



## 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 malondialdehyde 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.

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### 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<sub>2</sub> 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 near-shore 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<sup>2+</sup> is a well known Fenton reactant which, if not tightly bound to ferritin Fe-storage protein, catalyzes the reduction of H<sub>2</sub>O<sub>2</sub> to the detrimental hydroxyl radical (OH·) and hydroxyl anion (OH<sup>-</sup>) (Gonzalez et al., 2008). The resulting Fe<sup>3+</sup> can be reoxidized mainly by interaction with O<sub>2</sub><sup>-</sup> released in small amounts by the mitochondria, or in large quantities during oxidative burst reactions. Aluminum (Al<sup>3+</sup>) is the most abundant metal in the earth crust and although not a transition metal and Fenton reactant, exacerbates Fe<sup>2+</sup> 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<sub>2</sub><sup>-</sup> 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) (Stoys 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<sub>2</sub>) 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 submerged 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.

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<sub>2</sub> at Jubany–Dallmann. Samples were transported to Bremerhaven, Germany and stored in liquid N<sub>2</sub> 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<sub>3</sub> (65%) and 1 ml subboiled HF (40%) for 2 h at 60 °C, 3 h at 100 °C and 5 h at 160 °C in closed teflon beakers. Afterwards the samples were heated in 5 ml ultrapure H<sub>2</sub>O 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<sub>3</sub>. 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 µg L<sup>-1</sup> to 10 mg L<sup>-1</sup>. Cd calibration was in a range from 0.1 µg L<sup>-1</sup> to 2 µg L<sup>-1</sup>.

### 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 18,000g 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<sub>2</sub>O<sub>2</sub> 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 15,000g 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 15,000g 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 7500g 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<sub>2</sub>-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<sup>-1</sup> 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<sub>2</sub> 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 20,000g 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).

## 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 concen-

**Table 1**

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

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

trations (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 ( $-333 \pm 6.97$  mV) in the intertidal than the sub-littoral ( $-359 \pm 4.03$  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 2**

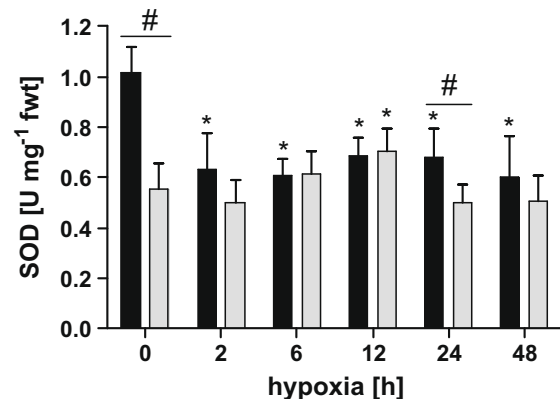
Superoxide dismutase (SOD) and Catalase (CAT) activities [ $\text{U} \times \text{mg}^{-1}$  fwt] in gill and digestive gland tissue of the Antarctic limpet *Nacella concinna*. Values were normally distributed and significant differences tested by unpaired *t*-test.  $n = 7$ –10, means  $\pm$  SD.

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

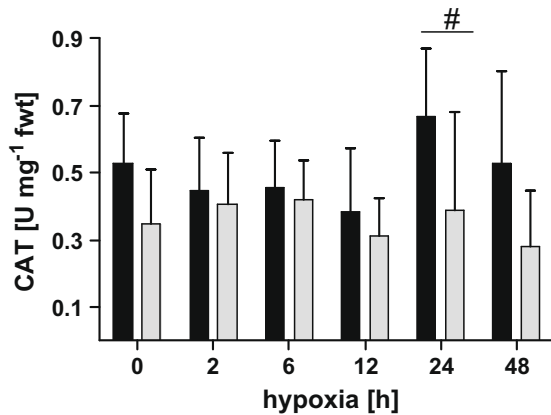
**Table 3**

Glutathione [ $\mu\text{mol} \times \text{g}^{-1}$  fwt] and ascorbate was measured in animals from 2007.

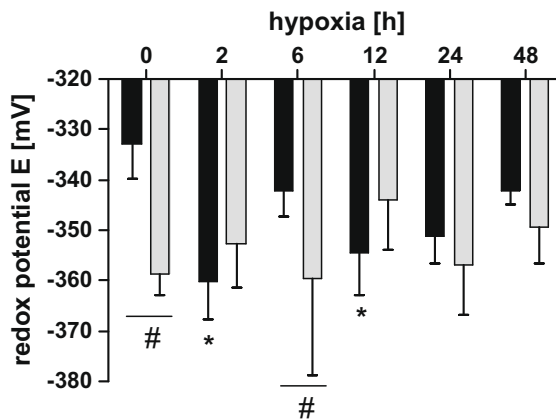
	Intertidal	Sub-littoral	<i>p</i> -Value
<i>(A) Gill</i>			
GSH	345.5 $\pm$ 61.04	390.2 $\pm$ 56.29	>0.05
GSSG	61.57 $\pm$ 51.36	64.42 $\pm$ 45.47	>0.05
GSSG/GSH	0.141 $\pm$ 0.06	0.136 $\pm$ 0.07	>0.05
<i>(B) Foot</i>			
GSH	450.8 $\pm$ 61.58	480.8 $\pm$ 144.4	>0.05
GSSG	34.7 $\pm$ 16.9	47.89 $\pm$ 26.22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



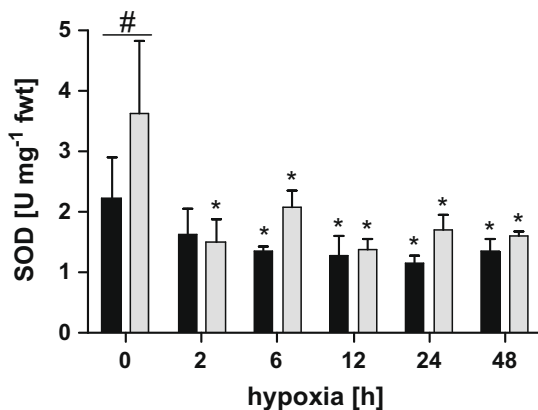
**Fig. 1a.** SOD activity in gills of *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Means  $\pm$  SD,  $n = 5$ –10. \* Significant difference from 0 h-value; one-way ANOVA. Intertidal  $p < 0.0001$ ;  $F = 12.55$ ;  $n = 35$ ; subtidal  $p = 0.0014$ ;  $F = 5.102$ ;  $n = 40$ . 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.



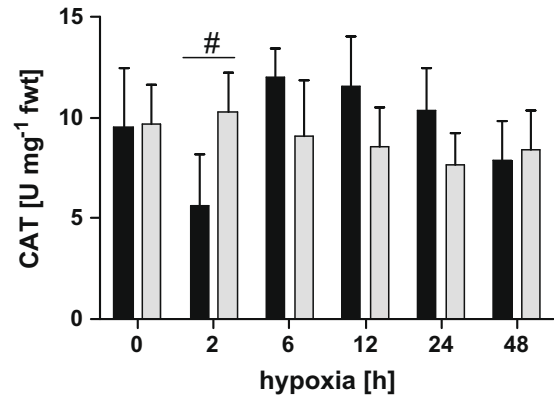
**Fig. 1b.** CAT activity in gills of *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Means + SD,  $n = 5-11$ . Subpopulation  $p = 0.001$ ;  $F = 11.76$ ,  $n = 81$  two-way ANOVA. # Significant difference between the subpopulations (two-way ANOVA, Bonferroni,  $p < 0.05$ ).



**Fig. 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:  $p = 0.0125$ ;  $F = 6.775$ ,  $n = 57$ .



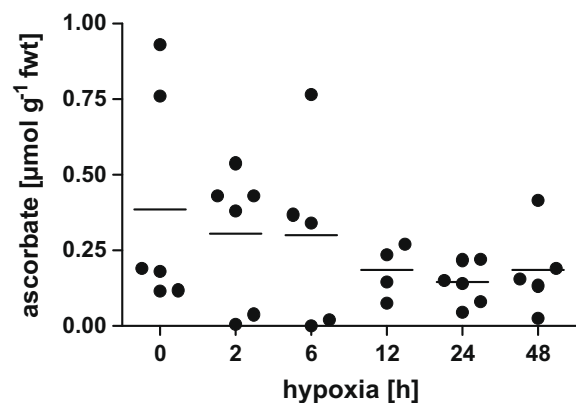
**Fig. 2a.** Superoxide dismutase activity in digestive glands of intertidal (black) and subtidal (grey) *Nacella concinna* during hypoxia incubation for several hours. Means + SD,  $n = 5-9$ . \* Indicates significantly lower activities than in the controls (one-way ANOVA). Intertidal  $p = 0.0002$ ;  $F = 6.976$ ;  $n = 37$ ; 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. 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.

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 ( $0.01 \pm 0.07 \mu\text{mol g}^{-1}$  fwt) than sub-littoral animal foot muscles ( $0.007 \pm 0.003 \mu\text{mol g}^{-1}$  fwt), but the difference was not statistically significant (Mann–Whitney test,  $p = 0.081$ ,  $U = 142$ ,  $n = 39$ ).

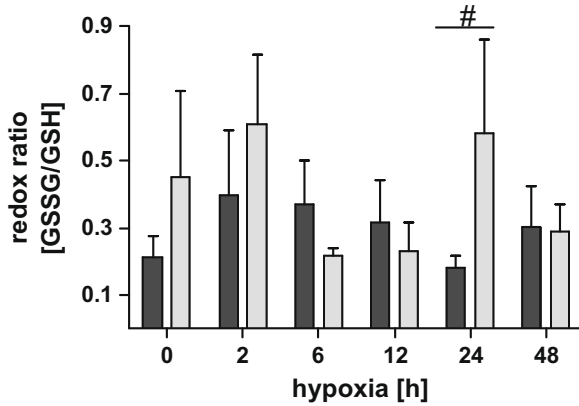


**Fig. 3.** Ascorbate concentration in foot muscle of intertidal *N. concinna* exposed to 2%-oxygen.

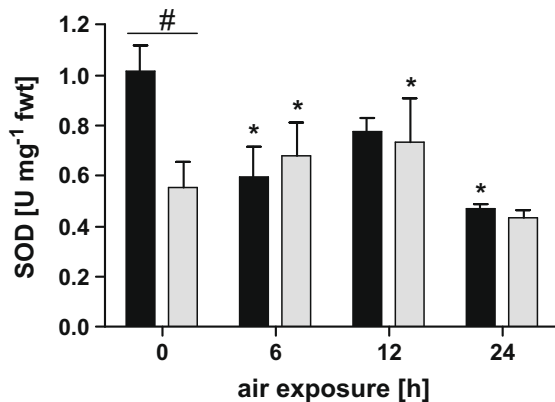
### 3.3. Effects of air exposure on tissue antioxidant parameters

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 sig-



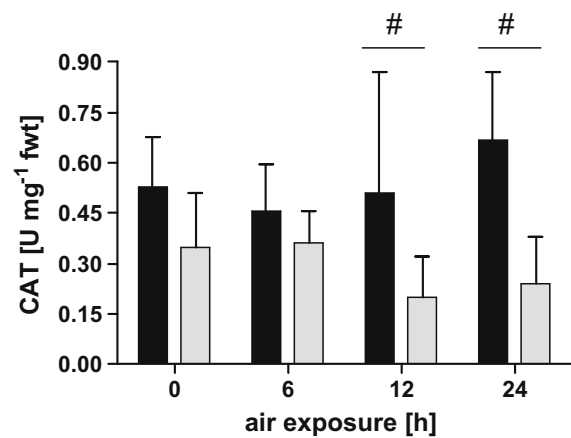
**Fig. 4.** Ratio of oxidized (GSSG) and reduced (GSH) glutathione (GSSG/GSH) in foot tissue of the Antarctic limpet *Nacella concinna* (black intertidal; grey subtidal) during hypoxia. Subtidal  $p = 0.0383$ ; KW = 11.75;  $n = 25$  (one-way ANOVA). Means + SD.  $n = 3-5$ . Time  $p = 0.0414$ ;  $F = 2.573$ ; 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.



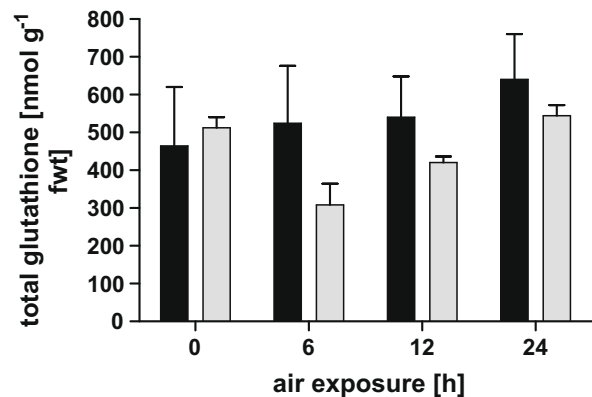
**Fig. 5a.** Superoxide dismutase activity in gills of intertidal (black) and subtidal (grey) Antarctic limpets *Nacella concinna* during several hours of air exposure. Mean values + SD.  $n = 6-10$ . \* Significantly different SOD activity to control animals. (ANOVA, Tukey if normally distributed and 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).

nificantly 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  $511.9 \pm 86.02 \text{ nmol g}^{-1} \text{ fwt}$  ( $n = 8$ ) in controls to  $335.2 \pm 118.2 \text{ nmol g}^{-1} \text{ fwt}$  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.

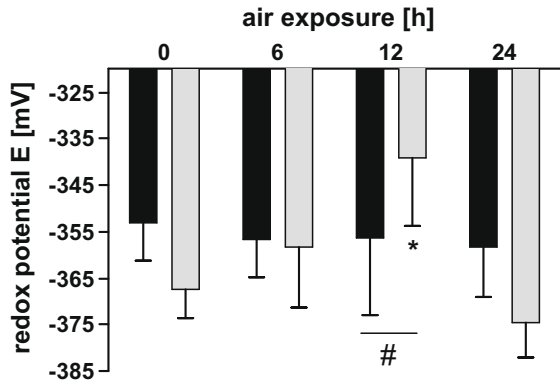
Tissue pH was measured in gills and foot tissue of sub-littoral and intertidal control animals in 2005 and 2007. After confirming that pH did not differ between control animals in both years, the values were pooled for each tissue and analysed for the general differences between subpopulations. In submersed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



**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).



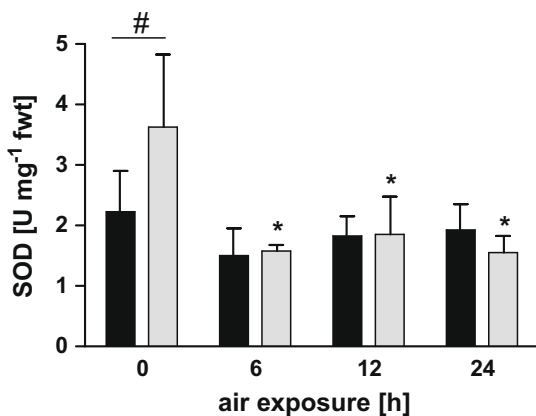
**Fig. 6a.** Glutathione concentration (GSH + 2GSSG) in gills of intertidal (black) and (grey) sub-littoral *Nacella concinna* during air exposure. Subtidal  $p = 0.0099$ ; KW = 11.36. Subpopulation  $p = 0.0078$ ;  $F = 7.838$ ; time  $p = 0.0103$ ;  $F = 4.289$ ;  $n = 46$ . Means + SD.  $n = 3-8$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



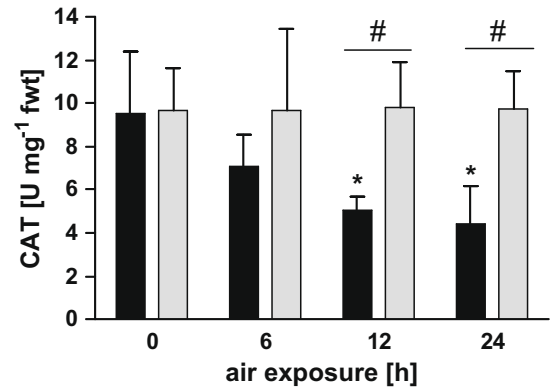
**Fig. 6b.** Redox potential in gills of intertidal (black) and (grey) subtidal *Nacella concinna* during air exposure. \* Significantly lower than in control group ( $p < 0.05$ , ANOVA, Dunn's Multiple Comparison Test). # Significant difference between subpopulations (two-way ANOVA, Bonferroni,  $p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

( $7.707 \pm 0.188$ ,  $n = 8$ ,  $t$ -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



**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 from 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).

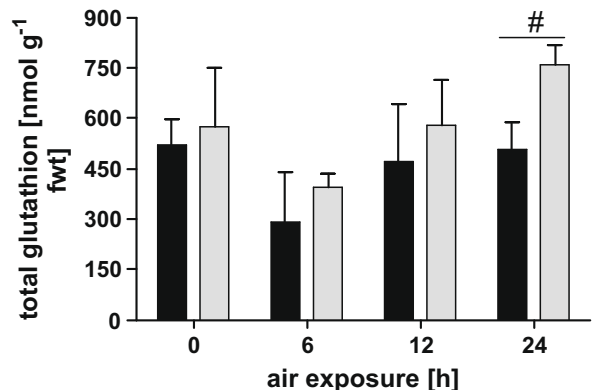


**Fig. 7b.** Catalase activity in digestive gland of the Antarctic limpet *Nacella concinna* from the intertidal (black) and the subtidal (grey) during several hours of air exposure. Mean + SD.  $n = 3-10$ . \* Significantly lower CAT activity than in control animals (ANOVA, Dunn's Multiple Comparison 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,  $p < 0.05$ ).

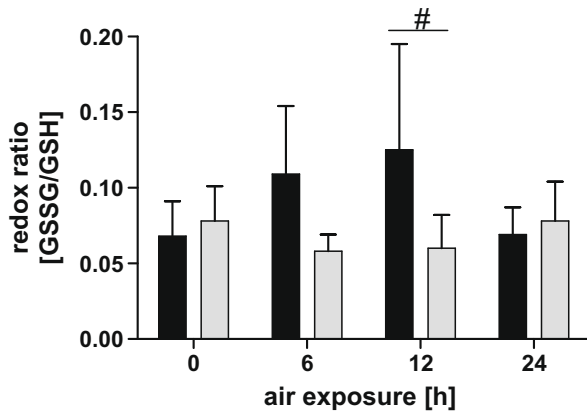
of air exposure. DG CAT activity (Fig. 7b) decreased significantly in the intertidal subpopulation during air exposure and remained constant in sub-littoral animals (subpopulation  $p < 0.0001$ ,  $F = 19.91$ ,  $n = 49$ ; interaction  $p = 0.024$ ;  $F = 3.491$ ; two-way ANOVA).

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 ( $0.1252 \pm 0.07$ ) compared to sub-littoral limpet foot muscles ( $0.0602 \pm 0.02$ ) ( $p < 0.05$ ; 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. 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.  $n = 3-8$ .



**Fig. 8b.** GSSG/GSH ratio in foot muscle of intertidal (black) and (grey) sub-littoral *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  $0.104 \pm 0.09 \mu\text{mol ascorbate g}^{-1} \text{fw}$ ;  $n = 27$ ; sub-littoral  $0.093 \pm 0.06 \mu\text{mol ascorbate g}^{-1} \text{fw}$ ;  $n = 27$ ; unpaired t-test  $p = 0.5949$ ). Malondialdehyde (mean  $\pm$  SD) was more concentrated in foot muscle of intertidal limpets ( $0.00869 \pm 0.002 \mu\text{mol g}^{-1} \text{fw}$ ,  $n = 29$ ) than of their sub-littoral congeners ( $0.00679 \pm 0.003 \mu\text{mol g}^{-1} \text{fw}$ ,  $n = 29$ ;  $p = 0.0066$ ;  $F = 8.066$ , 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  $\text{H}_2\text{O}_2$  and, in so doing, presumably minimize activation of radical chain reactions and Fenton catalyzed hydroxyl radical ( $\text{OH}^\cdot$ ) 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 sys-

tem 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  $\text{PO}_2$  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 ( $E_{\text{mV}}$  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 ( $\uparrow$ ) or decrease ( $\downarrow$ ) of enzyme activity. In case of pHa and redox potential ( $E_{\text{mV}}$ ) " $\downarrow$ " indicates acidification/reduction. No change observed is indicated as " $\leftrightarrow$ " and an initial decrease with subsequent slight recovery of a parameter by " $\downarrow\uparrow$ " and " $\uparrow\downarrow$ " stands for early transient decrease followed by recovery.

	Intertidal		Sub-littoral	
	Air	Hypoxia	Air	Hypoxia
SOD (gill)	$\downarrow$	$\downarrow$	$\uparrow$	$\uparrow$
CAT (gill)	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$	$\leftrightarrow$
$E_{\text{mV}}$ (gill)	$\leftrightarrow$	$\downarrow$	$\downarrow\uparrow$	$\leftrightarrow$
pH (gill)	$\leftrightarrow$	$\leftrightarrow$	$\downarrow\uparrow$	$\leftrightarrow$
SOD (DG)	$\leftrightarrow$	$\downarrow$	$\downarrow$	$\downarrow$
CAT (DG)	$\downarrow$	$\uparrow$	$\leftrightarrow$	$\leftrightarrow$



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.

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