawater for between 10 days and 3 weeks before experiments were started. Two different experiments were conducted: hypoxia incubation and exposure to dry air.

For the hypoxia incubation, 10 sub-littoral or 15 intertidal animals were placed into glass jars filled with natural seawater that was adjusted to 2 kPa PO_2 by bubbling the water with a N_2/O_2 mixture (Air Liquide, Krefeld, Germany). Oxygen concentration was controlled using a fiber optical system of PreSens (Precision Sensing GmbH, Germany). The jars were placed in a temperature controlled water bath at 0°C. After 0, 2, 6, 12, 24 and 48 h of hypoxia incubation, the animals were killed and immediately dissected. During the second experiment, animals were exposed to dry air for 0, 6, 12, 24 h. Limpets were placed individually on plastic dishes over dry silica pearls in a desiccator. The desiccator was maintained in snow and covered with dark cloth to minimize impact of light and to keep temperatures at 0°C. Experiments were started in the evening, as the animals were observed

to be more active during the day. No animal died during the incubations. Digestive gland, gills and foot tissue of animals from both experimental set-ups were freeze-clamped, stored in liquid nitrogen and transported to the Alfred-Wegener Institute, Bremerhaven at -196°C in a nitrogen atmosphere.

RNA isolation and determination of Hypoxia inducible factor (HIF-α) sequence

Total RNA was extracted from frozen digestive gland tissue using TRIzol (Invitrogen, Karlsruhe, Germany). The frozen tissue (50-100 mg) was ground under liquid nitrogen, homogenized in the 50-fold volume of Trizol, incubated for one hour at room temperature and subsequently processed according to the manufacturer's instructions. The remaining DNA was digested by incubation with DNase (5 U μl^{-1}) (Invitrogen) for 15 min at room temperature, and the resulting RNA was further purified using RNeasy-Mini-Kit (Qiagen Hilden, Germany) following "RNeasy Mini Protocol for RNA Clean up". RNA concentrations and quality were determined spectrophotometrically (Nanodrop ND-1000, PecLab). Measurement of RNA quality resulted always in $A_{260/280}$ ratios > 2. Integrity of the RNA was checked using a bioanalyser (Agilent Technologies, Waldbronn, Germany).

1 µg total RNA was reverse transcribed with random decamer (6 pmol, Invitrogen), 0.625 mM of each dNTP (desoxynucleotide triphosphate), 10 mM DTT (dithiothreitol), 2.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris/HCl (pH 8.4) and 20 U Superscript reverse transcriptase III (Invitrogen, Germany) at 42°C for 65 min, followed by heat inactivation at 70°C for 20 min.

Amplification of resulting cDNA was carried out in a gradient thermocycler (Tgradient, Biometra, Göttingen, Germany) with 0.05 U/µl Taq-polymerase for fragments < 1000 bp (Eppendorf, Hamburg, Germany) or SuperTaq Plus polymerase for fragments > 1000 bp (Applied Biosystems, Darmstadt, Germany), 0.5 µM of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris/HCl (pH 8.4).

First PCR primers (see Table 1) for *Nacella concinna* were designed from a HIF-1α fragment we obtained of the common limpet (*Patella vulgata*, E. Weihe, D. Abele, M. Lucassen, unpublished) by aligning published fish and invertebrate HIF-1α nucleotide sequences (*Danio rerio* AY326951; *Palaemonetes pugio* AY655698; *Caenorhabditis elegans* NM075607; *Daphnia magna* AB425958) and performing nested PCR with touch down program (see below). Subsequent PCRs were performed for completing the sequence, using "primer walking" with one *N. concinna* specific primer and a degenerated primer designed on the basis of sequence segments conserved between published vertebrate and invertebrate HIF-1α sequences.

Primers for the actin-fragment, which was used as endogenous control in the real-time PCR, were designed from a published sequence of the bivalve *Dreissena polymorpha* (HF082863). PCR program 35 cycles: 94°C 4 min; 94°C 45 s; 55°C (±6°C) 1 min; 72°C 30 s, and a final prolongation step of 8 min at 72°C. MacVector 9.5 (Accelrys Software Inc., San Diego) was used for primer design, assemblage and sequence analysis.

For determination of the remaining end parts of the gene, rapid amplification of cDNA ends (5´RACE and 3´RACE) was carried out using First Choice RLM-RACE Kit following the manufactorer´s instructions (Applied Biosystems), SuperTaq Plus DNA Polymerase (Applied Biosystems), primers listed in Table 1, and the following touch down program for PCR: 10 cycles: 94°C 4 min; 94°C 45s; 64°C (± 6°C) 80s (-1°C per cycle); 68°C 2 min; 25 cycles: 94°C 45s ; 57°C (±6°C) 1min 20s; 68°C 2 min.

Table 1: List of all primers used.

Primer	Sequence	Position	Purpose
	Patella vulgata		
HIF-DF1	SMAAAGRAAARGAGRSGGAAYTCGG	171 - 195	Outer RT-PCR
HIF-DB2	CCARCGGMRTYTCRATGTTSGARGG	845 - 821	Outer RT-PCR
HIF-DB3	TCTCVACRTAGGTGWACTTCATRTCC	697 - 571	Outer RT-PCR
HIF-DF2	TCGGAMARGCGSAAGGAGAAVTCCCG	170 - 195	Inner RT-PCR
HIF-DB1	AGYTCVTCRTGRTCGCASGGGTG	567 - 545	Inner RT-PCR
PvHIF-F4	TCTGTGTTCAAAGGCTCTGGAAG	404 - 428	RT-PCR
PvHIF-F1	AAAACGGAAGGAGAAATCCCG	174 - 195	RT-PCR
DmHIF-B4C	GTCTGGAMCCAMRCAWAACCWCC	1089 - 1067	RT-PCR
PvHIF-5´-RB4	CCTCTGTTTCTTTACTCCGACGAC	233 - 210	5´RACE
PvHIF-5´-RB5	CCTTCCAGAGCCTTTGAACACAG	429 - 407	5´RACE
	Nacella concinna		
PvHIF-F3N	CGGTGTCGTCGGAGTAAAGAAAC	219 - 231	RT-PCR
PvHIF-B1N	TAGTTCAATCTGCTGTATGCCCAG	517 - 494	RT-PCR
NcHIF-F23	AGAAACTGTTGCCAAGTATCTGGG	475 - 498	RT-PCR
NcHIF-B41	TTCTCAACAACATGGCTGTCCAG	999 - 977	RT-PCR
HIF-CT-F2	TCTTGACAGCAACACCTTTCTTACC	841 - 865	Outer RT-PCR
HIF-CT-F4	CAGCCATGTTGTTGAGAACGC	982 - 1002	Inner RT-PCR
HIF-CT-B1	TGAACRGGRGCRTTBACTTC	2298 - 2279	RT-PCR
NcHIF-5´-RB5	TGGCTTGTAATACTGGTTGGCAG	288 - 266	5´-RACE
NcHIF-5´-RB10	TGCTTTTGAACACAGGGCGTC	421 - 401	5´-RACE
NcHIF-3´-R8	TATTTGAAGTGCCTCCCCAGC	1875 - 1895	3´-RACE
NcHIF-3´-R12	CCACCTCTAATAGGGATGAGC	2072 - 2092	3´-RACE
NcHIF-3´-R1	CAATCGCTATCATACCTACTTCCG	2122 - 2145	3´-RACE
ACT-F1	GWGTGATGGTTGGTATGGGWCAG		RT-PCR
ACT-B2	GCKGTGGTTGTGAAWGARTARCC		RT-PCR

PCR-products were separated in a 1.3% agarose gel (45 mM Tris-Borat, 1 mM EDTA, pH 8,3) containing 0.1 µg/ml ethidium bromide. Fragments of appropriate size were purified by QIAquick gel extraction kit (Qiagen) and cloned using TOPO TA cloning kit (plasmid vector: pCR4-TOPO; chemically competent *Escherichia coli* TOP10; Invitrogen) following the manufacturer´s instruction. Six to eight clones were selected for overnight liquid culture in LB-medium (10g/l trypton, 5 g/l yeast extract, 10 g/l NaCl in H_2O) containing 100 µg/ml ampicillin. Cloned plasmids were isolated using the Qiaprep Spin Miniprep kit (Qiagen). The

presence and size of inserts was checked by restriction digestion with *Eco*R1 (Invitrogen). For each fragment, DNA sequencing of positive clones of both strands was outsourced to (Martinsried, Germany).

The assembly of the pherograms was carried out in MacVector 9.5 (Accelrys Software Inc., San Diego). The double-stranded HIF sequence of *N. concinna* was translated into amino acids and aligned to 60 HIF protein sequences (isoforms 1, 2 and 3) using the MUSCLE algorithm set to a maximum of 8 iterations as implemented in Geneious Pro 4.7.6 for OS X (Drummond et al. 2009). The resulting protein alignment was 1172 amino acids long, but contained gap-rich and poorly aligned regions. A preliminary analysis showed that the HIF isoform 1 sequences of vertebrates clustered together with strong support and that the amino acid variation within this group was small compared to the variation within the invertebrate HIF or among HIF isoforms (data not shown). The dataset was subsequently reduced to 39 taxa by removing 22 vertebrate HIF isoform 1 sequences.

Phylogenetic analysis

In order to verify if our protein was indeed a molluscan HIF sequence, only well-aligned regions of the original alignment were extracted and used for phylogenetic analyses. A sequence similarity threshold ≥ 60% (BLOSUM62) corresponded to four well-defined regions with 493 amino acids in total. The delimitation of the conserved areas of the alignment and the resulting tree topologies depended only to a small degree on the exact setting of the similarity threshold. A suitable model amino acid subsitutions over time was determined with a likelihood ratio test (LRT) using PROTTEST (Abascal et al 2005) and a corresponding tree topology was determined by calculating a Maximum Likelihood tree using the parameters of the LRT in PhyML (Guindon & Gascuel 2003). Because all known HIF sequences comprised the ingroup and the evolutionary ancestry of HIF is not unambiguously known, the polarity of the tree was determined by midpoint rooting (Hess & De Moraes Russo 2007).

RealTime PCR

10 ng of total RNA, isolated from digestive gland, were used as template for reverse transcription to cDNA using the high-capacity cDNA archive kit (Applied Biosystems).

The target gene HIFα and the reference gene actin were amplified from 6 individuals of each incubation group, using gene specific primers and probes (Table 2) and TaqMan universal PCR master Mix (Applied Biosystems). Primers and probes were designed using the customer design service of Applied Biosystems. Quantitative real-time PCR was carried out

using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA) and its universal PCR protocol.

Table 2: Primer and reporter (dye FAM) used for real-time PCR.

Name	Sequence	Purpose
NcHIF-a-ANYF	GATTATCTATCAGTTATTTGAACTTGTGTAATATTTTGGATT	Forward Primer
NcHIF-a-ANYR	CAGGGCGTCCATTTTCTTTTCTT	Backward Primer
NcHIF-a-ANYM1	CTGCTGCTGAAACTGA	Reporter
NcActin-ANYF	CCAGAAGAACATCCCGTCCTT	Forward Primer
NcActin-ANYR	GGTCATCTTTTCTCGGTTGGCTTTA	Backward Primer
NcActin-ANYM1	AAGCCCCACTCAACCC	Reporter

Each plate set-up consisted of cDNA from 12 individuals, representing 2 experimental groups, and one additional sample that was always added as internal standard in order to calibrate the efficiency of amplification between PCR runs. Each HIF and actin amplification was done in triplicate. Each primer set was checked to ensure no unspecific reactions and full efficiency.

For the calculation of relative expression levels under hypoxia conditions x compared to control 0, the comparative Ct method was used (formula: $2^{[(Ct_{HIF\text{-}1\alpha,x} - Ct_{actin,x}) - (Ct_{HIF\text{-}1\alpha,0} - Ct_{actin,0})]}$), where Ct correspond to the threshold cycle number.

Immunoprecipitation and immunoblot analyses

Primary antibodies were produced in two rabbits (#2096; #2097) by Biotrend Chemikalien GmbH (Germany) based on the deduced *N. concinna* HIF-1α amino acid sequence CTLTSKGRNVNLKSA (position 179 – 193) located between the two PAS domains.

Protein extracts for immunoprecipitation and immuno blotting were prepared from flash-frozen tissues. 100 mg tissue pieces were homogenized in a glass homogenizer on ice in RIPA buffer (1:10 w:v) containing 50 mM Tris/HCl pH 7.8, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton, 0.5% sodium deoxycholate, 0.1% SDS (sodium dodecylsulfate), 5 µl ml^{-1} protease inhibitor cocktail (Sigma, P8340), 5 µl ml^{-1} antipain, 1 mM Na_3VO_4, 1 mM DMOG (Dimethyloxaloylglycine), 0.5 mM phenylmethylsulphonyl fluoride, 1 mM dithiohtreitol. After 10 min incubation on ice, the homogenates were centrifuged 10 min at 1000 g and 0°C.

The supernatants were incubated with 0.5 µg of protein specific antibody of animal #2097 over night at 2°C. On the following day, incubated samples were added to 30µl pre-washed Sepharose CL-4B beads (Sigma, P3391), having protein A immobilized, and incubated for 3 h at 2 °C. The immunoprecipitate bound to the beads was collected by 5 min centrifugation at 1000 g, and 2 °C, and washed three times with ice-cold buffer containing 100 mM KCl, 0.2 mM EDTA and 20 mM Tris/HCl pH 7.8. The interacting proteins were detached from the beads by suspending the pellet in 60µl of the sample loading buffer, containing 62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% glycerol and 5% β-mercaptoethanol and incubating for 10 min at 75°C under slight agitation.

Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to a PVDF (Polyvinylidene difluoride) membrane (Bio-Rad, Germany). Membranes were blocked in 5% non-fat dry milk in TBS-T (Tris-buffered saline with 0.1% Tween 20) for 1 h at room temperature and incubated with the primary antibody of animal #2096 over night at 2°C. The further processing and detection of the antibodies using the ECL system and a cooled CCD-camera system (LAS-1000, Fuji, Japan) was done essentially as described earlier (Deigweiher et al. 2008). Protein levels in each sample were expressed relative to the signal intensity of the antibody used for immunoprecipitation.

Measurement of organic acids

The foot tissue was ground in liquid nitrogen and homogenized in the 3-fold volume (w/v) of 7% perchloric acid (PCA). After centrifugation (25 min, 25 000 *g, 4°C for all 3 centrifugation steps), the pH in the supernatant was adjusted to pH 2-3 with 3M NaOH. Samples were centrifuged again, and the supernatant collected and frozen at -20°C to precipitate residual NaCl. Thawed samples were centrifuged again, and the supernatants injected into the HPLC (high performance liquid chromatography).

Organic acids were separated on a BioRad Aminex HPX-87H column (300 x 7.8 mm) by isocratic elution with 2.25 mM H_2SO_4, flow rate 0.6 ml/min, at 60°C column temperature and UV detection at 210 nm. Authentic standards for calibration were succinate (S2378; Sigma), D(-)lactate (L1000; Sigma), fumarate (F1506; Sigma), acetate (24,285-3; Aldrich), and propionate (P1880; Sigma).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 Software. Previously data were tested for normality (Gaussian distribution) by D'Agostino & Pearson omnibus normality test. Parameter changes within one subpopulation were analyzed by one-way ANOVA (Kruskal-Wallis test for not Gaussian distributed data) and Tukey post-hoc tests for normally

distributed data, whereas Dunn´s test was used if normality was not given. Two-way ANOVA with Bonferroni post-hoc test was used to analyse differences between the two subpopulations. T-test was used to compare two single data groups, depending on normality unpaired t-test with Welch´s correction (or Mann-Whitney test).

Results

Cloning and sequencing of HIF-α from *Nacella concinna*

When we started our project, no molluscan HIF-α sequence, and only few invertebrate sequences were available. Overall the conservation of the HIF-α in the animal kingdom is limited to specific regions of the protein (see discussion). Therefore we constructed a matrix of outer and inner primers (designed from distantly related species within the animal kingdom) and performed nested PCR with touch down program as described above. This primer matrix was used with cDNA from two bivalve and two limpet species. The primer combination of HIF-DF1, HIF-DB2 and HIF-DB3 as outer primer and HIF-DF2 and HIF-DB1 as inner primer resulted in a 397 bp fragment only in the limpet *Patella vulgata*. This fragment was enlarged by RT-PCR and 5´RACE (primers see Table 1) resulting in a 978 bp fragment. This first molluscan HIF-1α fragment was then used as a template to construct primers (PvHIF-F3N, PvHIF-B1N) for the Antarctic limpet, the entire mRNA sequence was then determined using "primer walking" and RACE techniques (primers see Table 1).

HIF-α sequence and molecular characterisation

The complete *N. concinna* HIF-α sequence consists of 3249 base pairs (bp) with one 2286 bp open reading frame (ORF) starting at position 113 and stopping at 2396, encoding a 762 amino acids (aa) protein, corresponding to a deduced molecular weight of 85.4 kDa. The two limpet nucleotide sequence fragments (ORF 978 bp) were 76% identical. The deduced amino acid sequences of both limpets had 84.7% identity and 90.2% similarity.

Further, the isolated *N. concinna* protein sequence has a close identity (45.1%; 62.5% similarity) with the sequence of the pacific oyster *Crassostrea gigas* (BAG85183), the only molluscan sequence which became recently available in public data bases. The overall identity with vertebrate sequences is moderate with about 28% identity to fish and 25% to the human HIF-1α sequence (similarity: 41 – 44% see Table 3). The basic helix-loop-helix (bHLH) and the two PER-ARNT-SIM (PAS) domains are more conserved than the C-terminus of the oxygen dependent degradation domain (ODD), which could not be clearly identified. The bHLH domain has a similarity of 93% with mammalian and amphibian sequences and 87% - 90.7% with fish. The PAS A domain has higher similarities (83% - 89%)

with vertebrates than the PAS B domain (80% - 82%). The N-terminal transactivation domain (N-TAD) seems to be lacking in the *N. concinna* sequence, whereas the C-terminal transactivation domain (C-TAD) exists and has a similarity of 72% – 76% with fish and 84% mammal sequences.

Table 3: Identities (%) above the empty row and similarities (%) below the empty row of HIF-1α protein sequence of different species.

	Nacella concinna	*Crassostrea gigas*	*Cancer magister*	*Zoarces viviparus*	*Pachycara brachychephalum*	*Carassius carassius*	*Rana temporario*	*Xenopus laevis*	*Homo sapiens*
Nacella concinna		45.1	22.8	28.3	28.0	28.7	28.1	28.1	25.8
Crassostrea gigas	62.5		21.2	27.5	27.0	28.2	26.2	25.3	25.3
Cancer magister	34.6	32.5		19.6	19.6	21.3	21.0	20.5	20.1
Zoarces viviparus	43.1	42.4	31.1		96.9	56.4	47.1	49.1	48.0
Pachycara brachychephalum	42.9	42.2	31.2	98.3		56.1	46.7	48.7	49.3
Carassius carassius	44.1	43.3	33.2	68.6	69.1		63.9	51.9	51.9
Rana temporario	42.8	39.5	32.1	59.2	59.6	63.9		79.9	64.3
Xenopus laevis	41.3	38.9	31.4	60.9	61.0	65.2	88.5		67.4
Homo sapiens	41.6	40.1	31.7	61.3	61.7	65.7	79.1	81.2	

The limpet HIF-α protein contains both functional proline residues (hydroxylation sites) within the ODD, as well as the functional asparagine residue in the C-TAD (Figure 1). The sequence findings presented here clearly document the limpet sequence belonging to the HIF-α family of transcription factors.

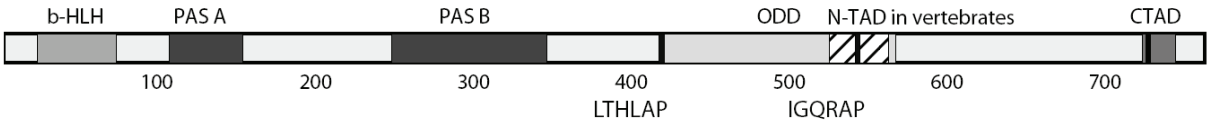

Figure 1: HIF-α protein sequence with domains deduced from cDNA sequence of *Nacella concinna*.

bHLH: basic helix-loop-helix domain, PAS A and PAS B: Per-ARNT-SIM, ODD: oxygen dependent degradation domain, CTAD: C-terminal transactivation domain. Black bars indicate functional hydroxylation sites. Motifs in front of the proline residues are written below the sequence. Striped box indicates the theoretical position of the N-TAD. Numbers below the sequence indicate the position of amino acid.

A phylogenetic tree based on the 493 amino acids that could be aligned reliably across all 39 taxa shows three distinct and well-supported clades, corresponding to the three isoforms of HIF and within isoform 1 well-supported clades containing vertebrates and mammals (Figure 2). All invertebrate HIF sequences including *Nacella concinna* in our study cluster together and form a fourth cluster that obtains a sistergroup position to all other known vertebrate HIFs (isoforms 1, 2, 3). The Antarctic limpet sequence clusters with high bootstrap support with the only other molluscan sequence, the Pacific oyster. The two molluscs in turn form a well-supported cluster together with all other invertebrate HIF sequences so far available. A full phylogeny of the taxa in our study is beyond the scope of this paper and would suffer from taxon sampling bias in favor of vertebrates, but our analyses confirm that the lower complexity of the invertebrate HIF as compared to vertebrates may indeed be the ancestral state.

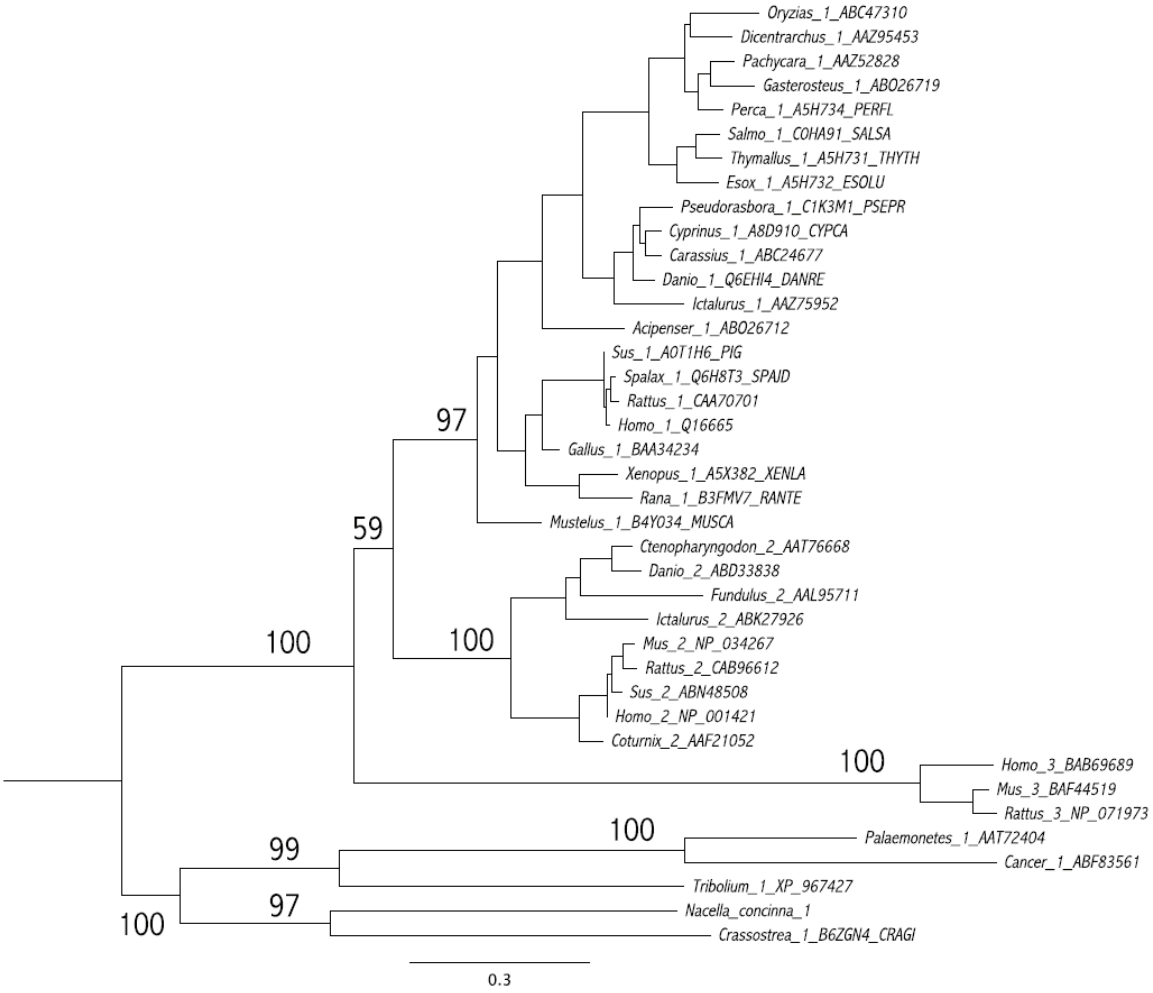

Figure 2: Maximum Likelihood tree of 39 HIF sequences, isoforms 1, 2 and 3.
493 amino acids, JTT model of amino acid replacement, discrete gamma, ncat=4, alpha=0.987, midpoint rooting. Numbers on internal branches denote bootstrap support calculated using the Neighbor Joining algorithm and the same substitution model, no bootstrap values inside vertebrate HIF isoforms is given due to space constraints. The terminal taxa are given in the format genus_HIFisoform_accessionnumber, e.g. Danio_2_ABD33838 stands for *Danio rerio*, HIF isoform 2, GenBank accession number ABD33838

HIF-α mRNA expression during hypoxia

HIF-α mRNA (Figure 3) was expressed under normoxic and hypoxic conditions (2 kPa oxygen) in digestive gland tissue of both *N. concinna* subpopulations. Given the large inter-individual variability neither the factor "subpopulation" nor hypoxia exposure over time (intertidal $p = 0.5762$; $F = 0.776$; $n = 31$; sub-littoral $p = 0.6611$; $F = 0.6541$; $n = 32$) had a significant effect on HIF-α expression.

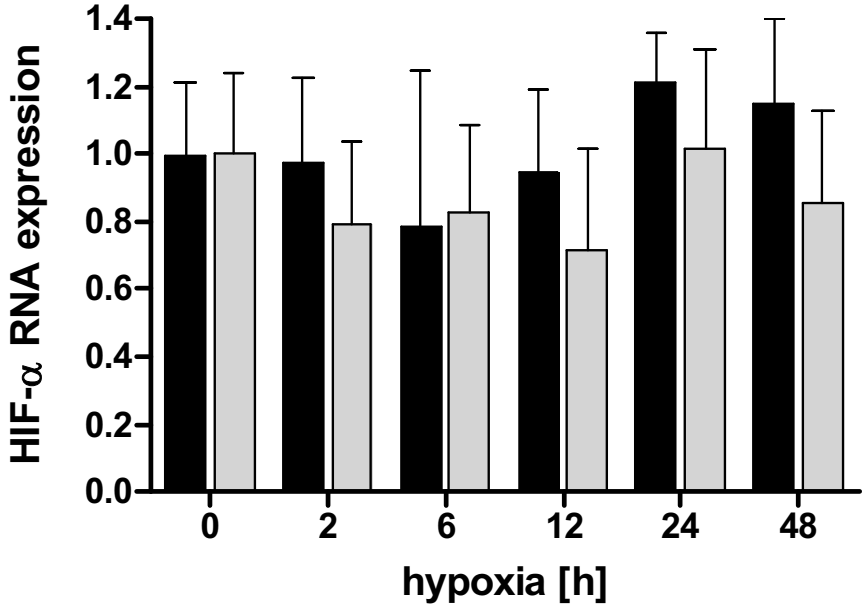

Figure 3: Relative amount of HIF-α mRNA in digestive gland tissue of intertidal (black) and sub-littoral (grey) *N. concinna* during hypoxia incubation normalized to sub-littoral controls.
Mean + SEM. N = 4 – 7. Interaction p = 0.8879; F = 0.3375; population p = 0.1573; F = 2.06; hypoxia p = 0.4259; F = 1.002 (two-way ANOVA). Intertidal p = 0.5762; F = 0.776; Sub-littoral p = 0.6611; F = 0.6541 (one-way ANOVA).

The overall extractable RNA levels did not change in either subpopulation during hypoxia. Intertidal limpets had always higher overall RNA content than sub-littoral animals (p = 0.0173; F = 5.964, n = 77; data not shown).

HIF-α protein expression during hypoxia and aerial exposure

For the quantification of protein expression, a peptide specific antibody was constructed, which recognized one protein band with a molecular mass between 95 and 100 kDa (Figure 4A) after enrichment by immune-precipitation. Signals of the respective size were lacking in reagent blanks containing only extraction buffer, beads and the antibody used for precipitation (Figure 4B).

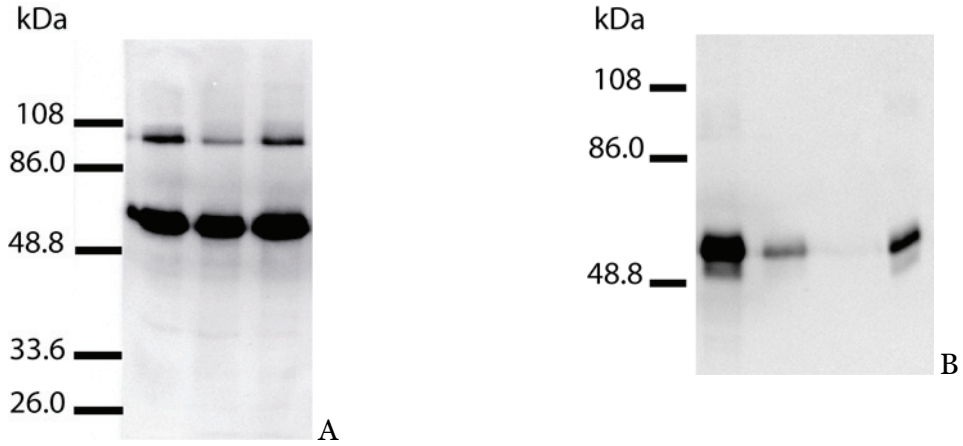

Figure 4: Immunoblots after immunoprecipitation.
A) Immunoblot showing the HIF-α protein (100 kDa), after immunoprecipitation, isolated from three different normoxic digestive gland tissues of *Nacella concinna*, each lane represents HIF-α detected in one single animal and the antibody used for immunoprecipitation (50 kDa).
B) Immunoblot showing immunoprecipitation only with the antibody but without tissue extract to verify that signal at 100 kDa results from the tissue extracts itself. First lane = the immunoprecipitate, second lane = supernatant after bead centrifugation, third lane = washing buffer of the beads, fourth lane = only antibody without immunoprecipitation.
Molecular size marker used for both immunoblots: prestained broad-range marker (Bio-Rad, München, Germany).

Comparing the HIF-α protein content in normoxic animals, we found significantly higher concentrations in foot muscle than digestive gland tissue in both limpet subpopulations. In intertidal limpets the foot muscle contained nearly the 10-fold (9.98-fold) amount of HIF-α protein ($p = 0.0014$; $t = 4.757$; $df = 8$) and in sub-littoral limpets about 10.6-fold ($p = 0.0023$; $t = 3.775$; $df = 13$).

In digestive gland of both subpopulations, the protein expression of HIF-α changed significantly during hypoxic incubation (intertidal $p = 0.0412$; $KW = 11.57$; sub-littoral $p = 0.0234$; $F = 3.269$). After 12h of hypoxia HIF-α protein levels were 12.9-fold (sub-littoral) and 2.8-fold (intertidal) elevated over the controls. At 24h and 48h protein levels returned to normoxic values (Figure 5, intertidal black bars, sub-littoral grey bars). Thus, hypoxia produces a strong effect on *N. concinna* HIF-α protein levels ($p = 0.0001$; $F = 6.371$) with the same expression time pattern but different levels of induction in both subpopulations (interaction $p = 0.0923$; $F = 2.012$).

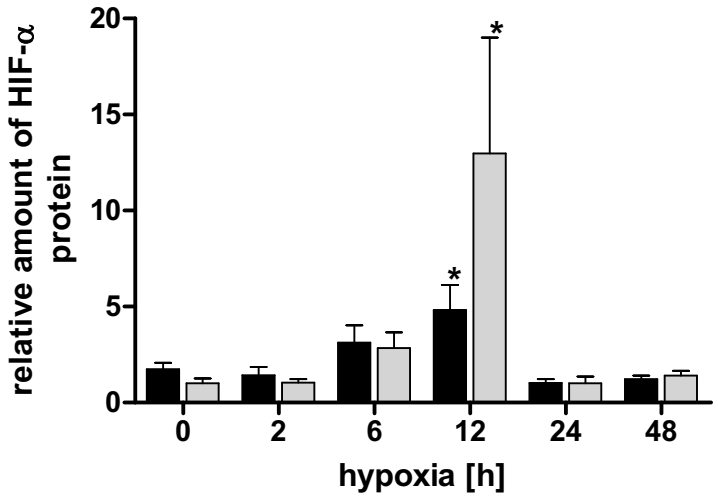

Figure 5: Relative amount of HIF-α protein in intertidal (black) nd sub-littoral (grey) *N. concinna* digestive gland tissue during hypoxia normalized to sub-littoral controls.
Mean + SEM. N = 4 – 8. * significant elevated HIF-α protein levels than in controls. Intertidal p = 0.0412; KW = 11.57; Sub-littoral p = 0.0234; F = 3.269.

Furthermore, experimental exposure to dry air had a significant effect (p = 0.0242; F = 3.375) on digestive gland HIF-α protein expression only in intertidal limpets, whereas HIF-1α levels in sub-littoral limpets remained unchanged (Figure 6). The interaction between "time of aerial exposure" and "subpopulation" was therefore highly significant (p = 0.0083; F = 4.294, DF= 3). After 12h of aerial exposure, intertidal limpets had accumulated significantly more HIF-α protein, 5-fold elevated over the intertidal controls in the digestive gland. These 12 h values were 7.3-fold higher than the HIF protein levels in sub-littoral limpet digestive gland after 12 h of air exposure (p < 0.001). The accumulation followed the same time pattern already seen under experimental hypoxia, and expression levels were back to control values after 24 h of air exposure.

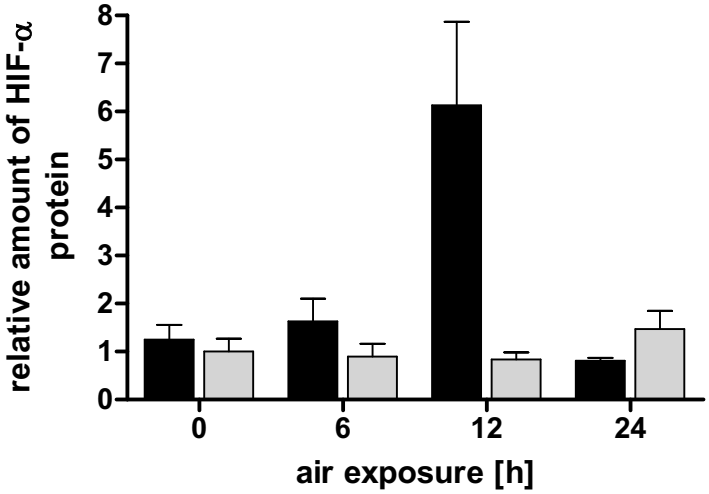

Figure 6: Relative amount of HIF-α protein in intertidal(black) and sub-littoral (grey) *N. concinna* digestive gland tissue during aerial exposure normalized to sub-littoral controls.
Mean + SEM. N = 5 – 15. Intertidal p = 0.0652; KW = 7.219; Sub-littoral p = 0.6668; KW = 1.568.

Anaerobic metabolism (organic acids) during hypoxia in foot muscle

The effect of hypoxia on organic acid concentrations in foot tissue of intertidal and sub-littoral *N. concinna* is shown in figure 7. Succinate and lactate concentrations increased significantly in limpets from the intertidal zone (succinate $p < 0.0001$; $F = 27.62$; $n = 41$; lactate $p < 0.0001$; $KW = 38.4$; $n = 43$) as well as the sub-littoral (succinate $p = 0.0004$; $KW = 22.86$; $n = 36$); lactate $p < 0.0001$; $KW = 32.45$; $n = 38$). After 12 h of hypoxia succinate levels were 7.4-fold (intertidal) and 13.9-fold (sub-littoral) elevated over normoxic controls with no significant difference between populations. Whereas intertidal limpets had significantly elevated succinate concentrations already after 2 h of incubation ($p < 0.05$), sub-littoral limpets showed a significant increase after 6 h ($p < 0.01$). After 24 h (intertidal) or rather 48 h (sub-littoral) succinate levels were back to control levels in both populations.

Lactate concentrations were significantly elevated in intertidal limpets after 12 h ($p < 0.05$) and in sub-littoral limpets after 24 h ($p < 0.001$).

In contrast, fumarate concentrations decreased in limpets of both subpopulations (intertidal: $p = 0.0049$; $KW = 16.8$; sub-littoral: $p = 0.001$; $KW = 20.5$). In sub-littoral limpets the decrease accounted even for 80% from 2.031 ± 1.04 [µmol g^{-1} fwt] to 0.3811 ± 0.23 [µmol g^{-1}

fwt] within 48 hours ($p < 0.05$). In intertidal limpets fumarate increased within the first two hours from 0.7989 ± 0.34 [µmol g^{-1} fwt] up to 1.98 ± 1.16 [µmol g^{-1} fwt] to decrease afterwards by more than 75% to 0.4659 ± 0.32 [µmol g^{-1} fwt].

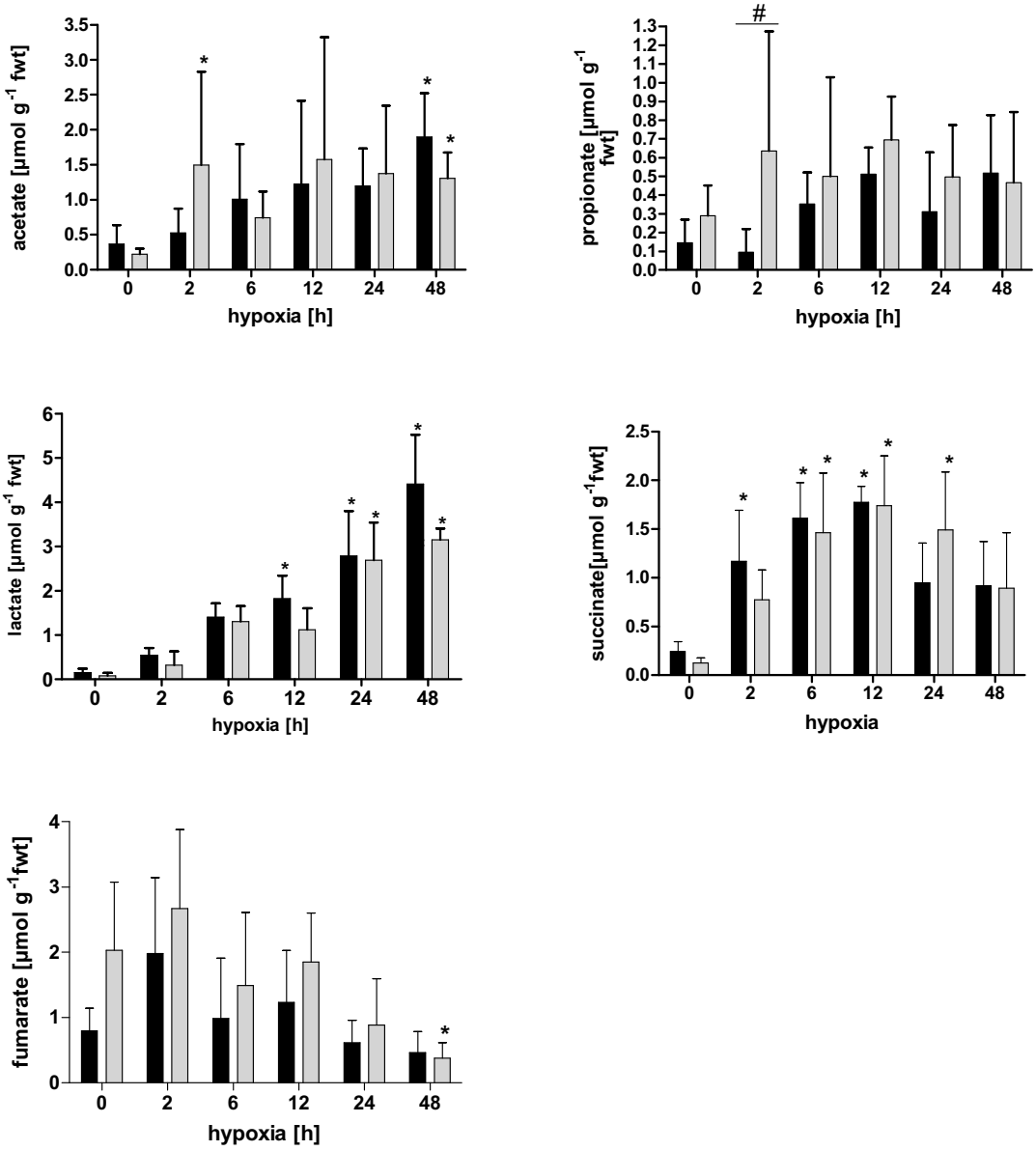

Figure 7: Concentrations of anaerobic metabolites in foot muscle of intertidal (dark) and sub-littoral (grey) *Nacella concinna* during hypoxia.
Data from November 2005. Metabolite concentrations in µmol mg^{-1} fwt. Mean values + SD, n = 5 - 10 per group. * indicates significantly different values from control value (Dunn´s Multiple Comparison Test). # indicates significant difference between the two subpopulations (Bonferroni).

Discussion

We report the first characterization of a HIF-α protein isolated from molluscs. The deduced amino acid sequence showed highest similarity to the HIF-α sequence of *Crassostrea gigas* (BAG85183) and is also similar to other published invertebrate sequences. It contains all characteristic motifs of a HIF-α protein, including the basic helix-loop-helix domain, responsible for dimerization of the α- and β- subunits and DNA-binding, the two PAS domains, which play an important role in target specification, dimerization and transactivation, the core region with the oxygen-dependent degradation domain (ODD), containing both essential prolines targeted by prolyl-hydroxylase enzymes (PHDs), and the C-TAD with the conserved asparagines residue targeted by FIH (factor inhibiting HIF). Only the N-terminal transactivation domain (N-TAD) of vertebrate HIF-1α could not be identified in the sequence alignments. The limpet HIF-α protein was detected by two polyclonal antibodies, constructed against the deduced peptide sequence, corresponding to position 179 – 193 of the protein. The antibody binding site is located between the two PAS domains, and shows conservation to vertebrate HIF-α isoforms. Only one out of 15 aa is substituted compared with either the oyster or fish sequences.

Both the ODD and TAD domains are negatively and independently of each other regulated by the cellular oxygen content (Mahon et al., 2001). Oxygen-dependent hydroxylation of the two functional proline residues in the ODD generates a binding site for the von Hippel-Lindau factor (pVHL), which serves as recognition unit for the E3 ubiquitin-protein ligase (Kamura et al., 2000) and targets HIF-α for ubiquitination and degradation via the proteasomal pathway (Bruick & McKnight, 2001; Ivan et al., 2001; Jaakkola et al., 2001). In *N. concinnna* the first proline residue (Pro^{420} corresponding with Pro^{402} in human HIF-1α) is located within the conserved and essential LTVLA**P** motif (LXXLA**P)** (Jaakkola et al., 2001) in the N-terminal ODD. The second proline (Pro^{541} corresponding to Pro^{564} in *H. sapiens*), is located within a less conserved region, the C-terminal ODD (IGQRA**P**FIP), which overlaps with the the N-TAD (residues 531-575) in vertebrates. This protein-TAD cannot be identified in either *N. concinna* or crustaceans (Li & Brouwer, 2007; Soñanez-Organis et al., 2009). Furthermore, the proline motif (IGQRA**P**FIP) in *Nacella concinna* and in other available invertebrate sequences has Arg or Lys in the place of the proximal Leu^{562} of the vertebrate sequence (vertebrates: LXX**L**AP; Nacella: IGQ**R**AP). Ivan et al. (2001) found this Leu^{562} residue in mammalian cell lines to be essentially required for hydroxylation of the Pro^{564}, and substitution of Leu^{562} by alanine abrogated Pro^{564} hydroxylation necessary for binding of pVHL. Contrary, Huang et al. (2002) concluded the leucine residue to play no major role with respect to the hydroxylation of the Pro^{564}, since substitution by alanine had no effect in their in vitro system. Arg and Lys, which substitute for Leu^{562} in the invertebrates (molluscs,

crustaceans and insects) are positively charged, bulky amino acids when compared to the hydrophobic Leu and Ala. We postulate that this sequence pattern has functional implications in invertebrates possibly causing less efficient binding of PHDs to the motif, which may modify the conditions for Pro^{564} hydroxylation and pVHL binding in the limpet ODD. It seems possible that the Leu^{562} substitution could support normoxic HIF-α protein levels in *N. concinna* (see below).

The second oxygen dependent regulation occurs in the C-TAD where hydroxylation of the functional asparagine (Asn^{725}, corresponding to Asn^{803} of *H. sapiens*) is catalyzed by an asparaginyl hydroxylase (FIH). FIH interacts with both HIF-α and pVHL to form a complex which prevents the p300/CBP transcriptional co-activator to bind to the C-TAD, and thereby impairs the transcriptional activation of HIF-α (Lando et al., 2002; Mahon et al., 2001). This Asn residue and the neighbouring Val^{724} (Val^{802} in human) which is important for positioning the Asn for hydroxylation (Linke et al., 2004) are conserved between limpets and mammals, indicating the mechanisms of gene transactivation by HIF to be conserved in evolution. Altogether, the sequence conservation of specific functional motifs and domains clearly confirms the limpet sequence to belong to the HIF-α family of transcription factors.

Phylogenetic analysis of available vertebrate and invertebrate HIF-α sequences, including the *N. concinna* sequence, revealed four prominent clusters separating vertebrate from invertebrate HIF-α and, further, mammals inside the vertebrates. The *N. concinna* protein clusters with all other invertebrate HIF sequences and appears genetically distant from vertebrate HIF sequences, including all three isoforms HIF-1α, HIF-2α and HIF-3α. A similar tree was already published from phylogenetic analysis of crustacean HIF-α (Li & Brouwer, 2007, Soñanez-Organis et al., 2009). Both phylogenetic trees suggest that multiple isoforms of HIF-α have evolved only in vertebrate fauna, including fish (Rahman & Thomas, 2007), to allow for rapid and organ specific oxygen transport requirements into different body regions of stenoxic organisms (Stroka et al., 2001). Unlike HIF-α, which exists in three different isoforms within the vertebrates, so far no HIF-α isoforms have been detected in invertebrates suggesting that the invertebrate HIF-α with its less sophisticated structure and regulation mechanism may represent the ancestral HIF-α of euryoxic animals. This is further supported by the ancestral position the invertebrate HIF occupies in the phylogenetic tree (Figure 2). It seems unlikely that other isoforms would have been overlooked with the applied methods, as we used a complex set of primers for functional domains conserved throughout the vertebrate isoforms (Table 1). Furthermore, the entire genome of the nematode *Caenorhabditis elegans* contains only one HIF-α isoform (WormBase: WBGene00001851), and also extensive sequencing of the oyster *Crassostrea gigas* (AB289857) has so far yielded only one HIF-α isoform.

If invertebrate HIF-α represents an ancestral form, the question arises whether this HIF-α is regulated by similar oxygen dependent cellular mechanisms as the vertebrate protein, and whether its function as oxygen-dependent transcription factor is similar in molluscs and vertebrates or mammals. To answer this question, we investigated HIF-α mRNA and protein expression levels in digestive gland of *N. concinna* after different periods of hypoxic exposure. Whereas mRNA levels, although variable in individual limpets, basically remained unchanged throughout the incubations at 2kPa oxygen, HIF-α protein accumulated upon after 12 h hypoxic exposure in both groups of sub-littoral and intertidal limpets. Constitutive mRNA levels during hypoxia exposure (2.5 and 1.5 ppm dissolved oxygen) were also reported from experiments with grass shrimp *Palaemonetes pugio* (Li & Brouwer, 2007) and the authors suggested (post)translational regulation of HIF-α in the crustacean, similar to mammals. Decreasing mRNA levels have been reported for gill and muscle tissues of the white shrimp *Liopenaeus vannamei* during hypoxia (Soñanez-Organis et al., 2009) in contrast to increased mRNA levels detected in anoxic insect larvae (Morin et al., 2005). In several studies exposing fish to different levels of hypoxia, changes of HIF mRNA are reported including up- and down-regulation in different tissues such as gonads and gills (Rahman & Thomas, 2007; Rissanen et al., 2006), with no change observed in the rest of the body. These rather contradictory results argue for different regulatory mechanisms in hypoxia-tolerant fish and invertebrates than in mammalian systems.

In contrast to many findings from mammalian systems, the *N. concinna* hypoxia inducible factor protein was clearly detectable under normoxic conditions. Likewise, normoxic HIF-1α protein expression was recorded in different organs of teleost fish (see Rissanen et al. (2006) for crucian carp; Heise et al. (2006) for North Sea eelpout; organs: liver, heart, gills and kidney). Conservation of the Leu^{562} within the HIF-1α degrading pVHL binding motif in the ODD of fish around the second 4-hydroxyproline (Pro^{564} in human) indicate that normoxic HIF-α levels in limpets not essentially limited to the presence of bulky, basic amino acids Asp or Lys at this position (see above). However, substitution of the functional Pro^{402} (of the human sequence) by leucine in the Antarctic eelpout (AAZ52828), indicate that pVHL dependent HIF-α degradation might be generally less important in invertebrates and fish compared to mammals. This is supported by a multiple alignment analysis of pVHL protein sequences of mammals, fish and insects, that moreover included the sequence of the worm *Caenorhabiditis elegans*, at least the "closest" relative to the limpets (Sikora & Godzik, 2004). Alignment analysis and mapping of conserved binding motives on the pVHL protein surface showed only 30% sequence identity between *C. elegans* and mammalian pVHL, and especially the pVHL α-domain that binds Elongin C and enables the interaction between the tumor factor and the E3 ubiquitin ligase complex was so divergent from the mammalian protein that, according to the authors, rapid oxygen-dependent (and also depending on

factors such as LON/MetAP2) proteasomal-degradation of HIF-1α would be non-functional in *C. elegans*. Instead HIF regulation by other mechanisms is postulated (Sikora & Godzik, 2004).

Still, HIF protein expression patterns observed in the limpets we exposed to hypoxia and to air are not fundamentally opposed to the patterns in mammalian cells, and oxygen was presumably be the major modulator of protein stability. Thus, protein levels detected with our antibodies reflect additional HIF stabilization over normoxic levels, following 12 h of hypoxia in both groups, and the only difference between both subpopulations was observed in the air exposure experiment, where HIF accumulated after 12 h only in intertidal limpets.

Over all, we could show that hypoxic gene regulation is initiated in Antarctic limpets, although after quite long exposure time, and the response during stressful air exposure is limited to the intertidal specimens. Sub-littoral limpets adapted to be permanently submerged in cold water with high and predictable oxygen concentration survived 48 h of 2% oxygen without a single death in our experiments and were able to up-regulate HIF protein levels, but failed to do so upon air exposure. The difference in HIF up-regulation between populations upon aerial exposure can be explained by the changes of the PO_2 in mantle cavity water we recorded during submergence and air exposure of individuals from both subpopulations (Weihe & Abele, 2008). These recordings in the top of the shell, and thus close to the limpets´ own sensory organs (Lindberg & Ponder, 2001), document a significant reduction of the mean PO_2 in intertidal limpets from 2.6 kPa (submerged) to 0.1 kPa (air exposed), whereas the sub-littoral animals maintained PO_2 at 1.3 kPa (submerged) and 1.5 kPa during air exposure (Table 4 of Weihe & Abele 2008). Thus, although both groups of limpets conserved the capacity for hypoxic gene regulation, the sub-littoral subpopulation lacks the adequate behavioural/physiological response to air exposure, which finally stabilize the hypoxic transcription factor.

Time pattern of the hypoxic response in limpets

Up-regulation of the HIF-α protein in digestive gland of limpets exposed to 2% O_2 took longer (12 h) than in mice, where HIF-α was stabilized in different tissues following 1h of systemic hypoxia exposure at 6% O_2 (Stroka et al., 2001). In the crucian carp, HIF-1α protein levels were significantly increased in different tissues following 6h of exposure of the fish to 6-8% O_2 (Rissanen et al., 2006). Although the authors did not test shorter incubation times, HIF-1α protein up-regulation was faster than in limpets, where we found only insignificantly higher protein levels after 6h. This indicates hypoxic gene transcription is not required at an early stage of the low oxygen survival program in limpets, but starts only, if the hypoxic state persists longer then usually experienced in the natural habitat (for example tidal

oscillations). Anaerobic metabolites, especially succinate from fumarate reduction and acetate started to accumulate already after 2 h of hypoxic exposure in limpet foot muscle (Figure 7). Part of the succinate is apparently converted to propionate in the mitochondria, but succinate levels remained high until 12 h of air exposure and then declined again, most pronouncedly in the intertidal limpets (after 24 h) and in both groups after 48 h of hypoxia. Elevated succinate (Selak et al., 2005) and fumarate levels (Pollard et al., 2005) inhibit HIF-α prolyl hydroxylases that results in the accumulation of HIF-α. High organic acid levels could thus be causal for inactivation of the PHDs, which would inhibit or reduce hydroxylation of the functional prolines in the HIF-α ODD and thus degradation of the oxygen regulated subunit in the limpets. A subsequent drop in succinate concentration to control level could then also explain the complete reversion of the HIF protein signal after 24h hypoxia in limpet DG. Alternatively, increasing evidence suggests a negative feedback of HIF-1 directly on PHD activity. Marxsen et al. (2004) found increased PHD expression induced by HIF-1 itself, which leads to enhanced HIF-1α degradation under prolonged hypoxia in human cells. The feedback mechanism can further be confirmed by metabolic modelling, suggesting PHD expression to be at least in part regulated via a HRE (see Kohn et al. 2004). The transitory character of HIF up-regulation in hypoxic tissues has already been reported from most systemic investigations of HIF-1 (Marxsen et al., 2004; Rissanen et al., 2006). It is mainly attributed to the induction of PHDs by HIF itself and seems to be necessary to prevent tumour formation by HIF over-expression in mammals (Kohn et al., 2004; Pugh & Ratcliffe, 2003).

Conclusion

To conclude, Antarctic limpets carry an ancestral HIF gene that contains all important functional regions of the later evolved mammalian sequence, with the exception of the N-terminal transactivation domain. Also, one suspicious amino acid exchange in the Pro^{564} ODD was recorded between limpets and mammals that may affect Pro^{564} hydroxylation by the PHD in limpets. In conjunction with existing data comparing mammalian and invertebrate pVHL protein conformation, the present sequence analysis suggests that the rapid oxygen-dependent degradation pathway of HIF-α in mammals might be less active and slower in hypoxia tolerant invertebrate fauna. Given that limpets are hypoxia tolerant, there would also be no need for a rapid gene-regulatory response to low oxygen. The relatively late onset of HIF-α protein stabilization after 12 h of hypoxia may thus reflect response in a later phase of hypoxic survival, in which metabolic resources that bridge low oxygen availability are becoming exhausted. Succinate that inhibits PHDs and induces HIF levels in a transgenic human cell model (Selak et al., 2005) rapidly accumulates in hypoxia exposed limpets and may represent a modulatory mechanism of HIF stabilization. This includes the downfall of

HIF levels after 24 h of hypoxia, when succinate concentrations also declined. Differences between intertidal and sub-littoral limpet HIF response were manifest during air exposure, where the sub-littoral specimens were not effectively able to close the shell and reduce mantle cavity water PO_2. This coincides with non-stabilized HIF protein levels as were found in (hypoxic) intertidal specimens. Thus, HIF may indeed be important during prolonged hypoxic conditions in organisms naturally facing fluctuating oxygen concentrations. A consequent behavioural response and metabolic adaptation may be key mechanisms in the intertidal specimens that enable onset of a genetic regulatory response under prolonged stress exposure.

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4 Additional Results

4.1 Adenylate concentrations during hypoxia

Total adenylate concentrations were measured in foot tissues of intertidal and sub-littoral *Nacella concinna* exposed to hypoxia (Table 4-1). Intertidal animals had higher adenylate concentrations than limpets from the sub-littoral ($p < 0.0001$; $F = 25.15$; $n = 60$ two-way ANOVA). This significant difference resulted from higher amounts of ATP ($p < 0.0001$; $F = 28.88$; $n = 69$) and ADP ($p = 0.003$; $F = 9.625$; $n = 67$). The later was also affect by hypoxia in both subpopulations ($p = 0.02$; $F = 2.945$; interaction $p = 0.45$; $F = 2.448$; two-way ANOVA). AMP concentrations in contrast did not differ between subpopulation nor was it affected by hypoxia. The energy charge (EC), calculated from ATP, ADP and AMP concentrations according to Atkinson (1968), was also elevated in limpets from the tidal zone compared with limpets from the sub-littoral (subpopulation $p = 0.011$, $F = 7.109$), which had going line with that higher AMP/ATP ratios than the intertidal animals ($p = 0.0029$; $F = 9.813$; $n = 61$ two-way ANOVA). The EC was not only affected by the subpopulation but also by hypoxia (hypoxia $p = 0.0247$; $F = 2.920$; $n = 51$; two-way ANOVA).

Table 4-1: Adenylate concentrations in foot muscle of intertidal and sub-littoral *Nacella concinna* during hypoxia.
Concentration in µmol g^{-1} FW. Hypoxia = 2% oxygen. Means ± SD. p-values indicate the factor exposure time or respectively subpopulation. EC = energy charge; Total = ATP + ADP + AMP.

intertidal	ATP	ADP	AMP	Total	EC	AMP/ATP
0 h	0.3206 ± 0.09	0.06575 ± 0.02	0.0188 ± 0.01	0.4282 ± 0.14	0.8605 ± 0.04	0.0634 ± 0.03
2 h	0.4425 ± 0.13	0.0786 ± 0.01	0.01733 ± 0.01	0.5455 ± 0.15	0.8924 ± 0.02	0.037 ± 0.014
6 h	0.3772 ± 0.1	0.0686 ± 0.01	0.01225 ± 0.004	0.4993 ± 0.1	0.9097 ± 0.01	0.028 ± 0.01
12 h	0.4133 ± 0.12	0.1025 ± 0.03	0.02775 ± 0.01	0.541 ± 0.19	0.857 ± 0.01	0.0665 ± 0.01
24 h	0.33 ± 0.08	0.07 ± 0.02	0.022 ± 0.01	0.4326 ± 0.09	0.8606 ± 0.04	0.0644 ± 0.04
48 h	0.4147 ± 0.09	0.0808 ± 0.01	0.02217 ± 0.01	0.4958 ± 0.08	0.8692 ± 0.03	0.05833 ± 0.03
p-value	0.3428	0.0315	0.1978	0.5769	0.1263	0.1117
F	1.182	2.952	1.596	0.7761	1.991	2.021
N	5 - 6	4 - 6	4 - 6	4 - 6	3 - 5	4 – 6

sub-littoral	ATP	ADP	AMP	Total	EC	AMP/ATP
0 h	0.2616 ± 0.03	0.08117 ± 0.03	0.03733 ± 0.02	0.3738 ± 0.06	0.8034 ± 0.05	0.1442 ± 0.09
2 h	0.3502 ± 0.1	0.08133 ± 0.03	0.022 ± 0.02	0.433 ± 0.17	0.8638 ± 0.03	0.06275 ± 0.04
6 h	0.2285 ± 0.1	0.045 ± 0.01	0.0148 ± 0.01	0.2586 ± 0.08	0.8683 ± 0.03	0.0946 ± 0.08
12 h	0.2267 ± 0.09	0.061 ± 0.02	0.02267 ± 0.01	0.3095 ± 0.12	0.8383 ± 0.03	0.0955 ± 0.03
24 h	0.2795 ± 0.1	0.05717 ± 0.02	0.0146 ± 0.004	0.3496 ± 0.13	0.8702 ± 0.05	0.0678 ± 0.05
48 h	0.2083 ± 0.06	0.05417 ± 0.02	0.01833 ± 0.01	0.2807 ± 0.07	0.8508 ± 0.01	0.09483 ± 0.08
p-value	0.1002	0.0331	0.1325	0.1932	0.1090	0.4744
F	2.055	2.84	1.880	1.613	2.091	0.9367
N	5 - 6	5 - 6	4 - 6	4 - 6	4 - 5	4 - 6

population	ATP	ADP	AMP	Total	EC	AMP/ATP
p-value	< 0.0001	0.003	0.6124	< 0.0001	0.0111	0.0029
F	28.88	9.625	0.2598	25.15	7.109	9.813
N	69	67	63	60	51	61

4.2 Ascorbyl radical content during hypoxia

The ascorbyl radical (•A) content in digestive glands of hypoxia exposed *Nacella concinna* was measured by Paula M. Gonzalez (University of Buenos Aires, Argentina) by electron paramagnetic resonance (EPR)-spin trapping. Measurements were performed at room temperature (18°C) using a Bruker (Karlsruhe, Germany) spectrometer ECS 106 with a cavity ER 4102ST. Homogenates were done as described in González et al. (2008) with the difference that the digestive gland tissue of *N. concinna* was homogenated 1:20 instead of 1:4 as *Mya arenaria*. For instrument settings see González et al (2008).

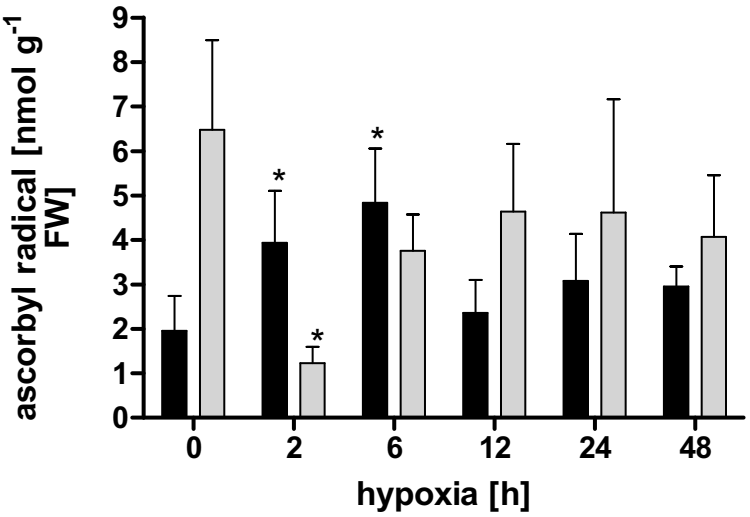

Figure 4-1: Ascorbyl radical concentration in the digestive gland of intertidal and sub-littoral *N. concinna* during hypoxia.
Concentrations in nmol g^{-1} FW. Intertidal = black bars; sub-littoral = grey bars. * indicate significantly different values from the control group. Means + SD. N = 4-6 limpets per group.

Sub-littoral limpets (6.477 ± 2.014 nmol g^{-1} FW) had significant higher ascorbyl radical concentrations than the intertidal limpets (1.955 ± 0.7862 nmol g^{-1} FW) under normoxic conditions ($p < 0.001$, Bonferroni) but decreased significantly ($p < 0.001$, Tukey´s test) temporary even below intertidal levels within the first two hours ($p < 0.01$, Bonferroni). Whereas intertidal limpets had significantly elevated •A levels after two and six hours ($p < 0.05$ and $p < 0.001$). •A concentrations were significantly changed by hypoxia in both subpopulations (intertidal $p = 0.0002$; $F = 7.022$; sub-littoral $p = 0.0005$; $F = 6.475$, one-way ANOVA).

5 Discussion

In the present work, the physiological and molecular response of intertidal and sub-littoral *Nacella concinna* to desiccation and hypoxia was examined. The aim of this thesis was to investigate whether the intertidal limpets developed an adaptation to the stress factors they may regularly face in their environmental.

Results from three publications and additional results will be reviewed and discussed.

5.1 Hypoxic Metabolism in *N. concinna*

Intertidal limpets are more active than their sub-littoral congeners, they have higher adenylate concentrations and a higher energy charge (EC) in their foot tissue (Publ. I; Table 5 and Table 4-1). Even under hypoxia they accumulated more lactate and succinate at a faster rate than sub-littoral limpets (Publ. III; Figure 7). During exposure to both stressors the EC remained constant in both subpopulations (Publ. I ; Table 5 and Table 4-1), which, on the one hand may be due to reduced metabolism and/or energy demand, and on the other hand due to elevated ATP turn over rates (Pasteur effect). Potentially this reduced energy demand caused the limpets to detach from the glass walls of the incubation jars after prolonged hypoxia exposure (personal observation). Despite this loss in adhesion they did not utilize the succinate-propionate pathway to gain an extra ATP molecule per mol of substrate. Another reason for the detachment behaviour could be a flight response against hypoxia as described by Davenport (2001) against freshwater. To verify whether this detachment is a result of depleted energy storages or a flight response, glycogen and aspartate concentrations could be examined.

The foot muscle of sub-littoral *N. concinna* turns anaerobic during aerial exposure as well as hypoxia. Comparing the anaerobiosis during both treatments reveals significant differences in the accumulation of lactate and succinate (Figure 5-1). Whereas both metabolites started to accumulate right from the beginning during hypoxia, succinate even faster than lactate, both metabolites increased significantly after only 12 h of air exposure. These data show that aerial exposure does not immediately lead to oxygen deprivation. This assumption is supported by oxygen measurements in the extrapallial water, which showed high oxygen concentrations in sub-littoral limpets during eight hours of air exposure (publ. I). It is very likely that the animals started to close their shells after prolonged exposure, to prevent further desiccation, resulting in declining oxygen concentrations in the inner shell water. In addition excessive muscle contraction due to clamping down against the substratum can result in functional anaerobiosis. After 12 h the limpets have very high glycolytic turn over rates (Pasteur effect), which can be deduced from the massive increase in lactate and

succinate. This is supported by the fact, that propionate was accumulated in 4 out of 9 limpets, whereas under hypoxic conditions, propionate accumulation was not found. Furthermore, unchanged low HIF-α protein levels in the digestive gland of sub-littoral limpets during the whole aerial exposure (up to 24 h) (Publ. III; Figure 6) indicate that oxygen concentrations in the extrapallial water are too high for HIF-α stabilisation, indicating that not the whole organism becomes anaerobic, which would be the case during environmental anaerobiosis. Taking all these facts together strengthen the proposition that the accumulation of anaerobic metabolites in foot tissue is presumably caused by functional anaerobiosis resulting from muscular contraction of the limpets, trying to clamp down against the substratum. The Antarctic limpet, a non-homing limpet, is not able not make a tight seal between shell and substratum (Davenport, 2001). This is especially so for sub-littoral limpets, which have a curved shell margin. These little gaps may allow oxygen to diffuse into the extrapallial water.

Although succinate levels had already decreased after 12 h of aerial exposure, they remained elevated for up to 24 h during hypoxia. In limpets, glycogen and aspartate are used for anaerobic energy production (Michaelidis & Beis, 1990), It is likely that glycogen and aspartate were already depleted by the extensive lactate and succinate production and by the increased aerobic metabolic rates within the first hours. This was previously described by Michaelidis and Beis (1990), who exposed the limpet *Patella caerulea* to air for up to 16 hours.

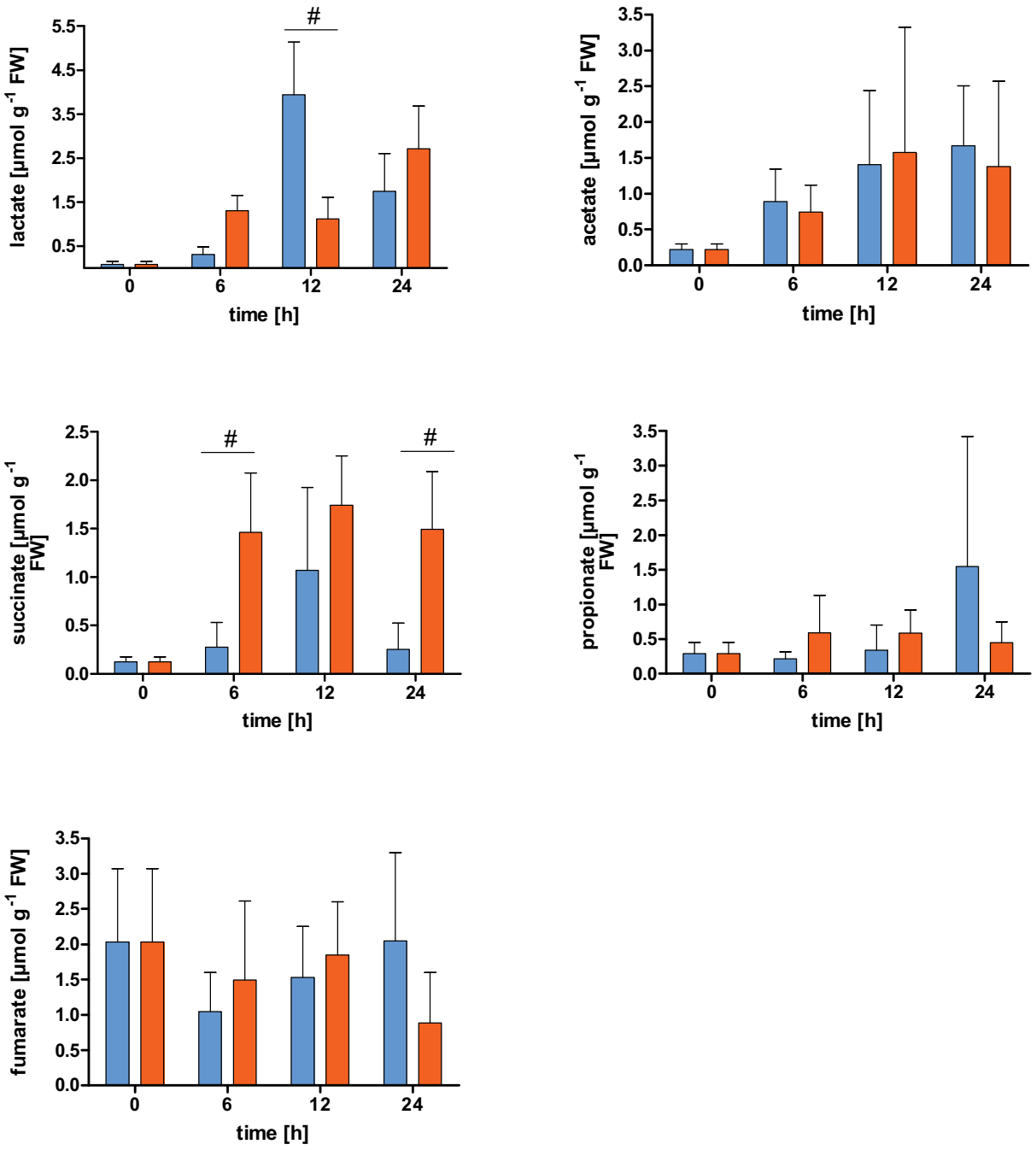

Figure 5-1: Anaerobic metabolites in foot muscle of sub-littoral *Nacella concinna*.
Concentrations in µmol g^{-1} FW. Blue bars: air exposure, orange bars: hypoxia. Means + SD. N = 5-9 limpets per group. # indicates significant differences ($p < 0.001$) between the two treatments.

In intertidal limpets the effect of desiccation and hypoxia is different. During aerial exposure the foot tissue remains aerobic (Publ. I; Figure 5), during hypoxia the anaerobic metabolites succinate and lactate were accumulated within the first hours (Publ. III; Figure 7). Therefore the accumulation pattern of lactate and succinate differed significantly ($p < 0.0001$) between the two treatments. In contrast acetate, propionate, and fumarate concentrations did not differ but were influenced by the exposure time (Figure 5-2).

Nevertheless, the possibility that inter-tidal limpets become oxygen limited in their natural environment seems to be quite unlikely.

These findings correspond to the study of Brinkhoff and colleagues (1983) who reported an accumulation of succinate in the common limpet *Patella vulgata* only under anoxic condition and not during air exposure. Moreover the foot muscle had a constant EC during both treatments, as seen for the sub-littoral limpets, indicating a reduced energy demand or Pasteur effect. The higher EC and lower AMP/ATP ratio seen after prolonged air exposure (12 h and 24 h) when compared to hypoxia (data not shown) indicates that for intertidal limpets aerial exposure is less energetically stressful. Presumably this is because they experience aerial exposure much more often in their natural setting than hypoxia.

These results also show that aerial exposure does not necessarily lead to hypoxia in inter-tidal limpets. It is very likely that the intertidal limpets take-up oxygen via the lateral margin of the foot and their pallial gills. During air exposure intertidal limpets exhibit elevated malondialdehyde concentrations in their foot muscle during air exposure compared with sub-littoral *N. concinna*. MDA is a marker for lipid peroxidation and together with a more oxidized GSSG/GSH ratio after 12 h of air exposure these findings support the hypothesis of oxygen up-take via the foot margin. McMahon (1988) reported temperate molluscs to have even higher respiration rates when air exposure than when covered by water. Despite their ability to take-up oxygen during aerial exposure, the oxygen content in the extrapallial water of exposed intertidal *N. concinna* was significantly lower than in submerged animals (Publ. I; Table 4). As the foot muscle of intertidal limpets remains aerobic, it may maintain the ability to contract the foot muscle even during prolonged air exposure and stay attached to the substratum. Elevated HIF-α protein levels in digestive glands of intertidal limpets after 12 h of air exposure (Publ. III; Table 6) may potentially be a result of the reduced oxygen concentrations in the surrounding extrapallial water. These results, in combination with the low water loss, argue for reduced or even ceased ventilation.

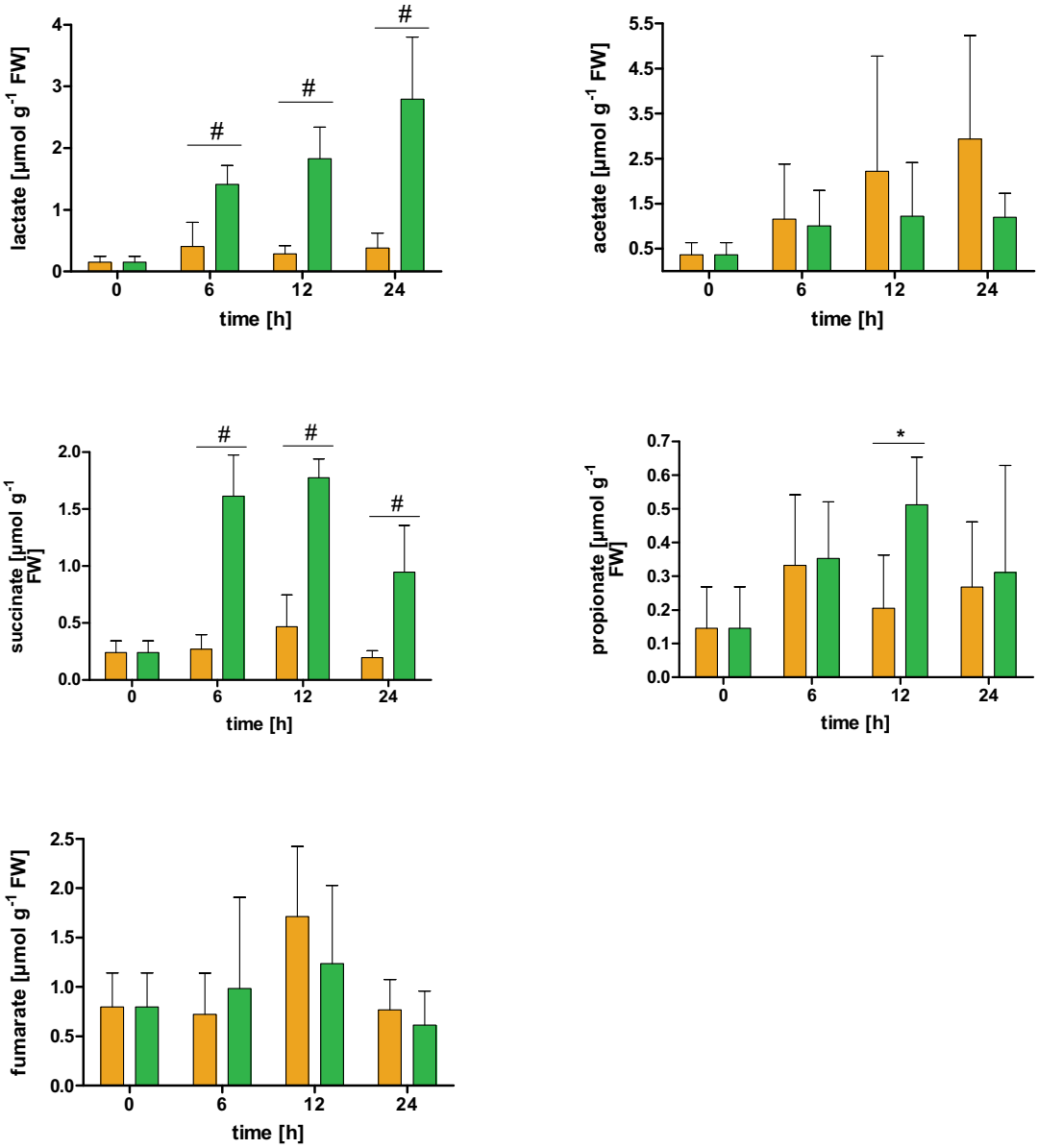

Figure 5-2: Anaerobic metabolites in foot muscle of intertidal *Nacella concinna*.
Concentrations in µmol g^{-1} FW. Yellow bars: air exposure, green bars: hypoxia. Means + SD. N = 5-10 limpets per group. # and * indicate significant differences between the two treatments (# $p < 0.001$; * $p < 0.05$).

5.2 The transcription factor HIF-α in *N. concinna*

This thesis presents the first characterization of a molluscan HIF-α, which was isolated from the Antarctic limpet *N. concinna*. Phylogenetic analyses of the deduced peptide sequence and available invertebrate and vertebrate sequences indicate invertebrates to possess an early ancestral version of the HIF-α protein. The evolution of different HIF-α isoforms (HIF-1α, HIF-2α, and HIF-3α) appears to have occurred only in vertebrates, including fish (Rahman &

Thomas, 2007). Invertebrate species including the intensively studied nematode *Caenorhabditis elegans*, the crustaceans *Palaemonetes pugio* (Li & Brouwer, 2007) and *Liopenaeus vannamei* (Soñanez-Organis et al., 2009) and the extensively sequenced oyster *Crassostrea gigas* (AB289857), were thus far reported to only possess one HIF-α isoform. Similarly, Leite et al. (2008) found only one prolyl hydroxylase (PHD) isoform in the marine protist *Perkinsus olseni*, and the same is reported from other ancient eukaryotes. However, in vertebrates up to four PHD isoforms have been described. This may indicate a co-evolution, as hypothesised by Bird (1995) for major evolutionary developments. Furthermore Bird (1995) reported the transition from invertebrates to vertebrates to be a period of macroevolutinary change, where biological complexity increased. He referred to the ancestral, monomeric globin of invertebrates, in contrast to the heterotetramer globin molecules in vertebrates, as an example for an increase in complexity in vertebrates.

The HIF-α protein of invertebrates lacks the N-terminal transactivation domain which is located within the C-terminal oxygen-dependent degradation domain of the vertebrate HIF-α protein. Nevertheless, all three functional hydroxylation sites (two proline and one asparagine residue) of the HIF- α subunit are present in the invertebrate HIF-α peptide sequences. Hydroxylation of the two proline residues (Pro^{402} and Pro^{564} in human) is the initial step of the proteasomal degradation of HIF-α (Ivan et al., 2001). The hydroxylation of Asn^{803} by FIH (factor inhibiting HIF) prevents transcriptional activation of HIF (Lando et al., 2002).

The proline motif (IGQRA**P**FIP) in *Nacella concinna* and other available invertebrate sequences exhibit an Arg or Lys in the place of the Leu^{562} of the vertebrate sequence (vertebrates: LXX**L**AP; *Nacella*: IGQ**R**AP). Ivan et al. (2001) described the proximal Leu residues to be essential for the proline hydroxylation, the initial step for HIF-α proteasomal degradation, as substitution of Leu^{562} by alanine inhibited proline hydroxylation. As the motif in front of the second proline (Pro^{564} in human) shows this potentially fundamental substitution the necessity of both proline residues in the degradation process of invertebrates' HIF-α is questionable. The finding of Heise et al. (2005), who reported the first of the two proline residues (Pro^{402} in human) to be substituted by a leucine in the Antarctic eelpout *Pachycara brachycephalum* (AAZ52828) supports this consideration and expands it for fish. Masson et al. (2001) indeed could show that both proline residues were hydroxylated independently from each other, and that the loss of one hydroxylation site resulted in reduced degradation of HIF-1α isolated from rabbit reticulocytes. Nonetheless substitution of the proline, or rather in the proline motif, may not account exclusively for HIF-α protein levels under normoxic conditions. Rissanen et al. (2006) also reported HIF-1α protein levels in normoxic tissues of crusian carp (*Carassius carassius*) which exhibit no amino acid exchange in the discussed regions.

The question that still remains open is why invertebrates and fish exhibit detectable HIF-α protein levels under normoxic conditions. Especially as iron and cadmium concentrations in digestive gland tissues of *N. concinna* (Table 5-1) are a multiple of the concentrations described to trigger HIF-1α degradation in cell lines (Chun et al., 2000; Li et al., 2006).

As oxygen concentrations in water are only 1/30th of oxygen in the same volume of air, aquatic invertebrates and fish are therefore adapted to lower oxygen concentrations (Randall et al., 1997). Therefore it is conceivable that in aquatic organisms the master regulator of hypoxic response is regulated in a different way. Especially in hypoxia-tolerant animals such as *N. concinna* and the crucian carp.

Sikora and Godzik (2004) investigated the von Hippel-Lindau protein (VHL), which binds to the hydroxylated HIF-α and initiates ubiquitilation subsequently leading to proteasomal degradation of HIF-α. They found VHL isolated from invertebrates to vary from vertebrates VHL, leading to the presumption of other HIF-α regulating mechanisms in lower organisms than the VHL mediated degradation. They postulated HIF-1 deactivation to be the primary factor in lower species, instead of the HIF-α degradation, which is the primary factor in human cells.

Taking together all of these results, the question arises whether HIF-α from the hypoxia-tolerant *N. concinna* is regulated in a similar oxygen-dependent manner as reported for mammals, or if other factors than oxygen play a crucial role for HIF-α degradation.

5.2.1 Regulation of HIF-α during stress

The transcription factor HIF (hypoxia-inducible factor) is a αβ heterodimer in which the stability of the α-subunit is regulated by oxygen-dependent posttranslational hydroxylation of two functional proline residues. This process targets HIF-α for ubiquitylation and subsequent proteasomal degradation. During the hydroxylation reaction of the prolyl hydroxylases (PHDs), molecular O_2 is split and one of the resulting oxygen atoms is used for the hydroxylation of HIF-α, whereas the other oxygen is used for the oxidative decarboxylation of 2-oxoglutarate to succinate and CO_2. During this reaction Fe^{2+} is oxidized to Fe^{3+}, which has to be reduced again to Fe^{2+} by ascorbate. Changes in the availability of the multiple cofactors iron, ascorbate, and 2-ooxoglutarate may modulate “oxygen sensing” by altering the rate of hydroxylation at any given oxygen concentration (Kaelin Jr & Ratcliffe, 2008).

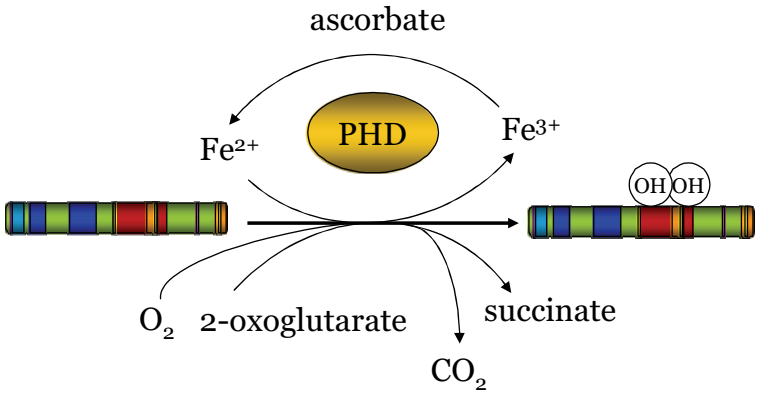

Figure 5-3: Reaction scheme of HIF-α hydroxylation by PHDs.
According to Kaelin Jr & Ratcliffe, 2008.

HIF-α protein levels in the digestive gland of both *Nacella concinna* subpopulations increased significantly after 12 h of hypoxia (2 kPa oxygen) (Publ. III; Figure 4) and after 12 h of air exposure in the intertidal subpopulation (Publ. III; Figure 5). Guzy and colleagues (2005) described elevated H_2O_2 (hydrogen peroxide) concentrations in the cytosol during hypoxia to have an inhibiting effect on the PHDs, and thus stabilizing the HIF-α protein. O_2^- (superoxide) is released in higher amounts during hypoxia by the reduced mitochondrial electron transport chain (Chandel et al., 1998; Guzy et al., 2005) and can be than dismutated to H_2O_2 superoxide dismutase (SOD) or nonenzymatically (Dröge, 2002). Additionally O_2^- can be produced by several enzymes such as NAD(P)H oxidases and xanthine oxidases. In *N. concinna* a nonenzymatically H_2O_2 formation seems to be most likely during hypoxia, as SOD activities decreased significantly during hypoxia (Publ. II; Figure 2a). The inhibiting effect of H_2O_2 may potentially result from decreasing Fe^{2+} concentrations due to elevated Fenton reaction rates, which oxidises Fe^{2+} to Fe^{3+}. If Fe^{2+} becomes insufficient PHD activities decrease and HIF-α accumulates. Cytosolic catalase (CAT) in contrast, reverses the effect of H_2O_2 on HIF-α and destabilises accumulated HIF-α (Guzy et al., 2005), probably due to its H_2O_2 degrading activity without utilizing any Fe^{2+}. Decreased CAT activities in the digestive gland of intertidal *N. concinna* after 12 h of air exposure could therefore theoretically have accelerated HIF stabilization. Under normoxia H_2O_2 and CAT were found to have no effect on HIF stabilization (Brunelle et al., 2005).

Ascorbate reduces Fe^{3+} to Fe^{2+} and thereby increases prolyl hydroxylation. Ascorbate concentrations in the foot tissue of *N. concinna* (intertidal 80 µM; sub-littoral 64 µM; calculated from data in Publ. II; Table 3) are lower than the K_m values of the PHD for ascorbate determined in mammalian cells (140 µM, Table 5-1) (Koivunen et al., 2004). Low ascorbate concentrations could result in an insufficient reduction of Fe^{3+} to Fe^{2+}, which could result in limited PHD activities. As HIF-α protein levels were measured in the digestive

gland, whereas ascorbate concentrations were determined in the foot tissue, the influence of ascorbate on HIF-α in *N. concinna* is not predictable under these circumstances. Both parameters need to be determined in the same tissue.

Succinate and lactate accumulated significantly in the foot tissue of both subpopulations during hypoxia (Publ. III; Figure 6). Succinate (Selak et al., 2005) and fumarate (Pollard et al., 2005) were described to impede PHD activity by inhibiting the decarboxylation of 2-oxoglutarate to succinate (product inhibition). The effect of fumarate, which is even higher than of succinate (Koivunen et al., 2007) (for K_i values see Table 5-1), is probably caused by its chemical similarity to succinate even though it is more rigid than succinate (Pollard et al., 2005). Presuming a comparable accumulation of anaerobic metabolites in the digestive gland as in the foot, at least during environmental hypoxia, increased succinate levels could potentially have accounted for HIF-α accumulation after 12 h of hypoxia. Succinate concentrations in normoxic limpets were below the K_i values described for mammalian cells (Table 5-1), but exceeded the K_i value of PHDs within the first two hours of hypoxia. The decrease of succinate levels after 24 h (intertidal limpets), or rather 48 h (sub-littoral limpets), may not have caused HIF-α degradation after 24 h of hypoxia exclusively, as succinate concentrations remained above the K_i values. However, increasing succinate levels could potentially have initiated the stabilisation of HIF-α whereas the degradation after prolonged hypoxia may have been triggered by another factor such as the negative feedback mechanism described by Marxsen and colleagues (2004), This will be discussed in more detail below. Fumarate concentrations in the foot tissue of both subpopulations were higher than the described K_i values during the whole incubation time and could thus have an inhibiting effect on PHD activity resulting in HIF-α stabilization, even under normoxic conditions. When discussing K_i values for succinate and fumarate it has to be considered that the existing information has been determined in mammalian cell lines (Pollard et al., 2005; Selak et al., 2005), where succinate concentrations can also increase during anaerobiosis, but probably not to an extent as in marine invertebrates (Starkov, 2009). Therefore it is very likely that K_i values of PHD for the organic acids may be higher in invertebrates such as *N. concinna*. Further studies, preferably with cells isolated from limpets, are required to investigate the direct effect of co-factors and products on HIF stabilisation, or rather PHD activities.

During aerial exposure only sub-littoral *N. concinna* accumulated significant succinate and lactate concentrations in the foot muscle (Publ. I; Figure 5). The foot of intertidal limpets in contrast remained aerobic. As discussed in the passage above the accumulation of organic acids in the foot tissue may have presumably resulted from functional anaerobiosis and therefore anaerobic conditions can not be assumed for the whole organism. This is supported by the PO_2 measurements in the extrapallial water of air exposed and submerged limpets.

These measurements showed a reduction of O_2 concentrations in intertidal limpets during aerial exposure, whereas sub-littoral limpets exhibited unchanged O_2 levels (Publ. I; Table 4). Since the digestive gland is enclosed by the extrapallial water, its O_2 concentrations may be influenced by O_2 levels in the water.

Taking these findings together HIF-α protein concentrations in the digestive gland during aerial exposure (Publ. III; Figure 5) seem to be mainly influenced by O_2 concentrations in the extrapallial water.

Table 5-1: Km and Ki values of parameters influencing prolyl hydroxylase activity.
a) Koivunen et al. (2004); b) Koivunen et al. (2007); c) Kaelin & Ratcliff (2008); d) Li et al. (2006); e) Chun et al. (2000)

	Human cells	Concentration in intertidal *N. concinna*	Concentration in sub-littoral *N. concinna*
O_2	K_m PHD: 100 µM [c]	means: 44 µM median: 58 µM max: 223 µM	means: 23 µM median: 22 µM max: 206 µM
Fe^{2+}	Threshold: 0.5 mM [d]	Iron: 1.22 mM	Iron: 11.95 mM
Cd	Threshold: 10 µM [e]	56 µM	68 µM
Ascorbate	K_m PHD: 140 µM [a]	80 µM	64 µM
Succinate	K_i PHD: 350 – 460 µM [b]	192 µM	100 µM
Fumarate	K_i PHD: 50 – 80 µM [b]	640 µM	1624 µM

The factors discussed so far could potentially have initiated the stabilisation of HIF-α, whereas the degradation after 12 h of stress exposure is presumably regulated by a different mechanism. After prolonged hypoxia, HIF-1 was found to increase the expression of the prolyl hydroxylases and initiate thereby its own destabilisation (Marxsen et al., 2004). The results of Benita and colleagues (2009), who identified the PHDs as target genes of HIF-1, strengthen the previously described theory of a “direct negative feedback mechanism”.

6 Conclusion and Perspectives

This study shows that *Nacella concinna* from sub-littoral zones are less resistant to aerial exposure than the intertidal subpopulation, which migrates every spring to the tidal zone for the summer months. To cope with aerial exposure, intertidal limpets have evolved a variety of behavioural, morphological, physiological and molecular strategies.

Intertidal *N. concinna* have steeper shells than their sub-littoral congeners and consequently a smaller circumference to volume ratio. The relatively smaller circumference presents a smaller surface of tissue from which water may be lost (Davies, 1969). In addition, shells with a higher shell height to shell length ratio facilitate heat loss by convection (Harley et al., 2009). This protects the limpets from the heat stress they may encounter during low tide when exposed to sun radiation.

In addition to shell morphology, intertidal *N. concinna* have evolved behavioural strategies to minimize water loss. They seem to reduce the ventilation rate (i.e. rate of extrapallial water exchange), as indicated by decreased O_2 concentrations in the extrapallial water. In contrast, sub-littoral limpets retain high extrapallial water oxygen concentrations and face a higher rate of water loss as a consequence. Differences in oxygen concentrations could also be a result of higher respiration rates in intertidal limpets than in sub-littoral *N. concinna* as described by Davies (1967). To verify the reason for the divergent oxygen concentrations in the extrapallial water respiration measurements are required.

Furthermore, intertidal limpets have the ability to take-up oxygen via the lateral margin of their foot. This enables the muscle to remain aerobic, while the foot of sub-littoral limpets becomes anaerobic during air exposure. The α-subunit of the transcription factor HIF (HIF-α) shows distinctly different stabilisation patterns in both subpopulations during aerial exposure. Whereas HIF-α accumulates in the digestive gland of intertidal *N. concinna* after 12 h of air exposure, it remains at control levels in limpets from the sub-littoral. In contrast oxidative stress parameters, or rather antioxidative enzyme activities, in the digestive gland and gills show a less pronounced difference between subpopulations during air exposure. Instead the oxidative stress response seems to be regulated in a more tissue-specific manner, probably due to *in-situ* differences such as metal concentrations and oxygen up-take.

Both subpopulations show similar stress responses to hypoxia on metabolic as well as molecular levels. Organic acids accumulate in foot tissues of both subpopulations in a similar way, the difference being a more intense accumulation in intertidal limpets. HIF-α is stabilized in the digestive gland of intertidal as well as sub-littoral limpets after 12 h of hypoxic stress and is down-regulated after an additional 12 h. These results suggest that the

intertidal limpets do not experience hypoxia more regularly than their sub-littoral congeners. Thus, hypoxia does not seem to be a consequence of aerial exposure. From this study it is apparent that the subpopulation used for the experiment has a major influence on the results obtained from environmental stressors such as aerial exposure.

Together, the results in this thesis demonstrate that intertidal *N. concinna* evolved adaptive strategies on behavioural, morphological, biochemical and molecular levels to inhabit the tidal zone. As glaciers retreat, intertidal zones are becoming increasingly ice-free and new intertidal areas emerge. The adaptations to air exposure outlined here may allow intertidal *N. concinna* to repopulate these new areas. Recent observations from Adelaide Island found *N. concinna* to stay in the intertidal covered by ice even in the winter months (Waller et al., 2006a). De Aranzamendi and colleagues (2008) recently found evidence for the onset of genetic divergence of the two subpopulations. However their study has been heavily debated due to their use of poor genetic markers. Nevertheless their data and the results of this thesis raise the question whether the Antarctic limpet *Nacella concinna* is diverging into two separated species similar to *Nacella deaurata* and *Nacella magellanica*, which inhabit different shore levels on the coasts of Patagonia and Tierra del Fuego in South America (De Aranzamendi et al., 2009; Malanga et al., 2004). This question can not be definitively answered in this thesis, but the results here show that the two subpopulations have two different physiological phenotypes. Further studies, such as the doctoral thesis of Kevin Pöhlmann (AWI), should clarify whether these differences are also mirrored at a genetic level.

The characterisation of the first molluscan HIF-α, isolated from *N. concinna*, clearly documents the expression of the HIF-α subunit in the hypoxia-tolerant limpet and its posttranslational regulation in a stress dependent manner. The results here indicate that HIF-α is probably regulated by the oxygen concentration. The phylogentic analysis of the HIF-α peptide sequence shows that invertebrates possess an ancestral version of the HIF-α protein. Furthermore, the evolution of different HIF-α isoforms appears to occur only in vertebrates. Similar results were found for the prolyl hydroxylases (PHD) (Leite et al., 2008). Therefore the investigation of the hydroxylation mechanisms of HIF-α in invertebrates or invertebrate cells would have the advantage of no interactions of different isoforms, as observed in mammalian cells.

In addition, further investigations are required to complete the picture of cell signalling, HIF-α regulation and functionality in early-evolved, hypoxia-tolerant invertebrates. As the co-factors of the HIF-α hydroxylation reaction, such as ascorbate, succinate and fumarate, were measured in different tissues than the HIF-α protein (due to small tissue sizes), considerations about regulatory mechanisms remain speculative. Further investigations, in

particular measurements of these co-factors in the same tissue as HIF-α, should be the next step in gaining information about potentially regulatory mechanisms. Even more precise would be to study HIF-α in cells. The use of cells isolated from *N. concinna* would allow the direct determination of possible effects and cellular concentrations of inhibitors and promoters of HIF-α degradation. Furthermore, investigations of DNA-binding and target gene expression are of great interest given that other HIF-α regulatory mechanisms, such as the transactivation, are presumed to play a dominant role in invertebrates (Sikora & Godzik, 2004).

Beyond that, the comparison of limpet species from different latitudes would be very interesting for an investigation of the polar adaptations of HIF-α, as has already been discussed in polar fish (Heise, 2005). Moreover a comparison of molluscs with different hypoxia-tolerance such as the very hypoxia-tolerant ocean quahog *Artica islandica* and the hypoxia-sensitive scallop *Pecten maximus* could reveal new insights into HIF-α regulation. It would be of great interest to investigate whether hypoxia-tolerance accounts for normoxic HIF-α protein levels and the delayed HIF-α accumulation in *N. concinna*.

Even though many questions remain unanswered concerning the HIF-α transcription factor and its regulation, an important first step has been taken, bringing significant insights into the regulation and expression of HIF-α in the Antarctic limpet *N. concinna*.

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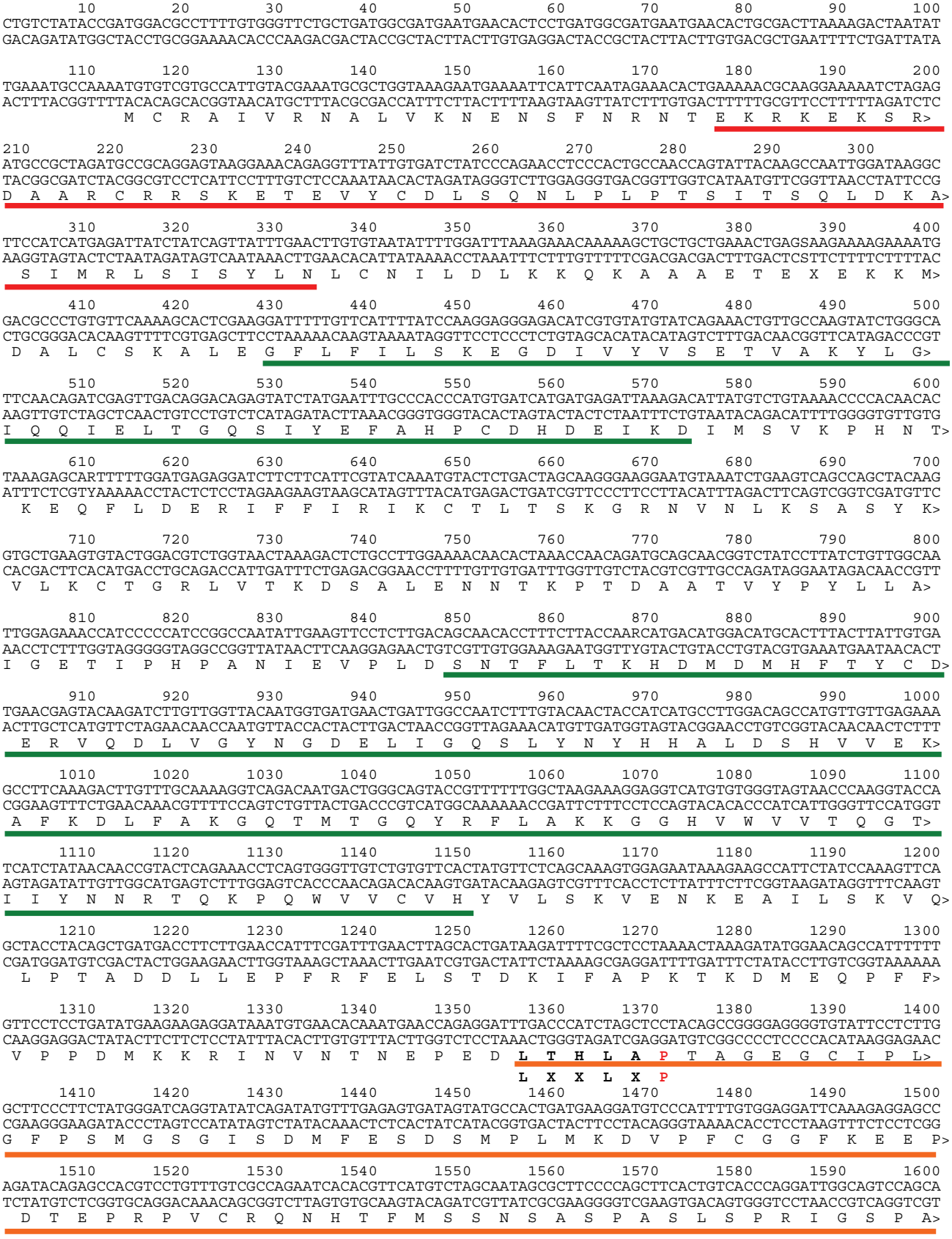
8 Appendix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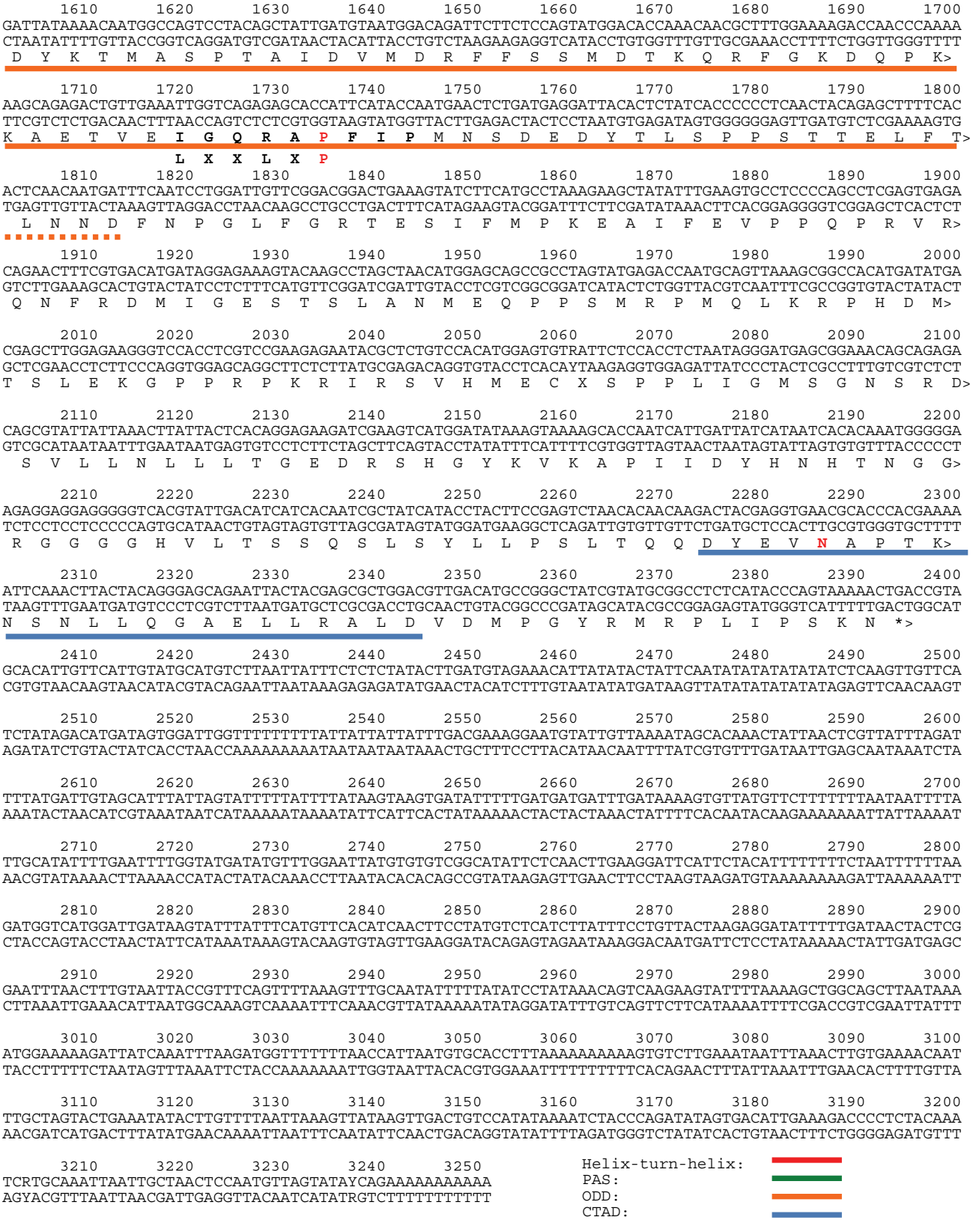

Figure 8-1: Primary sequence and deduced protein sequence of *N. concinna* HIF-α cDNA

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Bremen, 17. Dezember 2009

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Erklärung gem. § 6 Abs. 5 der PromO der Uni Bremen (vom 14. März 2007)

Ich erkläre hiermit,

1. dass ich die vorliegende Dissertation ohne unerlaubte Hilfe angefertigt habe,

2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe

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Ellen Weihe