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Mediterranean Marine Science

Vol. 11, 2010



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https://doi.org/10.12681/mms.76

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To cite this article:

CAPARKAYA, D., CENGIZ, S., DINCEL, B., DEMIR, S., & CAVAS, L. (2010). The Effects of UV Exposure on the Antioxidant Enzyme Systems of Anemones. *Mediterranean Marine Science, 11*(2), 259-276. doi:https://doi.org/10.12681/mms.76

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Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson)
The journal is available on line at http://www.medit-mar-sc.net

Research Article

The Effects of UV Exposure on the Antioxidant Enzyme Systems of Anemones

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Received: 23 October 2009; Accepted: 8 April 2010; Published on line: 22 October 2010

Abstract

Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) which are housekeeping enzymes protect cells from the harmful side effects of reactive oxygen species (ROS). *Anemonia sulcata* var. *smaragdina* and *Anemonia rustica* are widely distributed along the Turkish coastlines of the Aegean Sea. Recent studies have shown that environmental stresses such as elevated temperature, ultraviolet light, pathogen infection and decreased salinity might cause well-known bleaching effects in *Anemonia* species. The effect of UV-light on antioxidant enzyme activities such as SOD, CAT, GSH-Px, protein levels and secondary pigments were determined in *A. sulcata* var. *smaragdina* and *A. rustica.* SOD, CAT, GSH-Px activities, protein levels and secondary pigments of these morphotypes were observed in both tentacles and columns separately. According to studies on bleaching, elevated UV radiation may cause the bleaching event in anemones as a stress factor. However, in the present study no bleaching event was observed in anemone samples even after they were subjected to 5 hours of UV-exposure intervals. Moreover, UV exposure did not change antioxidant systems remarkably. However, more investigations are still needed to obtain the complete picture of the effects of UV-light on the cellular pathways of cnidarian–algal symbiosis.

Keywords: *Anemonia sulcata; Anemonia rustica;* Antioxidant enzymes; Bleaching; Lipid peroxidation.

Introduction

The sea anemone *Anemonia viridis* (Forsskål) (Cnidaria, Anthozoa) is a common species for the Mediterranean Sea and it is abundant along these shores. It is characterised by a short column and long green tentacles (PICTON and MORROW, 2007). Two well-known morphotypes of *Anemonia viridis* have been previously named as *Anemonia sulcata* var. *smaragdina* and *Anemonia rustica,* but these names are accepted as synonyms of *A. viridis* nowadays. The colour differences among these morphotypes are caused by the green fluorescent protein (GFP)-like proteins and pink pigments in *Anemonia sulcata* var. *smaragdina* (WIEDENMANN *et al.,* 2000; LUKYANOV *et al.,* 2000; SHAGIN

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et al., 2004; WIEDENMANN *et al.,* 2004; OSWALD *et al.,* 2007). Due to having these proteins and pink pigments, green tentacles with pink tips are specific to *Anemonia sulcata* var. *smaragdina,* while in *Anemonia rustica* they are an ordinary brown colour (GOSSE, 1860; LEUTENEGGER *et al.,* 2007). Both morphotypes contain an alga commonly known as zooxanthellae in a symbiotic form. This association is considered to be mutualistic as the anemone provides a hosting place, a protection from herbivory and inorganic nutrients required for photosynthesis, while the alga provides oxygen and carbohydrate-based products to the anemone as a product of photosynthesis (MUSCATINE *et al.,* 1979; FALKOWSKI *et al.,* 1984; MULLER-PARKER *et al.,* 1990; FURLA *et al.,* 1998; GROVER *et al.,* 2002; SABOURAULT *et al.,* 2009). Environmental stresses such as strong light, UV radiation, elevated sea temperature and pollution cause well-known bleaching effects in *Anemonia* species. Bleaching effects result in whitening of cnidarian symbiotic tissues and induce symbiont photoinhibition. RICHIER *et al.* studied the effects of temperature and increased UV radiation in the symbiotic cnidarian *Anthopleura elegantissima* transcriptome. The results of this study corroborate that the stress response results in overproduction of the ROS in the aerobic metabolism (RICHIER *et al.,* 2006; RICHIER *et al.,* 2008).

The ROS are overcome by antioxidants under normal conditions. Enzyme-based antioxidants play important roles in scavenging ROS (HALLIWELL & GUTTERIDGE, 1999). Superoxide dismutase (SOD) catalyses the dismutation reactions of the superoxide radical into hydrogen peroxide. The formed hydrogen peroxide is also a dangerous molecule for the cell and must be converted to a harmless molecule. This transformation is done by catalase (CAT). CAT decomposes hydrogen peroxide to molecular oxygen and water. Organic hydroperoxides are decomposed by glutathione peroxidase (GSH-Px). However, the overproduction of ROS results in deleterious effects such as lipid peroxidation (LPO) of polyunsaturated fatty acids (PUFA), DNA strand breaks and protein oxidation (HALLIWELL and GUTTERIDGE, 1999). The role of ROS in the bleaching phenomenon of anemones has been investigated by few researchers so far (RICHIER *et al.,* 2006; LEUTENEGGER *et al.,* 2007). The antioxidant enzyme activities, lipid peroxidation levels and also photosynthetic pigments were determined dependent on UV exposure time in the present study.

Materials and Methods

Sample collection and preparation

The specimens were collected from shallow waters (0.2- 0.5 m) of Gümüldür coastline. The coordinates were 38˚ 02’ 39.79" N and 27˚ 00’ 51.93" E. The habitats of sea anemones were rocky and no microclimatic habitats were observed in the collection site as mentioned by LEUTENEGGER *et al.* (2007). The samples were transferred to laboratory in natural seawater in plastic bags. The tentacles from three individuals were immediately cut at the base. Both tentacles and column samples were stored at -80 ˚C until used. These samples were labelled as the control group. UV-exposed experimental groups were created on the basis of different exposure time and each of them contained three individuals. These groups were exposed to UV-Lamb (Slyvania, G30W). The stress periods were set as 5 hours and samples were collected at hourly intervals. 0.10 gram samples were put into eppendorf tubes which contained 1 mL of phosphate buffer

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(pH 7.2, 50 mM). The samples were homogenized with Ultra-Turrax T8 IKA-Werke homogenizator in 15-seconds intervals, for 30 seconds as homogenization time. The homogenates were centrifuged in a refrigerated centrifuge (Hettich 32R) at 10,000 rpm for 10 min at +4˚C to remove the cell debris. Then supernatants were used for measuring protein content and enzyme activities.

Protein Assay

Total protein concentrations in supernatants were determined through the method reported by BRADFORD (1976). Bovine serum albumine (BSA) was used as the standard for calibration curve.

Superoxide dismutase (SOD) activity determination

SOD activity was assayed by using a commercial kit produced by RANDOX (SD 125). The assay principle is based on the inhibition of occurrence formazan dye formed in the reaction of a superoxide radical. Superoxide radical anions are formed by the xanthine-xanthine oxidase system. The formed superoxide radicals attack INT (2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium) to give a formazan dye. As SOD is an inhibitor of this reaction, the SOD enzyme activity is expressed via its inhibition rates. One unit of SOD is that which causes a 50% inhibiton of the rate of reduction of INT under the conditions of the assay. The absorbance of occurred formazan dye was measured at 505 nm against air in UV-VIS 1601 Schimadzu Spectrophotometer at 37˚ C.

Catalase (CAT) activity determination

CAT activity was determined by the AEBI method (1985, which measures the decrease in absorbance of H_2O_2 at 240 nm. The reaction mixture (1mL) contained 50 mM phosphate buffer, pH 7.0 and 10 mM H_2O_2 (AEBI, 1985). 1 IU/mL of CAT activity was expressed as decomposition of 1 mL of hydrogen peroxide (10 mM) in one minute. The absorbance at 240 nm was measured in a UV-VIS 1601 Schimadzu Spectrophotometer at 25˚C.

Glutathione peroxidase (GSH-Px) activity determination

GSH-Px activity was assayed by using a commercial kit produced by RANDOX (RS 505). GSH-Px catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione peroxidase and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form by glutathione reductase (GR) with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

Lipid peroxidation (LPO) level determination

The method described by YAGI (1994) was used to estimate the lipid peroxidation level in the samples. It was expressed as nmol MDA/g wet weight.

According to this method the samples are homogenized in KCl to aggregate the proteins. The membrane lipids are dissolved in SDS (sodium dodecyl sulfate) and a peroxidation reaction achieved by acetic acid. The coloured complex which was measured at 532 nm was formed with thiobarbituric acid.

Chlorophyll a-b and total carotenoids determinations

Chlorophyll a-b and total carotenoids levels were determined via the method published by LICHTENTALER and WELLBURN (1985) and DERE *et al.* (1998), with slight modifications. A 0.1 g sample was homogenized by using an Ultra-Turrax T8 IKA-Werke homogenisator. After addition

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of 100% acetone into the samples, these samples were vortexed vigorously for one minute. The homogenates were centrifuged for 5 min at 2500 rpm, 20˚C in a refrigerated centrifugator (Universal 32R, Hettich Zentrifuggen). The spectrum scanning was done between 350-800 nm in UV-VIS 1601 Schimadzu Spectrophotometer. The maximum absorbances were recorded at 470, 645 and 662 nm which were in association with the total carotene, chlorophyll b and chlorophyll a, respectively. The chlorophyll a, b and total carotenoids levels were estimated by using the following formulas:
Chlorophyll a = 11.75 A662 - 2.350 A645
Chlorophyll b = 18.61 A645 - 3.960 A662
Total Carotenoids = ((1000 A470 - 2.270 Chlorophyll a - 81.4 Chlorophyll b)) / 227

Statistical Analysis

Comparisons were statistically analyzed by ANOVA followed by the Tukey test. The statistical significance was set at 0.05. All numerical data were given as mean ± S.E.M. The values were the means of three separate experiments. The correlation test was applied for possible relationships between species.

Results

The protein concentration of both tentacles and columns are presented in Figure 1. A remarkable difference in the protein levels of tentacles of *A.sulcata* var. *smaragdina* is observed between the control group and the experimental groups ($p<0.05$). A similar difference is observed only for the 2nd hour group for the column of *A. rustica* ($p<0.05$). According to the results of a statistical test which was conducted to point out the differences between two morphotypes that were exposed to UV light with the same duration, it can be said that only the control groups (in tentacles) show a statistical difference in the protein levels ($p<0.05$). Similar results are also observed in control groups and 1 - 2 hour UV-exposed experimental groups for the protein levels of columns (Fig. 1, a and b) ($p<0.05$).

Figure 2a and b show the SOD activities in tentacles of *A.sulcata* var. *smaragdina* and *A. rustica.* According to these figures, there is a statistical difference between the SOD activities of *A. sulcata* var. *smaragdina* and *A. rustica* which was exposed UV light for 1 hour ($p<0.05$). It was interesting to note that there were no statistical differences among SOD activities in the tentacles of the control and experimental groups.

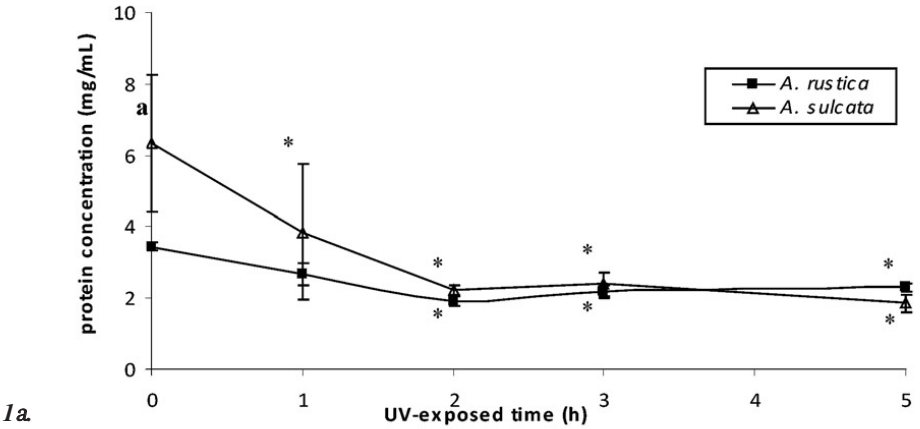
SOD activities in the columns of *A. sulcata* var. *smaragdina* and *A. rustica* are shown in Figure 2c and 2d. It can be said that from the results of these figures, the SOD values in the columns are significantly different from each other, although they show a similar trend.

The results related to CAT activities in tentacles and columns are presented in Figure 3. Volumetric CAT activities in tentacles have noticeable differences for 1, 3 and 5 hour UV exposed groups between two morphotypes ($p<0.05$). A sharp decrease occurs by the 3rd hour for *A. sulcata* var. *smaragdina* and as a result of this, significant differences between control group and 2, 3 and 5 hour UV-exposed groups are observed ($p<0.05$).

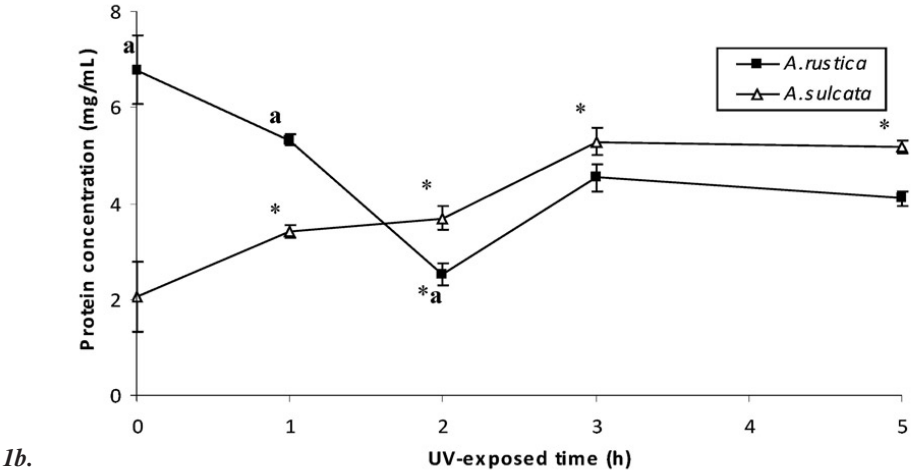
According to Figure 3b which shows the specific CAT activities of tentacles, the two morphotypes show the same tendency but *A. sulcata* var. *smaragdina* exhibits a sharp change in the 3rd hour, creating a significant difference between the two species for the 3rd and 5th hours ($p<0.05$).

According to Figure 3c which demonstrates volumetric CAT activity in columns of *A. sulcata* var. *smaragdina* and *A. rusti-*

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1a.



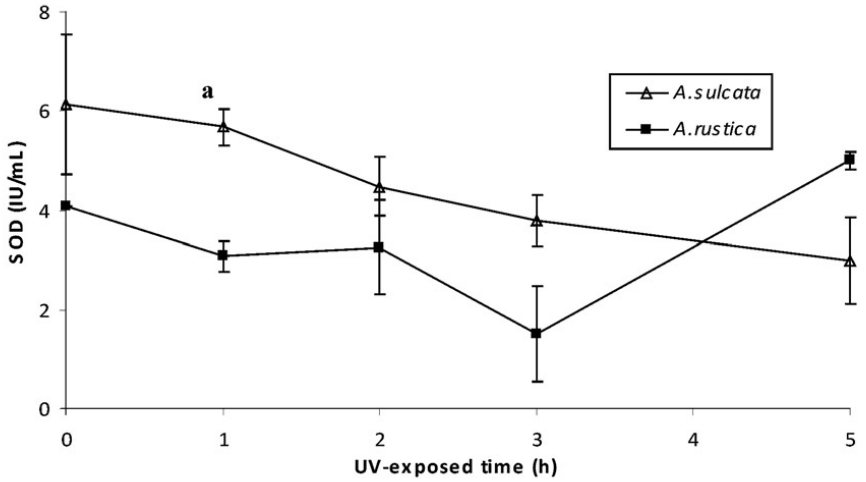
1b.

Fig. 1: Protein concentration of tentacles **(a)** and columns **(b)** of UV-exposed *A.sulcata* var. *smaragdina* and *A.rustica.* Error bars show ±S.E.M. The results are the means of three different experiments. All the groups are compared with control groups and experimental groups exposed at the same time. Asterisks (*) on the error bars show the statistical difference compared to control groups in *A.rustica* and *A.sulcata* ($p<0.05$). The letter (a) shows the statistical difference between the morphotypes which were exposed to UV light for the same length of time ($p<0.05$).

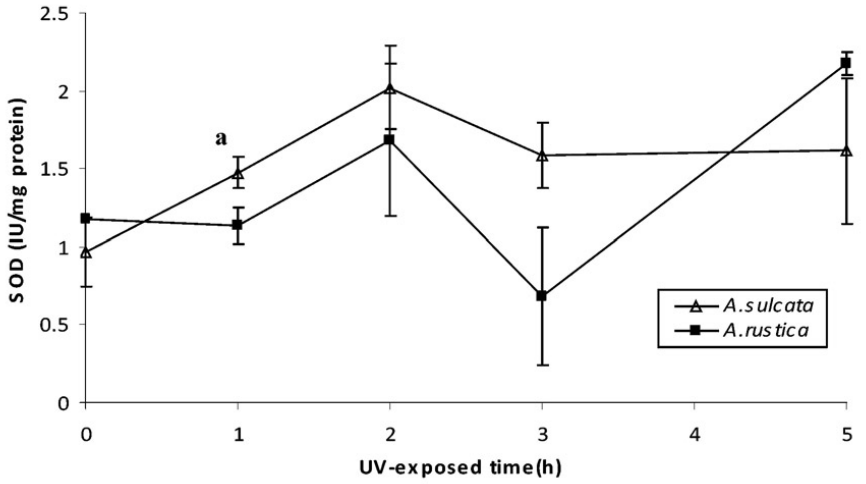
ca, the values observed for the 2, 3 and 5 hour UV-exposed groups show statistical differences between two morphotypes ($p<0.05$). Considerable differences are observed at 1, 2 and 3 UV-exposed groups of *A. sulcata* var. *smaragdina* from the control samples. A similar explanation is also valid for *A. rustica* except for 2,3 and 5 hour UV-exposed groups ($p<0.05$). Control groups of *A .sulcata* var. *smaragdina* and *A. rustica* show statistical differences for the CAT activities of columns (Fig. 3d) ($p<0.05$).

The results for GSH-Px are depicted in Figure 4. No significant change in the vol-

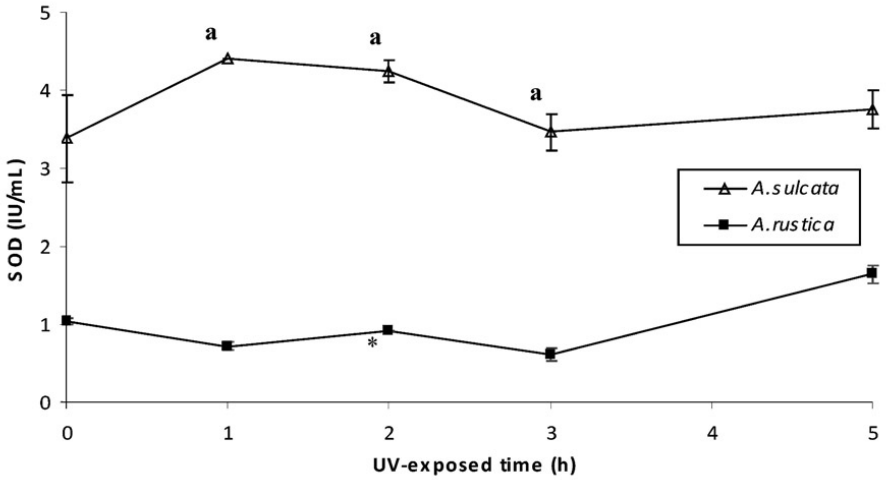
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2a.

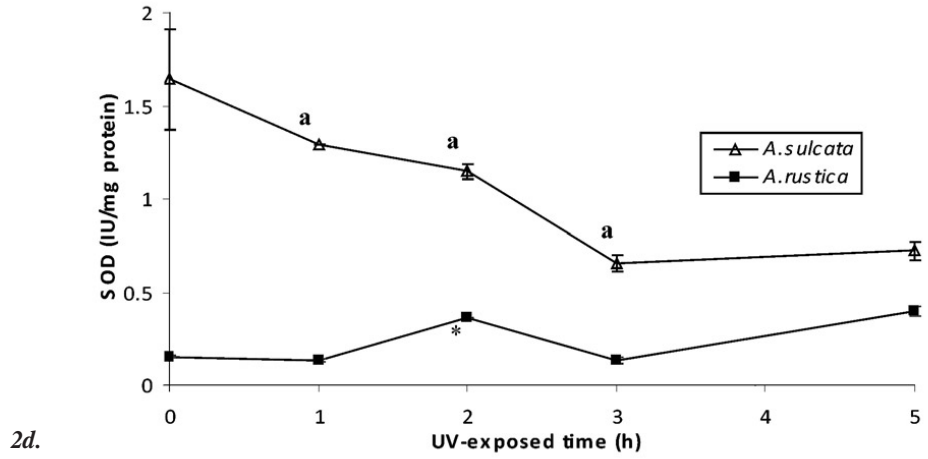


2b.



2c.

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2d.

Fig. 2: *Superoxide dismutase* (SOD) activities of UV-exposed *A.sulcata* var. *smaragdina* and *A.rustica.* **(a)** shows volumetric activities in tentacles and **(b)** shows specific activities in tentacles. **(c)** shows volumetric activities in columns and **(d)** shows specific activities in columns. Error bars show ±S.E.M. The results are the means of two different experiments. All groups are compared with control groups and simultaneous experimental groups. Asterisks (*) on the error bars show the statistical difference compared to control group in *A.rustica* ($p<0.05$). The letters (a) show the statistical difference between experimental groups exposed at the same time ($p<0.05$).

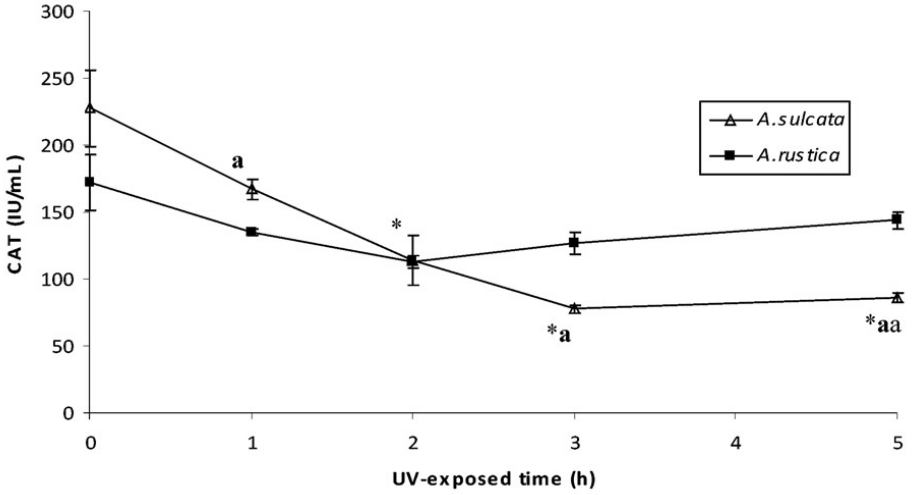
umetric GSH-Px activities of tentacles is observed among the control and experimental groups ($p>0.05$). On the other hand, a significant difference between 5 hour UV-exposed *A. sulcata* var. *smaragdina* and *A. rustica* is determined ($p<0.05$). No significant correlation is found between the volumetric activity values of the two morphotypes ($r=0.11$, $p>0.05$) (Fig. 4a). As can be seen in Figure 4b; the two morphotypes show a similar trend up to the third hour and significant differences are observed at the 5th hours for *A. sulcata* var. *smaragdina* and *A. rustica* ($p<0.05$).

In the columns, there is no difference in volumetric GSH-Px activity between *A. sulcata* var. *smaragdina* and *A. rustica* (Figure 4c). In the light of specific GSH-Px activity results, it can be noted that *A. rustica* shows a peak at the 2nd hour of UV-exposure. There is a statistical difference between the control groups of these morphotypes. Significant differences at the 1st and 3rd hours for *A. sulcata* var. *smaragdina* and at the 2nd hour for *A. rustica* are determined by comparing both of these morphotypes' experimental groups to control groups (Fig. 4d).

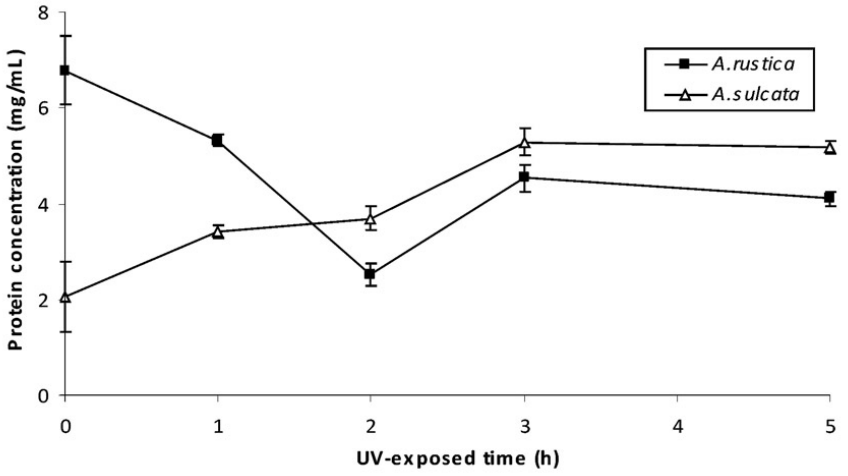
LPO levels of these morphotypes versus UV exposure time are plotted in Figure 5. According to Figure 5a, there is a statistical difference between 1 hour UV-exposed morphotypes, as they show a great discrepancy in their LPO levels. The LPO level at 1 hour UV-exposed *A. sulcata* var. *smaragdina* is higher than that of the control group ($p<0.05$). LPO levels in columns of *A. sulcata* var. *smaragdina* increase sharply in one hour, then decrease with the increasing UV-exposure time. On the other hand, no significant change is observed for the LPO levels of the columns of *A. rustica* (Fig. 5b).

Photosynthetic pigments such as chlorophyll a, b and total carotenoids are also in-

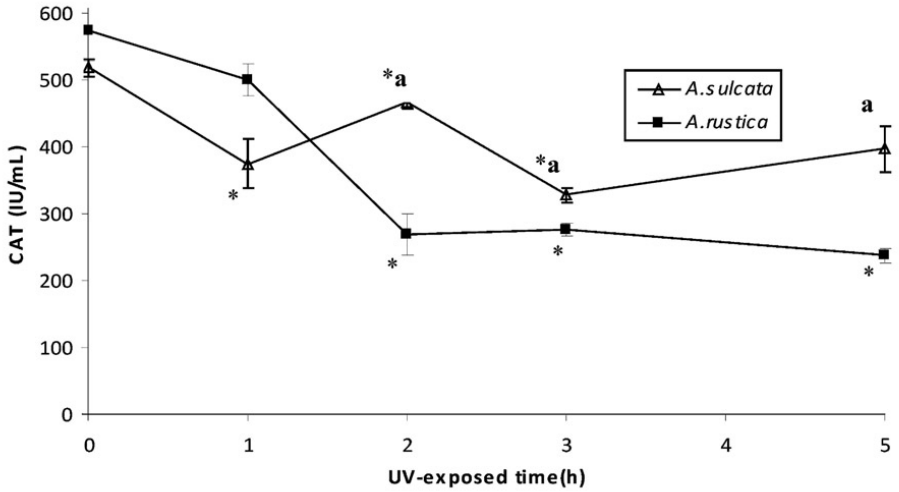
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3a.

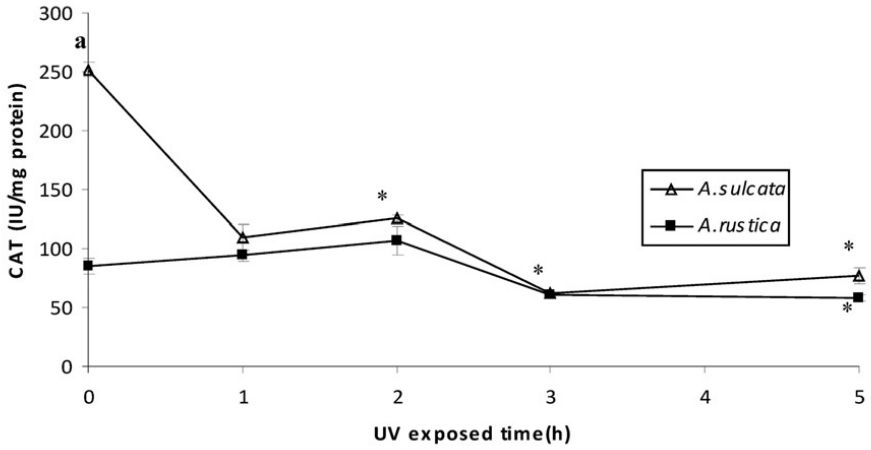


3b.



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3d.

Fig. 3: CAT activities of UV-exposed *A.sulcata* var. *smaragdina* and *A.rustica.* **(a)** shows volumetric activity in tentacles and **(b)** shows specific activities in tentacles. **(c)** shows volumetric activity in columns and **(d)** shows specific activities in columns. Error bars show ±S.E.M. The results are the means of three different experiments. All groups are compared with control groups and experimental groups exposed at the same time. Asterisks (*) on the error bars show the statistical difference compared to control group *A.sulcata* and *A.rustica* ($p<0.05$).
The letters (a) show the statistical difference between same time experimental groups ($p<0.05$).

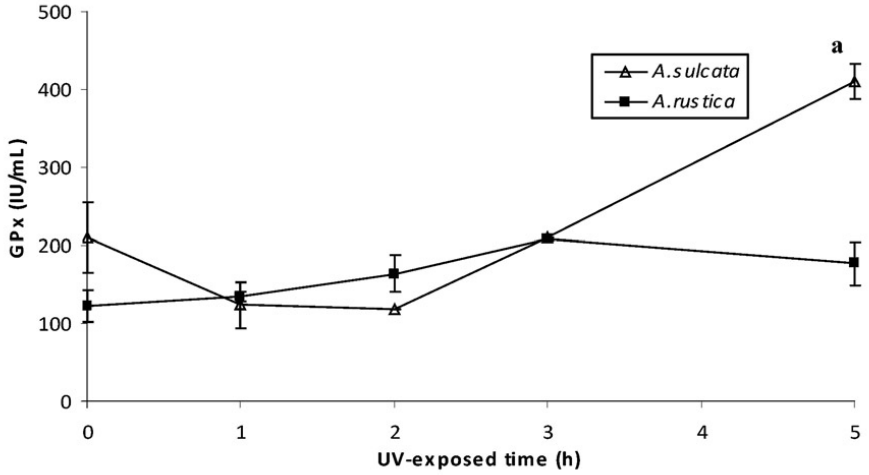
vestigated in the present study. According to Figure 6a which is related to chlorophyll a level, these species show the same tendency with changing UV exposure but the level for control groups of *A. sulcata* var. *smaragdina* is a bit higher than that of *A. rustica.* According to Figure 6b there is no significant difference between the chlorophyll b levels of these morphotypes. Total carotenoids exhibited the same trends as chlorophyll a (Fig. 7).

Discussion

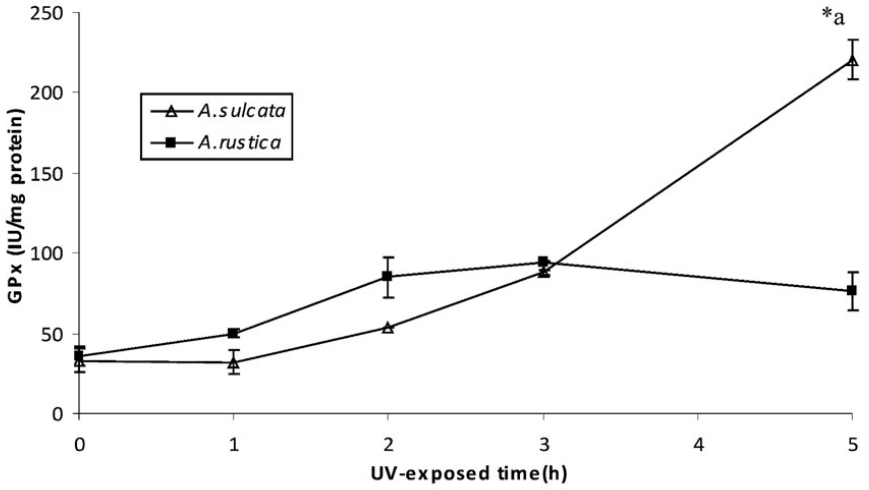
SOD, CAT, GSH-Px, protein concentrations, LPO levels and photosynthetic pigments were investigated in the body of *A. sulcata* var. *smaragdina* and *A. rustica* collected from Gümüldür region of Izmir (Turkey) province. Research into anthozoans has shown that several endogenous and exogenous factors cause overproduction of ROS in the cell (LEUTENEGGER *et al.,* 2007; LESSER and SHICK, 1989; DOWNS *et al.,* 2002; TCHERNOV *et al.,* 2004; SMITH *et al.,* 2005). The cnidarians, like other aerobic organisms possess comprehensive enzymatic and non-enzymatic antioxidant defences to cope with overproduced ROS (RICHIER *et al.,* 2008). In the present study, since no remarkable bleaching events were witnessed for the period between September 2006 and September 2008, we wanted to analyse and compare antioxidant enzyme activities in two well-known morphotypes of *A. viridis; A. rustica* and *A. sulcata* var. *smaragdina* from the Aegean Sea along Turkish coastlines. The study also aimed to present the antioxidant responses of two variations of *A. viridis* against UV radiation.

According to our results, there is a significant negative correlation between protein concentrations and specific SOD ac-

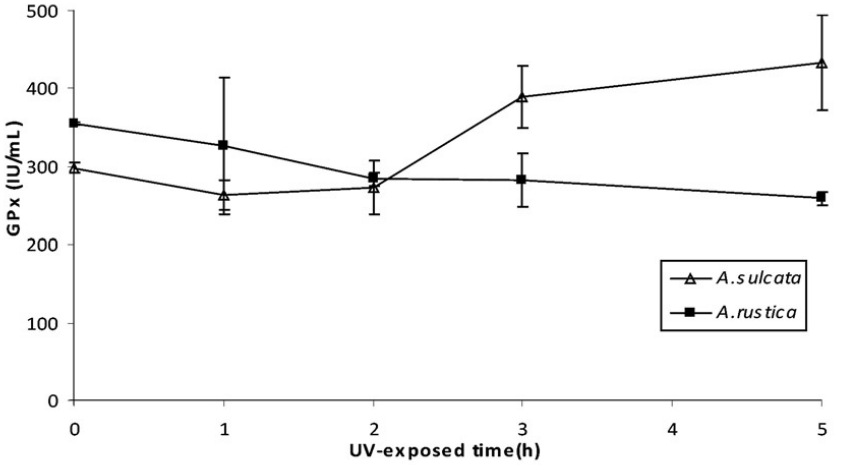
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4a.

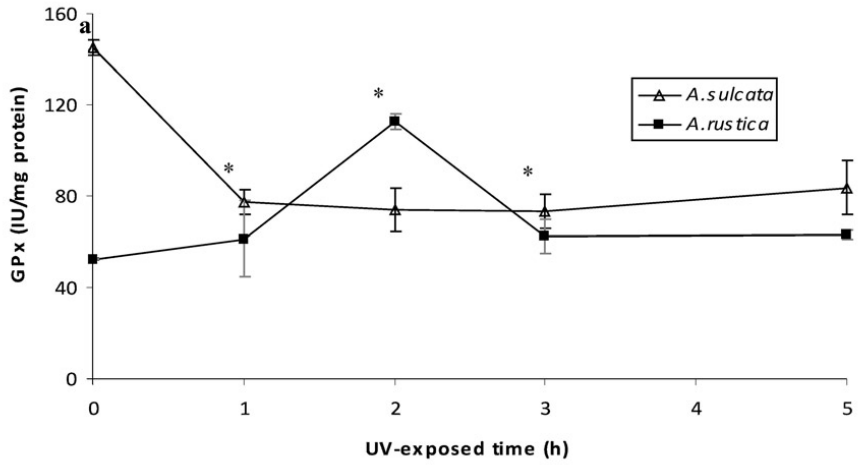


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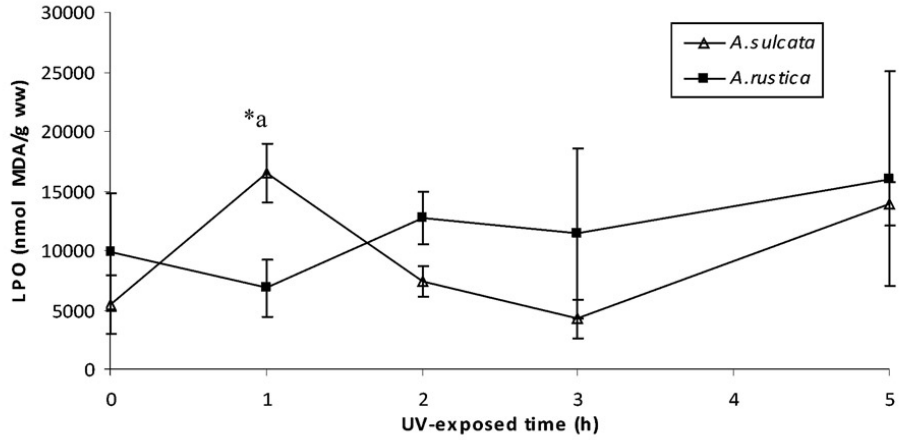


4d.

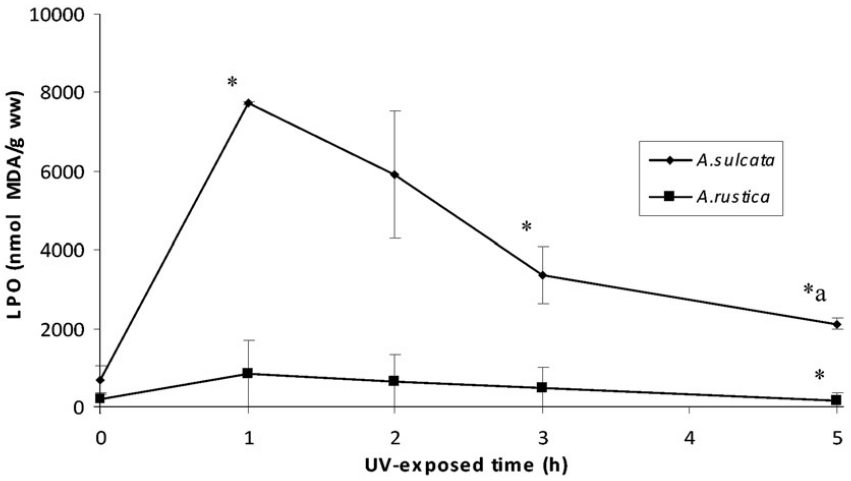
Fig. 4: Glutathione peroxidase (GSH-Px) activities of UV-exposed *A.sulcata* var. *smaragdina* and *A.rustica.* **(a)** shows volumetric activities in tentacles and **(b)** shows specific activities in tentacles. **(c)** shows volumetric activity in columns and **(d)** shows specific activities in columns. Error bars show ±S.E.M. The results are the means of two different experiments. All groups are compared with control groups and experimental groups exposed at the same time ($p>0.05$). Asterisks (*) on the error bars show the statistical difference compared to the control group *A.sulcata* and *A.rustica* ($p<0.05$).

tivity in the tentacles of *A. sulcata* var. *smaragdina* ($r=0.878$, $p<0.05$). Similarly, correlations were found among chlorophyll a and SOD-CAT activities in the tentacles of *A. sulcata* var. *smaragdina* ($r=-0893$, -0.925, $p<0.05$, respectively). It was very interesting to note that no correlation was found for the above- mentioned relationships in *A. rustica.* The existence of the correlations among SOD-CAT activities and photosynthetic pigments might be explained by the relationship of these pigments and ROS. According to photosynthetic process in higher plants, secondary pigments play important roles in scavenging overproduced ROS (BOYER, 2006). The bonds in the photosynthetic pigments in anemones might have been protected by other antioxidant molecules such as vitamins and glutathione etc, inasmuch as no remarkable differences were observed in the concentrations of photosynthetic pigments. The existence of relationships related to enzymes and photosynthetic pigments in *A.sulcata* var. *smaragdina* and also absence of these relationships in *A.rustica* might be explained by the existence of different regulatory mechanisms in these morphotypes. Significant correlation ($r=0.908$, $p<0.05$) between SOD and CAT activities in the column of *A.sulcata* var. *smaragdina* might show that these two enzymes work together in the columns. Moreover, a remarkable correlation ($r=0.922$, $p<0.05$) existed between CAT and GSH-Px activities. These results may reveal that only one peroxidase is not enough in the decomposition of overproduced hydrogen peroxide by SOD activities. Therefore, two peroxidases, CAT and GSH-Px, may play a synergistic role in the sea anemones. Observation of increased specific GSH-Px activities in the tentacles of both *A.sulcata* var. *smaragdina* and *A.rustica* can be explained by possible overproduced lipid hydroperoxides. In contrast to the sea anemone population in Gümüldür, we have recent-

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5a.

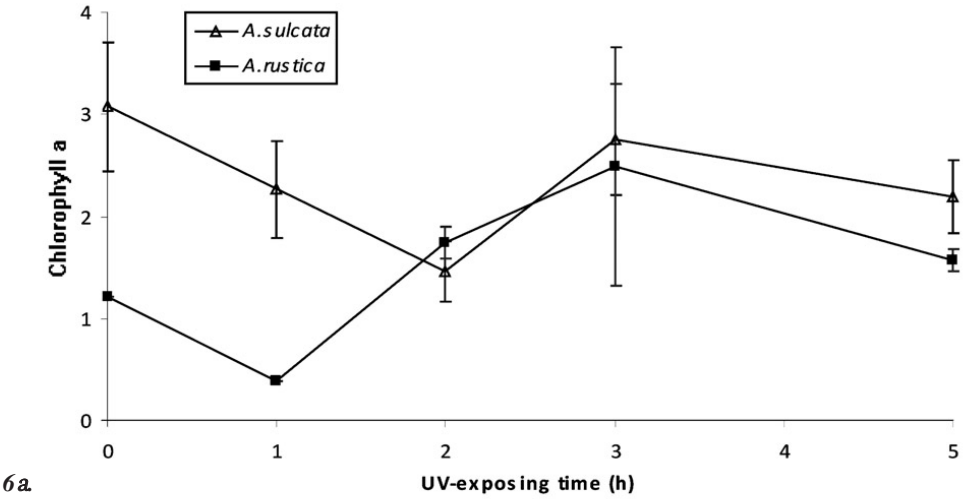


5b.

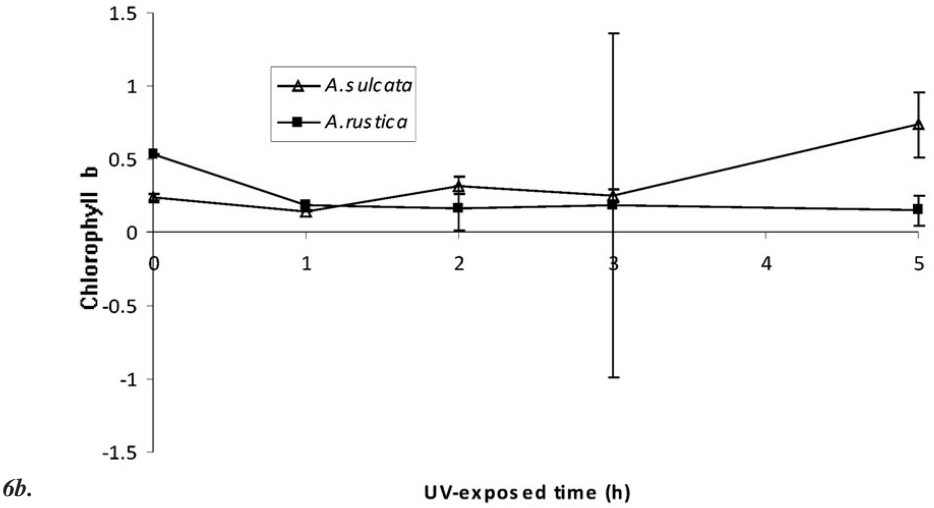
Fig. 5: **(a)** Lipid peroxidation levels in tentacles of *A.sulcata* var. *smaragdina* and *A.rustica.* **(b)** Lipid peroxidation levels in columns of *A.sulcata* var. *smaragdina* and *A.rustica.* Error bars show ±S.E.M. The results are the means of three different experiments. Asterisks (*) on the error bars show the statistical difference compared to the control group in *A.sulcata* ($p>0.05$). The letter (a) shows the statistical difference between same time experimental groups ($p<0.05$).

ly observed bleached anemones which live in shallow waters in Dikili. According to preliminary results, this bleaching event can be associated with the higher sea water temperature. There are many scientific reports on the antioxidant enzyme activities of sea anemones in the literature. Early attempts (DYKENS, 1984; DYKENS and SHICK, 1984; SHICK *et al.,* 1995; TYTLER and TRENCH, 1988) at the determination of antioxidant enzyme activities in sea cnidarians were carried out by using cell-free ex-

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6a.



6b.

Fig. 6: **(a)** Chlorophyll a amount in tentacles of *A.sulcata* var. *smaragdina* and *A.rustica.* **(b)** Chlorophyll b amount in tentacles of *A.sulcata* var. *smaragdina* and *A.rustica.* Error bars show ±S.E.M. The results are the means of three different experiments.

tracts as applied in the present study. Among the early attempts at antioxidant enzyme activities in sea cnidarians, DYKENS and SHICK (1984) investigated the antioxidant enzyme activities, SOD and CAT, in *Anthopleura elegantissima* which was subjected to sunlight to generate hyperbaric oxygen level. They found 590% and 100% increases in the SOD and CAT activities, respectively, of shade-adapted aposymbiotic *Anthopleura elegantissima* (DYKENS *et al.,* 1992). DYKENS *et al.* (1992) also underlined that SOD and CAT enzyme activities in *Anthopleura elegantissima* are associated

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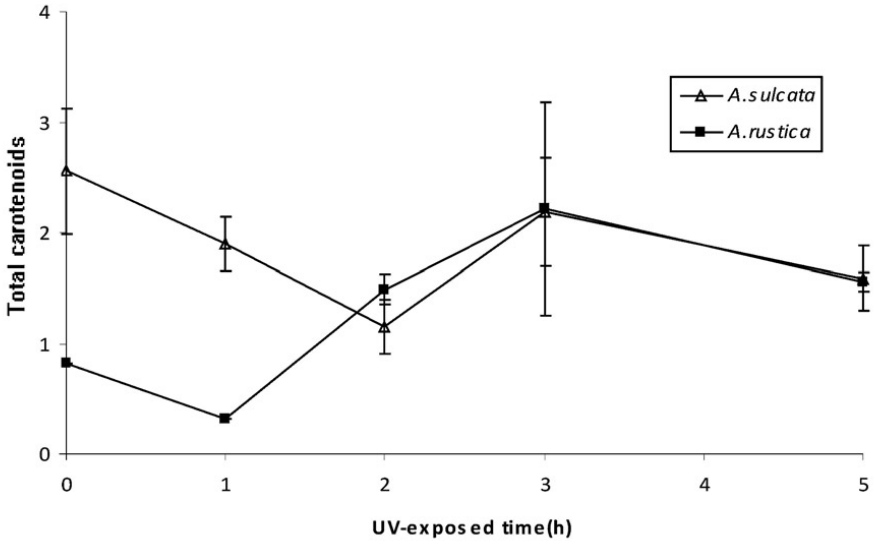


Fig. 7: Total carotenoid amount in tentacles of *A.sulcata* var. *smaragdina* and *A.rustica.* Error bars show ±S.E.M. The results are the means of three different experiments.

with the chlorophyll content of endosymbionts where the SOD and CAT are localised. HAWKRIDGE *et al.* (2000) studied the localisation of antioxidant enzymes in the sea anemone *Anemonia viridis* and the coral *Goniopora stokesi.* HAWKRIDGE *et al.* (2000) showed that SOD localized on the ruptured threads and shafts of b-mastigophore in *Anemonia viridis.* Existence of SOD and CAT in the symbiotic algae was also shown by researchers. However, the gold-labelling method that they used has some limitations because mammalian antibodies were used for sea anemones. In a very recent study, RICHIER *et al.* (2006) investigated the effects of thermal stress on the symbiosis breakdown in the sea anemone, *Anemonia viridis.* They found a link between bleaching and apoptotic process in the sea anemone. Due to the comparable values among antioxidant enzyme activities in the present study and also the absence of any remarkable bleaching events reported so far, it can be said that the production of ROS is got rid of by sea anemones in the Aegean Sea by their antioxidant system. On the other hand, inasmuch as the overproduction of ROS in aerobic organisms is dependent on not only endogenous factors but also exogenous factors such as pollution, pathogenity and abiotic factors etc., regular measurement of the antioxidant status of sea anemones is strongly warranted for the population health of sea anemones to estimate a possible bleaching event.

Conclusion

The present study demonstrates the antioxidant enzyme activities in two well-known sea anemones, *Anemonia rustica* and *Anemonia sulcata* var. *smaragdina* from the Aegean Sea along Turkish coastlines. These enzyme activities may be considered as indicators of the health of the cnidaria-related ecosystem. The antioxidant enzyme systems in sea anemones can also be defined as ROS scavenging systems. The increases in these en-

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zyme activities show the overproduced ROS level in the metabolism. Since these increases are generally caused as a result of environmental stresses such as UV radiation, pathogens, or elevated temperature, they can be taken as indicators of the healthy association between anemones and zooxanthella. In the present study no bleaching event was observed in anemone samples even when they were given 5 hours of UV-exposure. Moreover, UV exposure did not change antioxidant systems remarkably. However, more investigations are still needed in order to obtain a complete picture of the effects of UV-light on the cellular pathways of cnidarian–algal symbiosis.

Acknowledgement

The authors thank ProjectAWARE for the partial financial support of the project (Grant Application number 639).

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