THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

UV-induced DNA damage in coral reef fish: Damage levels and protection mechanisms

Christoph Braun

Dipl. Biol. (BSc. Hons. 1st class)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2015

School of Biomedical Sciences

Abstract

Ultraviolet radiation (UVR) reaching the surface of earth has been recognized as a major environmental stressor for marine organisms due to the potential of UVB (280 - 315 nm) to induce DNA damage such as cyclobutane pyrimidine dimers (CPDs) that can lead to cell death. Despite inhabiting a UV-rich environment, the levels of DNA damage in reef fish and factors influencing these levels are unknown. Whether reef fish are able to avoid UVR and repair UV-induced DNA damage is unclear despite the importance of these protection mechanisms in other animals. The presence of UV-absorbing Mycosporine-like Amino Acids (MAAs) in the external mucus of reef fish has been confirmed, however the efficiency of these compounds in preventing CPDs has not been studied. The aim of this PhD was to assess the impact of ambient and elevated levels of UVR on reef fish in terms of UV-induced DNA damage, and to evaluate the protective mechanisms available to fish with and without UV vision.

In order to determine the net level of DNA damage in skin samples of 15 species of fish from the reefs surrounding Lizard Island, a CPD-specific antibody was used in an ELISA. Analysis using a boosted regression tree shows that the most important factors governing CPDs were species and size, with higher damage being detected in smaller individuals. Other factors such as family, depth and the presence or absence of UV vision contributed the least to the variation in damage. The length of exposure to natural levels of UVR over the course of a day was found to have no significant influence on net DNA damage levels, which were relatively low *in situ*, compared to levels that were detected in later experiments using elevated UVR.

The first protection mechanism, behavioural avoidance to UVR, was tested using behavioural experiments in which fish with (*Pomacentrus amboinensis*) and without UV vision (*Thalassoma lunare*) were given a choice between UV-protected and UV-exposed compartments. Additionally, foraging behaviour of settlement-stage larvae of *P. amboinensis* was determined under ambient levels of UVR. Automated analysis of video footage using MatLab shows that neither species showed a specific avoidance response to varying levels of UVB. Although *P. amboinensis* showed a preference for deeper sections of the experimental tanks, fish spent equal amounts of time in exposed and protected compartments. The foraging activity and distance to shelter of *P. amboinensis* that were exposed to UVR were significantly reduced compared to fish that were observed under light conditions that lacked UVR.

Next, the efficiency of natural sunscreens, MAAs, in preventing CPDs was tested in *P. amboinensis* and *T. lunare*. The levels of MAAs in the mucus of the two species were either reduced or maintained during captivity before exposure to a short pulse of high UVR. Spectrometric measurements of light transmission through mucus samples collected after irradiation were used to quantify the amount of MAAs available for protection. In both species, DNA damage levels in skin samples from UVR exposed individuals was higher than in control groups that were exposed to light lacking the UV component. Spectrometric measurements of external mucus of both species revealed a clear link between higher mucus absorbance, i.e. MAA levels, and lower DNA damage levels. Furthermore, a significant increase in mucus absorption was observed in *P. amboinensis* after UV exposure.

The last protection mechanism, the ability to revert DNA damage via photoreactivation and dark repair was investigated in four species (*P. amboinensis, Pomacentrus moluccensis*, *Lethrinus variegatus* and *Siganus corallinus)*. All species examined showed significant increases of DNA damage after exposure to elevated UVR levels. Interspecific variation in the susceptibility to UVR was observed, with *L. variegatus* showing the highest damage levels. Significant reductions in DNA damage levels were found in *P. moluccensis* and *L. variegatus* that were exposed to photoreactivating light after the initial damage accumulation. Individuals of *P. moluccensis* that were shielded from any light exposure post UVR exposure also showed less DNA damage at the end of the experiment.

This is the first study to address levels of UV-induced DNA damage in reef fish under natural conditions as well as under elevated doses of UVR that could occur in a changing climate. The relatively low levels of CPDs in a diverse group of reef fish indicate that current levels of UVR pose only low threat and underline the importance of protection mechanisms against UVR. Increases in UVR could have an impact not only on adult fish capable of adjusting their protection mechanisms, but also settlement stage larvae which show some of the highest levels of DNA damage *in situ* as well as mortality during slight increases of UVR. Whether these and other effects such as decreased foraging activity have broader implications on the recruitment and reproduction of reef fish and the community structure on coral reefs needs to be examined in the future.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer - reviewed papers

Siebeck, U. E., O'Connor, J., Braun, C., & Leis, J. M. (2014). Do human activities influence survival and orientation abilities of larval fishes in the ocean? *Integrative zoology* (in press).

Conference abstracts

Braun, C., Reef, R. & Siebeck U.E. (2011). The effect of UV radiation on reef fish: avoidance, DNA damage and DNA repair. 3MT® presentation, International Postgraduate Symposium, School of Biomedical Sciences, Brisbane, Australia.

Eckes, M., Siebeck, U. E., Dunn, S., Braun, C. & Dove, S. (2012). Prevention and repair of ultraviolet induced DNA damage in fish. Oral presentation, International Coral Reef Symposium, Cairns, Australia.

Braun, C., Reef, R. & Siebeck, U.E. (2012). How do reef fish cope with harmful UVB radiation? Poster presentation, International Postgraduate Symposium, School of Biomedical Sciences, Brisbane, Australia.

Braun, C., Reef, R. & Siebeck, U.E. (2013). Mycosporine-like Amino Acids provide a rapid mechanism for UV protection in reef fish. Poster presentation, International Postgraduate Symposium, School of Biomedical Sciences, Brisbane, Australia & Australian Coral Reef Society conference, Sydney, Australia.

Braun, C., Reef, R. & Siebeck, U.E. (2014). Natural sunscreens in coral reef fish prevent UV-induced DNA damage. Oral presentation, Otago University Postgraduate Symposium, Dunedin, New Zealand.

Braun, C., Reef, R. & Siebeck, U.E. (2014). Natural sunscreens really do work: slip, slop, slap on the Great Barrier Reef. Oral presentation, Australian Marine Sciences Association student conference, Dunwich, Australia.

Braun, C., Reef, R. & Siebeck, U.E. (2014). Natural sunscreens really do work: UV protection in reef fish. Oral presentation, International Conference on the Biology of Fish, Edinburgh, Scotland.

Siebeck, U. E., Parker, A. & Braun, C. (2014). The impact of UVR on reef fish – the good and the bad. Australian Coral Reef Society conference, Brisbane, Australia.

Publications included in this thesis

No publications included.

Contributions by others to the thesis

U. E. Siebeck was significantly involved in the conception and planning of experiments and design of the whole study, catching of reef fish for chapter 2 and helped with revisions and interpretations of all chapters.

R. Reef assisted with establishing the assay for the DNA damage analysis, provided resources and technical assistance as well as feedback and revisions for chapters 1, 2, 4 and 5. R. Reef also helped with measurements of light intensity in chapter 2 and 5.

N. Rosic provided the measurements of MAAs in algae and fish food in chapter 4 as well as discussion and feedback on the manuscript.

Y. Reshitnyk wrote and improved the MatLab script for the analysis of video data in chapter 3, and provided feedback and drafts for the method section of chapter 3.

A. Parker, C. Newport and S. Van-Eyk assisted in aquarium maintenance and fish keeping, as well as discussions and feedback on this thesis.

J. Leis donated larvae for chapters 2 and 5, and gave feedback and discussion on chapter 1.

S. Blomberg provided assistance in analysing the data of chapters 3 and 4.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

Uli Siebeck and Ruth Reef, I am more than thankful for the help and assistance you have given me in the last years. You have trusted me to work independently, had always an open ear for questions and gave me the feedback and motivation I needed. Thank you!

A very special thank you must go out to Jeff Leis, without whose help and kind donation of specimens and equipment this study would not have been possible. I count myself lucky that I have had the opportunity to work with you and have learnt a great deal about larvae, fieldwork and life.

The directors as well as past and present staff of the Lizard Island Research Station: Anne Hoggett and Lyle Vail, Marianne and Lance Pearce, Cassy Thompson and Bruce Stewart, thank you for your support, patience, help in the workshop, hospitality, walks up Mt Cook, afternoon fishing trips and always giving your best no matter what the circumstance or weather to make sure the that field trips are still my favourite part of my work.

Jack O'Connor, Viktor Nilsson, Simone Bosshard, Nicola Fraser, Johanna Werminghausen, Alex Vail, Katia Nicolet, Gay Marsden, Amanda Hay and Andrea Schuessler; a massive thank you. I would not have kept on going, finish this project and keep my sanity without your help. Thank you for motivating me, getting me out of bed at sunrise, listening and talking to me, helping me to find solutions for tricky aquarium problems, boat rides, chasing fish and sometimes catching them, BBQs, late night walks across Lizard and for simply being awesome human beings.

My family at the opposite end of the globe for their love and support, encouraging me to pursue my ambitions and for being always only a phone call away.

My colleagues, labmates and friends, Cait Newport, Amira Parker, Sarah Van-Eyk and Yarema Reshitnyk for looking after my fish no matter how many times I had to ask. Thank you for being there for a laugh, coffee and banana bread, the many discussions about work and life and afternoons at the Red Room.

At the School of Biomedical Sciences, I am grateful for the help from everyone at the workshop and Jon Zenta for designing custom contraptions of any kind. Adam and Nigel for keeping cool when field season meant another shipment of liquid nitrogen and everyone at the Finance department for your quick responses and patience in dealing with me. Fiona Gilloway, thank you for keeping an eye on deadlines and for the open door. It makes a real difference to be able to talk to someone in person when dealing with all things admin.

I would like to express my thank you to Cameron Veal, who kindly allowed me to use an adapted version of a figure from his PhD thesis. In addition, he taught me how to use power tools underwater without chopping off my finger, and was a great source of wisdom in discussions in my first year. Thanks!

I would also like to acknowledge the financial support I have received during my PhD from the UQ International Fellowship, the SeaWorld Research and Rescue Foundation and the Australian Coral Reef Society.

Keywords

ultraviolet radiation, DNA damage, cyclobutane pyrimidine dimer, reef fish, natural sunscreens, avoidance behaviour, repair mechanisms, baseline, enzyme-linked immunoassay

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060205, Marine and Estuarine Ecology, 60%

ANZSRC code: 060201, Behavioural Ecology, 20%

ANZSRC code: 060199, Biochemistry and Cell Biology not elsewhere classified, 20%

Fields of Research (FoR) Classification

FoR code: 0602, Ecology, 80%

FoR code: 0601, Biochemistry and Cell Biology, 20%

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LIST OF ABBREVIATIONS

Ultraviolet	UV
Ultraviolet radiation	UVR
Particulate organic matter	POM
Dissolved organic carbon	DOC
Coloured dissolved organic matter	CDOM
Cyclobutane-pyrimidine dimer	CPD
Pyrimidine (6-4) pyrimidone photoproduct	64 PP
Flavin-adenin dinucleotide	FADH-
Nucleotide-excision repair	NER
Base-excision repair	BER
Enzyme-linked immunosorbent assay	ELISA
Phosphate buffered saline	PBS
Boosted regression tree	BRT
Great Barrier Reef	GBR
Lizard Island Research Station	LIRS
Area under the curve	AUC
Mycosporine-like Amino Acid	MAA
Reactive oxygen species	ROS
UV absorbing compounds	UVAC

CHAPTER ONE

General Introduction

UVR on Earth's surface and in the marine environment

The spectrum of the sun consists of a wide array of radiation, including Gamma and X-rays (> 10 nm), Ultraviolet (UV) radiation (10 – 400 nm), visible light (400 – 700 nm), infrared (700 – 1000 nm), as well as radio- and microwaves (> 1 mm). UV radiation (UVR) is divided into three spectral regions (International Commission on Illumination, CIE): UVC (190-280 nm), UVB (280–315 nm) and UVA (315–400 nm). No UVC radiation reaches Earth's surface due to absorption by the ozone (O_3) layer and the atmosphere (Madronich et al. 1998), which also reduces the amount of UVA and UVB (Diaz et al. 2000). Changes in the dose and spectra of UVR reaching Earth were critical for the development of early life on Earth (Cockell 1998), when UVC and some UVB were blocked from reaching the surface of the Earth by the forming atmosphere and ozone layer. In the tropics (0°- 30° latitude) today, more UVR reaches the Earth than in temperate regions, due to i) a lower zenith angle and with that a shorter light path, which in turn leads to less attenuation (Frederick et al. 1989) and ii) a thinner ozone layer (Baker et al. 1980, Fleischmann 1989, Banaszak & Lesser 2009 and references therein). Hence, tropical regions experience the highest doses of UVR (McKenzie et al. 2003) and overall solar radiation (Wild et al. 2005) on the planet, even when compared to UV values experienced in regions close to the Antarctic during ozone hole times (Diaz et al. 2001). UVR levels in the tropics show relatively small seasonal variations compared to temperate regions, but remain strongly affected by cloud cover and pollution (Whitehead et al. 2000). The reduction in ozone layer thickness and the resulting increase of UVR reaching Earth are well documented in polar regions (Madronich et al. 1998) and were not thought to affect tropical regions. However, long term data show that both ozone levels (Randel 2007) and cloud cover (Masiri et al. 2008) are decreasing in the tropics. This is reflected by a recent model predicting increasing UVR levels in the tropics (Watanabe et al. 2011).

Radiation that hits the surface of the world's oceans will either be reflected, or will penetrate the water column to a certain depth. Depending on its wavelength (Jerlov 1976), light is attenuated due to absorption and scattering processes. This attenuation is governed by the amount of particulate organic matter (POM), dissolved organic carbon (DOC) and coloured dissolved matter (CDOM) in the water, which greatly varies depending on many parameters including geographic location, season and distance from shore (Booth & Morrow 1997). The

attenuation of light with depth is described by the diffuse attenuation coefficient K_d (Kirk 1994). Together with the depth at which 10% of surface UV radiation is still present ($Z_{10\%}$ UVR), the K_d value is an important tool used to characterize the amount of UVR at a given location. In general, UVR is attenuated faster than blue or green light, and UVB radiation disappears from the water column faster than UVA (Hargreaves 2003), as indicated by increasing K_d values with decreasing wavelength in the UV (Fig. 1.1). Biologically significant amounts of UVB penetrate the water to depths of at least 20 m (Dunne & Brown 1996, Vasilkov et al. 2001, Tedetti & Sempéré 2006). At the Great Barrier Reef (GBR) off the east coast of Australia, clearer waters are found further offshore, with northern regions characterized by lower K_d values for UV and visible light than southern regions and lagoons (Veal 2011) and hence UVR penetrates deeper into the water column.

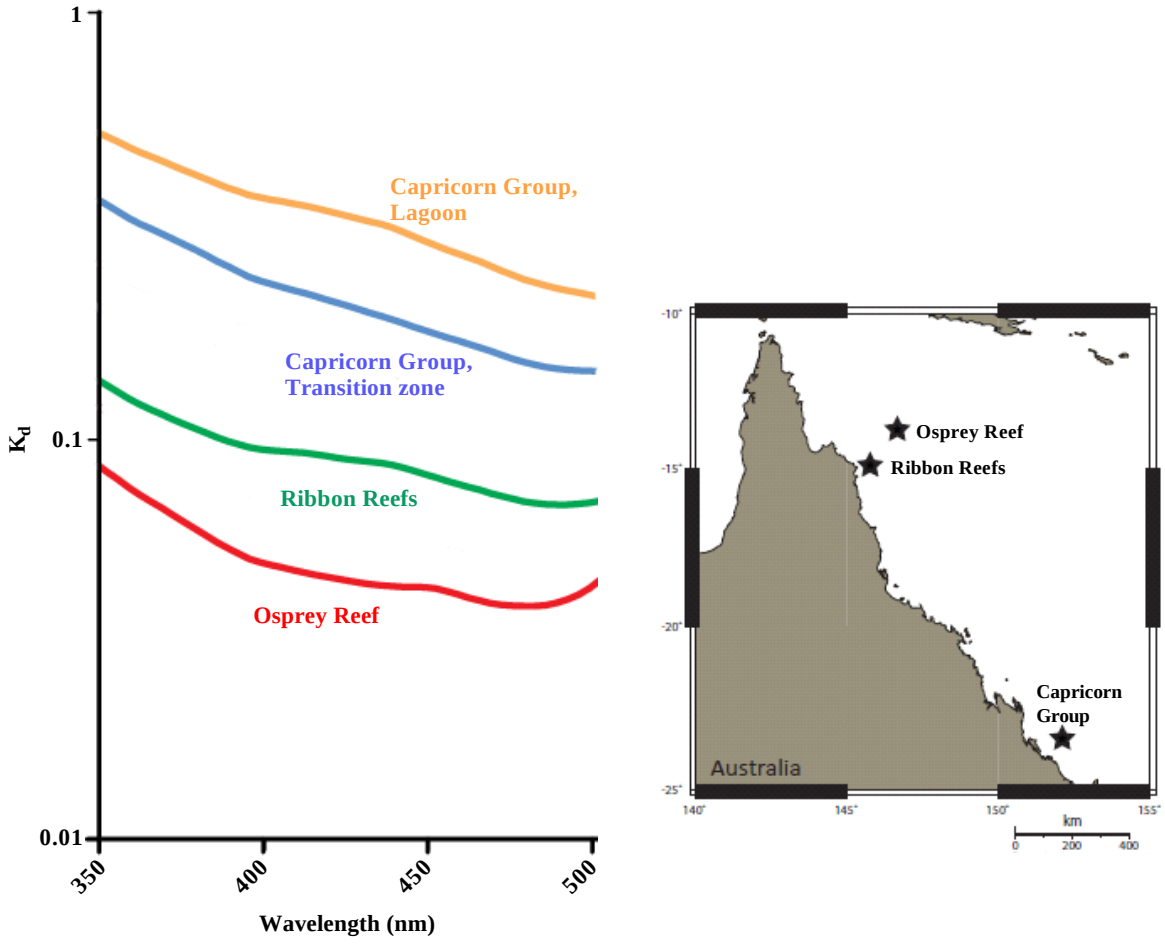


Figure 1.1: The diffuse attenuation coefficient (K_d) as a function of wavelength computed for four locations in the Great Barrier Reef. Clearer water is indicated by lower K_d values in northern and offshore locations. Adapted with kind permission from Veal (2011). Note the y-axis is on a logarithmic scale.

UV-induced DNA damage

Exposure to the high energy contained in short wavelength UV radiation is deleterious to biological systems in a multitude of forms: it affects the structures of living cells by damaging proteins and lipids in membranes and chromatophores, and impedes the function of vital processes such as photosynthesis and the replication of cells (Häder & Sinha 2005). Since UVR affects the most basal of biological processes, UVR sensitivity is universal to all living organisms and has been a major forcing variable in the evolution of life on Earth. For example, the significance and severity of UVB as an environmental stressor has been extensively studied in amphibians. Here, the negative effects include reduced survival and growth (Alton et al. 2010), increased susceptibility to disease (Kiesecker & Blaustein 1995) and behavioural changes (Kats et al. 2000). Together with anthropogenic and natural stressors, current and increasing levels of UVB radiation are thought to be responsible for the large scale decline of amphibians across the globe (Bancroft et al. 2008).

One of the most severe effects of UVR stem from the direct absorption of UVR by the DNA of living cells, which absorbs strongest in the short, high energy wavelength spectrum, with an absorption maximum at 260 nm (de Gruijl 2000).The absorption of a UVB photon leads to the dimerisation of adjacent pyrimidine (Cytosin (C) and Thymin (T)) bases (Setlow 1962). The primary form of such dimers are cyclobutane pyrimidine dimers (CPDs, Fig. 1.2a), which generally account for up to 80% of the lesions (Lo et al. 2005), and are characterized by a cyclobutyl ring between the 5' and 6' carbons of adjacent pyrimidine bases (either TT or TC). The effect of the formation of CPDs is often described as "nicks" or "bends" (Wang & Taylor 1991, Kim et al. 1995) in the DNA. CPDs are formed exclusively by UVR, specifically UVB, making them an ideal target to study the effects of this radiation. The second most common form of UV induced DNA damage are the pyrimidine (6-4) pyrimidone photoproducts (64 PPs, Fig. 1.2b), which form between the 6' and 4' carbons of neighbouring C and T bases after exposure to wavelengths > 290 nm and accounted for roughly 20% of UV-induced DNA damage quantified by Lo et al. (2005). The transcription of the DNA sequence by RNA polymerase II stalls at damaged sites (Mitchell et al. 1989) and blocks the transcription of genes, hence interfering with replication of cells (Protić-Sabljić & Kraemer 1985). Unrepaired, UVB-induced DNA damage ultimately leads to mutations through the insertion of incorrect nucleotides at damaged sites by low fidelity polymerases (Friedberg 2003) and eventually apoptosis if the mutation load is too high or in a vital gene (Browman et al. 2003, Lo et al. 2005). At sites where polymerase stalls, higher torsion can lead to strand breaks

(Karentz 2014). The prevention of replication due to double-strand breaks at UV-damaged sites in the DNA is also seen as a major trigger for apoptosis (Batista et al. 2009).

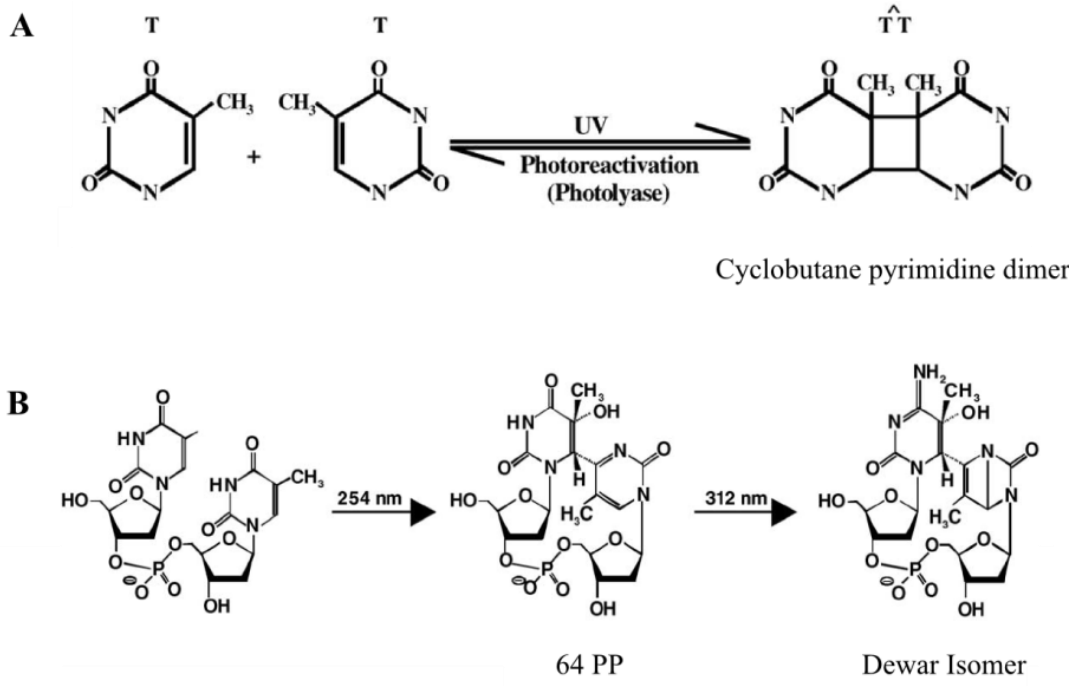


Figure 1.2: Structures of the two major types of DNA lesions that form upon the absorption of photons in the UV range: A) CPD lesion between two Thymine bases, and B) 64 PP and its' Dewar Isomer, also between Thymine bases. Modified from Häder and Sinha (2005).

UVR-specific DNA damage has been well documented in marine zooplankton and invertebrates (Häder et al. 2007, Banaszak & Lesser 2009, Lamare et al. 2011). UVR in the marine environment is shaping the distribution of organisms along a vertical gradient (Halac et al. 2014) and in turn drives the evolution of tolerance mechanisms in anthozoans (Siebeck 1988). DNA damage (Buma et al. 2001) and even cell death (Agustí et al. 2010) can be detected along a depth- and therefore dose gradient in Antarctic phytoplankton and Mediterranean cyanobacteria. The general consensus is that UVR-specific DNA damage is dose dependent (Malloy et al. 1997, Buma et al. 2003) and thus decreases with water depth and increases with length of exposure (Frederick et al. 1989, Jeffrey et al. 1996). A similar correlation of lower DNA damage with increasing sampling depth as well as diel patterns of UV damage have been found in bacterioplankton from tropical waters (Jeffrey et al. 1996), demonstrating the global impact of UVR on the smallest marine inhabitants. Even current ambient levels of UVR can lead to mortality in exposed zooplankton (Al-Aidaroos et al.

2014). Invertebrates, especially echinoderms, have been the subject to a number of studies detailing the impact of DNA damage and oxidative damage on their development, growth and survival as well as their protection mechanisms (Lamare et al. 2011). Similar to what was found for planktonic organisms, increases in UV dose lead to increased DNA damage in echinoderms, but also to the induction of repair mechanisms and external protection. Geographically, tropical species are at higher risk of DNA damage than their temperate or polar counterparts (Lamare et al. 2007).

In addition to DNA dimers caused by UVB radiation, the main focus of this thesis, exposed organisms face a number of other forms of UV damage, such as the formation of reactive oxygen species (ROS). These radical forms of oxygen, e.g. hydrogen peroxide (H_2O_2) and superoxide radicals ($O_2^{-\bullet}$), are formed by UVA directly or through intermediate molecules and have the potential to damage DNA (reviewed by Banaszak & Lesser (2009)). The result of oxidative damage ranges from increased metabolism rates and activity of protective enzymes in zooxanthellae (Lesser et al. 1990), to reduced photosynthesis rates of zooxanthellae in their coral hosts (Lesser 1996) to depth dependent DNA damage in larvae of sea urchin (Lesser 2010). Interestingly, due to the higher prevalence and lower attenuation of UVA compared to UVB, the amount of DNA damage related to UVA was higher than UVB-induced dimers. This evidence highlights the need to not discount other forms of DNA damage than CPDs. The scope of this thesis however, lies on the establishment of methods to quantify levels of UVB induced CPDs in reef fish and to explore the efficiency of protection mechanisms against only one type of damage.

Only a few studies have investigated natural damage levels in fish. Malloy (1997) found a correlation between UVB dose and CPD levels in eggs, but not in larvae of the ice fish *Chaenocephalus aceratus*. Vetter (1999) detected diel cycles of DNA damage in northern anchovy (*Engraulis mordax*) eggs and larvae under attenuated UVR, however also noted a high mortality under full solar radiation. However, no extensive studies on natural damage levels for reef fish of any developmental stage exist to date. The only study examining a reef fish (Eckes 2009) conclusively showed that *Thalassoma lunare*, (Labridae), has the ability to repair DNA damage and that the number of apoptotic cells, the endpoint of DNA damage, was significantly reduced under *experimental* conditions that lacked UVB radiation. The level of DNA damage that was induced in this study was several times higher than the damage recorded at the beginning of the experiment, and it is unknown, whether this relatively lower damage level represents the *in situ* conditions accurately. The lack of knowledge on naturally

occurring damage levels of reef fish, and the interplay between damage levels and protection mechanisms that counteract or limit DNA damage is unsatisfactory and need to be studied to understand the effects of UVR on reef fish biology and ecology. Results from studies examining UV damage and the levels of relevant UV protection in corals (Shinzato et al. 2011a) and anemones (Cubillos et al. 2014) indicate that these processes are dynamic both on short term and long time scales and to a certain degree adaptive. The results summarized in this thesis will add significantly to the growing body of understanding of the effects of UV radiation.

Benefits of UV exposure, UV vision and communication

Exposure to the incident UVR has shaped the evolution of life on Earth (Rothschild 1999), leading to a wide range of complex adaptations. Not only does UVR lead to negative effects such as DNA damage, UVR can also play significant roles by influencing predator-prey relationships, herbivory and the productivity of ecosystems as a whole (Caldwell et al. 1998, Paul & Gwynn-Jones 2003, Häder et al. 2007). Plants utilize UVR to generate pigments (Cashmore 1997) and increase resistance to pathogens and herbivores (Bornman et al. 2015). Additionally, UVR exposure can reduce the activity of human-specific bacteriophages (Diston et al. 2014) and decreases parasitism (Overholt et al. 2011) in aquatic organisms. In humans, UVR exposure increases the production of the neurotransmitters serotonin and melatonin (Mead 2008) and is needed to produce Vitamin D (Holick 1989, 2003, Holick 2004), a lack of which can lead to reduced bone growth and Rickett's disease.

UVR, particularly UVA, is used for behaviours such as foraging and visual communication. Visual sensitivity to UVA light has been demonstrated in a wide range of terrestrial (Tovée 1995) and aquatic (Losey et al. 1999, Leech & Johnsen 2003) animals. Not surprisingly, the use of UV wavelengths in animal behaviours are incredibly diverse and range from honeybees that use reflective patterns on flowers to find their targets (Johnson & Andersson 2002) to birds of prey that home in on the UV reflecting urine of their prey (Probst et al. 2002). UV vision is present in a large number of species of reef fish (Siebeck & Marshall 2001, 2007) from different lifestages (Siebeck & Marshall 2007). In Pomacentrids, UV- reflecting signals have been shown to be important for a number of behaviours, such as territorial interactions and species recognition (Siebeck 2004, Siebeck et al. 2010, Siebeck 2014), and contrast enhancement in the UV range is used in prey detection (Job & Bellwood 2007). These reef fish belong to the most common inhabitants of coral reefs, and the importance of

Pomacentrids as keystone species is recognized widely (e.g. Hixon (1983)). However, in order to see and signal in UVR, light must be able to pass through the eye and skin tissue and could thus cause significant damage to the DNA and other cellular components. It is currently unknown what costs are involved in UV vision and UV signalling, i.e. in having UV transparent ocular media and external mucus layers.

Adaptations to harmful UVR

Avoidance

Behavioural avoidance of biologically effective levels of UVR is the easiest possibility to minimize UV-induced damage (Roy 2000): (i) it prevents the need for costly repair of damage and (ii) does not involve the uptake and maintenance of sunscreens in the mucus (see below), since no damage occurs in the first place. Basal avoidance behaviour such as migration to deeper habitats in microbial and archaebacterial mats was necessary in the evolution of life (Cockell 1998, Rothschild 1999). Many invertebrates, such as phytoplankton and microbes show negative phototaxis to high light fluxes and therefore higher levels of UVR (Bebout & Garcia-Pichel 1995, Alonso et al. 2004, Gleason et al. 2006, Halac et al.) or display covering behaviours (Dumont et al. 2007).

While there is evidence for UVR avoidance behaviour in freshwater fish (e.g. preference for UVR-reduced treatment in salmon (Kelly & Bothwell 2002), nest site depths correlate with UVR levels in yellow perch (Williamson et al. 1997) and many other aquatic organisms (reviewed by Leech and Johnsen (2003)), the UV avoidance abilities of reef fish have not been described in greater detail. A prerequisite for UV avoidance is to be able to sense it e.g. by UV vision, which is common in pomacentrids such as *Pomacentrus amboinensis* (Siebeck & Marshall 2001). Fish photoreceptors may be maximally sensitive to the UVA, blue and green parts of the spectrum, however secondary maxima (beta-bands) of these photoreceptors exist that absorb UVB (Siebeck 2013) and may trigger an avoidance response. The limiting factor is whether the ocular media (cornea, lens and humours) are UVB transparent (Siebeck & Marshall 2001). In most pomacentrids, the ocular media transmit all wavelengths above 310 nm. Avoidance behaviour to current or elevated UVR levels has not been tested in reef fish yet, although the main prerequisite for the existence of such behaviour is fulfilled.

Protection

Protection from harmful UVR in marine organisms can arise from physical barriers and from UV-absorbing compounds, e.g. carotenoids (Mathews-Roth 1997) or Mycosporine-like amino acids (Shick & Dunlap 2002). 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 (Karentz 2001). These compounds cannot be synthesized by metazoans and are taken up through their diet (Bentley 1990). In teleosts fish, MAAs are found in eggs (Plack 1981), larvae (Lesser et al. 2001) and in the ocular media and external mucus of reef fish (Dunlap et al. 1989, Eckes et al. 2008). The efficacy of these natural sunscreens in preventing UV- induced DNA damage has not directly been shown (Bandaranayake 1998). However, cleavage delay in sea urchin larvae with reduced MAA levels seem to confirm MAAs as true sunscreens, as does the prevention of sunburn in mice, where MAAs where applied topically (de la Coba et al. 2009). There is evidence that MAAs act as external sunscreens, since their concentration in the mucus correlates with UV levels in the fishes' habitat (Zamzow 2007, Zamzow et al. 2013) and is strongly reduced under UV-lacking conditions (Zamzow 2004). Similar relationships from invertebrates (Banaszak et al. 1998, Lamare et al. 2004) also strongly suggest a protective function of MAAs against the negative effects of UVR. Fish that have UV vision/communication would need to reduce their MAA content in order to send and receive their signals, which could explain differences in mucus absorbance between a pomacentrid and a labrid species (Zamzow & Siebeck 2006). A direct link between UV-induced DNA damage and MAA levels has not been established to date.

Repair

Seen as the last line of defence, repair of damaged DNA is achieved by either restoring the original binding between bases, or replacement of damaged nucleotides. The process of restoring damaged sites is called photoreactivation (Kelner 1949). This process is facilitated by photolyases, a class of enzymes found in terrestrial and marine organisms ranging from archae (Shirley 1996) to most vertebrates (Thoma 1999, Sinha & Hader 2002), but not placental mammals (Ley 1993, Yasui 1994). Photolyase repairs damaged DNA by using UVA and blue light photons as energy donors to activate a cofactor (flavin-adenin dinucleotide, $FADH^-$) which splits the CPD or 6-4PP and returns the bases to their normal conformation (Sancar 2003). Specific photolyases exist for CPDs and 64 PPs, and repair rates for CPDs are generally higher than for 6-4PPs (Mitchell et al. 2001). Two other ways to repair damaged

DNA, nucleotide-excision repair (NER) and base-excision repair (BER) are often summarized under the term "dark repair", since no light is needed to activate the repair mechanism (Bootsma & Hoeijmakers 1996). Here, the damaged site is excised (an oligonucleotide for NER and a single base for BER), and undamaged bases are inserted. These repair processes are highly conserved in eukaryotes and involves a complex machinery of more than 30 proteins (Häder & Sinha 2005). As with the other defence mechanisms, our knowledge of reef fishes' ability to repair DNA damage via photolyases or dark repair is limited. The only evidence comes again from Eckes (2009), who showed a significant decrease in the number of dead cells in tissue of UVB irradiated fish that had been exposed to photoreactivating light compared to fish deprived of these wavelengths. Similar results from polar and temperate species (Regan et al. 1982, Malloy et al. 1997, Vetter et al. 1999) and freshwater fish (Ahmed & Setlow 1993, Uchida et al. 1995, Meador et al. 2000, Zeng 2009, Mitchell et al. 2014), show the importance of the photolyase and dark repair systems across habitats with extremely different radiation characteristics.

Significance and aims

As coral reef fish live in a UV rich environment, they have had to evolve protection mechanisms against damaging UVR, but some also exploit this part of the suns' spectrum for visual communication despite the risk of damage. Recently, changes have been observed in stratospheric ozone (Wild et al. 2005), pH and phytoplankton levels (Hoegh-Guldberg & Bruno 2010), and water clarity is possibly increasing through ocean acidification (Williamson & Zagarese 2003). In addition, a changing climate leads to habitat loss due to declines in coral cover and structure complexity (Hoegh-Guldberg et al. 2007). As a consequence coral reef organisms are increasingly more exposed to UVR (Harborne 2013). It is currently unknown however, how reef fish may be able to cope with changing levels of UVR exposure, or how plastic their protection mechanisms are in response to different environmental conditions. Recent evidence however, suggests that the effects of climate change can act in a synergistic fashion with UVB in fish (Cramp et al. 2014) and bacteria alike (Li et al. 2014). Despite the extensive literature on the consequences of exposure to UVR and protection mechanisms in other animals and plants, our knowledge about these important issues is limited in reef fish. UV-induced DNA damage and eventual risk factors such as depth of habitat or developmental stage have not been measured and evaluated in reef fish, and even though fish with UV vision and communication have been the subject of several studies, it is unknown whether these fish have the ability to detect and avoid harmful levels of UVR. DNA repair mechanisms have

been studied in coral reef organisms but not teleost fish from these habitats. Although the presence of MAAs has been comparatively well characterized in reef fish, the potential of these compounds to protect against UV-induced DNA damage is unknown.

The overall aim of this thesis is the measurement of the impact of ambient and elevated levels of UVR on reef fish in terms of DNA damage (CPDs), and to evaluate the protective mechanisms available to fish with and without UV vision. Further, this study aims to measure UV-induced DNA damage in reef fish from the Great Barrier Reef as it occurs *in situ*, thus providing an understanding of the effectiveness of protection mechanisms that are available for reef fish. In chapter two, this "net" DNA damage, or residual level, is characterized in species with different lifestyles, life histories and different visual abilities, i.e. fish with UV vision and without, therefore covering a wide range of species. Early developmental phases, i.e. settlement stage larvae are included to assess whether net DNA damage in younger fish is different from older individuals. Sampling across depth and exposure gradients will reveal whether a dose effect exists in coral reef fish. Overall, it is hypothesized that increased UV exposure leads to higher levels of UV-induced DNA damage.

Further, the presence and efficiency of each of the three protection mechanisms, DNA repair, UV avoidance and protection using MAAs, is examined in separate chapters, using representative species that vary in their visual abilities and therefore are hypothesized to use different strategies to achieve UV protection. Chapter three examines the ability of two species of reef fish, *P. amboinensis* and *Thalassoma lunare*, to behaviourally avoid exposure to harmful levels of UVB radiation in an experimental arena. Additionally, the impact of UVB radiation on the foraging behaviour of settlement-stage larvae of *P. amboinensis* is investigated. It is hypothesized that i) fish with UV vision such as *P. amboinensis* are able to actively avoid areas of UVB radiation in contrast to fish without UV vision, i.e. *T. lunare*; and ii) that the foraging activity of P. amboinensis would be reduced under UVB irradiation compared to light conditions which lacked UVR. In chapter four, the effectiveness of MAAs in preventing UV-induced DNA damage is tested in *P. amboinensis* and *T. lunare* by exposing fish with varying MAA content to a UVB pulse that led to the formation of CPDs. This enabled to test the hypothesized sunscreen function of MAAs, specifically that a higher concentration of MAAs in the external mucus layer of reef fish results in less DNA damage. Concluding the assessment of the three commonly hypothesized protection mechanisms, the fifth chapter examines the ability of settlement stage larvae of reef fish to repair UV-induced DNA damage via photorepair and dark repair. It is hypothesized that the accumulated CPDs

are removed faster using the light activated mechanism of photorepair, whereas dark repair would be less efficient in removing these dimers. Chapter six concludes this thesis with a summary of the main findings and a synthesis of the efficiency of the protection mechanisms in fish with and without UV vision. The results are discussed also in terms of climate change and an outlook for future studies is presented.

This study is the first to address these questions in coral reef fish from the Great Barrier Reef and take into account present as well as elevated UVR levels, which could affect this area that is of great natural and economic value.

CHAPTER TWO

A baseline for UV-induced DNA damage in fish from the Great Barrier Reef

Abstract

Ultraviolet radiation (UVR) induces DNA damage and can severely impact the development and behaviour of exposed organisms. In order to make predictions about the impact of UVR on animal populations in the future, it is important to be aware of the current levels of DNA damage. The aim of the study was to determine the net level of DNA damage present in reef fish exposed to natural levels of UVR. DNA was isolated from skin and tissue samples from a total of fifteen species of reef fish collected from the reefs surrounding Lizard Island on the Great Barrier Reef, Australia. Using CPD-specific antibodies in an enzyme-linked immunosorbent assay (ELISA), DNA damage was detected in fish from different life stages and life histories. Additionally, the influence of size, UVR exposure time and depth were studied in detail in two species of Pomacentrids. Analysis using a boosted regression tree shows that the most important factors governing UV induced DNA damage in reef fish are species and size, whereas other factors such as family, the depth where the fish were caught and the presence or absence of UV vision contribute the least to the variation in DNA damage. The length of exposure to natural levels of UVR over the course of a day was found to have no significant influence on net DNA damage levels in *Pomacentrus amboinensis*, as did the depth where the closely related *P. moluccensis* were caught. On the contrary, significantly higher levels of DNA damage were found in smaller *P. moluccensis* than in larger conspecifics. Overall, DNA damage levels were relatively low *in situ*, compared to DNA damage levels that were detected in later experiments using elevated UV levels.

Introduction

Fish are exposed to multiple environmental stressors that have the capacity to inflict DNA damage (Kurelec et al. 1989, Reichert et al. 1998, van der Oost et al. 2003, Häder & Sinha 2005, Valavanidis et al. 2006). Ultraviolet radiation (280 - 400 nm wavelength, UVR) has been recognized as one of the most significant evolutionary drivers in the marine environment (Cockell 1998) and is responsible for the formation of cyclobutane pyrimidine dimers (CPDs) in the DNA of exposed organisms. CPDs are a major contributor to the negative effects of UVR exposure, which include stalled transcription and replication of DNA(Protić-Sabljić & Kraemer 1985, Lesser et al. 2001), the arrest of the cell cycle and the induction of apoptosis (Sinha & Häder 2014). The quantification of CPDs using a variety of techniques (Gel fragmentation, RIA, ELISA) has been used in numerous studies in laboratory experiments as well as *in situ* studies in fish (Applegate & Ley 1988, Armstrong et al. 2002, Mitchell et al. 2014) and has proven to be a powerful method to assess the severity and degree of UV damage.

In the tropics, where levels of UVR are higher than in temperate regions, the effects of UVR exposure and DNA damage, which are known to be dose dependent in tropical and polar invertebrates (Lamare et al. 2007), could be severe due to the accumulated UVR dose. The exposure to UVR has been shown to severely affect swimming abilities, alter the behaviour and increase mortality in juvenile fish (Leech & Johnsen 2003). Besides effects on individuals, UVR exposure and its consequent negative effects has the potential to alter plankton composition and disrupt primary production (Häder & Sinha 2005) at an ecosystem level.

The use of thick protective shells in benthic organisms and natural sunscreens that block UVR in marine animals from diverse classes such as anthozoans, echinoderms and teleosts attest to the need of protection from UV-induced DNA damage on a coral reef (Eckes et al. 2008, Lamare et al. 2011, Rosic & Dove 2011). Two other UV protection mechanisms, DNA damage repair via photoreactivation and behavioural avoidance, are commonly hypothesized to exist in marine organisms including reef fish (Buma et al. 2003, Leech & Johnsen 2003). Taken together, the general notion in the literature (e.g. Leech (2003)) is that reef fish should be well protected due to their evolutional history in the tropics. However, recently, the occurrence of melanoma in coral trout (Sweet et al. 2012), an ecological and commercial key species (Harrison et al. 2012), has been linked to UVR after a range of other pathogens were

ruled out. It therefore appears that fish are vulnerable to UVR despite the various protection mechanisms.

In order to make an appropriate statement about the levels of DNA damage in reef fish several risk factors that could affect the residual or background level of damage *in situ*, i.e. the net amount of DNA damage in reef fish under consideration of all possible protection mechanisms (protection, DNA repair and avoidance), need to be considered. Water depth where fish have their habitat is an obvious factor due to the attenuation of UVR (Buma et al. 2001, Ylönen et al. 2005, Olson et al. 2006). Size and age are a key factor in amphibians and fish (Bancroft et al. 2007, 2008), with early developmental stages being usually at a higher risk of DNA damage. At this critical time, the larvae of many reef fish species undergo a pelagic phase and spend a significant amount of time at a shallow depth (< 20 meters (Leis 2004)), therefore being exposed to high levels of UVR (Jerlov 1976). Additionally, many Pomacentridae have UV transparent ocular media (Siebeck & Marshall 2001) at an early life stage, and less protection using MAA sunscreens (Zamzow & Losey 2002) than other reef fish such as Labridae. These two factors could result in higher levels of DNA damage in species that use UV signals in order to communicate with conspecifics (Siebeck 2004). UV-induced DNA damage has further been shown to be dose dependant (Malloy et al. 1997, Lamare et al. 2007), often fluctuating between relatively high and low levels in a diel manner, indicating the presence of repair mechanisms. The accumulation of net DNA damage over time however (Mitchell et al. 2009), shows that sunscreens, DNA repair and behavioural avoidance are not able to provide complete protection from UVR. Considering the high amount of UVR that reaches coral reefs (Dunne & Brown 1996) compared to temperate regions, it is surprising to see that UV-induced DNA damage and its impacts on coral reef fish has been addressed only marginally in a small number of studies (Zamzow 2004, Sweet et al. 2012).

This study presents the first measurements of relative levels of CPDs in 15 species of reef fish from the Great Barrier Reef (GBR). The following work acts as a valuable and comprehensive baseline of UV-induced DNA damage in the light of possible increases of UVR at coral reefs due to climate change (Vasilkov et al. 2001, Watanabe et al. 2011) and the loss of habitat complexity (Munday et al. 2008). Species included Pomacentrids which use UV signals in visual communication as well as wrasses, which are known to have a high amount of UV absorbing compounds in their external mucus. Additionally, fish like Lethrinids, Lutjanids and larger Serranids (e.g. *Plectropomus leopardus*, coral trout) with a contrasting lifestyle

compared to the small meso-predators and omnivorous and planktivorous Damselfish were included in this survey. Early developmental stages of reef fish from light trap catches were also included in this study due to the potential for higher DNA damage in young fish. Further, the *in situ* CPD levels in *Pomacentrus moluccensis* along a depth and size gradient were included as well as the DNA damage that accumulated during exposure to natural UVR over one day in the closely related *Pomacentrus amboinensis*.

Methods

Study location

All fish were caught on the reefs surrounding Lizard Island (14°40'44.74"S; 145°26'46.25"E, Fig.2.1), situated approximately 30 kilometres offshore in the GBR, under permits to UES and CB (GBRMPA: G11/34453.1; General Fisheries Permit: 162472; Ethics committee of The University of Queensland: SBMS/091/11). Larvae from light trap catches were kindly donated by Dr. Jeff Leis of the Australian Museum, Sydney. The experiment for the day series of *P. amboinensis* was carried out at Lizard Island Research Station (LIRS) under Ethics Permit SBMS/091/11 to UES and CB.

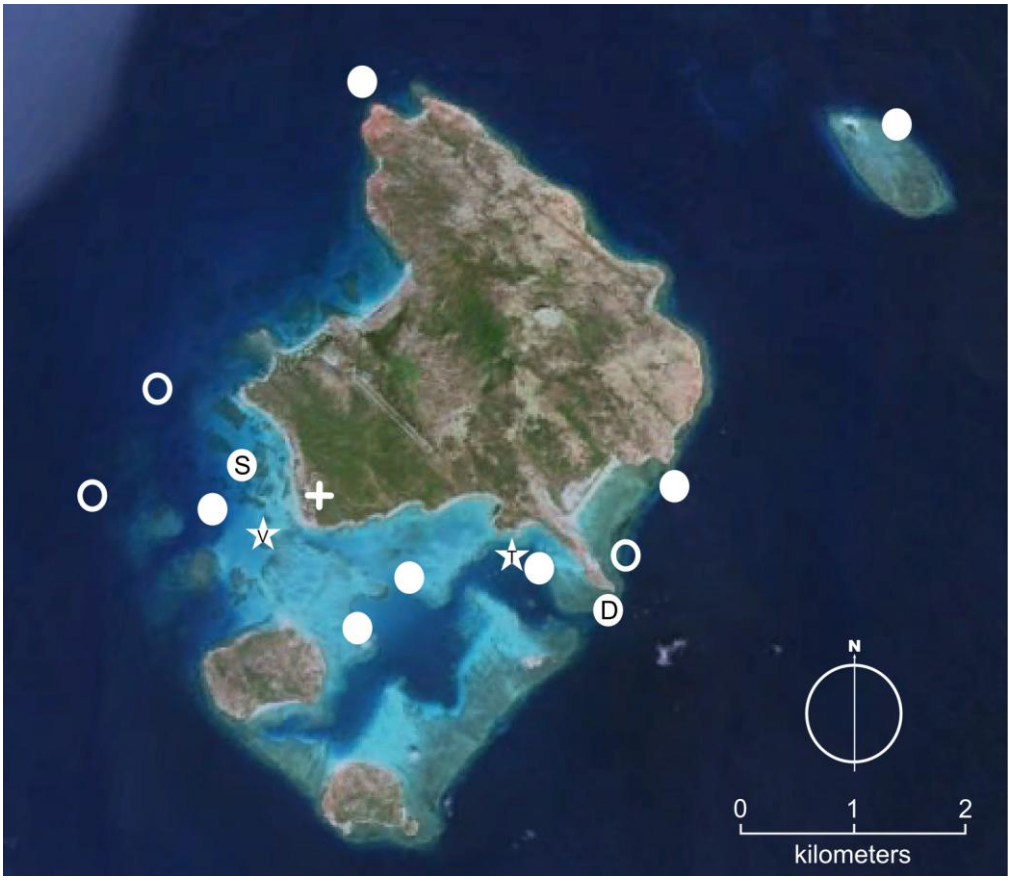


Figure 2.1: Satellite image of Lizard Island and surrounding reefs, upper right corner: MacGillivray Reef. The open circles represent the locations at which light traps were deployed, while the filled circles indicate the reefs where fish were caught using SCUBA and hook and line methods. The sites of the size and depth series are indicated by a filled circle and a letter (D = depth series, S = size series). Trawler Beach and Vicky's Reef are indicated by a white star and a letter (T = Trawler Beach, V = Vicky's Reef), where measurements of irradiance at different depths were made. The cross marks the position of Lizard Island Research Station. Image modified from GoogleEarth, © 2014 Digital Globe.

Baseline of in situ CPD levels

All fish for the measurement of *in situ* DNA damage were caught between November 2011 and April 2014 on several trips to LIRS (n = 4). Settlement stage larvae (see Table 2.1) were caught using light traps deployed overnight several hundred meters offshore from Lizard Island. Light traps were retrieved shortly after sunrise and whole light trap catches were brought back to the station in several buckets (10 l) with aerated seawater. Larvae were sampled from these catches within one hour after collection of the traps and euthanized in an ice-seawater slurry. Whole fish were packed between Whatman filter paper wrapped in aluminium foil and stored in liquid nitrogen. For transport back to the University of Queensland, the samples were transferred to dry shipper primed with liquid nitrogen and upon arrival stored at -80 °C for DNA damage analysis. Adult fish were either caught on SCUBA using hand or barrier nets or caught with hook and line (see Table 2.1 for details) and euthanized in an ice-seawater slurry. While some of the smaller fish (e.g. *Pomacentrus amboinensis*, *Labroides dimidiatus*) could be stored as a whole specimen similar to the settlement stage larvae, skin and tissue samples were cut from larger species like *Plectropomus leopardus* or *Thalassoma lunare* (see Fig. 2.2 and Table 2.1 for details). These samples were taken after euthanization from a dorsal area close to the head and were up to 21cm^2 in size for larger specimens like the Lutjanids and Lethrinids. Storage and transport of these samples was the same as with whole specimens. For the DNA damage analysis, the larger portions of skin and tissue were subsampled using a Harris UniCore (diameter 5 mm), which resulted in an area of skin that was not larger than those of the larvae, which were cut along a diagonal line (Fig. 2.2) to yield enough tissue.

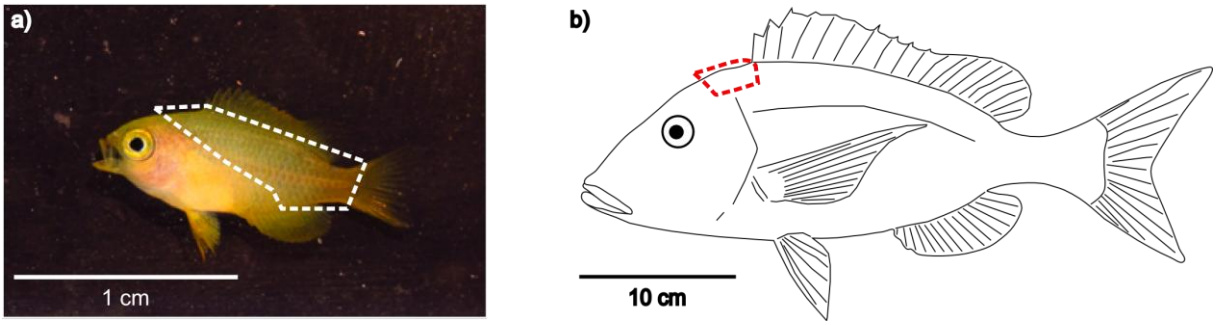


Figure 2.2: Schematic representation of body areas of fish that were sampled for CPD quantification. In a) settlement stage larvae, here *Pomacentrus moluccensis*, an area comprising most of the dorsal tissue, including ventral tissue behind the anus was used for DNA extraction and damage analysis. The fins and intestines were not used. b) In fish larger than settlement stage larvae, for example *Lethrinus nebulosus* (modified from Cada, L.A., www.fishbase.org), a section of skin and tissue was cut from a dorsal part of the body near the head and later subsampled using a tissue core.

Depth and size series in Pomacentrus moluccensis

For the depth (n = 30) and size (n = 39) series, *P. moluccensis* were collected from reefs at Lizard Island in January 2014 and April 2013, respectively. Specimens for the depth series were collected on SCUBA using hand nets and a clove oil solution (clove oil x ethanol x seawater 10 x 10 x 100) as anaesthesia and euthanized as described above. The standard length (SL) of the fish was 4.41 (+/- 0.87 StDev). These fish were caught from three different reefs, which were at a depth of 2 to 4 meters (n = 11, category "0 – 5 meters"), approximately 7 to 8 meters (n = 10, category "5 – 10 meters") and 12 to 15 meters depth (n = 9, category "> 10 meters"). These three reefs were separated from each other and neighbouring reefs. Given the territorial nature of *P. moluccensis* it can be assumed that the sampled fish were confined to their respective reefs. The fish caught from the shallowest depth (0 – 5 meters) were also included in the measurement of the baseline DNA damage. The fish for the size series were caught on another, detached shallow reef (< two meters) while snorkelling using hand nets and clove oil. The mean SL was 3.45 cm (1.503 cm StDev) ranging from 1.1 cm to a maximum size of 5.5 cm. The size of the fish in the three groups were: large group (>4 cm, n= 19): 4.78 cm, STDev: 0.39; medium group (2-4 cm, n=10): 2.8 cm, STDev: 0.64 cm; small group (0-2 cm, n=10): 1.15 cm, STDev: 0.085cm.

Dayseries of Pomacentrus amboinensis

In order to track the level of DNA damage over the course of a day, fish were exposed to natural solar UVR in experimental tanks at LIRS. Adult *P. amboinensis* (n = 30, SL: 4.41 cm +/- 1.01 cm StDev) were caught on SCUBA using hand nets (without clove oil) in March 2013. Fish were placed overnight in holding tanks (8 l volume) connected to the station's flow-through seawater system. Approximately one and a half hours before sunrise the morning following capture, the fish were transferred to the five experimental tanks (40 x 30 x 30 cm), with 6 fish per tank. These tanks were placed under open sky and connected to the same seawater system and were additionally aerated. Four of the five tanks were covered with a UV-transparent acrylic sheet (OP4, Acrylite) in order to expose the fish to UVR, while one tank was covered with UV-blocking acrylic (OP3, Acrylite), which acted as a control for UV-induced DNA damage. One individual was sampled from each tank at 0800, 1200, 1600, 2000, 2400 and 0400 (the next day) hours and euthanized as described above. Skin samples from the dorsal area just distal of the head were taken, frozen in liquid nitrogen and stored at -80°C until analysis of DNA damage.

UV measurements

The underwater UV spectrum (280 – 400 nm) was measured at Trawler Beach in the Lizard Island lagoon (Fig. 2.1) at 1 nm intervals, a site typical for the area with a shallow reef in one to three meters depth and a sloping sandy bottom to about ten meters depth. Additional measurements were made at a site near Vicky's reef, a shallow reef near the exit of the lagoon. Irradiance was measured using an OceanOptics USB2000 (OceanOptics, Dunedin, FL, U.S.A.) spectrometer in April 2013 on a sunny cloudless day around midday. The spectrometer was combined with a custom-made underwater housing and a one meter long fibre optic cable with a diameter of 200 µm and a cosine corrector (both OceanOptics). The spectrometer was calibrated against an OceanOptics DH-2000 lamp. Ten replicate measurements of downwelling irradiation were taken at the surface and at a depth of two and six meters. After subtraction of a dark measurement and averaging of the ten replicates, the attenuation coefficient K_d (Kirk 1994) at a given wavelength was calculated using the equation

$$(1) \qquad I_{d\lambda} = I_{0\lambda} e^{(-K_d)z}$$

where I_λ describes the irradiance at depth d and wavelength λ, $I_{0\lambda}$ stands for the irradiance at the surface and at wavelength λ, and z is the depth in the water column in meters. A low K_d indicates a high water clarity and less absorption at the given wavelengths. In turn, a high K_d indicates water of a lower clarity. In this case, the K_d is calculated per wavelength using the measurements above the surface and at two metres water depth. Together with the depth (D) at which 1% and 10% of surface UVR are still present, calculated with the equation

$$(2) \qquad D = \frac{\ln\left(\frac{I_D}{I_0}\right)}{-K_d}$$

where I_D/I_0 is either 0.1 or 0.01 and stands for 1% and 10 % of surface UVR, these two deductions are an important tool used to characterize the amount of UV radiation that can be found in a certain location. During the dayseries experiment, the incident UVB radiation was measured at the experimental tanks using a portable solarmeter (Solartech Inc., Harrison, MI, U.S.A., model 6.0) with a range from 280 – 322 nm and a peak response at 300 nm. The device has a resolution of 0.1 $mW*m^{-2}$ and takes three measurements each second. The meter was held clear from any obstruction (e.g. the researchers' head or body) and ten replicate measurements were taken in quick succession.

DNA damage analysis

In order to extract genomic DNA from all samples, a DNeasy® Blood and Tissue kit (Qiagen, Venlo, The Netherlands) was used according to the manufacturers' instructions, following the method of Mori (Mori et al. 1991). The still frozen larvae were cut along the area indicated in Figure 1a and immediately transferred into two ml of lysis buffer in order to prevent the degradation of DNA. The integrity of the DNA was visually inspected using gel electrophoresis and quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmingtion, DE, USA). For the quantification of CPD lesions only structurally intact DNA (i.e. one non-fragmented band on the gel) with an absorption coefficient 260/280 nm >1.8 was used. All samples were brought to a final concentration of 0.2 µg/ml diluted in 1 x phosphate buffered saline (PBS).

UV-induced DNA damage, i.e. CPD lesions, were detected in an enzyme-linked immunosorbent assay (ELISA) using the TDM-2 primary antibody (Cosmo Bio, Tokyo, Japan). All assays were performed on 96-well plates (TPP, Trasadingen, Switzerland; and Costar, Corning Inc., Corning, NY, U.S.A.) read out in a 96-well plate reader (Model 680,

Bio-Rad, Berkeley, CA, U.S.A.) at 492 nm. The mean values of triplicate samples of each fish were used as the statistical replicate. A standard curve (Fig. 2.3) generated from salmon testes DNA (170 µg/ml in 1xPBS, Sigma-Aldrich) was included in each assay and allowed for comparison between individual 96-well plates. This standard was generated by exposing the solution in a six-well plate to UVR (280-400 nm) emitted by two UVB lamps (2x GL20SE lamps, Sankyo Denki, Kanagawa, Japan, distance from well plate 12 cm) for up to 20 minutes. The lamps' total UVR dose was 23.5 $W*m^{-2}$, which was measured with a USB2000 spectrometer (OceanOptics, Dunedin, FL, U.S.A.) calibrated to an OceanOptics DH-2000 lamp. At the surface of the six-well plate, the dose of UVB (280 – 315 nm) irradiation was 0, 1.7, 4.2, 5.9, 8.4, 12.6 and 16.9 $kJ*m^{-2}$; and the dose of UVA (315 – 400 nm) irradiation was 0, 1.1, 2.8, 3.9, 5.6, 8.4 and 11.3 $kJ*m^{-2}$ at the respective intervals of 0, 2, 5, 7, 10, 15 and 20 minutes. The standard solution was then diluted in 1 x PBS to 0.2 µg/ml to match the concentration of the samples extracted from the fish. In addition to the triplicates of the DNA standard and the isolated DNA samples, a triplicate blank (1 x PBS) was included in all assays. After reading out the optical density of a plate, background fluorescence was excluded by subtracting the average blank OD values from the average OD values of standard and samples. The DNA damage levels were then expressed as a proportion of the average of the three highest values of the standard curve. For statistical analysis, the DNA damage was arcsine transformed to normalize the proportional data.

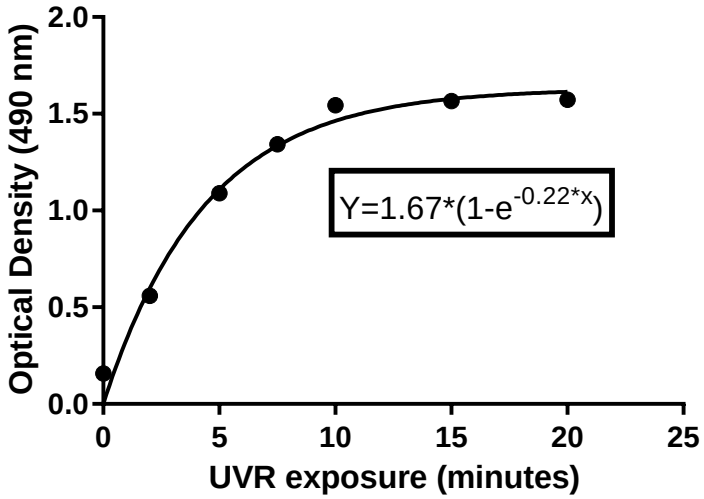


Figure 2.3: The standard curve for UV induced DNA damage (CPDs) generated by irradiating a DNA solution (from salmon testes) for up to 20 minutes with UVR. The average of the last three timepoints (10, 15 and 20 minutes) was used to normalize each sample.

Optical density measured at 490 nm (y axis) is plotted for each timepoint (x axis) that the DNA solution was exposed to UV radiation.

Statistical analysis

The results of the baseline measurements of CPD levels were not directly compared to each other or between different species due to the heterogeneity of the dataset, e.g. different sampling techniques, uneven sample size, different sampling dates and a high degree of uncertainty when considering movements of some fish in the water column. Therefore, the CPD levels presented here should rather be treated as reference for future studies. Nevertheless, a boosted regression tree (BRT, (Elith et al. 2008)) can incorporate these factors and make predictions about the relative influence of each factor on the measured variable (DNA damage) in a heterogeneous, relatively large dataset. BRT's are a predictive data mining tool, robust against outliers, missing values and different categories of data. A prediction of how much influence a certain factor (e.g. UV vision) has on the measured variable is achieved by splitting random subsamples of a dataset into a large number of very simple sequential decision trees. With each step that the model takes, the ability of the factors to predict the observation is improved and finally expressed as a percentage. In this study, the *gbm* model (Elith et al. 2008) with a learning rate of 0.01, a tree complexity of 5 and the predictors "Species", "Family", "size class", "depth class", "larvae or adult", and "UV vision/signalling" were used to generate 2700 trees with a total deviance of 0.015, resulting in a training correlation of 0.48. The analysis was performed using RStudio version 0.98.953 (RStudio 2014). Additionally, the mean DNA damage of the Baseline samples was compared to a theoretical value of 0 using a one-sample t-test to investigate whether the DNA damage was significantly higher than zero. The data of the depth-, size- and dayseries were analysed using non-parametric methods Kruskal-Wallis test with Dunn's test for multiple comparisons as well as linear regressions using GraphPad Prism 6.

Results

Baseline of in situ CPD levels

UV-induced DNA damage in the form of CPDs was detectable in all species examined (Table 1) and was significantly different from zero in 11 out of 15 species. The highest mean damage (0.076 +/- 0.084 StDev) in adult fish was found in *Lethrinus nebulosus*, while in settlement stage larvae *P. moluccensis* (0.109 +/- 0.006 StDev) had the highest damage. Variation among and within species was high (Fig. 2.4), while no particular pattern between species or families, or species with and without UV vision seems visible. Settlement stage larvae appeared to have higher DNA damage than adult fish. These trends are confirmed by the results of the BRT analysis, which describes the influence of the individual species on the net DNA damage as the single most influential factor with 73% (Fig. 2.5 and Table 2.2), while size class and lifestage (larvae or adult), two factors that are closely linked to each other, are the second and third most influential predictors of DNA damage. The depth at which the fish were caught (1.3%) did not play a large role influencing CPD levels, as did the family of the individuals (1.2%). Whether or not a species had UV vision (0.7%) had the least influence on net DNA damage in all fish sampled. Overall, levels of DNA damage were low, between 0. 5 and 10 percent of the DNA damage standard, with an overall mean of 0.045 (SD +/- 0.024), which represents just under 5 percent of the DNA damage standard. This level of damage is several times less than what was observed in adult and larval stage fish under artificial UVR exposure (chapter 4 and 5).

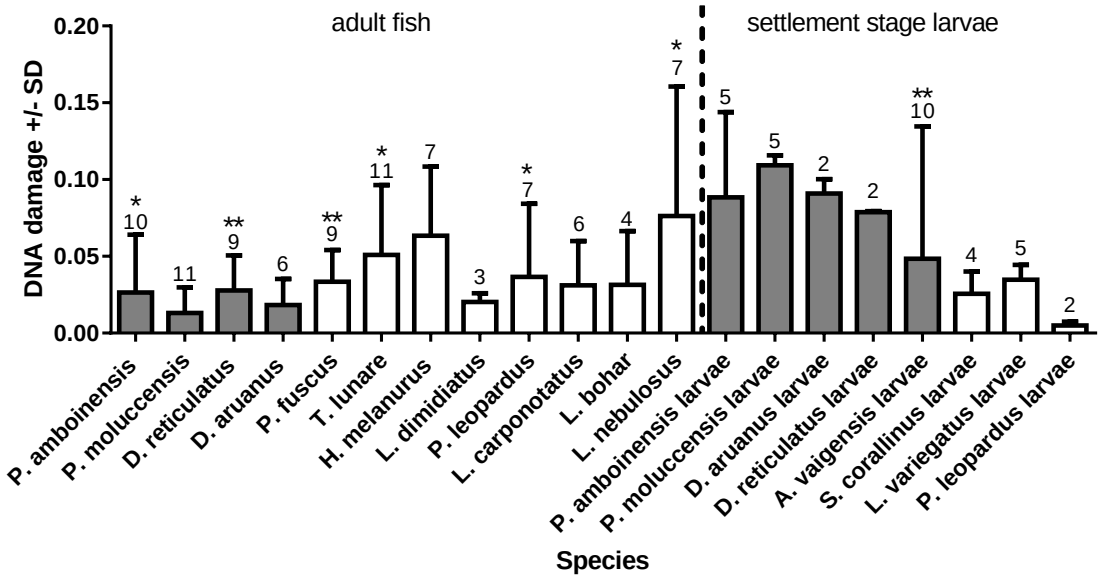


Figure 2.4: Levels of *in situ* CPD levels in fish from Lizard Island. Error bars indicate one standard deviation, while the numbers above the bars show the sample size for each species. Grey bars indicate a species with UV vision/signalling, open bars stand for species without UV vision/signalling. The asterisks above the bars indicate significant differences in DNA damage from zero: * = 0.05 > P > 0.01; ** = 0.01 > P > 0.001. Values on y-axis have been logit- transformed.

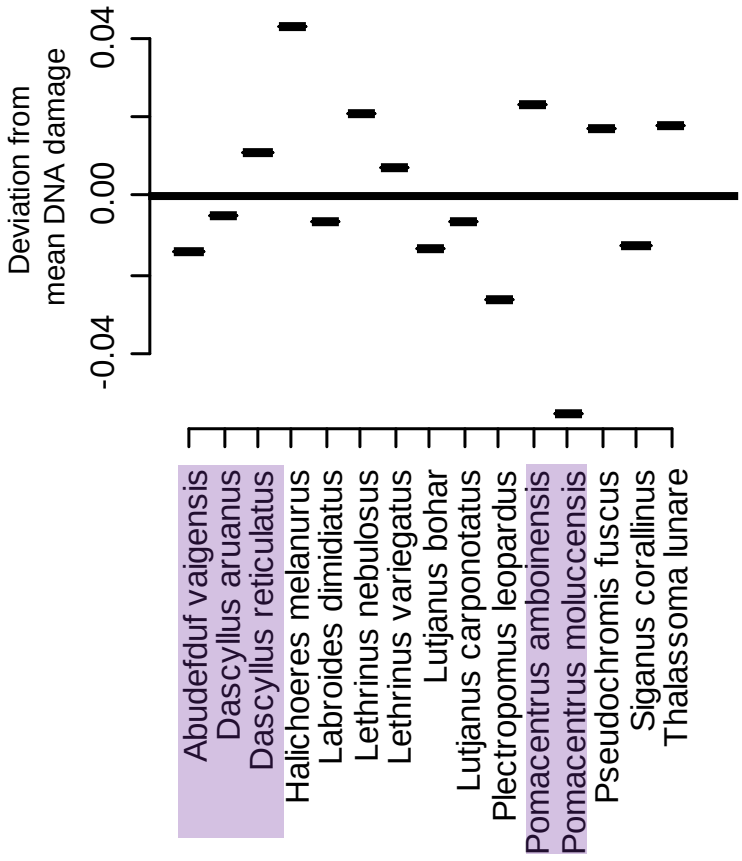


Figure 2.5: Partial dependance plot of the factor "species" created by the BRT model on mean DNA damage (zero on Y- axis at with logit scale) in reef fish from Lizard Island. Species with UV vision/signalling are highlighted.

Table 2.1: Overview of the species collected as well as details on collection and sampling method. The column "UV vision" is based on Siebeck (2000, 2001, 2007). Significant P values (bold) indicate that the mean DNA damage is significantly different from zero in a one-sample t-test.

Species	n	Lifestage	Sampling	Size class	Depth caught	Collection method	Family	Date sampled	UV vision	CPD level (StDev)	P
Pomacentrus amboinensis	10	Adult	Skin sample	2 - 20 cm	< 2 m	SCUBA	Pomacentridae	03/13	yes	0.026 (0.038)	0.0534
Pomacentrus amboinensis	5	Larvae	whole fish	< 2 cm	< 2 m	light trap	Pomacentridae	11/11	yes	0.088 (0.056)	**0.0236**
Pomacentrus moluccensis	11	Adult	skin sample	2 - 20 cm	< 2 m	SCUBA	Pomacentridae	01/14	yes	0.013 (0.017)	**0.0274**
Pomacentrus moluccensis	5	Larvae	whole fish	< 2 cm	< 2 m	light trap	Pomacentridae	11/11	yes	0.109 (0.006)	**< 0.0001**
Dascyllus reticulatus	9	Adult	skin sample	2 - 20 cm	< 2 m	SCUBA	Pomacentridae	11/11 & 01/14	yes	0.028 (0.023)	**0.0066**
Dascyllus reticulatus	2	Larvae	whole fish	< 2 cm	< 2 m	light trap	Pomacentridae	11/11	yes	0.079 (0.001)	**0.004**
Dascyllus aruanus	6	Adult	skin sample	2 - 20 cm	< 2 m	SCUBA	Pomacentridae	01/14	yes	0.018 (0.017)	**0.0451**
Dascyllus aruanus	2	Larvae	whole fish	< 2 cm	< 2 m	light trap	Pomacentridae	11/11	yes	0.091 (0.009)	**0.0463**
Abudefduf vaigensis	10	Larvae	whole fish	2 - 20 cm	< 2 m	light trap	Pomacentridae	01/14	yes	0.048 (0.086)	0.1088
Thalassoma lunare	11	Adult	skin sample	2 - 20 cm	< 2 m	SCUBA	Labridae	03/13	no	0.051 (0.046)	**0.0041**
Halichoeres melanurus	7	Adult	skin sample	2 - 20 cm	2 - 5 m	SCUBA	Labridae	12/11	no	0.063 (0.045)	**0.0097**
Labroides dimidiatus	3	Adult	skin sample	2 - 20 cm	2 - 5 m	SCUBA	Labridae	12/11	no	0.020 (0.006)	**0.0264**
Plectropomus leopardus	7	Adult	skin sample	> 20 cm	2 - 5 m & 5 -10 m	hook and line	Serranidae	12/11 & 01/14	no	0.037 (0.048)	0.0894
Plectropomus leopardus	2	Larvae	whole fish	> 20 cm	< 2 m	light trap	Serranidae	01/14	no	0.005 (0.003)	0.2237
Lutjanus carponotatus	6	Adult	skin sample	> 20 cm	5 - 10 m	hook and line	Lutjanidae	12/11 & 01/14	no	0.031 (0.029)	**0.0461**
Lutjanus bohar	4	Adult	skin sample	> 20 cm	5 - 10 m	hook and line	Lutjanidae	11/11	no	0.031 (0.035)	0.1712
Lethrinus nebulosus	7	Adult	skin sample	> 20 cm	2 - 5 m	hook and line	Lethrinidae	11/11	no	0.076 (0.084)	0.0541
Pseudochromis fuscus	9	Adult	skin sample	2 - 20 cm	2 - 5 m & 5 -10 m	SCUBA	Pseudochromidae	11/11 & 01/14	no	0.033 (0.021)	**0.0013**
Siganus corallinus	4	Larvae	whole fish	2 - 20 cm	< 2 m	light trap	Siganidae	12/11	no	0.026 (0.015)	**0.0398**
Lethrinus variegatus	5	Larvae	whole fish	2 - 20 cm	< 2 m	light trap	Lethrinidae	12/11	no	0.035 (0.010)	**0.0013**

Table 2.2: Relative influence of the predictors on the measured variable "DNA damage". The analysis via boosted regression trees used six predictors (species, size class, development stage (larvae or adult), depth, family and the ability to see and communicate with UV signals) in order to determine their influence on the amount of DNA damage in skin samples.

Predictor	Relative influence (%)
Species	73.9
Size class	17.7
Larvae or adult	5.1
Depth class	1.3
Family	1.2
UV vision/signalling	0.7

Depth and size series in Pomacentrus moluccensis

The DNA damage did not vary among three groups of *P. moluccensis* caught at three different depths (Fig. 2.6a, Kruskal Wallis test, P = 0.85) and did not decrease with increasing depth (linear regression: R^2 = 0.038, F = 1.09, Df = 1, 28; P = 0.31). The mean DNA damage was 0.013 (+/- 0.017 StDev) for the shallow group, 0.009 (+/- 0.009 StDev) for the fish caught between five and ten meters, and 0.007 (+/- 0.007) for the group of fish caught below ten meters of depth. In contrast to the depth series, the level of DNA damage did vary significantly between three groups of *P. moluccensis* that differed in size (Fig 2.6b, Kruskal-Wallis test, P = 0.007, Dunn's multiple comparisons $\alpha = 0.05$). DNA damage was highest in the smallest cohort of fish (0.065 +/- 0.05 StDev), and significantly different from the medium (0.014 +/- 0.018 StDev) and the largest group (0.013 +/- 0.018 StDev). There was a significant, negative correlation between size and DNA damage (linear regression: R^2 = 0.31, F = 16.96, Df = 1,37, P = 0.0002).

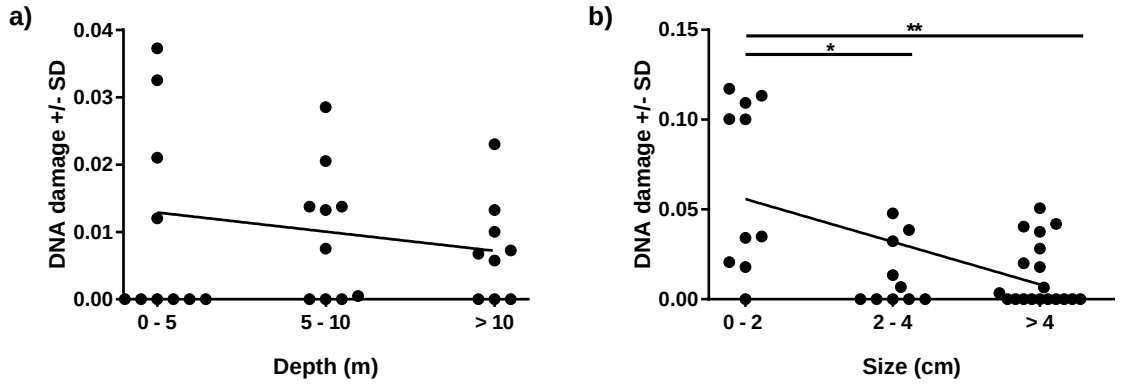
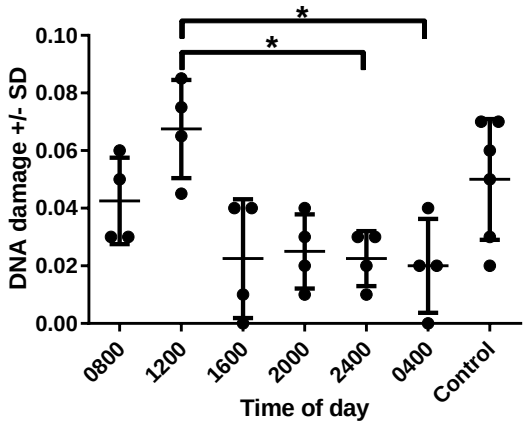


Figure 2.6: DNA damage profiles of *Pomacentrus moluccensis* across a depth (a) and size (b) gradient. The linear regression for the depth series was not significant (P = 0.3, y = -0.0006x = 0.013), while the linear regression for the size series was significant (P = 0.0002, y = -0.01x + 0.056). The asterisks indicate the significance level for the Dunn's multiple comparison tests: * = 0.05 > P > 0.01, ** = 0.01 > P > 0.001.

Dayseries of Pomacentrus amboinensis

Although the levels of UVB irradiation measured at water surface of the experimental tanks increased during the day (Fig. 2.7), the mean levels of DNA damage from groups sampled during the day did not vary significantly from the control group (Dunn's multiple comparison test, $\alpha = 0.05$, all $P > 0.05$). However, the means differed significantly from each other (Kruskal-Wallis test, P = 0.019), and the highest amount of DNA damage (0.068 +/- 0.017 StDev) occurred at noon, when the UVB dose was highest (7.07 $W*m^{-2}$). This highest damage was significantly different from the mean damage at 2400 and 0400 hours (Dunn's multiple comparison test, P = 0.04 and 0.02, respectively), but was not different from the control group (Dunn's multiple comparison test, $P > 0.05$). Table 2.5 gives the accumulated and daily dose of UVB irradiation during the dayseries experiment. It is evident that the daily dose is typical for a tropical location (see Lamare et al.(2007)), with the last timepoint having received a total of 172 $kJ*m^{-2}$ of UVB, and that nearly half the daily dose is reached shortly before solar noon which occurred at 1221 at this day.



Time	UVB (W/m²)
0800	1.64
1000	2.24
1200	7.07
1600	1.13
1800	0

Figure 2.7: DNA damage levels in adult *Pomacentrus amboinensis* at different times of the day, and UVB irradiation measured at the surface of the experimental tanks. The DNA damage for four replicate fish per timepoint are plotted, the control shows the DNA damage for five replicates. The asterisks indicate significant differences ($\alpha = 0.05$, $P < 0.05$) between groups. The UVB irradiance at the day the sampling took place is shown, which was measured at five times using the portable Solarmeter.

UV measurements

The spectra (Figs. 2.8a and 2.8c) show the measured irradiance at surface and at two depths (two and six meters) at Trawler Beach and Vicky's Reef. Irradiance at wavelengths below 310 nm was excluded due to a large proportion of noise in the data. As expected, irradiance decreased with depth (Fig. 2.8a) and absorption was stronger at shorter wavelengths in the UV spectrum as indicated by higher K_d values (Fig. 2.8b, Table 2.3). However, irradiance levels at Vicky's Reef were about a magnitude lower than at Trawler Beach, but also stronger attenuated as reflected by higher K_d values. The K_d values in the measured UV range lie between 0.658 and 0.034 for Trawler Beach and indicate water of high clarity at this site. In contrast, the K_d values for Vicky's Reef range from 1.773 to 0.522. The depth Z_1 in the UVB range lies between seven and eight meters (310 and 315 nm respectively), at Trawler Beach and less than five meters at Vicky's Reef. The UVA penetration into water at Trawler Beach was considerably higher with values between approximately 12 to more than 135 meters depth (Z_1), compared to the Z_1 maximum depth of less than ten meters recorded at Vicky's Reef. The characteristics of these measurements are reflected by the doses of UVB and UVA that were calculated and are shown in Table 2.4. The location with the higher water clarity, Trawler Beach, is receiving around 5 $W*m^{-2}$ just below the surface, while the more opaque

site, Vicky's Reef, is receiving only a fraction of that irradiance (0.62 W*m^{-2}). A higher proportion of UVA to UVB is visible at Trawler Beach than Vicky's Reef, which is reflected by the higher K_d values in the UVA range (Fig. 2.8d).

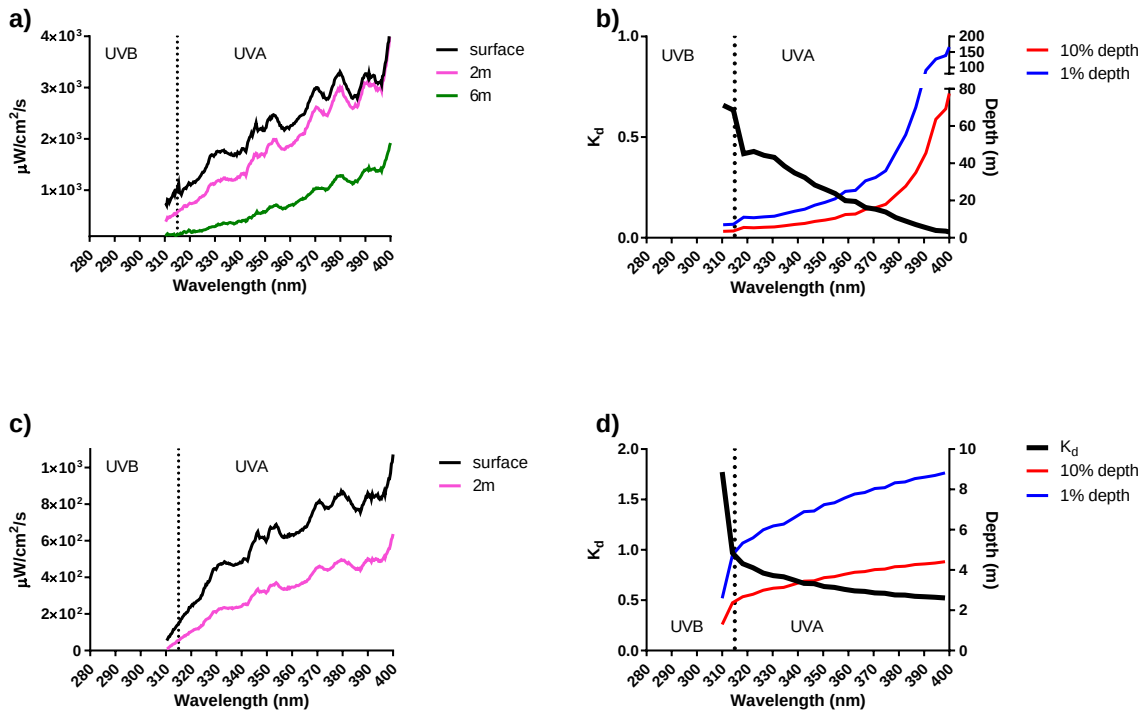


Figure 2.8: Underwater light spectrum in the UV range at Trawler Beach (a, b) and Vicky's Reef (c, d) in the Lizard Island lagoon. Three measurements of UV irradiance were taken at Trawler Beach (a), and one at the surface and one at depth are shown for Vicky's Reef (c). The resulting attenuation coefficients (K_d) and the depths at which 1% and 10% of the surface irradiance are still present are plotted in b (Trawler Beach) and d (Vicky's Reef).

Table 2.3: Attenuation coefficient K_d, and the depths at which 10 and 1 % of surface UV are present, Z_{10} and Z_1 respectively, for Trawler Beach and Vicky's Reef in the UVR range.

Location	Wavelength (nm)	310	315	320	340	360	380	400
Trawler Beach	**K_d**	0.658	0.584	0.394	0.299	0.182	0.103	0.034
	Z_{10}	3.497	3.943	5.842	7.701	12.620	22.357	67.711
	Z_1	6.994	7.885	11.685	15.403	25.239	44.713	135.422
Vicky's Reef	**K_d**	1.773	0.961	0.815	0.679	0.597	0.553	0.522
	Z_{10}	1.299	2.394	2.824	3.394	3.858	4.167	4.413
	Z_1	2.6	4.79	5.648	6.787	7.715	8.334	8.827

Table 2.4: Irradiance of UVB and UVA at different depths at Trawler Beach and Vicky's Reef. The spectra in Figure 2.8a) and 2.8c) were integrated for the UVB (280 315 nm) and UVA (315 – 400 nm) range. All measurements were made around midday. Doses are in $W*m^{-2}$.

Location	Depth	UVB (280 - 315 nm)	UVA (315 - 400 nm)	Ratio UVB/UVA (%)
Trawler Beach	**below surface**	4.95	197.94	2.50
	2m	2.8	166.54	1.68
	6m	0.72	64.41	1.12
Vicky's Reef	**below surface**	0.62	53.34	1.16
	2m	0.2	29.2	0.68

Table 2.5: Accumulated doses during the dayseries experiment (see Fig 2.7). No measurement was made at the first timepoint (0630) which was shortly before sunrise (0633). Sunrise and sunset (1808) as well as solar noon (1221) were provided by www.timeanddate.com for the nearest location (Cairns) for the 18th of August 2012. Note that the UVB irradiance is measured in $W*m^{-2}$, while the accumulated dose is given in $kJ*m^{-2}$.

Time	UVB Irradiance ($W*m^{-2}$)	Accumulated dose in $kJ*m^{-2}$	% of total daily dose
0630	0	0.00	0.00
0800	1.64	6.48	3.75
1000	2.24	27.54	15.94
1200	7.07	77.76	45.00
1600	1.13	166.32	96.25
1800	0	172.80	100.00

Discussion

In the last decades, UVR levels, especially in the southern hemisphere, have increased drastically due to anthropogenic influences (Herman 2010). Simultaneously, our knowledge about the effects of current and increased UVR levels on marine organisms has grown substantially and been the subject of a number of excellent reviews (e.g. Lamare (2011, 2014), Karentz (2014)), however, our knowledge about the effects of UVR on reef fish is limited. The aim of the study was to provide a baseline measurement for UV- induced DNA damage, measured as relative CPD quantity in fish from the Great Barrier Reef (GBR). The influence of the variables fish size and capture depth, species, family, whether fish were captured as settlement-stage larvae or adults, as well as the ability to communicate with UV signals on the prevalence of UV- induced DNA damage was evaluated. Furthermore, the relative amount of DNA damage was tracked over time to reveal whether daily changes in UV correlate with damage levels.

This is the first study to show the occurrence of the most common type of UV-induced DNA damage, CPDs, in coral reef fish from the GBR. The detection of CPDs in fish skin and tissue samples from the field is important considering the environmental changes that organisms on coral reefs are facing. Knowing the current level of impact of UVB radiation (280 - 315 nm), is essential for future studies to enable comparisons, and acts as a "Baseline" of net UV-induced DNA damage. This study shows that CPDs are present in all 15 species from 7 families of teleosts examined here. The most influential factor governing the level of damage was the factor "species" with an effect of nearly 75%, while the factor "family" only accounted for 1.5% of the variance, painting a similar picture to other kinds of DNA damage caused by pollutants that have been previously examined (Reichert et al. 1998). Several species had greater deviation from the mean DNA damage than others, e.g. *Halichoeres melanurus*, *Lethrinus nebulosus*, *Pomacentrus amboinensis*, *Pseudochromis fuscus* and *Thalassoma lunare* showed a higher than average DNA damage, whereas lower DNA damage was observed especially in *Pomacentrus moluccensis*, *Plectropomus leopardus* and *Siganus corallinus*. In other studies differences in sensitivity to UVR between species has been attributed to differences in pyrimidine base composition (Kellogg & Paul 2002) and variation in anthropogenic influences such as deforestation of habitats along the distribution of a species (Mitchell et al. 2014). Since the genetic sequences of the species studied here are yet unknown and there was only a single location (Lizard Island) were fish were collected,

the specific factors influencing the higher and lower levels of DNA damage in these species remain to be determined in future studies.

The two factors determining over 20% of variation in DNA damage were the size of individuals (17.7%) and whether the fish were sampled as settlement stage larvae (5.1%). The risk of higher DNA damage in smaller individuals is reflected in the size series of *P. moluccensis*, where individuals smaller than two centimetres, which are effectively recently settled fish had the highest amount of DNA damage. Prior to settlement, many larvae of coral reef fish are found in top ten metres of the water column (Leis 2004), where UVR is highest. The impact of UVR on early development stages of marine organisms has been documented before (Lesser et al. 2001, Bancroft et al. 2007, Mitchell et al. 2009, Sucré et al. 2012, Fukunishi et al. 2013a) and this study confirms that coral reef fish larvae are no exception. It is further unclear whether protection mechanisms such as Mycosporine-like Amino Acids (MAAs, see chapter 4) are present in reef fish larvae, however UV-absorbing compounds are present in eggs (Plack 1981) and ovaries and are thought to be passed on to eggs by female fish (Zamzow 2004). DNA repair mechanisms (see chapter 5), specifically photoreactivation and dark repair are present in larvae of some species such as *P. moluccensis*, however their ability to restore UV-induced DNA damage is relatively poor compared to other marine organisms such as corals (Reef et al. 2009) and could lead to the comparatively high CPD levels in younger and smaller fish. In some samples collected for the size series and baseline study, no damage at all could be detected, suggesting a combination of protection mechanisms (avoidance, repair and protection) at work. None of the species sampled are strictly nocturnal, which would prevent exposure to UVR, but most fish used in this study, including *P. moluccensis*, live in close association to corals such as *Acropora*. The inclusion of individuals with a greater preference for staying hidden amongst coral branches could also lead to the large variation in DNA damage observed here and the occurrence of samples with no detectable DNA damage.

Depth had a relatively low level of influence on the amount of DNA damage, at least for the 15 species included in the baseline study. These results were supported by the depth series of *P. moluccensis*, which also showed very little variability with depth. This is surprising since it was expected that fish in shallower water show more CPDs. There was a clear difference in the attenuation properties (K_d and the Z_{10} and Z_1 depths) between two sites near the Lizard Island lagoon, the comparatively clear site at the entrance to the lagoon near Trawler Beach where fish for this series were sampled, and the relatively opaque site at Vicky's Reef, a point

where the prevalent currents and tides sweep water out of the lagoon. This shows that apart from a depth gradient, site specific differences in UVR attenuation are encountered by reef fish. In support of this finding, it has been shown that the amount of UV absorbing sunscreens is correlated with depth (Zamzow & Losey 2002) and water clarity (Zamzow 2007, Eckes et al. 2008), so that the impact of UV-induced DNA damage in shallower reefs and in clearer waters could be negated by increased protection, or at least be kept at an "acceptable" background level. In addition to protection from UVR by natural sunscreens, the large proportion of photoreactivating wavelengths (> 400 nm) which facilitate DNA repair in shallow water could aid fish in achieving low levels of DNA damage. The presence of photoreactivating light has been shown to be instrumental for repair of DNA damage induced by ecologically relevant doses of UVB radiation (Olson & Mitchell 2006, Mitchell et al. 2009). Higher rates of damage repair could also explain the occurrence of a larger proportion of samples with no measurable damage in shallow water (Smith et al. 1992). Although not specifically tested, the results presented here indicate a significant role of photorepair in the determination of UV-induced DNA damage levels in reef fish.

UV signalling and the presence of UV vision did not play a major role in determining DNA damage in the species included in the Baseline study, although a lower amount of UV absorbing compounds in the mucus and UV transparent ocular media are necessary to enable this mode of communication, and fish using this "secret communication channel" have been hypothesized to be at higher risk from UV exposure (Eckes et al. 2008, Siebeck 2014, Siebeck et al. 2014). DNA damage, especially in adult fish with UV vision and communication (e.g. *P. amboinensis*) was not higher than in fish from the same habitat and depth (e.g. *T. lunare*). A greater spread across families that have the potential for UV signalling (Siebeck & Marshall 2001, Siebeck & Marshall 2007) could potentially shed more light on this issue, as this study only included Pomacentridae as a representative for this factor. However, the influence of the phylogenetic classification "family" also showed very low influence on the amount of DNA damage in reef fish, so that DNA damage across several families with UV vision can be expected to show a low level of variation.

Protection mechanisms like MAAs and most likely DNA repair could be responsible for the consistently low levels of DNA damage in *P. amboinensis* that were exposed to high levels of UVB radiation over the course of the day. Here, DNA levels first increased towards noon, but remained similar to the background levels of DNA damage in animals that were shielded from UVR. Unlike the clear diel cycles in DNA damage profiles found in ice fish larvae

(Malloy et al. 1997) or plankton (Boelen et al. 2002), DNA damage in the adult *P. amboinensis* remained relatively low. This suggests extremely efficient protection (chapter 4) and repair (chapter 5) mechanisms in this species. The dose of UVB that accumulated over the course of a day (up to 172.8 $kJ*m^{-2}$) are in agreement with other reported measurements at tropical locations (Lamare et al. 2007). It has been reported that in comparison with inhabitants from temperate and polar regions, DNA damage rates in tropical locations are lower despite higher doses of UVB irradiation. With the data presented here, such comparisons can now be made with teleost fish in future studies.

Recently it has been suggested by Sweet et al (2012) that UVR exposure and UV-induced DNA damage could be the likely cause of melanoma in coral trout (*Plectropomus leopardus)* from the southern Great Barrier Reef. In the samples collected near LIRS, no evidence was found for higher than normal levels of DNA damage in adults of this species, and the two larval specimens had the lowest levels of DNA damage of all the species examined. Other long-lived predatory species such as those belonging to the families Lethrinidae and Lutjanidae showed a similar picture in terms of DNA damage, giving rise to the assumption that these species are not at a higher risk from UVR exposure than others. Also, no external signs of melanoma or changes in skin colouration or ulcers were observed in any of the individuals included in this study. The cause of the high rate of melanomas in wild population of reef fish therefore remains unknown for now, but could well be explained by considering the potential for genetic mutations (Sweet et al. 2012) and their influence on the rate of occurrence of melanomas in reef fish.

The overall low levels of DNA damage under natural conditions suggest that coral reef fish are well adapted to the current conditions and the amount of UVR exposure can be well managed. However, the trend for higher DNA damage in smaller fish, i.e. settlement stage larvae indicates that during early development, fish are at higher risk from UVR exposure. To further elucidate the effects of UVR exposure on fish larvae, DNA damage accumulation and repair were directly investigated in chapter 5.

CHAPTER THREE

Behavioural changes of reef fish during UVR exposure

Abstract

Behavioural avoidance to UV (280 – 400 nm) radiation (UVR), which has the potential to induce DNA damage, has been hypothesized to act as a possible protection mechanism in fish. UV vision is seen as a prerequisite for such a behavioural reaction, and is common among many coral reef fish which live in the UV-rich environment of the shallow tropical waters. In order to reveal whether a behavioural avoidance response to UVR exists in reef fish, experiments were designed in which fish with UV vision (*Pomacentrus amboinensis*) and without UV vision (*Thalassoma lunare*) were given a choice between UV-protected and UV-exposed compartments. Additionally, the boldness and foraging behaviour of settlement-stage larvae of *P. amboinensis* were determined under elevated levels of UVR. Using an automated analysis of video footage using MatLab this study shows that neither species displayed a specific avoidance response to varying levels of UVB, and fish spent equal amounts of time in exposed and protected compartments. However, *P. amboinensis* showed a preference for deeper sections of the experimental tanks. No preferences for either exposed or protected compartments or a specific depth in the experimental tanks was found for *T. lunare*. The foraging activity and distance to shelter of newly settled *P. amboinensis* that were exposed to an ambient dose of UVR was significantly reduced compared to fish that were observed under light conditions that lacked UVR. This effect was found to be UV-specific since fish that were exposed to light that did not contain UV but had the same intensity showed no reduction in their behaviours. The findings that are presented here challenge the general notion that fish with UV vision are able to seek protection by choosing areas that are lacking UVR. However, the preference for deeper sections of the experimental tanks as well as the changes in the behaviour of juvenile *P. amboinensis* highlight the negative effects of UVR exposure on coral reef fish.

Introduction

Invisible to humans and ranging from 280 to 400 nm in wavelength, ultraviolet (UV) radiation has shaped the evolution of life on Earth for billions of years (Cockell 1998, Rothschild 1999). The ozone layer blocks UVC radiation (190 – 280 nm), and only UVA (315 – 400 nm) and the more energetic UVB (280 – 315 nm) wavebands reach Earth's surface (Frederick et al. 1989). Due to the ozone layer's distribution, UV radiation is generally higher in tropical regions both on Earth's surface (McKenzie et al. 2003) and in the marine environment (Vasilkov et al. 2001).

Exposure to UVR has multiple negative effects in marine organisms, such as the formation of radical oxygen species (ROS) which in turn damage enzymes, lipids, membrane proteins and DNA (reviewed by Banaszak and Lesser (2009)). ROS are mostly generated by UVA, while the absorption of UVB predominantly leads to the formation of dimers in the DNA (Friedberg 2003). These cyclobutane pyrimidine dimers (CPDs) lead to mutations and cell death if unrepaired (Ikehata & Ono 2011) and have been linked to reduced growth (Lesser et al. 2001), formation of melanomas (Sweet et al. 2012) and increased mortality (Dong et al. 2007, Tucker et al. 2010, Fukunishi et al. 2013b) in fish from temperate and tropical waters.

In order to counteract these negative effects, three protection mechanisms have been discussed in the literature: i) protection using natural sunscreens (Dunlap & Shick 1998), ii) DNA damage repair via photoreactivation and excision repair (Sinha & Hader 2002) and iii) avoidance behaviour in response to UV radiation exposure (Leech & Johnsen 2003). Behavioural avoidance can be seen as the ideal way to prevent DNA damage (Roy 2000), since it precedes the need for both DNA repair and protection with sunscreens. Avoidance behaviours to UV radiation have been shown in a range of aquatic organisms, and include the movements of bacteria (Bebout & Garcia-Pichel 1995), negative phototaxis in zooplankton (Pennington & Emlet 1986, Alonso et al. 2004, Hansson et al. 2007), movement in leeches (Jellies 2014), settlement in coral larvae (Gleason et al. 2006) and covering behaviours of sea urchin (Adams 2001, Verling et al. 2002, Dumont et al. 2007).

In fish, UV avoidance behaviours have been associated with a preference for deeper nest sites in bluegill (Gutiérrez-Rodríguez & Williamson 1999). More specifically, Kelly and Bothwell (2002) showed that salmon preferred UV-reduced and protected environments particularly at high solar irradiances, and Ylönen (2004) described UV avoidance behaviour in coregonid

larvae that chose deeper habitats in lakes with a low attenuation of UVR. However, these studies did not control for brightness so that behavioural reactions could have been triggered by a general high intensity solar irradiance or experimental treatment rather than high intensity UV radiation (Ylönen et al. 2005). UV avoidance could be achieved by organisms with the ability to perceive or detect UV radiation visually. Light detecting structures as simple and rudimentary as oocelli or photoreceptors in the pituitary gland might be sufficient. UV transparent ocular media common in many reef fish and the presence of UV sensitive photoreceptors have led to the hypothesis that reef fish may be able to visually detect dangerous levels of UVR and therefore regulate their exposure to UVR (e.g. Siebeck (2014)). Another possible, however unexplored, way which could induce avoidance behaviour is based on physiological changes since UV-induced DNA damage itself or increased damage repair, both lead to the activation of many pathways including cell cycle arrest and apoptosis (Sinha & Häder 2014).

Despite living in the UV rich environment of coral reefs (Dunne & Brown 1996), UV avoidance behaviours of reef fish have not been addressed so far. This lack of knowledge becomes more striking when one considers that many reef fish, among them many Pomacentridae, have UV-transparent ocular media (Siebeck & Marshall 2001) and photoreceptors with UV-sensitive visual pigments (McFarland & Loew 1994, Losey et al. 1999). In contrast, wrasses of the genus Thalassoma, for example, generally do not have ocular media that allow the transmission of wavelengths below 400 nm (Siebeck & Marshall 2000, 2001). Whether species with UV vision, which are able to detect UVR, are also able to correctly ‘interpret’ certain UVR levels as dangerous and therefore avoid or reduce exposure, is unknown.

Besides the many direct and indirect negative effects of UVR exposure, some positive aspects of UV exposure have been shown to occur such as the production of Vitamin D in microorganisms and the accumulation in the food chain (Lock et al. 2010), correct spicule formation in Ascidians (Hirose et al. 2006) and a reduction in parasitism (Overholt et al. 2011) in *Daphnia*. Sticklebacks show a preference for UV-rich habitats when displaying shoaling behaviour (Modarressie et al. 2006). In Pomacentrid fish, UV vision and UV-reflecting skin patterns play an important role in communication (Siebeck 2004, Siebeck et al. 2010), using a part of the available light spectrum that many predators cannot see (Siebeck 2014). UVR, more specifically UVA, has further been shown to assist in foraging by

increasing contrast detection of prey items in all lifestages from larvae and juveniles to adult fish (McFarland & Loew 1994, Johnsen & Widder 2001, Job & Bellwood 2007). Whether an elevated dose of UVB in the light environment could possibly counteract any of these benefits of UVR exposure in coral reef fish is not known, but changes in swimming behaviour during UVR exposure have been demonstrated in juvenile trout (Alemanni et al. 2003).

The damselfish *Pomacentrus amboinensis* and the wrasse *Thalassoma lunare* occur on the same shallow coral reefs along Australia's Great Barrier Reef (Allen et al. 2003), where increases of UVR have occurred in the last decade (Masiri et al. 2008). The presence of UV sunscreens in the external mucus layer of both species (Zamzow & Siebeck 2006) and a UV specific upregulation of these sunscreens in *P. amboinensis* (chapter 4) demonstrate the need for UV protection in these species.

Given their different visual abilities in the UV range these two species present an ideal system to test for a UV-specific avoidance response and altered feeding and activity behaviours during UV-exposure in reef fish. Two hypotheses shall be tested: i) in contrast to the wrasse *T. lunare*, the damselfish *P. amboinensis* shows a UV specific avoidance response by preferring an environment that lacks elevated, dangerous levels of UVB, and ii) the activity and feeding behaviours are negatively influenced, i.e. reduced in juvenile *P. amboinensis* under exposure to an acute dose of UVB.

Methods

The experiments were conducted in an aquarium facility at the University of Queensland with *Pomacentrus amboinensis* and *Thalassoma lunare* attained from a commercial aquarium trader (both species, Cairns Marine, Cairns, Australia), and at Lizard Island Research Station (LIRS) with specimens (*P. amboinensis* only) that were caught using hand nets while SCUBA diving at Lizard Island (145°26'31"E, 14°40'7"S) with permits to UES and CB (GBRMPA: G11/34453.1; General Fisheries Permit: 162472; Ethics committee of The University of Queensland: SBMS/091/11).

Experiment 1: Avoidance of UVR

Experimental setup and holding conditions

Both, at LIRS and the University of Queensland, specimens were held in experimental tanks (60 x 25 x 40 cm length width height, Fig. 3.1) connected to flow through seawater systems. The water depth was 30 cm and the temperature in the tanks was 22 °C (UQ) and 23.5 °C (LIRS) and did not differ between individual tanks. The tanks were divided into two equally sized parts using a grey opaque sheet of PVC (0.5 x 25 x 40). This divider had a PVC tube (diameter six cm, length ten cm, cut length wise to create a tunnel) glued to the bottom edge which acted as shelter and simultaneously made it possible for the fish to move freely between the two sections of the tank. One aquarium light housing that could hold up to two fluorescent tubes (Sankyo Denki, Kanagawa, Japan) was suspended 20 cm above water level. On top of the tank (approx 10 cm above water level) the sections were covered with either one sheet of UV- transmitting acrylic (OP4, Acrylite) or a combination of the UV transmitting acrylic and one sheet of UV- blocking material (description see below in "light treatments and filters"), which resulted in the treatment condition "UV+" and "UV-" in the opposite compartments of the experimental tank.

Experimental procedure

Fish were acclimatised for up to three (*P. amboinensis*) to five days (*T. lunare*) in the experimental tanks before testing began. During this time, fish were fed several times per day, in the case of *P. amboinensis* with fish flakes (TetraMin, Melle, Germany) and in the case of *T. lunare* with cut prawn. When the fish were feeding readily and showing foraging

behaviour in both sides of an experimental tank, as well as accepting the PVC tube in the middle of the divider as shelter, the experiment started. Fish were fed several hours before testing, and any leftover food was removed by siphoning before observations were made. During this acclimation period, the UV lights above the tanks were turned off, so that the illumination contained only light with wavelengths > 400nm.

In a first run of experiments, *P. amboinensis* (conducted at LIRS, n = 16) were observed in both a high and low dose UVR condition (see section on light treatments below for details) for one hour on two subsequent days. The starting condition (high or low dose) was randomized. After the first 30 minutes of observation, the "UV+" and "UV-" filter combinations were changed so that both the left and the right side of the experimental tank were exposed to or shielded from UVR once. Which side was exposed or shielded first was decided by coin toss. As damselfish are able to see UV light, the two treatments would have differed to the fish in both, light spectrum and brightness. Separate cohorts of *P. amboinensis* were therefore used in a second run of experiments (conducted at UQ, n = 12,) in which the brightness difference between the UV+ and UV- treatment was controlled, i.e. equalized.

T. lunare was tested for UV avoidance under both the low and the high dose. All other procedures followed the same protocol as in *P. amboinensis*. During the experiments, the fish were filmed for 60 minutes (two observation periods of 30 minutes) using a HD video camera (Sony HDR-CX410VE (25 fps)) on a tripod placed perpendicular approximately 1.5 metres away from the tank, facing the long side. The experimenter was not present in the room during the test period. Each observation period started one minute after the UV lights above the tanks were turned on or the filter positions were changed.

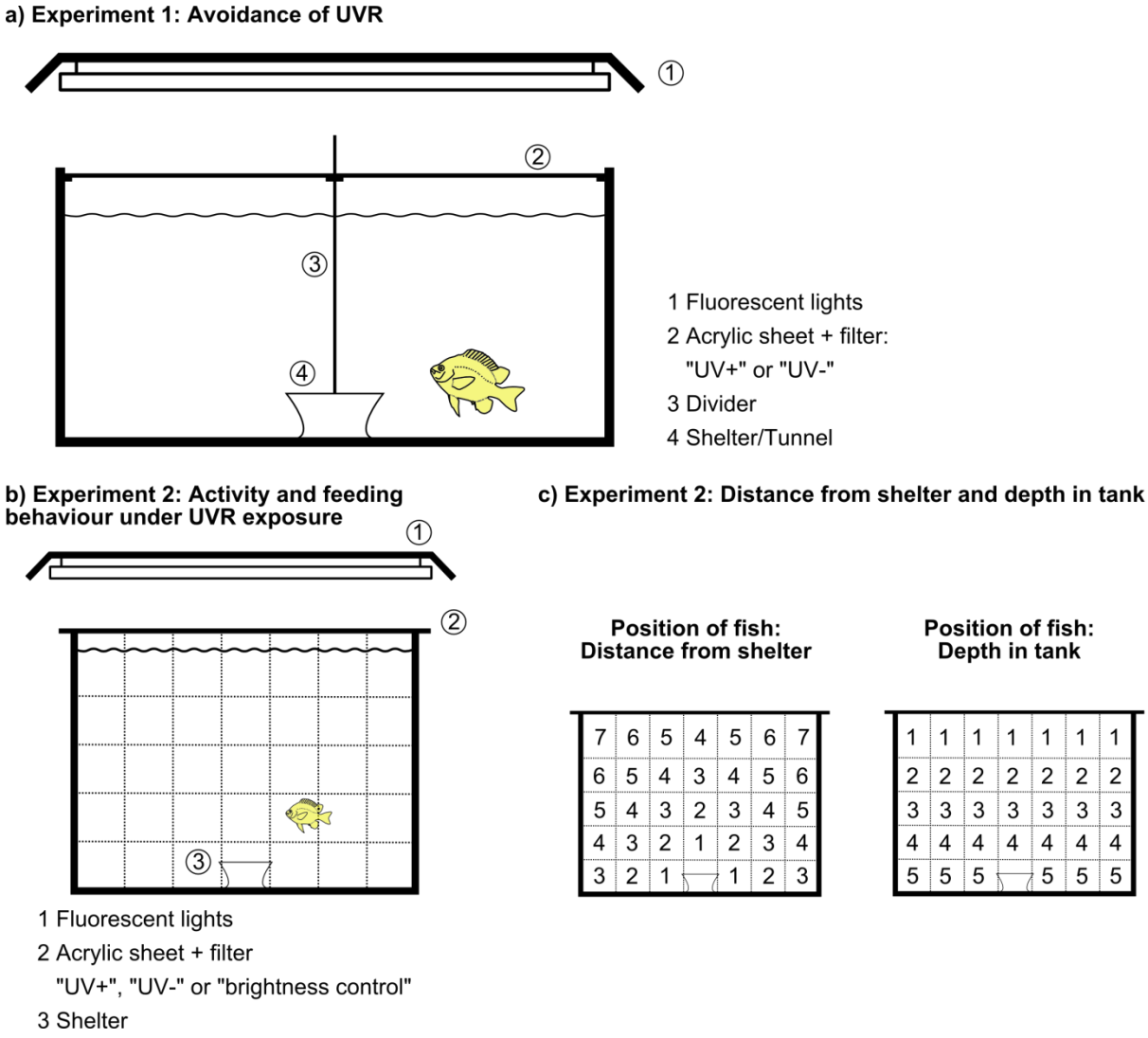


Figure 3.1: Schematic representation of the tanks used in the experiments. **a)** Experimental setup used in experiment 1 testing for UV avoidance with *P. amboinensis* and *T. lunare*: A set of fluorescent UVB lamps is suspended approximately 20 cm above the water level. The combination of an acrylic sheet and filter foil rests approximately 10 cm above the water level. The tank is divided into two equally sized parts by a sheet of grey PVC with a shelter that also enables the fish to freely move between the two compartments. **b)** Experimental setup used in the experiment 2 investigating the activity of juvenile *P. amboinensis* under different UVR regimes. The fluorescent lamps are suspended approximately 15 cm above the water surface, which is completely covered by a combination of an acrylic sheet and filter. A PVC tube acts as shelter and is placed in the centre of the tank. **c)** The thin dotted lines mark the coordinates which enabled the observer to score the position of the fish, i.e. the distance from the shelter, and the activity (by line crossings) of the fish during the experiment. The lines were painted on the front side of the tank with a water-resistant black marker.

Light treatments

In order to block UVR in one section of the tank (= treatment condition "UV-"), the acrylic lid was covered with one layer of LEE 226 filter (LEE filters, Andover, Hampshire, UK, 50% transmission = 410 nm). For the experiments that balanced the brightness (in terms of photons) in both sections of the tank (= treatment "brightness control, BC"), the side of the tank that was exposed to UVR was covered using a combination of an OP4 acrylic sheet and a UVR transparent neutral density filter (overhead projector transparency, EXP500 OHP, printed with 25 % greyscale using a laser printer (Brother HL 2140). In the "UV+" treatment the tank was covered only with the OP4 acrylic sheet. The transmission of the acrylic sheet and the different filter combinations (Fig. 3.2) was measured using an OceanOptics JAZ spectrometer equipped with a 1000 µm optic fibre and a cosine corrector.

Measurements of UVB irradiance were made using a digital UV meter ("Solarmeter" Model 6.0 (UVB) by Solartech Inc. USA, peak response 300nm, spectral response 280- 320 nm, accuracy +/- 10% Ref NIST). Ten measurements were made per light treatment (Fig. 3.2) at different positions under the respective acrylic sheet and filter combinations. All measurements were made with the sensor facing the light source horizontally at water level. The UVB irradiance for the "UV+" treatments was 5.2 and 2.4 $W*m^{-2}$, for the high and low dose respectively. The average irradiances in the brightness control treatments were overall lower because of a loss of UVB below ~ 305 nm due to the filter material. The average UVB irradiance was 1.77 $W*m^{-2}$ and 0.7 $W*m^{-2}$ in the high and low dose conditions, respectively. No UVB was detected in the "UV-" treatments which had a LEE226 filter additionally to the OP4 acrylic.

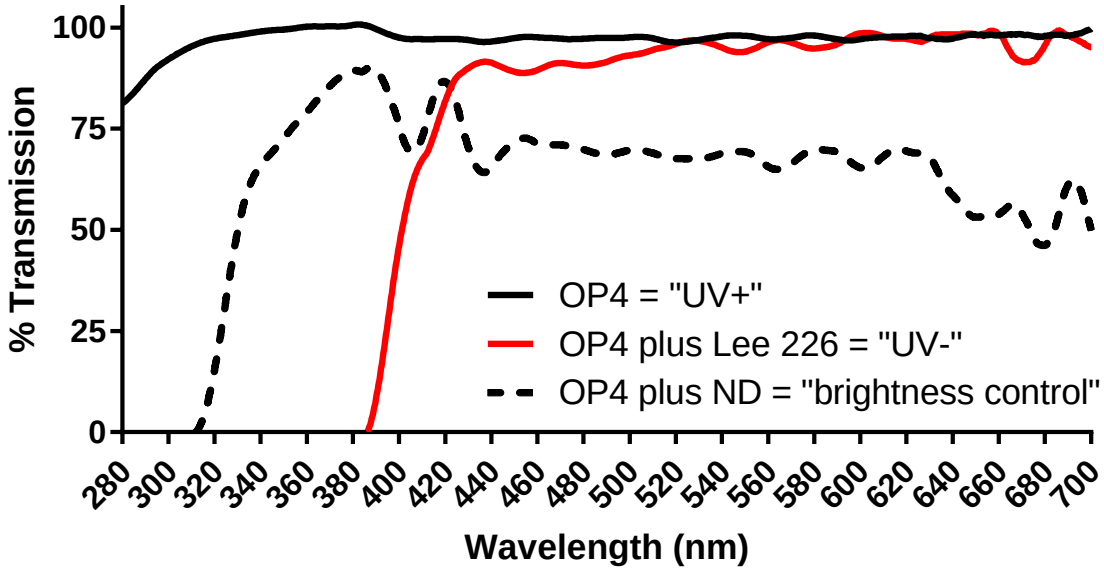


Figure 3.2: Relative transmission of light emitted from the fluorescent UVB lamps in the treatments. The acrylic OP4 sheet transmitted most light including UVB (solid black line), and was used to create the "UV+" compartment of a tank in both the low and high dose experiments under the conditions that were not controlled for in brightness. Fish were shielded from UVR when a Lee 226 filter was added to the acrylic ("UV-", solid red line). A combination of OP4 acrylic and a neutral density filter (dashed black line) created light conditions (UV+ brightness) in a compartment of the tank that exposed fish to some UVB and had the same brightness (number of photons) as the "UV-" compartment.

Video and statistical analysis

Video files were converted to .avi format and cut to 30 minutes of length. The movement of the fish in the tank was tracked using a custom written MatLab script. In the first step, the frame of the video was cropped such that only the border of tank was in view, thus aligning the PVC divider to the centre of the frame. Since fish generally moved around the tank, their contribution within a single frame is negligible relative to the average of the whole video. This allowed for us to determine the background by averaging each pixels colour value for all frames to obtain a background (the empty tank). Following removal of the background, a threshold value was found such that when each image was binarized, only the profile of the fish was remaining within the frame. The centroid of this area was calculated to determine the fish's location in the tank, with the constraint that if the area of the profile falls below the fish's smallest profile based on orientation, its position was unknown. This would only occur

if the majority of the fish was inside the shelter and was recorded as blank position. These events were classified as “hiding”. When the fish was outside the shelter, its position was scored and classified into one of four quadrants, i.e. “upper half UV+”, “lower half UV+”, “upper half UV-” and “lower half UV-”, depending on the treatment (i.e. side of the tank).
The program then stepped through every 50 frames of the video file so that the location of the fish was determined approximately every two seconds for 30 minutes of one file. Finally, the location scores of the fish in all counted frames were summed. The time that each fish spent in a quarter was then expressed as a percentage of the total time of the trial, and averaged over both runs for each fish (reversed positions of the “UV+” and “UV-“ treatments. The resulting data (brightness controlled experiment: n = 12; brightness not controlled experiment: n = 16) for the relative time spent in each quarter were tested for normal distribution (Shapiro-Wilk normality test) and used as the response variable in repeated measurements one-way ANOVAs for each experiment (low and high dose brightness controlled, low and high dose not brightness controlled). Post hoc tests controlled for multiple testing (Tukey’s HSD, significance level 0.05) and determined whether significant preferences of fish for a certain compartment of the tanks existed. Additionally, the mean time that the fish spent in the “UV+” half of the tank, combining the values of the upper and lower quarter, was compared to a theoretical value of 0.5 using a one sample t-test. A significant difference then indicates a preference or avoidance for the area that was exposed to UVR.

Experiment 2: Activity and feeding behaviour under UVR exposure

Experimental setup

The experiment was conducted at the Lizard Island Research Station in March 2014 with juvenile *P. amboinensis* (n = 36, SL = 19.4 mm, StdDev 2.9 mm) collected from the reefs surrounding the station. The newly settled juvenile fish were collected from small patch reefs in the Lizard Island lagoon using hand nets while SCUBA diving. During a short acclimation phase, which lasted two to three days, fish were held individually in a plastic container (5 l Volume) with a seawater flow through at ambient temperature and a shelter tube and fed artemia larvae twice a day. The experiment was performed in a 28 x 20 x 16 cm glass tank (water depth = 19 cm, Fig. 3.1) that had a 7 x 5 grid marked on the front side, using a water resistant black pen. An aquarium light holding two fluorescent tubes (2x GL20SE lamps, Sankyo Denki, Kanagawa, Japan) was suspended 15 cm above water level. The experimental

tank was shielded from other tanks in the same room with a black shade cloth suspended on a frame at a distance of about 50 cm from the tank. A small window cut into the front side of this curtain was used to film and observe the behaviour of the fish in the tank during the experiment. A video camera (Sanyo Xacti (29 fps)) was placed behind the curtain and was used to film the fish during the experiment.

Light treatments

The light treatments ("UV+", "UV-", "brightness control", Fig. 3.2) were set up by using the same combination of acrylic, filters and lights as in the avoidance experiments. The UVB irradiance measured at water surface using the digital UV meter was 5 $W*m^{-2}$ in the "UV+", and 0.2 $W*m^{-2}$ in the "brightness control" treatment. No UVB could be detected in the "UV-" treatment. These conditions are comparable to UVB irradiances measured at just below surface level at Trawler Beach (4.95 $W*m^{-2}$) and light conditions as they can be found in 2 meters depth at the less clear site of Vicky's Reef (0.2 $W*m^{-2}$), see also chapter 2, Table 2.4.

Experimental procedure

Before each fish was tested in the experimental tank, the water was exchanged and left to settle for a few minutes. The lamps above the tank were turned on and a fish was placed in the tank. For the first 15 minutes the fish was recorded on video but no additional observations were made since preliminary experiments showed that *P. amboinensis* took up to ten minutes to start exploring the novel situation of the tank and the shelter. After 15 minutes, a "baseline" period started for which the activity of the fish was scored in terms of line crossings and the position, i.e. the distance of the fish from the shelter and depth. After this, five ml of seawater containing approximately 50 artemia larvae were released into the tank at the top centre using a syringe and a plastic tube and the activity and the position of the fish were scored again. After this "feeding" period, which lasted another 15 minutes, the fish was removed from the tank and the water exchanged before another fish was tested.

The activity of the fish was scored during the trials by recording the number of times a line of the 7 x 5 grid was crossed. This was done by the observer watching the fish on the screen of the camera behind the curtain during the experiment. The distance from the shelter and depth of the fish were scored every ten seconds of the "baseline" and "food" periods by noting the coordinates of the 7 x 5 grid. This was done after the trials by converting the video files into a

sequence of photographs taken every ten seconds using Freestudio v. 5.0.28 (dvdvideosoft.com).

A total of 12 fish were tested in each of the three light treatments (see below). The fish that were tested in the high UVB "UV+" treatment were euthanized after the experiment in an ice/seawater bath (Ethics committee of the University of Queensland, Brisbane: SBMS/091/11) and immediately stored in liquid nitrogen for analysis of UV- induced DNA lesions. DNA damage from this group was compared to net DNA damage levels of settlement stage larvae (n = 5, caught in November 2011, chapter 2) of the same species and of the closely related *P. moluccensis* (n = 10, caught in April 2013, size series chapter 2, cohort 0 – 2 cm).

DNA isolation and damage quantification

The analysis of cyclobutane pyrimidine dimers (CPDs) induced by UVR followed the procedure described in chapter 2 and Mori et al. (1991). Briefly, genomic DNA was extracted from up to 25 mg of skin and tissue from the dorso-lateral body area of fish using the DNeasy® Blood and Tissue kit (Qiagen) following manufacturers' instructions. A scalpel blade was used to shave off a section of skin and tissue (<1 mm thick) from the still specimens. The sample was immediately transferred into a 2 ml tube containing the lysis buffer to prevent DNA degradation. Following isolation, the integrity of the DNA was visually inspected using gel electrophoresis. Additionally, DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmingtion, DE, USA) and diluted in 1 x PBS to a final concentration of 0.2 µg/ml. Only structurally intact DNA samples (i.e. one non-fragmented band on the gel) with an absorption coefficient 260/280 nm >1.8 were used for damage analysis.

CPD lesions were detected by using the TDM-2 primary antibody (Cosmo Bio, Tokyo, Japan) 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. DNA samples were loaded in triplicate and the mean value was used as the damage level for the 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. In addition to the triplicates of the DNA standard and the isolated DNA samples, a triplicate blank (1x PBS) was included in all assays. Background fluorescence was excluded by subtracting the average blank OD values from the average OD values of 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 and arcsine transformed before statistical analysis.

Statistical analysis

The total number of line crossings and the mean scores for the depth and distance from shelter were analysed using repeated two-way ANOVA's to account for both parts of an observation period ("baseline" and "food") in each of the three light treatments ("UV+", "brightness control" and "UV-"). The change in activity of fish between the "baseline" and "food" periods was calculated by subtracting the total number of line crossings in the "baseline" period from the total number of crossings in the "food" period, and then dividing the product from the total number of crossings in the "food" period. The change in activity was analysed using a one-way ANOVA. All analyses of variance were followed by post hoc tests to correct for multiple testing and to determine differences between treatments. Data for one fish in the UV- treatment were excluded due to a camera malfunction that led to an incomplete recording of the observation period. The amount of DNA damage in the fish that were in the "UV+" treatment and possible correlations with the other factors that were assessed (activity, depth in the tank and distance from shelter) were investigated using non-parametric spearman-rank correlations. Additionally, the amount of DNA damage that accumulated was compared to DNA damage of individuals of a closely related species (*P. moluccensis*), which were caught at Lizard Island.

Results

Experiment 1: Avoidance of UVR

P. amboinensis, UV+ versus UV-

The time *P. amboinensis* spent in a quarter of the experimental tanks varied significantly between the quarters (RM ANOVA: $F_{3,15}$ = 11,45, P = 0.0004) in the low dose (2.4 W*m^{-2}) experiment (Fig 3.3a). The relative time (+/- SD) spent in the "lower UV-"(0.35 +/- 0.09) quarter was significantly longer (Tukey's HSD, P < 0.02) than in the "upper UV-" (0.16 +/- 0.1) quarter. The difference between the "lower UV+" (0.31 +/- 0.12) and the "upper UV+" quarter (0.17 +/- 0.08) was not significant (Tukey's HSD, P < 0.052). Additionally, the "lower UV+" quarter was more often frequented than the "upper UV-" (Tukey's HSD, P = 0.04). Similarly, the time spent in the "lower UV-" quarter was significantly longer than in the "upper UV+" quarter (Tukey's HSD, P = 0.0016). Fish spent equal time in the "UV+" and "UV-" half of the tank (P = 0.59, Table 3.1) at a low dose of UVB (2.4 W*m^{-2}). When exposed to a higher dose of UVB (5.2 W*m^{-2}), there was no longer a clear preference for the deeper areas (Fig 3.3b), however fish spent slightly more time (Tukey's HSD, P = 0.048) in the "lower UV+" (0.31 +/- 0.12) compared to the "lower UV-" quarter (0.24 +/- 0.09). This is also evident by the significantly larger proportion of time spent in the "UV+" half compared to the "UV-" half (P = 0.035, Table 3.1) of the tank.

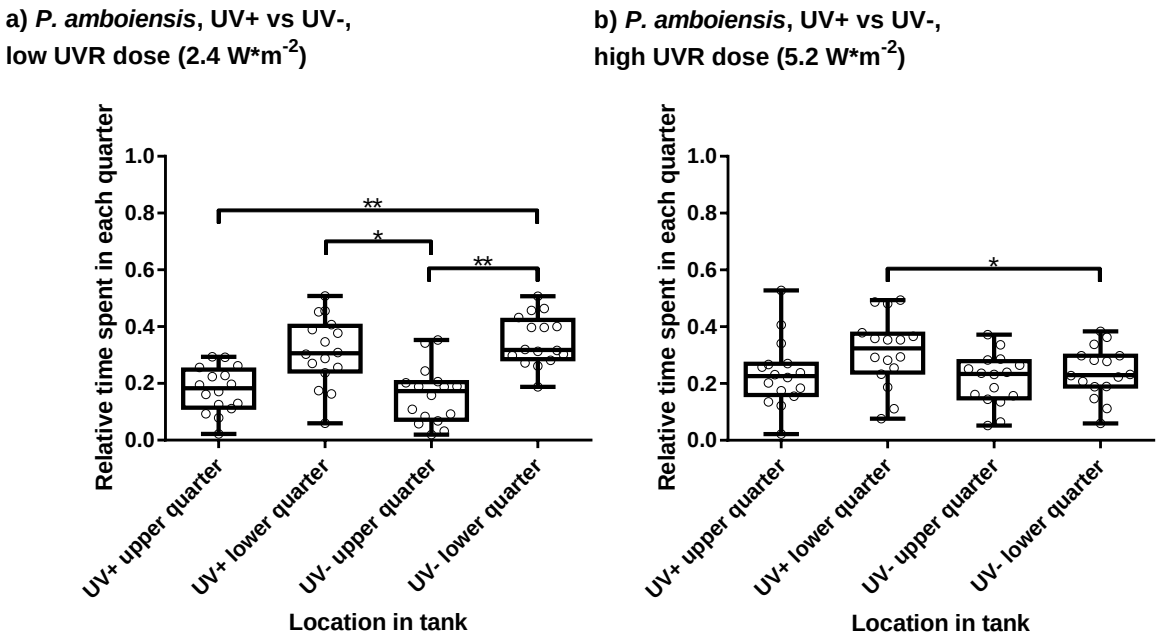


Figure 3.3: The relative time fish spent in each quarter of the experimental tank. Each circle represents the mean time of one individual in the respective quarter. The error bars indicate the standard deviation. The irradiance of UVB in the "UV+" half measured at water surface was a) 2.4 W*m^{-2} in the low dose experiment and b) 5.2 W*m^{-2} in the high dose experiment. Asterisks: *: 0.05 > P > 0.01; **: 0.01 > P > 0.001

P. amboinensis, UV+ versus UV-, brightness controlled

Similar to the results of the previous experiments that were conducted under a low dose of UVR, the time that *P. amboinensis* spent in each quarter of the tank when the number of photons was balanced between the "UV+" and "UV-" compartments varied significantly (RM ANOVA: $F_{3,11}$ =, P = 0.0001). In this instance, the UV dose was also relatively low at 0.7 W*m^{-2}. The fish spent relatively more time (+/- SD) in the "lower UV+" (0.45 +/- 0.09) and "lower UV-" (0.36 +/- 0.08) quarters (Tukey's HSD, P < 0.001) than in the respective quarters in the upper half of the tank ("upper UV+" quarter: 0.11 +/- 0.08; "upper UV-" quarter: 0.08 +/- 0.04). Additionally, the "lower UV+" quarter was more often frequented than the "upper UV-" quarter (Tukey's HSD, P = 0.0001). Similarly, time spent in the "lower UV-" quarter was significantly longer than in the "upper UV+" quarter (Tukey's HSD, P = 0.0005) and the "upper UV-" quarter (Tukey's HSD, P = 0.0001). A significant preference for the "UV+" half of the tank was observed (P = 0.006, Table 3.1). When exposed to a higher dose of UVB (1.7 W*m^{-2}), a clear preference for deeper areas of the tank was no longer visible (Fig 3.4b), and the relative time that fish spent in all areas of

the tank did not differ significantly between the four quarters. Contrary to the lower dose experiment, no significant difference in the time that fish spent in either the "UV+" or "UV-" half of the tank (P = 0. 67, Table 3.1) was found when fish were exposed to a high dose of UVB.

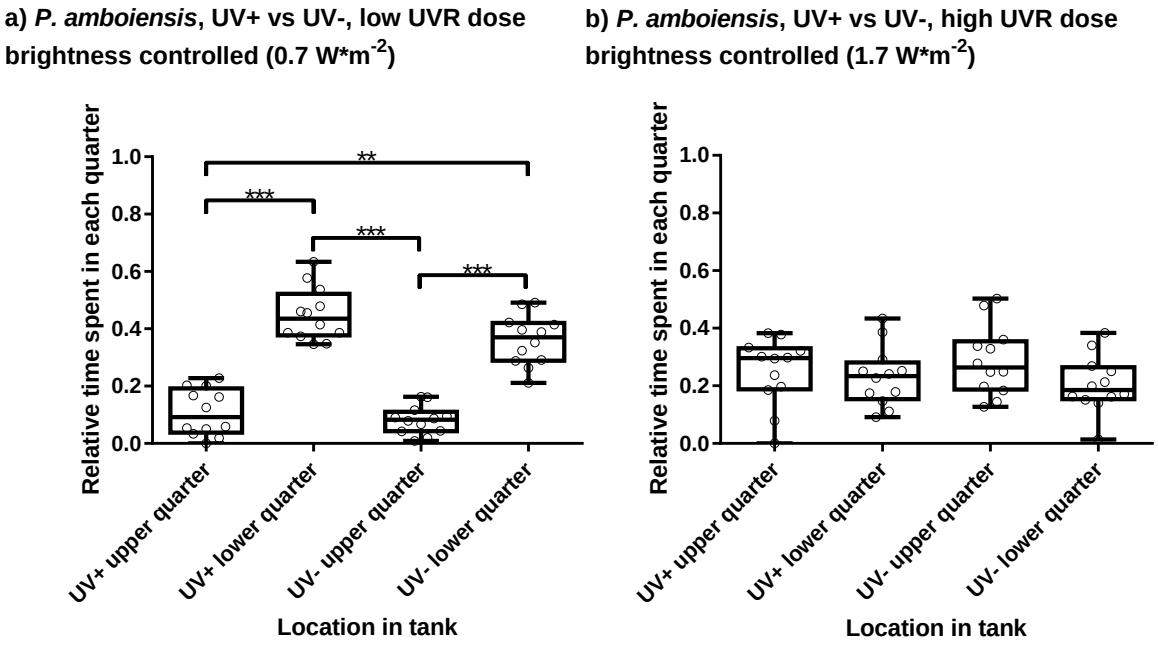


Figure 3.4: The relative time fish spent in each quarter of the experimental tank is shown. Each circle represents the mean time of one individual in the respective quarter. The irradiance of UVB in the "UV+" quarter measured at water surface was a) 0.7 $W*m^{-2}$ in the low dose experiment and b) 1.77 $W*m^{-2}$ in high dose experiment. The error bars indicate standard deviation, and the asterisks show the significance level: **: 0.01 > P > 0.001; ***: 0.001 > P > 0.0001.

T. lunare, UV+ versus UV-

No significant difference in the relative time that fish spent in the four quarters of the experimental tank was detected for *T. lunare*, irrespective of the UV dose. Again, a significant preference (P = 0.04, Table 3.1) for the "UV+" half of the tank was observed at a low dose of UVB.

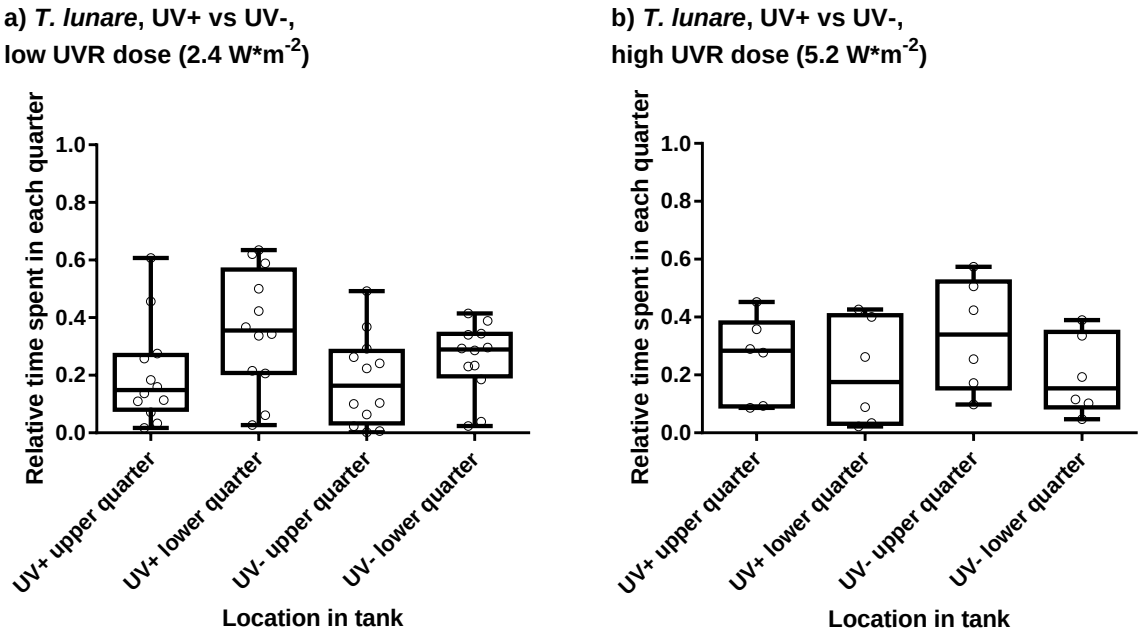


Figure 3.5: The relative time *T. lunare* spent in each quarter of the experimental tank. Each circle represents the mean time of one individual in the respective quarter. Error bars indicate standard deviation. The irradiance of UVB in the "UV+" quarter measured at water surface was the same as for *P. amboinensis*: a) 2.4 W*m^{-2}in the low dose experiment and b) 5.2 W*m^{-2} in high dose experiment.

Table 3.1: The mean time (+/- SD) that all fish spent in the "UV+" or "UV-" half of the tank. Significant P values of a two-tailed t- test are highlighted in bold letters and indicate whether the "UV+" section of the tank was frequented more often than chance (0.5). Fish did not avoid the UVB exposed sections of the tank, since the means in significant comparisons were larger than 0.5.

Experiment	UVB dose	Species	N	UV+	UV-	P(UV+ vs 0.5)	UVB avoidance?
UV+ vs. UV-	**Low**	***P. amboinensis***	12	0.56 (0.06)	0.44 (0.06)	**0.006**	no
Brightness controlled	**High**		12	0.51 (0.14)	0.49 (0.1)	0.67	no
UV+ vs. UV-	**Low**	***P. amboinensis***	16	0.49 (0.08)	0.51 (0.07)	0.59	no
Brightness not controlled	**High**		16	0.55 (0.08)	0.45 (0.08)	**0.035**	no
	Low	***T. lunare***	12	0.56 (0.09)	0.44 (0.09)	**0.04**	no
	High		6	0.46 (0.08)	0.54 (0.08)	0.348	no

Experiment 2: Activity and feeding behaviour under UVR exposure

Activity

The activity of juvenile *P. amboinensis*, measured by the number of line crossings was significantly influenced by the light treatments (RM-ANOVA: $F_{2,32}$ = 8.119, P = 0.0014). Activity was significantly reduced in the ”UV+” group compared to the “UV-” and “brightness control” groups (Holm-Sidak post-hoc test, adjusted P for multiple testing: 0.0002 and 0.0001, respectively; Fig. 3.6a) in the observation period “food”. The addition of the artemia food particles itself did not have a significant effect on the activity of fish ($F_{1, 32}$ = 0.05233, P = 0.8205), however the interaction term was significant ($F_{2, 32}$ = 4.543, P = 0.0183). The change of relative activity between the two observation periods “baseline” and “food” was significantly lower in fish that were in the “UV+” treatment (ANOVA: $F_{2, 32}$ = 5.340, P = 0.01; Dunnett’s Post hoc adjusted P for multiple testing: < 0.05; Figure 3.6b) compared to both the groups that were shielded from UVR (“UV-“) or exposed to the brightness control treatment.

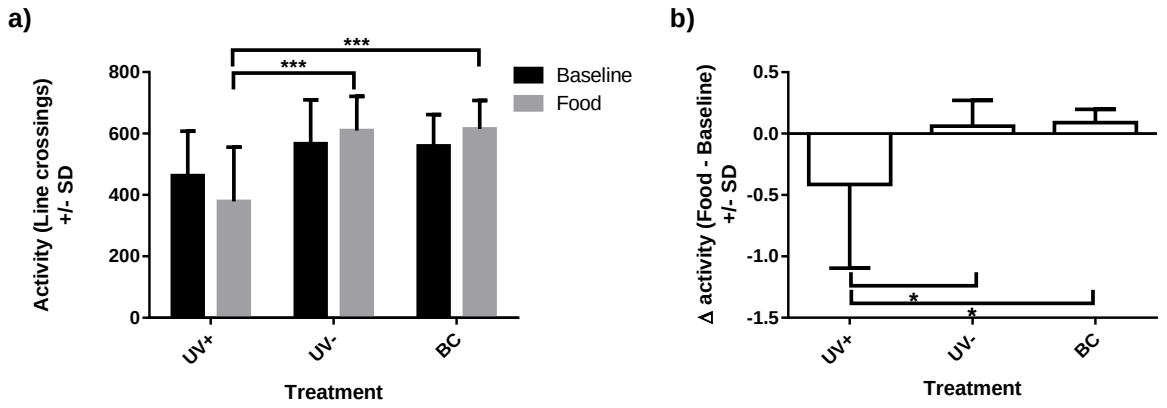


Figure 3.6: The activity of fish under different light treatments during the observation periods. a) The total number of line crossings of *P. amboinensis* for each light treatment. b) The relative change in activity between the “baseline” and the “food” observation periods. The error bars indicate the standard deviation while the asterisks give the significance level: *: 0.05 > P > 0. 01; ***: 0.001 > P > 0.0001. BC = Brightness control.

Distance from shelter and depth in tank

The depth at which fish were observed (Fig 3.7a) did not differ significantly between light treatments (RM-ANOVA: $F_{2,32}$ = 2.332, P = 0.113) and the effect of food was also not significant (RM-ANOVA: $F_{1,32}$ = 0.1846, P = 0.6703), with no significant interaction being detected.

The mean distance of fish from shelter did not differ significantly between the light treatments (RM-ANOVA: $F_{2,32}$ = 0.4419, P = 0.647), but was significantly affected by the addition of food (RM-ANOVA: $F_{1,32}$ = 12.64, P = 0.001), with the position of the fish in the "UV+" treatment being significantly closer to the shelter when food was present (Sidak's multiple comparison test, P > 0.05).

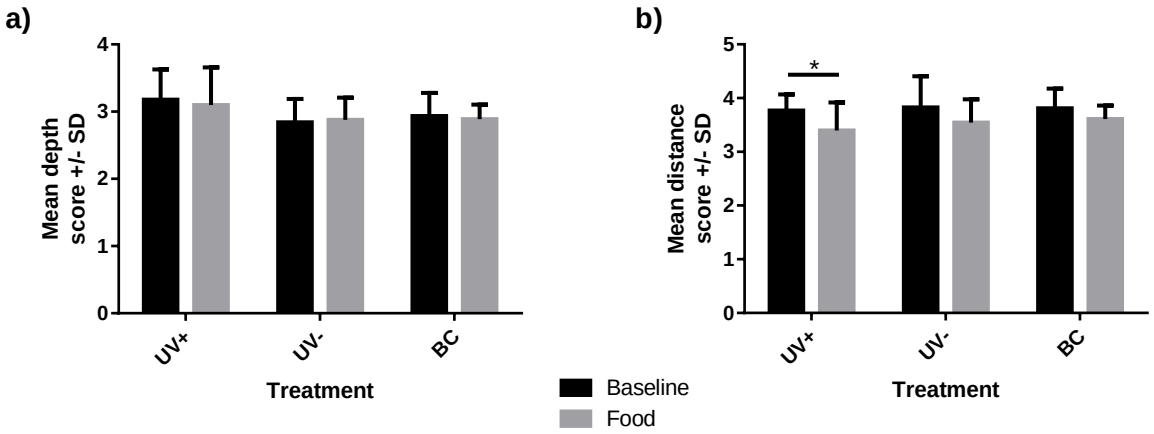


Figure 3.7: The mean depth (a) and mean distance from the shelter (b) at which the fish were observed in the tank +/- standard deviation. The asterisk indicates a significance level of 0.05 > P < 0. 01. BC = Brightness control.

DNA damage of fish in the "UV+" treatment

The mean UV-induced DNA damage was significantly higher (t-test, t = 6.973, df = 20, P = 0.0001) in juvenile *P. amboinensis* (1.031 +/- 0.125 SEM, n =12) in the "UV+" treatment compared to the net DNA damage in the closely related *P. moluccensis* of this size at Lizard Island (see chapter 2, 0.065, +/- 0.139, n = 10). The DNA damage that had been induced during this experiment was also significantly higher (Mann Whitney U, P = 0.0003, U = 0) than the net DNA damage in larvae of *P. amboinensis* (0.089 +/- 0.025 SEM, n = 5) from Lizard Island. The individual DNA damage did not correlate significantly with any of the factors (total line crossings, depth in the tank, distance to shelter) in separate Spearman rank correlations (not shown).

Discussion

UV avoidance has been proposed as an important mechanisms for the control of UV exposure (Kelly & Bothwell 2002, Leech & Johnsen 2003, Ylönen et al. 2004) and thus also the control of UV damage. Here, this hypothesis is tested in two species of reef fish, one with the ability to see UV light, *Pomacentrus amboinensis*, and one without, *Thalassoma lunare*. No direct evidence was found for UV avoidance as neither species avoided the UV illuminated half in favour of the UV protected half of the experimental arena. In a second experiment it was found that foraging and general activity patterns of juvenile *P. amboinensis* were indeed influenced under light conditions with UVR compared to treatments that lacked UVR.

Experiment 1: Avoidance of UVR

The results of two separate behavioural experiments, and with two different doses of UVB, show that neither *P. amboinensis* nor *T. lunare*, two species of reef fish that differ in their visual abilities in the UV range (Siebeck & Marshall 2000, 2001), display a specific avoidance response to UVR. As *P. amboinensis* can see UV light, an additional control had to be conducted in which the brightness of the two light environments (UV+ and UV-) was equalised in order to control for the possibility that the fish showed a response to the brightness rather than spectral composition of the light. No difference in the use of the two compartments was found which makes a visually triggered, wavelength specific avoidance behaviour in this species unlikely.

Rather than showing a preference for the "UV-" compartment, both species spent more time in the "UV+" section of the tank, therefore preferring an environment that was exposed to damaging levels of UVB. Irrespective of the dose used, and under conditions that differed in the number of photons (i.e. brightness not controlled), *P. amboinensis* spent more time in the "UV+" halves of the tanks. The irradiance of UVB used (5.2 $W*m^{-2}$) is comparable to levels of UVB measured at water surface on Lizard Island during the austral summer, which can reach up to 7 $W*m^{-2}$ (chapter 2, Dayseries). Exposure to a similar dose of artificial UVR led to a significant increase in DNA damage in both species of fish (chapter 4). The absence of a clear avoidance behaviour in the current experiment suggests that a "recognition" or "classification" of the UV environment as damaging or dangerous does not take place, or if such a categorization does take place, no avoidance action is taken. Conversely, under conditions that did not differ in the number of photons between the "UV+" and "UV-" compartments of the tank, *P. amboinensis* preferred the "UV+" compartment during the lowest UVB irradiance (0.7 $W*m^{-2}$) used in this study, which is naturally occurring in their habitat (chapter 2). The proportion of UVR is higher at crepuscular conditions at

dusk and dawn (Zepp et al. 2008) and coincides with times when courtship and territorial interactions have been observed in this species (Thresher 1984). Compared to the case of *P. amboinensis* which preferred the exposed compartment at two extremely different doses of UVB, *T. lunare* showed a preference for the "UV+" compartment at an intermediate irradiance of 2.4 $W*m^{-2}$. These results suggest that fish with UV vision, i.e. *P. amboinensis*, do not avoid potentially dangerous UVR levels under the experimental conditions used here. This stands against the general notion that fish with UV sensitive visual systems avoid UVR (Leech & Johnsen 2003), as shown by Ylönen (2004) and Kelly (2002) in experiments that were not controlled for brightness.

On the other hand, *P. amboinensis* displays a depth seeking behaviour that is unspecific to the presence or absence of UVR, since the preference for deeper areas of the tank was visible in both compartments of the experimental setup, irrespective of whether the area of the tank was exposed or protected to UVR or controlled for in brightness. However, this behaviour was only present at comparatively low doses of UVR within the experimental regime (i.e. the two experiments that were either controlled for in brightness or differed in this aspect). Since the experimental sequence of testing fish in high and low doses of UVR was randomized, a sequence effect or habituation to one level or to the other can be excluded. A possible explanation of this phenomenon could be the different origin of the fish that were used in the two experiments that differed in brightness. Fish used in the experiment where brightness was equal in both compartments were sourced from the commercial aquarium trade, and those fish used in the experiment where brightness did differ were caught at Lizard Island. The time that individuals of the latter group spent in captivity (four to five days in total) was considerably less than fish sourced from the aquarium trade (several weeks). Fukunishi showed that wild sea bream had a higher tolerance to exposure to high doses of UVB (2013b) than hatchery- sourced fish. Whether longer time in captivity is responsible for the observed differences in behaviour to UVR exposure, similar to changes in sunscreen defence (Zamzow & Siebeck 2006), needs to be examined in detail in the future.

Nevertheless, this type of depth seeking behaviour can be considered as an unspecific form of UV avoidance behaviour since deeper areas generally contain less UVR (Jerlov 1976). This behaviour was not displayed by *Thalassoma lunare*, a species that in contrast to *P. amboinensis* does not have UV vision (Siebeck & Marshall 2001). Since the natural sunscreen defence is lower in *P. amboinensis* compared to *T. lunare* (chapter 4, (Zamzow & Siebeck 2006) but the *in-situ* rates of UV-induced CPDs do not differ between these two species (chapter 2), depth seeking behaviour in order to avoid harmful UVR could provide *P. amboinensis* with a distinct advantage in the prevention of UV- induced DNA damage or at least achieve the same level of DNA damage as *T. lunare*. This depth seeking behaviour could have a significant impact on the habitat choice and

survival (Olson & Mitchell 2006, Olson et al. 2008) of fish that live in deeper habitats. Further, as a species that uses UV-reflecting facial signals in order to communicate with conspecifics (Siebeck 2004, Siebeck et al. 2010), deeper habitats with less UV could mean a less effective use of this secret communication channel (Parker unpubl.), if UVR do not increase. However, considering the importance of UV facial signals displayed by *P. amboinensis* in territorial interactions, it might not be a preferred option of individuals to avoid UVR and trade off less UVR in deeper water against reduced communication. As the combined impacts of climate change, which in turn are predicted to lead to higher levels of UVR (Watanabe et al. 2011), increase pressure on coral reef systems and their inhabitants at critical points of their lifecycle (Munday et al. 2008, Munday et al. 2010, Siebeck et al. 2014), the altered depth for the preferred habitat could also impact community structure of reef inhabitants and their predator-prey relationships (Tucker & Williamson 2011). The disruption of the depth seeking behaviour of *P. amboinensis* at higher doses of UVR indicates that behavioural changes under future climate scenarios with higher UVR in the marine environment are possible.

Experiment 2: Activity and feeding behaviour under UVR exposure

In juvenile *P. amboinensis* that have recently settled in the coral reef environment, it was found that exposure to a high dose of UVB radiation led to reduced foraging and a closer association with the shelter. The UV dose used in this experiment occurs at the study location at the water surface at midday (chapter 2). These conditions seem extreme, but shallow reefs in the Lizard Island lagoon can be exposed for several hours during spring tides. These results clearly highlight that UVR affects the behaviour of a reef fish and is another example of the negative effects of UVB rich light on marine organisms. Altered behaviour under UVR has also been found by Holtby and Bothwell (2008) who showed a reduction in feeding in juvenile coho salmon during UVR exposure. In their experiments, the UVA waveband was thought to be responsible for the reduced number of feeding strikes. In this study the UVB waveband was mainly responsible for the reduction in activity and the closer association with a shelter since the measured behavioural parameters in the "brightness control" treatment, which contained a significant proportion of UVA, did not differ from the "control" treatment.

Conversely, the presence of UVR, specifically the less energetic and less dangerous UVA, has also been implicated to enhance foraging of juvenile and larval Damselfish (Job & Bellwood 2007) which have UV transparent ocular media early in their development (Siebeck & Marshall 2007). UV light has been suspected to positively influence the abundance of *Chromis viridis* (Ponton et al. 2012) in its coral reef habitat. No negative effects of UVA exposure were reported in the studies by

Job and Bellwood (2007) or Ponton et al. (2012). However, their experimental conditions either lacked UVB radiation (Job & Bellwood 2007), or were not addressed (Ponton et al. 2012). Since UVB reaches the depth (chapter 1) where pre- and post-settlement stage larvae and juvenile *P. amboinensis* can be found (Leis 1991, Leis et al. 1996, Leis 2004), the present results indicate the trade off from UVR exposure on these fish: with a decrease in feeding activity under high UVB fluxes and the induction of DNA damage on one side, and the facilitation of communication (Siebeck 2014) and prey detection (Job & Bellwood 2007) in the UVA waveband on the other.

The complex relationship between communication using a "secret channel" of the available sunlight and the negative effects of UVB suggest that a trade off exists between communication and the need for protection. It appears that *T. lunare* and indeed *P. amboinensis* do not actively avoid UV-exposed areas in their environment, possibly due to a lack of detection ability by the former and the need to use the available UVR for communication by the latter. At the same time, acute UVB exposure can decrease activity during feeding and increase DNA damage, a clear indicator that protection using other means (such as sunscreens) is needed for reef fish. Future studies should address where the balance lies between successful communication between individuals and the exposure to levels of UVR in the environment that lead to behavioural changes such as depth seeking behaviour and reduced activity. Further, it needs to be determined at which UV levels communication breaks down in order to better understand the impacts that rising UVR levels on the Great Barrier Reef could have on reef fish such as *P. amboinensis*.

CHAPTER FOUR

Mycosporine-like Amino Acids provide a rapid response mechanism for UV protection in some reef fish

Abstract

Mycosporine-like Amino Acids (MAAs) are ultraviolet (UV) - absorbing compounds commonly found in marine organisms and the external mucus of fish. MAAs are thought to act as sunscreens by preventing the damaging effects of UV radiation, however, direct evidence for their protective role has been missing. This study shows that MAAs provide UV damage protection in two species of reef fish, *Pomacentrus amboinensis* and *Thalassoma lunare*. For both species, the amount of UV induced DNA damage sustained following the exposure to a one hour pulse of high UV radiation was negatively correlated with mucus absorbance, a proxy for MAA concentration. Furthermore, a rapid and significant increase in MAA concentration was observed in *P. amboinensis* following UV exposure. 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.

Introduction

Ultraviolet radiation (UVR, 280 - 400 nm), specifically short-wavelength UVB radiation (280 - 315 nm) causes damage to the DNA such as the formation of cyclobutane pyrimidine dimers (CPDs) between adjacent pyrimidine bases (Setlow 1962) leading to structural changes in the DNA double helix, which can inhibit polymerases thus arresting replication and transcription of the DNA sequence (Mitchell et al. 1989). If left unrepaired, UV-induced DNA damage can lead to mutations (Ikehata & Ono 2011) and apoptosis of affected cells (Browman et al. 2003, Lo et al. 2005). In fish, the effects of UVR exposure include damage to tissues of the skin (Armstrong et al. 2002) and even the brain (Vehniäinen et al. 2012), increased mortality (Dong et al. 2007, Fukunishi et al. 2013a, 2013b) and behavioural changes in trout (Alemanni et al. 2003) and salmon (Kelly & Bothwell 2002).

In the tropics, where levels of UVR are among the highest on Earth (Banaszak & Lesser 2009), the clear and shallow waters around coral reefs allow UVR to penetrate farther than in other aquatic ecosystems (Jerlov 1976, Dunne & Brown 1996) leading to a high potential for UVB induced DNA damage. Due to changes in ozone levels (Wild et al. 2005), aerosols, green house gases and cloud cover (Masiri et al. 2008, Watanabe et al. 2011) and loss of coral shelter due to increased cyclone intensity (Knutson et al. 2010) UVR around coral reefs is likely to continue to increase (Watanabe et al. 2011).

Protection from harmful UVR in marine organisms can arise from physical barriers (e.g. shells and scales) and from UV-absorbing compounds like carotenoids (Mathews-Roth 1997) and Mycosporine-like amino acids (MAAs; (Shick & Dunlap 2002). 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 (Karentz 2001). This variety of MAA compounds (Rosic & Dove 2011) is synthesized by microbes, fungi and plants via the shikimate pathway (Bentley 1990) and alternatively the pentose phosphate pathway (Balskus & Walsh 2010, Spence et al. 2013). Although some of the genes from the shikimate pathway have been found in the sea anemone *Nematostella vectensis* (Starcevic et al. 2008) and corals (Shinzato et al. 2011b) it is generally thought that MAAs cannot be synthesized by animals (Bentley 1990) and are likely of dietary origin (Karentz et al. 1991). MAAs can be found in fish eggs (Plack 1981), larvae (Lesser et al. 2001) and in the ocular media and external mucus of reef fish (Dunlap et al. 1989, Zamzow & Losey 2002).

MAAs are widely recognized to act as sunscreens due to their absorbance properties and the tissues in which they are found (Karentz et al. 1991, Bandaranayake 1998, Karentz 2001, Banaszak & Lesser 2009), and their ability to prevent sunburn when topically applied to the skin of mice (de la Coba et al. 2009). MAAs have been shown to protect against cleavage delay in sea urchins (Adams & Shick 1996) and there is circumstantial evidence that MAAs may also have a protective function in reef fish (Eckes 2009). MAA concentration in the mucus, and hence the mucus absorbance of UVR, correlates with the levels of UVR levels in the fishes' habitat. Therefore, mucus absorbance is often used as a proxy for overall MAA levels (Zamzow 2007, Eckes et al. 2008). 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 (Zamzow 2004).

The following experiments test the sunscreen hypothesis, specifically that a higher level of MAA protection leads to reduced UV- induced DNA damage. To achieve this, fish with different levels of the mucus UV absorbance (i.e. MAA levels) were exposed to a high pulse of UVB radiation in order to induce UV-specific DNA damage in the skin. If MAAs indeed acted as sunscreens, it can be expected to find higher DNA damage (CPDs) in fish exposed to the "UVB pulse" that had lower MAA concentrations in their mucus.

Methods

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. 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 plastic sealable bag with little seawater and gently placing the bag on a plastic 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 (2006) 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.).

Holding conditions

Test 1 – natural MAA levels: All fish were randomly assigned to the following treatments and holding conditions. Thirty *P. amboinensis* and twenty *T. lunare* were subjected to the treatments of 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 MAA 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. The aim of this was to manipulate mucus MAA levels to increase their variability within each species. This was done so that UV-specific DNA damage following the UVR treatment could be analysed in relation to a wider range of mucus MAA levels. Previous studies showed that both, MAA-rich food and exposure to UV is required to maintain high mucus UV absorbance (Zamzow 2004). Here all fish were fed the same diet (MAA rich food (see below and Fig. S 4.1) and relied on the presence/absence of UV for the manipulation of mucus MAA levels. The fish were randomly assigned to a plastic tank (same dimensions per species as above) which was linked to the seawater

flow-through system of LIRS and contained a small PVC pipe that served as shelter. *Pomacentrus amboinensis* were either shielded from natural sunlight (inside an air-conditioned aquarium room of LIRS) or exposed to natural sunlight, with equal number of fish (n = 10) being held in each condition. The aquaria exposed to natural sunlight were placed on 2 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 in the tanks in each holding condition was recorded every 15 minutes by an immersed HOBO Datalogger (Onset Computer Corporation, Bourne, MA, U.S.A.) in an additional tank. Mean temperature in the tanks outside was 29.53 °C (SD ± 1.57), which was slightly higher than in tanks inside (28.79 °C, SD ± 0.59) and the water temperature in the Lizard Island Lagoon (28.52 °C, SD ± 0.24; measured by the AIMS float at 0.6m, data provided by Integrated Marine Observing System).

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 (Carefoot et al. 2000). 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 4.1).

Experimental treatments (UV exposure challenge)

The exposure to one of three light treatments (see below) was conducted inside one of the aquarium facilities at LIRS. Each treatment 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, ten *P. amboinensis* were challenged with exposure to a high dose of UVB radiation (treatment "UVB +"). Ten control individuals (treatment no UVB "UVB -") were exposed to the fluorescent lights of the room only and handled in the same way as the treatment fish (Fig. 4.1). In order to ensure that any changes in UV-induced DNA damage and mucus absorbance levels that might be observed 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, 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, and five individuals each to the "UVB +" and "UVB -" treatment after ten days in captivity. 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 (see below).

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) of $7.74*10^{15}$ $photons*cm^{-2}*s^{-1}$ was detected at the water surface. 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. 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. No UVR (UVA and UVB) was present in this treatment, and the photon flux (280 – 700 nm) was measured at $1.08*10^{14}$ $photons*cm^{-2}*s^{-1}$.

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 no UVR could be detected with the Jaz spectrometer. The photon flux (280 – 700 nm) in the "brightness control" treatment was $7.48*10^{15}$ photons*cm^{-2}*s^{-1} (Fig. 4.1).

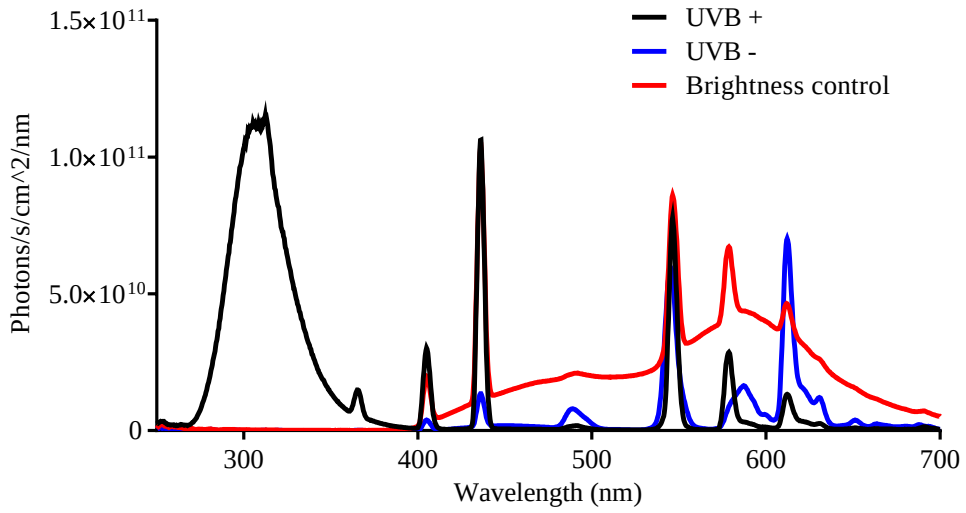


Figure 4.1: Photon flux in the light treatments of the experiment. Note the large proportion of UVB (280 – 315 nm) radiation in the "UVB +" compared to the "UVB –" treatment. This also required a larger proportion of visible light in the "brightness control" treatment to equalise overall photon flux. Thick line: "UVB +", dashed line: brightness control, thin line: "UVB –".

Mucus absorbance analysis

Mucus was taken from both flanks of the fish using a dull scalpel blade (Zamzow & Losey 2002) 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 (2002). 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 (Zamzow & Losey 2002, Zamzow & Siebeck 2006, Zamzow et al. 2013). 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 eventual 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. Since the mucus not only contains MAAs, but also a suite of endogeneous proteins and lipids (Shephard 1994) and external compounds such as bacteria (Carreto & Carignan 2011) which could all possibly add to the absorbance of the mucus in the UV range, the measurement of "sunscreens" in the mucus using the technique described here includes all possible compounds. Therefore the term "sunscreens" in this chapter refers to UV absorbing compounds (UVAC).

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. (1991).

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.

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.

Linear regressions (and in the case of *T. lunare* an additional Spearman-rank correlation) were used to test whether decreased mucus absorbance was linked to higher amount of UV-induced DNA damage. GraphPad Prism 6 and JMP 10 were used in this analysis.

Results

Pomacentrus amboinensis

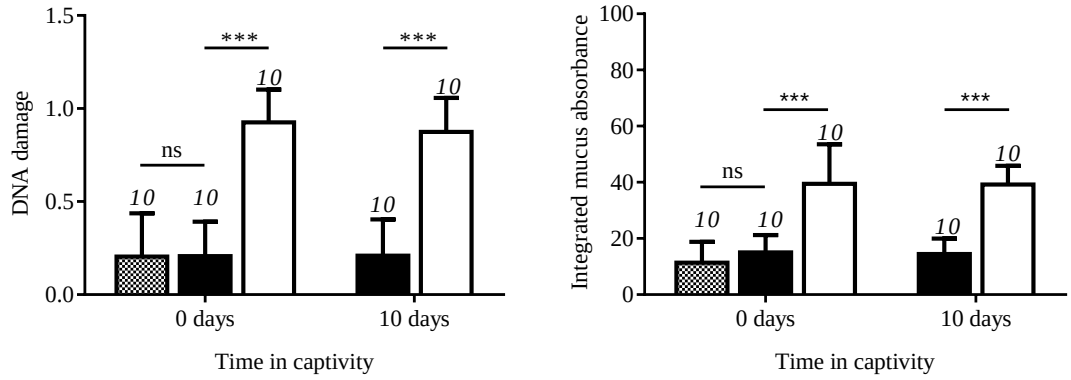
The treatments of the UV challenge had a highly significant effect on DNA damage (ANOVA, $F_{1,36}$ = 139.9, P < 0.0001) and the integrated mucus absorbance (ANOVA, $F_{1,36}$ = 47.22, P < 0.0001). Both measured variables were significantly higher in the "UVB +" compared to the "UVB-" treatment (Dunnett's, P < 0.0001, Figs. 4.2a and 4.2b). The time the fish spent in captivity had no significant effect on DNA damage or integrated mucus absorbance (P > 0.05, Figs. 4.2a and 4.2b, and also Figs. 4.3a and 4.3b). 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. 4.2a) or mucus absorbance (ANOVA, $F_{1,18}$ = 1.43, P = 0.25, Fig. 4.2b) 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. 4.3a and 4.3b). The SD error (omitted in Fig. 4.3 for clarity) overlapped slightly between "UVB +" and "UVB -" fish at 0 days (Fig. 4.3a), and did not overlap at wavelengths > 290 nm after ten days in captivity (Fig. 4.3b). However, the "brightness control" and "control" treatments appeared very similar (Fig. 4.3a) and the SD error overlaps across the spectrum.

The DNA damage of fish was negatively correlated with the integrated mucus absorbance (Fig. 4.4a) in fish of the "UVB +" treatment (linear regression, n = 20, R^2 = 0.34, P = 0.007), but not in fish of the "UVB -" treatment (linear regression, n = 20, R^2 = 0.06, P = 0.3).

a) *P. amboinensis*: DNA damage

b) *P. amboinensis*: integrated mucus absorbance



c) *T. lunare*: DNA damage

d) *T. lunare*: integrated mucus absorbance

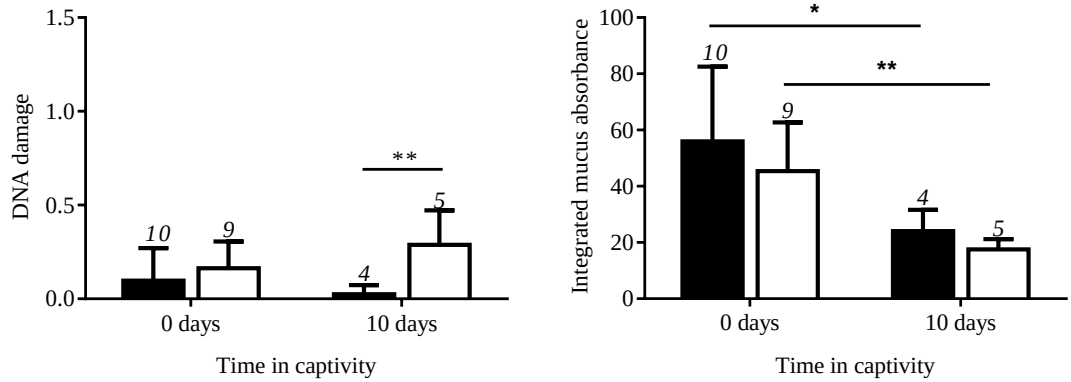


Figure 4.2: Relative DNA damage in skin samples (a,c) and integrated mucus absorbance (b,d) of *P. amboinensis* (a,b) and *T. lunare* (c,d) after exposure to one of three treatments ("UVB +", "UVB -" and "brightness control") measured within 24 hours of capture ("0 days") and after ten days in captivity ("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 (*: $0.01 < P < 0.05$; **: $0.001 < P < 0.01$; ***: $0.001 < P < 0.0001$).

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. 4.2c) 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. 4.2d). 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. 4.3c and 4.3d) 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. 4.3). 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 = 20, R^2 = 0.24, P = 0.07, Fig. 4.4b). No significant relation between DNA damage and integrated mucus absorbance was observed in "UVB –" animals (linear regression, n = 20, R^2 = 0.11, P = 0.25, Fig. 4.4b).

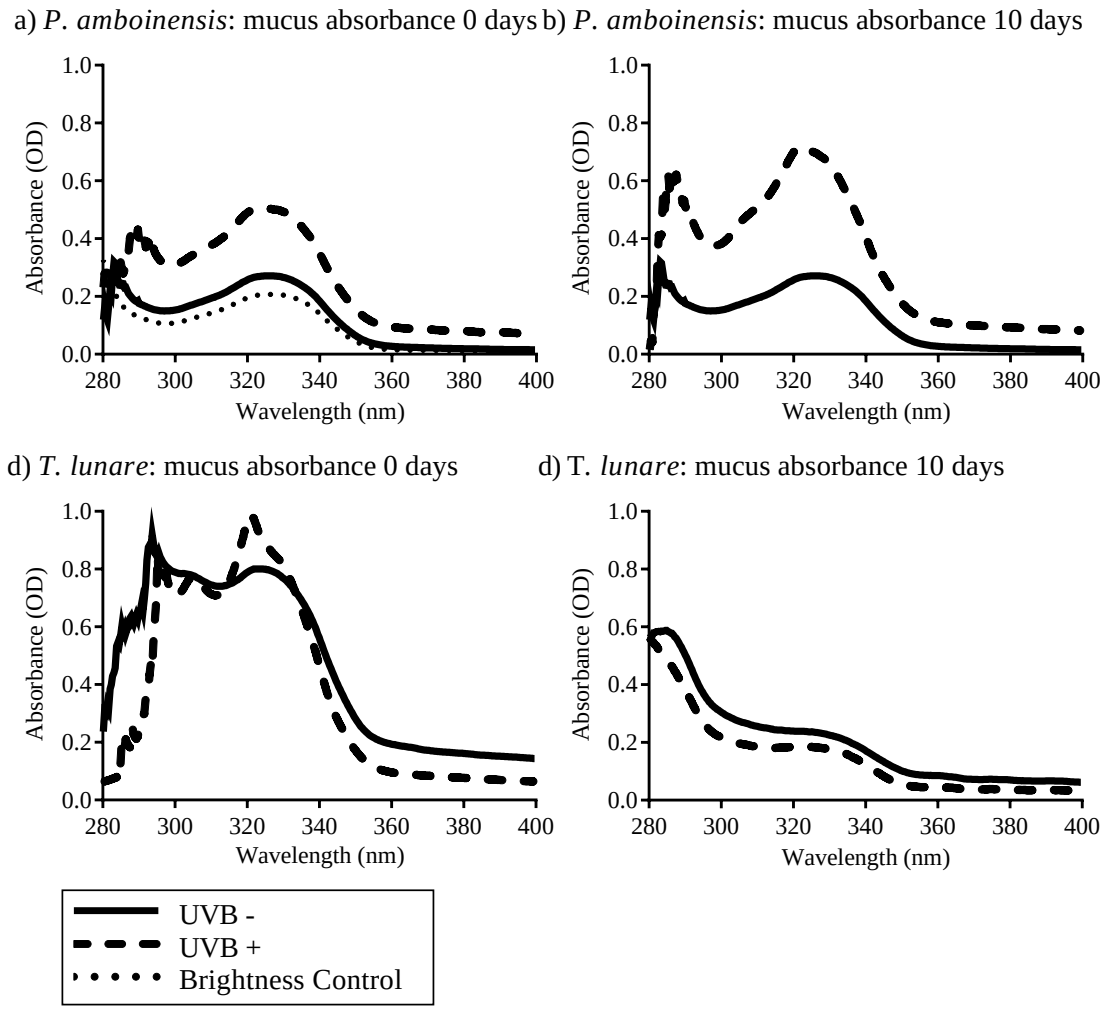


Figure 4.3: Mean absorbance curves of mucus samples of *P. amboinensis* and *T. lunare*. Mucus was sampled after exposure to one of the three treatments: "UVB -" (solid lines), "UVB +" (dashed lines) and in the case of *P. amboinensis*, the "brightness control" (dotted line). Mucus absorbance for *P. 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.

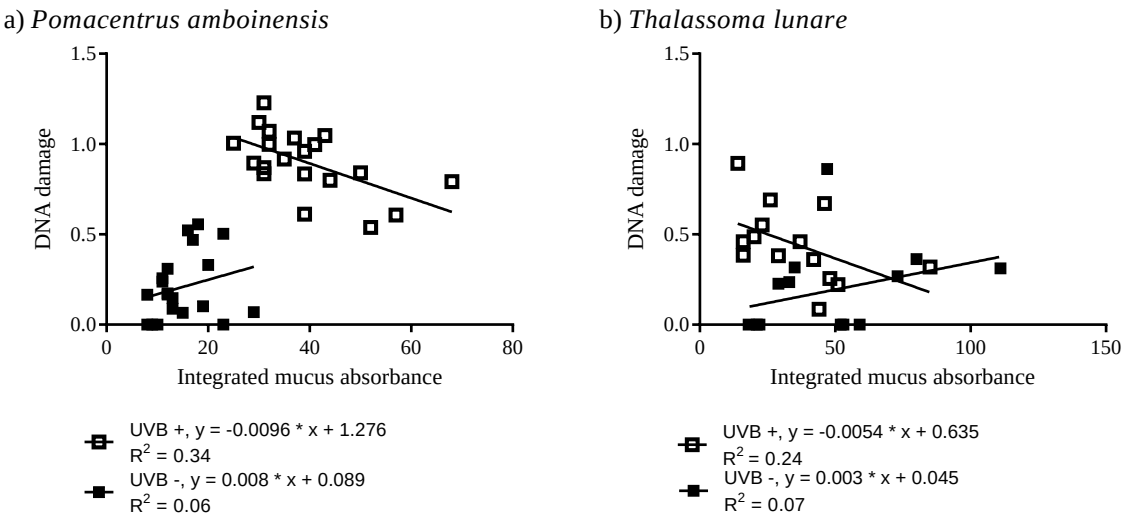


Figure 4.4: Relationship between DNA damage levels and mucus absorbance in fish exposed to various treatments a) DNA damage significantly increased with decreasing mucus absorbance in fish exposed to the UV pulse treatment (linear regression P = 0.007) but not in "UVB -" fish (P = 0.3). b) The linear regression for the "UVB +" exposed *T. lunare* was not significant (P = 0.07), however a Spearman-rho rank correlation was significant (P = 0.01). A linear regression for the "UVB -" group was not significant (P = 0.25). Open squares: "UVB pulse", solid squares: "UVB -".

Discussion

Mycosporine-like Amino Acids (MAAs) are widely recognized to act as sunscreens, however direct evidence for a protective function that actually reduces or mitigates the negative effects of UVR exposure in fish 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 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. Fish exposed to the "UVB –" treatments had variable levels of MAA levels but overall low DNA damage. Additionally it was discovered that in *P. amboinensis*, but not *T. lunare*, MAA quantity rapidly increased following a 1 hr pulse of UVB light, therefore potentially increasing the amount of MAA protection in their mucus.

Similar protective functions of MAAs have previously been shown only in invertebrates. Adams (1996) 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 (1998) 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.

The experiments were designed to manipulate MAA levels found in the external mucus of fish to achieve high variability in MAA levels prior to exposure to UV radiation. In particular, it was attempted to lower the mucus absorbance by exclusion of UVR in captivity, while keeping the diet rich in MAAs. After ten days, lower mucus absorbance was indeed achieved in *T. lunare*, which was in concordance with previous studies (Zamzow 2004, Zamzow & Siebeck 2006) and the general idea that UVR exposure is necessary for MAAs to be sequestered into the external mucus layer. 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 1hr UVB pulse certainly 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 at 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 (Zamzow 2004). 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 or a visible change in the shape of the absorbance curves in fish that were exposed to the brightness control treatment. Further, the observed increase of mucus absorbance in *P. amboinensis* within one hour of exposure is the fastest MAA related response described in a reef fish. Changes in mucus absorbance, mediated by MAAs after changes to the UVR regime, have been documented before (Zamzow & Losey 2002, Zamzow 2004, Zamzow et al. 2013), but usually are only detected after several days or weeks. Similarly, the accumulation of MAAs in algae (Karsten et al. 1999), diatoms (Helbling et al. 1996) and corals (Shick et al. 1999) due to UVB exposure occurs over longer time periods.

The lamps that were used to induce the DNA damage and the fish that changed their mucus absorbance were exposed to, had an uneven spectrum in the UV range, i.e. a spectrum with two peak emissions at 310 and 360 nm. Further, the lamp of the JAZ spectrometer used to measure UV absorbance in the mucus samples emits an even spectrum throughout the UV range, without any major peaks. It could be argued that the change in the mucus absorbance that was observed in P. amboinensis could have been influenced by an uneven distribution of light in the exposure treatments or the light present during the measurement of the samples. It seems most certain that UV exposure influenced the mucus absorbance, but is unlikely that the peaks of the UV lamps of the treatments biased the changes, since there is no correspondence 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. What speaks against a possible influence of the light source of the spectrometer on the measured mucus absorbance is clearly the fact that mucus samples of control animals and native fish from the field did not show such a change in their mucus absorbance.

The increase in mucus absorbance in *P. amboinensis* did not prevent higher levels of DNA damage compared to controls not exposed to UVR, but it is difficult to tell how much more DNA damage would have been induced by the exposure to the UVB pulse if this increase had not taken place. The formation of CPDs, the most common type of UV- induced DNA lesions (Sinha & Hader 2002), occurs nearly instantaneously upon irradiation with UV light (Schreier et al. 2007). Any protection by MAAs 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 (Regan et al. 1982, Olson & Mitchell 2006, Groff et al. 2010, Fukunishi et al. 2013b).

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 (Carefoot et al. 2000) and gut (Zamzow et al. 2013), 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.

MAAs 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 (Grutter 1996b, Marnane & Bellwood 1997) but certainly not fast enough to prevent the formation of CPDs. MAAs originating from bacteria with a functioning shikimate pathway transferred into fish mucus (Carreto & Carignan 2011) 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 (Shephard 1994) and also possesses antibacterial properties (Ebran et al. 1999, Subramanian et al. 2008), 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* (Siebeck & Marshall 2000, 2001), where mucus absorbance must be modulated otherwise (Zamzow 2004, Zamzow et al. 2013). For *P. amboinensis*, the ability to modulate the UV absorption properties of their external mucus layer might be essential in order to successfully send and receive their UV signals for communication (Siebeck 2004, Siebeck et al. 2010). *T. lunare* had an up to 63% higher mucus absorbance than *P. amboinensis*, confirming previous findings of family differences in MAA levels (Zamzow & Losey 2002).Insofar as this presents a trade off between communication with UV signals (at times of low UV and low MAA levels) and protection from UV radiation at times of high UV and high MAA levels is unknown and needs to be examined in detail.

Variable MAA protection could 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 (Harborne 2013). Changes in the UV regime over larger timescales and magnitudes facilitated by climate change (Masiri et al. 2008) are currently ongoing on the Great Barrier Reef. 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 (Hoegh-Guldberg et al. 2007) 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.

Supplementary Material

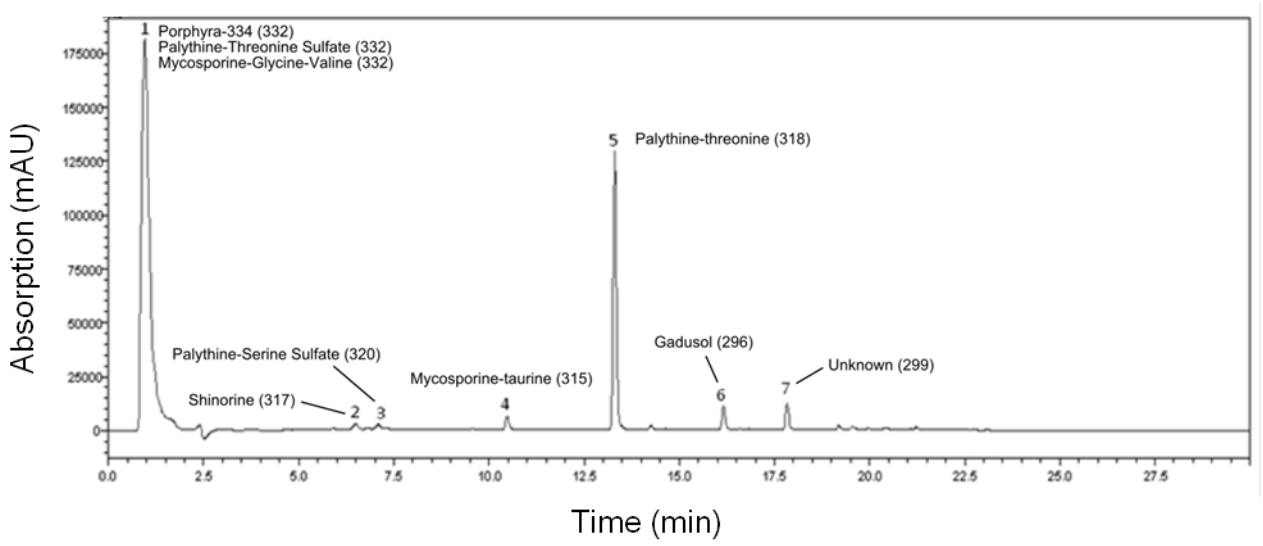


Figure S 4.1: HPLC-MS 330 nm chromatogram of methanol extracts from the red alga *Acanthophora spicifera* used in the food mix during the experiment. The peaks correspond to different MAAs and their wavelength of maximum absorption (in brackets). Peak identification was based on absorbance maxima, retention times and mass of $[M+H]^+$.

CHAPTER FIVE

Evidence for repair of UV-induced DNA damage in coral reef fish via photolyase and dark repair

Abstract

The ability to remove Cyclobutane-pyrimidine dimers (CPDs) from UV exposed DNA is crucial for the survival of organisms that live in UV rich environments. DNA repair via photoreactivation and dark repair was investigated in four species of reef fish, for which DNA repair has been poorly studied. For this approach, settlement stage larvae were used, a life stage which previously showed higher susceptibility to UVR than adult fish. Accumulation and removal of CPDs from skin and tissue samples was tracked using ELISA. All species examined (*Pomacentrus moluccensis*, *Pomacentrus amboinensis*, *Lethrinus variegatus* and *Siganus corallinus*) showed significant increases of DNA damage and mortality after exposure to UVR levels that were up to 120 % higher than ambient levels of UVR at water surface. Interspecific variation in the susceptibility to UVR was observed, with *L. variegatus* showing significantly higher damage levels than the other species. Significant reductions in DNA damage levels were found in *P. moluccensis* and *L. variegatus* that were exposed to photoreactivating light for up to eight hours after the initial damage accumulation. This strongly indicated the presence of an active photolyase system in reef fish. Evidence for dark repair was only found in one species (*P. moluccensis*), again highlighting differences in vulnerability to UVR between species. The high mortality of fish under increased UVR levels and the incomplete removal of CPDs suggest that future increases in UVR could seriously affect larvae of coral reef fish and that UVR protection must be achieved with the help of other mechanisms such as MAA sunscreens in order to explain low levels of DNA damage in samples from the field.

Introduction

Ultraviolet radiation (UVR, 280-400 nm), specifically short-wavelength UVB radiation (280-315nm) can cause damage to the DNA of exposed organisms. One of the main types of UV-induced DNA damage are cyclobutane pyrimidine dimers, CPDs, which form between adjacent pyrimidine (T or C) bases (Setlow 1962). CPD formation causes structural changes in the DNA double helix which can inhibit polymerases thus arresting replication and transcription of the DNA sequence (Mitchell et al. 1989). Unrepaired, UVB- induced DNA damage can lead to further mutations (Friedberg 2003) and apoptosis of affected cells (Browman et al. 2003, Lo et al. 2005). The negative effects of UVB exposure are well documented in the early developmental stages of aquatic organisms. They include delayed growth (Hernández Moresino et al. 2011), severe malformation and increased mortality (Dong et al. 2007). Additionally, behavioural changes (increased swimming: Alemanni et al.(2003), avoidance of UVR: Kelly & Bothwell (2002)) have been reported for salmonid juveniles, which can visually detect UVR as juveniles (Novales-Flamarique & Hawryshyn 1994, Deutschlander et al. 2001). Generally, the eggs and larvae of marine organisms seem to be more susceptible to the negative effects of UVB radiation than later life stages (reviewed by Bancroft et al. (2007).

In the tropics, where levels of UVR are among the highest on Earth (Banaszak & Lesser 2009), the clear and shallow waters around coral reefs allow UVR to penetrate further than in many other aquatic ecosystems (Jerlov 1976, Dunne & Brown 1996) leading to a high potential for UVB induced DNA damage. As an example, 10 % of UVB (315 nm) are still present at 4 m depth at the Lizard Island field site in the northern Great Barrier Reef (GBR) lagoon (chapter 2, Trawler Beach). Not surprisingly, many reef fish invest in UV-absorbing compounds (Shick & Dunlap 2002) like Mycosporine-like Amino Acids,(MAAs) which are thought to act as natural sunscreens (Eckes et al. 2008). However, despite the physiological costs of UV exposure, some reef fish, e.g. *Pomacentrus amboinensis*, rely on UV light as a means of communication (Siebeck 2004) and therefore do not block UV from entering their eyes (Siebeck & Marshall 2007) or from interacting with their skin. It thus follows that fish with UV communication could suffer higher rates of UV induced DNA damage than fish without such communication, unless these fish are able to compensate for the lack of MAAs e.g. through behavioural avoidance or efficient DNA repair.

The occurrence of CPDs triggers the activation of two main repair pathways (Sinha & Häder 2014) which either restore the original binding between bases (photoreactivation) or replace the damaged nucleotides (excision repair). Photolyase enzymes utilise UVA and blue light as energy donors to activate a cofactor (flavin-adenin dinucleotide, FADH-) which splits the dimer site and returns the

bases to their normal conformation (Sancar 2003). Photolyases are found in terrestrial and marine organisms ranging from archaea (Shirley 1996), invertebrates (Isely et al. 2009, Lamare et al. 2011) to most vertebrates (Thoma 1999, Sinha & Hader 2002), but not in placental mammals (Ley 1993, Yasui 1994). The other repair pathway commonly referred to as excision or dark repair, does not require the energy of photons (Bootsma & Hoeijmakers 1996). In this mechanism the damaged site is excised and undamaged bases are inserted. These repair processes are highly conserved in eukaryotes and involve a complex machinery of more than 30 proteins (Häder & Sinha 2005).

Both, photoreactivation and dark repair have been studied in marine organisms such as coral planulae (Reef et al. 2009), echinoderms (Lamare et al. 2006) and fish from polar (Malloy et al. 1997) and temperate (Regan et al. 1982, Vetter et al. 1999, Olson & Mitchell 2006) environments. The reduction of DNA damage levels by photoreactivation is usually greater than by dark repair (Sinha & Häder 2014), highlighting the importance of this pathway to aquatic animals from a diversity of phyla and habitats. Surprisingly, the repair pathways in coral reef fish larvae have not yet been studied, although larval fish are known to be exposed to high levels of UVR (Dunne & Brown 1996).

The aim of this study was to track the accumulation and removal of UVR- induced CPDs in the DNA of settlement stage coral reef fish larvae. It was hypothesized that both repair pathways were present in the four species of coral reef fish that were used in this experiment: *Pomacentrus moluccensis, P. amboinensis, Lethrinus variegatus* and *Siganus corallinus.* These species were chosen mainly due to their abundance in light trap catches during the study period, but are also representatives of different trophic niches: two small omnivore/planktivores with UV vision (*P. moluccensis* and *P. amboinensis*), a predator of small benthic invertebrates without UV vision (*L. variegatus*) and an herbivore, also without UV vision (*S. corallinus).*

Methods

Study location and sample collection

Settlement-stage larvae of *P. moluccensis*, *P. amboinensis*, *L. variegatus* and *S. corallinus* from light trap catches were donated by Dr. Jeffrey Leis. Light traps were deployed within 500 m of the beach at Lizard Island (14°40'44.74"S; 145°26'46.25"E) on the Great Barrier Reef, Australia in January 2012 (*P. moluccensis*, *L. variegatus* and *S. corallinus*) and November 2013 (*P. moluccensis* and *P. amboinensis*). The species of interest for this study were sorted from the catch using small hand nets and then transferred into the tanks used for irradiation in the experiment. All procedures were approved by the Ethics committee of the University of Queensland, Brisbane (SBMS/091/11).

Holding conditions and experimental procedure

Presettlement larvae of *P. moluccensis* (n = 139), *L. variegatus* (n = 144) and *S. corallinus* (n = 132) were used in the first experiment (experiment 1, high UVB dose: 13.4 $W*m^{-2}$) conducted in January 2012. DNA damage was induced by irradiating the fish with UVB for four hours ("damage phase") and followed by another four hours during which the fish were exposed to either photoreactivating light or were subject to a dark repair treatment ("repair phase", see Fig. 5.1a). This experiment was repeated in November 2013 with a lower dose (experiment 2, low dose, 6 $W*m^{-2}$) and shorter exposure to UVB (2 hrs) using *P. moluccensis* (n = 138) and *P. amboinensis* (n = 138) larvae. Following the damage phase, fish were given up to 24 hours to repair the DNA damage (Fig. 5.1b).

During both experiments and both the damage accumulation and repair phase, fish were randomly assigned to ten 15 x 20 x15 cm tanks with 13 to 15 individuals per tank. As for the possibility of behavioural protection during the experiment, the tanks used during the exposure treatments were chosen to prevent the possibility of shade seeking or covering under lids, in corners or water inlets. The aim here was to eliminate most other protection mechanisms in order to only investigate the DNA repair mechanism. The treatments (see below) were "damage" and "control" during the damage accumulation phase, and "photolyase repair", "dark repair" and "control" during the repair phase (Fig. 5.1). Fish were sampled randomly from the tanks as the number of tanks differed between species and the numbers sampled differed between timepoints (Tables 5.1 and 5.2). Seawater was gravity fed into all tanks to provide a continuous water supply. Oxygen was provided by an air bubbler in each tank. Water temperature was measured at one-minute intervals by

immersing a HOBO® Datalogger (Onset Computer Corporation, Bourne, MA, U.S.A.) in one tank of each treatment during a trial run.

In the high dose experiment (experiment 1) replicates from individual tanks were sampled at the following time-points: 0, 30, 60, 90, 120, 150, 180, 240, 255, 270, 300, 360 and 420 minutes (Fig. 5.1a), with 0 marking the start of the experiment. In the low dose experiment (experiment 2), replicates were sampled at 0, 90, 120, 135, 150, 180, 360 and 600 minutes (Fig. 5.1b), with 0 marking the start of the experiment. Experiment 2 was planned to last up to 24 hours. However, due to mortality of fish in the UVB irradiated tanks (not in control tanks), the experiment was terminated early. In both experiments, three to six replicates per timepoint were sampled from each treatment group (control, damage, photolyase and dark repair, see Tables 5.1 and 5.2 for details).

Sampled fish were euthanized in an ice/seawater bath and immediately frozen in liquid nitrogen. The samples were then stored at -70°C until further analysis (see below).

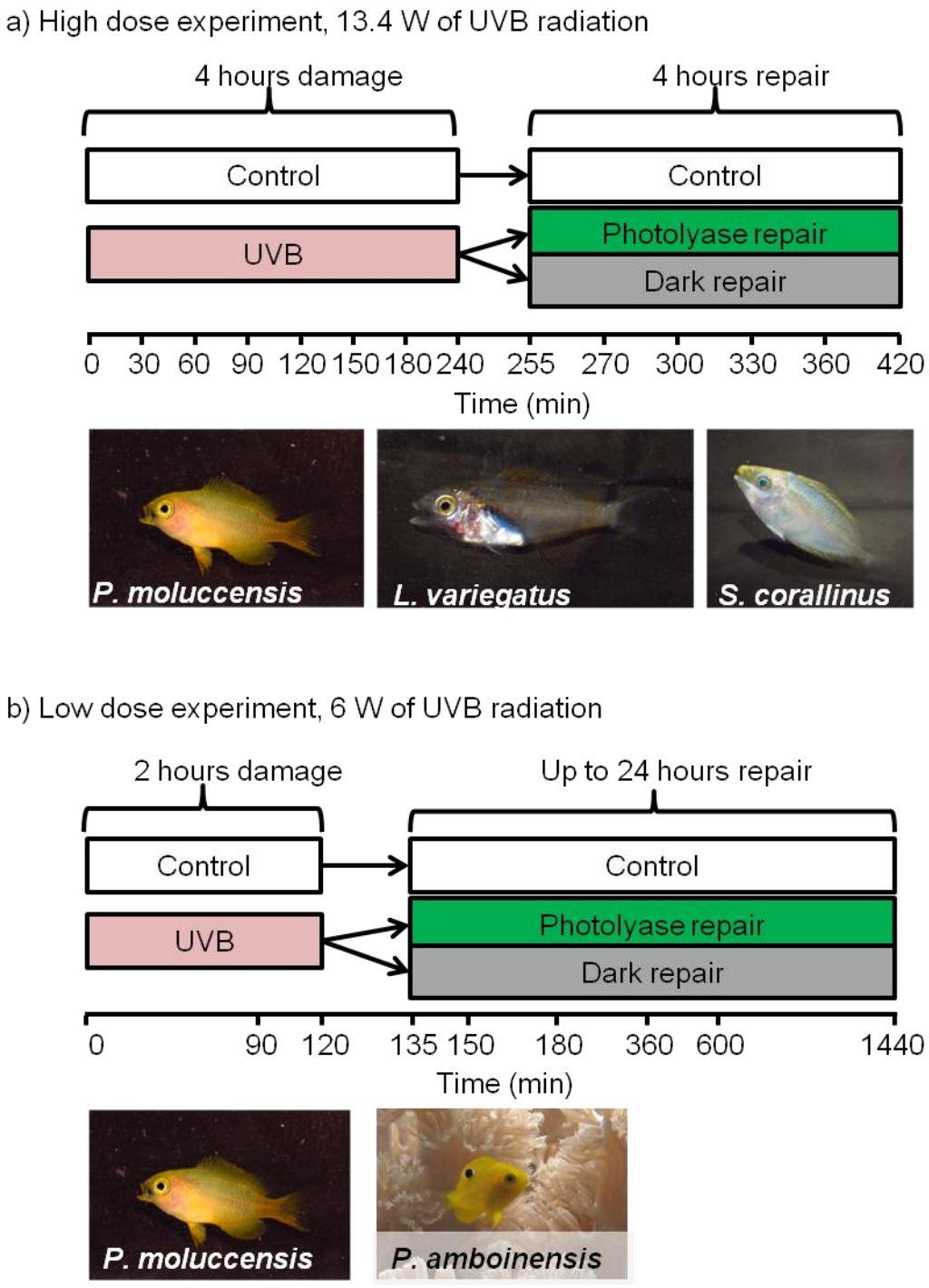


Figure 5.1: The four study species and a schematic representation of the "Control" and "UVB" treatments during the damage accumulation phase of the experiment, followed by the "Control" and the two repair treatments "Photolyase repair" and "Dark repair" in the repair phase. Each value on the time axes represents a sampling point in each treatment.

Light treatments

Artificial illumination was used during the UVB damage phase (2x GL20SE lamps, Sankyo Denki, Kanagawa, Japan) and also for the repair phase (2x Sylvania Premium Daylight deluxe FL36W lamp, Osram, Danvers, MA, U.S.A.). The fluorescent tubes were used in commercially available aquarium light fittings and suspended 25 cm above the experimental tanks. The relative intensity and spectra of both types of lamps are shown in Figure 5.2. Absolute irradiance of the UV lamps was measured using a Jaz spectrometer (OceanOptics, Dunedin, Fl, U.S.A.) with a 1000 µm fibre and a cosine corrector, calibrated to an OceanOptics DH-2000 lamp. The lamps used to induce DNA damage delivered mainly UVB radiation with an absolute irradiance of 13.4 $W*m^{-2}$ and 6 $W*m^{-2}$ for experiments 1 and 2 respectively. Comparison with field measurements show that the UVB irradiance in experiment 1 was roughly 2.4 times higher and in experiment 2 similar compared to the UVB radiation at water surface on a sunny cloudless day at noon at Lizard Island during the austral summer (5.65 $W*m^{-2}$). The total accumulated doses of UVB were 192.9 $kJ*m^{-2}$ and 43.2 $kJ*m^{-2}$, respectively. This roughly correlates to an irradiance that is present at noon (4.95 $W*m^{-2}$, chapter 2, Table 2.4, see also Table 2.5 for a daily dose) over the course of 10 and 2 hours, respectively. The fish in the "damage" treatment were exposed to the full spectrum of radiation (UV and PAR, 290 – 700 nm) during the damage accumulation phase of both experiments (Fig. 5.2). The fish in the "control" treatment were not exposed to UV radiation as the light was filtered by a sheet of UV-opaque Perspex (Acrylite OP4) and therefore only contained wavelengths between 400 and 700 nm.

In experiment 1 the fluorescent lamps used during the repair phase delivered photoreactivating light (PAR, 400 - 700 nm) at 41 $\mu mol*m^{-2}*s^{-1}$ measured with a LI-1400 datalogger and LI-190 Quantum PAR sensor (both LI-COR, Lincoln, NE, U.S.A.). All measurements were made at the water surface of the experimental tanks (20 x 15 x 12 cm, water depth 12 cm) 25 cm below the lamps. In experiment 2, the tanks were exposed to natural sunlight under a UV-shielding shade cloth during the repair phase. The mean PAR intensity was 517 $\mu mol*m^{-2}*s^{-1}$ (SD 98. $\mu mol*m^{-2}*s^{-1}$) and was measured using an OceanOptics JAZ spectrometer (Dunedin, FL, U.S.A). In both experiments, the containers holding "dark repair" groups were placed into a black plastic bin (70 x 40 x 40 cm) with the lid closed.

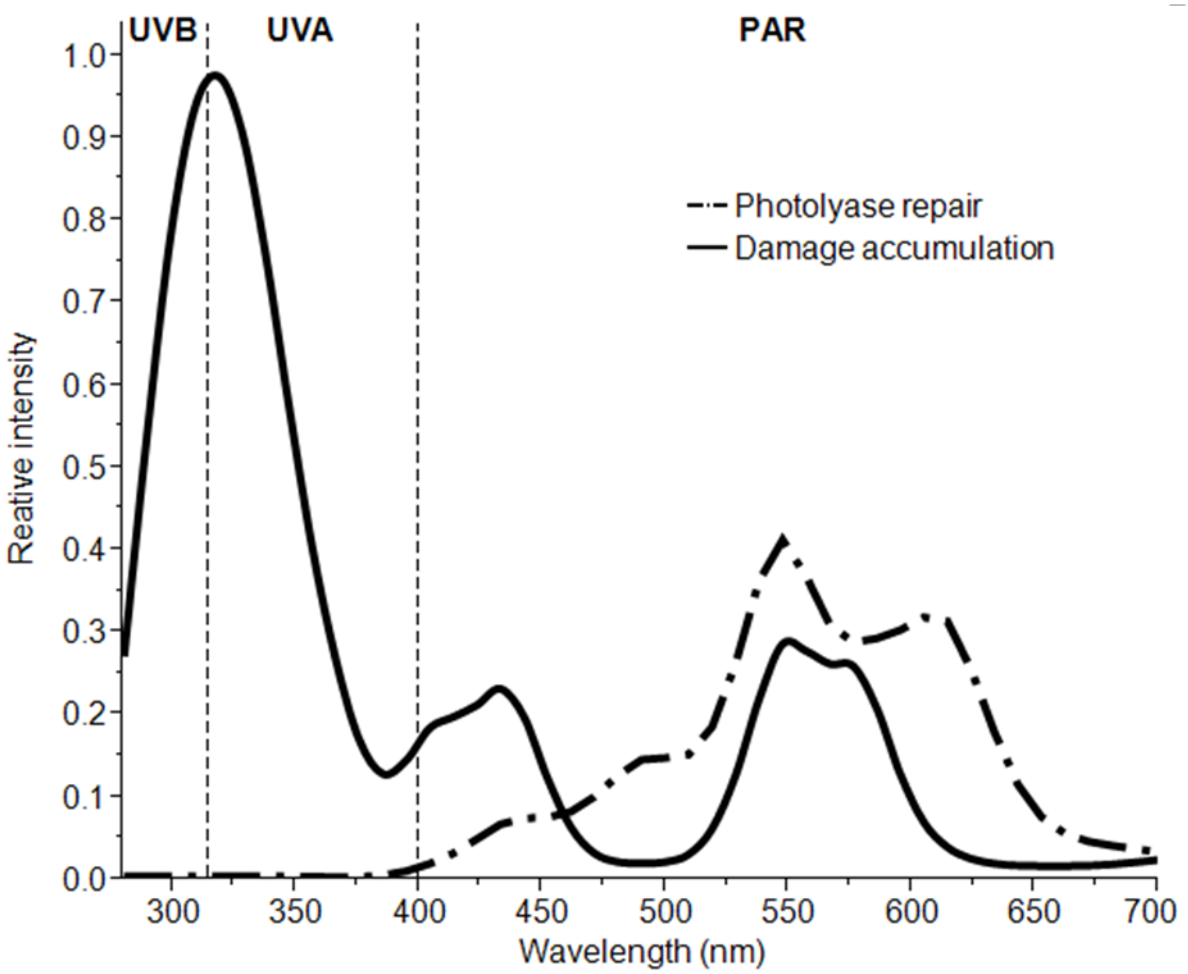


Figure 5.2: Light spectra of the two treatments, damage accumulation and damage repair. Note the high proportion of UVB/UVA light in the damage accumulation treatment and the absence of UVR in the damage repair treatment. Spectral data (counts/ wavelength) were normalized by setting the highest value equal to 1.

DNA isolation and damage quantification

The DNA isolation and quantification of DNA damage in the form of CPDs was carried out as described in chapter 2. The same standard was used in the ELISA assay. The method followed the protocol described by Mori et al. (1991). Briefly, genomic DNA was extracted from up to 25 mg of skin and tissue from the dorso-lateral body area of all larvae using the DNeasy® Blood and Tissue kit (Qiagen) following manufacturers' instructions. A scalpel blade was used to shave off a section of skin and tissue (<1 mm thick) from the still frozen larvae. The sample was immediately transferred into a 2 ml tube containing the lysis buffer to prevent DNA degradation. Following isolation, the integrity of the DNA was visually inspected using gel electrophoresis. Additionally,

DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmingtion, DE, USA) and diluted in 1 x PBS to a final concentration of 0.2 µg/ml. Only structurally intact DNA samples (i.e. one non-fragmented band on the gel) with an absorption coefficient 260/280 nm >1.8 were used for damage analysis.

CPD lesions were detected by using the TDM-2 primary antibody (Cosmo Bio, Tokyo, Japan) 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. DNA samples were loaded in triplicate and the mean value was used as the damage level for the fish, which was the statistical replicate. In order to compare damage levels between assays, i.e. 96-well plates, a DNA standard (chapter 2) was included with each assay. In addition to the triplicates of the DNA standard and the isolated DNA samples, a triplicate blank (1 x PBS) was included in all assays. Background fluorescence was excluded by subtracting the average blank OD values from the average OD values of 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 and arcsine transformed before statistical analysis.

Results

Mean water temperature (± 1 standard deviation) was 29.4 °C (± 0.26 °C) for the control treatment, 29.5 °C (± 0.28 °C) in the damage accumulation treatment, 29.3 °C (± 0.18 °C) and 29.3 °C (± 0.15 °C) in the photolyase and dark repair treatments, respectively.

Damage accumulation

Experiment 1: high dose experiment

During the damage accumulation phase, DNA damage in all three species was significantly higher at all timepoints compared to the respective control animals (Fig.5.3, Fisher's LSD, all pairs P< 0.005, Table 5.1). The initial damage levels of fish at timepoint 0 did not differ from the unirradiated control samples for either species (Wilcoxon signed ranks test, all P> 0.05, not shown).

The mean DNA damage increased 21.8- fold in *Pomacentrus moluccensis*, 17.6- fold in *S. corallinus* and 31.7- fold in *Lethrinus variegatus* after 240 minutes of exposure to UVR in the damage accumulation phase of the experiment (Fig. 5.3). Mean DNA damage varied significantly between the three species (Kruskal-Wallis test, H = 8.363, df = 2, P = 0.0056, Fig 5.5a). Specifically, *L. variegatus* showed significantly higher DNA damage levels than *S. corallinus* (Dunn's multiple comparison test, adjusted P = 0.0015), with other comparisons between the three species being not significant (Dunn's multiple comparison test, adjusted P = 0.0015).

Experiment 2: low dose experiment

The mean DNA damage of *P. moluccensis* increased 24.6- fold, and 11.9- fold in *P. amboinensis* after 120 minutes of UV exposure. The DNA damage of *P. moluccensis* and *P. amboinensis* did not differ significantly after the damage accumulation phase (Mann-Whitney, U = 14, P = 0.5714, Figure 5.5b). Again, DNA damage of fish at timepoint 0 did not differ from the control samples (Wilcoxon signed ranks test, all P> 0.05, not shown).

The DNA damage levels of *P. moluccensis* at the end of the damage accumulation phase in the high and low dose experiments did not differ (Mann-Whitney, U = 8, P = 0.242) from each other.

Repair

Experiment 1: high dose experiment

Significant decreases in DNA damage were observed in the photolyase treatment after 270 and 420 minutes for *P. moluccensis* when compared to the damage levels at the end of the damage accumulation period at the 240 minute timepoint (Dunnett's test, P = 0.0244 and P = 0.0442, respectively). After 360 minutes, *L. variegatus* also showed a significant reduction in DNA damage levels in the photolyase treatment. In the dark repair treatment, only *P. moluccensis* showed a significant reduction in DNA damage after 330 and 420 minutes (Dunnett's test, P = 0.0306 and P = 0.0126, respectively). All other comparisons between damage levels at timepoints during the repair phase and the damage sustained at the end of the accumulation phase were not significantly different (Dunnett's test, P> 0.05).

At the 330 and 360 timepoints, dead *L. variegatus*, (n= 15) were found in the dark and photolyase repair treatments. Fish were determined to be dead when no sign of gill movement could be detected. No mortality occurred in *S. corallinus*, *P. moluccensis* or in the control treatments. Dead fish were excluded from the damage/repair experiment since too much time elapsed before fish could be stored in liquid nitrogen, and degradation of DNA was likely. Therefore, additional replicates were sampled from the remaining fish. Due to mortality occurring at the 330 and 360 minute time-points and the resulting additional sampling of replicates, the experiment for *L. variegatus* was terminated after 360 minutes.

Experiment 2: low dose experiment

Only *P. moluccensis* showed a significant reduction in DNA damage, which occurred after 360 and 600 minutes in the photolyase treatment (Dunnett's test, P = 0.0118 and P < 0.0001, Fig. 5.4). Similarly to experiment 1, this experiment had to be terminated early for *P. moluccensis* after 600 minutes and for *P. amboinensis* after 360 minutes, since all remaining fish in the photolyase and dark repair treatments died. Again, no mortality occurred in the control treatments.

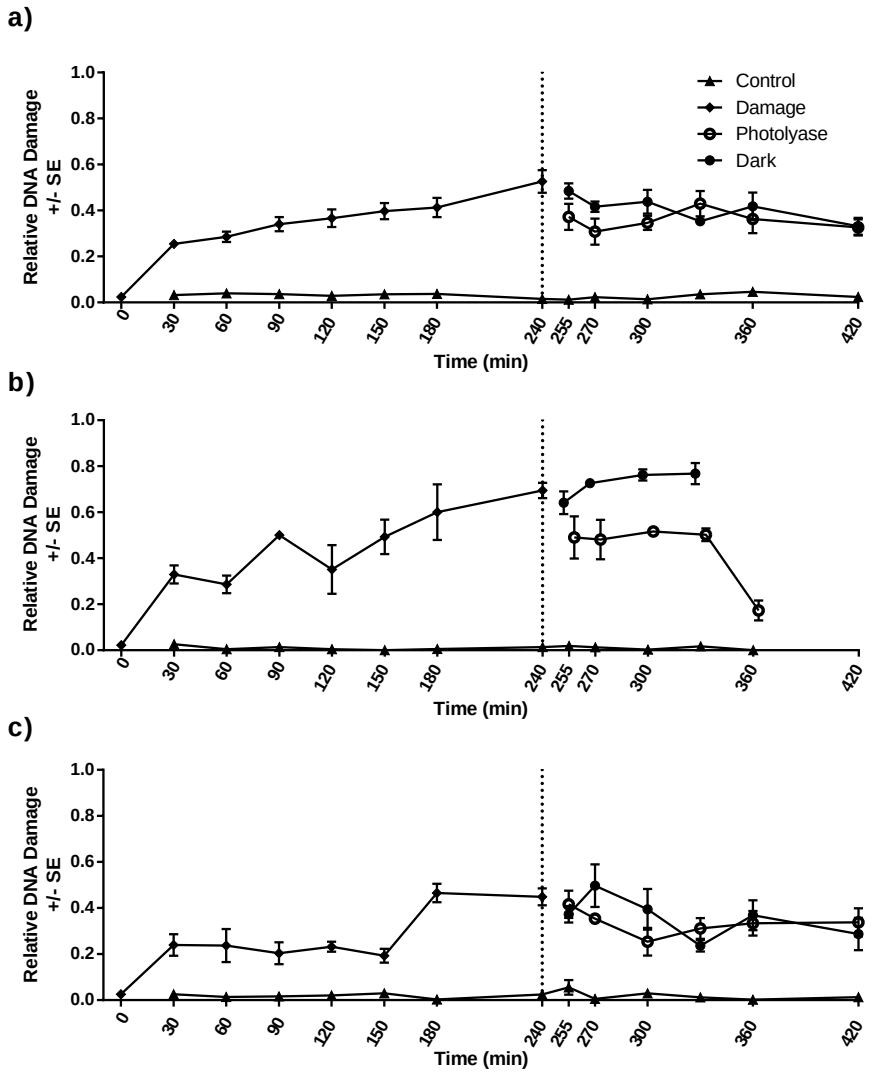


Figure 5.3: Mean DNA damage levels (CPDs expressed as OD units, +/- 1 SE) during the damage accumulation and repair phases in the high dose experiment. *P. moluccensis* (a) and *Lethrinus variegatus* (b) show a dose-response like increase in DNA damage over time, whereas DNA damage in *Siganus corallinus* (c) seems to increase in two distinct steps, after 30 and 180 minutes of exposure. A significant negative, trend in DNA damage is visible in *P. moluccensis* and *L. variegatus*, but not in *S. corallinus*.

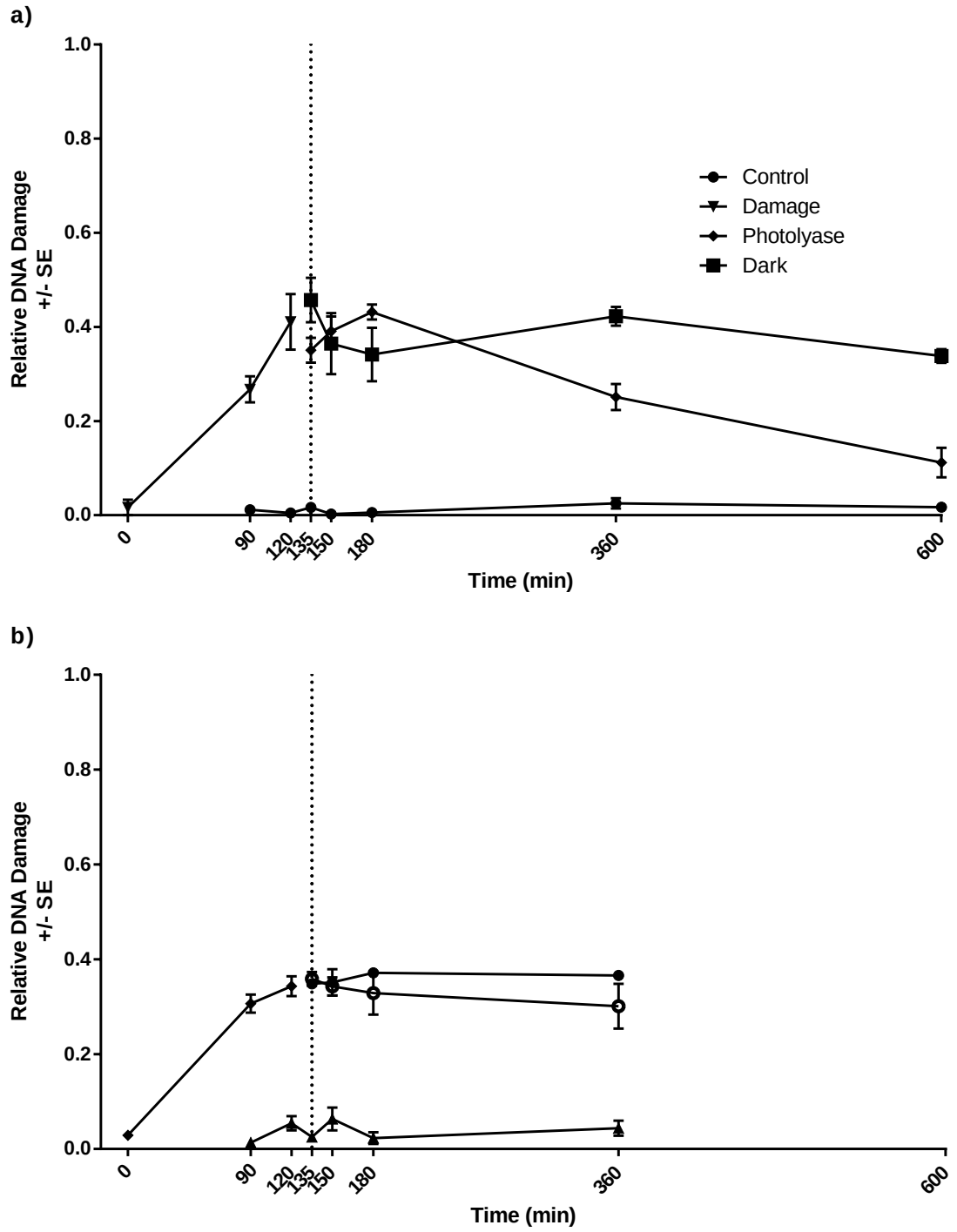


Figure 5.4: Mean DNA damage levels (CPDs expressed as OD units, +/- 1 SE) during the damage accumulation and repair phases in the low dose experiment. *P. moluccensis* (a) shows a significant reduction in DNA damage in the photolyase treatment after 300 and 600 minutes, in contrast to *P. amboinensis* (b) where DNA damage levels remain high even after four hours (timepoint 360 minutes) of exposure to photoreactivating wavelengths.

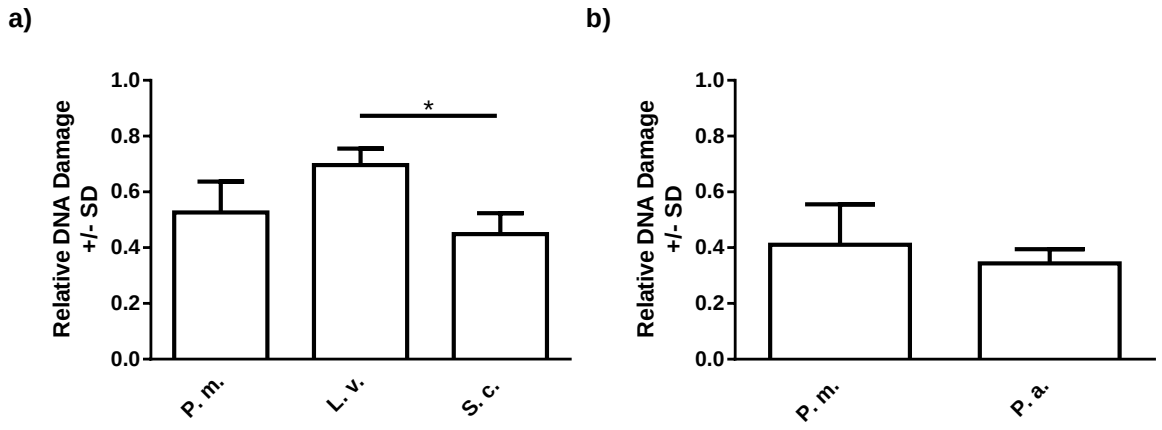


Figure 5.5: DNA damage of skin samples from reef fish larvae after irradiation with UVB for 240 (a) minutes in the high dose experiment and 120 (b) minutes in the low dose experiment. Error bars are 1 standard deviation from the mean, the asterisk indicates the significance level (adjusted for multiple testing, Dunn's $P < 0.05$). DNA damage is higher in *Lethrinus variegatus* (L.v.). than in *P. moluccensis* (P.m.) and *Siganus* corallinus (S.c.), which do not differ significantly from each other. No significant difference in DNA damage was observed between *P. moluccensis* and *P. amboinensis* (P.a.) in the low dose experiment (b).

Table 5.1: Statistical results (Fisher's LSD test) of comparisons of DNA damage between fish of the UVB exposed treatment and respective control groups in the damage accumulation phase and comparisons of DNA damage of fish from both repair treatments versus DNA damage levels at the end of the damage accumulation period (Dunnett's test).

High dose Experiment

Damage accumulation (Fisher's LSD test versus Control at each timepoint)					
Species	**Time (min)**	**Mean Difference**	**n_{Damage}**	**$n_{Control}$**	**P**
P. moluccensis	30	0.1005	5	3	**0.0002**
	60	0.131	5	3	**< 0.0001**
	90	0.1859	5	3	**< 0.0001**
	120	0.2119	5	3	**< 0.0001**
	150	0.2425	5	3	**< 0.0001**
	180	0.2591	5	3	**< 0.0001**
	240	0.3718	5	3	**< 0.0001**
L. variegatus	30	0.2873	4	3	**0.0012**
	60	0.2796	4	3	**0.0015**
	90	0.4783	4	3	**< 0.0001**
	120	0.3438	4	3	**0.0002**
	150	0.4929	4	3	**< 0.0001**
	180	0.5914	4	3	**< 0.0001**
	240	0.6724	4	3	**< 0.0001**
S. corallinus	30	0.2145	4	4	**< 0.0001**
	60	0.2238	4	4	**< 0.0001**
	90	0.188	4	4	**0.0001**
	120	0.2114	4	4	**< 0.0001**
	150	0.1638	4	4	**0.0006**
	180	0.4626	4	4	**< 0.0001**
	240	0.424	4	4	**< 0.0001**

Low dose Experiment

Damage accumulation (Fisher's LSD test versus Control at each timepoint)					
Species	**Time (min)**	**Mean Difference**	**n_{Damage}**	**$n_{Control}$**	**P**
P. moluccensis	90	0.2556	6	6	**< 0.0001**
	120	0.406	6	6	**< 0.0001**
P. amboinensis	90	0.2931	6	6	**< 0.0001**
	120	0.2893	6	6	**< 0.0001**

Bold values indicate significant differences ($P < 0.05$)

Table 5.2: Statistical results (Dunnett's test) of comparisons of DNA damage between fish of the photolyase and dark repair treatments and the damage levels at the end of the damage accumulation phase (240 and 120 minutes for the high and low dose experiments, respectively).

High dose Experiment

Damage repair (Dunnett's test versus damage levels at 240 minutes)					
Species	**Time (min)**	**$n_{(Photorepair)}$**	**$P_{(Photorepair)}$**	**$n_{(dark\ repair)}$**	**$P_{(dark\ repair)}$**
P. moluccensis	255	5	0.1653	5	0.9531
	270	5	**0.0244**	5	0.2707
	300	5	0.0804	5	0.4773
	330	5	0.59	5	**0.0306**
	360	5	0.1302	5	0.2839
	420	5	**0.0442**	5	**0.0126**
L. variegatus	255	4	0.1041	4	0.68
	270	4	0.0848	4	0.9214
	300	3	0.2332	4	0.4963
	330	4	0.132	4	0.4235
	360	3	**0.0001**	-	-
	420	-	-	-	-
S. corallinus	255	4	0.9934	4	0.9006
	270	4	0.589	4	0.9872
	300	4	0.0515	4	0.9761
	330	4	0.2445	4	0.1216
	360	4	0.4005	4	0.8815
	420	4	0.4416	4	0.3205

Low dose Experiment

Damage repair (Dunnett's test versus damage levels at 120 minutes)					
Species	**Time (min)**	**$n_{(Photorepair)}$**	**$P_{(Photorepair)}$**	**$n_{(dark\ repair)}$**	**$P_{(dark\ repair)}$**
P. moluccensis	135	6	0.6134	6	0.9368
	150	6	0.993	6	0.9375
	180	6	0.991	6	0.7577
	360	6	**0.0118**	6	0.9999
	600	6	**<.0001**	6	0.7276
P. amboinensis	135	6	0.992	6	0.9988
	150	6	1	6	0.9912
	180	6	0.9931	6	0.6069
	360	6	0.7686	6	0.7557
	600	-	-	-	-

Bold values indicate significant differences ($P < 0.05$)

Discussion

Exposure to UVR in the tropical marine environment leads to the accumulation of DNA damage. In order to minimize DNA damage levels, protection and repair mechanisms have evolved and are being utilized across the plant and animal kingdoms (Karentz 2014). The aim of this study was to test whether coral reef fish repair UV-induced DNA damage via photoreactivation and/or dark repair. In *Pomacentrus moluccensis* both repair pathways are shown to be present which led to a significant reduction of CPDs after exposure to relatively high (both repair modes) and low doses (only photoreactivation) of UVB. Further the presence of photoreactivation was confirmed in settlement-stage larvae of *Lethrinus variegatus*. Contrary to the hypothesis, dark repair cannot be confirmed in *L. variegatus* and either one of the repair pathways in *Siganus corallinus* and *Pomacentrus amboinensis*.

Fish of all four species used in this study showed evidence of rapidly increasing DNA damage, with some variation in the kinetics of this accumulation. None of the species studied within the time provided in the experiment and under both UVB and PAR scenarios were able to restore their relatively low levels of UV-specific DNA damage observed prior to the experiment. Similar to findings by Mitchell (2014), this study also shows that coral reef fish larvae differ in their sensitivity to UVR in terms of CPD levels, with one species, *L. variegatus* showing significantly higher levels of DNA damage than *S. corallinus* under the same illumination conditions. The occurrence of mortality in only one species (*L. variegatus*) in the high dose experiment, as well as the mortality that occurred in *P. moluccensis* in the low dose experiment (but not in the high dose conditions) further highlights differences in sensitivity to the experimental UVR conditions, similar to findings in fish (Mitchell et al. 2014) and amphibians (Blaustein et al. 1996, Smith et al. 2000). The high levels of mortality that were observed especially towards the end of the low dose experiment prevented an assessment of the repair abilities of settlement stage larvae of *P. moluccensis* and *P. amboinensis* over a full day.

In the first experiment fish were exposed to a very high dose of UVR (2.4 times higher than the incident midday dose at Lizard Island), with an equal amount of time (four hours) after the damage accumulation to allow for either photoreactivation or dark repair. Before the repair phase, all fish showed significantly higher DNA damage levels compared to levels at the start of the experiment, which are representative of DNA damage levels from the field. Interestingly, DNA damage in *L. variegatus* after 240 minutes of UV exposure was significantly higher than in *S. corallinus*, and coincided with mortality in this species during the second half of the experiment. As expected significantly lower levels of DNA damage in the photolyase and dark repair treatments, indicating

functioning repair pathways, were found in two species, *P. moluccensis* and *L. variegatus*. In the photoreactivating treatment in *P. moluccensis* however, the significant reduction of DNA damage at the 270 timepoint (and at the 330 timepoint in the dark repair treatment) was followed by a period where DNA damage levels showed no significant difference with the levels at the end of the damage accumulation period, before being significantly reduced again after 420 minutes. This could possibly be due to relatively low sampling numbers, however, clear DNA repair has been shown with similarly low replicates (Malloy et al. 1997).

To test for the possibility that the high dose experiment had caused to much physiological stress on the animals, the UVB dose was reduced (to a level comparable to the incident dose at Lizard Island), the dose of photoreactivating light during the repair phase was increased and the time for repair was extended. The amount of DNA damage did not vary between *P. moluccensis* and *P. amboinensis* after two hours of UVR exposure to the low dose, and was only marginally less than the DNA damage levels after four hours of exposure in the high dose experiment. Again, the repair capabilities differed between species, even between the two closely related Pomacentrids that were used in the second experiment. Although *P. moluccensis* was able to significantly repair UV-induced DNA damage in the photoreactivation treatment, no dark repair was observed in this species, and *P. amboinensis* showed repair in neither treatment. Similar to Experiment 1, high mortality of the UV-exposed fish prevented an assessment of DNA damage for the planned time period of 24 hours. It seems likely at this stage, that due to the significant doses of UVB and UVA which fish were exposed to, and the resulting multiple effects such as oxidative damage, DNA damage and the physiological stress of the resulting repair pathways (Lesser et al. 2001), a threshold was exceeded, resulting in mortality of the experimental specimens.

Our observation of a significant reduction of CPDs within a short time of exposure to photoreactivating wavelengths, is comparable with findings of Regan et al. (1982) and Kienzler (2013) who demonstrated DNA repair using cell lines of labrid and salmonid fishes, respectively. Further, photolyase repair was also observed by Malloy et al. (1997) in icefish eggs and larvae, Dong et al. (2007) in zebrafish larvae, Meador et al. (2000) in platyfish, Applegate and Ley in minnow (1988), and is likely to be the cause of diel cycles of CPDs in anchovy larvae reported by Vetter et al. (1999). The relatively low efficiency of the CPD repair pathways observed in three different species of reef fish from a UVR rich environment at an early life history stage, e.g. the pelagic larvae, seems puzzling. Olson (2006) documented a lack of photoreactivation in trout larvae after exposure to a high dose of UVB (138 $kJ*m^{-2}$), which was still lower than the dose used here (up to 192.8 $kJ*m^{-2}$). However, a later study by Mitchell (Mitchell et al. 2009) confirmed the existence of a photolyase system when the dose of the UVB radiation used to inflict DNA damage

was reduced, and ample time for repair (up to 48 hours) was provided. The highly significant reduction of DNA damage via photoreactivation in the low dose experiment at least in one species (*P. moluccensis*) is similar to these observations.

It is possible that the very high dose of UVB radiation combined with the short time (4 hours) allowed to repair the inflicted DNA damage masked the detection of a functioning repair system in this study due to a saturation, or at least a very high level of DNA damage. The high levels of UVR in the first experiment could also have led to damage of the repair system itself, leading to reduced rates of photoreactivation (Armstrong et al. 2002, Lo et al. 2005) and mortality (Daly 2012). DNA repair is turned off in cells that are beyond repair and in which apoptosis has been initiated, further decreasing the rate of repair (Sinha & Häder 2014). However, the step-like way in which DNA damage accumulated in *S. corallinus* and *L. variegatus* hints that repair mechanisms, possibly both photoreactivation and excision repair pathways like nucleotide- and base-excision repair were at work under the prevalent damaging and photoreactivating wavelengths. Mitchell (2009) observed saturation of DNA damage in trout larvae, and suggests that in such a case a steady-state between damage induction and repair is reached. In both *L. variegatus* and *S. corallinus* DNA damage reached a saturation level after 30 minutes, before further increases occurred. In *S. corallinus*, another saturation point was reached after 180 minutes of UVB exposure, shortly before the light regime was switched to only include photoreactivating wavelengths. Several studies have shown that DNA repair via photolyase is either constitutive (Vetter et al. 1999) or can be induced and enhanced by exposure to photoreactivating wavelengths before UV exposure (Yasuhira et al. 1991, Wiegand et al. 2004). However, since specimens used in this study were caught using light traps that emit photoreactivating (400 - 700 nm) light (Fisher & Bellwood 2002), a lack of prior exposure can be excluded as a cause of the low repair rates observed here. While prior exposure to the very high levels of UVR used in the first experiment seems unlikely in the GBR, the dose used in the second experiment (6 $W*m^{-2}$) is commonly encountered in tropical regions (see chapter 2 and Lamare et al. (2007)). Therefore, at least the fish used in the second experiment were likely to have been exposed to a comparable dose of UVR before, and their protective systems should have been adapted to this kind of stress. Further studies in this field should include improved light conditions which take into account the accessory role that UVA light plays in the process of photorepair (Sancar 2003). The blocking of UVA light in both experiments in this chapter might have prevented higher rates of photorepair and led to the high rates of mortality in some species. However, as the significant reduction of DNA damage via photorepair in *P. moluccensis* and *L. variegatus* show, a lack of UVA does not prevent photorepair completely. Species- specific differences in using UVA and/or blue light to repair DNA damage via photorepair should be investigated in the future. Repair

genes, both for dark repair and photoreactivation, are ancient and well conserved (Eisen & Hanawalt 1999), and the genes coding for photolyase are present in a number of teleosts (Oliveri et al. 2014), rendering it unlikely that reef fish larvae do not possess the photolyase pathway to repair CPDs. Photolyase has been shown to be the main pathway for organisms to repair UV-induced DNA damage (Reef et al. 2009), and dark repair can take significantly longer to show a reduction on accumulated DNA damage. It is possible, that no dark repair was observed in L. variegatus because the observation time was not long enough, and should have been at least 12 hours (see experiment 2, *P. moluccensis*). Future studies should take note of the observations made here and increase the observation time in order to solve the question of the "lacking" dark repair in reef fish. Settlement-stage larvae of reef fish would certainly benefit from the ability to repair DNA damage, since the three genera used in this experiment are found in the upper two meters of the water column (Leis 1991, Leis et al. 1996, Fisher & Bellwood 2002, Leis 2004), where UVR is highest.

Melanin absorbs UVR (Cockell & Knowland 1999) and screens the DNA from the damaging effects of UVR, so that different melanophore densities in these species could be a likely cause for the differences in DNA damage and mortality observed in the experiments. Since *Lethrinus* sp. is more translucent (i.e. less pigmented, Fig 5.2) than *S. corallinus* and *P. moluccensis* and therefore possibly less protected, the same UVR dose could lead to more serious consequences, e.g. mortality. Similarly, higher mortality after UVR exposure was reported for less pigmented red sea bream *Pagrus major* compared to more pigmented black sea bream *Acanthopagrus schlegeli* (Fukunishi et al. 2013b). Variation in susceptibility to UVR exposure was also reported by Armstrong et al. (2002) in several strains of Japanese medaka larvae, *Oryzias latipes*. CPD levels were found to be higher in wild type strains, which contained more melanophores, possibly due to a sensitizing effect for DNA damage by melanin. Cells containing more melanophores show increased numbers of free radicals (Hill et al. 1997) and therefore increase UV toxicity in exposed cells and tissues. These contradicting results clearly demonstrate the need for an assessment of pigmentation in further studies on UV induced DNA damage in reef fish.

Further studies are also needed to elucidate the functionality of the UV damage repair systems in reef fish. Specifically, even lower, more natural doses of UVR should be considered in order to detect the repair of UV induced DNA damage in reef fish, since the species used in the experiments showed a high sensitivity to increased UVR.

CHAPTER SIX

General Discussion

Ultraviolet radiation (UVR) reaching the surface of Earth has been recognized as a major evolutionary factor for terrestrial and marine organisms alike (Bancroft et al. 2007, 2008). DNA damage as the result of UVR exposure has received much attention in the literature over the last three decades (Sinha & Hader 2002, Buma et al. 2003, Friedberg 2003, Banaszak & Lesser 2009, Dahms & Lee 2010, Lamare et al. 2011, Daly 2012, Karentz 2014, Sinha & Häder 2014). In addition to DNA damage originating from both UVB and UVA irradiation, physiological stress as a result of repair pathways and oxidative damage caused by UVA exposure are contributing to UV stress. Coral reef fish must protect themselves against high doses of UVB and the associated DNA damage to thrive in the clear and shallow waters. Behavioural avoidance of UVR is seen as the first line of defence at least in fish which are able to visually detect UV light (Leech & Johnsen 2003). The second protection mechanism exploits the UV-absorbing characteristics of Mycosporine-like Amino Acids (MAAs), which are omnipresent in the marine environment (Karentz et al. 1991, Dunlap & Shick 1998, Shick & Dunlap 2002). The third protection mechanism is DNA repair via photoreactivation and dark repair, which has been shown to exist in various freshwater and temperate marine fish species (Ahmed & Setlow 1993, Uchida et al. 1995, Malloy et al. 1997, Vetter et al. 1999, Lesser et al. 2001, Armstrong et al. 2002, Mitchell et al. 2009, Zeng 2009, Mitchell et al. 2014).

Despite coral reef fish inhabiting a UV-rich environment and the number of studies concerned with their ecology and ability to withstand other environmental stressors, such as elevated temperature (Hughes et al. 2003, Biro et al. 2010, Donelson et al. 2010, Donelson et al. 2012) or changes in pH (Munday et al. 2008, Munday et al. 2009, Nilsson et al. 2012), our knowledge about the impact of UVR on reef fish is very limited. The overall aims of this project were therefore to assess the impact of ambient and elevated levels of UVR on reef fish in terms of UVB- specific DNA damage, and to evaluate the possible protective mechanisms available to fish with and without UV vision.

Key findings

This study finds that *in situ* levels of DNA damage in more than a dozen species are low compared to the DNA damage levels that were induced by exposure to artificial UVB. Additionally, evidence is provided that two of three protection mechanisms, DNA repair and protection with MAAs, are present and in some species effective in defending against UV induced DNA damage.

Although DNA damage, measured in terms of CPDs, in samples from the field was generally low, variation between individuals was high. The low level of damage suggests that reef fish are well adapted to the UVR levels they are currently facing on the northern Great Barrier Reef. Upon closer examination of the potential factors leading to the observed variability, species and size were found to be most important while capture depth and UV vision had the least influence. These results lead to several interesting questions. What are the underlying causes for the differences in CPD levels between species? Why is there a trend for more DNA damage in smaller fish, i.e. in early development stages? Why do fish with UV vision and UV signalling (and therefore reduced MAA protection) not experience higher levels of DNA damage compared to MAA protected fish? Also, what is the cause of the breakdown of the defences, as is now evident by the high damage levels and mortality that occurred in experiments with artificial, elevated levels of UVR?

Factors influencing UV-induced DNA damage

The most important factor in defining the variation in DNA damage in the sampled reef fish was species, but not family, which speaks against a phylogenetic predisposition for a certain level of damage. Hence, the effects of UVR are different in closely related species as well as distant relatives, and besides size, must be influenced by other factors. Life history differences or different life styles that determine exposure time to UVR for example, exist between the study species, and most certainly contribute to the observed variation in DNA damage. Ambush predators such as *Plectropomus leopardus* which are more active late in the day or at night would be expected to have less DNA damage because they seek shelter under corals or in caves during the day (Kerry & Bellwood 2012, Wen et al. 2013) compared to species more active during the day, such as *Thalassoma lunare*. MAA protection was shown to be crucial in determining the amount of DNA damage (chapter 4), so that differences in sunscreen protection certainly contributed to the variation in DNA damage. The influence of diet on sunscreen protection in reef fish has been shown to be substantial (Carefoot et al. 2000, Zamzow 2004). Whether MAAs accumulate in the same rate in herbi-, omni- or piscivorous reef fish has not been studied, and there was no assessment of current MAA status in the fish sampled for the Baseline study. Another factor that could indirectly lead to differences in DNA damage between species is sex. MAAs occur in higher quantities in ovaries and eggs compared to testes and sperm (reviewed by Shick & Dunlap (2002)), therefore leading to different levels of UV protection. The sex of the fish that were sampled was not determined, which could have led to an uneven distribution of males versus females between the species. Further, differences in habitat depth come to mind. Lethrinidae prefer shallow sandy and vegetated habitats inside the lagoon (Heithaus 2004, Gomelyuk 2009), whereas coral trout (*Plectropomus* sp.) can

often be found on deeper reefs (Leis & Carson-Ewart 1999, Heupel et al. 2010). However, the role of depth on the amount of DNA damage has shown to be minor in this study, both in the overall analysis as well as the depth series of *Pomacentrus moluccensis*. The different sampling locations on the other hand, could well have lead to different levels of DNA damage between species, as the water clarity measured at two sites at a distance of only a few hundred meters varied strongly, and the UV dose in clearer waters (Trawler Beach) was several times higher than at the more turbid site (Vicky's Reef). The influence of water clarity and UV dose is correlated with differences in UV protection of parrotfish (Zamzow 2007). Whether the differences in DNA damage and high variability between species observed here can be explained by the role of microenvironments and habitat choice (e.g. reef flat versus reef crest; live coral versus rubble) needs to be examined in detail in the future, and should be accompanied by simultaneous measurements of both UV dose and MAA protection under consideration of the sex of the sampled fish.

Why do smaller fish at an earlier developmental stage have higher levels of DNA damage compared to larger fish or later life stages? The results presented here, especially in chapters 2 and 5, are in concordance with previous findings which have shown a more severe impact of UVR in earlier developmental stages (Vetter et al. 1999, Olson et al. 2006, Bancroft et al. 2007, Vehniäinen et al. 2012) compared to adult individuals. The pelagic nature of pre-settlement reef fish larvae (Leis & Carson-Ewart 2000) which are exposed to higher UV levels compared to post-settlement larvae on coral reefs (Jerlov 1976, Eckes 2009, Veal 2011), could result in the higher DNA damage levels. High DNA damage in several species of larvae (*Pomacentrus* sp., *Abudefduf vaigensis*, *Dascyllus* sp.) usually found in the upper water column (Leis 1991) support this hypothesis. Further, the larvae of many species often appear to be near transparent, with reduced pigmentation and scales compared to adult fish. This can lead to the penetration of UVR into deeper layers of tissue such as the brain, (Vehniäinen et al. 2012), showing the potential for severe effects of UV exposure in young fish. Whether such damage could lead to altered development and behavioural changes such as observed here (chapter 3) remains to be studied. Whether increasing levels of UVR could therefore influence recruitment and successful settlement of reef fish needs to be addressed in the future. However, the results of chapters 3 and 5 suggest that exposure to current surface levels of UVR ($5 – 7\ W*m^{-2}$) severely impact the development into adult fish through changes in behaviour, foraging, increased DNA damage and the associated high mortality. Any change in climate, water clarity and habitat that could increase the probability of increasing UVR levels on coral reefs must therefore be regarded as highly concerning.

Role of UV vision and communication in avoidance behaviour

Surprisingly, the results showed that fish with reduced external UV protection (i.e. fish with UV vision and displaying UV signals for communication) did not have higher DNA damage compared to fish not engaged in UV communication. One possible explanation is that fish with UV vision achieve the same amount of UV damage as UV-blind heterospecifics by avoiding exposure to high levels of UVR. The role that UV avoidance plays for *in situ* DNA damage levels is yet unknown, but the results of chapter 3 suggest that reef fish do not actively avoid UVR. In contrast, it seems that under certain conditions, Pomacentrids even prefer areas with higher UVR compared to areas that do not contain UVR, despite the potential for UV-induced DNA damage. Pomacentrids are not only able to forage under UV only conditions but also improve their foraging efficiency (Job & Bellwood 2007, Ponton et al. 2012), which could explain the preference for an area rich with UV. For a fish with UV vision, any area completely devoid of UV might appear "unnatural". Given the presence of the two other protection mechanisms, DNA repair and external MAA protection, behavioural avoidance might not be necessary, but also rather unpractical in a real-life coral reef setting. Considering behaviours such as territorial interactions and foraging, of finding and attracting mates, which require to be more or less exposed, having to hide from UV under corals or in crevices would be counterintuitive (Wishingrad et al. 2014). The slight trend for preferring deeper areas which was observed in *P. amboinensis* could serve as a suitable alternative to hiding or seeking shelter in areas that lack UVR completely, as damaging UVB radiation is attenuated in the water column relatively fast in comparison to UVA (Veal 2011). Further, the potential for altered behaviour under UVR exposure was evident in juveniles with reduced foraging activity (chapter 3), comparable to negative effects of UVR reported in freshwater fish (Alemanni et al. 2003, Holtby & Bothwell 2008). Additionally to the immediate damage to DNA during UV exposure, the decreased activity could lead to slower growth due to a lower food intake (Fukunishi et al. 2013a) and decreased survival rate in the population if the effects of UVR on behaviour and activity are different in predatory species (Fukunishi et al. 2012). Whether this could lead to changes in an even broader context (e.g. recruitment) on a coral reef needs to be examined, but evidence for the wide-ranging effects of UVR exist (Paul & Gwynn-Jones 2003, Häder et al. 2007, Karentz 2014, Bornman et al. 2015).

Protection by natural sunscreens

Neither, *P. amboinensis* (UV vision, MAA poor mucus) and *T. lunare* (UV-blind, MAAs) showed a UV avoidance reaction. Yet, how can the nearly identical levels of DNA damage in samples collected from the field and from the same habitat be explained? The results presented in chapter 4

support the important protective role of MAAs in achieving these *in situ* levels, since higher mucus absorbance, i.e. MAA levels, were linked to lower DNA damage levels. Importantly, a significant increase in mucus absorption was observed in *P. amboinensis* after UV exposure. The ability to modulate the mucus absorbance could enable Pomacenetrids to communicate with their UV signals while having reduced UV absorbing compounds in their mucus, and in time of high UVR in the environment, e.g. low tide or midday on cloudless days, to increase the level of sunscreens. Additionally, *P. amboinensis* might be able to minimize the trade-off between UV communication and UV protection by having an UV protection mechanism that is highly flexible. Since the change in mucus absorbance was UV specific, and occurred only in the species with UV vision, it is intriguing to speculate that this response could be triggered visually. However other triggers such as DNA damage itself, which activates a multitude of pathways (Sinha & Häder 2014) or a non-visual "recognition" of increased UVR through light receptors in the pituitary gland could be possible. The pathways for this reaction are unknown at this point, but merit further investigation. The increased levels of DNA damage in adult fish exposed to a UVR dose similar to levels measured at the water surface again demonstrate the vulnerability of reef fish to increases in UVR and indicate the limitations to the DNA repair mechanism.

DNA repair

Similar DNA damage levels in fish with and without UV vision could also be achieved by differences in the efficiency of DNA repair pathways. At the same time, variation in DNA repair could explain some of the variation in damage levels. An indication for the presence of DNA repair has already been gained for adult *P. amboinensis* in chapter 2. Since avoidance of UVR or seeking shelter from UVR by swimming deeper was prevented, the constantly low levels of DNA damage after midday must have been achieved otherwise. Protection using adjustable levels of sunscreen (chapter 4) might have prevented further increases of DNA damage. The reduction in DNA damage in samples taken after midday however, can only be explained by the presence of efficient DNA repair mechanisms.

In chapter 5, it was therefore examined how reef fish with (*P. amboinensis*, *P. moluccensis)* and without (*Siganus corallinus*, *Lethrinus variegatus*) UV vision and communication repair UV-induced DNA damage. There was not only variation in the accumulated DNA damage, but also considerable variation in the repair abilities, which shows differential vulnerability to elevated UVR exposure between the species examined. A breakdown of the UV protection mechanisms indicated by the high rates of mortality occurred in all four species. This suggests that the UVR exposure comparable with midday surface irradiance levels was beyond a general sensitivity threshold. It is

possible that damage to proteins, especially those responsible for photoreactivation was responsible for the lack in repair (Daly 2012) and mortality. Thus, the mortality that was observed in these experiments was caused by damage to DNA via exposure to UVR and the formation of CPDs, as well as the follow-on effects of UVR exposure. These indirect effects likely included oxidative damage and associated damage to DNA, proteins, lipids, increased physiological stress due to the activation of repair enzymes and enzymes such as superoxide dismutase, which protects against oxidative damage.

Even with lower UV doses and under a very high PAR flux, therefore excluding possible effects of insufficient light available for photoreactivation, only approximately 60 % of the initial DNA damage was repaired. Compared to other fish larvae and even eggs (Vetter et al. 1999), in which up to 80 % of DNA damage was repaired quickly (< 6 hours), the lack of a clear, swift reduction in CPDs in more than one species is remarkable. Phylogenetic differences in repair abilities similar to those found by Olson, Mitchell and colleagues (Olson & Mitchell 2006, Mitchell et al. 2014) are plausible, given the variation in repair via either pathway in closely related species such *P. moluccensis* and *P. amboinensis*. Although DNA repair in reef fish larvae and most likely in adults is present and certainly plays an important role in the reduction of UV-induced DNA damage, the relatively low efficiency of photoreactivation, the apparent lack of dark repair and the variation in the repair abilities could be responsible for variation in damage levels between species and higher damage levels found in larvae.

Key conclusions

This is the first study to address levels of UV-induced DNA damage in reef fish under natural conditions as well as under elevated doses of UVR that could occur in a changing climate. Although the current threat from CPDs in reef fish appears to be low, the variation of damage between species and the higher levels of DNA damage in settlement stage larvae reveal potential risk groups if UVR rises as predicted (Watanabe et al. 2011). The relatively low levels of DNA damage in a diverse group of reef fish underlines the importance of certain protection mechanisms against UVR. Protection using MAAs and DNA repair via photoreactivation are the likely cause of unchanged levels of DNA damage in *P. amboinensis* despite exposure to UVR over the course of a day. Significant reductions of DNA damage in settlement stage larvae confirm the presence of photoreactivation and dark repair, two DNA repair mechanisms previously not demonstrated in reef fish. Simultaneously, the absence of repair in other species as well as the occurrence of mortality highlights the need for further study and improvement of the experimental conditions when using

settlement stage larvae. The lack of a specific UV avoidance response in a species of reef fish with UV vision contradicts previous literature that predicted UV avoidance as a first line of defence against UV-induced DNA damage. Given the ubiquitous presence of UVR on a shallow coral reef however, constant avoidance of light seems to be counterintuitive and would interfere with essential behaviours such as foraging or territorial interactions. Altered behaviours of juveniles under UVR exposure, however, are in concordance with previous findings, emphasizing the negative effects of UVR on early developmental stages.

UV-absorbing MAAs, by far the most studied protection mechanism in reef fish, can now be confirmed to protect against CPDs since higher levels of sunscreens led to less DNA damage in two species. The discovery of changes in mucus absorbance after a short period of UVR exposure suggests that sunscreens play the most important role in UV protection in the absence of UV avoidance and only insufficient DNA repair. Whether the ability to adapt their sunscreen protection to elevated levels of UVR could benefit certain species if UV levels increase in the future is currently unknown and needs to be examined.

This study presents initial evidence that exposure to damaging levels of UVR can present fish with one or more trade-offs, whether it is the prevention of DNA damage via an upregulation of MAAs (chapter 4) and the ability of Damselfish to communicate with UV signals; UV damage and changes in foraging behaviour (chapter 3); the ability to repair DNA damage and the associated physiological costs (chapter 5). However, these apparent trade-offs were not tested in this thesis explicitly, but future studies will no doubt benefit from the first steps that have been made by characterizing the negative impact in form of UVB induced DNA damage on reef fish.

Synergistic effects of UV and other environmental stressors influenced by climate change: prospects for future studies

Further studies in this area will benefit significantly from the insights gained here, and should take into account the presence of DNA repair and adaptive sunscreens in their assessment of DNA damage in reef fish. In contrast to amphibians, where the effects of a combination of UV and other stressors are well known (e.g. Kiesecker and Blaustein (1995), Long et al. (1995) and Bancroft et al. (2008)), relatively little is known about synergistic effects in the tropical marine environment (but see Lesser et al. (1990) for a study on coral bleaching). On the individual level, the ability of reef fish to withstand UV exposure during development and the impact of UVR on young reef fish should be examined in further detail and under consideration of other stressors (elevated

temperature and pH) which could act in synergy with UVR (Cramp et al. 2014, Li et al. 2014). Cramp (2014) showed that UVR in combination with elevated temperature increases parasitism and therefore risk of disease in freshwater fish. Parasites infect reef fish of all life stages at a high rate (Grutter 1996a) and impact local populations (Finley & Forrester 2003) and reduce fitness parameters such as swimming speed (Ostlund-Nilsson et al. 2005). How will reef fish, especially the early developmental stages, fare in a warmer, potentially more UV transparent environment, when these stressors are added to the current situation? It has been argued that synergistic effects of climate change will also change the behaviour, i.e. orientation of reef fish larvae from the pelagic to the reef environment (Siebeck et al. 2014), and that the effects could be additive, if not synergistic. Ocean acidification and changes in pH are being studied intensively in the past decade, and several studies have shown changes in fish behaviour as a result (Munday et al. 2009, Nilsson et al. 2012) of exposure to predicted pH levels . Li et al. (2014) demonstrated negative effects of UVR and pH changes on photosynthetic diatoms. Since these single celled organisms are an important source of MAAs (Helbling et al. 1996), downstream effects on reef fish and sunscreen protection should be investigated. These stressors and their interactions with each other must be properly understood in order to make sound assessments on the health of coral reefs. It is important to further elucidate the changes in sunscreen protection in reaction to elevated UVR observed in *P. amboinensis*, and how this change is facilitated physiologically, which MAAs are involved, and whether other species are able to react in a similar manner. Improvements in the design of the light environment used for DNA repair studies as well as the incorporation of genetic methods in order to track the expression and activity of repair enzymes (Isely et al. 2009, Reef et al. 2009) will be necessary to shed more light on the mechanisms of repair and the breakdown of these defences in reef fish.

More work is also needed to assess the impact of UVR on reef fish at a larger scale rather than a single location. Although many of the changes resulting from anthropogenic influence on the GBR are associated with decreases in water quality and clarity (e.g. dredging and dumping of dredge-spoil, coastal development, increased run-off from agriculture), there exists the possibility that changed weather patterns, ocean acidification and habitat destruction could lead to increased levels of UVR on coral reefs. These changes are likely to affect not only a single location or species, but will play out on an ecosystem level, therefore showing the need for further study in this area.

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