



## Review

# Formation of reactive species and induction of antioxidant defence systems in polar and temperate marine invertebrates and fish

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**Abstract**

High oxygen solubility at cold-water temperature is frequently considered to be responsible for an apparently elevated level of antioxidant protection in marine ectotherms from polar environments. However, tissue oxidative stress is in most cases a function of elevated or variable  $pO_2$ , rather than of an elevated tissue oxygen concentration. This review summarizes current knowledge on pro- and antioxidant processes in marine invertebrates and fish, and relates reactive oxygen species (ROS) formation in polar ectotherms to homeoviscous adaptations of membrane and storage lipids, as well as to tissue hypoxia and re-oxygenation during physiological stress.

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*Keywords:* Oxidative stress; Antioxidants; Low temperatures; Marine invertebrates; Fish**Contents**

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## 1. Cellular mechanisms of reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation in marine ectotherms

The term ROS refers to oxygen free radicals, partially reduced intermediates of the 4 electron reduction of oxygen

to water: superoxide anions ( $O_2^{\cdot-}$ ) and hydroxyl radicals ( $\cdot OH$ ), as well as the non-radical active species, such as hydrogen peroxide ( $H_2O_2$ ). If these noxious oxygen derivatives are not controlled by antioxidant defence systems, oxidative stress occurs (Sies, 1985). Oxidative stress is a state of unbalanced tissue oxidation, involving enhanced intra- and extracellular ROS production, peroxidation of lipids, proteins, and DNA, and often causes a general disturbance of the cellular redox balance, i.e. the ratio of reduced to oxidized glutathione (GSH/GSSG) and the NADH/NAD ratio. Oxidative stress has been related to

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many pathophysiological states, e.g. ischemia–reperfusion injury, hyperoxia, but also to hypoxia, iron overload and intoxication (Di Giulio et al., 1989; Staniek and Nohl, 1999; Nohl et al., 1993).

The mitochondria are thought to consume over 90% of the cellular oxygen in unstressed cells and are considered the major sites of aerobic cellular ROS production (Boveris and Chance, 1973; Staniek and Nohl, 1999; Lenaz, 1998; Han et al., 2001). While there is no doubt that mitochondrial electron transport chains *in vitro* convert around 2% of the oxygen consumed to univalently reduced superoxide anions, the extent to which this happens *in vivo* and the rate of escape of radicals to the cytoplasm is still under debate (Gnaiger et al., 2000; St-Pierre et al., 2002; Guidot and McCord, 1999). Moreover, ROS are also produced by the microsomal systems of the endoplasmic reticulum (Chu and La Peyre, 1993; Winston et al., 1996), and by various enzymatic oxidase reactions.

Univalently reduced  $O_2^{\cdot-}$  are reduced to uncharged  $H_2O_2$  either spontaneously or by superoxide dismutase (SOD).  $H_2O_2$  diffuses freely through mitochondrial and cellular membranes. If  $H_2O_2$  is not enzymatically decomposed, it can be converted to the very short-lived and highly aggressive  $\cdot OH$ , via the transition metal catalyzed Fenton reaction (Halliwell and Gutteridge, 1985). The Fenton reaction is a driving force in tissue damage and apoptosis, and presumably involved in ROS signaling functions. In order to control ROS production, aerobic cells are endowed with an array of antioxidant enzymes which either convert  $O_2^{\cdot-}$  to  $H_2O_2$  (SOD), convert  $H_2O_2$  to water and oxygen (catalase, CAT), or use  $H_2O_2$  to oxidize substrates (peroxidases, e.g. glutathione peroxidase). The enzymatic antioxidant system is supplemented by small molecule antioxidants such as glutathione, vitamins E, A, and C, urate, biliverdin among others (Di Giulio et al., 1989; Storey, 1996).

Compared to the many publications describing pro- and antioxidant systems of vertebrates, studies of ROS related processes in marine ectotherms are still scarce, although highly warranted. Oxidative stress is increasingly studied in marine invertebrates used as sentinel organisms for monitoring pollution in coastal as well as in more remote environments (Viarengo et al., 1990; Kirchin et al., 1992; Regoli, 1992; Palace and Klaverkamp, 1993; Pellerin-Massicotte, 1994; Ahn et al., 1996; Regoli et al., 1997, 1998a,b; Angel et al., 1999). However, the basal dynamics of oxidative stress in marine invertebrates are not well understood. These animals preserve a high surface to volume ratio and, in contrast to air breathing animals, diffusive oxygen uptake over the body surface is important. Many aquatic invertebrates are oxygen conformers, i.e. oxygen consumption varies as a function of the environmental oxygen partial pressure (Abele et al., 1998a; Nikinmaa, 2002), and since some of these species are highly sensitive to higher environmental oxygen, they colonize sedimentary, low oxygen environments (Tschischka et al., 2000; Abele, 2002).

This review describes the main intracellular and extracellular mechanisms involved in metabolic formation of ROS as a function of environmental oxygen levels, as well as the radical neutralizing antioxidant systems in marine ectotherms. It compares oxidative stress in tissues of temperate and polar marine ectotherms, to explore whether life at the permanently cold-water temperatures and high oxygen concentrations of polar aquatic systems is associated with increased tissue oxygenation, and whether this causes higher oxidative stress levels in polar ectotherms.

Nitric oxide (NO), a free radical molecule, can be formed from endogenous or exogenous NO donors or from L-arginine by the activity of the enzyme nitric oxide synthase (NOS, EC 1.14.13.39) (Knowles, 1997). Isoforms of NOS have been isolated from fish (Olsson and Holmgren, 1997; Nilsson and Söderström, 1997 for review) and invertebrates (mainly insects and molluscs) and partly sequenced (Moroz et al., 1996; Martinez, 1995; Jacklet, 1997; Arumugam et al., 2000). Calcium–calmodulin dependence and cofactor requirements are conserved in both phylogenetic groups (Cox et al., 2001). Data on NO signaling in diverse phyla suggest that a common ancestor had the ability to use NO signaling and that it conferred high adaptive value (Olsson and Holmgren, 1997; Jacklet, 1997). Among the physiological functions ascribed to NO in marine invertebrates and fish, its neurotransmitter function and its role in cellular immune defence are the most outstanding (Cox et al., 2001; Arumugam et al., 2000). In marine and freshwater molluscs, NO signaling is involved in muscle contraction and relaxation, mucus secretion and excretion, and in triggering feeding behavior (Moroz et al., 1996). However, high concentrations of NO have cytotoxic effects as they inhibit a number of cellular processes, such as DNA synthesis and mitochondrial respiration (Bolaños et al., 1995; Cleeter et al., 1994; Lizasoain et al., 1996; Brown and Cooper, 1994). Some of these effects may be direct and others arise from the reaction of NO with  $O_2^{\cdot-}$  to peroxynitrite (Beckman et al., 1990), indicating that oxygen and nitrogen radicals are highly interactive at the cellular level (Taha et al., 1992).

## 2. Elevated oxygen solubility in cold seawater and changes of tissue oxygen conductance as possible causes for elevated oxidative stress in polar marine ectotherms

Oxidative stress phenomena have recently become of greater interest in polar physiology. In many current papers, the argument is put forward that due to higher oxygen solubility in cold seawater and body fluids of ectothermal animals (Wells, 1986), polar invertebrates and finfish experience elevated rates of cellular ROS formation (Viarengo et al., 1995, 1998; Ansaldo et al., 2000). Indeed, the solubility and the concentration of oxygen in sea water increase by 40% between 15 and 0 °C. Oxygen solubility in

aqueous cytosol will be less influenced by temperature because of the high content of solutes (Sidell, 1998), but higher steady state oxygen concentrations are to be expected in tissues of polar ectotherms. Especially in sluggish benthic invertebrates with low rates of oxygen consumption in the cold, high tissue oxygen concentrations can be expected. On the other hand, cold temperatures reduce oxygen conductance. According to Sidell (1998), the oxygen diffusion constant ( $-kO_2$ ) for the cytosol of marine fish decreases by 1.6% per 1 °C of cooling as tissue viscosity increases. Increased mitochondrial surface area and mitochondria volume density in Antarctic and sub-Antarctic fish may therefore help to overcome thermal limitations of diffusive oxygen supply (Guderley and St-Pierre, 2002). This brings us to the question: do higher tissue oxygen concentrations as such support elevated steady state levels of ROS production in polar marine invertebrates? Many enzymatic and chemical ROS forming processes are  $pO_2$  dependent, and higher rates of ROS formation from these processes are expected as  $pO_2$  increases. However, clear experimental proof for higher basal ROS formation rates, based on high tissue oxygen solubility at low temperature, without a concomitant increase of  $pO_2$ , has still not been obtained.

Higher tissue concentrations of dissolved oxygen may enhance the risk of lipid hydroperoxide formation and exacerbate lipid radical chain propagation. To achieve homeoviscous adaptation of membrane transport, including oxygen diffusibility at low temperatures, polar invertebrates (de Moreno et al., 1998; Falk-Petersen et al., 2000) and fish (Guderley and St-Pierre, 2002) tend to have higher degrees of unsaturation in membrane and storage lipids (reviewed by Storelli et al., 1998). In some polar fish, a better oxygen conductance in muscle cells is achieved by incorporation of lipid droplets, to enhance oxygen solubility and overcome reduced diffusion slow down and intracellular oxygen inhomogeneity in the cold (Desaulniers et al., 1996). This applies especially to icefish, but also to the less vascularized glycolytic tissues of red blooded fish species (Sidell and Hazel, 1987; Desaulniers et al., 1996; Sidell, 1998, for review). Polyunsaturated fatty acids (PUFA) are easy targets for ROS driven oxidation, and once the process of lipid radical formation is started, higher lipid unsaturation and high oxygen concentrations will both enhance the velocity of lipid radical chain reactions. Thus, as a major drawback, homeoviscous adaptation and higher cytosolic oxygen solubility in the cold may render polar animals more susceptible to lipid radical chain propagation, and prolonged half life of free radicals in the cold may facilitate the oxidative stress situation to adjacent tissue areas.

Comparing rates of lipid radical generation with iron-citrate as the radical chain initiator in digestive gland extracts of a polar (*Laternula elliptica*) and a temperate mud clam (*Mya arenaria*), we detected one order of magnitude higher rates of lipid hydroperoxide formation in the polar bivalve. The electron paramagnetic resonance (EPR) meas-

urements were carried out at 2 °C for the polar and 15 °C for the temperate clam (Estevez et al., 2002), and the overall lipid content was similar (10% of tissue dry weight) in both species. These results support the hypothesis that a higher lability of mitochondrial membranes and storage lipids contributes to elevated lipid radical formation in polar invertebrate species. This increased susceptibility may present no problem under unstressed conditions, but could become a major obstacle under any form of physiological hazard, leading to enhanced cellular ROS production. It may represent an explanation for the higher levels of antioxidants in some polar benthic invertebrates, as depicted in Section 4. Thus, polar ectotherms may be more vulnerable than temperate ectothermic animals to accelerated free radical production under physiological stress such as warming or UVB-exposure.

### 3. Reactive oxygen species formation in marine ectotherms: effects of low temperatures and hypoxic conditions

According to the general perception of body temperature effects on biochemical as well as enzymatically catalyzed cellular reactions, ROS production rates in marine invertebrates should be much reduced when compared to endotherms with a constant body temperature above 35 °C. Moreover, oxygen turnover and mitochondrial densities are much higher in most cell types of vertebrates (Guderley and St-Pierre, 2002) as compared to marine invertebrates. However, as stated by Brand (2000), ROS production is not a linear function of the electron transport rate, but can be modulated by other features of the electron transport system. An interspecies comparison (Table 1) shows that absolute rates of ROS production by marine invertebrate mitochondria are much lower than rates in insect and mammalian mitochondria. However, the conversion of oxygen to  $H_2O_2$  in invertebrate mitochondria in vitro amounts to between 3% and 7% under state 4 respiratory conditions (i.e. in the presence of substrate and the absence of ADP) (Heise et al., 2003; Keller et al., 2004) and clearly exceeds the maximal rates of 2–3% reported from mammals and insects (Farmer and Sohal, 1989).

Mitochondrial ROS production has been shown to depend on the magnitude of the mitochondrial membrane potential ( $\Delta\Psi_m$ ) in vertebrates (Korshunov et al., 1997; Brand, 2000) and invertebrates (the polychaete *Arenicola marina*, Keller et al., 2004), and to be kept at low levels under well coupled state 3 conditions (i.e. ADP stimulated oxidative phosphorylation). Elevated in vitro ROS production occurs upon transition from states 3 to 4, i.e. after complete consumption of ADP (Loschen et al., 1971; Boveris et al., 1972) and is attributed to the high proton potential and the increasingly reduced state of the complex III ubiquinone pool under non-phosphorylating conditions. In state 4, mild uncoupling by proton leakage through the

Table 1

Literature data of the ranges of oxygen radical formation reported in animal tissues from marine invertebrates to warm blooded species

Species tissue	Substrate	Respir. state	Temperature [°C]	ROS [nmol H <sub>2</sub> O <sub>2</sub> mg <sup>-1</sup> prot min <sup>-1</sup> ]	Source
<i>Marine invertebrates</i>					
<i>M. arenaria</i> mantle	malate	3	10	0.05–0.13	Abele et al., 2002
<i>M. arenaria</i> mantle	malate	4+	10	0.04–0.11	Abele et al., 2002
<i>L. elliptica</i> gill	pyruvate	3	1	0.04–0.09	Heise et al., 2003
<i>L. elliptica</i> gill	pyruvate	4+	1	0.03–0.06	Heise et al., 2003
<i>A. marina</i> body wall	succinate	3	10	<0.01	Keller et al., 2004
<i>A. marina</i> body wall	succinate	4	10	0.03–0.10	Keller et al., 2004
<i>Other</i>					
<i>Musca domestica</i>	n.g.	4	n.g.	0.8–2.0	Sohal, 1991
Rat liver	succinate	3	21–23	0.08	Boveris et al., 1972
Rat liver	succinate	4	21–23	0.4–0.5	Boveris et al., 1972
Rat liver	malate glutamate	3	21–23	0.08	Boveris et al., 1972
Rat liver	malate glutamate	4	21–23	0.19	Boveris et al., 1972
Mammal liver	n.g.	4	n.g.	0.01–0.15	Sohal et al., 1995
Rat heart	succinate	3	25	0.3–0.4 (nmol O <sub>2</sub> <sup>-</sup> )	Nohl et al., 1978
Rat heart	succinate	3	37	0.5	Hansford et al., 1997
Pigeon heart	succinate glutamate	4	n.g.	0.64–0.7	Boveris and Chance, 1973
Pigeon heart	malate glutamate	4	n.g.	0.45–0.8	Boveris and Chance, 1973

All data were obtained in vitro with mitochondrial isolates, which implies not at physiological oxygen tension. Measurement temperature, respiratory substrate and the respiratory state, in which the data were obtained, are indicated. n.g.: information not given in the paper.

inner mitochondrial membrane dissipates  $\Delta\Psi_m$ , whereby decreasing ROS formation and cellular oxygen content (Skulachev, 1996, 1998; Brand, 2000). As a rule of thumb, coupling (RCRs) tends to be lower in invertebrate than in vertebrate mitochondria (Pörtner et al., 2000; Tschischka et al., 2000), indicating less efficient oxidative phosphorylation, which corresponds to the lower scope for activity in these animals. This correlates with higher percent conversion of oxygen to ROS as ATP synthesis decreases. In vitro rates of ROS production by mitochondria from polar and temperate ectotherms are similar, even when the mitochondria are assayed at habitat temperature (Heise et al., 2003; Table 1). Although measurements in Table 1 were carried out in normoxic buffers and can be considered as vastly hyperoxic compared to in vivo conditions, they indicate that both types of mitochondria have the same capacity to produce ROS.

Another question is, what happens under environmental stress? In marine invertebrates from typically low oxygen sedimentary habitats, several forms of physiological stress, including critical warming, lead to functional tissue hypoxia, as ventilation and circulation fail to cover tissue oxygen demand (Pörtner, 2002). Following a hypoxic episode, oxygen radicals are released from ubisemiquinone during tissue re-oxygenation (Boveris and Chance, 1973; Loschen et al., 1973; Boveris and Cadenas, 1975; Duranteau et al., 1998) or, in an alternative model, directly during the hypoxic state (Chandel et al., 1998; Chandel and Schumacker, 2000). Thus, marine ectotherms, which undergo frequent episodes of environmental and physiological hypoxia, are likely to receive elevated levels of

ROS formation during or on recovery from physiological stress.

Exposure to critical warming, accompanied by a state of functional hypoxia, was found to increase lipid peroxidation in marine mollusks from Antarctic and from North Sea environments, and also produces a response of the tissue antioxidant defence system (Abele et al., 1998b, 2001, 2002; Heise et al., 2003). Under severe heat stress above the critical temperature ( $T_c$ : defined by onset of temperature induced anaerobiosis, Pörtner, 2001), bivalve mitochondria were progressively uncoupled presumably because of heat and ROS-mediated membrane damage. Progressive mitochondrial ROS formation accompanied this heat induced uncoupling effect as phosphorylating efficiency decreased (Abele et al., 2002). While state 3 respiration was less efficient at critically high temperatures, non-phosphorylating state 4 oxygen consumption increased, as the inner mitochondrial membrane became more leaky and more oxygen was univalently reduced to superoxide. This illustrates how critical warming stress may exacerbate cellular oxidative stress in marine ectotherms.

Investigations of mitochondrial cold compensation in polar and subpolar benthic invertebrates and fish frequently find higher mitochondrial volume density and peripheral localization of mitochondria in the cells of polar, compared to animals from warmer environments (Sommer and Pörtner, 2002; Johnston, 1981; Johnston et al., 1998; Guderley and St-Pierre, 2002 for review). Both strategies increase tissue aerobic capacity and reduce the diffusion distances and consequently cellular gradients of oxygen and substrates in the polar animals. Long diffusion distances



might not only limit mitochondrial energetic functioning (Guderley and St-Pierre, 2002), but may also create cellular gradients and limit oxygen supply to some mitochondria, and thereby exacerbate the danger of cellular ROS production under physiological stress.

Contrary to physiological hypoxic stress, several benthic marine invertebrates exhibit a positive energy balance under environmental hypoxia as compared to normoxic conditions (Theede, 1973; Oeschger, 1990; Gerlach, 1993; Abele et al., 1998a). These animals have retreated to—or actually never left—low oxygen and predominantly anoxic niches. Usually, these animals are oxyconforming and metabolic slow down occurs as environmental  $pO_2$  decreases (Taylor, 1976; Oeschger, 1990; Tschischka et al., 2000).

During exposure to short and prolonged critical hypoxia, the extremely hypoxia tolerant bivalve *Astarte borealis* activated important antioxidant enzymes (SOD, CAT, glutathione peroxidase; Abele-Oeschger and Oeschger, 1995) to prevent radical mediated damage. In the same study, the less tolerant lugworm, *A. marina*, did not show a comparable response of its antioxidant defence. Both animals suffered elevated ROS production in their body fluids under hypoxia and sulfidic hypoxia. Here, ROS were produced from autoxidation of both animals' complex hemoglobins in a Haber-Weiss reaction which produces methemoglobin and superoxide.

In the polychaete *Heteromastus filiformis* from the North Sea intertidal, catalase activity was induced during anoxia and 200 Torr hyperoxia (Abele et al., 1998a). A head-down sediment deposit feeder, *Heteromastus*, is highly tolerant of hypoxia and sensitive to full oxygenation. This worm offers a perfect model of an organism spanning the sediment redox cline, between the oxygenated surface and the oxygen free deeper horizons. It seems possible that free radical production is related to the vertical  $pO_2$  and pH gradients in *H. filiformis* and that ROS originate from hemoglobin autoxidation in the more acidic head end of the worms (Abele et al., 1998a).

$H_2O_2$  was also found to accumulate in the coelomic fluid of the hypoxia tolerant sipunculid worm, *Sipunculus nudus*, under severe hypoxia (1.3 kPa) and hyperoxia (>40 kPa). Antioxidant enzyme activities (catalase and SOD) being lowest at 7 kPa and, thus, in the  $pO_2$  range of moderately oxygenated sediments (Buchner et al., 2001) indicate that the worms were facing oxidative stress at both  $pO_2$  extremes. By contrast, the periwinkle (*Littorina littorea*) exposed to hypoxia by Pannunzio and Storey (1998), failed to express more antioxidants, while it increased the concentrations of the non-enzymatic antioxidant glutathione, which may be energetically “cheaper” for the animals.

The above observations of ROS production at high and, interestingly, at low  $pO_2$  extremes led to the concept that spatial and temporal fluctuations of hypoxic/oxic exposure can cause lower animals to beef up their antioxidant defence system, by voluntarily inducing hypoxia to increase stress defence (Abele, 2002).

#### 4. Adjustment to permanent cold of antioxidant defence in marine invertebrates and fish

Elevated susceptibility of polar animals to oxidative stress would create a need to adjust antioxidant defence systems to function at low temperatures. Some enzymatic systems including antioxidant enzymes (AOX), like superoxide dismutase, display temperature optimum curves with a maximal activity within the habitat temperature range in temperate ectotherms (Abele et al., 2002). Thus, maximal antioxidant activities in polar invertebrates should occur at or close to 0 °C. On the other hand, adjustment might also consist in an increased synthesis of AOX proteins to compensate for a temperature induced loss of activity in the cold. Table 2 compares overall activity of the two major antioxidant enzymes in polar and temperate molluscs. Whereas catalase activities ( $2H_2O_2 \rightarrow 2H_2O + O_2$ ) were very heterogeneous with high and low activities in both climatic groups, SOD activities ( $2O_2^{\cdot-} + 2H^+ \rightarrow 2H_2O_2$ ) were consistently higher in the polar animals, when compared at a common assay temperature. All SOD data in Table 2 were obtained according to Marklund and Marklund (1974) in our laboratory (assay  $T$ : 20 °C). Data from other studies are difficult to compare, as results are often not provided in convertible units. However, a survey of AOX activities in polar and Mediterranean molluscs by Regoli et al. (1997) supports the idea, with significantly higher SOD activities in gills of the Antarctic scallop *Adamussium colbecki* when compared to the Mediterranean bivalves *Mytilus galloprovincialis* and *Pecten jacobaeus* (assay  $T$ : 19 °C). These authors also found higher activities for other AOX including catalase, glutathione reductase, and glutathione peroxidase.

However, this conclusion may not be valid for all tissues as Regoli et al. (1997) and Viarengo et al. (1995) with the same polar scallop stock show lower SOD activities in digestive gland of *A. colbecki* as compared to Mediterranean scallops. Accordingly, levels of the oxidative stress indicator malondialdehyde (MDA) were significantly higher in digestive gland homogenates of the polar compared to the Mediterranean scallop (Viarengo et al., 1995). The reason for the discrepancy between the results for gill and digestive gland remains speculative; however, they might relate to still lower levels of environmental pollution in Antarctica compared to the Mediterranean, which may affect oxidative stress levels in the molluscs' digestive glands.

Temperature optimum curves of two AOX (catalase and glutathione *S*-transferase) in *A. colbecki*, as well as for *M. galloprovincialis* digestive gland by Regoli et al. (1997) display no activity decrease from 30 to 0 °C for the polar scallop, whereas in the Mediterranean blue mussel, both AOX activities clearly decrease at lower temperatures. Constant temperature activity relationships for catalase were recorded in gill and mantle tissue of the Antarctic clams *Yoldia eightsi* and *L. elliptica*. By contrast, in the temperate bivalve *M. arenaria* from the German Wadden Sea coast, catalase activity declined by about 50% between 20 and 10 °C

Table 2

Catalase and superoxide dismutase activities in tissues of temperate and polar mollusc species compared at an assay temperature of 20 °C

	Catalase	SOD
<b>Temperate molluscs</b>		
<i>Cerastoderma edule</i>	135.14±70.8	2.58±0.6
<i>Mya arenaria</i>	26.3±17.7	2.26±1.1
<i>Scrobicularia plana</i>	127.0±57.02	2.51±1.9
<b>Polar molluscs</b>		
<i>Nacella concinna</i> (sublittoral)	44.5±21.9	3.80±2.2
<i>Nacella concinna</i> (intertidal)	140.2±39.4	4.45±2.8
<i>Tonicella marmorea</i> <sup>a</sup>	11.4±1.3	8.81±1.4
<i>Margarites helicinus</i> <sup>a</sup>	37.5±2.6	11.30±2.0
<i>Yoldia eightsi</i> <sup>b</sup>	79.4±16.3	18.75±1.2

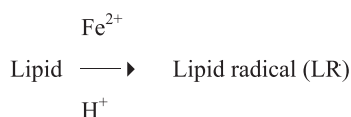
Measurements were conducted with whole animal homogenates in *T. marmorea* and *M. helicinus*, because the animals were too small for dissection. In all other cases, data are for gill tissues. Data in U mg<sup>-1</sup> protein, n=6–10.

<sup>a</sup> Philipp and Abele, unpublished thesis.

<sup>b</sup> Abele et al., 2001.

in vitro. Thus, apparently in vitro activity in this temperate animal displays some temperature dependency. In vitro temperature incubations carried out for *M. arenaria* SOD activity showed a fast denaturation of the enzyme above the maximal habitat temperature (18–20 °C) with an activity loss by 40% at 25 °C and 70% at 30 °C (Abele et al., 2002).

Levels of small molecular antioxidants in polar and temperate scallops are essentially similar, both for glutathione in gills and digestive gland (Regoli et al., 1997), as well as for glutathione,  $\alpha$ -tocopherol and  $\beta$ -carotene content in digestive gland (Viarengo et al., 1995). Significantly higher  $\alpha$ -tocopherol and  $\beta$ -carotene contents were, however, found in digestive gland material of the sessile Antarctic bivalve *L. elliptica* compared to the temperate soft-shell clam *M. arenaria* (Estevez et al., 2002). Using electron paramagnetic resonance analysis, we detected higher lipid radical content in the polar clam and related the higher lipid radical formation rates to an elevated content of iron (II) in tissues of *L. elliptica*. Iron reductase activity was significantly higher in the digestive gland of the polar clam, so that inert Fe (III) could be readily converted to the catalytically active Fe (II). Fe(II) initiates formation of highly toxic OH<sup>•</sup>-radicals from H<sub>2</sub>O<sub>2</sub> via Fenton-type reactions and, moreover, exacerbates lipid peroxidation (Puntarulo and Cederbaum, 1988):



This study showed that sediment dwelling polar invertebrates from Antarctic coastal areas can be subjected to natural ROS inducing factors. Higher loads of transition metal sediment deposits (24.2 mg Fe/g DW; Ahn et al., 1996), originating from volcanic islands as compared to Dorum Wadden Sea sediments (7.5 mg Fe/g DW; Estevez et al., 2002), are likely one of these factors. In spite of higher

antioxidant defence levels, lipid radical formation in tissue homogenates of *L. elliptica* was far higher than in the temperate mud clam. Thus, it seems that the protective effect was still insufficient, to mop up the bulk of free radicals produced in the Antarctic bivalve.

High temperatures represent another physiological stress which fosters elevated ROS levels and induced antioxidant enzymes in marine ectotherms (Di Giulio et al., 1989; Abele-Oeschger et al., 1994, 1997; Abele et al., 1998a). Investigations of oxidative stress response upon exposure to acute ( $\leq 48$  h) and gradual (7–10 days) warming within and above the habitat temperature range have recently been conducted in polar and boreal molluscs. Antarctic limpets of the species *Nacella concinna* from a subtidal Antarctic stock were exposed to temperatures between  $-2$  and  $+1$  °C (habitat temperature range) and to acute heat stress of up to 9 °C (Abele et al., 1998b). Catalase activity, assayed at 20 °C, was only mildly induced by warming *N. concinna* to 9 °C, the critical temperature ( $T_c$ ) of that species. Superoxide dismutase (SOD) activities increased at 4 °C, but at  $T_c$  the enzyme proved heat labile either due to denaturation or delayed synthesis in heat stressed animals (assays at 20 °C). If assays were carried out at each of the different exposure temperatures, to evaluate the antioxidant protection available to the animal at that particular temperature, the temperature effect on both AOX was marginal between 4 and 9 °C. As a consequence, oxidative stress parameters like lysosomal membrane labilisation and accumulation of neutral lipids in the limpets' tissues showed a drastic response under heat stress, when compared to the control group. Thermal sensitivity of SOD but not of catalase activity, and a concomitant increase of lipid peroxidation upon exposure to acute and acclimated warming above  $T_c$

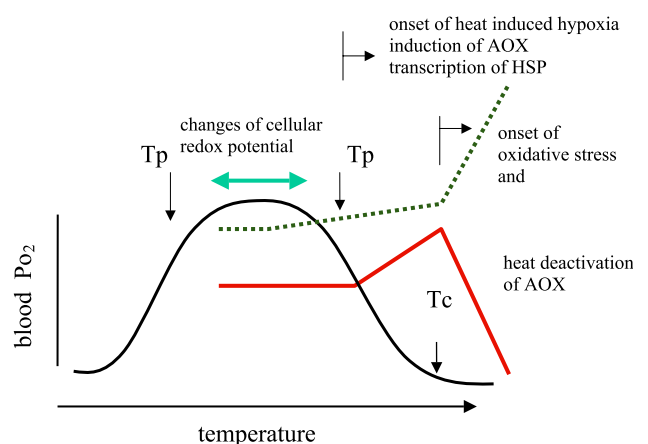


Fig. 1. Model of thermal tolerance thresholds in marine ectotherms based on Pörtner (2001) and their implication for oxidative stress and antioxidant defence systems.  $T_p$ : pejus temperature which marks limitations of aerobic scope and onset of blood or hemolymph hypoxia. This is accompanied by increased production of ROS and induction of AOX (antioxidant enzymes).  $T_c$ : critical temperature where anaerobic metabolism is activated to support survival, while at least SOD activity declines and oxidative stress markers accumulate in the tissues.

were further detected in the polar mud clam *Y. eightsi* (Abele et al., 2001).

Temperate intertidal species like the soft shell clam *M. arenaria* from the German Wadden Sea show little change of oxidative stress parameters in response to fluctuating environmental temperatures. Stepwise warming within the habitat temperature range did not elevate enzyme activities or increase lipid peroxidation markers such as MDA. Exposure to acute heat stress by direct transfer of live animals from below (18 °C) to above habitat temperatures (25 °C) significantly increased catalase activity, while lipid peroxidation did not change (Abele et al., 2002). So,  $T_c$  for these animals had obviously not yet been reached at 25 °C, and both the major AOX could be mobilized, to suppress oxidative stress.

It seems characteristic of both polar and temperate molluscs studied so far that pro- and antioxidant processes are balanced below  $T_c$ , whereas above  $T_c$  at least SOD denatures and compensation fails. At some point, vital functions are so impaired that, according to Pörtner's (2001) model of thermal tolerance, animals enter a state of temporary survival, from which they cannot return to their normal activity. We extend this model to oxidative stress parameters, as antioxidants are clearly induced in the range above pejus temperature ( $T_p$ ), which mark the limits of optimal oxygenation of body fluids, but below the critical temperature of a species, as displayed in Fig. 1. Beyond the  $T_c$ , antioxidant enzymes denature, while heat shock proteins (HSP) may come into play as suggested by Pörtner (2001), in a final effort to prolong passive survival.

Immediate early heat stress response in time scales of minutes to hours was studied in the marine sponge *Suberites domuncula* (Bachinski et al., 1997). Parallel to the induction of heat shock proteins (HSP70), the authors detected a major decrease of glutathione *S*-transferase activity by 40% after 5 min of heat stress, whereas the concentration of glutathione was reduced by 50% after 15 min of warming from 21 to 31 °C.

Thus, antioxidant enzymes and glutathione seem to be promising biomarkers for immediate early (min) and acute (up to 48 h) heat stress in marine ectotherms. It is unclear, however, from these experiments, whether the observed reduction in AOX activity at high temperatures is due only to thermally induced protein unfolding, or whether it reflects a general metabolic disturbance, affecting also synthesis of relevant antioxidant systems.

Little work has been devoted to temperature effects on oxidative stress parameters in marine finfish. An early study comparing superoxide dismutase and catalase activities in liver, heart, and muscle tissue of Mediterranean and polar fish species by Cassini et al. (1993) found no statistical difference for SOD activity between both climatic groups. By contrast, catalase activity was significantly higher in all tissues of the Mediterranean fishes. Within the Antarctic species, the red blooded fish had consistently higher SOD

and catalase activities in all tissues compared to the hemoglobin deficient icefishes. Ansaldo et al. (2000) confirmed higher SOD activities in blood of notothenoids when compared to icefish, and related this to the presence of hemoglobin and thus higher oxygen carrying capacity in notothenoid red blood cells. Interestingly, icefish had significantly higher SOD activity in gills as compared to the notothenoids, while catalase activity was significantly higher in red blooded than in icefish gills. This finding is difficult to interpret without deeper knowledge of the dynamics of radical formation in both types of gills. Hemoglobin catalyses reduction of superoxide anions ( $O_2^{\cdot-}$ ) to  $H_2O_2$  in a methemoglobin producing autoxidation reaction (Winterbourn, 1985; Abele-Oeschger and Oeschger, 1995). Thus, it seems mandatory for hemoglobin-rich fish, to keep  $H_2O_2$  concentrations under enzymatic control. By contrast, any  $O_2^{\cdot-}$  that is produced in an icefish gill can only leave that gill by diffusion to the surrounding water if converted to  $H_2O_2$  (Wilhelm-Filho et al., 1994) by SOD catalysis. These fishes would therefore need high amounts of SOD active at low temperatures. Native Cu,Zn SOD, isolated from liver of Antarctic icefish, proved highly conservative with respect to amino acid sequence, as well as to catalytic and biochemical properties (pH, anionic strength) and especially heat resistance, when compared to bovine or shark Cu,Zn SOD (Natoli et al., 1990). Apparently, little or no modification of the enzyme molecular structure has occurred in response to evolutionary cold adaptation in icefish.

Homeoviscous adaptation to permanent cold in Antarctic fish comprises elevated content of polyunsaturated fatty acids (PUFA) in plasma membranes to ensure membrane fluidity at low temperatures (Storelli et al., 1998). At the same time, a higher PUFA content implies an elevated risk of oxidative stress, as PUFA are primary targets for ROS. ROS react by removing a proton from the conjugated double bond system, whereby creating a peroxy radical ( $LOO\cdot$ ), which then initiates lipid peroxidation chain reactions (Porter, 1984; Halliwell and Gutteridge, 1985). This process is generally described as lipid peroxidation, and vitamin E ( $\alpha$ -tocopherol) is the most powerful lipid soluble antioxidant, which the fishes absorb with their herbivorous prey and which scavenges lipid radicals and thereby prevents initiation of radical chain reactions (Yamamoto et al., 2001). Gieseg et al. (2000) have compared plasma vitamin C and E levels in Antarctic and temperate finfish and found five to six times higher vitamin E content in the Antarctic species. Apparently, homeoviscous membrane adaptations and the accumulation of lipid droplets in muscle cells increase oxidative stress levels in polar fish to such an extent that they sequester vitamin E at higher concentrations. Vitamin C levels were significantly higher in only one out of two Antarctic, as compared to the temperate New Zealand, fish species. Most probably this relates to the role of the water soluble vitamin C as a secondary quencher of emerging vitamin



E<sup>-</sup>radicals in the cell membranes of the fishes. Both vitamins were stable during 7 days of experimental heat stress (7 to >18 °C) in the stenothermal banded wrasse (*Notolabrus fucicola*) although the fishes were not fed during the temperature incubations. Thus, heat stress does not enhance degradation of cellular and dissolved vitamins (Giese et al., 2000).

The recent discovery of a “marine derived tocopherol” (MDT), an  $\alpha$ -tocopherol derivative with high antioxidant potential, especially at low temperatures (Yamamoto et al., 2001), supports the view that adaptation to permanently cold environments may imply higher basal oxidative stress levels. MDT is widely distributed among tropical and cold-water marine fish. It occurs along with  $\alpha$ -tocopherol in almost all tissues of the fishes, with highest concentrations per wet mass found in liver and gonads, as well as transferred to the eggs.

A comparison of tropical and cold-water finfish yielded clear indication of higher MDT concentrations in the cold adapted species, which the authors explained with the higher vulnerability of these species to lipid peroxidation in membranes and storage oils. Moreover, MDT was found to be of greater mobility in viscous lipid-rich solutions than in aqueous media and may be the preferable and more effective antioxidant in cold species, where diffusion is limited by high cytoplasm viscosity, as mentioned before.

## 5. Conclusions

Life under permanent cold-water conditions in polar habitats causes reduced activity and lower metabolic rates in marine invertebrates and finfish. This results in lower rates of reactive oxygen species (ROS) formation in mitochondria of polar ectotherms, as these are regular by-products of cellular respiration.

However, cellular ROS production could actually be higher in cells of polar, compared to temperate, ectotherms under environmental stress. Even compared at the respective habitat temperature, ROS production rates per mg of mitochondrial protein were similar in polar and temperate bivalve mitochondria (Table 1), although specific respiration is higher in the latter. If it should turn out that higher mitochondrial densities are a common feature of cold adaptation in polar invertebrates and fish, these mitochondria might actually produce more ROS under stress, rendering polar animals prone to suffer elevated oxidative injury.

To some degree, oxygen flux in tissues of polar fish is facilitated by higher lipid contents and a better oxygen solubility, counteracting the rise of cytoplasmic viscosity in the cold. Homeoviscous adaptations of membrane fluidity involve higher lipid unsaturation and, again, may render cells of polar species more vulnerable to oxidative injury. Enhanced lipid unsaturation exacerbates lipid radical formation, which fosters lipid peroxidation chain reactions.

As a response, polar fish and some invertebrates sequester higher levels of lipid soluble antioxidants, especially vitamin E into lipid-rich tissues.

Comparisons of enzymatic antioxidants showed higher superoxide dismutase activities in polar compared to temperate mollusc species, but it is unclear whether the enzyme activity is highly operative at low temperatures. This is however the case with catalase activity in polar bivalves, which is practically constant between 0 and 30 °C. This leads us to conjecture that some molecular adaptations may have taken place to compensate for antioxidant enzyme activity at low temperatures.

However, the basic premise and conclusion of this review is that polar animals, although highly adapted to cold environments and well balanced with respect to pro- and antioxidant processes, are immensely sensitive to oxidative stress, and thus to any physiological stress provoking higher ROS formation rates (e.g. heat stress, UVB-radiation, hyperoxia, hypoxia, intoxication) or an impairment of their antioxidant system (e.g. heat stress, starvation).

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