The Antarctic 'ozone hole' combined with no sea ice causes severe oxidative damage in echinoid embryos

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Over the past three decades, the 'ozone hole' has caused a transient increase in the levels of ultraviolet B radiation (UV-B, 280 to 320 nm) reaching Antarctic coastal marine ecosystems[1]. The direct effect of this enhanced UV-B on pelagic organisms remains unclear, for few studies have examined *in situ* the responses of Antarctic marine organisms in direct relation to the 'ozone hole'. Here we show that the presence of the 'ozone hole' over McMurdo Sound, Antarctica, during a two-week period in 2008 resulted in unequivocal increases in oxidative damage and developmental abnormality in embryos of the sea urchin *Sterechinus neumayeri* Meissner (Echinoidea: Echinidae) growing in open waters. We show that although embryos have a limited capacity to increase the activities of protective antioxidant enzymes, increased UV-B exposure caused a very large increase in oxidative damage to proteins and lipids. Importantly, we show that embryo damage, resulting from the presence of the 'ozone hole', is largely mitigated by sea ice, with embryos beneath the ice protected from UV-B and hence oxidative damage. As the ozone hole is now expected to persist for a further 80 years[2], during which time significant reductions in sea ice coverage are expected around the Antarctic continent due to global warming[3], our findings sound a warning that the

coincidence of the two phenomena (high UV-B and open water conditions) will provide a window when significant damage to marine ecosystems may occur.

Recent increases in biologically harmful UV-B radiation penetrating the waters surrounding Antarctica, as a result of stratospheric ozone depletion, are well documented[1]. However, despite a number of excellent studies, the ways in which biological systems are responding to this increase are not adequately understood. Experimental evidence suggests that exposure to UV-B can reduce primary productivity[4], affect reproduction and development[5,6], and increase the mutation rate in phytoplankton, macroalgae, and in the eggs and larval stages of fish and other aquatic animals[7,8,9]. With the ozone hole now forecast to remain for longer than first predicted, and with climate change threatening to reduce sea-ice concentration, the impacts of increased UV-B on Antarctica's unique marine ecosystems could become critically important.

We examined oxidative stress and developmental abnormalities in *Sterechinus neumayeri* embryos in response to short-term fluctuations in stratospheric ozone concentrations, from both sea ice-covered and sea ice-free water columns. The production of reactive oxygen species (ROS) commonly occurs when organisms are exposed to solar UV-B radiation[10] so is a key cellular indicator of a UV-B induced stress.

Experiments were undertaken in McMurdo Sound (77°51.618'S, 166°40.660'E) over two consecutive 4-day periods in the spring of 2008 (26 – 30 October and 1 – 5 November). During this period, a 'hole' in the ozone layer covering 17 million km^2 was directly over the Antarctic continent. The 'hole' was located south of the study site in McMurdo Sound during the first experimental period, but was directly overhead of the study site during the second experimental period (Fig. 1). Ozone concentrations over McMurdo Sound ranged from 233 to 312 DU, with an average of 283 DU during the first experimental period, decreasing to between 166 and 269 DU with an average of 210 DU during the following 4-day experimental period (Table 1). Concurrent with changes in ozone concentrations were changes in ambient irradiances, the most noticeable being an increase in incident UV-B during the second experimental period (Table 1). Maximum UV-B increased 1.7-fold, total UV-B dose increased 3.8-fold and

the ratio of UV-B to visible light (measured from 400 – 600nm) doubled. Attenuation of light through the open water column was measured at midday using a LiCOR Li1800UW spectroradiometer, and indicated radiation levels at 1m were 24.6% of surface UV-B, 27.1% of surface UV-A and 28.4% of surface PAR. Irradiances were not measured beneath the sea ice in this study, however previous spectroradiometer readings from beneath sea ice (2.5 m thickness) at the same location and time of year, record under-ice irradiances of 0.29% of surface UV-B, 0.99% of surface UV-A and 1.1% of surface PAR[11].

In both experiments, 1-day old *S. neumayeri* embryos, obtained by standard *in vitro* spawning and culturing techniques[11], were placed *in situ* in inert plastic bags attached to racks, with various plastic filters used to adjust the radiation spectrum to which embryos were exposed. At the termination of each experiment embryos were recovered and immediately returned to the laboratory where a sub-sample was scored under a dissecting microscope for abnormal development, while the remainder of the sample was frozen and stored at -80°C for later quantification of oxidative damage and antioxidant enzyme activity.

Our experiment was designed to test the interaction of ozone depletion and sea ice cover on oxidative stress and abnormal embryonic development. We found that abnormality, and oxidative damage increased greatly in embryos exposed to UV-B (UV-T treatments) in the open water experiments compared to embryos protected from UV-B by artificial filters (UV minus (UV-O) and UV-B minus (UV-A)), or by sea ice (Fig. 2). While the activities of protective antioxidant enzymes increased significantly in embryos exposed to full UV-B, the increases were relatively small and were insufficient to protect the embryos from oxidative damage (Fig. 3). In addition, both abnormality and oxidative damage were clearly dose dependent, as although the same trends were observed for both experiments, embryos exposed to the higher ambient UV-B levels, found during the period of lower ozone column, had a significantly higher proportion abnormality and much greater oxidative damage. Specifically, in embryos exposed to UV-B in the first open water experiment (average of 283 DU, Total UV-B dose = 783.04 kJ m^{-2}), there was a 1.8-fold increase in the proportion of abnormal embryos, and a 1.8 and 2.5-fold increase in oxidized lipids and proteins respectively (Fig. 2), compared to the UV-O controls. In comparison, during the period of lower

ozone concentrations (average of 210 DU, Total UV-B dose = 204.4 kJ m^{-2}) in the second experiment, there was a 2.5-fold increase in the proportion of abnormal embryos, and 2.4 and 4.4-fold increases in oxidized lipids and proteins respectively (Fig. 2) in embryos exposed to UV-B. There was no difference in our measures of damage or enzyme activities in embryos on racks beneath the sea ice. The above results indicate that (1) UV-B induced oxidative damage occurs in dose-dependent (column ozone concentration-dependent) manner and (2) that sea ice plays a critical role in protecting embryos from fluctuations in ambient UV-B levels brought about by the presence or absence of the 'ozone hole'.

These findings raise a critically important question: what role might oxidative stress play in a changing Antarctic environment? A wide variety of marine taxa have been shown to mitigate oxidative damage via biochemical mechanisms[6]. In the present study we showed that the proportional increase in the activities of these antioxidant enzymes, in embryos exposed to full UV-B in open water conditions compared to those under the sea ice, was minor compared with the increases observed in oxidative damage to proteins and lipids. These results imply that while the activities of protective antioxidant enzymes do increase during times of enhanced UV-B stress, that this increase is not sufficient to provide protection from severe oxidative damage. In addition to high levels of UV-B, Antarctic marine organisms are challenged by low temperatures and high oxygen concentrations. The capacity to respond to oxidative stress may already be impaired in Antarctic marine organisms due to the inhibitory effects of low water temperatures on enzyme activities and metabolism[12].

Studies have shown that respiration in marine organisms declines at reduced temperatures[13]. Many marine organisms, adapted for life at low temperatures, have cellular modifications, including increased mitochondrial density and/or changes in plasma and mitochondrial membrane properties, such as the degree of lipid saturation, that help them to function at low temperatures. Such modifications have been shown to increase ROS production, particularly under conditions of stress[13] and hence a reduced capacity to scavenge ROS combined with a greater potential for ROS production could make some Antarctic marine organisms especially sensitive to environmental stresses with the potential to increase cellular ROS levels.

While enhanced UV-B will affect marine ecosystems at all levels, the impact may be more influential at certain times in a species life-history, particularly the embryonic and larval stages of marine invertebrates due to their small size, high transparency, rapid cell division and distribution near the ocean surface[14]. Antarctic larval forms in the Southern Ocean environment may be even more at risk than their temperate and tropical counterparts due to their polar adaptations, such as slow development and an isolated evolutionary history. Furthermore, many invertebrate spawning times coincide directly with the spring-time development of the 'ozone hole' making them highly susceptible to damage[4]. As embryos represent a key life-history stage, lower survival rates will reduce the long-term viability of affected populations. If, as our results suggest, the normal development of the embryos of sea urchins is dependent upon sea ice acting as a UV-B screening filter, protecting them from potentially damaging increases in UV-B caused by the presence of the ozone hole, significant reductions in sea ice coverage brought about by global warming could potentially have a significant impact on important and sensitive Antarctic ecosystems.

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Methods

Extractions. Lipid hydroperoxide samples were mixed with 600µl of methanol:chloroform (2:1) and left to stand for 1 minute. 400µl of chloroform was then added to this solution and shaken for 30 seconds. 400µl of deionised water was added to this and shaken for a further 30 seconds before placing tubes at 4°C overnight to allow the phases to separate. 50µl of the Chloroform phase was then transferred to a microtitre plate for analysis. Samples used for analysis of protein carbonyls and enzyme activities were mixed with 300µl of potassium phosphate buffer (pH 7.0) (containing 0.1mM Na_2 EDTA, 1% PVPP, 1nM PMSF and 0.5% TritonX-100) for approx 30 seconds then centrifuged (1,300*g*) for 5 minutes at 4°C. Aliquots of 50µl were stored at -80°C until assays were conducted.

Damage Assays. Lipid hydroperoxides were quantified using the ferric thiocyanate method described by Mihaljevic *et al*. (1996). Protein carbonyls were determined via reaction with 2.4-dinitrophenylhydrazine (DNPH) as described by Reznick and Packer, (1994). Both assays were adapted for measurement in a microplate reader.

Antioxidant enzyme Assays. Superoxide dismutase (SOD) (EC 1.15.1.1) was assayed using the microplate assay described by Banowetz *et al*. (2004) with minor modifications. Catalase (CAT) (EC 1.11.1.6) was assayed using Maral *et al*. (1977)'s chemiluminescent method, adapted according to Janssens *et al*. (2000). Protein was quantified according to the assay described by Fryer *et al*. (1986). All assays were carried out using a PerkinElmer (Wallac) 1420 multilabel counter (Perkin Elmer, San Jose, California, U.S.A.), controlled by a PC, and fitted with a temperature control cell and an auto-dispenser. Data were acquired and processed using the WorkOut 2.0 software package (Perkin Elmer, San Jose, California, U.S.A.).

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Authour Contributions K.N.L and M.D.L designed the study and conducted field experiments in the Antarctic. K.N.L. and D.J.B. carried biochemical analyses. All three authors discussed results and prepared the manuscript.

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Lister_Fig1.pdf

Ozone concentrations (Dobson units – DU) over the Antarctic continent on 26 October and 3 November 2008. The location of the 'ozone hole' (where ozone concentrations were < 200 DU as indicated by blue/purple shading) tended to be south of the experiments deployed in McMurdo Sound during the first 4-day experimental period (26 – 30 October) and overhead during the second experimental period (1 – 5 November). Table 1 reports details of overhead ozone conditions during the two experimental periods.

Lister_Fig2.pdf

Proportion of abnormal development (a), protein carbonyls (b) and lipid hydroperoxides (c) as a function of experimental period, sea ice coverage and light treatment in *Sterechinus neumayeri* embryos. *In situ* exposures were undertaken by out-planting bagged embryos (1L of filtered seawater containing 25 embryos ml^{-1}) onto experimental PVC racks (31 × 26 cm horizontal dimensions) at one metre down a moored rope. Each rack was covered by a plexiglass light filter that either (i) removed UV-B (50% transmittance cut-off at 370nm, 0.1% UV-B transmittance) but retained a proportion of UV-A (46.5% transmittance) and PAR (77.9% transmittance), (ii) removed both UV-B and UV-A (50% transmittance cut-off at 407nm, 0.0% UV-B, 5.2% UV-A and 81% PAR transmittance) or (iii) transmitted the full light spectrum (80.6% UV-B, 84.6% UV-A and 84.5% PAR transmittance). Three replicate racks (one for each light treatment) were located in the open water location (open water treatment) and nine replicate racks (three for each treatment) were located under the annual sea ice of two metres thickness (sea ice treatment). Experiments were run over two consecutive periods that represented higher column ozone concentrations (green columns, 26 – 30 October) and lower column ozone concentrations (blue

columns, 1 – 5 November). Proportion of abnormal development was scaled to one for the no-UV control treatments (UV-O), and an example of normal versus abnormal embryos is shown (a). For all columns in the open water and under ice experiments mean ± s.e.m was calculated from four and three replicates respectively. Statistically significant differences between treatments and experimental periods were tested using a two-way ANOVA. 'Open water' and 'sea ice' experiments were analysed separately. Within each graph, bars sharing the same letter are not significantly different (at a significance level of 0.05).

Lister_Fig3.pdf

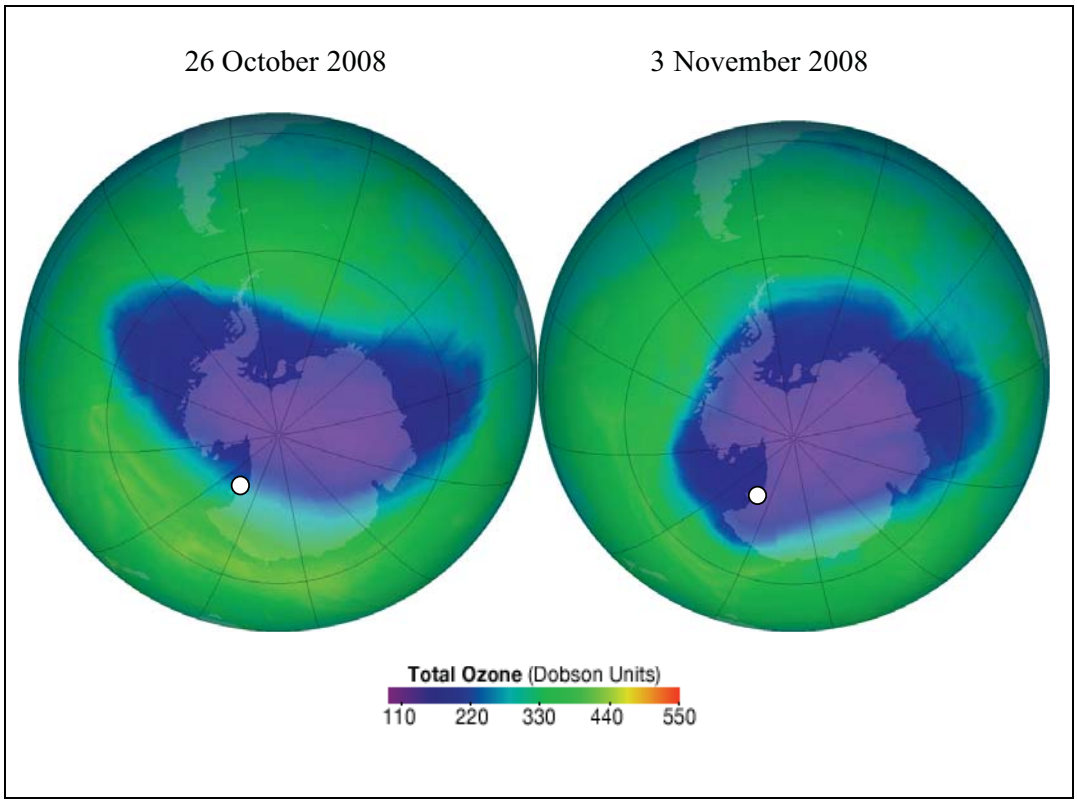
Superoxide dismutase and catalase activities as a function of experimental period, sea ice coverage and light treatment in *Sterechinus neumayeri* embryos. The experimental design and conditions are described in Fig. 2. Within each graph, bars sharing the same letter are not significantly different (at a significance level of 0.05).

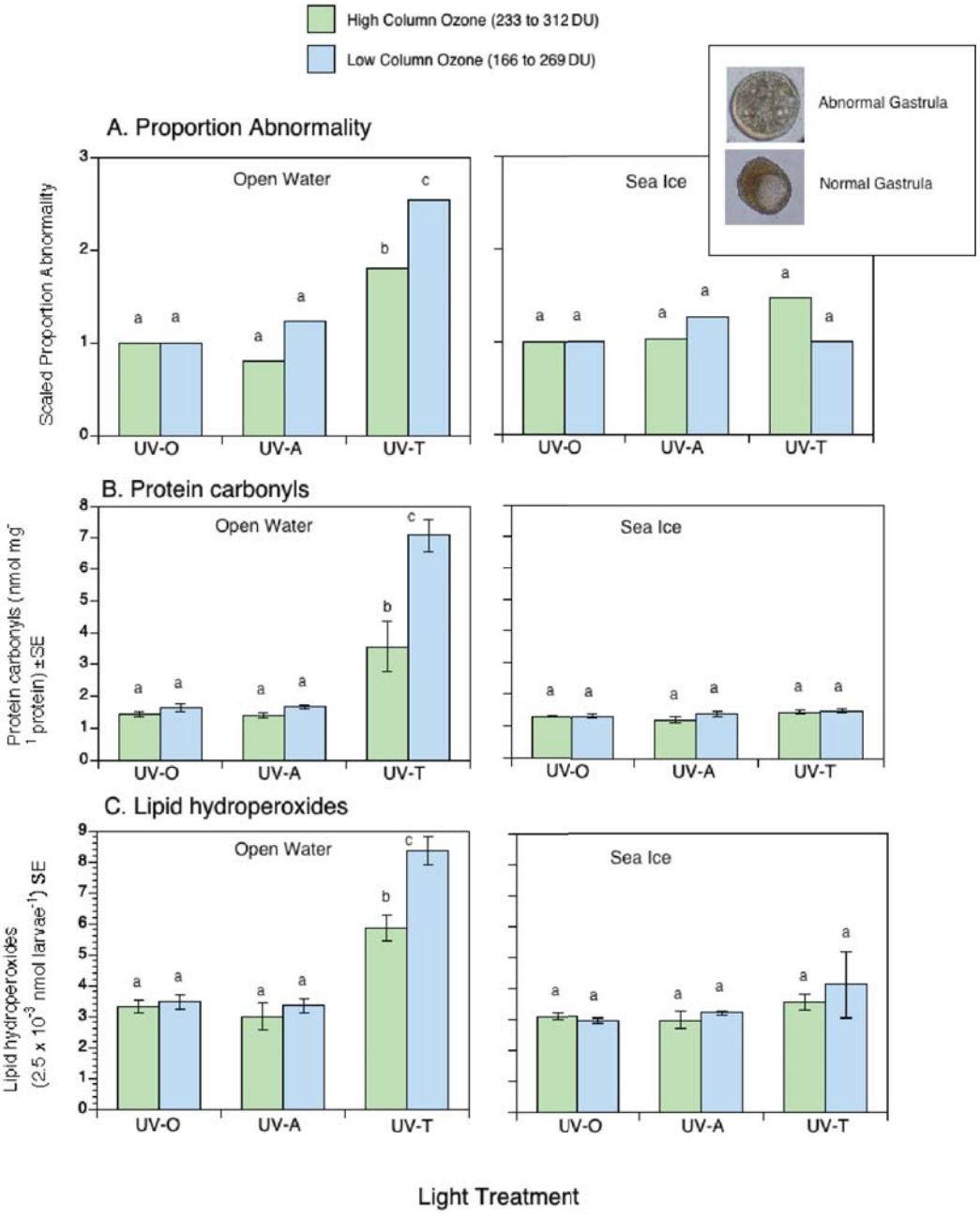
Lister_Table1.pdf

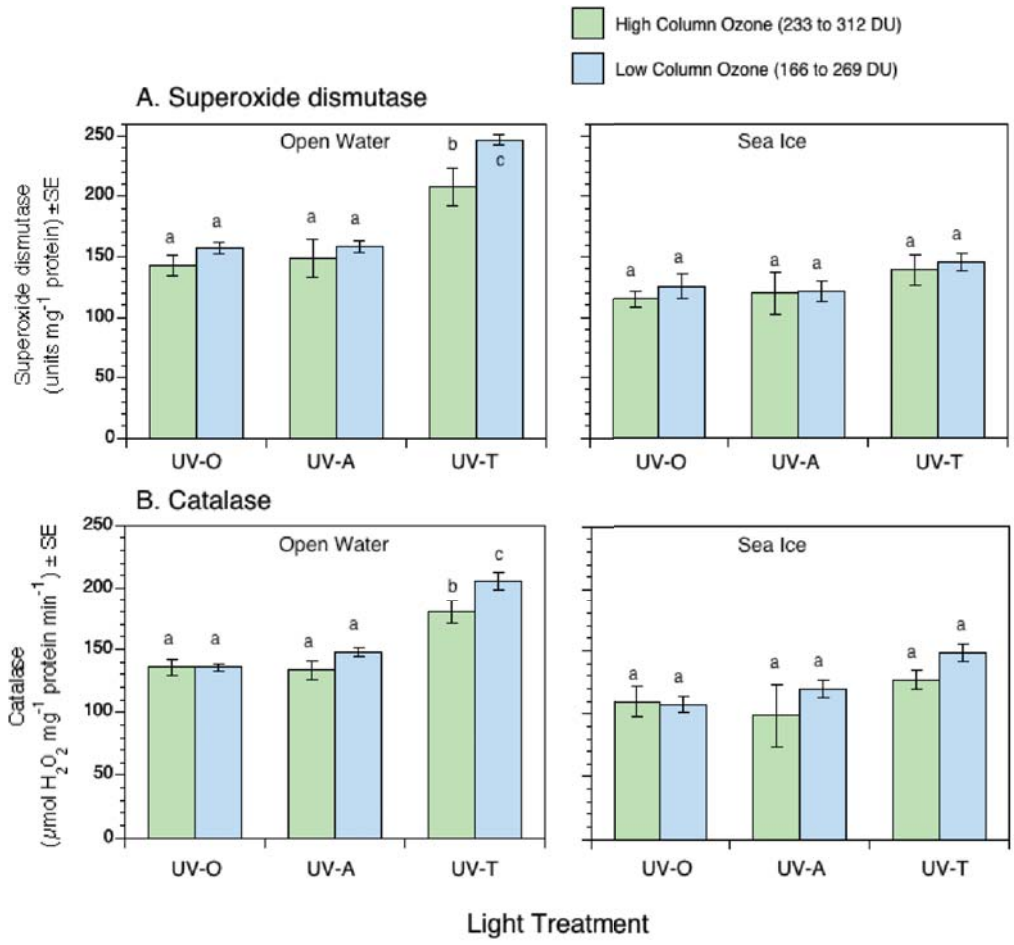
Atmospheric ozone concentrations (Dobson units - DU) over McMurdo Sound (77°51.618'S, 166°40.660'E) from 26 to 30 October, and 1 to 5 November 2008. (b) Surface irradiances, mean daily dose and total dose of UV-B (290-320nm), UV-A (320-400nm) and visible light (400-600nm) over McMurdo Sound during the two experimental periods.

	Max ozone (DU)	Min ozone (DU)	Mean ozone (DU)
26 - 30 Oct	312	233	283
1 - 5 Nov	269	166	210

	Max. irradiance ($W\ m^{-2}\ /s^{-1}$)		Mean daily dose ($kJ\ m^{-2}$)		Total dose ($kJ\ m^{-2}$)	
	26 - 30 Oct	**1 - 5 Nov**	**26 - 30 Oct**	**1 - 5 Nov**	**26 - 30 Oct**	**1 - 5 Nov**
UV-B	1.05	1.80	34.15	54.52	204.45	783.04
UV-A	32.81	32.89	1104.72	1281.62	4806.08	18407.27
VIS	194.19	159.71	5338.52	5998.20	22493.23	86149.08
UVB:PAR	0.01	0.02				


26 October 2008
3 November 2008
Total Ozone (Dobson Units)
110
220
330
440
550


High Column Ozone (233 to 312 DU)
Low Column Ozone (166 to 269 DU)
Abnormal Gastrula
Normal Gastrula
A. Proportion Abnormality
Scaled Proportion Abnormality
Open Water
Sea Ice
B. Protein carbonyls
Protein carbonyls (nmol mg-1 protein) ±SE
C. Lipid hydroperoxides
Lipid hydroperoxides (2.5 x 10-3 nmol larvae-1) SE
UV-O
UV-A
UV-T
Light Treatment


High Column Ozone (233 to 312 DU)
Low Column Ozone (166 to 269 DU)
A. Superoxide dismutase
Open Water
Sea Ice
Superoxide dismutase (units mg-1 protein) ±SE
250
200
150
100
50
0
a
a
a
a
b
c
a
a
a
a
a
a
UV-O
UV-A
UV-T
B. Catalase
Catalase (µmol H2O2 mg-1 protein min-1) ± SE
Light Treatment