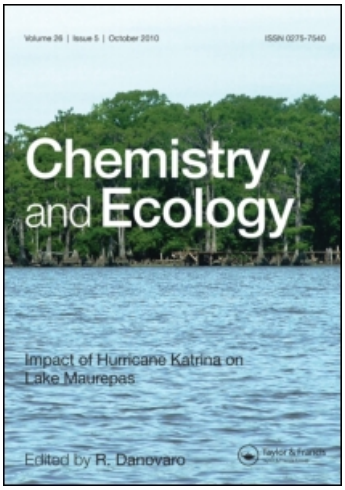

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Oxidative challenge is an important factor affecting the adaptive strategies of Antarctic fish, but data on antioxidant defences in these organisms remain scarce. In this investigation, individual antioxidants and the total oxyradical scavenging capacity (TOSC) were characterised in three notothenioid species, *Trematomus bernacchii*, *Trematomus hansoni* and *Trematomus newnesi*; seasonal fluctuations were further analysed in *T. bernacchii* sampled during different periods of the reproductive cycle, ice melting and phytoplanktonic blooms. The overall results revealed only limited differences between the three notothenioids, with greater TOSC values in *T. hansoni* and *T. newnesi*. The capacity for decomposing hydrogen peroxide via catalase was not particularly enhanced in these fish, in contrast to the prominent role of the enzyme in Antarctic invertebrates. An alternative antioxidant strategy, based on the efficiency of low molecular mass scavengers was suggested, especially for *T. bernacchii* which had higher levels of glutathione and glutathione reductase; the diet composition of the investigated species might explain the differences in tissue antioxidants. Oxidative stress responses revealed almost constant values between November and January in *T. bernacchii*, a quite unusual and unexpected result considering the marked changes occurring in several biological and environmental factors. In this respect, the antioxidant efficiency of *T. bernacchii* would counteract the naturally elevated environmental prooxidant conditions and the associated potential increase in oxidative challenge, i.e. spawning period, sea-ice melting, phytoplanktonic development and the seasonal increase in cadmium bioavailability at Terra Nova Bay.

Keywords: *Trematomus*; oxidative stress; antioxidants; total oxyradical scavenging capacity; seasonal fluctuations; adaptation

1. Introduction

The family of Nototheniidae is the most abundant and conspicuous group of Antarctic fish, with 12 genera and 49 species distributed among continental shelves and sub-Antarctic islands [1]. The coastal fish fauna of Terra Nova Bay (Ross Sea) is largely dominated by the genus *Trematomus* [2], including species with benthic (*Trematomus bernacchii*, *Trematomus hansoni*, *Trematomus nicolai*, *Trematomus pennelli*, *Trematomus scotti* and *Trematomus tokarevi*), epibenthic (*Trematomus eulepidotus*, *Trematomus lepidorhinus* and *Trematomus loennbergii*) and semipelagic (*Trematomus newnesi*) habits [3, 4].

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Antarctic fish, isolated for several million years, have adapted to extreme environmental conditions characterised by low seawater temperature and elevated solubility of dissolved oxygen, which increases by 40% between 15 and 0 °C [5]. Under such conditions, the oxidative challenge is an important factor affecting the metabolic adaptive strategies of these organisms for which high tissue oxygen concentrations can be expected. The risk of susceptibility to pro-oxidant attack is also exacerbated by lipid droplet accumulation in cytosol, elevated unsaturation of membrane lipids and the higher density of mitochondria [5]. The prolonged half-life of oxyradicals at low temperature and the slow cellular turnover of proteins and lipids have been indicated as additional pro-oxidant factors which impose long-term antioxidant protection on these cellular components [6,7].

The capability of Antarctic species to counteract oxidative challenge is of particular importance to better understand the role of antioxidant defences in response to cold environments. In this respect, one of the objectives of this study was the characterisation of basal antioxidant efficiency in three notothenioid species, *T. bernacchii* and *T. hansoni*, which are the most common benthic fish in the area of Terra Nova Bay, and the semipelagic *T. newnesi*, typically associated with the water column.

The efficiency of antioxidant defences can be influenced by seasonal changes in both environmental factors and metabolic activities that are particularly marked for Antarctic species, i.e. those related to food availability, reproductive cycles and gonad development. Specific local features might further contribute to modulate the basal oxidative challenge in Antarctic organisms, for example, the elevated natural levels of cadmium at Terra Nova Bay [8, 9]: in this area, the recurring formation of a coastal polynia and consequent up-welling phenomena determine a cadmium enrichment in surface waters before the algal bloom, which is then responsible for the elevated transfer of this element to marine organisms [8]. Pro-oxidant effects of cadmium have been investigated in *T. bernacchii*, showing an elevated complexity of responses, with interactions and cascade effects difficult to predict, and influencing the metabolism of xenobiotics and susceptibility to different forms of toxicity [10]. Considering the effects that pollutants have on the biochemistry and endogenous redox status, variations in antioxidant defences are widely accepted as biomarkers revealing potentially deleterious consequences of chemicals mediated by the enhanced formation of reactive oxygen species [10–12].

Understanding natural changes in antioxidant efficiency in fish can also be useful for the proper interpretation of field results, to discriminate between the onset of biological disturbance and natural variability. In this study, the seasonality of oxidative stress biomarkers could be analysed in *T. bernacchii* sampled in three different periods of the Antarctic summer, which corresponded to various phases of the reproductive cycle, ice melting and phytoplanktonic blooms.

The oxidative parameters examined in the three Nototheniidae species were a battery of antioxidant defences including: catalase, which reduces hydrogen peroxide to water; glutathione *S*-transferases (GST), a family of isoenzymes involved in detoxification reactions of electrophilic compounds; glutathione reductase, responsible for the conversion of oxidised glutathione GSSH to its reduced form; Se-dependent and Se-independent glutathione peroxidases, which detoxify both hydrogen peroxide and organic hydroperoxides with GSH as the cofactor; and levels of total glutathione, a cofactor of antioxidant enzymes and a direct scavenger of reactive oxygen species. The integrated biological significance of individual antioxidants and susceptibility to oxidative stress conditions were evaluated using the total oxyradical scavenging capacity (TOSC) which quantifies the whole capability of tissues to neutralise different forms of reactive oxygen species, such as peroxyl and hydroxyl radicals [6, 13–16].

The overall results obtained in this study were expected to extend our basal knowledge on the biological importance of antioxidant systems in Antarctic species, providing an useful insight into seasonal variability in *T. bernacchii*.

2. Materials and methods

2.1. *Specimen collection*

Sexually mature specimens of *T. bernacchii*, *T. hansoni* and *T. newnesi* were sampled by hook in November 2003, during the XIX Italian Antarctic Expedition from Tethys Bay, a pristine area close to the Italian Base 'Mario Zucchelli' at Terra Nova Bay. For *T. bernacchii*, organisms could be sampled during different seasonal periods of the reproductive cycle and food availability: in addition to those collected in November, corresponding to the spawning period before phytoplanktonic development, other specimens were caught in December and January, after sea-ice melting, during the main and second algal blooms. Immediately after collection, livers were dissected, frozen in liquid nitrogen and stored at $-80\,^{\circ}\mathrm{C}$ until analyses.

2.2. *Antioxidant defences*

Enzymatic antioxidants were measured in liver samples homogenised (1 : 5 w/v) in 100 mM Tris/HCl buffer pH 8.0, 0.1 mM phenylmethanesulphonyl fluoride (PMSF), 0.1 $\mathrm{mg \cdot mL^{-1}}$ bacitracin, 0.008 $\mathrm{TIU \cdot mL^{-1}}$ aprotinin, 3% NaCl, and centrifuged at 100,000 ***g*** for 1 h at $4\,^{\circ}\mathrm{C}$. Measurements were made with a Varian (model Cary 3) spectrophotometer at a constant temperature of $18\,^{\circ}\mathrm{C}$, as detailed elsewhere [10]. Catalase (EC 1.11.1.6) was measured by the decrease in absorbance at 240 nm ($\varepsilon = 0.04\,\mathrm{mM^{-1} \cdot cm^{-1}}$) due to H_2O_2 consumption (12 mM H_2O_2 in 100 mM Na-phosphate buffer pH 7.0). Glutathione reductase (EC 1.6.4.2) activity was followed by the oxidation of NADPH at 340 nm during the reduction of GSSG (extinction coefficient, $\varepsilon = 6.22\,\mathrm{mM^{-1} \cdot cm^{1}}$). The assay conditions were 100 mM Na-phosphate buffer pH 7.0, 1 mM GSSG and 60 μM NADPH. GST (EC 2.5.1.18) was determined at 340 nm using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate ($\varepsilon = 9.6\,\mathrm{mM^{-1} \cdot cm^{-1}}$). The assay was carried out in 100 mM Na-phosphate buffer pH 6.5, 1.5 mM CDNB, 1 mM GSH. Glutathione peroxidase (GPx) activities were measured in a coupled enzyme system in which NADPH is consumed by glutathione reductase to convert the formed GSSG to its reduced form. The decrease in absorbance was monitored at 340 nm ($\varepsilon = 6.22\,\mathrm{mM^{-1} \cdot cm^{-1}}$) in 100 mM K-phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 1 mM NaN_3 (for hydrogen peroxide assay), 2 mM GSH, 1 unit glutathione reductase, 0.24 mM NADPH and 0.5 mM hydrogen peroxide or 0.8 mM cumene hydroperoxide as substrates for the Se-dependent and the sum of Se-dependent and Se-independent forms. The rate of the blank reaction was subtracted from the total rate.

Levels of total glutathione were measured after homogenisation (1 : 5 w/v) in 5% sulphosalicilic acid with 4 mM EDTA; samples were maintained for 45 min on ice and centrifuged at 37,000 ***g*** for 15 min; the resulting supernatants were assayed enzymatically, as reported elsewhere [10].

2.3. *Total oxyradical scavenging capacity*

To measure TOSC, samples were homogenised as described previously for enzymatic analyses, except that PMSF and bacitracin were not added to the buffer. The TOSC assay measures the ability of cellular antioxidants to inhibit the oxidation of 0.2 mM α-keto-γ-methiolbutyric acid to ethylene gas in the presence of different forms of oxyradicals, artificially generated at a constant rate [17, 18]. Peroxyl radicals (ROO·) were generated by the thermal homolysis of 20 mM 2-2′-azo-bis-(2methylpropionamidine)-dihydrochloride (ABAP) in 100 mM potassium phosphate buffer, pH 7.4. Hydroxyl radicals (HO·) were generated from the Fenton reaction of iron–EDTA

(1.8 μM Fe^{3+}, 3.6 μM EDTA) plus ascorbate (180 μM) in 100 mM potassium phosphate buffer [18]. Ethylene formation in control and sample reactions was analysed at 10–12 min intervals by gas-chromatographic analyses and TOSC values were quantified from the equation: $\text{TOSC} = 100 - (\int \text{SA} / \int \text{CA} \times 100)$, where $\int$SA and $\int$CA are the integrated areas calculated under the kinetic curve produced during the reaction course for sample (SA) and control (CA) reactions [17]. An experimental TOSC ranging from 0 to 100 indicates, respectively, no inhibition of ethylene formation ($\int \text{SA} / \int \text{CA} = 1$) and maximum scavenging capacity with no α-keto-γ-methiolbutyric acid oxidation (SA = 0). The specific TOSC (referred to 1 μg of protein) was calculated by dividing the experimental TOSC values by the μg of proteins contained in the assay.

Protein concentrations were determined using the Lowry method with bovine serum albumin (BSA) as standard.

2.4. *Statistical analyses*

Differences in antioxidant parameters between species or seasonal periods were tested by analysis of variance (ANOVA). The homogeneity of variance was analysed by Cochran's *C*, and post-hoc tests (Newman–Keuls) were used to discriminate between means of values.

3. Results

Comparison of the three notothenioids, *T. bernacchii*, *T. hansoni* and *T. newnesi*, revealed some species-specific differences in the levels of antioxidant defences (Figure 1).

Catalase activities in *T. bernacchii* were $55.1 \pm 5.17\,\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, significantly lower than in *T. newnesi* ($76.1 \pm 19.1\,\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) and *T. hansoni* ($85.7 \pm 11.9\,\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$).

By contrast, *T. bernacchii* exhibited higher levels of glutathione reductase (5.22 ± 1.91 nmol · g tissue) and total glutathione ($1.07 \pm 0.16\,\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$), $\sim 45\%$ greater than values observed in the other notothenioids.

No significant differences among the species were obtained for the other glutathione-dependent enzymes, i.e. GST (average activity $148 \pm 23.7\,\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$) and glutathione peroxidases; for the latter enzymes, activities ranged between 48.3 and 99.8 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for Se-dependent forms, and between 67.1 and 103 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ for the sum of Se-dependent and Se-independent forms.

The TOSC results indicated a significantly lower capability to neutralise oxyradicals in *T. bernacchii*; TOSC values toward peroxyl and hydroxyl radicals were, respectively, 255 ± 59.1 and 336 ± 37.6 UTOSC · mg protein^{-1} in *T. bernacchii*, 392 ± 31.5 and 562 ± 75.0 UTOSC · mg protein^{-1} in *T. hansoni* and 373 ± 59.6 and 492 ± 65.5 UTOSC · mg protein^{-1} in *T. newnesi*.

The integration between individual antioxidants and measurement of TOSC toward both peroxyl and hydroxyl radicals was further characterised in *T. bernacchii* sampled in different seasonal periods (Figure 2).

No significant temporal changes were observed for enzymatic activities of catalase, glutathione reductase, GST, Se-dependent and Se-independent glutathione peroxidases, and levels of total glutathione, with almost constant values measured at all sampling times from November to January.

Similarly, the ability to scavenge peroxyl and hydroxyl radicals did not reveal any change during various periods, confirming the absence of seasonal fluctuations in the antioxidant status of *T. bernacchii* throughout the Antarctic summer.

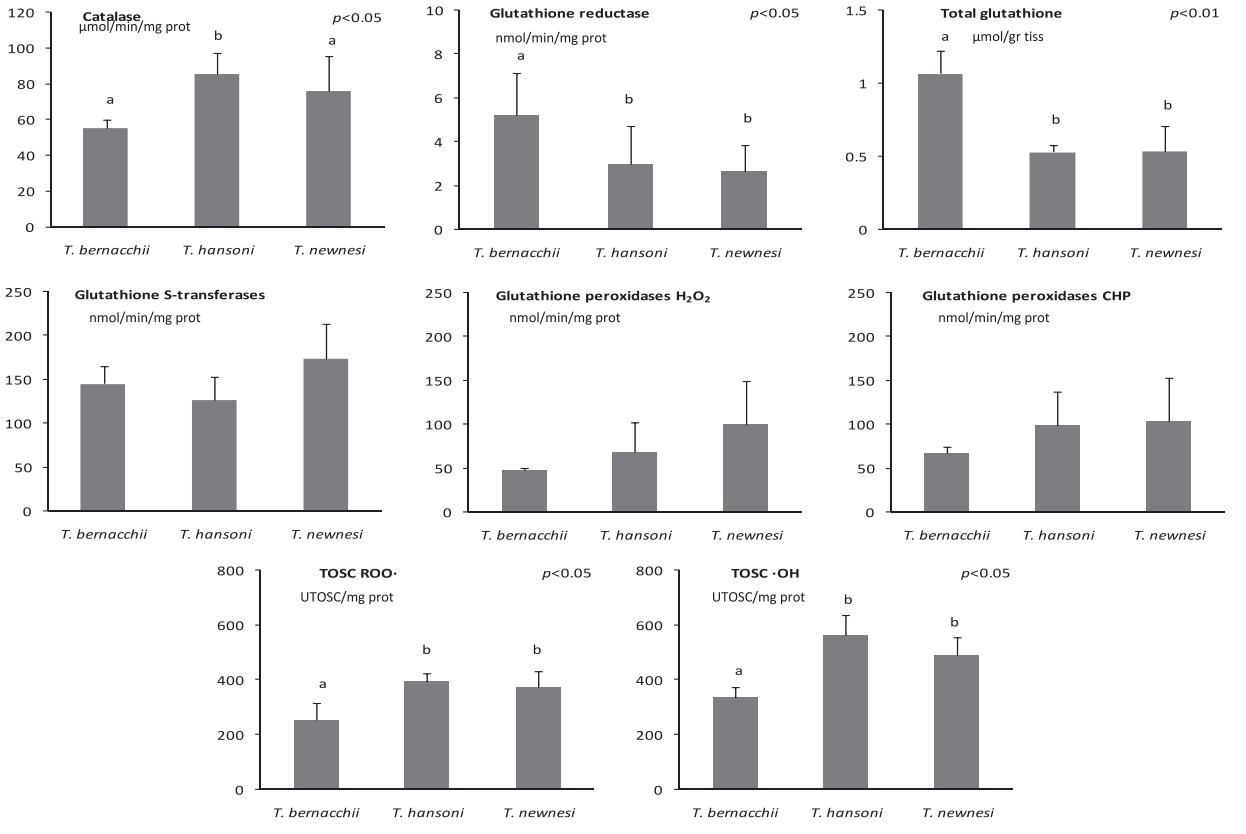

Figure 1. Antioxidant parameters: catalase, glutathione reductase, total glutathione, glutathione S-transferases, sum of (Se)-dependent glutathione peroxidases (H_2O_2) and (Se)-dependent and (Se)-independent glutathione peroxidases (CHP) and total oxyradical scavenging capacity towards peroxyl radicals (TOSC ROO·) and hydroxyl radicals (·OH) in the livers of different *Trematomus* species (mean ± SD; $n = 10$). The p values are reported for significant variations, and different letters indicate significant differences between groups of means (post hoc comparison).

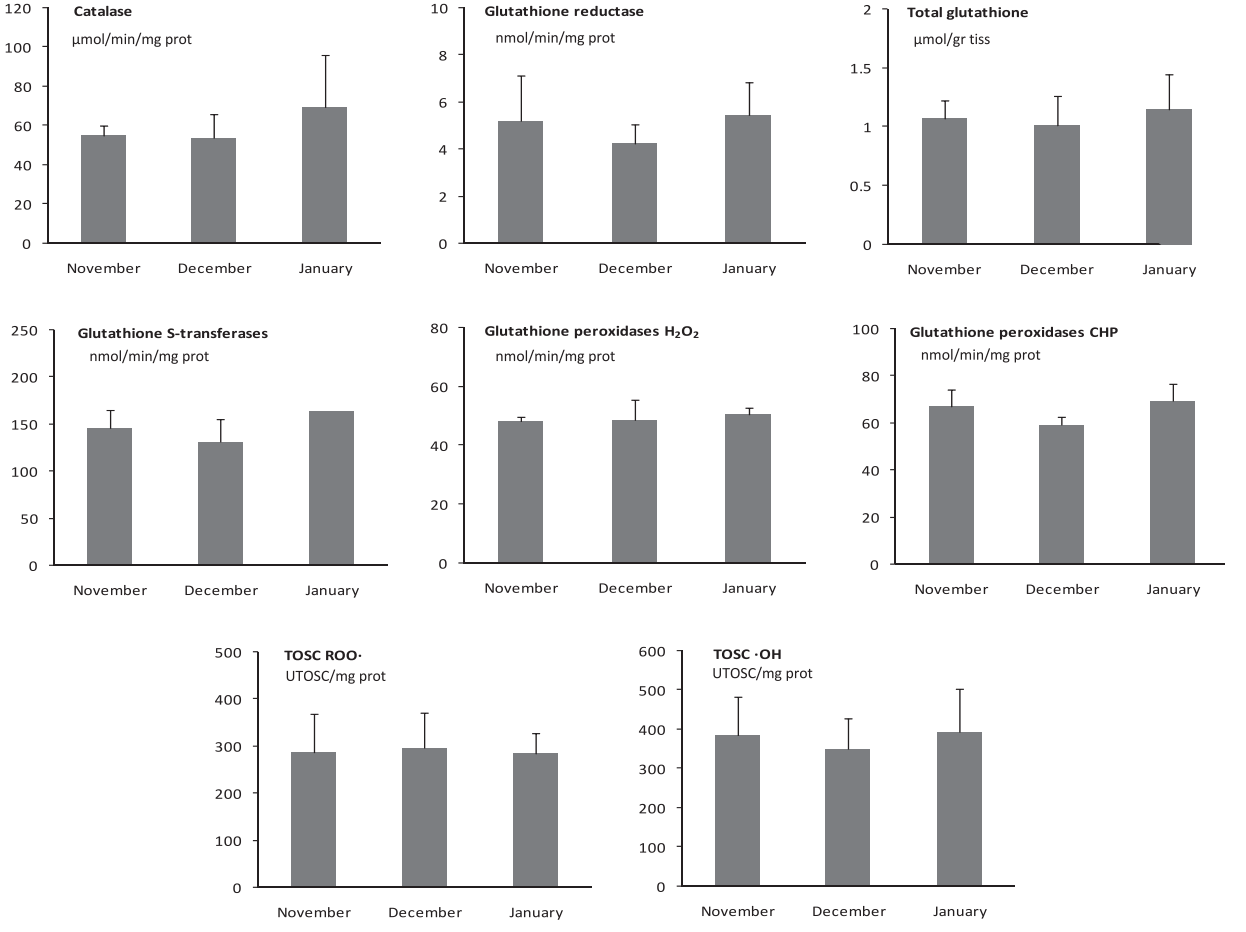

Figure 2. Antioxidant parameters: catalase, glutathione reductase, total glutathione, glutathione S-transferases, sum of (Se)-dependent glutathione peroxidases (H_2O_2) and (Se)-dependent and (Se)-independent glutathione peroxidases (CHP) and total oxyradical scavenging capacity towards peroxyl radicals (TOSC ROO·) and hydroxyl radicals (·OH) in livers of *Trematomus bernacchii* sampled during different seasonal periods (mean ± SD; $n = 10$).

4. Discussion

Data on antioxidant defences are scarce for Antarctic fish and this study was designed to extend our baseline knowledge on three typical notothenioids, *T. bernacchii*, *T. hansoni* and *T. newnesi*. Although these species are sympatric in Terra Nova Bay, their different feeding habits and distribution contribute to the maintainance of limited interspecific competition [19, 20]. The rock cod *T. bernacchii* lives on the bottom and is primarily a benthic feeder, eating sedentary and moving prey, with a varied diet including scallops, fish eggs, algae, krill and polychaetes. *T. hansoni* eats juvenile fish, fish eggs, algae, polychaetes, krill, amphipods, anemones and gastropods, taking more prey from the water than other primarily benthic feeding fish like *T. bernacchii*. Finally, *T. newnesi* is the only cryopelagic species associated with the underside of the sea ice [21–23], showing a marked trophic plasticity with variability in feeding search and diet diversity [23]. The investigated species are also distinguished by their reproductive cycles with a spawning time following a chronological sequence: for *T. bernacchii* it occurs in late spring/early summer between October and December, whereas *T. hansoni* spawns between January and February, and *T. newnesi* in autumn between March and April [24].

The overall results obtained in this study showed quite similar levels of antioxidant defences in the three notothenioid species, although with some significant differences. Higher levels of catalase were measured in liver of *T. hansoni* and *T. newnesi* compared with *T. bernacchii*, but activities measured for this enzyme were lower than those generally obtained in temperate organisms [25–27]. This result is in agreement with previous studies, reporting lower values of catalase in red-blooded Antarctic fish than in white-blooded and temperate species like the Mediterranean gobiid *Zosterisessor ophiocephalus* [28, 29]. Such findings indicate that the capacity for decomposing hydrogen peroxide via catalase is not enhanced in Antarctic fish, contrasting with the prominent role demonstrated for this enzyme in Antarctic invertebrates in which the elevated activities of catalase have been identified as one of the main adaptations to counteract high oxygen levels and the formation of hydroxyl radicals [5, 30].

An alternative biochemical strategy, based on the efficiency of low molecular mass scavengers, could be hypothesised to counteract pro-oxidant challenge in the investigated fish. In particular, *T. bernacchii* showed a higher glutathione content than *T. hansoni* and *T. newnesi*, and also a more elevated glutathione reductase activity, the enzyme responsible of the conversion of oxidised glutathione (GSSH) to its reduced, functionally active form (GSH). Compared with other Antarctic fish, *T. bernacchii* has previously been shown to also contain higher concentrations of ascorbic acid and vitamin E [31, 32], suggesting an antioxidant potential based on low molecular mass scavengers which can guarantee more flexibility than enzymes toward a wider spectrum of pro-oxidant molecules [13]. The intake of such antioxidants is mostly related to diet [33, 34] which for *T. bernacchii* from Terra Nova Bay is composed almost exclusively of *Adamussium colbecki*, known to contain high levels of glutathione, carotenoids and vitamin E [30, 33]. By contrast, the other notothenioids have a more opportunistic diet based on a wide variety of invertebrates and vertebrates for *T. hansoni*, and planktivory species that vary in different seasonal periods for *T. newnesi* [23, 35]; this feeding variability might be at least partly responsible for differences in the tissue composition of antioxidants between various species and the more elevated total oxyradical scavenging capacity in *T. hansoni* and *T. newnesi* compared with *T. bernacchii*. Overall, the results obtained in this study revealed a high capability to neutralise both peroxyl and hydroxyls radicals in the three species of genus *Trematomus* in respect to the scavenging capacity of oxyradicals characterised in several marine organisms [26, 36].

One of the objectives of this study was the assessment of seasonal variations of antioxidant efficiency which could be investigated only in *T. bernacchii*. Although the concept of seasonality is restricted to the limited temporal window of the Antarctic summer, this period is characterised by marked variations in biological and environmental factors which are known to modulate oxyradical

metabolism in marine organisms. Significant seasonal variations in antioxidant defences in temperate fish have been associated with the phase of the reproductive cycle, feeding regimes and changes in seawater temperature [27, 36, 37]. In this work, *T. bernacchii* showed quite constant values for all individual antioxidants measured between November and January, and the same results were obtained for the total oxyradical scavenging capacity. Although individual antioxidants are characterised by an elevated sensitivity in revealing a varied pro-oxidant challenge, TOSC is less sensitive but nevertheless important for understanding the biological significance of antioxidant variations and oxidative challenge [6, 13, 18, 26, 38]. In this respect, the efficiency of the antioxidant system in *T. bernacchii* appeared able to counteract the naturally elevated environmental pro-oxidant conditions and the potential increase of oxidative challenge associated to the spawning period or to the consequences of sea-ice melting.

Among these, the massive release of inorganic nutrients, algal development and photo-activation of dissolved organic matter have previously been shown to enhance pro-oxidant pressure and antioxidant efficiency in embryos of the Antarctic silverfish *Pleuragramma antarcticum* [39]. Another example of natural oxidative challenge is the seasonal development of phytoplanktonic assemblages which determine symbiotic associations of diatoms with Antarctic sponges, and specific adaptations of their antioxidant defences in response to increased levels of photosynthetically produced oxygen [40]. Phytoplanktonic blooms have been further shown to influence the feeding regime and the oxyradical scavenging capacity in the Antarctic scallop *A. colbecki* which exhibit a significant seasonal increase of antioxidant efficiency [41].

The development of phytoplankton is also the principle event modulating the biotic transfer of elevated natural basal levels of cadmium at Terra Nova Bay. In November, before ice melting and phytoplanktonic blooms, seawater cadmium concentrations at Ross Sea are $\sim 0.7\,\mathrm{nmol \cdot L^{-1}}$, whereas those typical of marine surface waters are generally $< 0.02\,\mathrm{nmol \cdot L^{-1}}$ [9, 42]; algae efficiently remove cadmium from the water column, representing an important source for filter-feeders such as *A. colbecki* and their direct consumers such as *T. bernacchii* [42]. As a result, cadmium levels in marine organisms from Terra Nova Bay are often 10–20-fold higher than values measured in similar species from temperate latitudes [8, 10, 43–45]. The enrichment of cadmium in Terra Nova Bay has no direct adverse consequences for the organisms [10], but some indirect or modulatory effects on oxidative parameters might have been expected. Similar effects may include the enhanced formation of several reactive oxygen species, like hydroxyl radicals, superoxide anions, nitric oxide and hydrogen peroxide [46], the interaction with $-$SH groups and the inhibition of various antioxidant defences [10]. The elevated cadmium background at Terra Nova Bay influences the high metal content in digestive gland of the scallop *A. colbecki*, which further increases during intensive algal development in December, with relevant effects on oxyradical scavenging capacity some weeks later [41, 43].

Also in *T. bernacchii*, hepatic concentrations of cadmium were shown to almost double from $9.2\,\mu\mathrm{g \cdot g^{-1}}$ dry weight in November, to $> 20\,\mu\mathrm{g \cdot g^{-1}}$ in December and January, after the algal blooms [12]. No variations were measured in the levels of metallothioneins (data not shown), suggesting that the elevated levels of these proteins, $\sim$ 4–5-fold higher than those observed in temperate species [27], may compensate for the naturally high concentrations and seasonal variations in cadmium bioavailability at Terra Nova Bay. Similarly, the constant values of antioxidant defences in *T. bernacchii* reflect an elevated adaptation of this Antarctic fish to the high and fluctuating levels of oxidative pressure experienced by the organisms during different seasonal periods. In this respect, the adaptation of *P. antarcticum* to the elevated oxidative challenge naturally experienced by the embryos, would explain the limited response of this species to pro-oxidant chemicals [39].

In conclusion, this study provided an additional contribution to characterisation of the antioxidant system in different *Trematomus* species. Such defences have an important role in the adaptation to extreme conditions, and their basal levels appear able to counteract the naturally

elevated and fluctuating oxidative challenge in the Antarctic marine environment. Considering the potential effects of both global change and anthropogenic pollutants, alterations in these biochemical responses may represent a useful tool for the early assessment of environmental disturbance. Baseline knowledge on key sentinel species is thus fundamental for the proper assessment of biological impact and species vulnerability and should be adequately addressed in a coordinated standardisation of biomonitoring procedures in these remote areas.

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