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Aspects of antioxidant status in the polychaete *Arenicola marina*: tissue and subcellular distribution, and reaction to environmental hydrogen peroxide and elevated temperatures

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ABSTRACT: Solar radiation leads to hydrogen peroxide (H_2O_2) accumulation in shallow intertidal surface waters during daytime tidal emersion periods. The lugworm *Arenicola marina* irrigates its sedimentary tubes with surface water containing variable H_2O_2 concentrations. We studied aspects of the antioxidant status of the intertidal polychaete *A. marina* in response to seasonal variations of oxidative stress in its environment. Antioxidant enzyme activities [superoxide dismutase (SOD), glutathione reductase (GR) and catalase] and vitamin E were concentrated chiefly in the chloragog and to a lesser extent in body wall tissue. Response to experimental H_2O_2 exposure (5 µmol l^{-1}) was confined to the chloragog tissue and consisted of elevated catalase activity. On a subcellular level, the major part of enzymatic antioxidants examined was found in the cytosolic (77% of SOD activity, 60% of GR activity, 41% of catalase activity, 87% of total glutathione) and the peroxisomal (56% of catalase activity) fractions, whereas the same antioxidants were virtually absent in mitochondria. Gradual acclimation of winter *A. marina* (5°C) to higher ambient temperatures (20°C) over 20 d resulted in a doubling of chloragog SOD activities, while catalase activity was not affected. Elevated *in situ* concentrations of photoproduced H_2O_2 during summer (1.7 µmol l^{-1}) coincided with a significant increase of chloragog catalase activities in young (winter: 317.97 ± 78.3 U mg^{-1} protein, summer: 783.41 ± 192.7 U mg^{-1} protein, means ± SD) and in adult (winter: 480.09 ± 160.1 U mg^{-1} protein, summer: 1165.5 ± 207.5 U mg^{-1} protein) lugworms. Seasonal differences in the SOD activities of adult worms (winter: 16.06 ± 4.69 SOD units mg^{-1} protein; summer: 23.29 ± 3.31 SOD units mg^{-1} protein) were attributed to the elevated sediment temperatures in intertidal areas during summer.

KEY WORDS: *Arenicola marina* · Hydrogen peroxide · Enzymatic antioxidants

INTRODUCTION

Oxygen radicals and hydrogen peroxide (H_2O_2) are cytotoxic oxygen derivatives, generated as by-products of various metabolic pathways in aerobic organisms. If these active oxygen species are not rapidly detoxified by cellular antioxidants, they may cause oxidative damage to lipids, proteins and nucleic acids (Gutteridge & Halliwell 1990, Kurata et al. 1993) and may fundamentally disturb vital cellular functions and energy homeostasis (Hyslop et al. 1988). Observable effects range from growth and developmental disturbances to carcinogenesis and cell death (Zoeger et al. 1992).

In recent years, accumulation of H_2O_2 as a consequence of UV-driven oxygen radical formation has been studied in various marine coastal environments (Cooper et al. 1988, Szymzcak & Waite 1988, Price et al. 1992). Oxygen radical formation in surface waters is

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a photodynamic process in which oxygen reduction is induced by solar radiation via excitation of photosensitizing dissolved organic matter (Cooper & Zika 1983, Zika et al. 1985, Petasne & Zika 1987, Fujiwara et al. 1993). Our own research has been focusing on H_2O_2 accumulation in intertidal sandflat areas of the German Wadden Sea. H_2O_2 is of considerable stability with a half-life of 60 h in filtered seawater (Petasne & Zika 1987) and may well reach micromolar concentrations in intertidal pools during summer ebb tides (Abele-Oeschger & Oeschger 1995b). In contrast, during the winter months H_2O_2 levels rarely exceed 0.5 µmol l^{-1}. Besides these seasonal fluctuations a considerable daily variability exists, which is related to both UVA and UVB radiation (D. Abele-Oeschger, R. Röttgers & H. Tüg unpubl.). Therefore, benthic fauna from intertidal environments is not only subjected to higher H_2O_2 concentrations, but also to short-term variations of active oxygen concentrations in intertidal surface water far higher than that ever encountered by sublittoral species. Like all aerobic organisms, intertidal animals have acquired various antioxidant defence systems to protect their tissues against oxidative damage. Enzymes catalyzing the breakdown of oxygen radicals and H_2O_2 serve as primary antioxidants. Superoxide dismutase (SOD; EC 1.15.1.1.) detoxifies internally produced superoxide anion radicals at the expense of H_2O_2 formation $2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$. Peroxidases and catalase form 2 classes of H_2O_2-metabolizing enzymes. Catalase (EC 1.11.1.6) removes H_2O_2 via $2H_2O_2 \rightarrow 2H_2O + O_2$. Among the low molecular weight antioxidants, vitamins and glutathione are considered to be important (Elstner 1990). The reduced form of glutathione (GSH) may either spontaneously or via glutathione peroxidase (GPX; EC 1.11.1.9.) catalysis be oxidized by H_2O_2 or organic peroxides to the oxidized glutathione (GSSG). Rereduction of GSSG is catalyzed by glutathione reductase (GR; EC 1.6.4.2.): $GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$.

The present paper is a study of aspects of the antioxidant system of the lugworm *Arenicola marina* from intertidal sandflats of the German Wadden Sea. This polychaete digs U-shaped burrows down to approximately 30 cm sediment depths and irrigates its burrows with oxic surface water containing seasonally variable H_2O_2 concentrations. It was the objective of this work to elucidate the status of the antioxidant enzyme activities of SOD, GR and catalase as well as the concentration of the low molecular weight antioxidants glutathione, vitamin A and vitamin E in different tissues and to localize the antioxidant enzyme activities within different subcellular fractions. The main aim was to investigate whether the seasonality of H_2O_2 in the habitat of lugworms is correlated with a seasonality of the respective antioxidant enzyme activities. A comparison between young and older *A. marina* was included, to account for age-related differences of antioxidant capacity in lugworm life cycles. For the seasonal comparison, it was necessary to know the enzymatic response of *A. marina* to increased H_2O_2 concentrations and to another seasonal variable factor, temperature, which was also investigated experimentally.

MATERIAL AND METHODS

Collection of animals and treatment. *Arenicola marina* specimens were collected in December 1993 and in June 1994 from an intertidal sandflat of the German Wadden Sea, near Bremerhaven. Polychaetes were transported to the laboratory in a container with sediment and seawater from the habitat for maximal 1 h. *A. marina* used for investigations of seasonal differences in the antioxidant status were always collected between 11:00 and 14:00 h when H_2O_2 accumulation in intertidal pools was reaching its daily maximum (Abele-Oeschger & Oeschger 1995b). Each time *A. marina* were sampled, temperature and H_2O_2 concentrations were measured in the overlying tide pool water. Young and adult lugworms were classified according to size and colour Young winter individuals were smaller than 5.5 cm, whereas young summer individuals had a maximal body length of 7 cm. All young individuals were light red. Adult individuals from both seasons were over 9 cm long and appeared dark red or black.

Enzyme activities and total glutathione concentrations were assayed in chloragog and body wall tissues separately. Separation was performed by cutting the body wall, removing the coelomic fluid and the blood vessels and emptying the gut. A middle section of the body wall and chloragog were washed in filtered seawater. SOD and uricase (EC 1.7.3.3.) activity, as well as vitamin concentration, were immediately measured in freshly sampled tissues, because we found reduced levels in shock-frozen and stored samples. All other enzymes, as well as total glutathione concentration were measured in samples previously shock-frozen in liquid nitrogen and stored at –80°C. For separation of cellular subfractions, freshly dissected chloragog tissue was used.

Experimental procedure. Hydrogen peroxide incubations were done by exposing *Arenicola marina* to 5 µmol l^{-1} H_2O_2 for 6 h in aerated seawater without sediment at a temperature of 10°C. Control *A. marina* were kept under the same conditions without H_2O_2. The H_2O_2 concentration in the incubation water was checked at hourly intervals and re-adjusted if neces-

sary. To study the effect of higher temperatures on the antioxidant defence of lugworms, *A. marina* were collected in January 1995 at an environmental temperature of 4°C. The *A. marina* were kept in aerated seawater with sediment from the sampling site. Under these conditions the water temperature was increased over 7 d to 10°C and over 20 d to 20°C. Control *A. marina* were kept at 5°C throughout.

Hydrogen peroxide measurements. Water for H_2O_2 analyses was collected directly into plastic centrifugation tubes and stored in darkness and on ice until analysis. The time span between water sampling and analysis was less than 1 h. H_2O_2 was measured fluorimetrically in a peroxidase catalyzed reaction, using scopoletin (7-hydroxy-6-metoxy-2H-benzopyran) as a fluorescence indicator dye (Pamatmat 1990). A Kontron (Zürich, Switzerland) SFM 25 fluorimeter was used at 365 nm excitation and 490 nm emission wavelength. To 3 ml of the water sample, 50 µl of potassium phosphate buffer (100 mmol l^{-1}, pH 7.0) and 20 µl of scopoletin solution (0.17 mg dissolved in 2 ml phosphate buffer) were added and, after stirring, the relative fluorescence was adjusted to a 100% reading The reaction was started with 20 µl of horseradish peroxidase (EC 1.11.1.7., Serva, Heidelberg, Germany: 592 U mg^{-1}, 1.25 mg dissolved in 1 ml phosphate buffer) and the subsequent fluorescence decrease recorded on a Kontron Plotter-800. After the reaction terminated, 20 µl of an H_2O_2 standard (0.4 nmol in purified Milli-Q water) were added and the fluorescence decrease recorded and used as an added standard for the calculation of the H_2O_2 concentration in the water sample. The H_2O_2 standard-solution was calibrated iodometrically.

Enzyme activities. ***Superoxide dismutase:*** Samples were homogenized in 0.05 mol l^{-1} Tris-succinate buffer (w/v = 1:3 to 1:5, pH 8.2). After centrifugation (14 500 × *g*, 15 min) enzyme activities were measured spectrophotometrically in the supernatant at 25°C according to Marklund & Marklund (1974). The assay is based on the ability of SOD to inhibit the autoxidation of pyrogallol in 0.05 mol l^{-1} Tris-succinate buffer (pH 8.2) aerated for 1 h prior to use. The addition of 50 µl pyrogallol solution (0.57 g l^{-1}) to 950 µl buffer resulted in an absorbance increase of 0.020 abs min^{-1} at 420 nm. At 25°C and pH 8.2, 1 unit of commercially available SOD (Fluka, Neu-Ulm, Germany) or 1 SOD unit in the samples inhibits this increase by 50% (= 0.010 abs min^{-1}). Inhibition was tested using an SOD standard from bovine erythrocytes (Fluka, 5000 U mg^{-1}).

Catalase: Samples were ground in liquid nitrogen and homogenized in 50 mmol l^{-1} potassium phosphate buffer (w/v = 1:4, pH 7.0) including 1 part of 1% Triton-X100 solution to 10 parts of buffer. After centrifugation (14 500 × *g*, 15 min), catalase activities were determined in the supernatant spectrophotometrically at 25°C according to Aebi (1985). The catalase assay was conducted in potassium phosphate buffer (50 mmol l^{-1}, pH 7.0) using H_2O_2 (12.1 mmol l^{-1}, extinction coefficient ($\varepsilon_{H_2O_2}$ = 0.041 mmol l^{-1} cm^{-1}) as substrate at 240 nm. The assay was tested with commercial catalase from Boehringer Mannheim, Germany (2600 U mg^{-1}). One unit catalase activity in samples decomposes 1 µmol l^{-1} H_2O_2 min^{-1} under the assay conditions applied.

Glutathione reductase: Samples were ground in liquid nitrogen and homogenized in 0.12 mol l^{-1} phosphate buffer (w/v = 1:3, pH 7.2). After centrifugation (14 500 × *g*, 15 min), GR activities were spectrophotometrically determined in the supernatant at 25°C and 340 nm. The assay is based on the oxidation of NADPH during the reduction of GSSG to GSH. GR activity was assayed in Tris buffer (0.1 mol l^{-1}, pH 8.2) to which ethylenediamine-tetraacetic acid (EDTA, 0.94 mmol l^{-1}), GSSG (4.6 mmol l^{-1}) and NADPH (0.16 mmol l^{-1}) had been added. The assay was tested with commercial GR from Boehringer Mannheim (120 U mg^{-1}). One unit GR activity in samples is defined as a decrease of 1 µmol l^{-1} substrate min^{-1} under the assay conditions applied.

Glutamate dehydrogenase: The mitochondrial marker enzyme GDH (EC 1.4.1.3.) was determined in whole tissue extracts and in different subcellular fractions following Abele Oeschger et al. (1994). In preliminary studies, the exact pH-optimum of *Arenicola marina* chloragog GDH was determined. GDH activity was measured spectrophotometrically at 340 nm. The triethanolamine buffer (TRA, 50 mmol l^{-1}) contained ammonium acetate (100 mmol l^{-1}), 2-oxoglutarate (20 mmol l^{-1}), ADP (1 mmol l^{-1}) and 0.15 mmol l^{-1} NADH (pH 7.95) at 25°C. One unit GDH activity in samples catalyzes the turnover of 1 µmol l^{-1} substrate min^{-1} under the assay conditions described.

Uricase: The activity of the peroxisomal marker enzyme uricase was measured in whole tissues and in different cell fractions modified after Vetter (1989). The assay is based on the photometrically measurable decrease of urate in a uricase catalyzed reaction: urate + $2H_2O$ + O_2 → allantoine + H_2O_2 + CO_2. The specific extinction coefficient (ε) for urate is ε_{urate} = 12.6 µmol l^{-1} cm^{-1} at 293 nm. In a pre-experiment, the exact pH optimum of uricase from *Arenicola marina* was determined. For measurements in whole chloragog and body wall tissue, freshly prepared tissues were homogenized in Tris-HCl buffer (0.1 mol l^{-1}, pH 8.1) containing dithiolthreitol (DTT, 10 mmol l^{-1}), EDTA (2 mmol l^{-1}), ethylene glycol-bis (β-amino ethyl ether)-tetraacetic acid (EGTA, 2 mmol l^{-1}) and phenylmethyl sulfonyl fluoride (PMSF, 1 mmol l^{-1}) as a trypsin inhibitor. After centrifugation (14 500 × *g*, 10 min) the uricase activity in the supernatant was assayed at 25°C

and 293 nm in borate buffer (0.15 mol l^{-1}, pH 9.1) containing PMSF (1 mmol l^{-1}) and uric acid (60 µmol l^{-1}) as substrate. One unit GDH activity in samples is defined as the decrease of 1 µmol l^{-1} substrate min^{-1} under the assay conditions described.

All enzymatic activities in whole tissues are related to tissue protein concentrations. Protein concentrations were determined in homogenates according to Bradford (1976).

Glutathione concentration. The total amount of GSH and GSSG was determined spectrophotometrically using 5,5-dithio-2-nitrobenzoic acid ('Ellmans reagent', DTNB) as described in Abele-Oeschger & Oeschger (1995a). The assay is based on the reduction of DTNB by GSH in the sample measurable at 405 nm. GSSG is reduced by adding excess GR to the assay. Deep-frozen tissue samples were ground in liquid nitrogen and extracted with perchloric acid (w/v = 1:4). After centrifugation (14 500 × *g*, 10 min) the supernatant was neutralized with $KOH/KHCO_3$. The concentration was measured in potassium phosphate buffer (100 mmol l^{-1}, pH 7.0) containing EDTA (1 mmol l^{-1}) to which 1 ml NADPH (0.13 mmol l^{-1}) and 3 ml DTNB (1 g l^{-1}) had been added. The reaction was started by adding 0.3 units GR (Boehringer Mannheim) and calibrated with a GSSG standard solution (10 µmol l^{-1}). Glutathione was calculated as µmol GSH + GSSG g^{-1} tissue fresh mass.

Vitamins. Vitamins A and E were determined as described in Lemaire et al. (1993). Tissues were ground in liquid nitrogen and homogenized with deionized water (w/v = 1:2). Then, 4 ml sodium dodecyl sulphate (SDS, 100 mmol l^{-1}), 8 ml of absolute ethanol and 1 ml heptane were added, mixed carefully and kept in the dark for 15 min to extract the lipid fraction. After centrifugation (12 000 × *g*, 15 min) the upper heptane phase was kept at −80°C to separate any residual aqueous phase. Heptane extracts were then concentrated by desiccation under an N_2 gas stream and resuspended in heptane at ¼ to ⅕ of the volume of the aqueous extract. Of the resulting heptane phase, 100 µl was used for vitamin A and E HPLC determination (Kontron, Data system 450) on a 2.5 µm Spherisorb RP-C18 column, using absolute methanol as eluent at a constant flow of 1.4 ml min^{-1}. Eluting peaks were detected with UV-vis monitor at 254 nm. Vitamin concentrations in the samples were calculated comparing to commercially available vitamin standards (SIGMA, Diesenhofen, Germany).

Separation of different cellular fractions. SOD, catalase, GR, the mitochondrial marker enzyme GDH and the peroxisomal marker enzyme uricase were determined in extracts of whole chloragog tissue and in isolated subcellular fractions. Additionally, the content of oxidized and reduced glutathione was measured in whole tissue and in the subcellular fractions. For cell fractionation, the chloragog tissues of 2 to 3 lugworms, amounting to a total of between 300 and 600 mg tissue fresh weight, were pooled. The material was homogenized (w/v = 1:10) according to a modified procedure after Livingstone et al. (1992) in 20 mmol l^{-1} Tris-HCl buffer (pH 7.35), containing 0.5 mol l^{-1} sucrose, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} dithiotriethol, 0.15 mol l^{-1} KCl and 0.2% bovine albumin (BSA) using a Potter-Elvehjem (Braun AG, Melsungen, Germany) homogenizer. The homogenate was centrifuged at 2000 × *g* for 15 min to remove cellular debris, at 12 000 × *g* for 30 min yielding the mitochondrial-peroxisomal fraction and at 100 000 × *g* for 90 min to sediment the microsomal fraction. The residual supernatant was of cytosolic origin. The mitochondrial-peroxisomal fraction and the microsomal fraction were resuspended in homogenization buffer. For SOD measurements, these fractions were resuspended in known volumes of Tris-succinate buffer (0.05 mol l^{-1}). After resuspension, every fraction was diluted to the volume of the original homogenate.

Calculation and statistics. Data are given as means ± standard deviations (SD). In Table 2, SOD activities are given as individual values. Differences between means were evaluated via Student's *t*-test.

RESULTS

Antioxidants in different tissues of lugworms and within chloragog subcellular fractions

Specific activities of SOD, catalase and GR and concentration of vitamin E were significantly higher in the chloragog, as compared to body wall tissue of adult lugworms. In contrast, neither total glutathione nor vitamin A concentration differed between the tissues. The peroxisomal marker enzyme uricase, which also functions as a major H_2O_2 source in purine catabolism, had about 10-fold higher activities in chloragog compared to body wall tissue (Table 1).

Results of the enzymatic and glutathione measurements in whole chloragog and in isolated subcellular fractions are summarized in Table 2. For the mitochondrial marker enzyme GDH, different pH optima are known for different animals (K. Tschischka pers. comm.). The same applies for the peroxisomal enzyme uricase. The GDH pH optimum in *Arenicola marina* chloragog was found to be 7.95, and for uricase at 9.5 (Fig. 1). Maximal activity of GDH, together with the highest uricase activity, was located in the mitochondrial-peroxisomal fraction, whereas the cytosolic and microsomal fractions displayed only minor activities of both enzymes. In

Table 1. *Arenicola marina*. Levels of specific SOD (SOD units), catalase, GR and uricase activity (U mg^{-1} protein), total concentration of glutathione (µmol g^{-1} fresh mass) and concentration of vitamin A and vitamin E (µg g^{-1} fresh mass) in chloragog and body wall tissue of adult lugworms during winter (means ± SD, n = 5 to 8). *Significant differences ($p < 0.05$) between the 2 tissues

	Chloragog tissue	Body wall tissue
SOD	17.84 ± 4.5 n = 5	5.73 ± 2.82* n = 5
Catalase	462 ± 230 n = 5	8.1 ± 2.4* n = 5
GR	0.033 ± 0.009 n = 5	0.0037 ± 0.0012* n = 5
Glutathione	0.00296 ± 0.001 n = 5	0.498 ± 0.156 n = 8
Vitamin A	2.64 ± 1.48 n = 3	0.19 ± 0.0963 n = 3
Vitamin E	92.39 ± 23.04 n = 3	22.55 ± 9.1* n = 3
Uricase	0.00296 ± 0.001 n = 5	0.00021 ± 0.0002* n = 5

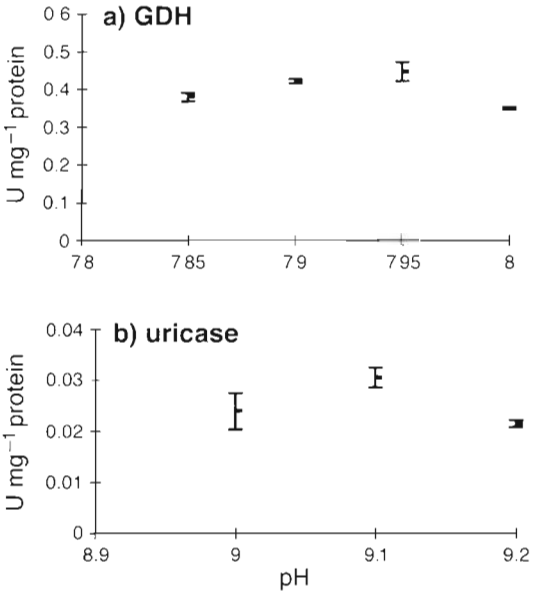

Fig. 1. *Arenicola marina*. Specific activities of (a) the peroxisomal marker enzyme uricase and (b) the mitochondrial marker enzyme GDH at different pH values (n = 3)

contrast, the major part of chloragog SOD (77%), GR (60%) and total glutathione (87%) were located within the cytosolic fraction. 56% of the total catalase activity was found in the mitochondrial-peroxisomal fraction, co-occurring with the highest uricase activities, whereas 41% of the catalase activity was cytosolic. Measurements of the microsomal fraction revealed a minor presence of antioxidant enzyme activities as well as minimal glutathione concentrations.

Effect of experimental H_2O_2 exposure on antioxidant defense

In a laboratory experiment during winter 1993-1994, *Arenicola marina* were exposed to an H_2O_2 concentration of 5 µmol l^{-1} for 6 h. The *A. marina* were collected at an ambient H_2O_2 concentration below 0.5 µmol l^{-1}. A significant response to experimental H_2O_2 exposure was found only in the catalase activity of the chloragog

Table 2. *Arenicola marina*. Sub-cellular distribution of specific activities of GDH, uricase, SOD, catalase and GR and of total glutathione in pooled chloragog tissues of adult lugworms. Upper data-sets give enzyme activities in U ml^{-1} extract (SOD activity in SOD units ml^{-1} extract) and glutathione concentration of each fraction in µmol l^{-1} Percentages of the activities measured in each subcellular fraction in relation to the summarized activity from all fractions are given in italics. Values are means ± SD (n = 3)

		Whole tissue	Mitochondrial fraction	Microsomal fraction	Cytosolic fraction	Summarized value for all fractions *% of all fractions to whole tissue*
GDH	U ml^{-1} extract	0.126 ± 0.017	0.089 ± 0.04	0.0016 ± 0.0006	0.009 ± 0.0065	0.0996
	%		*89.35*	*1.61*	*9.04*	*79.05*
Uricase	U ml^{-1} extract	0.0486 ± 0.0057	0.032 ± 0.01	0.00089 ± 0.0004	0.0024 ± 0.0012	0.0353
	%		*90.65*	*2.52*	*6.8*	*72.63*
SOD	SOD units ml^{-1} extract	16.44	2.97	0.48	11.3	14.75
	%		*20.14*	*3.25*	*76.61*	*89.72*
Catalase	U ml^{-1} extract	116.9 ± 24.6	49.71 ± 4.24	2.83 ± 0.99	36.54 ± 16.6	89.08
	%		*55.8*	*3.18*	*41.02*	*76.2*
GR	U ml^{-1} extract	0.0878 ± 0.013	0.032 ± 0.0084	0.0013 ± 0.001	0.0503 ± 0.0064	0.0836
	%		*38.28*	*1.56*	*60.17*	*95.22*
Glutathione	nmol l^{-1} extract	11 ± 4	0.49 ± 0.54	0.66 ± 1	7.7 ± 3.3	8.85
	%		*5.54*	*7.46*	*87.01*	*80.45*

Table 3. *Arenicola marina*. Specific activities (U mg^{-1} protein) of SOD (SOD units), catalase, GR and total glutathione concentration (µmol g^{-1} fresh mass) in chloragog tissue of adult lugworms in November–December 1993 with 5 µmol l^{-1} H_2O_2 (6 h) and without H_2O_2 exposure (means ± SD, n = 5 to 8). *Significant differences ($p < 0.05$) between H_2O_2 exposed and control lugworms

	Chloragog tissue	
	5 µmol l^{-1} H_2O_2 (6 h)	Controls
SOD	14.02 ± 2.13 n = 6	17.84 ± 4.5 n = 5
Catalase	877 ± 307* n = 7	462 ± 230 n = 5
GR	0.0417 ± 0.013 n = 8	0.033 ± 0.009 n = 5
Glutathione	0.680 ± 0.174 n = 8	0.534 ± 0.238 n = 8

tissue (Table 3). In contrast, SOD and GR activities, as well as total glutathione, were unaffected. In body wall tissue, no significant effect of H_2O_2 on either of the 3 antioxidant enzymes or the glutathione concentration was observed.

Seasonal variability of H_2O_2 accumulation in intertidal surface water and of antioxidant enzymes in young and adult lugworm chloragog tissue

To clarify whether the antioxidant potential in the chloragog tissue of lugworms undergoes changes corresponding to the seasonality of H_2O_2 levels in intertidal surface waters, chloragog tissues of lugworms were studied with respect to the seasons. In addition, this study was conducted with respect to 2 different age classes of lugworms, to check for age-dependent antioxidant capacities. The seasonal variability of H_2O_2 concentrations at the intertidal sampling area has been recorded during the last 4 yr, including the 7 mo of sampling in the present study. H_2O_2 levels at the sampling site showed a clear-cut seasonality with elevated levels of up to 5 µmol l^{-1} during the summer months (April–October) and lower levels of up to 0.5 µmol l^{-1} surface water during winter (December–March) (D. Abele-Oeschger, R. Roettgers & H. Tüg unpubl.).

SOD and catalase activities differed significantly between winter and summer *Arenicola marina*. During summer, both young and adult lugworms had higher catalase activities; adult lugworms, moreover, displayed increased SOD activities. In contrast, neither GR activity nor the glutathione concentration showed differences between summer and winter *A. marina* of either age group (Fig. 2). Whereas the total concentration of glutathione did not differ between age groups, the antioxidant enzyme activities were indeed significantly different. During both seasons, catalase activities were found to be higher in adults than in the young. However, younger lugworms displayed significantly higher GR activities than adult individuals. During winter, SOD activities of young individuals were also found to be significantly increased compared to adult individuals (Fig 2).

Effect of experimental temperature increase on antioxidant defense

Elevated SOD activities in lugworm chloragog tissue, measured during the summer months, are not explained by the increased ambient H_2O_2-levels, because H_2O_2 per se did not induce SOD activity in the H_2O_2 exposure experiment. To check for a possible effect of elevated temperatures on SOD levels in lugworm tissue, we performed a second laboratory experiment with adult lugworms during winter 1995. A

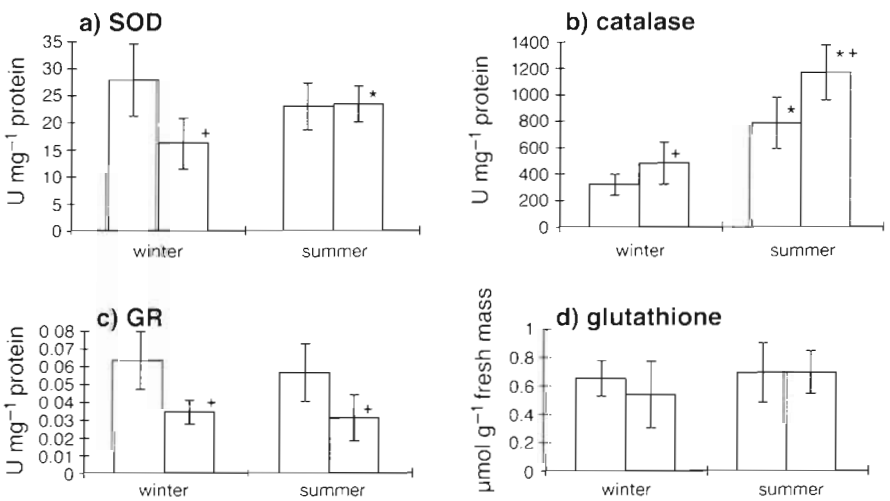

Fig. 2. *Arenicola marina*. Specific activity (U mg^{-1} protein) of (a) SOD (SOD units), (b) catalase, (c) GR activity and (d) total glutathione (µmol g^{-1} fresh mass) in chloragog tissue of freshly captured young (white bars) and adult (filled bars) worms during winter 1993–1994 and summer 1994. Mean $[H_2O_2]$ was 0.4 ± 0.3 µmol l^{-1} during winter and 1.7 ± 0.3 µmol l^{-1} during summer. Mean water temperatures were 7°C during winter and 19°C during summer. *Significant differences ($p < 0.05$) between winter and summer individuals in each age group; +significant differences ($p < 0.05$) between young and adult individuals. Values are means ± SD (n = 6 to 10)

Table 4. *Arenicola marina*. Specific activities (U mg^{-1} protein) of SOD (SOD units) and catalase in chloragog tissue of adults in January–March 1994 under different temperature conditions (means ± SD, n = 5 to 7). *Significant difference ($p < 0.05$) from control lugworms (5°C)

	8 d at 10°C	Controls	20 d at 20°C	Controls
SOD	11.27 ± 3.2 n = 6	17.54 ± 6.7 n = 5	23.34 ± 6.21* n = 6	14.22 ± 3.11 n = 5
Catalase	210.6 ± 64.6 n = 7	229.7 ± 27.2 n = 5	282.04 ± 56.3 n =6	249.1 ± 87.7 n =5

gradual temperature increase from 5 to 20°C over a period of 20 d resulted in elevated SOD activities in the chloragog tissue of the lugworms, while chloragog catalase activities were unaffected (Table 4).

DISCUSSION

In this paper, we describe characteristic features of the antioxidant status of the polychaete *Arenicola marina*. Specific questions were: what are the levels of antioxidant protection in different tissues and in subcellular fractions? Do ambient H_2O_2 and increasing temperatures influence the antioxidant status of *A. marina*? Does the apparent seasonality of the H_2O_2 concentrations in the Wadden Sea surface water of the intertidal correlate with the variability of the antioxidant status of *A. marina*? And, are age-related differences observable in the antioxidant status of lugworms?

Characterizing the antioxidative status in *Arenicola marina* tissues and subcellular fractions

Deducing from the antioxidant enzyme levels in chloragog and body wall tissue, the main prooxidative pressure arises within the chloragog tissue. Specific activities of the antioxidant enzymes were 3- (SOD), 10- (GR) and 57-fold (catalase) higher in chloragog in comparison to *Arenicola marina* body wall. The concentration of vitamin E was 4-fold higher in chloragog than in body wall tissue. The 10-fold higher uricase activity in chloragog is a marker for a high density of peroxisomes, and implies that a number of prooxidative catabolic reactions are also located within this tissue. The chloragog of annelids is a storage tissue with a high glycogen and lipid content (Cuénont 1898, d'Hertling 1923, Semal-van Gansen 1956), its function being comparable to the digestive gland of molluscs and crustacean as well as the vertebrate liver (Urich 1958). High catalase activity has been detected in the chloragog of a terrestric annelid, the oligochaete *Lumbricus terrestris* (Prentø & Prentø 1984, Prentø 1987). The authors discussed these results in relation to the responsibility of the chloragog for the destruction of superoxide anion radicals and H_2O_2 from the interaction between blood hemoglobin and molecular oxygen. Analogous to this, the well-perfused lugworm chloragog is confronted with oxidative stress from hemoglobin autoxidation in blood especially under hypoxic conditions (Abele-Oeschger & Oeschger 1995a). Higher antioxidant enzyme activities occur also in the digestive gland of *Mytilus edulis* compared to other tissues. This has been related to xenobiotic detoxification involving cytochrome P-450 and cytochrome P-450 reductase (Livingstone et al. 1990). It seems likely that these processes will also lead to elevated antioxidant enzyme activities in the *A. marina* chloragog.

The distribution of enzyme activities in subcellular fractions of the chloragog tissue provides further insight into the nature of the oxidative stress affecting the *Arenicola marina*. Of the measured GDH activity, 90% was found in the 2000 to 12 000 × *g* pellet. The same fraction also contained the major part of the peroxisomes as indicated by the presence of 90% activity of the peroxisomal marker uricase. This shows that the 2000 to 12 000 × *g* pellet represents a combined peroxisomal and mitochondrial fraction. Hence, the catalase activity found in this fraction is likely to be mainly, if not entirely, of peroxisomal origin. The microsomal fraction also contained minor amounts of peroxisomal uricase and catalase activities. However, the ratio of catalase to uricase activity, which was twice as high compared to the mitochondrial-peroxisomal fraction, could be an indication that some of the catalase activity is of microsomal origin. SOD and, moreover, the major part (60%) of the GR-activity seem to be located mainly in the cytosol. A similar distribution of SOD and catalase activities were found in subcellular fractions of the digestive gland of the mussel *Mytilus edulis* (Livingstone et al. 1992). In addition, the authors determined the form of SOD in the subcellular fractions and found the major part of CuZn-SOD in the cytosolic fraction, whereas the 2000 to 12 000 × *g* pellet contained both CuZn- and Mn-SOD. According to Abele-Oeschger (1996) 60 ± 13% of total SOD from unfractionated lugworm chloragog belongs to the CuZn-form, whereas 40 ± 10% of SOD is in the Mn-form. Mitochondrial SOD is classified following Fridovich (1978) as Mn-form, whereas the cytosol can contain both CuZn- and Mn-SOD. Taken together, our results and the study of Abele-Oeschger (1996) indicate that the SOD in the mitochondrial-peroxisomal fraction could be of the Mn-form, whereas the cytosolic fraction pre-

sumably contains both the CuZn- and the Mn-form. This indicates that the antioxidant system in *A. marina* chloragog consists of cytosolic SOD, detoxifying oxygen radicals at the expense of H_2O_2 formation, followed by the detoxification of H_2O_2 via cytosolic and peroxisomal catalase, and, to a minor extent, via glutathione and glutathione reductase. Consequently, only about 20 to 40% of total SOD activity, at most about 40% of the available GR and an as yet unknown amount of glutathione peroxidase constitute the enzymatic mitochondrial antioxidant system in *A. marina* chloragog tissue.

What triggers antioxidant protection under experimental conditions?

H_2O_2 exposure resulted in a significant increase of the catalase activity in chloragog tissue under experimental conditions. A slight, though statistically non-significant increase was also observed within GR activity (Table 3), whereas SOD levels were not affected. Evidently, catalase is indicated to detoxify externally produced, as well as internally generated, H_2O_2 and can thus be induced by environmental H_2O_2 stress. Our experiment shows that even under winter conditions *Arenicola marina* is able to face variable H_2O_2 concentrations and to respond to them by a rapid increase of its H_2O_2-decomposing potential. SOD converts internally produced superoxide radicals and is thus not affected by external H_2O_2 under experimental conditions. In contrast, only the SOD activities were affected by gradual increasing ambient temperatures (Table 4). Elevated temperatures have an increasing effect on aerobic metabolic rates (Schmidt et al. 1992) and as determined by Baumfalk (1979) a temperature increase from 6 to 22°C entailed 6 times higher ventilation rates in *A. marina*. Increased aerobic metabolic rates will presumably enhance superoxide radical formation leading to higher SOD activities. This effect became apparent in the temperature experiment with adult winter *A. marina*, as well as the seasonal comparison of SOD activities in the chloragog tissue of adult lugworms *in situ*.

What triggers antioxidant defense in the natural environment, and do different age groups have different potentials to react?

During summer, when the ambient H_2O_2 levels in the Wadden Sea intertidal reach their maximum values (D. Abele-Oeschger, R. Roettgers & H. Tüg unpubl.) both age groups of lugworms exhibit significantly increased catalase activities, compared to winter data. In contrast, no differences in GR activities between winter and summer *Arenicola marina* were found. Together with the results from the laboratory experiments, this provides evidence that the *A. marina* react to elevated H_2O_2 concentrations prevailing in their environment during spring and summer with increased catalase activities. Only in adult lugworms did SOD activity increase in the summer. The temperature experiment showed that elevated temperatures during summer could be one explanation for the higher SOD activities in adult lugworms. A similar seasonality in antioxidant status was described for the digestive gland of mussels (Viarengo et al. 1991a) and for another intertidal polychaete *Nereis diversicolor* (Abele-Oeschger et al. 1994).

Age-related differences occurred mainly with respect to the catalase activities, which were higher in older compared to younger lugworms, and to GR activities, which were in fact higher in younger compared to older lugworms. As both enzymes are involved in the detoxification of H_2O_2, the question arises whether this cannot be seen as a mere compensatory effect: catalase substituting the declining GR activity with increasing age of the individual? However, more than 400 units catalase decompose far more substrate than 0.063 units GR. A more likely explanation would be that GR forms a pair of coupled antioxidants with SOD in subcellular fractions where catalase is absent, to detoxify H_2O_2 molecules originating from SOD catalysis of internally produced superoxide radicals and re-reduce lipid peroxides. Young lugworms had high SOD activities also during winter, presumably due to higher aerobic metabolic rates of younger individuals, as described by Krüger (1958). The elevated catalase activities in the chloragog of older worms are contradictory to an age-related development of antioxidant defence in *Mytilus edulis* as described by Viarengo et al. (1991b), who found decreasing catalase activities in older mussels. However, Viarengo et al. (1991b) could have used an exact time scale and found decreasing catalase activities in animals older than 6 yr. In contrast, we do not know the exact age of the adult lugworms, so it may well be that *A. marina* older than the specimens analyzed in this study may have lower activities. On the other hand, the elevated catalase activities in adult *A. marina* may be the result of long-term adaptation to increased ambient H_2O_2 summer concentrations in the Wadden Sea intertidal environment, and therewith also an age-related characteristic of lugworm antioxidant protection. In further studies of antioxidant status of marine invertebrates, seasonality and age-dependent variations must be considered.

Conclusion

Being a member of the benthic infauna *Arenicola marina* is frequently confronted with low oxygen partial pressures in its burrow waters (Völkel et al. 1995). However, the polychaete is evidently in need of the same level of antioxidant protection as any other aerobic invertebrate species living under fully oxidized conditions above the sediment surface. The oxygen radical stress which *A. marina* apparently encounters is partly produced by elevated H_2O_2 levels and elevated temperatures in tide pool and burrow water during summer. On the other hand, the rapid alterations of the environmental oxygen availability, due to tidal immersion and emersion periods, frequently accompanied by formation of H_2S within the burrow (Völkel et al. 1995), may lead to internal production of oxygen radicals via hemoglobin autoxidation as described by Abele-Oeschger & Oeschger (1995a), or via the xanthine oxidase (EC 1.2.3.2) / dehydrogenase reaction (Chance et al. 1979). The role of the mitochondria of *A. marina* as yet another source of oxygen radicals under hypoxia and hyperoxia conditions will have to be studied in future research.

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LITERATURE CITED

Abele-Oeschger D (1996) A comparative study of superoxide dismutase activity in intertidal and subtidal marine invertebrates with respect to sulphide exposure. J Exp Mar Biol Ecol 197:39–49

Abele-Oeschger D, Oeschger R (1995a) Hypoxia induced autoxidation of hemoglobin in the benthic invertebrates *Arenicola marina* (Polychaeta) and *Astarte borealis* (Bivalvia): possible effect of hydrogen sulphide. J Exp Mar Biol Ecol 187:63–80

Abele-Oeschger D, Oeschger R (1995b) Antioxidant protection in 3 different developmental stages of polychaete spawn. Ophelia 43(2):101–110

Abele-Oeschger D, Oeschger R, Theede H (1994) Biochemical adaptations of *Nereis diversicolor* (Polychaeta) to temporarily increased hydrogen peroxide levels in intertidal sandflats. Mar Ecol Prog Ser 106:101–110

Aebi HE (1985) Catalase. In: Bergmeyer HU (ed) Methods of enzymatic analysis, Vol VIII. Verlag Chemie, Weinheim, p 273–286

Baumfalk YA (1979) On the pumping activity of *Arenicola marina*. Neth J Sea Res 13:422–427

Bradford MM (1976) A rapid and sensitive method for the quantitation microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chance B, Sies H, Boveris A (1979) Hydroperoxide metabolism in mammalian organs. Physiolog Rev 59(3):527–605

Cooper WJ, Zika RG (1983) Photochemical formation of hydrogen peroxide in surface and ground waters exposed to sunlight. Science 220:711–712

Cooper WJ, Zika RG, Petasne RG, Plane JMC (1988) Photochemical formation of H_2O_2 in natural waters exposed to sunlight. Environ Sci Technol 22:1156–1160

Cuénot L (1898) Etudes histophysiologiques sur les oligochètes. Arch Biol (Liège) 15:79. In: Lindner E (1965) Ferritin und Hämoglobin im Chloragog von Lumbriciden (Oligochaeta). Z Zellforsch 66:891–913

D'Hertling H (1923) Untersuchungen über die Harnstoffbildung beim Regenwurm. Zool Jb, Abt allg Zool u Physiol 58:57–68. In: Lindner E (1965) Ferritin und Hämoglobin im Chloragog von Lumbriciden (Oligochaeta). Z Zellforsch 66:891–913

Elstner FE (1990) Der Sauerstoff. Wissenschaftsverlag, Mannheim

Fridovich I (1978) The biology of oxygen radicals. Science 201:875–880

Fujiwara K, Ushiroda T, Takeda K, Kumamoto Y, Tsubota H (1993) Diurnal and seasonal distribution of hydrogen peroxide in seawater of the Seto Inland Sea. Geochem J 27:103–115

Gutteridge JMC, Halliwell B (1990) The measurement of mechanism of lipid peroxidation in biological systems. TIBS (Trends Biochem Sci) 15:129–135

Hyslop PA, Hinshaw DB, Hasley WA, Schraufstätter IU, Sauerheber D, Spraggs RG, Jackson JH, Cochrane CG (1988) Mechanisms of oxidant-mediated cell injury. J Biol Chem 263:1665–1670

Krüger F (1958) Zur Atmungsphysiologie von *Arenicola marina* L. Helgol Meeresunters 6:193–200

Kurata M, Suzuki M, Agar NS (1993) Antioxidant systems and erythrocyte life-span in mammals. Comp Biochem Physiol 106 B:447–487

Lemaire P, Viarengo A, Canesi L, Livingstone DR (1993) Prooxidant and antioxidant processes in gas gland and other tissues of cod (*Gadus morhua*). J Comp Physiol B 163: 477–486

Livingstone DR, Garcia Martinez P, Michel X, Narbonne JF, O'Hara SCM, Ribera SD, Winston DW (1990) Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. Funct Ecol 4:415–424

Livingstone DR, Lips F, Garcia Martinez P, Pipe HK (1992) Antioxidant enzymes in the digestive gland of the common mussel *Mytilus edulis*. Mar Biol 112:265–276

Marklund S, Marklund G (1974) Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 47:469–479

Pamatmat MM (1990) Catalase activity: a variable affecting H_2O_2 distribution in Kiel Bight. Meeresforschung 32: 261–275

Petasne RG, Zika RG (1987) Fate of superoxide in coastal seawater. Nature 325(5):516–518

Prentø P (1987) Distribution of 20 enzymes in the midgut region of the earthworm, *Lumbricus terrestris* L, with particular emphasis on the physiological role of the chloragog tissue. Comp Biochem Physiol 87a(1):135–142

Prentø P, Prentø A (1984) Crystalline catalase from the earthworm *Lumbricus terrestris* (Oligochaeta: Annelida): purification and properties. Comp Biochem Physiol 77b(2): 325–328

Price D, Worsfold PJ, Mantoura RFC (1992) Hydrogen peroxide in the marine environment: cycling and methods of analysis. Trends Anal Chem 11(10):379–384

Schmidt R, Schiedek D, Schöttler U (1992) Der Einfluß der Temperatur auf den anaeroben Energiestoffwechsel von *Arenicola marina* L. Polychaeta. Verh Dtsch Zool Ges 1992. Gustav Fischer Verlag, Stuttgart, p 167

Semal-van Gansen P (1956) Les cellules chloragogènes des lombriciens. Bull Biol 90:353–356. In: Lindner E (1965) Ferritin und Hämoglobin im Chloragog von Lumbriciden (Oligochaeta). Z Zellforsch 66:891–913

Szymczak R, Waite TD (1988) Generation and decay of hydrogen peroxide in estuarine waters. Aust J Mar Freshwater Res 39:289–299

Urich K (1958) Über die Funktionen des Regenwurm-Chloragogs, insbesondere über Fettresorbtion und Fettspeicherung bei *Lumbricus terrestris* L. Z Vgl Physiol 41: 342–363

Vetter RA (1989) Untersuchungen zur Funktion der Uratoxidase als Sauerstoff-Sensor bei der Strandkrabbe *Carcinus maenas* L. MSc thesis, Heinrich Heine University, Düsseldorf

Viarengo A, Canesi L, Pertica M, Livingstone DR (1991a) Seasonal variations in the antioxidant defence system and lipid peroxidation of the digestive gland of mussels. Comp Biochem Physiol 100C(1/2):187–190

Viarengo A, Canesi L, Pertica M, Livingstone DR, Orunesu M (1991b) Age-related lipid peroxidation in the digestive gland of mussels: the role of the antioxidant defence systems. Experientia 47:454–457

Völkel S, Hauschild K, Grieshaber MK (1995) Sulfide stress and tolerance in the lugworm *Arenicola marina* during low tide. Mar Ecol Prog Ser 122:205–215

Zika RG, Moffett JW, Petasne RG, Cooper WJ, Saltzman ES (1985) Spatial and temporal variations of hydrogen peroxide in Gulf of Mexico Waters. Geochim Cosmochim Acta 49:1173–1184

Zoeger D, Beyersmann D, Rensing L, Hagemann M (1992) Streßverarbeitung in der Zelle. Nat Rundsch 1:9–17

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