Assessing ultraviolet radiation-induced DNA damage and repair in field-collected *Aiptasia pallida* using the comet assay

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Abstract. Ultraviolet radiation (UVR)-induced DNA damage and repair in freshly-collected *Aiptasia pallida* from Walsingham Pond, Bermuda were examined using the comet assay. In addition, animals were screened for the presence of mycosporine-like amino acids (MAAs). We found that field anemones produce relatively large quantities of four MAAs (mycosporine-2-glycine, mycosporine glycine, shinorine and Porphyra-334). Additionally, field anemones were capable of fully repairing DNA damage incurred during a 12-h exposure to UVR and PAR. It appears that *A. pallida* initiates nucleotide excision repair (NER) during the first 2 h of recovery in the dark. The majority of DNA strand breaks (SB) formed from NER are repaired within an 8-h recovery period. These results suggest that the ability of *A. pallida* to repair DNA damage and / or protect itself from the detrimental effects of UVR are important factors for its survival.

Keywords: Comet assay, DNA damage, *Aiptasia pallida*, Ultraviolet radiation.

Introduction

Tropical littoral zones experience some of the highest doses of ultraviolet radiation (UVR) on Earth (Frederick et al. 1989; McKenzie et al. 2003). Consequently, sedentary marine invertebrates in these habitats are exposed to biologically harmful levels of ultraviolet-A (UVA, 320-400nm) and ultraviolet-B (UVB, 280-320nm) (Shick and Dunlap 2002). Exposure to UVA and UVB can detrimentally affect survival, growth, and reproduction of shallow-water marine invertebrates from oxidative stress that results in structural damage to proteins and lipids and physiological impairment at the cellular level (Stochaj et al. 1994).

DNA damage is arguably the most harmful effect of UVR. Exposure to UVR generates the formation of cyclobutanepyrimidine dimers (CPDs) and 6-4 photoproducts (Ananthaswamy 1997).

DNA lesions are repaired via enzymatic mechanisms such as nucleotide excision repair (NER), base excision repair (BER) (Kantor 1995) and photolyase activity (photoreactivation).

In addition to UVR exposure, single strand breaks are caused during oxidative stress when reactive oxygen species attack the backbone of DNA. Double strand breaks, which are the most severe form of DNA damage, can occur if two single strand breaks are nearby one another (Kantor 1995).

Symbiotic marine cnidarians are especially susceptible to these stresses in that UVR often co-occurs with intracellular hyperoxic stress (due to photosynthesis by zooxanthellae) and elevated environmental temperatures. These factors contribute synergistically to create oxidative stress and have been linked to bleaching events (Lesser 1997).

Organisms employ behavioral and physiological adaptations to defend themselves against the harmful effects of solar radiation. The most commonly reported defense against UVR exposure is the production of mycosporine-like amino acids (MAAs).

While many studies show that organisms produce MAAs in response to UVR exposure, few have demonstrated a link between DNA damage and MAA concentration.

The use of the comet assay has been suggested as a sensitive and rapid technique for the detection of DNA strand breaks caused by genotoxins in the environment and has recently been adapted for use in studies with cnidarians (Michelmore and Hyatt 2004). Rinkevich et al. (2005) were the first to employ the comet assay specifically to investigate UVR exposure and DNA damage in corals (*Stylophora pistillata*).

Here we present evidence that field-collected specimens of *Aiptasia pallida* efficiently repair DNA damage incurred during a 12-h exposure to photosynthetically available radiation (PAR) and UVR.

Material and Methods

Collection of A. pallida

Specimens of *A. pallida* were collected at 15:00 hrs from Walsingham Pond, Bermuda and transported to the Bermuda Institute of Ocean Sciences (BIOS) on the 19th June 2007. Surface water temperature and salinity readings at the time of collection were 26 °C and 37 ppt, respectively. Upon return to the BIOS station the anemones were maintained on an indoor seawater table for no longer than 16 hrs before being exposed to PAR and UVR.

Experimental design

Field-collected anemones were randomly transferred into four 100 x 50 mm Pyrex crystallization dishes (n=5 per dish) and maintained in an outdoor seawater flow-through tank. In addition to the 4 UVR exposed dishes, a control group (n=3) was included which was not exposed to UVR. For the control, individual anemones were placed in a 30 ml beaker and nested into the crystallization dish. To block UV transmission the control dish was completely covered with 3 layers each of Mylar film (a UVB blocker) and C3a film (a UVA blocker).

Due to shading from mangrove trees at Walsingham Pond, light readings were lower than those at BIOS. At Walsingham Pond UVB measured 1.6 W m^{-2} and PAR 1104 µmol photons m^{-2} s^{-1}, whereas at the BIOS station unshaded UVB measured 2.51 W m^{-2} and PAR 1742 µmol photons m^{-2} s^{-1}. Therefore, animals were exposed to a reduced level of PAR and UVR to better represent the radiation found in their typical habitat of Walsingham Pond (Fig. 1).

In order to reduce PAR and UVR the holding tank which contained dishes of anemones was covered with a sheet of Solacryl® SUVT (Spartech Polycast) (which allowed UV and PAR transmission) and one layer of black fiberglass screen.

Anemones were subjected to 12 hrs of PAR and UVR (Fig. 1); receiving an approximate UVB dose of 24 kJ m^{-2}. Sunrise was at approximately 06:00 hrs and sunset not until 20:00 hrs. To cease exposure after 12 hrs, we covered the flow-through tank with 2 layers of neutral density filter to mimic sundown at 18:00 hrs. In this way, samples remained in the dark after PAR and UVR exposure for recovery periods of 0, 2, 4, 6, and 8 h. There were 4 replicate anemones for each recovery time. Animals were randomly chosen by removing 1 individual each from the 4 UV-exposed dishes. These individuals were processed immediately to determine the extent of DNA damage incurred via the comet assay. A control group (n=3) was also immediately processed after 12 h of PAR but in the absence of UVR.
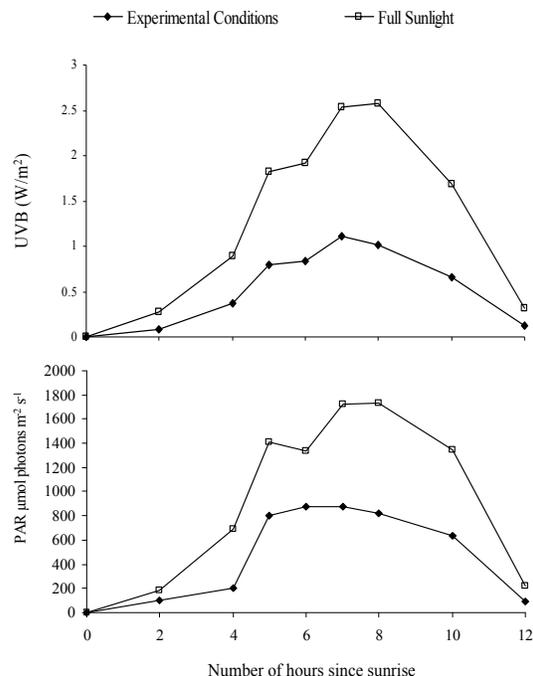

Figure 1: Light regime on 20 June 2007 at the BIOS. Field-collected symbiotic *A. pallida* were subjected to reduced PAR and UVR compared to full sunlight to mimic conditions observed at Walsingham Pond, Bermuda.

Assessing DNA damage using the comet assay

Host nuclei were isolated immediately following UVR exposures and subsequent recovery periods. This process occurred in a darkened room with no fluorescent lighting.

Anemones were carefully blotted with a Kimwipe to remove excess mucus then transferred to a Potter-Elvehjem homogenizer and homogenized in 1 ml of buffer (1x PBS + 0.25% sucrose; 100 µM EDTA (ethyl-enediaminetetra-acetic acid) pH 8; and 10% DMSO (dimethyl sulfoxide). Homogenization was achieved after 7 strokes of the Teflon pestle while rotating it 360° during each stroke.

The homogenate was passed through a cell strainer (50 µm) and carefully transferred using a Pasteur pipette onto a chilled 20% sucrose pillow to form a second layer. The cell suspension and pillow were centrifuged at 1340*g* for 2 min, which pelleted algal cells. The nuclei formed a surface layer on the pillow, which was transferred into a clean centrifuge tube and centrifuged at 4000*g* for 2 min to pellet the nuclei. The isolated nuclei were re-suspended in 0.7% low melting-point agarose.

Gel-bond™ slides were pre-coated with 150 µl 1% normal melting-point agar and covered with a glass cover-slip. Once the gels solidified the cover-slips were removed and 70 µl of nuclei suspension were layered onto the pre-coated Gel-bond™ slides, a new cover slip was added and the agarose containing

the nuclei allowed to solidify on ice in the dark for at least 15 min.

The cover-slips were removed and the slides transferred to a lysis solution (10% DMSO, 2.5 M NaCl, 100 mM EDTA, 10 mM Tris; pH 10) on ice and in the dark for 10 min. Following lysis the slides were immersed in alkaline unwinding buffer (300 mM NaOH, 1 mM EDTA; pH >10) on ice and in the dark for 5 min to unwind super-coiled DNA. Comet formation was achieved via electrophoresis in alkaline unwinding buffer at a constant amperage of 300 mA for 5 min.

Following electrophoresis, the slides were neutralized with three rinses in 0.4 M Tris; pH 7.5 for 10 min each, dehydrated with 95% ethanol for 5 min and allowed to air dry at room temperature for at least 24 h.

Dried gels were stained with 40 µl ethidium bromide solution (20 µg/ml)and examined under 400x magnification using a Zeiss Axioskopp epifluorescent microscope (excitation filter 510-560 nm green light, barrier filter 590 nm).

Digital images of the nuclei were taken with a Nikon, Coolpix 995 and analyzed using TriTek Comet Score™ (Freeware v.1.5). At least 50 nuclei per animal were analyzed for tail length (µm), and % DNA in tail and expressed as tail moment (i.e. the product of the two measurements). Results are expressed as means ± standard error of the mean (SEM).

MAA analysis

A sample of anemones (n=3) was immediately extracted for MAA content. Anemones were blotted dry and then homogenized in 1 ml 100 % methanol. A 100 µl sample was taken and diluted with deionized water for protein analysis (Bradford, 1976). The remainder was extracted for MAAs.

The homogenate was transferred into a microcentrifuge tube and extracted for 1 h in the dark at 4 ºC. Following centrifugation the supernatant was withdrawn and set aside. The pellet was extracted two more times. The pooled supernatant of individual anemones was passed through a SepPak C18 cartridge to remove pigment and lipid contaminants.

A spectrophotometric scan (280-400 nm) of the pooled extract (5x dilution) for each individual was performed on a Spectronic Genesys 5 UV-Vis to ascertain the presence of UV absorbing compounds. The pooled extracts were then evaporated to dryness using a Savant Speed-Vac SC100 and stored at -20 ºC until the samples were analyzed for MAA content via liquid chromatography followed by photodioarray and mass spectrometric analysis.

Statistical analysis

One-way ANOVA and post hoc multiple mean comparisons were performed on untransformed data for tail moment using SPSS v.15.0.

Results

MAA analysis

Spectrophotometric scans of methanolic extracts of field-collected anemones showed mean absorbance increased substantially in the range at which MAAs would be detected (302 nm – 360 nm) (Fig. 2). Chromatograms from methanolic extracts of anemones indicated the presence of UV-absorbing compounds (Fig. 3). Further investigation of extracts via photodiode array and mass spectrometry revealed measurable quantities of up to 4 MAAs: mycosporine-2-glycine; mycosporine-glycine which co-eluted with shinorine; Porphyra-334 and traces of 2 additional MAAs: usujirene / palythene and palythine.
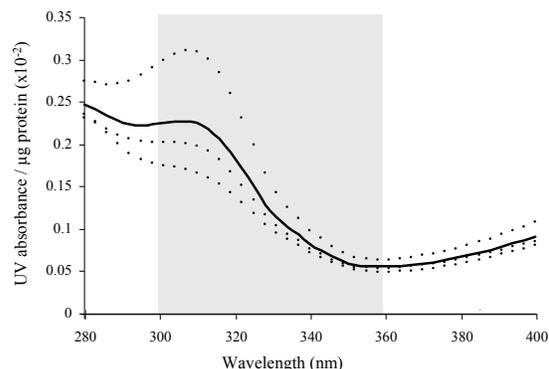

Figure 2: Spectrophotometric scan of 100% methanolic extracts from field-collected anemones. The shaded area represents the range at which MAAs absorb UVR. The solid black line represents the mean absorbance, dotted lines represent the scans of three individual anemones.
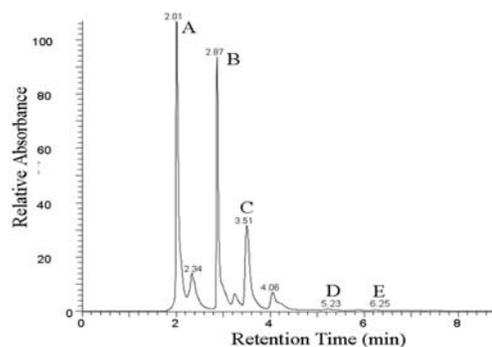

Figure 3: Chromatogram of methanolic extract from *A. pallida* Peak identifications: A - mycosporine-2-glycine; B_1 and B_2 - mycosporine-glycine which co-eluted with shinorine; C – Porphyra-334; D - usujirene / palythene and E – palythine.

Recovery Response

A one-way ANOVA indicated that DNA SB measured as tail moment varies significantly with recovery time ($p = 0.009$). Post Hoc multiple comparisons (Tukey HSD) revealed significant differences between repair times (Fig. 4).

While not statistically significant, tail moment increased by 127% in anemones which received 0 h recovery time compared to control organisms. This difference likely results from SB caused by a combination of UVR and oxidative stress, due to the fact that control groups did not have the added stress of UVR.
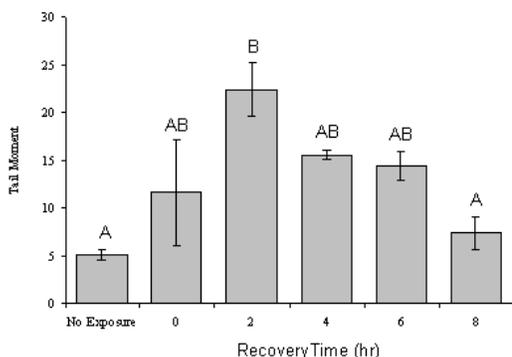

Figure 4: Genotoxic damage and DNA repair evaluated in isolated nuclei from field-collected *A. pallida* using the comet assay. The control group (n=3) received no UV exposure. Bars represent means ±SEM (n=4 anemones with 2 replicate gels per individual). Matching letters indicate non-significant differences at $\alpha = 0.05$

After a two-hour recovery period, tail moment doubled from that at the 0 h recovery time. This lag in time between UVR exposure and the subsequent increased number of breaks in the DNA indicates that excision of photoproducts is well underway at 2 h post-exposure.

The DNA SB at the 4 h recovery time dropped by 29%. However, the decrease in DNA SB from 2 h to 4 h is not statistically significant. This drop in DNA SB is likely attributed to the cells' gradual removal and repair of damage at specific sites of the DNA molecule.

There was not a further significant drop in DNA SB at the 6 h recovery time. However, after 8 h of recovery, tail moment was not significantly different from either controls or pre-exposure levels. This indicates that field-collected animals are capable of repairing DNA damage to a level equivalent to pre-exposure conditions within the course of a night.

Discussion

Spectrophotometric scans of extracts from field-collected anemones indicated peak absorbance at 310 nm, which implies the presence of relatively large quantities of mycosporine-glycine ($\lambda_{max} = 310$ nm).

Further investigation of these compounds via LC/MS confirmed initial scans and identified measurable quantities of 2 MAAs: mycosporine-2-glycine and mycosporine-glycine, with smaller amounts of shinorine and Porphyra-334. Traces of usujirene / palythene and palythine were also found. Similar to our findings, Stochaj (1989) reported the presence of mycosporine-2-glycine, mycosporine-glycine and shinorine in *A. pallida* from Bermuda but did not find Porphyra-334. Banaszak et al. (1998) also reported the presence of only mycosporine-glycine and shinorine in *A. pallida* from Belize.

The production of MAAs as a defense mechanism against UV damage is well-studied in symbiotic marine cnidarians (Dunlap and Chalker 1986, Drollet et al. 1997, Banaszak et al. 1998). In a recent study, Torregiani and Lesser (2007) observed that corals taken from a depth of 1m had very low concentrations of MAAs and high CPD accumulation.

A defining factor for coral survival depends on the efficiency with which they repair DNA damage. Lesser and Farrell (2004) reported that host DNA damage in the coral *Montastraea faveolata* is intensified under high solar irradiance and thermal stress due to higher oxidative stress and CPD formation. Therefore, the ability to repair such damage is vital. Our investigation shows that field-collected *A. pallida* also exhibit an increase in DNA SB post-UVR exposure (Fig. 4). This is likely, in part, due to base and nucleotide excision repair. The literature regarding UV-induced DNA SB and repair in cnidarians is extremely limited; only one other study of this nature has been reported (Rinkevich et al. 2005). Similar to our investigation, Rinkevich et al. employed the comet assay to demonstrate an increase of DNA SB in a branching coral following a 1 h recovery period immediately after UVR exposure. This increase in DNA SB was attributed to BER and NER. However, a single repair time of 1 h is not sufficient to follow the time course of repair. The repair experiment in our investigation explores multiple recovery periods which allows for a better understanding of the course of DNA repair.

While future UVB levels are difficult to predict, current estimates suggest that it will take at least half a century before UVB levels return to pre-ozone depletion levels. Moreover, for the next century the ozone layer will be at its most vulnerable (Madronich et al. 1998). Our research offers a technique which is easily employed in field-collected samples and could be used to identify increasing DNA damage in stressed corals. This assay will better equip scientists trying to monitor the health of corals, predict bleaching events, or further study the effects of UVR exposure and elevated sea temperatures.

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