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Seasonal Acclimatization of Antioxidants and Photosynthesis in *Chondrus crispus* and *Mastocarpus stellatus,* Two Co-Occurring Red Algae With Differing Stress Tolerances

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Abstract. *Mastocarpus stellatus* and *Chondrus crispus* are red macroalgae that co-dominate the lower rocky intertidal zones of the northern Atlantic coast. *M. stellatus* is more tolerant than *C. crispus* of environmental stresses, particularly those experienced during winter. This difference in tolerance has been attributed, in part, to greater contents or activities of certain antioxidants in *M. stellatus.* We compared the photosynthetic capacities and activities of three antioxidant enzymes—superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reductase (GR)—as well as the contents of ascorbate from fronds of *M. stellatus* and *C. crispus* collected over a year. Photosynthetic capacity increased in winter, but did not differ between species in any season. The activities of the three antioxidant enzymes and the contents of ascorbate were significantly greater in tissues collected during months with mean air and water temperatures below 7.5 °C ("cold" months; December, February, March, April) than in months with mean air temperatures above 11 °C ("warm" months; June, July, August, October). Overall, *C. crispus* had significantly greater SOD and APX activities, while *M. stellatus* had higher ascorbate contents. Species-specific differences in GR activity depended upon mean monthly temperatures at the time of tissue collection; *C. crispus* had higher activities during cold months, whereas *M. stellatus* had higher activities during warm months. Taken together, these data indicate that increased ROS scavenging capacity is a part of winter acclimatization; however, only trends in ascorbate content support the hypothesis that greater levels of antioxidants underlie the relatively greater winter tolerance of *M. stellatus* in comparison to *C. crispus.*

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Abbreviations: APX, ascorbate peroxidase; FW, fresh weight; GR, glutathione reductase; ROS, reactive oxygen species; SOD, superoxide dismutase.

Introduction

Mastocarpus stellatus (Stack. in With.) Guiry and *Chondrus crispus* Stackhouse are morphologically similar red macroalgae that occupy the lower rocky intertidal zone of exposed regions of the northern Atlantic coast. *M. stellatus* is more tolerant than *C. crispus* of environmental stresses, particularly those experienced during winter. Photosynthesis recovers more quickly in *M. stellatus* than in *C. crispus* after a bout of freezing in a laboratory setting (Dudgeon *et al.,* 1989, 1995). Frond bleaching and declines in photosynthesis and growth occur in *C. crispus* after long-term experimental exposure to periodic freezing, whereas these parameters remain unaffected in *M. stellatus* (Dudgeon *et al.,* 1990). Thalli of *C. crispus* become larger than those of *M. stellatus,* but the stipes are not as strong, leading to greater biomass loss during winter (Dudgeon and Johnson, 1992; Pratt and Johnson, 2002).

Collén and Davison (1999) reported that a greater capacity to scavenge reactive oxygen species (ROS) might contribute to the relatively greater stress tolerance of *M. stellatus.* ROS are reduced, and highly reactive, species of molecular oxygen such as superoxide and hydrogen peroxide, which are capable of damaging essential macromolecules (Halliwell and Gutterridge, 1999). ROS are by-products of bioenergetic pathways, particularly photosynthesis; however, exposure to environmental perturbations such as thermal stress, especially chilling, can greatly increase the rate of their formation (Niyogi, 1999). This is because

chilling inhibits the enzyme-catalyzed reactions of the Calvin cycle, but has a far smaller effect on photosynthetic light absorption and electron transport (Baker, 1994; Wise, 1995). Therefore, chilling leads to an increase in the absorption of light that exceeds the capacity for photosynthetic utilization (*i.e.,* excess light), and ROS production correlates with excess light absorption (Logan *et al.,* 1998a; Niyogi, 1999).

The primary mechanism for minimizing ROS-mediated cellular damage involves antioxidants, which are enzymes and low-molecular-weight compounds that detoxify ROS (reviewed in Asada, 1999). In the chloroplasts of photosynthetic tissues, superoxide can be safely converted to water *via* the combined activities of superoxide dismutase (SOD; EC 1.15.1.1), which catalyzes the disproportionation of two superoxides to molecular oxygen and hydrogen peroxide; and ascorbate peroxidase (APX; EC 1.11.1.11), which catalyzes the reduction of hydrogen peroxide to water, utilizing ascorbate as a reductant. Chloroplasts possess multiple mechanisms that recycle oxidized ascorbate, including one that couples the oxidation of glutathione to ascorbate reduction. Oxidized glutathione, in turn, is reduced by glutathione reductase (GR; 1.6.4.2).

M. stellatus, in comparison to *C. crispus,* was reported to possess larger pools of ascorbate and β-carotene (a lipid-soluble antioxidant) and greater activities for certain antioxidant enzymes, including GR (Collén and Davison, 1999). These species-specific differences were based upon measurements of fronds harvested in late summer and early autumn. This is relevant because levels of antioxidants undergo seasonal acclimatization. In photosynthetic tissues of algae and plants, acclimatization to colder temperatures generally involves several-fold increases in the activities and contents of antioxidants (Anderson *et al.,* 1992; Logan *et al.,* 1998b, 2003; Mallick and Mohn, 2000; Collén and Davison, 2001). Therefore, the levels of antioxidants in algae harvested when water temperatures are near their peak (late summer) may not be indicative of those in winter. We expanded upon those previous studies by comparing SOD, APX, and GR activities as well as ascorbate contents from *M. stellatus* and *C. crispus* collected on nine occasions over the course of a year. Since photosynthetic activity can influence the rate of ROS production, we also measured the photosynthetic capacities of the two species in all four seasons.

Materials and Methods

Site description and collection protocol

Mastocarpus stellatus (Stack. in With.) Guiry and *Chondrus crispus* Stackhouse were collected about 500 m northeast of the Giant's Stairs on Bailey Island, Maine (43°43′N 69°59′W). All collections were made from a single mixed stand of the two species on an exposed rock face in the intertidal zone. For the measurements of antioxidants, collections were made in 2001 on 16 March, 23 April, 29 June, 27 July, 14 August, 22 October, and 22 December, and in 2002 on 7 February, 25 March (collections are identified by month in the figures and tables, with March 2001 preceding March 2002). The algae were harvested at low tide near noon on clear sunny days. Samples were placed in plastic bags with paper towels moistened with seawater, transported to the laboratory in no more than 45 min, and stored in liquid nitrogen until analysis.

For measurements of photosynthesis, samples were collected from the same site in 2002 on 12 and 18 September (data combined) and 18 December, and in 2003 on 4 March and 20 July. Algae were collected in the afternoon in September and December; collected near dawn in March; and in mid-morning in July. Samples were handled as above and maintained at room temperature in September and July and on ice in December and March. All photosynthetic measurements were completed within 12 h of collection, during which time no systematic loss of photosynthetic activity was observed (data not shown).

Tissue extraction

Frozen frond tissue (0.3–0.6 g fresh weight (FW)) for assays of SOD and GR activity were ground in a prechilled mortar with a small amount of acid-washed sand and extracted with 1.5 ml 50 m*M* potassium phosphate buffer with 0.1 m*M* EDTA, 0.3% (w/v) Triton X-100, and 4% (w/v) soluble PVP-10, pH 7.6, as in Logan *et al.* (1998b). Samples for assays of APX activity were ground in 1.5 ml of the same buffer, with the addition of 1.3 m*M* ascorbate, as in Logan *et al.* (1998b). Samples for assays of ascorbate content were ground in 1 ml 6% H_2SO_4. All samples were centrifuged at 9300 $\times$ *g* for 10 min at 4 °C, and the supernatant was collected and stored on ice until measured. Samples used to measure SOD activity were dialyzed using Slide-A-Lyzer dialysis cassettes (10,000 MWCO, Product # 66425, Pierce Biotechnology, Inc, Rockford, IL). The pH of extracted samples used to measure ascorbate was raised to between 1 and 2 with the addition of Na_2CO_3, as in Logan *et al.* (1998b).

Antioxidant enzymes and ascorbate

All enzyme assays were performed at 25 °C in a temperature-controlled cuvette of a DU640 spectrophotometer (BeckmanCoulter, Fullerton, CA). Total assay volume for SOD, GR, and APX was 2 ml. Total assay volume for ascorbate was 1 ml.

To measure SOD activity, an NADH/phenazine methosulfate superoxide-generating system was employed. SOD activity was monitored as the inhibition of cytochrome *c* reduction. The SOD assay buffer consisted of 50 m*M* potassium phosphate, 0.1 m*M* EDTA, 0.01 m*M* cytochrome *c,*

0.1 m*M* NADH, pH 7.6. To this solution was added 200 μl of sample extract, and the change in absorbance at 550 nm was followed for 1 min after the addition of a sufficient volume of 1 μ*M* phenazine methosulfate to bring about a control rate of cytochrome *c* reduction of 0.125 absorbance units min^{-1}. SOD activity was corrected for the rate of nonspecific cytochrome *c* reduction, which was determined prior to the addition of phenazine methosulfate.

APX activity was quantified as the rate of ascorbate oxidation. The APX assay buffer consisted of 50 m*M* Hepes-KOH, 0.1 m*M* EDTA, 0.5 m*M* ascorbate, pH 7.6. To this was added 40 μl of sample extract, and the change in absorbance at 290 nm was measured for 1 min after the addition of 8 μl of 50 m*M* H_2O_2 (Logan *et al.,* 1998b). APX activity was corrected for the rate of nonspecific ascorbate oxidation, which was determined prior to the addition of H_2O_2.

GR activity was quantified as the rate of NADPH oxidation. The GR assay buffer consisted of 100 m*M* Tris-HCl, 1 m*M* EDTA, 0.5 m*M* oxidized glutathione, pH 8.0. To this was added 100 μl of sample extract, and the change in absorbance at 340 nm was measured for 1 min after the addition of 20 μl of 5 m*M* NADPH (Logan *et al.,* 1998b). GR activity was corrected for the rate of nonspecific NADPH oxidation, which was determined in the absence of oxidized glutathione.

The ascorbate assay buffer consisted of 200 m*M* sodium acetate, pH 5.6; 100 μl of pH-adjusted sample extract was mixed with 900 μl assay buffer, and the change in absorbance at 265 nm was recorded after reaction with 2 units ascorbate oxidase (Logan *et al.,* 1998b). Ascorbate content was determined by comparison to a standard curve.

Measurements of photosynthesis

Frond tissue (0.10–0.12 g FW) was placed in the chamber of a gas-phase oxygen electrode (model LD-2, Hansatech, King's Lynn, Norfolk, UK) on a pad moistened with seawater. Photosynthesis was measured in an atmosphere of humidified 5% CO_2, 21% O_2, and the balance N_2. The temperature of the frond tissue was maintained at 20 °C (the optimum temperature for both species, according to Mathieson and Burns (1971)), *via* the use of a circulating water bath. Tissues were exposed to progressively increasing light intensities for an overall period of about 12 min until the measuring intensity of 1700 μmol photons m^{-2} s^{-1} (saturating light) was reached. Measurements were made after the frond tissue had achieved steady state and rates of oxygen production were linear. Respiratory oxygen consumption was measured by darkening the sample. Gross photosynthetic capacity was calculated as the sum of net photosynthetic oxygen evolution and respiratory oxygen consumption.

Mean monthly temperatures

Temperature data were obtained from the Central Maine Shelf buoy (E0110) (43°42′43″N 69°21′20″W) of the Gulf of Maine Ocean Observing System (GoMOOS) for dates as far back as July 2001, with a gap at Buoy E0110 from October 2001 through December 2001 (Table 1).

Statistical analysis

Differences in antioxidant activities and contents were assessed by using temperature data, and photosynthetic capacities by mean monthly air and water temperature (Table 1). Months with mean monthly air and water temperatures greater than or equal to 11 °C (June, July, August, October) were pooled as "warm" months, and months with mean monthly air and water temperatures less than or equal to 7.5 °C (December, February, March, April) were pooled as "cold" months. ANOVA was followed by *a posteriori* Fisher's protected least significant difference (PLSD) tests when there was a significant interaction between species and temperature. These statistical analyses were followed by *a posteriori* Fisher's PLSD for planned monthly pairwise comparisons of means for each species. All statistics were performed using the statistical software program Statview

Table 1

Mean air temperature and mean water temperatures at 1 m depth for each month in which fronds were collected; all data from buoys in the Gulf of Maine Ocean Observing System (GoMOOS)

	2001							2002	
	Mar.[1]	Apr.[1]	June[1]	July[2]	Aug.[2]	Oct.[3]	Dec.[3]	Feb.[2]	Mar.[2]
Mean Air Temperature (°C)	1.4 ± 0.6	4.1 ± 0.7	11.7 ± 0.4	16.5	17.3	11.0 ± 0.1	3.9 ± 0.2	1.1	2.4
Mean Water Temperature (°C)	3.7 ± 0.5	4.0 ± 0.5	11.0 ± 0.3	15.6	16.1	11.9 ± 0.5	7.5 ± 0.02	5.1	4.7

[1] Mean (± SE of the mean of the means) using data that was averaged from 2002, 2003, and 2004 for GoMOOS Buoy E0110.
[2] Means from GoMOOS Buoy E0110.
[3] Mean (± SE of the mean of the means) using data that was averaged from the two GoMOOS Buoys that are closest to E0110 for that same year.

ver. 5.0.1 (SAS Institute, Cary, NC). Significance refers to $P \leq 0.05$. All statistics are given in the figure legends.

Results and Discussion

The activities of the three measured antioxidant enzymes (SOD, APX, and GR) and the contents of ascorbate were significantly greater in tissues collected during "cold" months (*i.e.,* mean monthly air and water temperatures less than 7.5 °C; December, February, March, April) in comparison to "warm" months (*i.e.,* mean monthly air temperatures greater than 11 °C; June, July, August, October) (Figs. 1–4). Similar up-regulation in antioxidant capacity has been reported for algae (Mallick and Mohn, 2000; Collén and Davison, 2001) and the leaves of many terrestrial plant species after the onset of winter (Anderson *et al.,* 1992; Logan *et al.,* 1998b) or experimentally imposed chilling (de Kok and Oosterhuis, 1983; Schöner and Krause, 1990; Mishra *et al.,* 1993; Xin and Browse, 2000). Cold-induced increases in the levels of antioxidant enzymes compensate for the effect of lower temperatures on their activities. Elevated levels of antioxidants may also be required in winter to cope with an enhanced rate of ROS formation that presumably results when chilling inhibits the use of light energy in the Calvin cycle.

When the data were pooled to examine antioxidant differences between species, *Chondrus crispus* had significantly greater SOD and APX activities than *Mastocarpus stellatus* (Figs. 1 and 2, respectively). *M. stellatus* had higher ascorbate contents than *C. crispus* (Fig. 4). Species-specific differences in GR activity depended upon mean monthly temperatures at the time of tissue collection; *C. crispus* had higher activities during cold months, whereas *M. stellatus* had higher activities during warm months (Fig. 3). Taken together, these data indicate that increased ROS scavenging capacity is a part of winter acclimatization; however, only trends in ascorbate content support the hypothesis that greater levels of antioxidants underlie the relatively greater winter tolerance of *M. stellatus* in comparison to *C. crispus.* Our approach does not allow us to examine the further possibility that either or both species expressed antioxidant enzyme isoforms with differing kinetic characteristics (*e.g.,* lower temperature optimum, or K_m) during colder months, as has been observed in some terrestrial plants (Guy and Carter, 1984).

We have expressed levels of antioxidants per unit frond fresh weight because we believe that this is the most functionally relevant basis of expression and because it allows direct comparison with previously published work (see, for example, Collén and Davison, 1999, 2001). Had we chosen to express our findings on the basis of frond chlorophyll content, seasonal changes in this parameter itself would have unduly influenced seasonal trends in levels of antioxidants. Fronds of *M. stellatus* tend to be thicker than those of *C. crispus* (Dudgeon and Johnson, 1992); therefore, antioxidant enzyme activities expressed per unit frond area may be greater in *M. stellatus* than in *C. crispus.*

Since photosynthetic activity can influence the rate of ROS production, we measured photosynthetic capacities at

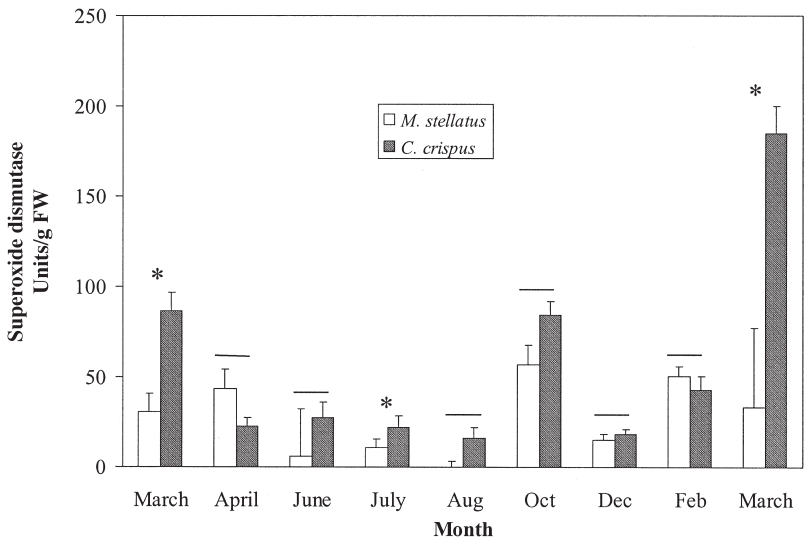

Figure 1. Superoxide dismutase activity, by month, for *Chondrus crispus* (dark bars) and *Mastocarpus stellatus* (white bars). When analyzed by mean monthly temperature, there were significant differences by temperature ($P_{1,86} = 0.003$) and by species ($P_{1,86} = 0.007$) with no significant interaction ($P_{1,86} = 0.7$): overall, both species had higher activities in the cold months, and *C. crispus* had higher activities than *M. stellatus.* A horizontal line indicates no significant difference ($P > 0.05$) between species in a given month, and an asterisk indicates significant difference ($P < 0.05$). Error bars are one standard error; $n = 5$.

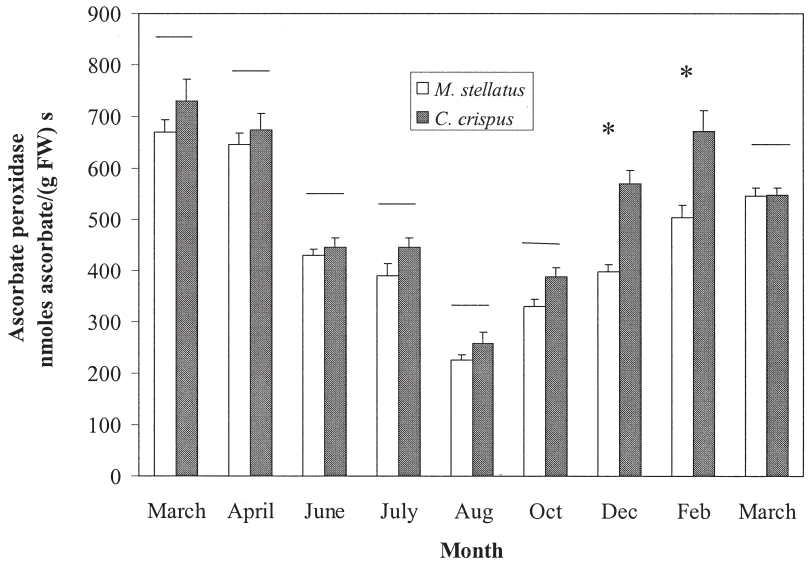

Figure 2. Ascorbate peroxidase activity, by month, for *Chondrus crispus* (dark bars) and *Mastocarpus stellatus* (white bars). When analyzed by mean monthly temperature, there were significant differences by temperature ($P_{1,106} < 0.0001$) and by species ($P_{1,106} = 0.004$) with no significant interaction ($P_{1,106} = 0.4$): overall, both species had higher activities in the cold months (especially in March and April), and *C. crispus* had higher activities than did *M. stellatus* (especially in December and February). A horizontal line indicates no significant difference ($P > 0.05$) between species in a given month, and an asterisk indicates significant difference ($P < 0.05$). Error bars are one standard error; $n = 5$–10.

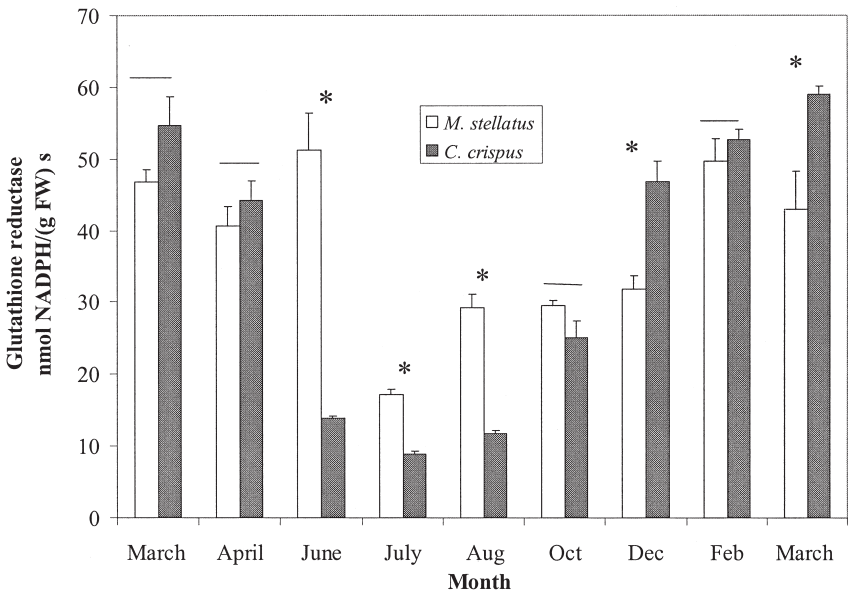

Figure 3. Glutathione reductase activity, by month, for *Chondrus crispus* (dark bars) and *Mastocarpus stellatus* (white bars). When analyzed by mean monthly temperature, there were significant differences by temperature ($P_{1,104} < 0.0001$) and by species ($P_{1,104} = 0.03$) with significant interaction ($P_{1,104} < 0.0001$): overall, both species had higher activities in the cold months, but *C. crispus* had relatively higher activities than did *M. stellatus* in cold months whereas *M. stellatus* had relatively higher activities than did *C. crispus* in the warm months (each *a posteriori* $P < 0.01$). A horizontal line over species in a given month indicates no significant difference ($P > 0.05$) between species in a given month, and an asterisk indicates a significant difference ($P < 0.05$). Error bars are one standard error; $n = 5$.

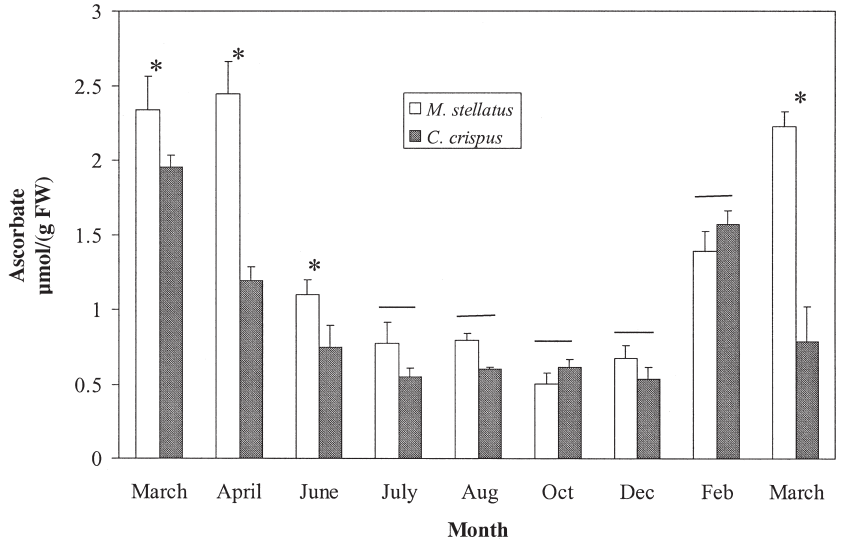

Figure 4. Ascorbate contents, by month for *Chondrus crispus* (dark bars) and *Mastocarpus stellatus* (white bars). When analyzed by mean monthly temperature, there were significant differences by temperature ($P_{1,125} < 0.0001$) and by species ($P_{1,125} = 0.002$) with no significant interaction ($P_{1,125} = 0.2$): overall, both species had higher contents in the cold months, and *M. stellatus* had higher contents than did *C. crispus* (especially in March and April). A horizontal line indicates no significant difference ($P > 0.05$) between species in a given month, and an asterisk indicates significant difference ($P < 0.05$). Error bars are one standard error; $n = 5$–10.

20 °C in all four seasons. Capacities for photosynthetic oxygen evolution underwent 3-fold increases from summer to winter in both species; however, statistically significant differences between species were not observed in any season (Table 2). Significant species-specific differences were also not observed when photosynthesis was measured at 5 °C from fronds collected in March, although rates were lower than at 20 °C (21 ± 7 and 25 ± 1 nmol O_2 (g FW)$^{-1}$ s^{-1} for *M. stellatus* and *C. crispus,* respectively; mean ± SD, $n = 5$).

Cold-induced increases in photosynthetic capacity have been reported for *M. stellatus* and *C. crispus* (Dudgeon *et al.,* 1995) as well as for winter cereal varieties and other terrestrial plants that actively grow in winter (Huner *et al.,* 1993). Such increases are interpreted as a means of compensating for the effects of low temperatures on enzyme activities. Increased photosynthetic capacity in winter may also have been influenced by greater nitrogen availability in this season (Topinka and Robbins, 1976).

Table 2

Photosynthetic oxygen evolution at 20 °C by month for Chondrus crispus *and* Mastocarpus stellatus

	nmol O_2 (g FW)$^{-1}$ s^{-1}			
Species	September	December	March	July
Mastocarpus stellatus	39 ± 6	59 ± 8	40 ± 7	22 ± 4
Chondrus crispus	41 ± 8	60 ± 4	46 ± 8	16 ± 4

Data are means ± standard deviation. There were significant differences in means by month ($P_{3,36} < 0.0001$) but not by species ($P_{1,36} = 0.50$), and there was no significant interaction ($P_{3,36} = 0.86$); all differences between months are significant (each *a posteriori* $P < 0.001$) except for between March and September (*a posteriori* $P = 0.49$); n = 6.

Previous reports that *M. stellatus* is relatively more resistant to freezing damage to the photosynthetic apparatus were based upon freezing regimens imposed experimentally in a laboratory (Dudgeon *et al.,* 1989, 1990, 1995). It is possible that conditions in the field around the time of our collections were not stressful enough for species to show differences in winter tolerance. Alternatively, since our measurements of photosynthetic capacity were performed in humidified air, whereas macroalgal photosynthesis is typically measured from submerged thalli, it may be that winter rates of photosynthesis for *C. crispus* and *M. stellatus* are more similar in air than they are in water. Intertidal algae may perform a significant proportion of total daily photosynthesis during exposure to air at low tide when light intensity is not attenuated by passage through seawater, and there can be species-specific differences in the rates of photosynthesis in air and in water. For example, *Ascophyllum nodosum,* an intertidal brown macroalgae that is also native to the north Atlantic coast, photosynthesizes at slightly greater rates when exposed than when submerged (Johnson and Raven, 1986), whereas the converse occurs in the green alga *Ulva* sp. (Johnson *et al.,* 1974; Quadir *et al.,* 1979)

The potential for tissue freezing may be the most stressful

aspect of the winter season, not cold temperatures *per se.* The tissue damage brought on by freezing is for the most part ultrastructural and results from extreme cellular desiccation induced by extracellular freezing or by the effects of intercellular ice formation (Nilsen and Orcutt, 1996). Antioxidants, on the other hand, protect cells from biochemical damage caused by ROS, whose production increases during imbalances within and between bioenergetic pathways (Wise, 1995). Therefore, one may not expect antioxidants to play a direct role in protection against freezing damage. In *Fucus vesiculosus,* experimental freezing led to an increase in the production of ROS only in thalli that had been acclimated to warm (20 °C) conditions; it did not affect ROS production in thalli that had been acclimated to 0 °C or 0 °C with daily exposure to −10 °C for 3 h (Collén and Davison, 2001).

If levels of antioxidants cannot fully explain the difference in winter tolerance between *M. stellatus* and *C. crispus,* what might? Resistance to freezing damage is greatly enhanced by the accumulation of a class of compounds known as cryoprotectants (Lillford and Holt, 2002). Cryoprotectants serve as osmolites that reduce the shrinkage cells experience when extracellular freezing causes water efflux. They may also stabilize proteins and membranes during cell collapse. Mannitol, a sugar alcohol, is among the most common cryoprotectants. *M. stellatus* contains mannitol, but *C. crispus* does not (Mathieson and Prince, 1973). Perhaps the presence of mannitol explains the relatively greater resistance of *M. stellatus* to freezing damage (Dudgeon *et al.,* 1989, 1990, 1995). This possibility merits more attention, including a comprehensive characterization of the tissue cryoprotectants in these two species.

Conclusions

In *Mastocarpus stellatus* and *Chondrus crispus,* as in other photosynthetic organisms, acclimatization to winter involved profound up-regulation in the capacity to detoxify ROS *via* antioxidants. In winter and early spring, when tolerance to chilling and freezing is important for survival, the activities of SOD, APX, and GR were greater in *C. crispus* than in *M. stellatus* and therefore cannot explain the relatively greater stress tolerance of *M. stellatus.* Of the measured antioxidants, only ascorbate content was significantly greater in *M. stellatus.*

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