**Effects of ultraviolet radiation (UVR) and other
environmental stressors on the development of intertidal
mollusc embryos**

by

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Figure 1 (a) Adult *Siphonaria australis* pulmonate limpets alongside newly deposited egg masses inhabiting the rocky intertidal zone at Point Halswell, Wellington, New Zealand.

ABSTRACT

Ozone depletion is a human-induced global phenomenon that allows increased ultraviolet radiation (UVR) to the Earth's surface. Although UVR is known to be harmful, relatively little is known about how increased UVR impacts natural ecosystems. Ecosystems in New Zealand are particularly at risk, because ozone depletion is much greater here, with levels of biologically harmful UVR up to two times greater than in northern latitudes. In the intertidal environment, potentially negative abiotic stressors are associated with low tide; and organisms inhabiting this environment are particularly vulnerable to UVR. Furthermore, embryos and larvae deposited in this habitat are especially susceptible to these stressors. The aim of this study is to identify the effect of UVR and other environmental stressors on the development of mollusc embryos in New Zealand.

Surveys of microhabitats in which egg mass deposition occurs, and what effect this site of deposition has on the survivorship of embryos, revealed that encapsulated embryos of the two common pulmonate limpets *Benhamina obliquata* and *Siphonaria australis* are highly vulnerable to the environmental stressors associated with different microhabitats. In particular, egg masses deposited in the sun for both species suffered high mortality. Although, *B. obliquata* is more susceptible to UVR damage than is *S. australis*, *B. obliquata* predominantly deposits egg masses in dry shaded microhabitats while *S. australis* deposits the majority of its offspring in sunny tidal pools, which surprisingly equated to highest embryonic mortality. Results of manipulative experiments reflected those found in the surveys: egg masses exposed to full spectrum

light incurred the greatest embryonic mortality; additionally environmental stressors (e.g. tidal pool conditions and desiccation) synergistically enhanced this mortality.

UVR in North America is significantly lower compared to New Zealand; this allowed a unique opportunity to use identical methods to examine the responses of ecologically similar, related species (bubble shell snails in the genus *Haminoea*), from two regions where UVR naturally differs. Results from surveys and manipulative experiments revealed that the New Zealand species *Haminoea zelandiae* suffered high embryonic mortality under full spectrum light and this mortality was enhanced by periods of desiccation. The North American species *Haminoea vesicula* also suffered significant mortality during periods of desiccation, but there were no signs of UVR damage. These results appear to be driven by species-specific vulnerability to these stressors and not to ambient UVR intensity in the regions at the time of study.

Relative concentrations of the chemical sunscreen compounds, mycosporine-like amino acids (MAAs), varied depending on several factors, but the biggest differences were among species. Analyses revealed that *B. obliquata* had the highest concentration of MAAs despite suffering high embryonic mortality when exposed to direct sunlight. MAA concentrations in *S. australis* were intermediate, with *H. zelandiae* having the lowest concentrations of all three species. MAA concentration for *B. obliquata* was dependent on stage of development and initial sun exposure at egg mass deposition site, suggesting interactions between MAAs, environmental conditions and embryonic development that need to be further explored. MAA concentrations were higher in *S. australis* egg masses deposited in spring compared to those deposited in early autumn, which may be driven by a shift in diet or nutrient levels. MAA concentrations did not

appear to be correlated with ambient levels of UVR or embryonic survival in *S. australis*. However, MAA concentrations were related to UV irradiance in both *Haminoea* species with higher MAA concentrations observed in egg masses initially deposited in the sun compared to those found in the shade.

Combined these results suggest: (1) increased UVR due to ozone depletion together with increases in temperatures due to climate change are likely to have strong impacts on the early life stages of these species, unless behavioural and physiological adaptations occur; (2) New Zealand species may be at particularly high risk from UVR damage compared to those from the Northern hemisphere; (3) the role of MAAs as photo-protection in these mollusc species is likely to be species specific, with a variety of abiotic and biotic factors influencing their uptake and sequestration. These experiments in part demonstrate how New Zealand's mollusc species are responding to human-induced changes in UVR levels.

Table of Contents

ACKNOWLEDGEMENTS.....	II
ABSTRACT.....	IV
TABLE OF FIGURES.....	X
LIST OF TABLES.....	XII
CHAPTER 1: GENERAL INTRODUCTION.....	14
CHAPTER 2: SYNERGISTIC EFFECTS OF ULTRAVIOLET RADIATION (UVR) AND CONDITIONS AT LOW TIDE ON BENTHIC EGG MASSES IN NEW ZEALAND.....	22
2.1 ABSTRACT.....	22
2.2 INTRODUCTION.....	23
2.3 METHODS.....	25
2.3.1 <i>Study species</i>	25
2.3.2 <i>Field surveys</i>	26
2.3.3 <i>Manipulative experiments</i>	27
2.3.4 <i>Environmental parameters</i>	31
2.3.5 <i>Statistical analysis</i>	31
2.4 RESULTS.....	32
2.4.1 <i>Field surveys</i>	32
2.4.2 <i>Manipulative experiments</i>	36
2.4.3 <i>Environmental parameters</i>	43
2.5 DISCUSSION.....	46
CHAPTER 3: VULNERABILITY OF BENTHIC MARINE EMBRYOS TO ULTRAVIOLET RADIATION: A COMPARISON OF NORTHERN AND SOUTHERN HEMISPHERE CONGENERS.....	53
3.1 ABSTRACT.....	53
3.2 INTRODUCTION.....	54
3.3 METHODS.....	57
3.3.1 <i>Study species</i>	57
3.3.2 <i>Study sites</i>	57

3.3.3 Field surveys	58
3.3.4 Manipulative experiments	59
3.3.5 Environmental parameters	62
3.4 RESULTS	64
3.4.1 Field surveys	64
3.4.2 Manipulative experiments	66
3.4.3 Environmental parameters	72
3.5 DISCUSSION	75
3.5.1 Conclusions	80
CHAPTER 4: CHEMICAL SUNSCREEN (MYCOSPORINE-LIKE AMINO ACIDS: MAAS)	
VARIATION IN MOLLUSC EMBRYOS FROM NEW ZEALAND AND USA.....	82
4.1 ABSTRACT	82
4.2 INTRODUCTION	83
4.3 METHODS	86
4.3.1 Study species	86
4.3.2 Study sites and egg mass collection	88
4.3.3 MAA analysis	89
4.3.4 Statistical analysis	90
4.4 RESULTS	91
4.4.1 Inter-specific variation and initial UVR/light exposure	91
4.4.2 Stages of development	93
4.4.3 Seasonal variation	96
4.4.4 Inter-hemispheric comparison	97
4.5 DISCUSSION	98
4.5.1 Inter-specific variation	98
4.5.2 Initial UVR/light exposure	101
4.5.3 Stages of development	103
4.5.4 Seasonal variation	104
4.5.5 Inter-hemispheric comparison	106
4.5.6 Conclusions	107

CHAPTER 5: GENERAL CONCLUSIONS.....	108
<i>5.1 Summary of findings.....</i>	<i>108</i>
<i>5.2 Future directions.....</i>	<i>112</i>
REFERENCES.....	114
APPENDICES.....	144
<i>Appendix 1: Siphonaria australis, experiment two-UVR and initial UVR/light exposure, (Run 2: 10th-14th March 2007).....</i>	<i>144</i>
<i>Appendix 2: Embryonic mortality throughout developmental stages of Siphonaria australis...</i>	<i>145</i>
<i>Appendix 3: The effects of ultraviolet radiation and low tide conditions on the development of the Northern hemisphere sea hare Phyllaplysia taylori.....</i>	<i>147</i>

Table of Figures

Figure 1 (a) Adult <i>Siphonaria australis</i> pulmonate limpets alongside newly deposited egg masses inhabiting the rocky intertidal zone at Point Halswell, Wellington, New Zealand.....	iii
Figure 2.1 <i>Siphonaria australis</i> embryos at early stage of development.....	28
Figure 2.2 Examples of viable and inviable veligers/embryos.....	30
Figure 2.3 Mean ($\pm$ 95% CI) percentage of inviable embryos of <i>Benhamina obliquata</i> and <i>Siphonaria australis</i> found in egg masses that were either submerged, desiccated or in tidal pool microhabitats at low tide and under different light exposure. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.....	34
Figure 2.4 Mean ($\pm$ 95% CI) percentage of inviable embryos of <i>Siphonaria australis</i> found in egg masses that were either submerged, desiccated or in tidal pool microhabitats at low tide and under different light exposure at Island Bay during two survey periods, either 7 th December or 9 th February. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses....	36
Figure 2.5 The effects of experimental treatments on embryonic mortality of (a) <i>Benhamina obliquata</i> and (b) <i>Siphonaria australis</i> . Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos in each light treatment (black bars are completely shaded, gray bars are UV-blocked and white bars are full spectrum) for each simulated low tide condition (submerged, desiccated, tidal pool) during two runs of each experiment (<i>B. obliquata</i> , either 16 th -20 th December 2006 or 11 th -15 th February 2007; <i>S. australis</i> , either 6 th -10 th February or 24 th -28 th September 2007).....	39
Figure 2.6 Effects of experimental light treatments (black bars are completely shaded, gray bars are UV-blocked and white bars are the full spectrum) and initial light exposure of egg masses (sunny or shaded microhabitats) on embryonic mortality of <i>Benhamina obliquata</i> and <i>Siphonaria australis</i> . Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos.....	43
Figure 3.1 Mean ($\pm$ 95% CI) percentage of inviable embryos of <i>Haminoea zelandiae</i> and <i>Haminoea vesicula</i> found in egg masses located in either desiccated or submerged microhabitats at low tide and under different light exposure. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.....	65
Figure 3.2 The effects of experimental treatments on embryonic mortality of (a) <i>Haminoea zelandiae</i> and (b) <i>Haminoea vesicula</i> . Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos in each spectral light treatment (black bars are completely shaded, gray bars are UV-blocked and white bars are the full	

spectrum) for each simulated low tide condition (submerged, desiccated, tidal pool).....68

Figure 3.3 The effect of diatoms on the viability of *Haminoea vesicula* embryos. Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos.....69

Figure 3.4 Effects of experimental light treatments (black bars are completely shaded, gray bars are UV-blocked and white bars are the full spectrum) and initial UVR exposure of egg masses (sunny or shaded microhabitats) on embryonic mortality of *Haminoea zelandiae* and *Haminoea vesicula*. Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos.70

Figure 3.5 Average daily UV indices (UVI) for Paraparaumu, New Zealand (black line) and Saturna Island, British Columbia, Canada (gray line), during spring and summer in each hemisphere. Plotted are daily UVI for 6 months. For New Zealand this is 1 September 2006–28 February 2007 and for Canada 1 March–31 August 2007. Note: these stations were the closest available in the dataset to the experimental locations.....74

Figure 4.1 Mean total MAA concentrations ($\pm$ 95% CI) for egg masses of New Zealand species *Haminoea zelandiae*, *Siphonaria australis* and *Benhamina obliquata* found in sunny and shaded sites. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.....90

Figure 4.2 The effects of stages of development on total MAA concentrations of (a) *Benhamina obliquata* and (b) *Siphonaria australis*. Plotted are mean total concentrations of MAAs ($\pm$ 95% CI) in each stage of development, and for *B. obliquata* the initial UVR/light exposure where egg mass was deposited. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses....93

Figure 4.3 Seasonal variation on total MAA concentrations of *Siphonaria australis* egg masses. Plotted are total concentrations of MAAs ($\pm$ 95% CI) during different seasons (early autumn: March 2007 and early spring: September 2007).....94

Figure 4.4 The effects of species (*Haminoea zelandiae* and *Haminoea vesicula*) and initial UVR/light exposure (sun or shade) on total MAA concentrations. Plotted are mean total concentrations of MAAs ($\pm$ 95% CI). Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.....96

List of Tables

Table 2.1 The percentage of <i>Benhamina obliquata</i> and <i>Siphonaria australis</i> egg masses found in different microhabitats and light exposure from combined survey locations.....	33
Table 2.2 Results of univariate ANOVA for the effect of microhabitat (submerged, desiccated or tidal pool at low tide), light exposure (sunny or shaded), and month of survey (December or February 2007) on embryonic mortality of <i>Siphonaria australis</i> egg masses at Island Bay. Significant factors ($p < 0.05$) are in bold.....	35
Table 2.3 Results of univariate ANOVA on the effect of experimental light treatment (full sun, UV-blocked, completely shaded), low tide conditions (submerged in flowing seawater, desiccated, and tidal pool), experimental run (December or February) and egg mass identity (nested within run) on embryonic mortality of <i>Benhamina obliquata</i> egg masses. Significant factors ($p < 0.05$) are in bold.....	38
Table 2.4 Results of univariate ANOVA on the effect of experimental light treatment (full sun, UV-blocked, completely shaded), low tide conditions (submerged in flowing seawater, desiccated, and tidal pool), experimental run (February or September) and egg mass identity (nested within run) on embryonic mortality of <i>Siphonaria australis</i> egg masses. Significant factors ($p < 0.05$) are in bold.....	41
Table 2.5 Results of univariate ANOVA for the effect of species, experimental light treatment (full sun, UV-blocked, completely shaded), initial UVR exposure at site of egg mass deposition (=Site: sunny or shaded), and egg mass identity (nested within species) on embryonic mortality of <i>Benhamina obliquata</i> and <i>Siphonaria australis</i> embryos.....	42
Table 2.6 Means of physical parameters ($\pm$ 95% CI) during the field surveys at Island Bay, and the experiments. Air and tidal pool temperatures during the surveys were recorded at low tide. Ambient seawater temperatures taken at VUCEL from 2 dates spanning the survey dates are included for comparative purposes. For the experiments, shown are means of each parameter over the course of each experiment using daily measurements taken at 2pm. Note there was no exposure to tidal pools in Experiment 2, so tidal pool temperature is not reported (NR=not reported). UVR data shown for the surveys encompasses the 5 dates prior to and including the survey date, and for the experiments, the 5 days of the experimental period.....	45
Table 3.1 The percentage of <i>Haminoea zelandiae</i> and <i>Haminoea vesicula</i> egg masses found in different microhabitats and light exposure from each survey location.....	64
Table 3.2 Results of univariate ANOVA for the effect of microhabitat (desiccated or submerged) and exposure (sunny or shaded) on embryonic mortality in	

Haminoea zelandiae and *Haminoea vesicula* egg masses from the field. Significant factors ($p < 0.05$) are in bold.....64

Table 3.3 Results from univariate ANOVA on embryonic mortality for experiment with *Haminoea vesicula* in WA, USA on experimental run (9-14 July and 17-22 July), spectral treatment (full sun, UV-blocked, and completely shaded) low tide conditions (desiccated, tidal pool, and control) and egg mass identity. Significant factors ($p < 0.05$) are in bold.....67

Table 3.4 Physical parameters during the different experiments. Shown are means of each parameter over the course of the experiment using daily measurements taken at 2pm, +/- 95% CI. Note there was no exposure to tidal pools in Experiment 2 for *Haminoea zelandiae*, so tidal pool temperature is not reported (NR=not reported). Salinities were not recorded during experiments conducted in New Zealand (ND=no data).73

Table 4.1 Results of univariate ANOVA for the effect of species (*Haminoea zelandiae*, *Siphonaria australis* and *Benhamina obliquata*) and initial UVR/light exposure at site of egg mass deposition (=Site: sunny or shaded) on total MAA concentration in egg masses from the field. Significant factors ($p < 0.05$) are in bold.....90

Table 4.2 Results from univariate ANOVA on total MAA concentration found in (a) *Benhamina obliquata* and (b) *Siphonaria australis* from the shade, egg masses during different stages of development (early, intermediate and late) and initial UVR/light exposure at site of egg mass deposition (=Site: sunny or shaded). Significant factors ($p < 0.05$) are in bold.....92

Table 4.3 Results of univariate ANOVA for the effect of species (*Haminoea zelandiae* and *Haminoea vesicula*) and initial UVR/light exposure at site of egg mass deposition (=Site: sunny or shaded) on total MAA concentration in egg masses from the field. Significant factors ($p < 0.05$) are in bold.....95

Chapter 1: General Introduction

Most marine invertebrates have complex life cycles with different modes of reproduction and development (Thorson 1950). It has long been recognised that presettlement processes occurring in early life stages significantly influence the population dynamics of marine species (Thorson 1950, Bousefield 1955, Efford 1970, De Wolf 1973). These early life stages are generally considered the most vulnerable stage of the life cycle (Thorson 1950) with mode of development significantly affecting the risk and vulnerability involved in reproduction and early development.

Invertebrates occupying the intertidal zone either free spawn gametes (with embryonic development occurring in the plankton), brood developing embryos, or produce benthic egg masses where embryos are enclosed within protective gelatinous coats or capsule walls that are attached to benthic surfaces (von Dassow and Strathmann 2005). Comparison between mortality rates of planktonic and benthic development suggest greater safety for encapsulated embryos than for pelagic embryos and larvae (Strathmann 1985, Rumrill 1990, Ohman and Hirche 2001, Lopez-Urrutia et al. 2004) with 99% of larvae of planktotrophic species dying before metamorphosis (Mileikowsky 1971). Planktonic larvae are at risk of starvation, predation and being washed away from suitable settling sites.

Depositing embryos in benthic egg masses is the parental care strategy of a variety of aquatic invertebrates. Many species have mixed life histories that incorporate aspects of both direct and pelagic developments (Mileikovsky 1971, Pechenik 1979). Embryonic development initially occurs in benthic egg masses and emerging swimming larvae subsequently spend several weeks feeding and growing in the plankton before

metamorphosing on the benthos (Thorson 1950, Pechenik 1979). Mixed life histories are considered to be beneficial as encapsulation retains embryos in the parental habitat until they are better able to cope with biotic and abiotic factors potentially encountered after hatching. However, this hypothesis assumes inconsequential prehatching mortality; an assumption that may not be valid, particularly for intertidal benthic egg masses.

Benthic egg masses deposited within the intertidal environment may be synchronously exposed to a variety of stressful conditions that can affect the development of encapsulated embryos (Hoffman et al. 2003, Przeslawski 2004a). Egg masses exposed to air are at risk of desiccation, whereas those submerged in still tidal pools are at risk of limited oxygen supply (Strathmann and Hess 1999) and embryos exposed to the sun are at risk of ultraviolet radiation (UVR) damage (Przeslawski et al. 2004). For benthic intertidal egg masses, a particularly great survival advantage must be associated with mixed development to offset the costs associated with exposure to intertidal environmental stresses (Pechenik 1979). Benthic development may also be energetically expensive. Not only does enough energy need to be furnished to the embryos to allow development to young adult status, but additional energy must go into egg capsules.

Species depositing benthic egg masses in the stressful intertidal environment can be expected to provide protection for their offspring. Exposure to stressors is often highly dependent on parental choice of deposition sites as well as the complex extra-embryonic structures that protect the embryos. Egg masses are often deposited at sites selected to reduce exposure to stress. For example, some species of snails deposit egg

capsules near anemones that protect them from predation from sea urchins (Shimek 1981). Fish eggs are guarded by males (DeMartini 1991, Hastings 1992, Warner et al. 1995) and squid eggs are deposited on the benthos subtidally, removed from solar radiation (Biggs and Epel 1991).

Many encapsulated embryos rely on a suite of mechanisms to protect them from the damaging effects of stress in the intertidal zone. For example, the gelatinous matrix in which eggs are deposited may reduce desiccation (Pechenik et al. 2003) and rate of salinity change (Woods and DeSilets 1997). Heat shock proteins may protect against thermal stress (Podolsky and Hoffman 1998) and chemical sunscreens against UVR damage (Carefoot et al. 1998, Wraith et al. 2006). However, not all animals may be able to produce protective structures that are effective throughout the adult's range of habitat (Strathmann and Strathmann 1989). Additionally, changes in environmental stressors due to global climate change and ozone depletion may have occurred over a relatively short evolutionary time period. Populations may not have had enough time to adapt to the increasingly stressful conditions brought about by these anthropogenic changes.

A variety of marine organisms that produce benthic egg masses often deposit them in sites exposed to UVR. UVR (280-400 nm) is beneficial and essential for many organisms. For example, longer wavelengths of solar radiation (UV-A) are used for vision, and plants need light for photosynthesis. However UVR—particularly ultraviolet-B (UV-B, 280-315nm)—induces deleterious effects in a range of living organisms including prokaryotic bacteria, plants, animals and humans (Sinha and Hader 2001). UVR negatively affects organisms in different ways, including: protein

destruction, reduction in growth and survival, pigment bleaching and photoinhibition of photosynthesis (Cullen et al. 1992, Sinha and Hader 1996, Sinha et al. 1996, Herndl 1997, Hader et al. 1998, Bornman and Teramura 1993, Klapner et al. 1996). The adverse effects of solar radiation on living systems are mostly attributed to the small amount of UV-B that is absorbed by cellular DNA. This effect has been identified in a variety of organisms such as bacteria, (Peak et al. 1984), cyanobacteria (Sinha et al. 2001), phytoplankton (Buma et al. 2001), macroalgae (Pakker et al. 2000), plants (Quaite et al. 1992), animals (Kripke et al. 1992), and humans (Stein et al. 1989).

There is conclusive evidence that the stratospheric ozone layer, which shields the Earth from the most biologically hazardous solar radiation, has become depleted. Anthropogenically released atmospheric pollutants such as chlorofluorocarbons, chlorocarbons and organo-bromides have catalysed this depletion and resulted in an increase in solar UV-B radiation reaching the Earth's surface (Blumthaler and Ambach 1990, Crutzen 1992, Kerr and McElroy 1993, Lubin and Jensen 1995). The Southern Hemisphere is particularly at risk to high irradiances of UVR compared to the Northern Hemisphere, in part due to the ozone hole observed over Antarctica (Farman et al. 1985), but is also explained by the closer Sun-Earth separation during the Southern Hemisphere summer (McKenzie 1991). For example, New Zealand and Australia typically receive almost twice the UVR during the summer months than similar latitudes in Europe and North America (Seckmeyer and McKenzie 1991, McKenzie et al. 1999a, b, Gies et al. 2004). Organisms in the Southern Hemisphere therefore can be predicted to be more adversely affected by UVR.

Ecological communities in both terrestrial and aquatic systems are influenced by UVR. This is evident through the molecular, physiological and behaviour strategies employed by organisms to repair, reduce or avoid UVR damage (Roy 2000). Some strategies include negative-phototaxis (Pennington and Emlet 1986, Adams 2001), non-enzymatic antioxidants (Dunlap et al. 2000), DNA repair (Kim and Sancar 1993) and chemical sunscreens (mycosporine-like amino acids: MAAs), (Shick and Dunlap 2002). Despite these strategies numerous studies have identified UV-B as an environmental stress having deleterious effects at all trophic levels in freshwater and marine ecosystems (see reviews by Hader et al. 1998, 2007, de Mora et al. 2000,, 2007). Although the damage to whole ecosystems still remains uncertain, evidence suggests that UV-B exposure may result in loss of biomass and changes to species composition (reviewed by Hader et al. 1998).

Effects of solar radiation on planktonic larvae and embryos have received considerably more attention (Thorson 1964, Damkaer et al. 1981, Hunter et al. 1982, Pennington and Emlet 1986) than the effects on benthic egg masses (but see Biermann et al. 1992, Przeslawski et al. 2004, Przeslawski 2005). Yet, benthic egg masses in the intertidal zone may be particularly vulnerable to UVR and other stressors, as there is often no medium to attenuate UV at low tide. Despite the relatively high levels of UVR in the Southern Hemisphere, little is known about the effect UVR has on New Zealand's marine environment (but see Lamare et al. 2004). Lack of research in this area makes it difficult to predict how organisms and communities will be affected by increased UVR (Lamare et al. 2004).

In addition to elevated levels of UVR, other abiotic variables may interact synergistically to affect the development of embryos in benthic egg masses. At low tide egg masses are often simultaneously exposed to a variety of physiologically stressful conditions including extreme salinities and heightened temperatures. These environmental stressors are often compounded by the effects of desiccation (e.g. Przeslawski et al. 2005b) and tidal pools at low tide. Yet the effect of multiple stressors on the survivorship of New Zealand intertidal species remains unclear. Previous studies examining the effect of environmental stress on embryonic development have focused on unrealistic periods of exposure (<1hour) (e.g. Przeslawski 2005). This study addresses this shortcoming by incorporating ecologically accurate exposure to low tidal conditions, including tidal pools.

Recent research has identified geographical hot spots, where summertime low tides coincide with the middle of the day when environmental stressors are at their peak (Helmuth et al. 2002). New Zealand's summertime low tides frequently occur in the middle of the day, when many species are reproductively active. New Zealand species are therefore exposed to extremes in anthropogenic stressors such as UVR at a time of day when other intertidal abiotic factors are greatest.

This study focuses on the effect of ecologically realistic exposure to UVR and low tide conditions on embryonic mortality of three intertidal mollusc species in New Zealand (*Siphonaria australis*, *Benhamina obliquata* and *Haminoea zelandiae*) and a congeneric species from the United States of America (USA) (*Haminoea vesicula*). *S. australis* occupies rocky intertidal shores throughout New Zealand (Jenkins 1983); while *B. obliquata* has a more restricted distribution, with populations existing in the

Otago (Borland 1950) and Wellington regions (Mestayer 1920). In comparison, both *Haminoea* species are found in sheltered sand-mudflat bays (Beeman 1968, Rudman 1971, Morris et al. 1980). All four species deposit their embryos in benthic gelatinous egg masses; however physical characteristics of the egg masses and modes of development differ among the species. *S. australis* and *H. vesicula* produce relatively small, thin egg masses while *B. obliquata* and *H. zelandiae* are much larger with generally more extra-embryonic matrices. The matrix of *B. obliquata* egg masses is much stiffer compared to the softer gelatinous extra-embryonic matrix of *H. zelandiae*. *H. zelandiae* is a direct developer and juvenile settlement occurs directly upon hatching. In contrast *S. australis*, *H. vesicula* and *B. obliquata* all have a mixed life history spending only a short period of time developing in the egg mass and comparatively a much longer period developing in the plankton. These species are all common and abundant herbivores in their relative intertidal communities. The effect of environmental stressors on early life stages is thought to be important both for the population dynamics of these species and for the structure of their respective communities.

The overall aim of this study is to examine the effects of UVR and other stressors occurring at low tide on the intertidal egg masses of these mollusc species. This aim is explored in three chapters. The first examines the effect of UVR and low tide conditions on embryonic development of the two New Zealand pulmonate limpets (*Siphonaria australis* and *Benhamina obliquata*), using manipulative experiments and field surveys of naturally deposited egg masses. The second chapter is an inter-hemispheric comparison using the same approaches to examine the effect of UVR and low tide conditions on the development of bubble shell snail embryos from the Southern

(*Haminoea zelandiae*) and Northern hemispheres (*Haminoea vesicula*). Both of these chapters also investigate the effect of intra-specific initial light exposure on embryonic development, and whether conditions and responses vary over time. Finally, the third chapter explicitly examines variability in natural chemical sunscreens (mycosporine-like amino acids: MAAs). MAAs are compared within and among species, over seasons, and the possible role these compounds have in mitigating the effects of UV damage in egg masses of *S. australis*, *B. obliquata*, *H. zelandiae* and *H. vesicula* are explored in light of the results from the previous chapters.

I hypothesise that the habitat in which egg masses from New Zealand are deposited will determine sensitivity to UVR. Specifically, egg masses deposited in direct sun will have higher concentrations of MAAs and lower embryonic mortality than those deposited in shade when exposed to further full spectrum light. Additionally, egg masses undergoing multiple environmental stressors simultaneously will have higher mortality than those subjected only to UVR. In *H. vesicula*, differences in sensitivities of egg masses laid in sun or shade will be less pronounced compared to *H. zelandiae* egg masses and will have lower MAA concentrations than those from New Zealand.

Please note: chapters of this research have been written as independent scientific papers and therefore there is some overlap in a quantity of material, particularly in the introductions.

Chapter 2: Synergistic effects of ultraviolet radiation (UVR) and conditions at low tide on benthic egg masses in New Zealand

2.1 ABSTRACT

The effects of ultraviolet radiation (UVR), desiccation and tidal pools on embryonic survival were examined in two common pulmonate limpets that lay intertidal benthic egg masses on rocky shores in New Zealand: *Benhamina obliquata* and *Siphonaria australis*. Although egg mass deposition sites were species-specific, for both species embryonic mortality was greater from egg masses in the sun compared to shade, and for *S. australis* there was also high mortality from egg masses in tidal pools or desiccated compared to those submerged in flowing seawater at low tide.

Experiments were conducted outdoors at different times of year to examine the effects of UVR exposure, and conditions at low tide for both species. Embryonic mortality was always greatest for egg masses exposed to full sun and lowest for those in completely shaded treatments. Mortality was enhanced if egg masses were in simulated tidal pools, or desiccated, but these responses were species-specific. Mortality was also greater when experiments were conducted during particularly hot, sunny periods. Finally, egg masses of both species that were initially deposited in the shade had greater mortality in response to UVR exposure compared to egg masses initially deposited in the sun. Results from this study suggest that the egg masses of these two species are highly vulnerable to UVR, as well as other intertidal environmental stressors. Embryos of both of these species are at risk of high mortality particularly during summer when

extreme conditions of UVR intensity and hot, sunny conditions coincide with low tide cycles.

2.2 INTRODUCTION

Ultraviolet radiation (UVR, 280-400 nm) can cause a range of deleterious effects on aquatic organisms, with early life stages at particular risk (reviewed by Hader et al. 2007). For example, exposure to UVR causes inhibition of photosynthesis and DNA damage to kelp zoospores (Wieneke et al. 2000). In yellow perch, 100% of eggs die when exposed to full solar UVR (Huff et al. 2004), and developing sea urchin embryos cells self-destruct in response to UVR damage (Lesser et al. 2003). UVR induced mortality in early life stages can have important implications for population dynamics of aquatic species; the global decline of amphibian populations may be due in part to mortality caused by UVR exposure during embryonic development (Blaustein and Kiesecker 2002).

Human-induced stratospheric ozone loss has resulted in elevated levels of UVR reaching the Earth's surface in recent decades, particularly for the mid-high latitudes of the Southern Hemisphere which were already exposed to higher levels of UVR (McKenzie 1991, McKenzie et al. 1999b). In New Zealand and Australia UVR is up to two times higher in the summer compared to similar latitudes in Europe and North America (Seckmeyer and McKenzie 1992, Gies et al. 2004, Lamare et al. 2004). Organisms in New Zealand may therefore be particularly vulnerable to the damaging effects of UVR, yet few studies have focused on risks to New Zealand's marine fauna (but see Lamare et al. 2004).

A variety of marine species deposit benthic egg masses throughout the intertidal zone, and recent research has been directed toward understanding what mechanisms allow early life stages to persist in this hazardous environment (reviewed by Przeslawski 2004a). Although adults can spawn in relatively stable environments where their offspring's risk to stressful environmental factors is reduced, many species routinely deposit their egg masses in sites where embryos experience periods of desiccation, UVR, temperature and osmotic stress (Benkendorff and Davis 2004). These environmental stressors can act synergistically to influence embryonic mortality in benthic egg masses on rocky shores at low tide (Przeslawski 2005, Przeslawski et al. 2005b). Tidal pools have been considered a refuge from stressful environmental conditions of rocky shores, however, a number of other environmental stressors likely occur in this microhabitat, particularly small volume pools, including extremes in salinity, water temperature and oxygen availability (reviewed by Metaxas and Scheibling 1993).

In this study, the effects of UVR, desiccation and tidal pools on the embryonic mortality were examined in two common pulmonate limpet species in New Zealand that lay benthic egg masses in the rocky intertidal zone, *Benhamina obliquata* and *Siphonaria australis*. Surveys were conducted to quantify egg mass deposition microhabitats in the field and any patterns of embryonic mortality associated with different microhabitats. Manipulative experiments were also conducted to determine the separate and combined effects of these conditions on embryonic mortality of these species.

Because UVR intensity can be dramatically altered according to the time of day, season, and cloud cover (McKenzie 1991), experiments were repeated at different times of year to examine whether natural temporal variability in climatic conditions can provide refuge from these stressors. Finally, there is evidence that species that deposit egg masses in sunny microhabitats are more likely to have innate protective mechanisms and therefore lower susceptibility to UVR, than species that exclusively deposit their eggs in shaded microhabitats (Przeslawski et al. 2004). Potential differential mortality due to site of egg mass deposition has not yet been examined within species that lay egg masses in both sunny and shaded microhabitats. Therefore the effect of initial light exposure of egg masses on embryonic mortality for both species was also examined.

2.3 METHODS

2.3.1 Study Species

Benhamina obliquata and *Siphonaria australis* are both species of pulmonate limpets inhabiting exposed and semi-exposed rocky intertidal shores throughout New Zealand (Jenkins 1983). *S. australis* occupies a wide vertical distribution, occupying low, mid and upper zones overlapping with *B. obliquata*'s range at the mid-intertidal level. Both species are hermaphrodites and produce gelatinous benthic egg masses. *S. australis* deposits numerous small, soft ribbon-shaped egg masses over the austral spring and summer months (September-April) (Knox 1955). *B. obliquata* deposits larger, stiffer gelatinous spiral-shaped egg masses, 2-3 coils per individual over a shorter breeding season (September- February) (Borland 1950). *S. australis* produces approximately 350,000 individually encapsulated embryos per season (Creese 1980), *B.*

obliquata egg masses house large numbers of transparent ovoid capsules each containing 10-30 eggs, with an estimated total of 45,000-190,000 eggs per mass (Borland 1950). Both *B. obliquata* and *S. australis* have a mixed life history where embryos spend 5-8 days developing on the benthos, followed by up to several weeks developing in the plankton as planktotrophic veligers (Borland 1950, Knox 1955).

2.3.2 Field Surveys

Surveys were conducted at low tide at six rocky intertidal sites in the Wellington region of New Zealand between 41°01' - 41°20' S and 174°42'-174°54' E (Makara Beach, Pukerua Bay, Moa Point, Island Bay, Point Halswell and Point Howard). A belt transect was established at each site in the mid-intertidal zone, 2m wide and 30m long, parallel to shore. Because the intertidal zone is relatively compressed in this region of New Zealand, the width of the transects spanned the high, mid and low littoral zones. Species identity (*Benhamina obliquata* or *Siphonaria australis*), exposure (sunny vs. shaded), and the microhabitat of deposition for every egg mass in the transect was recorded. Shaded microhabitats were either under overhangs of rocks or in deep crevices, remaining shaded at all times during the day. Comparatively sunny sites were typically on the top of rock. Egg mass microhabitats were assigned to one of three categories: desiccated on rock substrate, submerged, or in a tidal pool. Egg masses in the submerged category were deposited low enough on the shore to remain covered by water at low tide. Ten egg masses were collected from each combination of exposure and microhabitat to assess embryonic mortality, from each of six sites for *S. australis* and four sites for *B. obliquata*. Egg masses were examined each at 100x magnification on a compound microscope at the Victoria University Coastal Ecology Laboratory (VUCCEL). The first 60 embryos in each egg mass were scored as either viable (i.e.

healthy and developmentally normal in appearance) or inviable (i.e. malformed, often a mass of unidentifiable tissue). Surveys were conducted during December 2006; the same survey methods were repeated again in February 2007 for *S. australis* at Island Bay only, during a period of particularly warm and sunny weather.

2.3.3 Manipulative Experiments

Experiment One- UVR and low tide conditions

In this experiment, the effect of UVR and simulated low tide conditions on embryonic mortality of both *Benhamina obliquata* and *Siphonaria australis* egg masses was examined. The same experimental methods on egg masses for each species were used, but to minimize the logistical difficulties, each experiment on each species was conducted separately.

Egg masses were collected from shaded microhabitats in the field, and examined to ensure that only those still at very early stages (blastula) of embryonic development were used in the experiment (Fig 2.1). Six egg masses, separated by substantial distances and assumed to be deposited by different individuals, were collected (except in the September run of *S. australis*, in which only three were collected). Each egg mass was divided into nine equal-sized sections and each section was placed in its own 100ml plastic container, to ensure each egg mass was represented in all treatments. Numerous small holes were drilled through each container to ensure egg masses were retained, but water was free to circulate. For the UVR treatment there were three levels: full spectrum light, UV-blocked, and completely shaded. Three segments of each egg mass were randomly allocated to each treatment. These treatments were established by applying plastics with the appropriate UVR absorbing properties to each container as

lids and affixed around the outside (full spectrum light: PVC, UV-blocked: polycarbonate, completely shaded: foil). The plastic containers were transparent to UVR radiation; each absorbing plastic was tested using a UV-1601 Shimadzu spectrometer. These containers were then placed outside in a shallow seawater aquarium with continuously flowing seawater in a sunny location at Victoria University Coastal Ecology Laboratory (VUCEL). Seawater was first filtered through a 10µm bag filter to remove particles.

Figure 2.1 *Siphonaria australis* embryos at early stages of development.

Three levels of simulated low tide conditions were established as well: constantly submerged in flowing seawater (a control), desiccated for three hours to simulate aerial exposure at low tide, and three hours exposure to non-circulating heated seawater to simulate a tidal pool. From each egg mass, one of each of the three sections in the respective light treatments was randomly allocated to one of these low tide conditions. At 11am each day the containers exposed to three hours of desiccation were drained and placed in a dry aquarium immediately adjacent to, and similar in all respects to, the control aquarium. The containers subjected to simulated tidal pool conditions were placed in a third aquarium, again immediately adjacent, to which

seawater that had been allowed to heat naturally in a black tub exposed to full sun for approximately three hours prior to the experiment was added. The remaining containers were left in the submerged circulating seawater aquarium, to act as a control, but were momentarily lifted and replaced to mimic the movement of the other containers to their treatments. At 2pm the desiccation and tidal pool treatments were returned to the control aquarium. All positions of containers in all aquaria were random and changed daily. The daily assignment of aquaria to either the desiccated or tidal pool treatment was also random. Each experiment ran for five days, the point at which hatching was imminent but had not yet occurred.

To analyze the embryonic mortality of each egg mass similar methods to Przeslawski et al. (2004) were followed. Each segment of egg mass was placed in a vial with 400µl of filtered seawater and was agitated by hand for two minutes, the egg mass was then suspended in water. From this, 5µl were analyzed microscopically until the whole sample had been examined and embryos were scored as viable or inviable, as defined above except in this case all viable embryos had developed a larval shell, or protoconch (e.g. Fig 2.2). The analysis of embryonic mortality occurred while the samples were fixed in 5% buffered formalin for *B. obliquata* during experiment one, but was conducted while the embryos were still alive for the other experiments. Experiments were conducted on 16th-20th December 2006 (Run 1) and repeated 11th-15th February 2007 (Run 2) for *B. obliquata*, and on 6th-10th February 2007 (Run 1) and 24th-28th September 2007 (Run 2) for *S. australis*.

Figure 2.2 Examples of viable and inviable veligers/embryos.

Experiment Two-UVR and initial UVR/light exposure

In this experiment the effect of UVR and initial light exposure of egg masses on subsequent embryonic mortality of both species was examined in one factorial design. *Benhamina obliquata* and *Siphonaria australis* egg masses were collected from shaded and sunny microhabitats. Six egg masses at early stages of development were selected for each species from each microhabitat, and each was divided into three sections. Experimental UVR treatments were established using identical methods as experiment one above with the containers constantly submerged in circulating seawater. Embryonic mortality was scored in the same way as experiment one; all embryos were alive when scoring occurred at the end of the five days. This experiment was conducted once, on 12th-16th January 2007 (but see Appendix 1).

2.3.4 Environmental Parameters

Air and water temperatures, and UV Index (UVI) data were obtained during the field surveys at Island Bay (7th December 2006 and 9th February 2007), and during the experiments. For the surveys, air temperatures were noted at five times over the course of several hours spanning the survey period, and the water temperature of tidal pools from which egg masses were sampled was also recorded (n= 10 tidal pools on each date). UVR measurements were obtained from Industrial Research Limited (IRL), which maintains a UV radiometer located <5km from VUCEL that records the UVI every minute. The mean maximum daily UVI and mean UVI from 10am-4pm over the five days preceding each survey were calculated.

For each experiment, water temperatures of all treatments, and the air temperature, were recorded at 2pm every day. The IRL UVI data was also used to calculate the mean daily UVI maximum and mean UVI from 10am- 4pm over the course of each experiment.

2.3.5 Statistical Analyses

For the field surveys, the percentage of egg masses in the different exposures and microhabitats was analysed statistically in SPSS using a non-parametric chi-square test for both *Benhamina obliquata* and *Siphonaria australis*, combining data from all sites for each species. The percentage of inviable embryos across the different microhabitats was analysed using univariate ANOVA, with the fixed factors exposure and microhabitat. An additional univariate ANOVA was conducted for *S. australis* egg masses from Island Bay only, which were surveyed twice, and month was added as a fixed factor to the model.

Experimental data for each species were analyzed using univariate ANOVA, and for the first experiment the fixed factors included UVR/light treatment, low tide conditions, and experimental run. In the model for the second experiment the factors were species, initial light exposure and UVR/light treatment. Egg mass identity was also included as a random factor in models for both experiments to account for potential variability among individuals. For experiment one, this factor was nested within experimental run.

Percentage data were arcsine square root transformed (as recommended by Zar 1984) and transformed data fulfilled assumptions of homogeneity (when examined using Levene's test) and normality (by examination of normal probability plots).

2.4 RESULTS

2.4.1 Field Surveys

Although these two species are generally found in similar rocky intertidal habitats, they deposited their egg masses in different microhabitats (Table 2.1). *Benhamina obliquata* egg masses were only found desiccated on the rock substrate, and never in tidal pools or submerged in freely flowing water (Table 2.1). Of these egg masses, a greater proportion were deposited in the shade than in the sun (Table 2.1, $\chi^2 = 17.07$, $p < 0.001$), and shaded egg masses had greater embryonic survival (Fig 2.3, $F_{1,42} = 23.45$, $p < 0.0001$). Although many fewer egg masses were deposited in sunny microhabitats, they suffered over 40% embryonic mortality, compared to less than 10% mortality from those in the shade (Fig 2.3).

By contrast, a significantly greater proportion of *Siphonaria australis* egg masses were found in the sun compared to the shade (Table 2.1, $\chi^2 = 73.38$, $p < 0.001$). A greater proportion of *S. australis* egg masses were also found in tidal pools compared to desiccated or submerged microhabitats (Table 2.1, $\chi^2 = 70.04$, $p < 0.001$), however substantial proportions of egg masses (i.e. $> 20\%$) were found in all three microhabitats. Although the greatest proportion of *S. australis* egg masses were deposited in tidal pools, this was the microhabitat that incurred greatest embryonic mortality, nearly 25%, whereas $< 5\%$ of those that remained submerged at low tide were inviable (Fig 2.3, $F_{2,344} = 31.993$, $p < 0.001$, Tukey HSD $p < 0.001$). Similar to *B. obliquata*, regardless of microhabitat, embryonic mortality was significantly greater from egg masses in the sun compared to the shade (Fig 2.3, $F_{1,344} = 28.923$, $p < 0.001$), with no interaction between the microhabitat and sun exposure ($F_{2,344} = 0.568$, $p = 0.57$).

Table 2.1 The percentage of *Benhamina obliquata* and *Siphonaria australis* egg masses found in different microhabitats and light exposure from combined survey locations.

Microhabitat	<i>Benhamina obliquata</i>	<i>Siphonaria australis</i>
Sunny	23%	64%
Shaded	77%	36%
Submerged	0%	22%
Desiccated	100%	35%
Tidal Pool	0%	43%

Figure 2.3 Mean ($\pm$ 95% CI) percentage of inviable embryos of *Benhamina obliquata* and *Siphonaria australis* found in egg masses that were either submerged, desiccated or in tidal pool microhabitats at low tide and under different light exposure. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.

When comparing embryonic mortality of *S. australis* from surveys in Island Bay only, in December with surveys in February during a warm sunny week, greater mortality was found in egg masses exposed to sun, and least mortality in egg masses that were submerged (Fig 2.4, Table 2.2) in both surveys. There was greater overall embryonic mortality in February, and a significant interaction between survey month and light exposure that arose because the magnitude of the effect of being exposed to the sun was greater in February compared to December, in all three microhabitats (Fig 2.4, Table 2.2).

Table 2.2 Results of ANOVA for the effect of microhabitat (submerged, desiccated or tidal pool at low tide), exposure (sunny or shaded), and month of survey (December or February 2007) on embryonic mortality of *Siphonaria australis* egg masses at Island Bay. Significant factors ($p < 0.05$) are in bold.

Source of Variation	MS	df	F	p
Month	0.785	1	4.311	0.040
Microhabitat	1.042	2	5.721	0.004
Exposure	2.831	1	15.548	<0.001
Month*Microhabitat	0.235	2	1.841	0.164
Month*Exposure	1.191	1	9.312	0.003
Microhabitat*Exposure	0.010	2	0.079	0.924
Month*Microhabitat*Exposure	0.029	2	0.225	0.799
Error	0.128	108

Figure 2.4 Mean ($\pm$ 95% CI) percentage of inviable embryos of *Siphonaria australis* found in egg masses that were either submerged, desiccated or in tidal pool microhabitats at low tide and under different light exposure at Island Bay during two survey periods, either 7th December or 9th February. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.

2.4.2 Manipulative Experiments

Experiment One- UVR and low tide conditions

For *Benhamina obliquata*, there was dramatically greater mortality in egg masses exposed to full spectrum light (60-100%), across microhabitat treatments, compared to those that were completely shaded or in UV-blocked treatments, in both experiments (Fig 2.5a, Table 2.3). Egg masses in tidal pools had overall higher mortality than those that were desiccated, and those in control, flowing seawater had very low mortality (Fig 2.5a, Tukey HSD $p < 0.001$). An interaction between light treatment and microhabitat arose because although there was significant mortality in

tidal pools even in shaded treatments, there was no mortality in shaded treatments in control egg masses (Fig 2.5a, Table 2.3). Overall however, mortality due to microhabitat was lower than that due to exposure to UVR. The highest mortality, nearly 100%, occurred in egg masses in the sun, exposed to simulated tidal pool conditions, whereas there was almost no mortality in egg masses in constantly submerged flowing seawater that were shaded (Fig 2.5a).

B. obliquata egg masses endured slightly higher embryonic mortality in February compared to December (Fig 2.5a, Table 2.3). Mortality due to full sun was similar in both runs, but there was greater death in the UV-blocked and shaded treatments in February, leading to the significant interaction between light treatment and run (Table 2.3). Significant interactions were also observed between the survey months, exposure to low tide conditions and light treatments (Table 2.3). These are driven by elevated mortality in the tidal pool treatment of the UV-blocked and shaded egg masses, suggesting that the tidal pool conditions were particularly severe in the February run.

Siphonaria australis's responses to experimental treatments were driven primarily by differences in the two runs (Fig 2.5b, Table 2.4). The interactions between run and the experimental treatments are driven by the fact that there was no effect of light or microhabitat on embryonic mortality in September, when overall mortality was low (Fig 2.5b, Table 2.4). However, in February, there was greater mortality (70-100%) due to exposure to full sun compared to either UV-blocked or completely shaded treatments (Fig 2.5b, Tukey HSD $p < 0.001$). In contrast to *B. obliquata*, during this same experiment there were relatively high and similar levels of mortality in desiccated and tidal pool treatments, even those that were shaded where roughly 60% of embryos

were inviable (Fig 2.5b). In the control treatment of flowing seawater there was almost no mortality of egg masses in the shade (Fig 2.5b).

Table 2.3 Results of ANOVA on the effect of experimental light treatment (full sun, UV-blocked, completely shaded), low tide conditions (submerged in flowing seawater, desiccated, and tidal pool), experimental run (December or February) and egg mass identity (nested within run) on embryonic mortality of *Benhamina obliquata* egg masses. Significant factors ($p < 0.05$) are in bold.

Source of Variation	MS	df	F	p
Run	0.321	1	149.299	< 0.001
Light Treatment	10.476	2	1200.048	< 0.001
Tidal condition	1.890	2	216.449	< 0.001
Run*Light Treatment	0.065	2	7.481	< 0.001
Light Treatment*Tidal condition	0.052	4	5.974	0.003
Run*Tidal condition	0.030	2	3.405	0.038
Run*Light Treatment*Tidal condition	0.034	4	3.902	0.006
Egg mass (Run)	0.002	10	0.246	0.990
Error	0.009	80

(a) *Benhamina obliquata*

(b) *Siphonaria australis*

Figure 2.5 The effects of experimental treatments on embryonic mortality of (a) *Benhamina obliquata* and (b) *Siphonaria australis*. Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos in each light treatment (black bars are completely shaded, gray bars are UV-blocked and white bars are full spectrum) for each simulated low tide condition (submerged, desiccated, tidal pool) during two runs of each experiment (*B. obliquata*, either 16th-20th December 2006 or 11th-15th February 2007; *S. australis*, either 6th-10th February or 24th-28th September 2007).

Table 2.4 Results of ANOVA on the effect of experimental light treatment (full sun, UV-blocked, completely shaded), low tide conditions (submerged in flowing seawater, desiccated, and tidal pool), experimental run (February or September) and egg mass identity (nested within run) on embryonic mortality of *Siphonaria australis* egg masses. Significant factors ($p < 0.05$) are in bold.

Source of Variation	MS	df	F	p
Run	8.085	1	288.213	< 0.001
Light Treatment	0.709	2	25.856	< 0.001
Tidal condition	1.095	2	41.466	< 0.001
Run*Light Treatment	1.491	2	56.456	< 0.001
Light Treatment*Tidal condition	0.022	4	0.838	0.507
Run*Tidal condition	0.907	2	34.363	< 0.001
Run*Light Treatment*Tidal condition	0.066	4	2.530	0.050
Egg mass (Run)	0.028	7	1.062	0.400
Error	0.026	56		

Note: In September $n = 3$ replicate egg masses, but $n = 6$ in February.

Experiment Two-UVR and initial UVR/light exposure

Similar to experiment one, there was a dramatic increase in mortality from egg masses exposed to full spectrum light compared to those in either completely shaded or UV-blocked treatments, which suffered almost no mortality (Fig 2.6, Table 2.5). Additionally, *Benhamina obliquata* embryos suffered higher overall mortality when exposed to full sun compared to *Siphonaria australis* (Fig 2.6, Table 2.5). Further, for egg masses exposed to full sun only, there was increased mortality (almost doubled for both species) if the egg masses had initially been laid in the shade compared to those that had been initially laid in the sun (Fig 2.6, Table 2.5).

Table 2.5 Results of ANOVA for the effect of species, experimental light treatment (full sun, UV-blocked, completely shaded), initial UVR exposure at site of egg mass deposition (=Site: sunny or shaded), and egg mass identity (nested within species) on embryonic mortality of *Benhamina obliquata* and *Siphonaria australis* embryos.

Source of Variation	MS	df	F	p
Species	0.037	1	10.169	0.010
Site	0.227	1	45.267	< 0.001
Light	3.657	2	727.73	< 0.001
Species*Site	0.002	1	0.403	0.529
Species*Light	0.064	2	12.833	< 0.001
Site*Light	0.233	2	46.361	< 0.001
Species*Site*Light	0.000	2	0.064	0.939
Egg mass (Species)	0.004	10	0.716	0.705
Error	0.005	50

Figure 2.6 Effects of experimental light treatments (black bars are completely shaded, gray bars are UV-blocked and white bars are the full spectrum) and initial light exposure of egg masses (sunny or shaded microhabitats) on embryonic mortality of *Benhamina obliquata* and *Siphonaria australis*. Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos.

2.4.3 Environmental Parameters

The surveys conducted in February (mid-summer) had higher air, water temperatures and mean UV Index (UVI) values compared to December (Table 2.6). Similarly, air, flowing seawater, and simulated tidal pool temperatures all peaked in the February (mid-summer) runs of experiment one for both species (Table 2.6), and were dramatically lower in the September run (early spring) of experiment one for *Siphonaria australis*. In all but the spring run, temperatures of simulated tidal pool temperatures were higher than flowing seawater treatments (Table 2.6). UVI values were similarly high during the experiments in December and February, but relatively

low in January over experiment two, and lowest in September (Table 2.6). The unusually low mean UVI in the January run was most likely due to heavy cloud cover on the 12th and 13th.

Table 2.6 Means of physical parameters ($\pm$ 95% CI) during the field surveys at Island Bay, and the experiments. Air and tidal pool temperatures during the surveys were recorded at low tide. Ambient seawater temperatures taken at VUCEL from 2 dates spanning the survey dates are included for comparative purposes. For the experiments, shown are means of each parameter over the course of each experiment using daily measurements taken at 2pm. Note there was no exposure to tidal pools in experiment two, so tidal pool temperature is not reported (NR=not reported). UVR data shown for the surveys encompass the five dates prior to and including the survey date, and for the experiments, the five days of the experimental period.

	Date	Temperature (°C)			UV Index (mW/m ²)	
		Air	Seawater	Tidal Pool	Daily Max	10am-4pm
(a) Field surveys	7th Dec 06	14 $\pm$ 0.37	15.5 $\pm$ 0.69	17.8 $\pm$ 1.18	10.53 $\pm$ 2.66	8.86 $\pm$ 2.86
	9th Feb 07	19 $\pm$ 0.78	17.4 $\pm$ 0.88	22.6 $\pm$ 1.82	12.11 $\pm$ 0.24	10.85 $\pm$ 0.09
(b) Experiments						
Exp. 1. <i>B. obliquata</i>	16-20 Dec 06	20.4 $\pm$ 2.09	18.4 $\pm$ 0.90	23.5 $\pm$ 1.28	12.48 $\pm$ 1.78	10.34 $\pm$ 1.78
Exp. 2 both species	12-16 Jan 07	20.6 $\pm$ 1.33	17.2 $\pm$ 0.91	NR	6.65 $\pm$ 1.58	5.55 $\pm$ 1.77
Exp. 1. <i>S. australis</i>	6-10 Feb 07	27.4 $\pm$ 1.00	19.0 $\pm$ 0.88	25.2 $\pm$ 1.69	11.77 $\pm$ 0.66	10.36 $\pm$ 0.95
Exp. 1. <i>B. obliquata</i>	11-15 Feb 07	23.8 $\pm$ 3.63	17.4 $\pm$ 1.33	24.1 $\pm$ 4.61	10.96 $\pm$ 1.04	9.18 $\pm$ 1.89
Exp. 1. <i>S. australis</i>	24-28 Sept 07	10.2 $\pm$ 1.67	9.92 $\pm$ 0.74	10.06 $\pm$ 0.74	5.30 $\pm$ 0.85	5.16 $\pm$ 0.10

2.5 DISCUSSION

This study demonstrates that for these two pulmonate limpet species, egg mass deposition site is extremely important in determining susceptibility of embryos to different environmental stressors. In this study, *Benhamina obliquata* predominantly deposited its offspring in shaded, desiccated crevices, which corresponded to the highest probability of survival. It is not known what drives choice of egg mass deposition site in this species, as *B. obliquata* egg masses are thought to be deposited at night at low tide (Borland 1950) when natural cues about exposure to sun are reduced. However, this microhabitat may also offer protections against biotic factors as crevices mid-high in the intertidal zone are often inaccessible to mobile predators (Rochette and Dill 2000).

Compared to *B. obliquata*, *Siphonaria australis* egg masses were not found predominantly in microhabitats that resulted in highest embryonic survival. Rather the opposite, the microhabitat where the greatest proportion of *S. australis* egg masses were found was tidal pools where egg masses suffered the greatest mortality. Further, egg masses were more likely to be found exposed to the sun than in the shade, even though there was high mortality associated with exposure to the sun. A possible obstacle to protecting benthic egg masses may be the scarcity of safe deposition sites (Wilson 1986; von Dassow and Strathmann 2005), particularly for homing species such as *Siphonaria* spp.; favourable sites may be scarce in the vicinity of a home scar and individuals may therefore be limited by availability. However, the heterogeneous topography of Wellington's rocky intertidal shores creates a high availability of shaded microsites. This is supported by the fact that *B. obliquata* and *S. australis* co-occurred at several sites surveyed here, and *B. obliquata* egg masses were not found in tidal pools in these same surveys, and were more often in shade. It is also possible that minimizing

exposure of embryos to environmental stressors is not the primary driver of adult choice of egg mass deposition site for either species, but that it is instead more closely tied to predator avoidance or adult life history (Spight 1977). Reducing the risk of biotic factors such as predation may outweigh the risk associated with abiotic factors. Macroscopic predation was rarely observed during these studies and previous research has found intertidal molluscs and their egg masses to be unpalatable to predators (Ocana and Emson 1999), although avoidance of microscopic predation may be affecting spawning site selection. For example, protists may not only consume embryos but also compete for valuable resources such as oxygen which may increase mortality (Booth 1995, Strathmann and Strathmann 1995, Cohen and Strathmann 1996). Further, adult spawning behaviour may be determined by optimisation of other life stages, for example, wave action may be more conducive to hatched veligers and these benefits may outweigh the risk of environmental stressors found at these sites (Przeslawski and Davis 2007).

The ability of *B. obliquata* to deposit offspring in favourable microhabitats may be a reflection of the relative energetic investment in the egg mass of this species. The relatively large egg masses of *B. obliquata* can require 6-8 hours to deposit and individuals are thought to only deposit 2-3 egg masses per season (Borland 1950); therefore there would be great selective pressure to develop behaviours that maximise protection of egg masses and thereby increase fitness. In comparison, individual *S. australis* produce vast numbers of small and energetically relatively inexpensive egg masses over several months in spring and summer (Knox 1955). Smaller egg masses equate to reduced numbers of egg masses exposed to abiotic stress. Results from this study show that environmental stressors that are great in mid-summer can be much reduced in early spring or late summer, so mortality may be highly seasonal.

Nevertheless, there must be a huge cost to reproductive success due to loss of embryos exposed to the sun in mid-summer, in particular for *S. australis*.

The findings of the field surveys were supported by the manipulative experiments where for both species the overwhelming effect on mortality was exposure to UVR. Similar results have been observed among benthic egg laying gastropods in Australia (Przeslawski et al. 2004). This study, however, has been the first to demonstrate the cost of tidal pool immersion on benthic egg masses, which was particularly strong for *S. australis* in the summer run. Tidal pools are considered refuges that protect against desiccation (reviewed by Metaxas and Scheibling 1993), and perhaps they are during non-summer months, as suggested by the trend towards lower mortality in tidal pools compared to desiccation treatments in the experiment conducted in early spring.

Synergistic interactions among environmental stressors in the intertidal zone can be extremely complex and have important implications on embryonic mortality and population dynamics of species (Przeslawski et al. 2005b). In small tidal pools, evaporation caused by high temperature results in increased salinity (reviewed by Przeslawski 2004a). In one study, egg masses exposed to high salinity (45 ppt) caused embryonic mortality to increase markedly (Przeslawski et al. 2005b). Also, oxygen availability in intertidal pools may interact significantly with UVR to cause embryonic mortality. For example, the development of echinoid eggs in still water, where oxygen availability may be limited, revealed slower development (Strathmann and Strathmann 1989). *S. australis* embryonic mortality in desiccated microhabitats was similar to that in tidal pools and may also be driven by salinity stress, as evaporation of capsular fluid will increase intracapsular osmotic concentration within an egg mass (Pechenik 1986).

Egg masses can display inter-specific variation size, shape and colour (Pilkington 1974). These variations are predicted to reflect evolutionary adaptations to maximise hatching success and reduce energy costs among stressful abiotic factors (Clark and Goetzfried 1978). The lower susceptibility of *Benhamina obliquata* egg masses to desiccation compared to *Siphonaria australis* is likely due to species-specific egg mass characteristics. Thickness of the egg mass wall, shape and size can profoundly affect the rate of water loss and therefore the survival of the encapsulated embryos (Rawlings 1995); egg masses like *B. obliquata* that have stiffer walls (Feder et al. 1982) and coiled in shape (Chambers and McQuaid 1994) are thought to reduce desiccation. Further, *B. obliquata* egg masses may be better suited to desiccated microhabitats as larger gelatinous egg masses may reduce stress associated with desiccation to internal embryos (Przeslawski and Davis 2007).

Results here show that initial exposure to sun to some degree mitigates effects of further UVR damage on egg masses for these two species. Similarly, a study in Australia found that among species, molluscs that deposit eggs exclusively in the shade have greater embryonic mortality when exposed to UVR than those that deposit eggs in the sun (Przeslawski et al. 2004). Przeslawski et al. (2004) considered that the *Siphonaria* spp. in their study deposited egg masses in the sun and, in contrast to this study, they found little embryonic mortality due to further exposure to UVR. Here, although egg masses of the two focal species had lower mortality if deposited in the shade, full spectrum light still caused significantly higher embryonic mortality compared to completely shaded spectral treatments irrespective of initial exposure. A possible explanation for the discrepancy in these findings is that although timing of the experiments in the other study is not specified, there may be a seasonal component to

UVR vulnerability, and perhaps that study was not conducted in mid-summer as the first run here was. This would be supported by the fact that in September *S. australis* in this study also had low mortality with no response to experimental manipulations to UVR.

There has been a great deal of focus on the role of chemical sunscreens such as mycosporine-like amino acids (MAAs) in mitigating effects of UVR (Shick and Dunlap 2002). However, preliminary MAA analysis on the egg masses from this study (Chapter 4), as well as analyses from gastropods in Australia (Przeslawski 2004b), have found no significant difference in MAA concentration between egg masses deposited in the sun vs. those from the shade (Chapter 4). Further, Wraith et al. (2006) explicitly tested embryonic mortality in egg masses with different natural levels of MAAs and found that these compounds were probably not the most important source of protection against UVR damage. Alternative protective mechanisms may involve DNA repair mechanisms (Kaufmann and Paules 1996, Epel et al. 1999), and UVR absorption in egg mass walls (Rawlings 1996). These may offer explanations as to species-specific vulnerability to UVR (as in this study *B. obliquata* was at greater risk than *S. australis*), however it is more challenging to speculate about what might drive the intra-specific differences in mortality, which here were dependent on initial sun exposure, and further study is required.

This study also clearly shows the important role that temporal variability in environmental stressors play in influencing embryonic survival of benthic-developing intertidal organisms. Survey and experimental results reveal that embryonic mortality was generally greatest in the middle of summer (i.e. February) when the environment was most stressful. One puzzling result however was found for *S. australis* egg masses

initially deposited in the shade, exposed to full sun, but submerged in flowing seawater, when comparing those treatments across experiment one and two. There was high mortality in February (experiment one) when UVI and water temperatures were high, particularly when compared to the identical treatment in September (experiment one) when UVR and temperatures were at their lowest and so was embryonic mortality. However, mortality in this treatment in January (experiment two) was unexpectedly high given the relatively low mean UVI for that experiment (6.7), which was higher than, but similar to, mean UVI in September (5.3) rather than the other values in December and February which were all >10. This unusually low mean UVI recorded for the January experiment is likely attributable to heavy cloud cover that occurred over two of the days of the experiment. The relatively high mortality of *S. australis* in January compared to September may be attributed to the variable UVR over the January experimental time period, or perhaps indicate a threshold response of exposure between UVI of 5-7. Alternatively, higher embryonic mortality in submerged egg masses exposed to the sun may indicate a synergistic response between exposure to UVR and elevated water temperatures, and these hypotheses will be the basis for future experiments.

Despite the substantial increases in UVR reaching the Earth's surface that have been occurring for over 30 years, particularly in the Southern Hemisphere, research investigating effects on New Zealand's marine fauna has been minimal (but see Lamare et al. 2004). Unlike other studies focusing on the detrimental effects on early development using artificial sources and extreme levels of UVR, the conclusions from this study are ecologically realistic representations of natural mortality for these two common species. Further, geographical shifts in the timing of summertime low tides coinciding with the middle of the day when conditions are most extreme, means that

organisms in some locations are exposed to much higher thermal stress than others (Helmuth et al. 2002). In New Zealand, summertime low tides commonly fall in the middle of the day, which means that organisms here are not only subjected to extremes of thermal stress, but simultaneously to high levels of UVR. This suggests that intertidal fauna in New Zealand and Australia may be particularly vulnerable to synergistic effects of UVR and other stressors, particularly in light of predicted climate change.

Chapter 3: Vulnerability of benthic marine embryos to ultraviolet radiation: a comparison of Northern and Southern hemisphere congeners

3.1 ABSTRACT

Ultraviolet radiation (UVR) is widely recognised as a stressor able to cause a range of deleterious effects across taxa, with early life history stages particularly at risk. Due to interhemispheric differences in UVR intensity, Southern Hemisphere species may be at greater risk from damage due to UVR stress than Northern species at similar mid-high latitudes. This study was designed to explicitly compare the vulnerability of benthic marine embryos that develop in the stressful intertidal zone using ecologically similar, congeneric species from similar latitudes in the Northern and Southern hemispheres. Field surveys and multi-factorial experiments were used to examine synergistic effects of UVR and other environmental stressors at low tide (e.g. increased temperatures, salinity) on the embryonic mortality of two tidal-flat dwelling bubble shell snail species: *Haminoea zelandiae* from New Zealand, and *Haminoea vesicula* from Washington, USA.

In the manipulative experiments, UVR induced embryonic mortality was high for *H. zelandiae*, whereas *H. vesicula* egg masses were only slightly affected. By contrast, desiccation was a greater source of mortality for *H. vesicula* than *H. zelandiae*, and simulated tidal pools slightly increased mortality only for *H. vesicula*. Surveys of naturally deposited egg masses supported the experimental results: for *H. zelandiae*, those in the sun had twice the mortality of those in the shade, whereas for *H. vesicula*, there was nearly 30% mortality for desiccated egg masses, but no additional mortality

due to UVR exposure. Additionally, there was evidence that egg masses initially deposited in the sun were less vulnerable to effects of UVR than those initially deposited in the shade.

Experiments were conducted in northern mid-summer, but austral late summer, and UV indices were similar in both locations. These results are therefore conservative for *H. zelandiae*, and suggest an important role of species-specific differences in vulnerability to different stressors, even for these very similar sister-species, which here may be related to development time or egg mass characteristics. This work supports other studies demonstrating that Southern Hemisphere species may be particularly at risk to UVR, but shows that vulnerability can be due to species-specific characteristics, and not only interhemispheric differences in UVR intensity.

3.2 INTRODUCTION

Ultraviolet radiation (UVR, 280-400 nm) is recognized as an important environmental stressor, and UV-B (280-315 nm) in particular can cause a range of deleterious impacts on organisms including damage to DNA, increases in disease or risk of infection, stunted and delayed development, induction of deformities, and mortality across a range of taxa (reviews by Day and Neale 2002, Paul and Gwynn-Jones 2003). Physiological, behavioural and molecular mechanisms to reduce, avoid or repair UVR damage indicates that UVR is a significant selective force in ecological communities (Hader et al. 2007). Despite many protective strategies against UVR, UVR damage occurs in numerous organisms in both aquatic and terrestrial ecosystems, including bacteria, protists, plants and both invertebrate and vertebrate animals (Williamson et al. 1994, Hader and Worrest 1997, Sommaruga et al. 1997).

Aquatic animals, particularly early life stages, are at high risk from UVR damage (reviewed by Hader et al. 2007). For example, damage from UVR to eggs and larvae has been identified as a contributor to dramatic world wide declines in amphibian populations in recent years (Kiesecker et al. 2001). A number of studies document effects of UVR on freshwater zooplankton, (e.g. reviewed by Marinone et al. 2006), as well as marine planktonic larvae. UVR exposure leads to delays in cleavage (Rustad 1975, Adams and Shick 1996,) and cell self-destruction (Lesser et al. 2003) in sea urchin embryos, and reduced survival in the eggs and embryos of crustaceans and fish (Kouwenburg et al. 1999, Browman et al. 2000, 2003, Ban et al. 2007).

Although the majority of marine animals have a planktonic larval stage, many coastal marine species (both invertebrate and fish) reproduce by depositing their offspring in benthic egg masses in the intertidal zone. However, the vulnerability of embryos developing in benthic egg masses, where there is no water to attenuate the transmission of UVR, has surprisingly gained much less attention (but see Biermann et al. 1992; Rawlings 1996; Przeslawski et al. 2004; Przeslawski 2005). The intertidal zone is highly heterogeneous and egg masses deposited here may be exposed to a variety of stressful environmental conditions including: desiccation, osmotic stress, temperature stress and UVR (reviewed by Przeslawski 2004a). UVR is likely to be particularly stressful because many species reproduce over spring and summer when UVR is at its annual maximum.

The Southern Hemisphere is particularly vulnerable to high intensities of solar UVR, due in large part to interhemispheric and seasonal patterns of ozone depletion and the Sun-Earth separation (McKenzie et al. 1999a,b.). In the summer, locations in

Australia and New Zealand often receive much higher levels of UVR, up to a factor of two, compared to similar mid to high latitudes in Europe and North America (Seckmeyer and McKenzie 1992, Gies et al. 2004, Lamare et al. 2004). In Australia, researchers have recently found strong effects of desiccation (due to aerial exposure at low tide) and UVR on embryonic mortality of some rocky shore gastropod species (Przeslawski 2005). However, despite UVR intensity differing between the Northern and Southern Hemispheres, the effect of UVR on developing embryos of ecologically similar species between these two hemispheres has yet to be explicitly compared. In this study the effects of naturally variable UVR and its interaction with other environmental stressors at low tide was examined on the embryonic mortality of two congeneric species of marine bubble shell snails (Order: Cephalaspidea): *Haminoea zelandiae* (Southern Hemisphere) and *Haminoea vesicula* (Northern Hemisphere). Further, previous research has found species-specific responses to UVR, based on whether species preferentially deposit egg masses in either sunny or shaded microhabitats (Przeslawski et al. 2004). Species that deposit eggs in shaded habitats suffered higher mortality when subjected to UVR than those that deposit eggs in sunny ones. Because egg masses of both of the focal species in this study can be found in sun and shade, I also tested whether conditions at the initial site of egg mass deposition (i.e. sunny vs. shaded) mediate intra-specific responses to UVR.

3.3 METHODS

3.3.1 *Study species*

Both *Haminoea* species are common in sheltered intertidal bays and harbours, and often abundant on sand or mudflats. *H. zelandiae* is distributed around the North Island and northern part of the South Island of New Zealand whereas *H. vesicula* is found along the Eastern Pacific from the Gulf of California to Alaska (Morris et al. 1980). Both species deposit gelatinous egg masses in spring and summer, attaching them to firm substrata such as eelgrass, algae, or shells (Strathmann 1987). *H. zelandiae* has direct development where embryos spend 10-15 days in the benthic egg mass and emerge as benthic juveniles (Rudman 1971). *H. vesicula* has a mixed life history where embryos initially spend between 5-14 days (Hurst 1967) developing within the egg mass then hatch as pelagic feeding larvae which then spend several weeks developing in the plankton before settlement to benthic habitat (Gibson and Chia 1989).

3.3.2 *Study sites*

Surveys of *Haminoea zelandiae* egg masses were conducted at the tidal mud flat in Pauatahanui Inlet, a large estuary situated on the southern end of the North Island of New Zealand (41°07'S, 174°55'E). The natural distribution of *Haminoea vesicula* egg masses was assessed at two different field sites (both soft-sediment tidal flats) on San Juan Island, Washington (WA), United States of America (USA): False Bay (48°29'N, 123°04'W) and Argyle Lagoon (48°31'N, 123°00'W).

I used similar methods described below in both New Zealand and the US to facilitate direct comparisons of results between the two locations.

3.3.3 Field Surveys

Surveys of *Haminoea zelandiae* egg mass deposition took place in a 2m wide, 30m long belt transect across the mid-high littoral zone at Pauatahanui mudflat, March 2007 (austral late summer/early autumn). Surveys of *Haminoea vesicula* were conducted at the two sites in Washington, USA, described above using the same method in July 2007 (mid-summer). Within each transect every egg mass found was assigned to one of two light exposures (exposed to sun or shaded, usually by algae or eelgrass) and one of two microhabitats at low tide (desiccated or submerged in a shallow tidal pool).

To determine if egg mass deposition site influences embryonic mortality, 10 egg masses from each combination of UVR exposure and microhabitat type (n = 40 egg masses total) were randomly collected. Egg masses were brought to the laboratory (Friday Harbor Laboratories (FHL) in Washington and the Victoria University Coastal Ecology Laboratory (VUCCEL) in New Zealand) where each egg mass was examined at 100x magnification with a compound microscope. To quantify the embryonic mortality within each egg mass similar methods to Przeslawski et al. (2004) were followed, examining the first 60 embryos of each egg mass and scoring each as viable or inviable. Viable embryos were developmentally normal in appearance. Inviolate embryos had either no developed features and consisted of encapsulated disjointed balls or they had developed cilia but the internal structures were malformed.

The percentage of egg masses found in different environments was analysed using a non-parametric chi square test. The percentage of inviable embryos was assumed to be equivalent to embryonic mortality, and was analysed using a two-factor univariate ANOVA, with the factors UVR exposure and microhabitat. Data were

arcsine square root transformed (as recommended by Zar, 1998), and transformed data fulfilled assumptions of homogeneity of variance (using Levene's test) and normality (by examination of normal probability plots).

3.3.4 Manipulative Experiments

Experiment One: UVR and low tide conditions

Six replicate egg masses (separated by a minimum of 5m and so assumed to be from six different individuals) were chosen from shaded microhabitats, all at early (blastula) stages of development, as determined by microscopic inspection (e.g. Fig 2.1). Each egg mass was divided into nine equal segments; each placed into individual 100ml plastic containers which had been drilled with numerous small holes to allow seawater to flow through, but retain the egg mass segment. These containers were then placed in a large shallow aquarium with flowing seawater, outside exposed to all day sun at VUCCEL or FHL. Three levels of spectral treatments were established: (1) full sun, (2) UV-blocked (UV-A and UV-B), and (3) completely shaded by attaching appropriate UVR absorbing plastics over the tops and sides of each individual container (full spectrum light: PVC, UV-blocked: polycarbonate, completely shaded: foil). The containers themselves were transparent to UVR. The absorbing properties of all materials used were verified with a UV-1601 Shimadzu spectrometer. For all six egg masses, three of the nine segments from the same egg mass were randomly assigned to each of these three spectral treatments.

Also, three levels of simulated low tide conditions were established: (1) constantly submerged in continuously flowing seawater (a control) (2) three hours in a dry aquarium, simulating desiccation at low tide, and (3) three hours exposed to non-circulating naturally heated seawater to simulate tidal pool conditions at low tide. For

each egg mass, one of the three segments in each light treatment was randomly assigned to one of the three simulated low tide conditions. This experiment was fully crossed such that each of the six replicate egg masses was represented once in every combination of UVR and low tide condition.

At 11am every day, the containers containing the egg mass segments in the desiccation treatment were drained of seawater and removed from the aquarium with flowing seawater and placed in an identical dry aquarium immediately adjacent to it. The containers of egg mass segments in the simulated tidal pool treatment were placed into a third identical aquarium also immediately adjacent, into which seawater was added that had been heating naturally in the sun in a black tub for approximately three hours. Each of the containers that remained submerged in the flowing seawater aquarium was picked up, allowed to drain, and replaced in the aquarium, to control for the physical movement of the other containers to the treatments. At 2pm all containers were placed back into the aquarium with flowing seawater. All aquaria were identical, and the desiccated and tidal pool treatments were randomly allocated to one of the two aquaria on each day. Seawater in all treatments (including the control aquarium) was filtered through a 10µm pore size bag filter before use.

This experiment was conducted once with *Haminoea zelandiae* for ten days in March 2007 (10th-19th) and twice with *Haminoea vesicula* for six days each in July 2007 (run 1: 9th-14th and run 2: 17th-22nd), in all cases the experiment ended before embryos began hatching. Run 1 was conducted during a very hot, sunny week, whereas run 2 was conducted during a cooler, rainy week to determine whether mortality responses to experimental treatments depended on weather. At the end of the experiment, embryonic mortality was scored of each egg mass following similar

methods to Przeslawski et al. (2004). Each segment of egg mass was agitated in 400µl filtered seawater until all embryos were suspended in water. From this, 5µl was analysed microscopically until the entire sample had been examined. Embryos were scored as viable or inviable, as defined above for the field surveys except in this case all viable embryos had developed a larval shell, or protoconch (e.g. Fig 2.2). Additionally while analysing viability of *H. vesicula* embryos in run 1, the presence of diatoms was also recorded, as these microorganisms may protect against harmful UVR by shading underlying embryos and possessing chemical sunscreens (mycosporine-like amino acids: MAAs).

Experiment Two: UVR and initial UVR/light exposure

In the second experiment the effect of UVR and the initial UVR/light exposure of egg masses, was explicitly examined in a crossed, two-factor design. For each species, six undeveloped egg masses were collected from sunny microhabitats and six from shaded microhabitats, this initial exposure at the site of deposition was the first factor. Each egg mass was divided into three segments, and placed in individual containers, similar to the first experiment. UVR was also manipulated the same way as the first experiment (three levels: (1) full sun, (2) UV-blocked light and (3) completely shaded). All containers were placed in an aquarium with flowing seawater, outside, exposed to natural full sun. This experiment was conducted once at VUCEL with *Haminoea zelandiae*, 10th - 14th March 2007 (concurrently with *Siphonaria australis*, Appendix 1), and once at FHL with *Haminoea vesicula*, 17th- 22nd July 2007. At the end of the experiment, viability of embryos was scored as described for experiment one.

For both experiments, embryonic mortality was analysed using univariate ANOVA. For the first experiment the factors were light treatment (three levels) and

low tide conditions (three levels). In the second experiment the factors were initial UVR/light exposure (two levels) and light treatment (three levels). For *H. vesicula*, because experiment one was conducted twice, experimental run was also included as a third factor for that species. Egg mass identity was included in all models as an additional random factor to account for potential variability in vulnerability among individual egg masses to experimental treatments. Diatom presence was also analysed using ANOVA, with the dependant variable as embryonic mortality and the fixed factor as presence of diatoms.

All percent mortality data were arcsine square root transformed prior to analysis (as recommended by Zar, 1998) and assumptions for homogeneity and normality were met for transformed data. Equality of variances was determined using Levene's test and normality by the examination of normal probability plots.

3.3.5 Environmental parameters

Air and seawater temperatures (New Zealand and USA) and salinity (USA only, using a salinity meter Orion Research Inc, model 1230, Beverly, MA, USA) were measured in all treatments at 2pm daily for the duration of each experiment. UVR was quantified in two ways. At FHL, a handheld UVX radiometer was used (UVP Inc, Upland, CA, USA) to directly measure UVR hourly 10am-4pm each day of the experiments in units of $\mu\text{W}/\text{cm}^2$. Both the daily maximum value and daily average UVR over 10am-4pm were examined, and calculated averages of these values over duration of each experimental run. These data were used to compare UVR between runs 1 and 2 at FHL only. This instrument was not available in New Zealand. Instead, to quantify UVR in New Zealand, I used readings from an Actinic UV radiometer with a diffuser, interference filter and a solar blind phototube (type SED 240/ACTS270W, International

Light Inc, Newburyport, MA, USA). This instrument is operated by Industrial Research Limited, and is permanently deployed in a location <5km from VUCEL, where the experiments were conducted. This instrument reports values of UV Index (UVI) every minute. UVI is a measurement of UVR intensity weighted by the response of human skin to different wavelengths. Hourly averages were calculated and used both the daily maximum UVI value, and the daily average UVI from 10am – 4pm.

Because of inherent differences between the two types of instruments, it was not possible to directly compare UVR between New Zealand and USA at the time of the experiments with these data. However, a database of UVI is available from the Tropospheric Emission Monitoring Internet Service (TEMIS, url: www.temis.nl/uvradiation/SCIA/stations_uv.html). To compare UVI at the time of the experiments in New Zealand and the US, two locations in the database that were close to my study locations were used (Saturna Island, Canada, Lat: 48.78, Long: -123.13; and Paraparaumu, New Zealand, Lat: -40.90 Long: 174.98), and quantified the average UVI values over the course of the experiments at each location.

To compare overall patterns of UVR over spring and summer (when many coastal species are reproductive), at the two locations, I also plotted daily UVI values from the same locations using the TEMIS database as above, for six months during spring and summer of each hemisphere: 1 March- 31 August 2007 for Northern, and 1 September 2006- 28 February 2007 for Southern.

3.4 RESULTS

3.4.1 Field Surveys

In New Zealand, *Haminoea zelandiae* egg masses were predominantly found in submerged shallow pools, rather than desiccated on the sand/mud substrate ($\chi^2 = 36.450$, $p < 0.001$; Table 3.1), and more were shaded rather than fully exposed to the sun ($\chi^2 = 28.196$, $p < 0.001$; Table 3.1). Desiccation and light exposure both increased embryonic mortality, but the effect of UVR exposure was greater, resulting in a 4-fold increase in embryonic mortality compared to shaded egg masses in each microhabitat (Fig 3.1, Table 3.2). The greatest mortality therefore resulted from egg masses that were desiccated on the mud and exposed to the sun, where on average 50% of embryos were inviable. By contrast, egg masses in shaded pools had on average <10% mortality.

A significantly higher proportion of *Haminoea vesicula* egg masses from False Bay were found in submerged ($\chi^2 = 69.179$, $p < 0.001$) and sunny ($\chi^2 = 44.026$, $p < 0.001$, Table 3.1) microhabitats. At Argyle Lagoon a greater proportion of egg masses were found in submerged microhabitats compared to desiccated ones ($\chi^2 = 13.226$, $p < 0.001$, Table 3.1), but there was no difference in the proportion of egg masses found in the sun vs. the shade ($\chi^2 = 0.078$, $p = 0.780$). There was a significant effect of microhabitat on *H. vesicula* embryonic mortality (Fig 3.1, Table 3.2). Egg masses found desiccated on sand bars at low tide suffered 3-fold higher mortality compared to egg masses in tidal pools (Fig 3.1). Unlike for *H. zelandiae*, however, exposure to sun did not result in greater embryonic mortality (Fig 3.1).

Table 3.1 The percentage of *Haminoea zelandiae* and *Haminoea vesicula* egg masses found in different microhabitats and light exposure from each survey location.

Microhabitat	Pauatahanui (NZ) <i>Haminoea zelandiae</i> n= 378	Argyle Lagoon (US) <i>Haminoea vesicula</i> n= 230	False Bay (US) <i>Haminoea vesicula</i> n= 916
Sunny	31%	59%	66%
Shaded	69%	41%	34%
Desiccated	28%	40%	31%
Submerged	72%	60%	69%

Table 3.2 Results of a univariate ANOVA for the effect of microhabitat (desiccated or submerged) and exposure (sunny or shaded) on embryonic mortality in *Haminoea zelandiae* and *Haminoea vesicula* egg masses from the field. Significant factors ($p < 0.05$) are in bold.

Source of Variation	<i>Haminoea zelandiae</i>				<i>Haminoea vesicula</i>			
	MS	df	F	p	MS	df	F	p
Microhabitat	0.505	1	47.812	<0.001	1.639	1	18.741	<0.001
Exposure	1.006	1	95.272	<0.001	0.122	1	1.390	0.243
Microhabitat *	0.011	1	1.038	0.315	0.001	1	0.009	0.926
Exposure								
Error	0.011	36			0.087	36

Figure 3.1 Mean ($\pm$ 95% CI) percentage of inviable embryos of *Haminoea zelandiae* and *Haminoea vesicula* found in egg masses located in either desiccated or submerged microhabitats at low tide and under different light exposure. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.

3.4.2 Manipulative Experiments

Experiment One- UVR and low tide conditions

Both UVR exposure ($F_{2,40} = 379.71$, $p < 0.0001$) and simulated low tide conditions ($F_{2,40} = 72.13$, $p < 0.0001$) had strong effects on mortality of *Haminoea zelandiae* embryos (Fig 3.2a). Embryos from egg masses exposed to full spectrum light suffered up to a 3-fold increase in mortality compared to egg masses exposed to either UV-blocked light or no light (Tukey HSD, $p < 0.001$) (Fig 3.2a). Embryonic mortality was significantly higher when egg masses were desiccated compared to egg masses exposed to a simulated tidal pool or those in constantly flowing seawater (Tukey HSD

$p < 0.001$, Fig 3.2a). Spectral treatment and low tide conditions interacted significantly to affect embryonic mortality of *H. zelandiae* egg masses ($F_{4,40} = 3.825$, $p = 0.01$) because exposure to full sunlight was detrimental for egg masses even in cool, running seawater, which otherwise had very low mortality. There was also a small, but significant difference among egg masses in mortality (egg mass identity: $F_{5,40} = 2.555$, $p = 0.043$), due primarily to slightly lower overall mortality from one egg mass compared to the others.

For *Haminoea vesicula*, the results were different (Fig 3.2b, Table 3.3). Although the weather was sunny and warm during the first run, and cooler and rainy during the second run, embryonic mortality was similar during both. There was higher mortality in egg masses exposed to full spectrum light compared to egg masses in the completely shaded treatment (Tukey HSD $p < 0.05$; Fig 3.2b); however the UV-blocked treatment had intermediate mortality, not significantly different from the others. Egg masses subjected to desiccation in the middle of the day suffered extremely high mortality, more than double that compared to egg masses from either the tidal pool or control treatment (Tukey HSD $p < 0.0001$; Fig 3.2b), and egg masses in tidal pools had greater mortality than those in the control treatment of flowing seawater (Tukey HSD $p < 0.05$; Fig 3.2b). Therefore, for *H. vesicula*, embryos in egg masses that were desiccated and in the sun suffered nearly 100% mortality across both runs combined (Fig 3.2b). Additionally, simple surveys identifying the presence of diatoms during run 1 of these experiments found higher embryonic mortality in egg masses with no diatom cover compared to those egg masses with diatoms present ($F_{1,52} = 23.236$, $p < 0.001$, Fig 3.3).

Table 3.3 Results from ANOVA on embryonic mortality for experiment with *Haminoea vesicula* in WA, USA on experimental run (9-14 July and 17-22 July), spectral light treatment (full sun, UV-blocked, and completely shaded) low tide conditions (desiccated, tidal pool, and control) and egg mass identity. Significant factors ($p < 0.05$) are in bold.

Source of Variation	MS	df	F	p
Run	0.0363	1	0.4149	0.534
Light Treatment	0.4722	2	4.4507	0.015
Low tide conditions	11.2784	2	106.31	<0.0001
Run*Low tide conditions	0.30683	2	2.8923	0.061
Run*Light Treatment	0.0384	2	0.3616	0.698
Light Treatment*Low tide conditions	0.1341	4	1.2639	0.291
Run*Light Treatment*Low tide conditions	0.1246	4	1.1742	0.329
Egg mass identity (Run)	0.0876	10	0.8254	0.6053
Error	0.1061	80

(a) *Haminoea zelandiae*

(b) *Haminoea vesicula*

Figure 3.2 The effects of experimental treatments on embryonic mortality of (a) *Haminoea zelandiae* and (b) *Haminoea vesicula*. Plotted are mean ($\pm$ 95% CI) percentage of invariable embryos in each spectral light treatment (black bars are completely shaded, gray bars are UV-blocked and white bars are the full spectrum) for each simulated low tide condition (submerged, desiccated, tidal pool).

Figure 3.3 The effect of diatoms on the viability of *Haminoea vesicula* embryos.

Plotted are mean (+/- 95% CI) percentage of inviable embryos.

Experiment Two-UVR and initial UVR/light exposure

Consistent with the previous experiment, *Haminoea zelandiae* embryos in this experiment also had dramatically greater mortality if exposed to full spectrum light compared to either UV-blocked or completely shaded treatments ($F_{2,25} = 36.723$, $p < 0.0001$; Tukey HSD $p < 0.001$) (Fig 3.4). Although egg masses initially deposited in the shade had slightly greater mortality than those from the sun, this effect was not significant ($F_{1,25} = 3.3769$, $p = 0.078$). There was no interaction between initial exposure of site of deposition and light treatment ($F_{2,25} = 0.7898$, $p = 0.465$) nor was there any effect of egg mass identity on embryonic mortality ($F_{5,25} = 0.6017$, $p = 0.699$).

By contrast, for *Haminoea vesicula* in this experiment, there was a significant effect of site of deposition on embryonic mortality ($F_{1,25} = 6.7807$, $p = 0.0153$) where egg masses deposited in shaded sites had greater mortality than those from sunny sites (Fig 3.4; Tukey HSD < 0.05). Also in contrast to experiment one for the same species, in this experiment there was no effect of light treatment on embryonic mortality ($F_{2,25} = 1.3112$, $p = 0.287$). There was also no interaction between the factors ($F_{2,25} = 0.8441$, $p = 0.4418$) and no effect of egg mass identity ($F_{5,25} = 1.5404$, $p = 0.213$).

Figure 3.4 Effects of experimental light treatments (black bars are completely shaded, gray bars are UV-blocked and white bars are the full spectrum) and initial UVR exposure of egg masses (sunny or shaded microhabitats) on embryonic mortality of *Haminoea zelandiae* and *Haminoea vesicula*. Plotted are mean ($\pm$ 95% CI) percentage of inviable embryos.

3.4.4 Environmental parameters

Conditions in New Zealand were similar for experiments one and two, with air and ambient seawater temperatures intermediate to the two runs of experiments at FHL (Table 3.4). For experiment one in New Zealand, temperatures in the simulated tidal pool conditions were significantly warmer than the flowing seawater ($F_{1,18} = 5.77$, $p = 0.027$), although the magnitude of the difference was not great. At FHL, both air ($F_{1,8} = 11.99$, $p = 0.009$) and water temperatures ($F_{1,16} = 16.82$, $p = 0.0008$) were higher during run 1 of experiment one compared to run 2 (Table 3.4). In both runs at FHL, temperatures in flowing seawater were lower compared to the simulated tidal pool ($F_{1,16} = 23.82$, $p = 0.0002$), with no interaction between the effects of run and circulation ($F_{1,16} = 2.67$, $p = 0.12$).

Salinity was higher during the first run of experiment one at FHL than the second run ($F_{1,16} = 84.42$, $p < 0.0001$, Table 3.4). There was no significant difference between tidal pool and flowing seawater treatments ($F_{1,16} = 2.79$, $p = 0.11$), however there was a highly significant interaction between run and circulation ($F_{1,16} = 21.97$, $p < 0.0001$) because salinity in the simulated tide pool was higher than ambient seawater during run 1 when it was warm and sunny, but similar in run 2 when it was cooler and rainy.

Mean UVI values from the TEMIS database were significantly higher in run 1 of experiment one at FHL than experiment one in New Zealand, or run 2 at FHL ($F_{2,19} = 5.851$, $p = 0.01$, Tukey HSD < 0.05), which were both similar to each other. At FHL, UVR measured with the handheld radiometer was nearly significantly higher during run 1 of experiment one compared to run 2 when comparing the mean over the course of the experiment of the daily maximum value ($F_{1,12} = 4.69$, $p = 0.051$) and the difference was

highly significant when comparing the 10am–4pm average values of UVR ($F_{1,12}$ 17.82, $p = 0.001$, Table 3.4). Daily UVI over spring and summer 2007 was much higher in New Zealand compared to USA, reaching a peak of 13.4 compared to 8.7 in the USA (Fig 3.5).

Table 3.4 Physical parameters during the different experiments. Shown are means of each parameter over the course of the experiment using daily measurements taken at 2pm, +/- 95% CI. Note there was no exposure to tidal pools in experiment two for *Haminoea zelandiae*, so tidal pool temperature is not reported (NR=not reported). Salinities were not recorded during experiments conducted in New Zealand (ND=no data).

Physical Parameter	<i>Haminoea zelandiae</i>		<i>Haminoea vesicula</i>	
	Exp 1	Exp 2	Exp 1 (Run 1)	Exp 1 (Run 2) & Exp 2
(a) Temperature (°C)				
Air	20.5 ± 1.41	21.4 ± 2.29	29.8 ± 2.43	17.36 ± 1.57
Flowing seawater	16.4 ± 0.99	16.6 ± 1.99	17.2 ± 5.79	12.58 ± 4.00
Simulated tidal pool	18.65 ± 1.55	NR	29.4 ± 5.21	18.66 ± 4.29
(b) Salinity (ppt)				
Flowing seawater	ND	ND	26.22 ± 0.92	20.88 ± 2.17
Simulated tidal pool	ND	NR	33.76 ± 3.91	17.30 ± 0.88
(c) UVR				
TEMIS (UVI)	7.18 ± 0.45	7.22 ± 0.75	8.13 ± 0.29	6.93 ± 0.58
*Radiometer:				
Daily max value	7.11 ± 1.43	7.29 ± 1.19	1146.7 ± 130.7	795.3 ± 399.2
10am- 4pm	4.48 ± 0.63	4.46 ± 1.58	863.6 ± 255.3	376.1 ± 220.3

* Note: the direct measurements of UVR are not comparable between New Zealand and USA because of differences in the instruments and units. The New Zealand values are reported as UV Index, the USA values are in units of $\mu\text{W}/\text{cm}^2$.

Figure 3.5 Average daily UV indices (UVI) for Paraparaumu, New Zealand (black line) and Saturna Island, British Columbia, Canada (gray line), during spring and summer in each hemisphere. Plotted are daily UVI for 6 months. For New Zealand this is 1 September 2006–28 February 2007 and for Canada 1 March–31 August 2007. Note: these stations were the closest available in the dataset to the experimental locations.

3.5 DISCUSSION

Although these two species are ecologically very similar and closely related taxa, in this study dramatic species-specific differences in vulnerability to different environmental stressors was found. The southern species *Haminoea zelandiae* had much higher embryonic mortality when egg masses were exposed to UVR than did the northern species *Haminoea vesicula*. By contrast, although desiccation increased mortality in both species, it was a much greater stressor for *H. vesicula* than for *H. zelandiae*.

This is particularly interesting because the UVR intensity was similar between the locations at the time the experiments were conducted; therefore these experimental results must be due to intrinsic differences between the two species, and not differences in UVR intensity between the locations at the time of the experiments. The UVR values reported here for New Zealand in late summer/early autumn were also much lower than the extreme values that can often occur in austral mid-summer in the same region (Fig 3.4). Given the high embryonic mortality during the time of these experiments, *H. zelandiae* embryos exposed to periods of full sun in summer would likely be at risk of very low survival. UVR has also been found to cause almost 80% mortality in Australian intertidal gelatinous egg masses exposed to full light, nearly double that of those that were shaded (Przeslawski et al. 2004), although UVR measurements were not reported in that study. Nevertheless, taken together these studies suggest that offspring of Southern Hemisphere gastropods that lay benthic egg masses exposed to the sun are extremely vulnerable to mortality by high UVR levels. By contrast, *H. vesicula* appears to be at relatively low risk of mortality due to UVR exposure, even under the sunniest conditions in mid-summer. Because cloud cover can substantially reduce UVR, and has been implicated in protecting other species (e.g. Kuhn et al. 2000), variable weather conditions may be an important source of protection for the highly vulnerable Southern species, *H. zelandiae*, but afforded no extra protection for *H. vesicula*.

It is unknown what would cause *H. zelandiae* embryos to have such greater vulnerability to UVR compared to the congeneric *H. vesicula*. Mycosporine-like amino acids (MAAs) can act as chemical sunscreens in many marine organisms (reviewed by Shick and Dunlap 2002) including encapsulated molluscan egg masses (Carefoot et al. 1998, Karentz et al. 2001, Przeslawski et al. 2005a). However, research as part of this study suggests no difference in MAA concentrations between these two species

(Chapter 4). Other possibilities include differences in UV-absorbing pigmentation or DNA repair abilities. I noted that *H. zelandiae* egg masses were nearly transparent whereas *H. vesicula* egg masses had a darker yellow coloration, but the potential differences in UV absorbing pigments in egg masses or embryos were not examined. The major difference in the development of these two species is that development time of *H. vesicula* can be up to twice as fast, and results in a planktotrophic veliger, whereas fully formed juveniles hatch from *H. zelandiae* egg masses. Perhaps faster development time is linked to reduced vulnerability to the damaging effects of UVR, but further research is required to test this hypothesis.

Both *H. zelandiae* and *H. vesicula* egg masses endured greatest embryonic mortality during periodic desiccation compared to submerged conditions. Several studies suggest that desiccation stress is a primary cause of embryonic mortality within egg masses deposited in the field (Emlen 1966; Spight 1977; Pechenik 1978). The desiccation time used in the experiment here (three hours) corresponds to realistic, average emersion times that egg masses experience in the field during low tides cycles. *H. vesicula* egg mass deposition is limited by the need to deposit on hard substrate (von Dassow and Strathmann 2005), also likely the case for *H. zelandiae*; therefore egg masses found desiccated on the sand or mud are likely a result of detachment and transport away from the initial site of deposition via water currents. Embryonic mortality associated with detached egg masses likely represents a substantial proportion of total natural embryonic mortality of bubble shell snail populations, as 30-40% of egg masses across both species were found detached and desiccated, and for *H. zelandiae*, being detached also likely increases transport away from sources of shade, increasing exposure to sun.

Although both species suffered significant mortality in desiccated environments, *H. vesicula* was particularly vulnerable; this may be explained by species-specific differences in extra-embryonic matrices of the egg masses. *H. vesicula* egg masses are thin with little gelatinous matrix surrounding the embryos, whereas *H. zelandiae* egg masses are thicker; suggesting that this egg mass is less prone to drying out at low tide. This sturdier egg mass composition may be adaptive for *H. zelandiae* as its offspring spends a comparatively longer period of time developing in the intertidal zone, whereas the thin egg masses of *H. vesicula* likely allow for better oxygen diffusion and promotion of synchronous development (Chaffee and Strathmann 1984).

Tidal pools are often considered a temporal refuge from desiccation at low tide; but these microhabitats may increase vulnerability to other stressors (Chapter 2). In small volume tidal pools evaporation may cause increased salinity, which can increase embryonic mortality in benthic molluscan egg masses (Przeslawski et al. 2005b). *H. vesicula* egg masses exposed to tidal pool conditions during the first experiment endured greater embryonic mortality compared to egg masses that were constantly submerged in flowing seawater, but the magnitude of this effect was smaller than that of desiccation. A number of environmental stressors including temperature, salinity and oxygen limitation may be interacting together to synergistically enhance embryonic mortality in this treatment for *H. vesicula* (e.g. Pechenik 1986). It is likely that the difference in control and simulated tidal pool conditions was largely driven by responses from run 1 when both salinity and water temperature were much higher in the simulated tidal pool compared to the flowing seawater control. Salinity in particular would have been extreme for this species because typical salinity around San Juan Island is relatively low, ranging from about 20-30ppt (C. O’Kelly pers. comm.).

In the field, egg masses of the New Zealand species, *H. zelandiae*, were predominantly deposited in microhabitats that corresponded to the highest embryonic survival, i.e. submerged in tidal pools and shaded by algae. Although a similarly high proportion of *H. vesicula* egg masses were also found submerged, they were also twice as likely to be found in the sun compared to *H. zelandiae*. In New Zealand however, exposure to sun resulted in much higher mortality for natural *H. zelandiae* egg masses, whereas for *H. vesicula* it was desiccation rather than UVR exposure that corresponded to greater mortality. This supports a previous study that also found high survival in *H. vesicula* egg masses collected from sunny sites (Biermann et al. 1992).

The greater proportion of *H. zelandiae* egg masses found in the shade compared to *H. vesicula* may be due to active egg deposition site choices by females; however evidence from gastropods in Australia suggests that many species do not adjust the seasonal timing or microhabitat location of their spawning to avoid physiologically stressful conditions (Przeslawski and Davis 2007). Alternatively, this result may also reflect a greater abundance of shade-providing substrates at Pauatahanui in New Zealand compared to the bays in Washington, with no active choice for shade made by females. At Pauatahanui, the shade was commonly provided by the red macroalgae *Gracilaria spp.* whereas in WA, it was provided by the green macroalgae *Ulva spp.*, however relative abundances of these algae were not measured.

For the Northern species *H. vesicula*, although there was no effect of UVR exposure on embryonic mortality from egg masses in the field, there was a small but significant increase in mortality from egg masses in the sun compared to those in the completely shaded experimental treatments. Interestingly, the UV-blocked treatment caused intermediate mortality. One explanation for the difference between experimental

and field results for this species is that what was considered “shaded” in the field provided only partial shade compared to the completely shaded experimental treatments.

Gastropod species that actively deposit egg masses exclusively in the shade have higher overall embryonic mortality in response to subsequent exposure to UVR compared to species that deposit egg masses in locations exposed to direct sunlight (Przeslawski et al. 2004). In this study, however, even within a species (*H. vesicula*) egg masses that were initially deposited in shaded microhabitats were more vulnerable to the damaging effects of UVR than those that were initially deposited in the sun. The results for *H. zelandiae* were also nearly significant, and the trend was the same. Microfouling organisms such as diatoms that only recruit to egg masses exposed to sunlight can themselves harbour MAAs, and may offer further protection from UVR by shading the embryos underneath irrespective of whether they contain MAAs (Riegger and Robinson 1997). The presence of diatoms in run 1 of the UVR-low tide condition experiment proved to be an important factor influencing embryonic development in *H. vesicula*, as the presence of diatoms was positively correlated to embryonic survivorship, with the absence of diatoms corresponding to highest embryonic mortality. Further, egg masses from the sun had higher concentrations of MAAs than those from the shade, although the origin of the compounds is not known and further work is needed (Chapter 4).

3.5.1 Conclusions

Increases in UVR over recent decades due to human-induced ozone depletion, have been particularly dramatic in the Southern Hemisphere, and will not likely subside for many decades (McKenzie et al. 1999a,b). UVR has the potential to interact

synergistically with other stressors to negatively impact populations and communities across a variety of systems in New Zealand, yet direct comparisons and assessment of risk of similar species from similar latitudes in the two hemispheres have not been made until now. Both bubble shell snail species in this study were vulnerable to desiccation stress, but only for the southern species *Haminoea zelandiae* was there additional high UVR-induced embryonic mortality. These results are conservative, and embryonic survival in this species is likely highly dependent on the shade-providing macrophytes in this tidal mudflat habitat, such as eelgrass and macroalgae. The reproductive success of New Zealand bubble shell snails may therefore be tightly linked to the dynamics of these biogenic sources of shade.

The results here are striking, even though these two sister-species are ecologically and morphologically very similar. The drivers behind the species-specific responses to stressors found here require further study, and may be tied to differences in life history strategies, development times and egg mass characteristics. Overall, these results support other recent work suggesting that Southern Hemisphere species may be particularly at risk from UVR, but in this case the risk is determined primarily by species-specific characteristics rather than direct interhemispheric differences in UVR intensity.

Chapter 4: Chemical sunscreen (mycosporine-like amino acids: MAAs) variation in mollusc embryos from New Zealand and USA

4.1 ABSTRACT

Natural chemical sunscreens (mycosporine-like amino acids: MAAs) have been suggested to provide protection against ultraviolet radiation (UVR) damage in many marine organisms. This study focuses on the role and variation of MAAs over several spatial and temporal scales. The total concentration of MAAs were examined in four intertidal molluscs; *Benhamina obliquata*, *Siphonaria australis*, *Haminoea zelandiae* (New Zealand) and *Haminoea vesicula* (USA) using high performance liquid chromatography (HPLC). In this study intra- and inter-specific MAA variation, MAA concentrations across embryonic development, and MAA concentrations during different seasonal UVR exposure were all specifically assessed.

In the New Zealand species, MAA concentrations were species-specific; the highest MAA concentrations occurred in *B. obliquata*, second highest in *S. australis*, with the lowest concentrations found in *H. zelandiae*. MAA concentrations also differed over development, with highest concentration of MAAs found in sunny *B. obliquata* egg masses at early stages of development. It remains unclear if total concentrations of MAAs are driven by ambient UVR levels, as MAA concentrations in *S. australis* were higher in early spring when UV indices (UVI) are relatively low. MAA concentrations were similar between the congeneric *Haminoea* species from samples that were taken where UVI were similar. The results of this study show that MAAs can vary within a species, over the course of development, and over time. These

results also support other recent work that has found that although MAAs are important in mitigating the deleterious effects of UVR in some species, alternative protective mechanisms are likely important for others.

4.2 INTRODUCTION

Ultraviolet radiation (UVR), including UV-A (320-400nm) and UV-B (280-320 nm) can have negative effects on the reproduction, behaviour and development of a variety of organisms including bacteria, phytoplankton, macroalgae and species of fish (Hader et al.1998; Carefoot et al.1998). The global increase in UV-B as a result of stratospheric ozone depletion is now well documented (Madronich et al.1998) and given the well-established seasonal depletion in atmospheric ozone levels over Antarctica, marine organisms occurring in the Southern Hemisphere are particularly susceptible to high levels of UVR exposure (Karentz 1991, 1994, McKenzie 1991, Seckmeyer and McKenzie 1992, Gies et al.2004). UVR levels follow a seasonal pattern as a result of zenith angle and day length with UV indices (UVI) reaching their highest in the middle of summer (Vanicek et al.2000), with UVR dose 5-10% lower in winter (Lamare et al.2004). The onset of global climate change and stratospheric ozone depletion has seen an increase in studies investigating the impact that UVR has on aquatic organisms (e.g. reviewed by Hader et al.1998, 2007).

Egg masses deposited in the intertidal region are subjected to stressful and variable environmental factors, including UVR and desiccation that can affect the development and survival of embryos (Spight 1977; Gosselin and Chia 1995, Chapters 2 and 3 of this thesis). Newly deposited egg masses may be particularly vulnerable to environmental stressors (Appendix 2). For example, heat shock proteins that protect

against temperature stress are expressed as embryos mature (Podolsky and Hoffman 1998, Podolsky 2000). UVR may present a particular problem to early life stages since the cell division cycle during the cleavage period is relatively short and often there are not mitotic checkpoints to ensure reliable transmission of DNA to daughter cells (Epel et al.1999).

There are many possible mechanisms to counteract UVR damage, including the possession of DNA repair enzymes, where DNA repair can occur even during short cell cycles (e.g. Blaustein et al.1994). However, strategies that prevent damage would seem preferable to repairing damage, such as anti-oxidants that protect against free radicals formed during UVR exposure (Epel et al.1999). Many organisms may avoid UVR exposure altogether, including adult gastropods that circumvent UVR through the presence of shells and opercula, while mobile organisms can actively prevent UVR damage via diurnal migrations and negative photo-taxis (Leech and Williamson 2001, Persaud et al.2003). Adult spawning behaviour can also minimise UVR exposure. A variety of gastropods deposit their egg masses under boulders where they are completely shaded from UVR (Benkendorff and Davis 2004) and some species of corals along the Great Barrier Reef in Australia undergo mass spawning at night (Babcock et al.1994), postponing trauma from eggs being exposed to UVR for the first several hours after spawning (Gulko 1995).

A variety of organisms use chemical compounds to mitigate the damaging effects of UVR. Terrestrial plants use flavonoids as a form of UVR protection (Stapleton and Walbot 1994), while mycosporine-like amino acids (MAAs) are found in many marine bacteria, algae and animals (reviewed by Shick and Dunlap 2002). MAAs encompass 19 known compounds that have UV absorption in the range of 310-360 nm,

and therefore are able to screen ambient UV-A and UV-B radiation (Garcia-Pichel et al.1993, Adams and Shick 1996; Shick and Dunlap 2002). The biosynthesis of MAAs uses the first steps of the shikimic acid pathway (Favre-Bonvin et al.1987): a biogenetic process only found in bacteria, fungi and algae and absent in metazoans (Bentley 1990, Bandaranayake 1998). Because animals are thought to lack the ability to synthesise MAAs, it has been suggested that they acquire them either through symbiosis (Dunlap and Chalker 1986), or diet (Shick et al.1992, Gleeson and Wellington 1993), although the understanding of the enzymatic and genetic basis of MAA synthesis is still very limited (Oren and Gunde-Cimerman 2007). MAAs have been found in early life stages of molluscs, echinoderms and corals (reviews by Shick and Dunlap 2002, Karentz et al.2001). The sea urchin *Strongylocentrotus droebachiensis* (Adams et al.2001) and the sea hare *Aplysia dactylomela* (Carefoot et al.1998) are both thought to sequester MAAs in spawn to minimise UVR damage to their offspring. An extensive study on 46 gastropod egg masses in Australia revealed that total MAA content in this group is species-specific depending on taxonomic group and diet (Przeslawski et al.2005a).

In this study similar approaches were used to determine inter-specific MAA variability from three focal mollusc species in New Zealand (the pulmonate limpets *Siphonaria australis*, and *Benhamina obliquata* and the opisthobranch bubble shell snail *Haminoea zelandiae*) as well as the congeneric bubble shell snail *Haminoea vesicula* from the west coast of the United States of America (USA). Further, based on results from chapters 2 and 3 where higher embryonic mortality was found from embryos in shaded microhabitats compared to sunny ones, MAA concentrations of egg masses deposited in the sun vs. shade for each species were also compared.

Results from a pilot study indicated that very early stages of development may be most vulnerable to UVR (Appendix 2). Therefore the hypothesis that MAA concentrations in egg masses would mature/increase with development was also explored. To identify if MAA concentrations differed according to seasonal variation in ambient UVR, MAA concentrations of *Siphonaria australis* egg masses were examined at two times of year. Finally I was particularly interested in the inter-hemispheric differences of MAA concentrations between two congeneric species found in the Southern and Northern Hemispheres, (*Haminoea zelandiae* and *Haminoea vesicula*), to help identify whether MAA concentrations are correlated with ambient UVR levels. This has yet to be explored in any previous study.

4.3 METHODS

4.3.1 Study Species

New Zealand: *Siphonaria australis* and *Benhamina obliquata* are pulmonate limpets occupying the mid-high littoral zone of the rocky intertidal zone (Jenkins 1983). Both species are found on sheltered and exposed shores around the Wellington region (Borland 1950). Spawning in *S. australis* occurs for a prolonged period in the spring and summer (September-April) where numerous small ribbon shaped egg masses are deposited in a variety of microhabitats with variable UVR exposure (Chapter 2). In contrast, *B. obliquata* deposits fewer large coiled shaped egg masses for a shorter breeding season (September-February) and are predominately deposited in dry shaded sites (Chapter 2). Siphonariids feed primarily on foliose macroalgae, although not exclusively as lichens, blue-green algae, microalgae and diatoms have also been found in gut content analyses (reviewed by Hodgson 1999). Both *B. obliquata* and *S. australis* have mixed life histories where embryos spend a relatively short period of time

developing in a benthic egg mass (5-6 days), but a much longer period developing in the plankton (~10 weeks).

The opisthobranch *Haminoea zelandiae* is found on tidal mudflats throughout the North Island and upper South Island of New Zealand where it produces numerous sausage shaped egg masses during summer and early autumn (Rudman 1971). These egg masses are predominately deposited in shaded tidal pools (Chapter 3), where they spend the duration of their development (<10days) until they emerge as juveniles. *H. zelandiae* feeds on both the microalgal film growing on the leaves of *Zostera spp.*, as well as *Ulva spp.* and filamentous algae such as *Enteromorpha spp.* (Rudman 2003).

USA: *Haminoea vesicula* similarly occupies soft sediment bays and is found along the north-eastern Pacific coast (Morris et al.1980) with large populations occurring in False Bay and Argyle Lagoon, WA, USA. *H. vesicula* deposits elongated egg masses on a variety of hard substrates during summer months (Strathmann 1987, von Dassow and Strathmann 2005) often exposed to UVR (Chapter 3, Biermann et al.1992). *H. vesicula* is thought to feed on *Ulva spp.* that densely populate False Bay and Argyle Lagoon (Gibson and Chia 1989). Unlike *H. zelandiae*, this species has a mixed life history where development in the egg mass lasts 6-7 days (Hurst 1967) and larvae continue to develop in the plankton until settlement (Gibson and Chia 1989).

4.3.2 Study Sites and Egg Mass Collection

New Zealand: *Siphonaria australis* egg masses from sunny and shaded microhabitats (n=50 from each) were collected from the exposed rocky intertidal zone at Island Bay, Wellington, NZ, in early autumn (March 2007). In September 2007, *S. australis* egg masses from only shaded micro-sites were also collected from this same

site and analysed microscopically and categorised according to stage of development (early (1-2 days), intermediate (3-4 days), late (5-6 days)). Early stages of development (1-2 days) were distinguished by newly deposited egg masses containing embryos not yet developed to the trochophore stage. Intermediate stages of development (3-4 days) were identified as trochophores or early veligers, often with visible movement. Late stages of development (5-6 days) were recognisable by dark late stage veligers close to hatching. Twenty-five shaded *S. australis* egg masses were collected from each stage of development. To determine MAA concentrations during two times of the year an additional 30 *S. australis* egg masses were also collected in March and again in September 2007 at Island Bay, from a combination of sunny and shaded sites.

Benhamina obliquata egg masses in sunny and shaded microhabitats (n=10 of each) were collected from the semi-exposed rocky intertidal at Makara in September 2007. *B. obliquata* egg masses were also collected and later categorised into the same 3 developmental stages as described above for *S. australis* (n= 8 egg masses from each stage of development from sun and shade). *Haminoea zelandiae* egg masses found in the sun and the shade (n = 50 of each) were also collected from the tidal mudflat at Pauatahanui during early autumn (March 2007).

USA: Similarly, 50 *Haminoea vesicula* egg masses were each collected from microhabitats exposed to the sun and the shade from the sand/mud flat at False Bay, WA, USA during the middle of summer in July 2007.

4.3.3 MAA analysis

All egg masses were frozen in a -80°C freezer. Once frozen, egg masses were then freeze dried, to quantify MAAs by dry weight, using a Sunvic freeze drier in New Zealand and a Virtus Sentry Freeze Mobile 12SL in USA.

Extraction: 0.3 g of each egg mass sample was weighed out and placed in a 15ml centrifuge tube. 3ml of 80% high performance liquid chromatography (HPLC) grade methanol was added. The sample was then sonicated and extracted for one hour. The supernatant was decanted into another tube. This was repeated three more times. To increase the probability of detecting MAAs per sample, three samples were mixed together and centrifuged at 5000 rpm for 1 minute to remove particular matter, reserving the supernatant. Each replicate sample contained 0.9g of dried egg mass which encompassed numerous egg masses dependant on weight of individual egg masses. Freeze-dried samples from USA were shipped chilled by air back to New Zealand where they were subsequently refrigerated for approximately two weeks until extraction and HPLC analysis took place.

High Performance Liquid Chromatography (HPLC): Dr. Kevin Mitchell conducted HPLC analyses on all the samples at Industrial Research Limited (IRL), New Zealand, using the following methods. All solutions were filtered with 0.45µm syringe filters before being subjected to HPLC analysis. Solutions were analysed by reverse-phase HPLC on a Philips Lichrosorb RP-8 (250x5mm I.D. 5 µm) using an Agilent 1100 column HPLC system equipped with auto sampler and diode array detector, and resultant data were stored and analysed using Agilent Chemstation software (version 8.03). Injection volumes of 10µl were used and elution was performed at a flow rate of 0.6ml min⁻¹ at 30°C. An isocratic mobile phase was employed consisting of 0.1%

aqueous acetic acid /methanol (7:3). Spectral data for all peaks were accumulated in the range 250-400nm and chromatograms were plotted and integrated at 320nm. MAA peaks observed were identified by examination of their online absorption spectra (typically MAAs possess a single maximum absorption in the range ca. 310-340nm).

Relative quantification was carried out by summation of peak areas for peaks identified as MAAs in each sample and average peak areas (mAu.sec.) were plotted.

4.3.4 Statistical Analysis

Statistical analyses were conducted in SPSS using univariate ANOVAs. In all cases, the dependent variable was total MAA concentration per 0.9g of dried egg mass. In the first analysis, to examine the concentration of MAAs across different initial light exposure and variation across species, fixed factors in the model included initial UVR/light exposure (sun or shade) and species (*Benhamina obliquata*, *Siphonaria australis* and *Haminoea zelandiae*). Second, to examine the concentrations of MAAs across stages of development, two individual univariate ANOVAs were run for the two different species (*B. obliquata* and *S. australis*). The only fixed factor for *S. australis* was stage of development (early, intermediate or late) as the samples were only collected from the shade. However, for *B. obliquata* the fixed factors included initial UVR/light exposure (sun or shade) as well as stage of development (early, intermediate or late). Third, to examine the variation of MAAs during different seasons in *S. australis* egg masses, the fixed factor consisted of season (early spring or early autumn). Finally, to examine MAA concentrations between *H. zelandiae* and *Haminoea vesicula*, fixed factors included initial UVR exposure (sun or shade) and species (*H. zelandiae* and *H. vesicula*) Data were log transformed to meet assumptions of equal variances

(examined using Levene's Test) and normality (determined by examination of probability plots).

4.4 RESULTS

4.4.1 Inter-specific variation and Initial UVR/Light Exposure

No difference was observed in MAA concentrations between egg masses initially deposited in the sun or the shade for any of the three species in this model (Fig 4.1, Table 4.1). MAA concentrations were species-specific. However MAA concentrations in *Haminoea zelandiae* egg masses were the lowest of the three (Tukey HSD $p < 0.001$, Fig 4.1), *Siphonaria australis* had intermediate MAA concentrations (Tukey HSD $p < 0.001$, Fig 4.1) and *Benhamina obliquata* had the highest MAA concentrations (Tukey HSD $p < 0.001$, Fig 4.1).

Table 4.1 Results of ANOVA for the effect of species (*Haminoea zelandiae*, *Siphonaria australis* and *Benhamina obliquata*) and initial UVR/light exposure at site of egg mass deposition (=Site: sunny or shaded) on total MAA concentration in egg masses from the field. Significant factors ($p < 0.05$) are in bold.

Source of Variation	MS	df	F	p
Site	0.008	1	0.215	0.644
Species	11.449	2	296.929	<0.001
Site*Species	0.069	2	1.781	0.174
Error	0.039	93

Figure 4.1 Mean total MAA concentrations (mAu.sec.: +/- 95% CI) for egg masses of New Zealand species *Haminoea zelandiae*, *Siphonaria australis* and *Benhamina obliquata* found in sunny and shaded sites. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.

4.4.2 Stages of Development

For *Benhamina obliquata*, MAA concentration varied over development, but was different depending on whether egg masses were in the sun vs. the shade (Fig 4.2a, Table 4.2a). The significant interaction between initial light exposure and stage of development arose because shaded egg masses had similar MAA concentrations across the developmental stages. However for those egg masses in the sun, MAA concentrations decreased over development, such that MAA concentrations in late stage embryos in the sun were less than 30% those of early ones (Fig 4.2a, Table 4.2a). However, concentrations of MAAs did not differ according to stage of development for shaded *Siphonaria australis* egg masses (Fig 4.2b, Table 4.2b).

Table 4.2 Results from ANOVA on total MAA concentration found in (a) *Benhamina obliquata* and (b) *Siphonaria australis* from the shade, egg masses during different stages of development (early, intermediate and late) and initial UVR/light exposure at site of egg mass deposition (=Site: sunny or shaded). Significant factors ($p < 0.05$) are in bold.

Source of Variation	Mean square	df	F	p
(a) <i>Benhamina obliquata</i>				
Site	0.026	1	1.632	0.214
Stage of Development	0.536	2	33.978	<0.001
Site*Stage of Development	0.121	2	7.669	0.003
Error	0.016	24		
(b) <i>Siphonaria australis</i>				
Stage of Development	0.001	2	0.099	0.907
Error	0.010	12

(a) *Benhamina obliquata*

(b) *Siphonaria australis*

Figure 4.2 The effects of stages of development on total MAA concentrations of (a) *Benhamina obliquata* and (b) *Siphonaria australis*. Plotted are mean total concentrations of MAAs ($\pm$ 95% CI) in each stage of development, and for *B. obliquata* the initial UVR/light exposure where egg mass was deposited. Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.

4.4.3 Seasonal Variation

Interestingly, MAA concentrations in *Siphonaria australis* egg masses in early spring (September 2007) were significantly greater ($F_{1,32} = 36.756$, $p < 0.0001$), almost 30% more than concentrations found in egg masses toward the beginning of autumn (March 2007) (Fig 4.3).

Figure 4.3 Seasonal variation on total MAA concentrations of *Siphonaria australis* egg masses. Plotted are total concentrations of MAAs ($\pm$ 95% CI) during different seasons (early autumn: March 2007 and early spring: September 2007).

4.4.4 Inter-hemispheric comparison

MAA concentrations in *Haminoea zelandiae* and *Haminoea vesicula* egg masses initially deposited in the sun were significantly higher than those found in the shade although there was no difference between the species and no significant interaction between species and initial light exposure (Fig 4.4, Table 4.3).

Table 4.3 Results of ANOVA for the effect of species (*Haminoea zelandiae* and *Haminoea vesicula*) and initial UVR/light exposure at site of egg mass deposition (=Site: sunny or shaded) on total MAA concentration in egg masses from the field. Significant factors ($p < 0.05$) are in bold.

Source of Variation	Mean square	df	F	p
Site	0.476	1	8.657	0.005
Species	0.173	1	3.147	0.083
Site*Species	0.067	2	1.226	0.274
Error	0.055	47

Figure 4.4 The effects of species (*Haminoea zelandiae* and *Haminoea vesicula*) and initial UVR/light exposure (sun or shade) on total MAA concentrations. Plotted are mean total concentrations of MAAs (+/- 95% CI). Open bars represent egg masses found in full sun, gray bars represent shaded egg masses.

4.5 DISCUSSION

4.5.1 Inter-specific variation

This study is consistent with previous studies confirming that MAAs are present in intertidal molluscan egg masses (Przeslawski 2004b, Przeslawski et al.2005a, Wraith et al.2006). A direct comparison of MAA concentrations in this study to other studies is unfortunately not possible due to variation in the units used to measure these compounds (Karentz 2001). To be able to compare the concentrations with other studies the use of a standard concentration would be required, which there was no access to at the time of this study.

Surprisingly, *Benhamina obliquata* had the highest concentrations of MAAs despite this species having the greatest embryonic mortality under full spectrum light and depositing the majority of its offspring in the shade (Chapter 2). Further, *B. obliquata* had higher concentrations of MAAs compared to *Siphonaria australis* despite *S. australis* displaying a greater resistance to UVR damage (Chapter 2). Therefore, no relationship appears to exist between MAA concentration and embryonic survivorship in *B. obliquata* or *S. australis*, although this was not explicitly tested. Similarly, MAA concentrations were not correlated to embryonic survivorship in two Australian gastropods *Bembicium nanum* and *Siphonaria denticulate*, with embryonic mortality low throughout a range of spectral treatments irrespective of MAA concentrations (Wraith et al.2006).

In contrast, *Haminoea zelandiae* suffered high UVR damage despite low UVR conditions (Chapter 3), which may be correlated to the overall very low MAA concentrations found in this study. Further, *H. zelandiae* encapsulated embryos are generally protected through spawning behaviour (Chapter 3), making protection associated with chemical sunscreens to a certain degree unnecessary. Although MAAs were detected in these egg masses, UVR damage may still occur. For example, decreases in cellular chlorophyll and Rubisco activities in the dinoflagellate, *Prorocentrum micans*, occurred despite the presence and accumulation of MAAs, and therefore it is largely unknown whether these compounds provide full protection against the damaging effects of UVR (Lesser 1996).

The difference in MAA concentrations observed across these three species may be dependant on the diet of the depositing adults, as MAAs are thought to be sequestered by adults into offspring (Carefoot et al.1998, Adams et al.2001). Some

mollusc and echinoderm eggs have been found to have higher MAA content if they are deposited by adults that consume food with high MAA concentrations compared to food with low concentrations of MAAs (et al.Carefoot et al.1998, 2000, Adams et al.2001, Lamare et al.2004). All of the molluscs in this study are herbivorous and consume foliose macroalgae. While the MAA content of the diet of these species is unknown; it could be hypothesised that *B. obliquata* and *S. australis* consume food with high concentrations of MAAs, such as red algae, whereas *H. zelandiae*'s diet may consist of food with relatively low concentrations of MAAs, such as green or brown algae (Wood 1987, Post and Larkum 1993, Karsten et al. 1998a, b, Hoyer et al. 2001, Lamare et al.2004).

Although MAA content is linked to adult diet, other factors may also influence the patterns observed in this study (Przeslawski 2004). MAA content can be related to phylogeny, for example MAA composition of the sea anemone *Anthopleura elegantissima* broadly reflected phylogenetic differences rather than environmental factors (Shick et al.2002) and complements of MAAs in Australian gastropod egg masses also reflected phylogenetic patterns (Przeslawski et al.2005a). MAAs are present in many marine organisms, and may have evolved from a common ancestor (Karentz 2001), perhaps appearing before the construction of the ozone layer when the Earth was exposed to UV-A, UV-B and harmful UV-C. This suggests that even species predominantly depositing eggs in the shade may coincidentally have high concentrations of MAAs as a result of diet (Cockell and Knowland 1999). MAA variation maybe both correlated with phylogeny and selective forces of present-day ecology. Therefore, it becomes difficult to determine if MAA concentrations are dependant on ecological factors tested in this study or whether they reflect phylogenetic differences, or

furthermore ecological factors not tested here, as variation due to phylogeny and ecological factors may not be mutually exclusive (Westoby et al.1995).

4.5.2 Initial UVR/Light Exposure

MAA concentrations did not differ between *Benhamina obliquata* and *Siphonaria australis* egg masses initially deposited in the sun and those in the shade, despite previous findings of increased embryonic mortality in shaded egg masses (Chapter 2 of this thesis). Similar results were found in Australia with no difference found in MAA concentrations in an inter-specific comparison of sunny vs. shaded molluscan egg masses, which also had similar trends in embryonic mortality (Przeslawski 2004b). Nor was there any significant correlation between MAA concentration and embryonic mortality of molluscan egg masses exposed to full spectrum and UV-blocked light treatments (Przeslawski et al.2005a). Considering that the majority of *B. obliquata* egg masses are deposited in shaded microhabitats where MAAs would be redundant, MAAs found in this species may be required during later life stages whilst developing in the plankton (Strathmann 1985, Strathmann 1987, Havenhand 1993). The dependence on MAAs as photo-protection in molluscs has been found to be species-specific; however, it is also highly probable that species also possess alternative mechanisms that minimize the negative effects of UVR (Wraith et al.2006). Other mechanisms that may act as alternate damage control strategies to attenuate initial UVR damage in *B. obliquata* and *S. australis* egg masses may include antioxidants (Dunlap et al.1999), metabolites such as carotenoids (see Bandaranayake 1998, Lamare and Hoffman 2004) or DNA repair enzymes (Carlini and Regan 1995).

In contrast to the two pulmonate limpets, both *Haminoea zelandiae* and *Haminoea vesicula* had significantly higher concentrations of MAAs in egg masses

found in the sun vs. those from the shade, suggesting MAA concentrations may be positively correlated with embryonic survivorship, considering embryonic mortality was generally higher in egg masses initially found in the shade (Chapter 3). The most likely explanation for intra-specific MAA variation according to initial light exposure at deposition site may be microfouling. Microfouling algae that only encrust egg masses exposed to sunlight can themselves harbour MAAs (Riegger and Robinson 1997), with MAA content in microalgae itself dependant on environmental UVR (Carreto et al.1990). These microalgae can live in the intra-gelatinous spaces surrounding the embryos and include marine diatoms living in the egg masses of molluscs (Buckland-Nicks et al.1973, Gibson and Chia 1989, Biermann et al.1992, Cohen and Strathmann 1996) and may operate in a nutritional mutualistic symbiosis where photosynthetic products are transferred from the algae to the embryo. Although egg masses were removed from their initial deposition site early in development, diatoms can colonise a new substrate in the marine environment within a matter of hours and can enter at the time of deposition (see Bookhout and Horn 1949, Cooksey and Wigglesworth-Cooksey 1995). Therefore, the higher MAA concentration observed in egg masses initially deposited in the sun may reflect additional MAAs present in diatoms inhabiting the egg masses, which may also be driving increased survivorship (Chapter 3). It could also be possible that MAA concentrations differ between adults of different nutritional histories as they select to deposit their eggs in appropriate sites. For example adults with a diet that is rich in MAAs may also tend to deposit their eggs in sunny locations, although this hypothesis requires further testing.

4.5.3 Stages of Development

MAA concentrations across different developmental stages are similar for *Siphonaria australis* and shaded *Benhamina obliquata* egg masses; suggesting that MAA content remains constant throughout embryonic encapsulation when embryos are in the shade. MAA content across developmental stages of macroalgae also indicate similar MAA concentrations (Hoyer et al.2001) despite spores being a sensitive developmental stage (Wiencke et al.2000). This result was unexpected as preliminary pilot studies on embryonic development of *S. australis* found that very early stages of development endured highest embryonic mortality when exposed to UVR (Appendix 2). However, MAAs in shaded egg masses are not drawn on to protect the embryos from UVR damage, suggesting that concentrations of these compounds may remain constant during development (Appendix 2). Similarly a study in Australia found no differences in total MAA concentrations between various developmental stages of numerous mollusc species, although initial UVR exposure was not examined across the developmental stages (Przeslawski et al.2005a).

In contrast, *Benhamina obliquata* egg masses in the sun showed a significantly higher concentration of MAAs during early development, compared to intermediate and late stages. The levels of some secondary metabolites may change over time, in the case of plants as a leaf develops, as the season changes, and even over the course of the daily light cycle (Chaves et al.1997, Riipi et al.2002); MAAs may do the same as an organism develops or the MAAs may be metabolised into something else as the egg mass develops (K. Mitchell, pers comm.). Therefore, *B. obliquata* egg masses in the sun may use MAAs to protect newly deposited embryos against UVR damage and then MAAs may get metabolised into other compounds as the vulnerability of embryos decreases. Further investigation would be required to determine if MAA concentrations

are correlated to vulnerability of early stages of embryonic development. The reduced MAA concentration observed in *B. obliquata* embryos may also be an artefact of normalising MAAs to dry weight, as increasing weights of embryos could lead to reduced concentration, rather than an actual decline in MAA concentration around the embryo.

4.5.4 Seasonal variation

Seasonal fluctuations of irradiance, nutrients and temperatures correlate strongly with seasonal cycles of growth and reproduction of many species, this is particularly relevant for organisms inhabiting the intertidal shore where the environment is relatively unpredictable (Gwinner 1986). *Siphonaria australis* suffered highest embryonic mortality during the middle of summer when environmental stressors are at their peak (Chapter 2). However, embryonic mortality of this species was still relatively high during March when UV indices are lower (Chapter 2), which could be correlated with lower concentrations of MAAs found during this period compared to September. Therefore, MAAs may be protecting *S. australis* embryos in the early spring, as they had both higher total MAA concentrations and were exposed to lower ambient UVR in early spring compared to late summer/early autumn (this study, and Chapter 2). Additionally, MAA concentrations in *Benhamina obliquata* egg masses were the highest in all four species and these egg masses were also collected in early spring. Observed seasonal variation in MAA concentrations may be attributable to changes in specific MAA compounds in parental food, for example, eggs collected in the late spring had a peak around 360nm, compared to 342 nm in the fall season (Epel et al.1999), however this would require further investigation using MAA standards.

Numerous studies have correlated MAA concentrations of algae and invertebrates with fluctuations in seasonal UVR intensities (e.g. Karentz et al.1991, Maegawa et al.1993, Molina and Montecino 1996, Drollet et al.1997, Michalek-Wagner 2001). For example, MAA concentrations in tunicate embryos (*Ascidia ceratodes*) during the winter months were reduced compared to concentrations found in the summer months (Epel et al.1999). Similarly the red alga *Bangia atropurpurea* forms and accumulates more MAAs under higher natural solar UVR (Karsten and West 2000). In New Zealand, some species of algae adjust MAA concentrations depending on ambient UVR, while MAA concentrations were not correlated with levels of UVR in other species (Lamare et al.2004).

However, if the change in MAA concentrations in *S. australis* during different seasons was attributable to UVR levels affecting the MAA concentrations in their diet then the expectation would be for MAA concentrations to be higher in late summer/early autumn when daily maximum UV indices are around 7, compared to 5 in early spring. It is possible that *S. australis*' diet may depend on the seasonal abundance of algae that may have different MAA concentrations irrespective of UV irradiance. Therefore, this seasonal change in MAA concentrations may reflect a shift in *S. australis*' diet between these two periods. Further, UV irradiance may not be the only factor influencing seasonal changes in biochemical defence systems but also variation in macronutrient (nitrate and phosphorus) levels in the seawater (Aguilera et al.2002), perhaps more nitrogen is available for MAA synthesis. Nutrient levels in the spring are generally higher than towards the end of summer, and may drive increases in MAA concentrations in algae during this early spring period, which may then be subsequently taken up by *S. australis*.

4.5.5 Inter-hemispheric comparison

MAA concentrations are generally higher in geographical locations where there are higher UVR intensities (Huovinen et al.2004, Lamare et al.2004); species of algae in high-solar latitudes exhibit more MAAs than individuals from low-solar latitudes (Karsten and West 2000). In cold-temperate and northern polar locations, MAA concentrations are almost half of those concentrations occurring in warm-temperate and southern polar regions (Karsten et al.1998a,b, Hoyer et al.2001). The two-fold higher MAA concentrations in algae from southern Spain compared to Arctic regions were explained by UVR intensities, which in Spain were double those of Arctic locations (Karsten et al.1998a).

This current study is unique in incorporating an inter-hemispheric comparison of MAA concentrations between two congeneric species (*Haminoea zelandiae* and *Haminoea vesicula*). Total MAA concentrations between these two species were relatively similar, despite New Zealand's higher ambient UVR levels (Seckmeyer and McKenzie 1991, McKenzie et al.1999 a,b). These findings may be attributable to the similarity of these species' diet, comprising *Ulva spp.*, which has been often found to have low concentrations of MAAs (e.g. Lamare et al.2004). Also, similar UVI values were recorded in Washington and Wellington, New Zealand, because the study occurred during the middle of the Northern Hemisphere's summer, but the end of the austral summer, when UV indices were comparable (Chapter 3). If MAA concentrations are positively correlated with UV irradiance in these species, then MAA concentrations observed in *H. vesicula* are at their peak, whereas concentrations found in *H. zelandiae* egg masses may be lower here compared to concentrations in the middle of summer when UVR is at its annual maximum, although this would require further investigation.

4.5.6 Conclusions

Stratospheric ozone depletion and an increase in biologically harmful UV-B is a serious threat to New Zealand's coastal marine populations with previous research (Chapter 2 and 3) identifying UVR as an environmental stressor capable of having strong negative effects on the development of mollusc embryos in New Zealand. This study demonstrates the variability in chemical sunscreens over spatial and temporal scales and is the first of many steps in identifying and understanding the mechanisms New Zealand's marine fauna have in place to mitigate UV-B damage. It remains unknown if MAAs in these molluscan egg masses are an evolved protective mechanism or a coincidental dietary benefit, or both. The magnitude of the effectiveness of MAAs as sunscreens for these organisms still remains unclear, but this study reveals that MAAs may be important to encapsulated embryos, although their role is likely species-specific.

Chapter 5: General Conclusions

5.1 SUMMARY OF FINDINGS

Anthropogenic changes to the Earth's environment are occurring at an unprecedented rate, with recent stratospheric ozone depletion resulting in increases in biologically harmful ultraviolet radiation (UV-B), as well as changes in temperature and sea level as a consequence of global climate change (Thomas et al. 2004, Schlesinger 2006, Kerr et al. 2007). Despite an increase in theoretical models predicting how ecosystems will respond to changes in the Earth's environment, realistic forecasts are limited by a lack of empirical data required to estimate organismal and population responses to the range and synergy of stresses that are most likely to increase (Osmonds et al. 2004, Parmesan 2006, Przeslawski et al. 2005b). This research is one of the first studies in New Zealand (but see Lamare et al. 2004) providing empirical data required to predict how New Zealand's marine coastal organisms are coping with anthropogenic changes in Earth's environment.

In this study I aimed to determine the effect of UVR and other environmental stressors at low tide on the development of New Zealand mollusc embryos. In New Zealand UVR dramatically increases embryonic mortality and is species specific, with embryonic mortality synergistically enhanced under variable low tide conditions (Chapters 2 and 3). Further, this study confirms the need to assess a suite of environmental stressors on the development of intertidal egg masses with significantly reduced embryonic mortality observed in egg masses exposed to spectral treatments in isolation (Chapters 2 and 3). The effects of UVR varied according to spawning habitat (Chapters 2 and 3) with intra-specific embryonic responses to UVR

detected in all four focal species. These intra-specific responses imply some variation in protection mechanisms against UVR damage. Survey results revealed that egg mass deposition site was species specific. Previous studies suggest that many species spawn in microhabitats that optimise embryonic survival (Pechenik 1978, D'Asaro 1986). However, while some results from this study were in consensus with this finding (*Benhamina obliquata*, *Haminoea zelandiae* and *Haminoea vesicula*), *Siphonaria australis* spawns in microhabitats that do not correspond to highest survival (Chapter 2). Alternative explanations for deposition sites may include species depositing their offspring in risky microhabitats to avoid predation, and hence the risk associated with predation should be higher than the risk currently observed under variable environmental stressors (Spight 1977, Menge 1978 a, b). Further, egg mass deposition occurring in exposed sites may optimise conditions of other life stages.

The research presented here suggests that UVR intensity may reduce severity of mortality in encapsulated embryos in New Zealand, with higher embryonic mortality observed in *S. australis* in the middle of summer compared to the beginning of spring when UV Indices (UVI) were much lower (Fig 2.3b, Chapter 2). UVR levels and temperature fluctuate during the year according to season, peaking in the summer months (McKenzie 1991, Fig 3.4, Chapter 3) as well as fluctuating due to weather patterns; for example, UVI are significantly reduced by cloud cover (McKenzie 1991). To combat the probability of UVR damage, egg mass deposition may occur in response to both spatial and temporal reductions in UVR where by more egg masses are deposited in early spring and under overcast conditions to seek temporal refuge from UVR trauma.

Previous research demonstrates that the Southern Hemisphere is particularly vulnerable to high incidences of UVR (McKenzie et al. 1999a, b). Further, intertidal environmental stressors are worsened by summertime low tides in New Zealand often coinciding with the middle of the day (e.g. Helmuth et al. 2002). Despite this uneven spatial distribution of UVR intensity, no research has included an inter-hemispheric comparison to determine the risk of New Zealand's marine fauna compared to elsewhere in the world. As hypothesised, the effect of spectral treatments in the US species *H. vesicula* was minimal compared to the New Zealand species *H. zelandiae*, despite experimental analysis corresponding to seasonally high UVI in USA (Chapter 3). This pattern may be driven by species-specific differences in vulnerability to UVR, including variation in physical and chemical characteristics of individual egg masses (Chapter 3). These results suggest that it is insufficient to rely on responses from species that seem similar and are even closely related, to predict risk.

Early life stages of these organisms may be especially vulnerable to environmental stress as they undergo sensitive developmental processes. Further, early life stages play a fundamental role in population dynamics (Eckert 2003, Giménez 2004) and the effects of stress can carry over into later life stages whereby direct and indirect effects of UVR damage can influence processes at the population and community levels (Pechenik 2006, Podolsky and Moran 2006). Therefore, continued ozone depletion and global climate change may seriously affect the community assemblages of the intertidal environment, although it is unknown if population decline in these species has occurred as a result of UVR damage since the ozone hole first appeared over Antarctica 30 years ago. Early studies have considered mixed life histories advantageous to planktonic development. However, this theory assumes insignificant prehatching mortality; an assumption that may not be valid with the onset

of climate change and increased ozone depletion, especially for intertidal egg masses where many species inhabiting this highly heterogeneous environment are already operating at the edge of their physiological tolerances (Helmuth et al. 2002, 2006).

MAA concentrations were species specific; with highest concentrations of MAAs found in *B. obliquata* egg masses that generally deposited offspring in the shade, suggesting that protection from UVR damage is provided both biochemically and through adult spawning behaviour. High concentrations of MAAs found in *B. obliquata* egg masses may be beneficial to hatched veligers that spend extended periods of time developing in the plankton and may be exposed to UVR before metamorphosing on the benthos (Strathmann 1985, Strathmann 1987, Havenhand 1993). MAAs may not be the sole protective mechanism mitigating the effects of UVR damage, and possible strategies that may work in tandem with MAAs include DNA repair enzymes (Rozema et al. 2002, Kim et al. 2004), antioxidants (Camus and Gulliksen 2005) and diatoms (Riegger and Robinson 1997). While MAA concentrations and other protective strategies may be present in this species, these mechanisms do not offer complete protection as evidenced by mortality observed in the field and the lab (Chapters 2, 3 and 4).

This research helps to demonstrate and predict the effects of anthropogenic ozone depletion and global warming by recognising potential synergistic relationships between environmental stressors that may further enhance the harmful effects of these phenomena. The knowledge obtained through this research will lead to better estimates of the impacts of global change on population dynamics of species as well as community composition and biodiversity. This knowledge is essential to inform and

drive policies to protect against further ecological harm (Ascher 2004, Nilsson et al. 2004, Baker 2005).

5.2. FUTURE DIRECTIONS

This study has shown that the responses to UV and other stressors may be governed by a variety of protective strategies controlled by complex relationships between abiotic and biotic factors. Further research to determine the role of MAAs and alternate mechanisms of protection is warranted. Identifying the mechanisms by which MAAs are obtained and sequestered into egg masses and what external cues influence these concentrations may also help explain the variation seen in these compounds. Controlled experiments should also be conducted to establish absolute thresholds to UVR intensity, and whether earliest stages are particularly vulnerable to the damaging effects of stress (see pilot study, Appendix 2), and finally, to look at the resulting hatchlings that survive after exposure and see if there are any sub-lethal carry-over effects on the performance of larvae or juveniles. Further investigation is warranted to assess the effect of UVR along a greater spectral gradient in numerous species from both hemispheres, and identify the amount of embryonic mortality incurred over the entire spawning season. For example, full spectrum light negatively affected the development of the sea hare *Phyllaplysia taylori* in additional pilot experiments at Friday Harbor Laboratories (FHL) (Appendix 3).

Increasing anthropogenic changes to the Earth's atmosphere may have important implications for aspects of New Zealand's diverse marine fauna not studied here. For example, synergistic interactions between increasing UVR, temperatures and

ocean acidification may have serious repercussions on other organisms that inhabit intertidal shores in New Zealand, unbalancing these intrinsically linked communities.

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Appendix 1: *Siphonaria australis* Experiment Two-UVR and initial UVR/Light exposure, (Run 2: 10th-14th March 2007)

Aim: To determine the effect of initial UVR/light exposure on *Siphonaria australis* embryonic viability when exposed to different light spectral treatments.

Methods: This experiment was conducted concurrently with *Haminoea zelandiae* egg masses (10th-14th March 2007, Chapter 3) and therefore followed identical methodology with egg masses initially sourced from the sun and the shade and then exposed to (1) full spectrum light, (2) UV-blocked light and (3) completely shaded.

Figure 1A Effects of experimental light treatments (black bars are dark, gray bars are UV-blocked and white bars are the full spectrum) and initial UVR/light exposure of egg masses (sunny or shaded microhabitats) on embryonic mortality of *Siphonaria australis*. Plotted are mean (+/- 95% CI) percentage of inviable embryos.

Appendix 2: Embryonic mortality throughout developmental stages of *Siphonaria australis*

Aim: This was a small pilot experiment that I conducted to determine if embryonic mortality in response to sun exposure changed over the course of benthic development.

Methods: Adult *Siphonaria australis* limpets were collected from the field and placed in aquaria with rocky substrate taken from the intertidal at Island Bay. One aquarium had a low level of circulating seawater to simulate low tide conditions; the other aquarium had a higher level of circulating seawater to simulate high tide. Limpets were tagged and an equal number of limpets (50) were placed in each tank. For the duration of the experiment, microhabitats of the newly deposited egg masses were noted (sun or shade, desiccated or submerged) and a small segment of the mass was removed and microscopically analysed, scoring the 30 embryos as viable vs. inviable at 100x magnification. The remaining egg mass was left to continue to develop in situ, and each (n = 30) was sampled daily over five days.

Figure 2A The percentage of inviable embryos observed over embryonic development (Day 1-5) in *Siphonaria australis* egg masses found in different microhabitats (triangle= desiccated and sun exposure, Diamond=submerged and sun exposure, cross=desiccated and shaded, square= submerged and shaded).

Appendix 3: The effects of ultraviolet radiation and low tide conditions on the development of the Northern hemisphere sea hare *Phyllaplysia taylori*

Aim: To determine the effect of UVR and low tide conditions on the development of *Phyllaplysia taylori* embryos.

Methods: Identical methods were used in this manipulative experiment as described in Chapter 2 and 3 examining the effect of embryonic survivorship under different spectral (three levels: (1) full spectrum light, (2) UV-blocked light and (3) completely shaded) and low tide treatments (three levels: (1) submerged in circulating seawater, (2) desiccated for three hours and (3) submerged in non- circulating seawater, simulating a tidal pool).

Figure 3A Effects of light experimental treatments (black bars are dark, gray bars are UV-blocked and white bars are the full spectrum) and for each simulated low tide condition (submerged, desiccated, tidal pool) on embryonic mortality of *Phyllaplysia taylori*. Plotted are mean (+/- 95% CI) percentage of inviable embryos.