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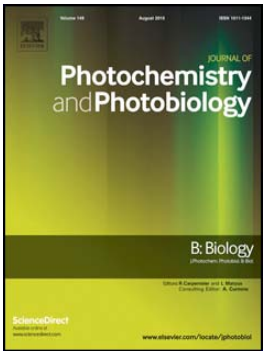

Ultraviolet absorbing compounds provide a rapid response mechanism for UV protection in some reef fish

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Ultraviolet absorbing compounds provide a rapid response mechanism for UV protection in some reef fish

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Abstract

The external mucus surface of reef fish contains ultraviolet absorbing compounds (UVAC), most prominently Mycosporine-like Amino Acids (MAAs). MAAs in the external mucus of reef fish are thought to act as sunscreens by preventing the damaging effects of ultraviolet radiation (UVR), however, direct evidence for their protective role has been missing. We tested the protective function of UVAC's by exposing fish with naturally low, *Pomacentrus amboinensis*, and high, *Thalassoma lunare*, mucus absorption properties to a high dose of UVR (UVB: 13.4 $W*m^{-2}$, UVA: 6.1 $W*m^{-2}$) and measuring the resulting DNA damage in the form of cyclobutane pyrimidine dimers (CPDs). s For both species, the amount of UV induced DNA damage sustained following the exposure to a one hour pulse of high UVR was negatively correlated with mucus absorbance, a proxy for MAA concentration. Furthermore, a rapid and significant increase in UVAC concentration was observed in *P. amboinensis* following UV exposure, directly after capture and after ten days in captivity. No such increase was observed in *T. lunare*, which maintained relatively high levels of UV absorbance at all times. *P. amboinensis*, in contrast to *T. lunare*, uses UV communication and thus must maintain UV transparent mucus to be able to display its UV patterns. The ability to rapidly alter the transparency of mucus could be an important adaptation in the trade off between protection from harmful UVR and UV communication.

Keywords: Ultraviolet absorbing compounds, Mycosporine-like Amino Acids; DNA damage; UV radiation; reef fish; sunscreens*; Pomacentrus amboinensis*

1 Introduction

Ultraviolet radiation (UVR, 280 - 400 nm), specifically short-wavelength UVB radiation (280 - 315 nm) causes damage to DNA. The formation of cyclobutane pyrimidine dimers (CPDs) between adjacent pyrimidine bases [1] is one of the key consequences of UVB exposure leading to structural changes in the DNA double helix, which can inhibit polymerases thus arresting replication and transcription of the DNA sequence [2]. If left unrepaired, UV-induced DNA damage can lead to mutations [3] and apoptosis of affected cells [4, 5]. In fish, the effects of UVR exposure include behavioural changes (e.g.in trout [6] and salmon [7]), damage to tissues of the skin (Japanese medaka [8]) and the brain (Northern Pike [9]), DNA damage [10] MAAs can be found in fish eggs [11], larvae [10] and in the ocular media as well as the external mucus of reef fish [12, 13]. and can lead to increased mortality (Zebrafish [14], Atlantic cod [15] and Sea Bream [16]).

In the tropics, where levels of UVR are among the highest on Earth [17], the clear and shallow waters around coral reefs allow UVR to penetrate farther than in other aquatic ecosystems [18, 19] leading to a high risk of UVB induced DNA damage. Due to changes in ozone levels [20], aerosols, greenhouse gases and cloud cover [21, 22] as well as loss of coral complexity due to increased cyclone intensity [23] and severe coral bleaching [24], UVR around coral reefs is likely to continue to increase [22]. These changes can be mediated wither directly by increases in irradiance [21, 22], or indirectly by the increases in water clarity and loss of shelter [24].

Protection from harmful UVR in marine organisms can arise from physical barriers (e.g. shells and scales) as well as from UV-absorbing compounds (UVACs) like carotenoids [25] and Mycosporine-like amino acids (MAAs), [26]. Over twenty MAAs with absorbance maxima between 309 and 360 nm have been found in the tissues of hundreds of marine species from all trophic levels and all latitudes, and together with Gadusol (absorbance maximum ~ 290 nm) cover the UVB and UVA spectrum [27]. This variety of MAA compounds [28] is synthesized by microbes, fungi and plants via the shikimate pathway [29] and alternatively the pentose phosphate pathway [30, 31]. Although some of the genes from the shikimate pathway have been found in the sea anemone *Nematostella vectensis* [32] and corals [33], MAAs cannot be synthesized by animals [29] and are likely of dietary origin [34, 35]. In reef fish, over 100 species (of 137 studied) show UV absorbing mucus [13], and the tissues where MAAs can be found are as varied as the number of compounds [36]. MAAs

have been detected in fish eggs [11], larvae [10] and in the ocular media as well as the external mucus of reef fish [12, 13], all tissues which are vulnerable and exposed to UVR.

MAAs as part of all UVACs in fish mucus are widely recognized to act as sunscreens due to their absorbance properties, the tissues in which they are found [17, 27, 34, 37], and due to their ability to prevent sunburn when topically applied to the skin of mice [38]. MAAs have been shown to protect against cleavage delay in sea urchins [39] and have recently been linked to reduced DNA damage in an intertidal gastropod [40]. In corals and other marine organisms, MAA concentrations in exposed tissues are linked environmental levels of UVR, as reviewd by Shick and Dunlap [26]. There is circumstantial evidence that MAAs may also have a protective function in reef fish [36]. The MAA concentration in the external mucus of reef fish correlates with the levels of UVR in their habitat [41, 42]. In captivity, the UVR absorbance of mucus of Hawaiian wrasse that were provided with an MAA-rich diet decreased under conditions that lacked UVR, suggesting that there is an energetic cost to the maintenance of MAA protection in the external mucus [43]. In the presence of UVR and under the same dietary conditions, MAA levels in mucus remained at pre-capture levels. The MAA profiles detected using laboratory methods (HPLC) and the absorbance of whole mucus samples measured in the field both vary between species and geographical locations [41, 44]. Mucus absorption has been established as a proxy for MAA concentration [42] in the external mucus of reef fish, and can be easily quantified in the field [45] by measuring UV absorbance in mucus samples.

Here, we address the sunscreen hypothesis, specifically that a higher level of UVACs lead to reduced UV- induced DNA damage. Therefore, fish with different known mucus absorbances were exposed to a high pulse of UVB radiation in order to induce UV-specific DNA damage in the skin. If UVACs, of which MAAs are an integral part, indeed acted as sunscreens, it is expected to find higher DNA damage (CPDs) in fish that have lower levels of UVACs in their mucus. Consequently, we tested for a sunscreen function of UVACs shortly after capture, assuming unchanged mucus absorbance, and after a period of captivity, which is shown to reduce mucus absorbance.

2 Methods

2.1 Location and experimental animals

The study was carried out at the end of the Australian summer in March and April 2013 at Lizard Island Research Station (LIRS, 14°40'5"S, 145°27'47"E). *Pomacentrus amboinensis* (Bleeker 1868, n = 50, SL = 6.9 cm, SD = 0.63 cm) and *Thalassoma lunare* (Linnaeus 1758, n = 30, SL = 11.08 cm, SD = 1.84 cm) were caught at two shallow (water depth < 2 m) sites inside the Lizard Island lagoon using hand and barrier nets. Both species were caught in locations that had a maximum depth of 2 metres. Both species are active during the day, and occur in the same habitat at Lizard Island. Damselfish such as *P. amboinensis* are highly territorial [46], and although wrasses such as *T. lunare* are more mobile than substrate-associated Damselfish, individuals were observed in the same habitat over several days (C. Braun, pers. observation). It is therefore highly likely that both species experience similar environmental levels of UVR. For the transport back to the research station by boat (< 5 min), the fish were held in plastic tanks (23x 21x 21 cm) filled with seawater and the lid closed. Upon arrival at the station, the size (SL) of the fish was measured to the nearest mm by transferring individuals to a sealable plastic bag with little seawater and gently placing the bag on a mat that had a ruler taped to it. Only fish of similar sizes (+/- 2 cm difference in SL) were used in the experiments since Zamzow and Siebeck [47] showed an effect of body length on mucus absorbance in *P. amboinensis*. All procedures were conducted with permission from the Queensland Government (General fisheries permit 162472 to U.E.S.), the Great Barrier Reef Marine Park Authority (permit G11/34453.1 to C.B. and U.E.S.) and the animal ethics commission of the University of Queensland (permit SBMS/091/11 to C.B. and U.E.S.).

2.2 Holding conditions

Test 1 – natural mucus absorbance levels: All fish were randomly assigned to the following treatments and holding conditions. Thirty *P. amboinensis* and twenty *T. lunare* were subjected to the UV challenge (see below) within 24 hours of capture. These fish were held in plastic tanks (*P. amboinensis*: 23x 21x 21 cm, water depth 20 cm; *T. lunare:* 40x 30x30 cm, water depth 20 cm) with flow through seawater inside an aquarium room of LIRS.

Test 2 – following manipulation of mucus absorbance levels: Twenty *P. amboinensis* and ten *T. lunare* were held in captivity for ten days before being subjected to the treatments of the

UV exposure challenge. These fish were different individuals than the fish that were used in Test 1. The aim of this was to manipulate mucus MAA levels to increase their variability within each species and hence manipulate mucus absorbance. Previous studies showed that both, MAA-rich food and exposure to UV is required to maintain high mucus UV absorbance [43]. Here all fish were fed the same diet (MAA rich food (see below and Fig. S 1) and relied on the presence/absence of UV for the manipulation of mucus absorbance levels.

The fish were randomly assigned to an experimental tank (same dimensions per species as above), which was linked to the seawater flow-through system and contained a small PVC pipe that served as shelter. *Pomacentrus amboinensis* were either shielded from natural sunlight (inside an aquarium room of LIRS), or exposed to natural sunlight, with equal number of fish being held in each condition. All ten individuals of *Thalassoma lunare* were held inside an aquarium, shielded from natural sunlight. The aquaria exposed to natural sunlight were placed on two benches which were aligned on an east-west axis to maximize sun exposure during the day and to prevent shading from nearby trees and buildings. On a daily basis, tanks were cleaned to prevent build-up of algae, detritus and leftover food.

The water temperature for each holding condition was recorded every 15 minutes by an immersed HOBO Datalogger (Onset Computer Corporation, Bourne, MA, U.S.A.), placed in an additional tank, which was also linked to the flow-through system. Mean water temperature for the outside condition was 29.53 °C (SD ± 1.57), which was slightly higher than for the inside condition (28.79 °C, SD ± 0.59) and the water in the Lizard Island Lagoon (28.52 °C, SD ± 0.24; measured by an oceanographic mooring (Australian Institute of Marine Science (AIMS), 14°68'S, 145°45'E at 0.6m, data provided by Integrated Marine Observing System (IMOS)).

2.3 Diet during captivity

Fish were fed twice daily with approximately 0.5 g food paste made of frozen prawns and whitebait, supplemented with 10% (w/w) ground *Acanthophora spicifera*, a rhodophyte rich in MAAs [48]. The algae were collected (GBRMPA permit G11/33857.1 to LIRS) from a shallow site (water depth < 2 m) in the lagoon. The food paste was prepared before the start of the experiment, aliquoted to small portions, and frozen at -20°C. A new aliquot was used each day and leftover food discarded. Characteristic signatures of eight known MAAs were detected in samples of *A. spicifera* using HPLC-MS (Fig. S 1).

2.4 Experimental treatments (UV exposure challenge)

The experiment was conducted inside the aquarium room. Each light treatment (see below) lasted one hour. Fish were placed individually in one of five plastic tanks (23x 21x 21 cm, water depth 20 cm) connected to the seawater flow-through system.

Within 24 hours of capture (test 1), ten *P. amboinensis* were challenged with exposure to a high dose of UVB radiation (treatment "UVB +"). Ten control individuals (treatment "UVB -") were exposed to the fluorescent lights of the room only and handled in the same way as the treatment fish (Fig. 1). In order to ensure that any changes in UV-induced DNA damage and mucus absorbance levels were due to an increase in UVB radiation and not brightness, an additional ten *P. amboinensis* were exposed to a light pulse that had the same amount of photons as the UVB challenge, but lacked any light below a wavelength of 400 nm (treatment "brightness control"). After ten days in captivity (test 2), five *P. amboinensis* held under exclusion of UVR and five individuals held under natural UVR were subjected to the "UVB +" and "UVB -" treatments.

For *T. lunare*, ten individuals each were exposed to the "UVB +" and "UVB -" treatment within 24 hours after capture (Test 1), and five individuals each to the "UVB +" and "UVB -" treatment after ten days in captivity (T est 2). One individual of the "UVB +" treatment (within 24 hours after capture) and one individual of the "UVB -" treatment (after 10 days in captivity) escaped from their tanks into the overflow area around the tanks during the 1 h UV challenge. Although these fish survived and were put back into the tanks, they were excluded from the analysis due to the extra handling stress they might have experienced.

Immediately after being subjected to one of the treatments, fish were euthanized in an ice bath, followed by sampling of mucus and skin tissue.

2.5 Light treatments

All measurements were made using an OceanOptics Jaz spectrometer, 1000µm optical fibre and a CC-3-UV-S cosine corrector (all OceanOptics, Ft Lauderdale, FL, U.S.A.), held horizontally just above the water surface facing the light source above the tanks. The spectrometer was calibrated against a DH-2000 Deuterium- Tungsten Halogen light source (OceanOptics, Ft Lauderdale, Florida, U.S.A.).

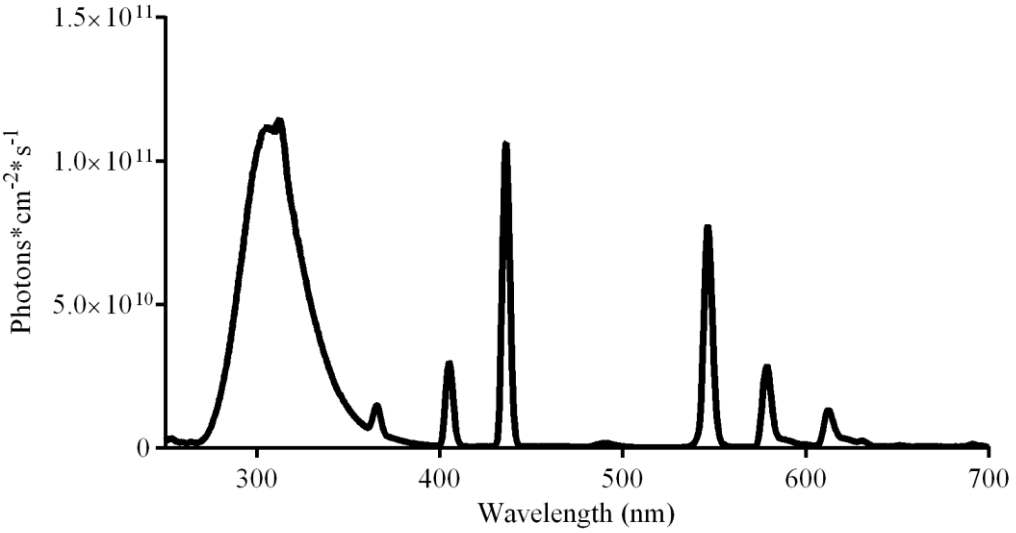
In the treatment "UVB +", three lamps with two fluorescent tubes each (Sankyo Denki G20T10E UVB, Kanagawa, Japan, peak emission at 310 nm) were used. The lamps were suspended 30 cm above the water surface of the tanks holding the individual fish. The UVB (280 - 315 nm) and UVA (315 – 400 nm) doses at the water surface were 13.4 and 6.1 $W*m^{-2}$, respectively. A total photon flux (280 – 700 nm, Fig 1a) of $7.74*10^{15}$ $photons*cm^{-2}*s^{-1}$ was detected at the water surface (Table S1 in supplementary material) The total UVB and UVA dose for the exposure of one hour were 48.2 and 21.9 $kJ*m^{-2}$, respectively. This UVB dose is the rough equivalent of a surface exposure of 2.7 hours around midday at Trawler Beach (Table 2 in supplementary material, for further details see Braun [49]). The animals used in this experiment were caught in this area, making this exposure environmentally relevant.

For the treatment "UVB -", illumination of the tanks was provided by four fluorescent tubes (Philips TLD 36W/950) on the ceiling above the exposure tanks. The photon flux (280 – 700 nm, Fig. 1b) was measured at $1.08*10^{14}$ $photons*cm^{-2}*s^{-1}$ (Table S1 in supplementary material). Spectral measurements confirmed that no UVR (UVA and UVB) was present in this treatment.

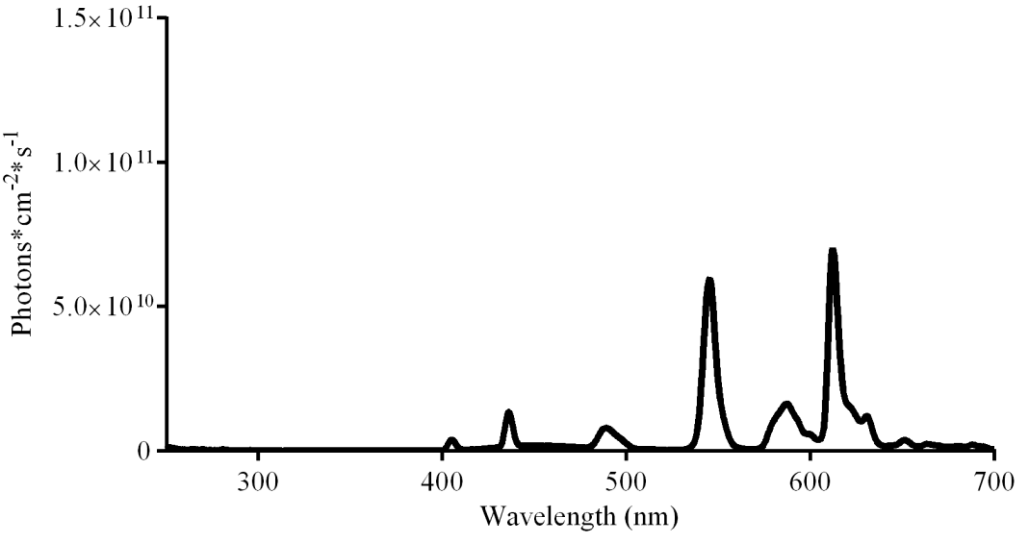
The light environment for the brightness control treatment was created by a combination of an AquaOne Daylight fluorescent tube (Arcadia, Redhill, Surrey, UK) and a UVB tube in each of the four lamps. The lamps were covered with one layer of UV-blocking filter material (LEE226, Andover, Hampshire, UK), and spectral measurement with the Jaz spectrometer confirmed that no UVR was present. The photon flux (280 – 700 nm) in the "brightness control" treatment was $7.48*10^{15}$ $photons*cm^{-2}*s^{-1}$ (Fig. 1c, Table S1 in supplementary material).

An overlay of figures 1a-c is shown in the supplementary material (Fig S2).

a) UVB +



b) UVB -



c) Brightness control

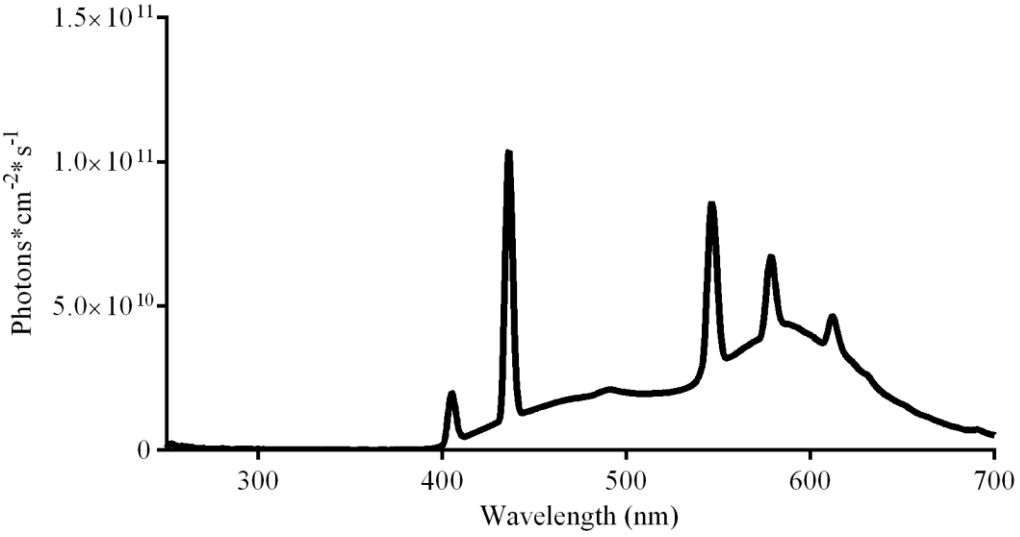


Figure 1: Photon flux in the light treatments of the experiment. **a)** Note the large proportion of UVB (280 – 315 nm) radiation in the "UVB +" treatment, **b)** compared to the "UVB –" treatment. c) The absence of UVR needed to be controlled with a larger proportion of visible light in the "brightness control" treatment to equalise overall photon flux.

2.6 Mucus absorbance analysis

Mucus was taken from both flanks of the fish using a dull scalpel blade [13] and stored in a 2 ml Cryotube on ice for absorbance measurements later on the same day. The UV absorbance (280 - 400 nm) of external mucus was measured following Zamzow and Losey [13]. This approach allows mucus samples to be measured shortly after their collection in the field and is a reliable method for the quantification of the mucus absorbance [13, 42, 45, 47]. Briefly, mucus samples were squashed between a UV-transparent slide (made from OP4 acrylic, T_{50} = 278 nm) and a coverslip (T_{50} = 290 nm). Two cover slips were glued to the slide and acted as spacers to standardize the mucus samples to a thickness of 0.25 mm. The slide was mounted on the stage of a modified microscope which held two UV-transmitting fibre optic cables (with a diameter of 200 µm) perpendicular to each other. Light from the Deuterium-Tungsten Halogen source of the Jaz spectrometer (OceanOptics, Ft Lauderdale, FL, U.S.A.) passed through the upper optic fibre through the slide and the mucus sample and into the lower fibre. Ten absorbance measurements against a blank (seawater plus slide) at different points of the mucus sample were averaged to account for heterogeneity of the mucus. The absorption data of the mucus samples were then integrated (AUC, area under the curve) between 280 and 400 nm using GraphPad Prism 6. Reef fish mucus not only contains MAAs, but also a suite of endogenous proteins and lipids [50] and external compounds such as bacteria [51]. All parts together possibly add to the absorbance of the mucus in the UV range. Hence, the measurement of "sunscreens" in the mucus using the technique described here includes all possible compounds, and the term "sunscreens" in this paper refers to all UV absorbing compounds (UVACs).

2.7 DNA damage analysis

After sampling of the mucus, the fish were immediately frozen in liquid nitrogen, and stored at -70°C until analysis of DNA damage. The procedure followed the protocol by Mori et al. [52].

Genomic DNA was extracted from up to 25 mg of skin and tissue from an area between the dorsal fin and the head of each fish. A scalpel blade was used to shave off a section of skin and tissue (< 1 mm thick) from the still frozen fish. Using the DNeasy® Blood and Tissue kit (Qiagen) following manufacturers' instructions, each sample was immediately transferred into a 2 ml tube containing the lysis buffer to prevent DNA degradation. Upon isolation, the integrity of the DNA was visually inspected using gel electrophoresis and the DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmingtion, DE, USA). The DNA was diluted in 1 x PBS to a final concentration of 0.2 µg / ml. Only structurally intact DNA samples (i.e. single band on the gel) and with an absorption coefficient (260 / 280 nm) > 1.8 were used for damage analysis.

To detect UV-induced CPD lesions, the TDM-2 primary antibody (Cosmo Bio, Tokyo, Japan) was used in an enzyme-linked immunosorbent assay (ELISA) read out in a 96 - well plate reader (Model 680, Bio-Rad, U.S.A.) at 492 nm. Mean values of triplicate DNA samples were used as the damage level for each fish, which was the statistical replicate. In order to compare damage levels between assays, i.e. 96 - well plates, a DNA standard was included with each assay. This standard was generated by irradiating a DNA solution (170 µg/ml in 1 x PBS from salmon testes, Sigma-Aldrich) in a 6 well plate for up to 20 minutes under two UVB lamps (2x Sankyo Genki GL20SE, peak emission at 310 nm, distance from well plate 12 cm). For the ELISA assay, the standard solution was then diluted in 1 x PBS to the same concentration as the DNA samples (0.2 µg / ml). Additionally to the triplicates of the DNA standard and the isolated DNA samples, a triplicate blank (1 x PBS) was included in all assays. This blank was then subtracted from the average of the OD values of all triplicates (standard and samples). OD values (i.e. DNA damage levels) were then normalized to the average of the three highest values of the standard curve.

2.7 Statistical analysis

For the statistical analysis, the normalized DNA damage values were arcsine transformed. First, it was tested for an effect of holding condition (shielded or exposed to natural sunlight) on the DNA damage and integrated mucus absorbance after the exposure to the different conditions in the UV challenge ("UVB +" and "UVB -") using two-way ANOVAs. This analysis was done for the cohort of *P. amboinensis* that was held in captivity for ten days. As there was no significant effect of holding condition on DNA damage ($F_{(1,16)} = 0.7$, $P = 0.4$) or

integrated mucus absorbance ($F_{(1,16)}$ = 4.2, P = 0.06), the data were pooled for further analysis.

Subsequently, a two-way ANOVA and post hoc tests (Dunnett's) were conducted for each species to test the effects of the UV challenge ("UVB +" and "UVB -") and time (zero days or ten days) in captivity on either DNA damage or integrated mucus absorbance. In the case of *P. amboinensis*, the brightness control treatment was excluded to keep the number of factors equal for the two time points (zero and ten days in captivity). The DNA damage and integrated mucus absorbance of the "brightness control" group were compared separately to the "control" treatment using an additional ANOVA.

The ratio of the integrated UVB/UVA absorbances were compared between treatment groups (UVB + versus UV -, and UV – versus brightness control) using a Mann-Whitney test. No significant differences were found in these ratios (all P > 0.5, see Table S3).

Linear regressions were used to test whether decreased mucus absorbance was linked to higher amount of UV-induced DNA damage. In the case of *T. lunare* an additional Spearman-rank correlation was performed, since the initial linear regression was not significant, possibly due to a low sample size. GraphPad Prism 6 and JMP 10 were used in this analysis.

3 Results

3.1 Pomacentrus amboinensis

The treatments of the UV challenge had a highly significant effect on DNA damage (ANOVA, $F_{1,36} = 139.9$, $P < 0.0001$) and the integrated mucus absorbance (ANOVA, $F_{1,36} = 47.22$, $P < 0.0001$). Both measured variables were significantly higher in the "UVB +" compared to the "UVB -" treatment (Dunnett's, $P < 0.0001$, Figs. 2a and 2b). The time the fish spent in captivity had no significant effect on DNA damage or integrated mucus absorbance ($P > 0.05$, Figs. 2a and 2b, and also Figs. 3a and 3b). No significant interactions between the treatments of the UV challenge and time in captivity were observed (DNA damage: ANOVA, $F_{1,36} = 0.22$, $P = 0.642$; integrated mucus absorbance: ANOVA, $F_{1,36} = 0.004$, $P = 0.951$).

There was no significant difference in either DNA damage (ANOVA, $F_{1,18} = 0.0006$, $P = 0.98$, Fig. 2a) or mucus absorbance (ANOVA, $F_{1,18} = 1.43$, $P = 0.25$, Fig. 2b) between the fish of the "UVB -" and "brightness control" treatments. An increase in average mucus absorbance of "UVB +" fish compared to the "UVB -" treatments is clearly visible in the absorption curves (Figs. 3a and 3b). The "brightness control" and "control" treatments appeared very similar (Fig. 3a). Figure S3 (supplementary material) shows the SD error, which was omitted here for clarity.

The amount of DNA damage was negatively correlated with the integrated mucus absorbance (Fig. 4a) in the "UVB +" treatment (linear regression, $n = 20$, $R^2 = 0.34$, $P = 0.007$), but not in the "UVB -" treatment (linear regression, $n = 20$, $R^2 = 0.06$, $P = 0.3$).

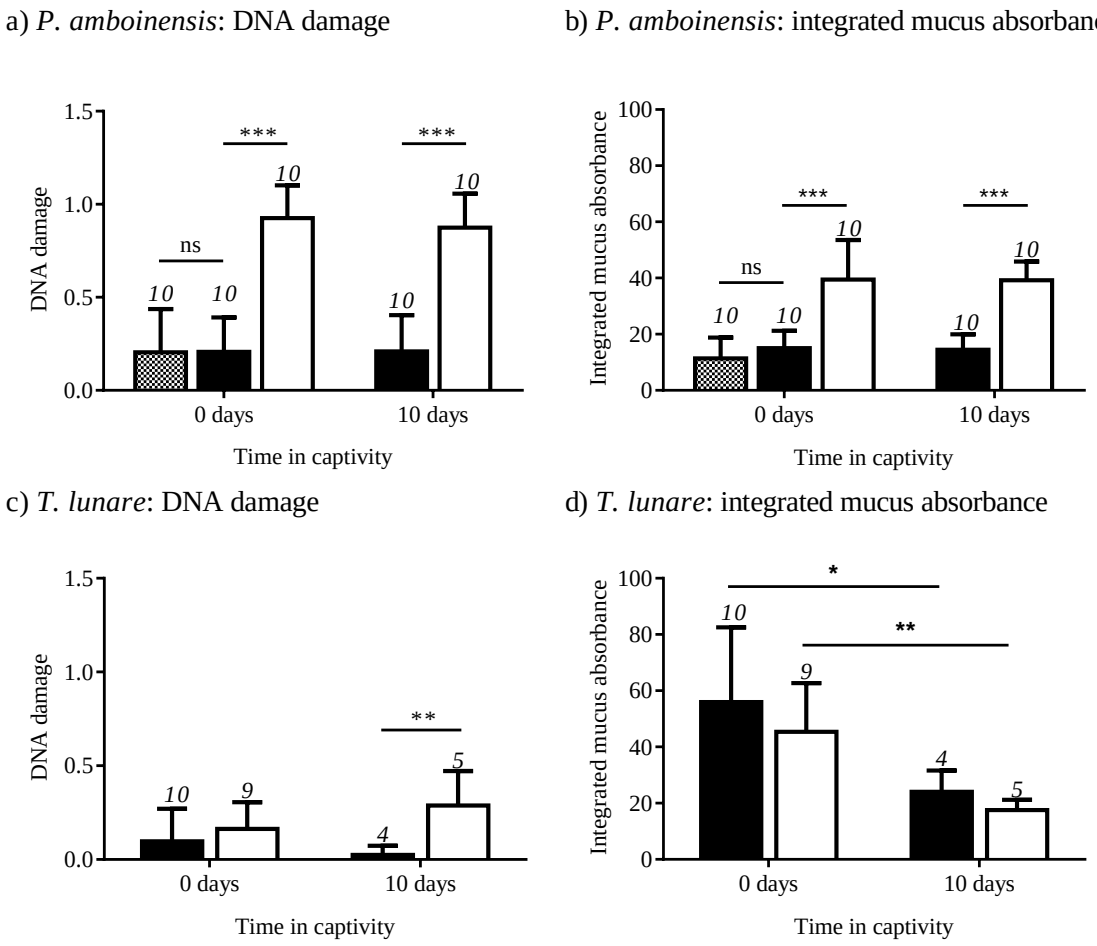


Figure 2: Relative DNA damage in skin samples (a,c) and integrated mucus absorbance (b,d) of *P. amboinensis* (a,b) and *T. lunare* (c,d) after exposure to one of three treatments ("UVB +", "UVB -" and "brightness control") measured within 24 hours of capture ("0 days") and after ten days in captivity ("10 days"). a) *P. amboinensis* exposed to the "UVB +" treatment had significantly higher DNA damage than fish from the treatments "UVB -" or "brightness control" at both timepoints. b) The same pattern was found for integrated mucus absorbance. c) DNA damage in *T. lunare* was significantly higher in fish of the "UVB +" treatment after ten days in captivity. d) Integrated mucus absorbance of *T. lunare* was significantly reduced after 10 days in captivity. Solid bars: "UVB -", open bars: "UVB +", chequered: "brightness control". Error bars indicate 1 SD from the mean. Numbers above bars indicate the total number of fish used in the respective treatment. Asterisks indicate significant Dunnett's post-hoc comparisons (*: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $0.001 < P < 0.0001$).

3.2 Thalassoma lunare

DNA damage was significantly affected by the UV challenge ($F_{1,24} = 12.23$, $P < 0.002$), with fish that were in the "UVB +" treatment showing significantly higher DNA damage levels than "UVB -" fish (Dunnett's $P < 0.0001$, Fig. 2c) after ten days in captivity. There was also

a significant effect of time spent in captivity on the integrated mucus absorbance ($F_{1,24}$ = 14.4, P < 0.0009), with mucus absorbance being significantly lower after ten days (Dunnett's, "UVB -": P = 0.04; "UVB +": P = 0.005, Fig. =2d). No significant effect of time spent in captivity on DNA damage of *T. lunare* was observed ($F_{1,24}$ = 0.02, P > 0.05). There were no significant interactions between the treatments of the UV challenge and time in captivity (DNA damage: $F_{1,24}$ = 3.134, P = 0.089; integrated mucus absorbance: $F_{1,24}$ = 0.067, P = 0.798).

The absorbance curves (Figs. 3c and 3d) of "UVB +" and "UVB -" fish did not differ upon visual inspection, with the SD error of the mean curves overlapping (omitted for clarity in Fig. 3, but see supplementary material, Fig S3). Similar to *P. amboinensis*, the DNA damage of exposed *T. lunare* was negatively correlated with the integrated mucus absorbance (Spearman rho -0.662, n = 14, P = 0.01), although the linear regression showed a non significant relationship (linear regression, n = 14, R^2 = 0.24, P = 0.07, Fig. 4b). No significant relation between DNA damage and integrated mucus absorbance was observed in "UVB –" animals (linear regression, n = 14, R^2 = 0.11, P = 0.25, Fig. 4b).

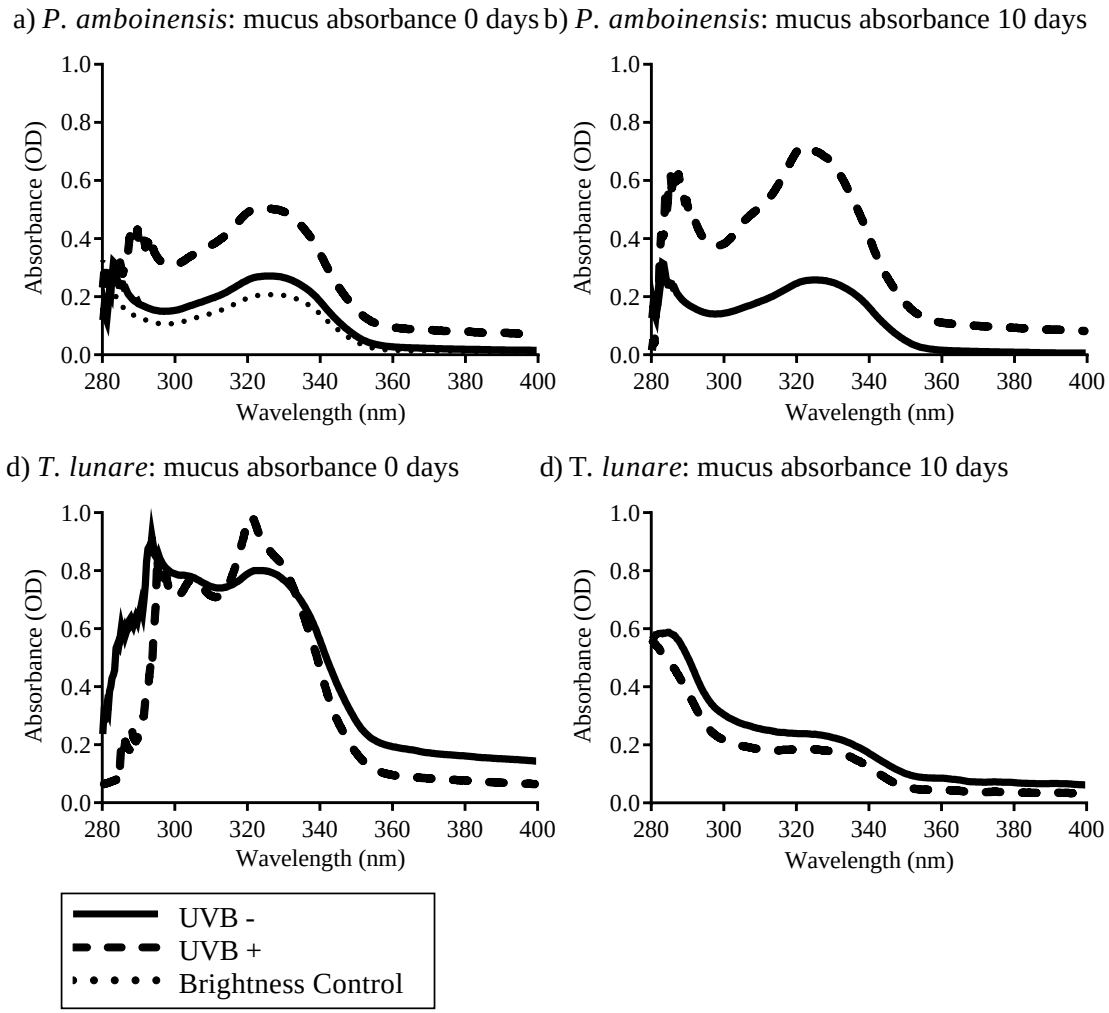


Figure 3: Mean absorbance curves of mucus samples of *P. amboinensis* and *T. lunare*. Mucus was sampled after exposure to one of the three treatments: "UVB -" (solid lines), "UVB +" (dashed lines) and in the case of *P. amboinensis*, the "brightness control" (dotted line). Mucus absorbance for *P. amboinensis* was higher across the spectrum in fish exposed to the "UVB +", both within 24 hours after capture (a) and after ten days in captivity (b). The "brightness control" treatment (a) was very similar to the "UVB -" treatment in terms of magnitude and shape. In contrast to *P. amboinensis*, mucus absorbance of *T. lunare* exposed to the "UVB +" treatment did not differ upon visual inspection and appeared very similar across the spectrum, both within 24 hours after capture (c) and after ten days in captivity (d). After ten days in captivity (d), a decrease in overall absorbance was visible in both "UVB -" and "UVB +" treated fish.

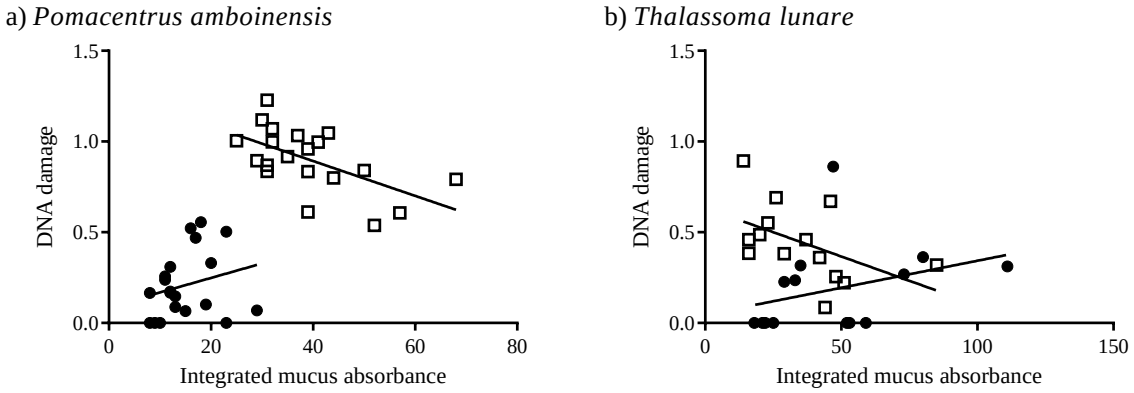


Figure 4: Relationship between DNA damage levels and mucus absorbance in fish exposed to various treatments a) DNA damage significantly increased with decreasing mucus absorbance in fish exposed to the UVB + treatment (linear regression, n = 20, R^2 = 0.34, P = 0.007) but not in "UVB -" fish (n = 20, R^2 = 0.06, P = 0.3). b) The linear regression for the "UVB +" exposed *T. lunare* was not significant (n = 14, R^2 = 0.24, P = 0.07), however a Spearman-rho rank correlation was significant (P = 0.01). A linear regression for the "UVB -" group was not significant (n = 14, R^2 = 0.11, P = 0.25). Open squares: "UVB +", solid circles: "UVB -".

4 Discussion

The UV-absorbing function of reef fish mucus has been relatively well studied [13, 36, 41, 42, 44, 47], however direct evidence for a protective, sunscreen function that actually reduces or mitigates the negative effects of UVR exposure in fish, specifically DNA damage, is missing from the literature. The results show that UVACs, of which MAAs are a crucial component, do indeed act as a first line of defence against UV- induced DNA damage in two species of reef fish, *Pomacentrus amboinensis* and *Thalassoma lunare*. When fish were exposed to a high dose of UVB radiation, the level of DNA damage in the skin of the fish was negatively correlated with the integrated mucus absorbance, which is an accepted proxy for the amount of MAAs present in the external mucus [42]. Fish exposed to the "UVB –" treatments had variable levels of UVACs levels but overall low DNA damage. Additionally, it was discovered that in *P. amboinensis*, but not *T. lunare*, mucus absorbance was significantly higher in UVB exposed fish compared to control groups, therefore potentially increasing the amount of UVAC and MAA protection in their mucus. The changes in mucus absorbance observed in this experiment were species-specific, and restricted to fish that were exposed to increased UVB light.

Similar protective functions of natural sunscreens have previously been shown only in invertebrates. Adams [39] showed that in larvae of the sea urchin *Strongylocentrotus droebachiensis*, lower MAA levels lead to longer delays in cleavage induced by acute UV exposure. Carefoot [53] observed reduced hatching in UV exposed *Aplysia* eggs, and higher MAA levels in spawn from UV exposed adults, however a definite protective function of MAAs could not be confirmed. Although we did not directly measure MAA content in mucus, our observations of mucus absorbance in relation to DNA damage strongly support a protective function of UVACs, and since MAAs are an integral part of fish mucus, also the hypothesis that MAAs serve as natural sunscreens.

The experiments were designed to manipulate mucus absorbance levels found in the external mucus of fish to achieve high variability in UVACs levels prior to exposure to UV radiation. In particular, to lower the mucus absorbance of one group of fish relative to that of another group through the exclusion of UVR light while keeping all other factors constant between the groups, including the MAA-rich diet. After ten days, lower mucus absorbance was indeed achieved in *T. lunare* that were held under the exclusion of UVR, which was in agreement with previous studies [43, 47] and the suggestion that UVR exposure is necessary for MAAs to be sequestered into the external mucus layer [41, 43]. However, no reduction in mucus

absorbance was observed in *P. amboinensis* over time, neither in fish that were held in conditions lacking UV, nor in fish exposed to natural sunlight for the ten day holding period. In the latter group, the increase in mucus absorbance due to the 1 hr UVB pulse possibly could have masked a decrease of mucus absorbance caused by the holding conditions. This is unlikely however, as the mucus absorbance of fish from the control group held in captivity for ten days was no different from that of the control group which was measured within 24 hrs of capture. A previous study showed that a reduction of MAAs can be induced in this species by using a MAA- free diet [43]. It seems that this species, which has naturally low MAA levels, maintains a minimum level of UV- protection in the presence of MAA-rich food, irrespective of ambient UVR levels.

The increase of mucus absorbance in *P. amboinensis* can be attributed to exposure to the UVR pulse which contained a high proportion of UVB light and therefore has a high potential to inflict DNA damage, rather than just an exposure to light with high intensity. This conclusion follows the fact that there was no significant increase in the integrated mucus absorbance, nor a visible change in the shape of the absorbance curves in fish that were exposed to the brightness control treatment.To our knowledge, the observed increase of mucus absorbance in *P. amboinensis* within one hour of exposure is the fastest change of such nature described in a reef fish. Changes in mucus absorbance, mediated by UVACs and/or MAAs after changes to the UVR regime have been documented before [13, 42, 43], but are usually only detectable after several days or weeks. Similarly, the accumulation of MAAs in algae [54], diatoms [55] and corals [56] due to UVB exposure occurs over longer time periods.

Most certainly, UV exposure influenced the mucus absorbance. The mucus absorbance curves of *P. amboinensis* appeared to be similar to the profiles detected by Eckes [36], who also documented a strong absorbance of mucus in the UVA range. Interestingly, the peaks of the UV lamps used in the treatments (310 and 360 nm) do not correspond with the mucus absorbance peaks (280 – 290 nm, representing most likely the MAA precursor Gadusol, and 320 – 340 nm, most likely representing the MAAs Palythine-threonine and Porphyra). This indicates that a defensive response using UV absorbing compounds is not directly matched to the environmental spectrum. However, the light spectrum used in these experiments does not recreate a natural exposure, like the one used by Zamzow [42]. Nevertheless, we did observe mucus absorbance increases in UVB exposed groups of fish, as did Zamzow [42], despite the different light sources that were used in the experiments.

Although UVB exposed *P. amboinensis* showed increased mucus absorbance, higher levels of DNA damage compared to a group of fish not exposed to UVR were still observed. However, it is difficult to know how much more DNA damage would have been induced by the exposure to the UVB pulse if this increase had not taken place, or mucus measurements in the same individuals were taken at the beginning of the treatment. However, such measurements require repeated handling of fish to sample mucus, which has been linked to disease in this species [47]. Our exposure regime was on a much smaller scale, and we intended to avoid any additional handling effects due to repeated sampling. The formation of CPDs, the most common type of UV- induced DNA lesions [57], occurs nearly instantaneously upon irradiation with UV light [58]. Any protection by UVACs in the mucus of *P. amboinensis* also did not prevent higher DNA damage levels (up to 58%) than in *T. lunare* and indicates interspecific variation in the susceptibility to UVR, which has been shown in both marine and freshwater fish [16, 59-61]. The fish used in this study differ in their diet and lifestyles, with *T. lunare* being a known diurnals piscivore, foraging for large amounts of time away from cover [62]. In contrast, *P. amboinensis* is a diurnal omnivore, andstrongly associated with sheltering habitats [46]. Both factors, diet and UV exposure are known to influence the levels of UVACs [43] and may have led to the observed susceptibility to DNA damage observed in the present study.

The mechanism of the regulation of MAA content in the mucus and their transport from the gut into the mucus layer is unknown. Potential storage locations in tissues such as the gonads [48] and gut [42], followed by transport to the mucus producing goblet cells in the epidermis seem possible, and could be responsible for the swift increase in mucus absorbance observed in the present study. Whether MAAs could also be stored in the mucus producing goblet cells for an even faster release than from the gut is unclear and needs further investigation.

UVACs in the mucus could also originate directly from the MAA rich food items in the gut, without previous storage in other tissues. Gut turnover rates in fish are possibly fast enough to process some MAAs to the mucus [63, 64] but certainly not fast enough to prevent the formation of CPDs. MAAs originating from bacteria with a functioning shikimate pathway transferred into fish mucus [51] cannot be discounted, and could potentially be responsible for the changes in mucus absorbance observed in this study. However, mucus is water soluble and constantly sequestered and replaced [50] and also possesses antibacterial properties [65, 66], therefore making an external source of MAAs in fish mucus a less likely explanation.

The modulation of mucus absorbance could be visually triggered, since *P. amboinensis* is able to see UV light in contrast to *T. lunare* [67, 68], where mucus absorbance modulation must be triggered otherwise [42, 43]. For *P. amboinensis*, the ability to modulate the UV absorption properties of their external mucus layer may be essential in order to successfully send and receive their UV signals for communication [46, 69]. The ability for fast UVAC modulation could be the result of a trade-off, which allows *P. amboinensis* to communicate in the UV as long as the UV damage can be kept at low levels but is induced once damage levels are too high, or UV exposure reaches critical levels. Initially at day zero, *T. lunare* had an up to 63% higher mucus absorbance than *P. amboinensis*, confirming previous findings of family differences in MAA levels [13]. At a later stage in the experiment, the high levels of mucus absorbance in *T. lunare* were lost and were up to 50 % lower than in *P. amboinensis* (cohorts exposed to UVB). Insofar as this presents a trade-off between communication with UV signals (at times of low UV and low UVAC levels) and protection from UV radiation at times of high UV and high UVAC levels is unknown and needs to be examined in detail.

Variable sunscreen protection could also provide an important selective advantage for reef fish to react quickly to increases in UV radiation over a short period of time with tidal movements and the movement of fish across a habitat [70]. Changes in the UV regime over larger timescales and magnitudes facilitated by climate change [21] are currently ongoing on the Great Barrier Reef, with less cloud cover increasing solar radiation and hence UV exposure. In this study, the dose of UVB radiation in the "UVB +" treatment (13.1 $W*m^{-2}$) was more than double the amount of UVB radiation measured at Lizard Island at midday in the austral summer (6 $W*m^{-2}$). However, the implications of increased UVR as an additional stressor in an ocean that is already impacted by warmer temperatures, higher acidity and a less complex habitat [71] are currently poorly understood. The relatively low levels of DNA damage in fish that were not UV challenged, compared to the sharp increase in DNA damage in fish exposed to the UVR pulse, indicate that at present, the level of UVR in their environment does not pose a significant threat and the negative effects of UV exposure are being held at bay by the protective function of their MAA sunscreens and other hypothesized UV protection mechanisms such as UV specific avoidance behaviour and DNA repair.

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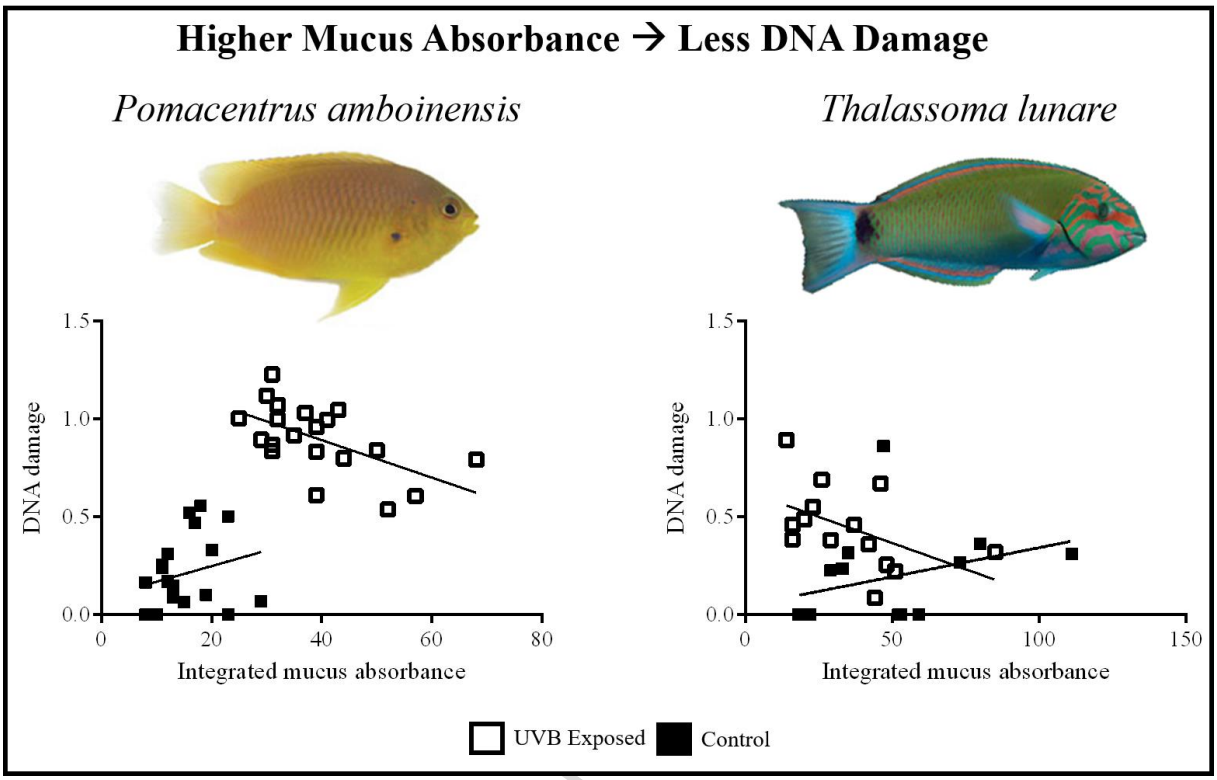

Graphical abstract

Highlights

- Ultraviolet absorbing compounds (UVACs) and MAAs have been dubbed nature's sunscreens
- The efficiency of MAAs to protect against UV induced DNA damage is yet unknown
- We induced DNA damage in reef fish with variable UVACs in their external mucus
- Fish with more sunscreens in their mucus had less DNA damage in skin samples
- An increase in mucus absorption when exposed to UV radiation was observed in one species