SUNBURNT SEA SNAILS:
THE ROLE OF ULTRAVIOLET RADIATION IN THE DEVELOPMENT OF ENCAPSULATED EMBRYOS FROM TEMPERATE ROCKY SHORES

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DOCTOR OF PHILOSOPHY

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by

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THESIS CERTIFICATION

CERTIFICATION

I, Rachel Przeslawski, declare that this thesis, submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Biological Sciences, University of Wollongong, is wholly my own work unless otherwise references or acknowledged. The document has not been submitted for qualifications at any other academic institution.

Rachel Przeslawski

29 March 2005

This thesis is dedicated to Mary Bissonnette without whom I never would have accomplished what I have today.
I'm only just starting to realise how much hard work and sacrifice you made over the years, and I'm truly grateful… thanks heaps Mom!

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I'm also thankful for all the support from my family over the years. Mom, Dad, Mark and Mike all helped instil the knowledge, natural curiosity, and persistence (stubbornness) that drive everything I do today. I'm particularly grateful to Patrick Falvey who supported me financially and emotionally during my research; without his help, this never would have been completed. I can't think of anyone else who would have run out on a roof in torrential rain with me to 'rescue' tiny snotty egg mass pieces in outdoor aquaria! Pat also used his bloody engineering skills for good by helping to construct many of the aquaria and tanks that were used in some of these studies.

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ABSTRACT

Ultraviolet radiation (UVR) is an important abiotic stressor for both aquatic and terrestrial organisms. The recent anthropogenic depletion of stratospheric ozone has resulted in elevated levels of potentially damaging UV-B to which organisms are exposed, and global climate change may also herald changes in environmental conditions, particularly temperature, precipitation, and sea level. Thus, organisms may be simultaneously exposed to variable environmental stressors. In the marine environment, embryos and larvae are likely the most vulnerable to the negative effects of these stresses. Moreover, intertidal organisms are particularly vulnerable to UVR because they occur in habitats where little or no UVR is absorbed by the water column, and the effects of UVR are coupled with other potential negative stresses associated with low tides. Surprisingly, little is known about the effects of potential interactions between environmental stressors on marine larvae, particularly involving UVR. This study aims to investigate the role of UVR in the development of encapsulated intertidal embryos through a series of six independent experiments that screen a large number of taxa.

In the first four experiments, I explored the direct effects of UVR in isolation and with other stressors (UVR/temperature/salinity and UVR/desiccation), as well as the indirect effects of UVR and fouling. Isolated effects of UVR were investigated on egg masses from 23 marine gastropod species collected from three intertidal habitats (full sun, partial shade, full shade) and exposed to four spectral treatments (full spectrum, no UV-B, no UVR, dark). Embryos from full shade habitats were significantly vulnerable to UVR while those from full sun habitats showed no significant mortality differences between spectral treatments.

Multifactorial experiments were then conducted in which encapsulated embryos of three common rocky shore gastropods were exposed to simultaneous combinations of (i) UVR, temperature, and salinity and (ii) UVR and desiccation. *Siphonaria denticulata* and *Bembicium nanum* embryos were expected to be tolerant to these negative interactions of stressors as they are routinely deposited on rock platforms exposed to solar radiation. In contrast, *Dolabrifera brazieri* embryos were predicted to be vulnerable to these stressors as they are deposited in shaded, submerged habitats. I

detected species-specific synergistic effects of these stressors, and increases in mortality and retardation of development were generally associated with the most physiologically stressful conditions. Based on laboratory results, embryos of *D. brazieri* were the most sensitive to all the stressors. In contrast, *S. denticulata* and *B. nanum* were vulnerable to negative effects associated with synchronous spectral, thermal, and salinity stress; but they were relatively tolerant to UVR and desiccation. Nevertheless, field results indicate that embryos of these species within desiccated habitats have a significantly higher mortality than those within submerged habitats, suggesting that development on rock platform surfaces may not be optimal for these embryos.

The indirect effects of UVR and fouling on encapsulated larval development were investigated on egg masses from 18 species cultured under three spectral treatments (full spectrum, no UV, dark). Algal fouling levels, protist colonisation, embryonic mortality, and encapsulation period were recorded, and I found that UVR inhibited algal growth and protist colonisation on egg mass surfaces. Although algal fouling was not directly related to embryonic mortality in most species, egg masses colonised by protists had a higher level of algal fouling; and overall, these egg masses had a significantly higher incidence of embryonic mortality.

In the last two experiments, I examined potential behavioural and biochemical protection afforded to encapsulated intertidal embryos against UV-induced damage. I conducted surveys of intertidal egg masses in south-eastern Australia over two years to determine if spatial and temporal variation in parental site selection could reduce potential environmental stress to encapsulated embryos. I predicted that egg masses would be predominantly deposited in shaded habitats not prone to environmental extremes. Furthermore, I anticipated that egg masses deposited on rock platform surfaces would be smaller and occur less frequently in these habitats during seasons of high environmental stress. As predicted, most species spawned under boulders, thereby minimising exposure to environmental stress. Analyses confirmed that summer had the highest UVR index, water temperature, and air temperature, as well as the lowest tides; but assemblages and abundances of egg masses on exposed rock platforms were highest during summer with no change in egg mass sizes. Thus, species spawning on rock platform surfaces do not seem to confer protection to their encapsulated offspring by avoidance of physiologically stressful times or conditions. Alternatively, one or more of

these potential stressors are beneficial to embryonic development, and these benefits outweigh negative effects. For example, high temperatures associated with direct sunlight may increase developmental rate and counteract any negative effects associated with UVR.

Potential biochemical protection against UV-induced damage was examined by quantifying potential chemical suncreens, mycosporine-like amino acids (MAAs), in intertidal egg masses from 46 mollusc species, two polychaete species, and one fish species from southeastern Australia. Analyses revealed that egg mass maturity and spawning habitat did not significantly affect MAA composition within egg masses. In contrast, adult diet, phylogeny, and viability significantly affected MAA composition. Herbivores had significantly higher levels of certain MAAs than carnivores, and viable egg masses had higher levels of some MAAs than inviable egg masses. MAAs also occurred in relatively high concentrations in molluscan egg masses when compared to adult molluscs and other common intertidal organisms. Despite the complexity of factors affecting MAA composition, the prevalence of MAAs in some species is consistent with protection afforded to offspring against negative effects of UVR.

Results from the experiments comprising this study indicate that encapsulated embryos use behavioural and biochemical protection against UVR and related stressors; and the presence and effectiveness of these mechanisms may be species-specific. Similarly, the species-specific effects of UVR highlight the importance of research on a range of species. Furthermore, the complex outcomes observed on applying multiple stressors could not have been predicted from examining environmental variables in isolation. Results from the single factor study here suggest that UVR does not negatively affect embryos of species that spawn in full sun, but multifactorial experiments revealed that UVR can indeed have negative effects when other stressors are considered. Hence, we may be dramatically underestimating the ecological impacts of climate change and stratospheric ozone depletion by failing to consider the complex interplay of combinations of environmental variables with organisms.

LIST OF FIGURES

ACRONYMS

CFC: chlorofluorocarbon

DOC: dissolved organic carbon

MAA: mycosporine-like amino acid

PAR: photosynthetically active radiation (visible light)

ROS: reactive oxygen species

UVR: ultraviolet radiation

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"Sunburnt Sea Snails: the role of UVR in the development of encapsulated embryos from temperate rocky shores"

* Independent manuscripts are enclosed in quotations

CHAPTER 1: Introduction[1]

"Human subtlety will never devise an invention more beautiful, more simple or more direct than does Nature, because in her inventions, nothing is lacking and nothing is superfluous."
Leonardo DaVinci

[1] Section 1.4 encompasses published manuscript:
Przeslawski, R. (2004) A review of the effects of environmental stress on embryonic development within intertidal gastropod egg masses. *Molluscan Research*, 24, 43-63

1.1 GENERAL INTRODUCTION

Ultraviolet radiation (UVR) is an important abiotic stressor for both aquatic and terrestrial organisms, comprising UV-A (315 – 400 nm), UV-B (280-315 nm) and UV-C (200-280 nm). It is assumed that UV-C played an important role in the evolution of early life since the Earth's surface was subject to high intensities of UV-C during the Archaen period 3.9 – 2.5 billion years ago (Cockell & Knowland 1999). However, since the formation of the ozone layer at the end of this period, UVR at the earth's surface has included only UV-A and UV-B (Kasting & Donahue 1980).

Incident solar radiation levels are affected by a range of variables that operate on both regional and global scales. Solar zenith angles and distance from Earth to sun account for global diurnal, seasonal, latitudinal, and altitudinal variation in UVR intensity and exposure. Excluding regional effects, UVR fluxes are generally higher at midday, during summer, at lower latitudes, and higher altitudes (Diaz *et al.* 2000). Other factors such as cloud cover and composition, tropospheric pollutants, and ozone layer integrity can affect surface UVR levels locally and regionally (Xenopoulos & Schindler 2001).

UVR levels at the surface of water are controlled by all the factors mentioned above, but the penetration of UVR into an aquatic environment depends on the properties of the water column. UVR transmission is reduced at depths, primarily due to dissolved organic carbon (DOC) compounds and suspended particles (Xenopoulos & Schindler 2001). Moreover, UVR decreases DOC levels because UV-B enhances degradation of DOC into smaller particles which can be consumed by microbes (Zagarese *et al.* 2001). In addition to DOC, waves may also slightly lower UVR transmission into aquatic habitats by reducing surface transmission (Jerome & Bukata 1998).

Of all the variables that affect UVR intensity, the ozone layer is the only one to result in a global anthropogenic change in surface UVR. The ozone layer is found in the stratosphere 10-50 kilometres above the earth's surface and comprises 90% of all ozone (O_3) in the atmosphere (Anderson & Sarma 2002). It shields organisms from the most biologically damaging UVR wavelengths by blocking all UV-C, much UV-B, and minimal amounts of UV-A. In the mid-1970s, researchers predicted the depletion of the ozone layer if the uncontrolled use of chlorofluorocarbons (CFCs) in industrial and

commercial applications continued (Molina & Rowland 1974). They hypothesized that UVR broke the bonds of stratospheric CFCs to release chlorine atoms that interrupted the continual cycle of ozone formation by bonding with oxygen atoms. Furthermore, they suggested that a single chlorine atom could destroy up to 100,000 ozone molecules (Molina & Rowland 1974). This prediction was validated in the early 1980s with the detection of a 20% decrease in ozone levels above Antarctica (Anderson & Sarma 2002). At first assumed to be based on erroneous readings (Gribbin 1988), the Antarctic ozone hole was later confirmed and determined to be caused by chlorine from CFCs (WMO 1988). Although not as dramatic as the depletion at the poles, further evidence of anthropogenic ozone depletion was also evident in non-polar regions (WMO 1988); and more recent reports indicate ozone losses in mid-latitudes of the southern hemisphere contribute to a year-round 6% increase in UV-B levels compared to those of the 1970s (WMO 1998). Numerous national and global policies, notably the Montreal Protocol signed in 1987, have been implemented to curb production of CFCs and associated chemicals (see Anderson & Sarma 2002). CFCs are to be completely phased out by 2010, and use of related hydrochlorofluorocarbons is to be stopped by 2040 (Madronich & Velders 1999), but the ozone layer is not predicted to recover to pre-1980s levels until 2050 (Hofmann & Pyle 1999).

Ozone depletion poses numerous risks to both terrestrial and aquatic organisms as a result of increased surface UVR intensity (reviewed by Haeder *et al.* 1998; Caldwell *et al.* 2003). UVR is absorbed by chromophores of proteins, nucleic acids, and other biomolecules. These molecules can be broken or transformed under high UVR flux resulting in a range of damaging effects including photoinhibition (Vincent & Neale 2000), carcinogenesis (Nairn *et al.* 1996), sterility or reduced fecundity (Karanas *et al.* 1981), developmental abnormalities (Adams & Shick 2001), cataracts (De Gruijl *et al.* 2003), deleterious behaviour (Kats *et al.* 2000), and overall impairment of biological function (reviewed by Haeder *et al.* 1998; Vincent & Neale 2000; Caldwell *et al.* 2003). Furthermore, UVR may indirectly harm organisms by accelerating the photochemical formation of reactive oxygen species (ROS). ROS can quickly react with many different compounds, resulting in potentially cytotoxic photoproducts (Vincent & Neale 2000).

Organisms can mitigate damage associated with UVR in three ways: avoid, reduce, or repair. First of all, some organisms avoid UVR altogether through opaque coverings

such as the leathery tunics of certain ascidians or the shells and operculi of some gastropods. Mobile organisms can actively avoid UVR via negative phototaxis or through diurnal migrations (Leech & Williamson 2001; Persaud *et al.* 2003). Sessile organisms can passively avoid UVR through habitat selection. For example, the undersides of boulders are often dominated by sessile invertebrates that settle consistently in shaded habitats (Irving & Connell 2002). Second, organisms can reduce the intensity of UVR exposure through various extracellular or intracellular screening agents (Roy 2000 and references therein). Of these, mycosporine-like amino acids (MAAs) are the most widely studied in aquatic environments. MAAs encompass nineteen known compounds with absorption maxima between 310 – 360 nm (Shick & Dunlap 2002), and they are almost ubiquitous in marine organisms, occurring in cyanobacteria, algae, invertebrates, and fish (reviewed by Karentz 2001). Finally, UVR damage can be mitigated through repair mechanisms. UVR-induced damage to DNA can be repaired through DNA repair enzymes such as photolyase; this enzyme is found in most organisms (Roy 2000), but photoreactivating activity seems to be species-specific (Karentz *et al.* 1991). In addition, antioxidants can limit damage done by ROS by detoxifying them or the resulting cytotoxic compounds (Roy 2000).

Intertidal organisms are particularly vulnerable to UVR because they occur in habitats where little or no UVR is absorbed by the water column. Furthermore, the effects of UVR in the intertidal do not occur in isolation; rather, they are coupled with other potential negative stresses associated with low tides such as desiccation and extremes in temperature and salinity. Embryos and larvae are likely the most vulnerable to the negative effects of these stresses and interactions among them as they are considered the most sensitive life stage of invertebrates (Spight 1975; Pechenik 1979; Pechenik 1987; Havenhand 1993). Embryonic development and survival is particularly important to the overall success of a population as it directly affects the viability of future generations. Although there has been a considerable amount of research on the effects of single abiotic factors on larval and embryonic development and survival, there is a paucity of knowledge about the effects of potential interactions between environmental stresses, particularly involving UVR.

Intertidal egg masses represent ideal models to study the effects of UVR and other environmental stresses on embryonic development and survival for several reasons. First of all, there is a diversity of taxa that routinely deposit egg masses in intertidal habitats, which allows for phylogenetic comparisons, analysis of interspecific variation, and generalizations based on results across numerous species. Most intertidal egg masses in southeastern Australia are deposited by a variety of gastropods, but some polychaetes and fish also encapsulate their offspring in egg masses. Second, intertidal species spawn in differentially stressed microhabitats; some spawn exclusively under submerged boulders, while others spawn in full sunlight (Benkendorff & Davis 2004). Therefore, comparisons between egg masses from these different environments can determine if mechanisms protecting against UVR and other stresses have evolved in species that consistently spawn in full sunlight. Finally, intertidal egg masses are identifiable, common, and conducive to both laboratory and field studies.

Previous research has catalogued many southeastern Australian egg masses to enable identification to a genus or species level (Rose 1985; Smith *et al.* 1989; Benkendorff 1999). Intertidal egg masses can be identified by associated spawning adults, or by characteristics such as colour and shape of the encapsulating structure. Morphologically, many egg masses are unique to a particular species and can be readily identified in the field (e.g. *Bembicium nanum, Dolabrifera brazieri, Dicathais orbita*). Many species regularly deposit egg masses along the southeastern coast of Australia during certain seasons, and egg masses are often abundant and easy to find. Embryos in egg masses are essentially sessile for the duration of their encapsulation, so field studies over the course of several days can be conducted with relative ease compared to those involving pelagic larvae. Similarly, encapsulated spawn is conducive to laboratory studies as embryos are in a fixed, yet easily manipulated location within the egg masses. Egg masses can also be easily divided into several pieces to enable replicate embryos to be placed in different treatments, thus reducing potential impact of variation between individual egg masses.

1.2 AIMS AND SIGNIFICANCE

This study aims to investigate the role of UVR in the development of encapsulated intertidal embryos through a series of six independent experiments that screen a large number of taxa. I sought to determine the effects of UVR on encapsulated embryonic development in the intertidal zone, as well as to identify and quantify effects of other environmental stressors that may interact significantly with UVR to affect development. Furthermore, I aimed to characterise potential protection afforded to encapsulated intertidal embryos against UVR-induced damage. I examined the potential for biochemical protection through mycosporine-like amino acids (MAAs) and behavioural protection through selection of spawning site. Finally, I sought to determine factors that influence the magnitude of UV-induced damage and protection, such as phylogeny and natural spawning habitat.

Results from this study will help predict the impacts of events associated with anthropogenic activity, such as the thinning of the ozone layer and global climate change, by investigating effects of factors associated with these phenomena (i.e. UVR and temperature). In addition, this study will highlight the importance of interactions and synergistic effects in intertidal environments. Considering effects of environmental stressors in isolation may underestimate the effects of both local and global change associated with anthropogenic and natural events, and it is hoped results from these experiments will corroborate this suggestion. Moreover, this study will contribute to our knowledge of the evolution of protective mechanisms against environmental stress by elucidating patterns of resistance to UVR-induced damage in intertidal organisms based on selection of spawning habitat, phylogeny, adult diet, and egg mass structure.

1.3 STRUCTURE OF THIS STUDY

This entire thesis encompasses a literature review and six experiments, each of which is presented as an independent manuscript. Thus, some repetition of material occur in Introductions and Discussions across manuscripts. Each manuscript has discrete hypotheses and aims, and these complement the overarching aims mentioned above. Most manuscripts included in this thesis have been accepted for publication in peer-

reviewed journals, and the relevant references are therefore included on the title page of each chapter containing the manuscripts. Within each manuscript, ‘study’ refers to the particular experiment(s) associated with only that section or chapter. References to other experiments are made by formal citation of the published or submitted work.

Chapter 1 is a general introduction to the concepts involved in this research (Section 1.1) as well as a presentation of the aims and significance of this work (Section 1.2). In addition, Chapter 1 contains a comprehensive literature review examining the effects of UVR, temperature, salinity, and oxygen availability on encapsulated molluscan development (Section 1.4). Importantly, the known relationships between these factors are also examined. Chapter 2 contains one manuscript that investigates the isolated effects of UVR on the embryonic mortality and encapsulation period of 23 intertidal gastropod species. Chapter 3 contains three independent manuscripts each based on experiments examining the effects of interactions between UVR and other potential stressors on encapsulated molluscan development. First, synergistic effects of UVR, temperature, and salinity are investigated on the mortality and developmental rate of three species of gastropod (Section 3.1). This is followed by examination of effects of interactions between UVR and desiccation on embryonic development of the same three gastropod species (Section 3.2). Finally, the effects of UVR and fouling on the mortality and developmental rate of 18 gastropod species are examined (Section 3.3). Chapter 4 contains two manuscripts based on research that investigates potential protective mechanisms against UV-induced damage to encapsulated intertidal embryos. The first part of this chapter examines parental site selection for egg mass deposition in relation to temporal and spatial variation in UVR exposure and other abiotic stressors (Section 4.1). The second part of Chapter 4 quantifies MAAs in egg masses from 46 species of mollusc, two species of polychaete, and one species of fish from southeastern Australia (Section 4.2). Chapter 5 represents a general conclusion and summary to the entire study, drawing together the results from all six experiments.

1.4 A REVIEW OF THE EFFECTS OF ENVIRONMENTAL STRESS ON EMBRYONIC DEVELOPMENT WITHIN INTERTIDAL GASTROPOD EGG MASSES

Gastropod egg masses are often deposited in the intertidal zone, where they are exposed to variable and often stressful environmental conditions that may affect the encapsulated embryonic development and survival of offspring. The present paper reviews data on developmental variation in gastropod egg masses owing to temperature, salinity, ultraviolet radiation (UVR) and oxygen availability. In general, increases in temperature or oxygen availability accelerate development, whereas UVR or extremes of salinity and temperature slow development or increase embryonic mortality. The relationships among these factors are discussed, as are their interactions with biotic factors, such as fouling, embryonic position within the egg mass and predation. One purpose of the present review is to raise awareness of these interactions so they become a focus for future research. Protective mechanisms of egg masses against environmental stresses are also reviewed.

1.4.1 INTRODUCTION

Intertidal organisms face a variety of selective challenges while reproducing. They must protect their offspring against the environmental extremes of the intertidal zone, as well as against risks of predation and infection. Many gastropods have adapted to these challenges by laying their eggs in benthic masses. Although gastropod egg masses include a striking array of varied structures, they can be divided into two general categories: capsular and gelatinous, as defined below (see Figures 1.1, 1.2).

Benthic egg masses are believed to provide protection to the developing embryos from environmental stresses and predation (Thorson 1950; Pechenik 1979; Strathmann 1985). Nevertheless, environmental factors still affect embryonic development, sometimes deleteriously. Gastropod intracapsular embryonic development and mortality may be influenced by temperature, salinity, ultraviolet radiation (UVR), oxygen availability, water flow, fouling, embryonic position, predation and parental history. These factors do not usually operate independently (Figure 1.3) and, consequently, confounding relationships should be considered when studying embryonic development within gastropod egg masses. Once it is known how embryos react to certain environmental factors, it may be possible to predict their response to the associated local and global environmental events, such as thermal fluctuations and the thinning ozone layer.

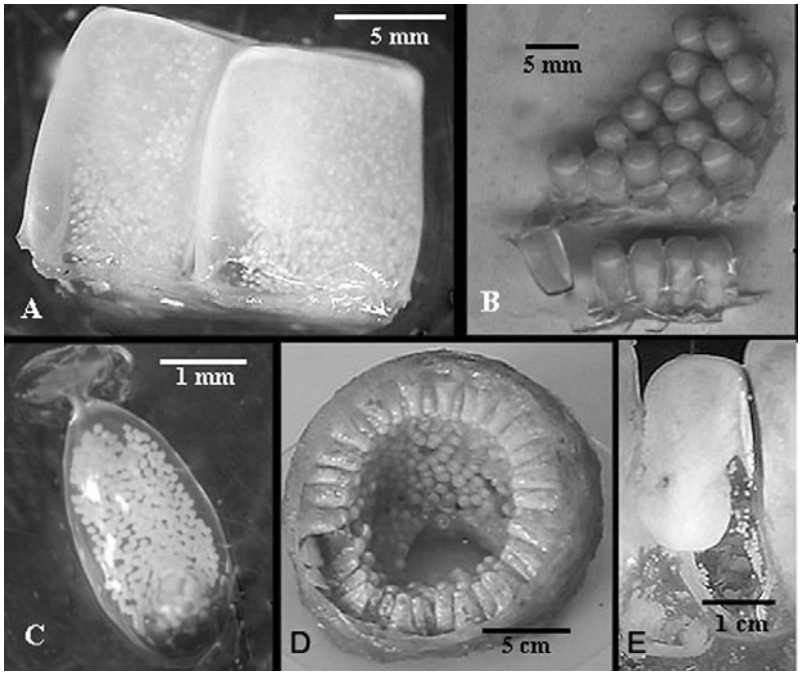


Figure 1.1 Capsular caenogastropod egg masses collected along the Illawarra coastline, NSW. A) *Dicathais orbita* capsules with swimming veligers. B) Part of *Ranella australasia* egg mass. C) *Mitra carbonaria* capsule containing trochophores. D) Intact *Cabestena spengleri* egg mass consisting of numerous capsules removed from brooding adult. E) *C. spengleri* capsule with only a few discrete eggs. The rest of the eggs have degenerated into a solid mass near the top of the capsule following exposure to sunlight.

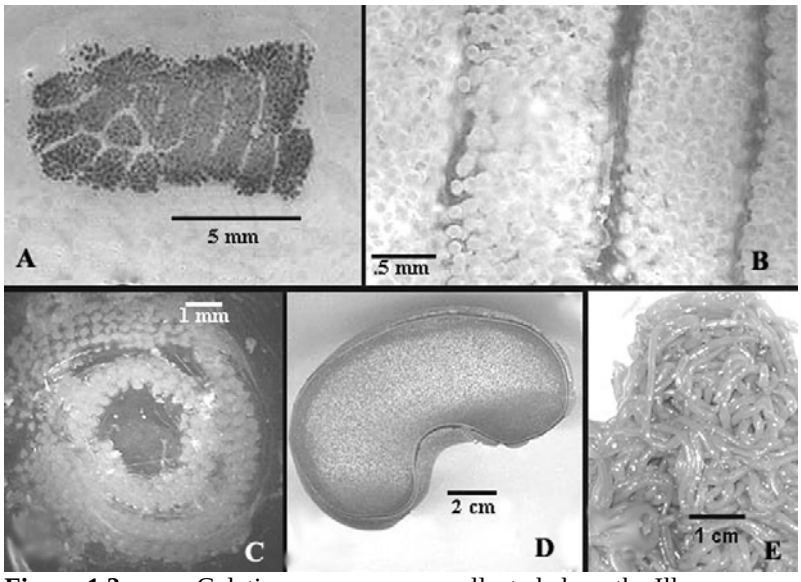


Figure 1.2 Gelatinous egg masses collected along the Illawarra coastline, NSW. A) *Bembicium nanum* egg mass with varying embryonic development. Peripheral embryos are more developed than central embryos as evidenced by their darker more developed shells. B) *Dolabrifera brazieri* egg mass with veligers. C) *Rostanga arbutus* egg mass with undeveloped eggs. D) *Polinices (Conuber)* spp. egg mass. E) *Dolabella auricularia* egg mass.

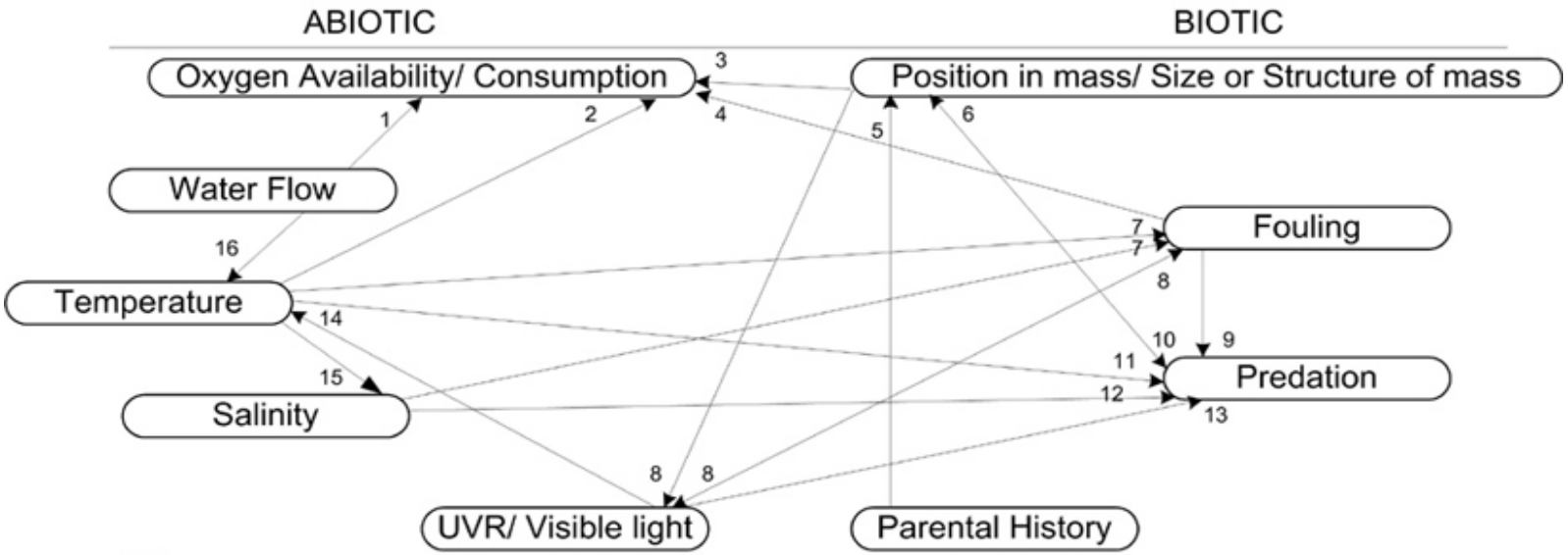


Figure 1.3 Interactions of abiotic and biotic variables that can affect embryonic development within molluscan egg masses. The relationships illustrated are by no means ubiquitous or exhaustive. Rather, each arrow represents a potential relationship that has been identified in at least one study, a representative of which is listed below the figure. A single arrow indicates a relationship is which one variable affects the other. A double arrow indicates a relationship is which both variables affect each other.

Previous reviews have been written about gastropod egg masses, but discussion relating to benthic egg masses is typically ancillary to theoretical discussion of general reproductive patterns (e.g. Thorson 1950; Gallardo & Perron 1982) or larvae (Pechenik 1987). Other reviews cover potential protective mechanisms of egg masses, but they do not focus on the effects of environmental stresses on the embryos within (e.g. Pechenik 1979; Eyster 1986; Rawlings 1999). Still other reviews on gastropod egg masses are confined to one geographic region (e.g. Strathmann 1987). Upon examination of the literature, very few studies were found that considered more than one environmental factor at a time (Table 1.1). Thus, there is a fundamental gap in our understanding of how confounding or interdependent environmental factors influence embryonic development in gastropod egg masses. The primary purpose of the present review is to consolidate available research about abiotic effects on gastropod egg mass development and to examine the complex relationships between abiotic factors, embryonic development and biotic factors, such as predation, fouling and embryonic position. It is hoped that this will help promote and guide future multifactorial research on embryonic development and mortality.

Table 1.1 Experiments on the developmental effects of various factors on encapsulated marine molluscan development. # refers to the number of mollusc species studied. Pos= embryonic position. Foul= algal or microfaunal fouling. Hist= parental history. Pred= predation.

Reference	**#**	**Abiotic**				**Biotic**			
		Temp	**Salinity**	**UV**	**O_2/ Flow**	**Pos**	**Foul**	**Hist**	**Pred**
Ganaros 1958	**1**	**x**							
Thompson 1958	**1**	**x**							
Scheltema 1965	**1**		**x**						
Scheltema 1967	**1**	**x**							
Hagerman 1970	**1**		**x**						
Spight 1975	**1**	**x**							
Switzer-Dunlap *et al.* 1977	**4**	**x**							
Pechenik 1978	**2**				**x**				
Dehnel *et al.* 1979	**1**	**x**							
Pechenik 1982	**3**		**x**						
Pechenik 1983	**1**		**x**						
Chaffee *et al.* 1984	**2**				**x**	**x**			
Rose 1986	**1**	**x**	**x**						
Roller & Stickle 1989	**1**	**x**	**x**		**x**				
Rawlings 1990	**1**								**x**

Rodriguez *et al.* 1991	**1**	**x**					
Biermann *et al.* 1992	**1**			**x**		**x**	**x**
Palmer 1994	**1**	**x**					
Booth 1995	**1**				**x**	**x**	
Strathmann *et al.* 1995	**3**				**x**	**x**	
Cohen *et al.* 1996	**2**			**x**	**x**	**x**	**x**
Rawlings 1996	**1**			**x**			
Richmond *et al.* 1996	**1**		**x**				
Woods *et al.* 1997	**1**		**x**		**x**	**x**	
Carefoot *et al.* 1998	**1**			**x**			
Podolsky *et al.* 1998	**1**	**x**					
Cancino *et al.* 2000	**1**				**x**		**x**
Podolosky 2002	**1**	**x**					
Pechenik *et al.* 2003	**1**	**x**	**x**		**x**		
Przeslawski *et al.* 2004	**23**			**x**			

Egg mass structure and phylogeny

Because the structure and composition of egg masses are related to their phylogeny, it is necessary to clarify the taxonomic stance adopted in the present paper. In this review, gastropod classification will follow (Beesley *et al.* 1998)(Table 1.2). Capsular egg masses are found among the Neritopsina and some Caenogastropoda (Figure 1.1; Table 1.2). Gelatinous masses are found among the Heterobranchia (Figure 1.2b,c,e) and among some Caenogastropoda (Figure 1.2a,d; Table 1.2). Some vetigastropods encase their eggs in jelly masses, although these often lack the organisation of heterobranch egg masses (Hickman 1992).

Capsular egg masses of many caenogastropods consist of multiple distinct capsules often connected to one another by a common basal layer (Figure 1.1b,d). The often tough leathery capsule wall characteristic of neogastropods is composed of several structurally and chemically distinct layers (Tamarin & Carriker 1967; LeBoeuf 1971), sometimes sealed with an apical plug (Sullivan & Bonar 1985). Eggs and embryos are located inside the capsule walls, where they develop within intracapsular fluid (Bayne 1968). Some species use non-viable eggs called nurse eggs to nourish developing embryos (Gallardo & Perron 1982; Hoagland 1986) (Figure 1.4).

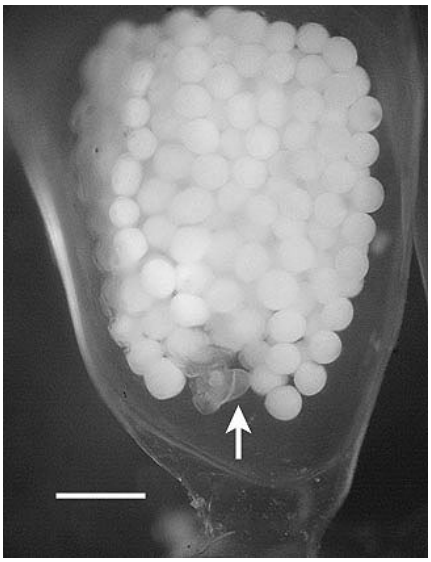


Figure 1.4 Egg capsule of the neogastropod *Mitra badia* collected from Bass Point, NSW. The arrow indicates the single developing veliger surrounded by nurse eggs. Scale bar represents 300 µm.

Neritid capsules have a calcareous apical capsule wall made with particles from a specialised crystal sac (D'Asaro 1986) (Figure 1.5) and thus differ from caenogastropod egg capsules. Despite the fact that *Nerita atramentosa* is one of the most common snails on the rocky shore of south-eastern Australia, there is no published research to date examining the effects of environmental stresses on embryos of neritid egg capsules. Regrettably, they will not be discussed any further in the present review other than to note that such research would be interesting because neritid capsules are often deposited high on the shore (Figure 1.5).

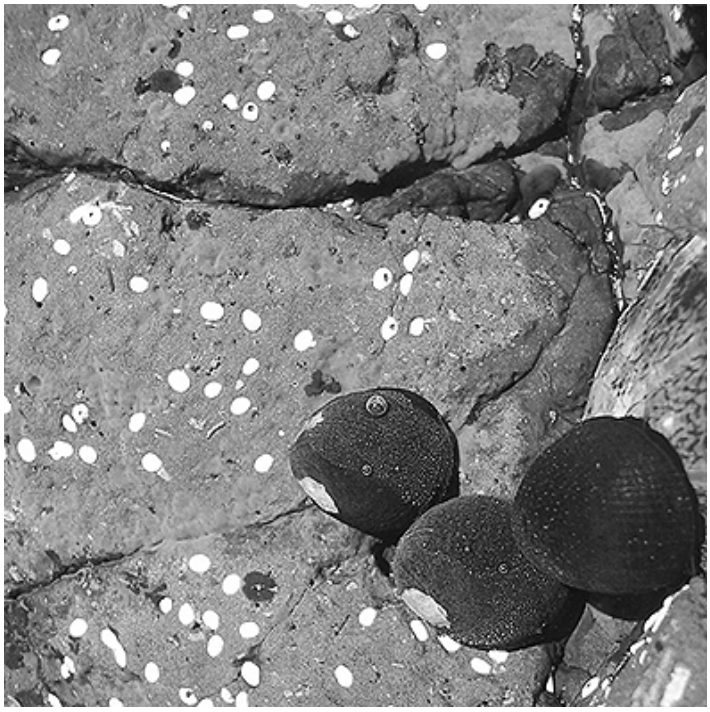


Figure 1.5 The common intertidal snail *Nerita atramentosa* with egg capsules. Egg capsules are deposited on rock platform surfaces where they are exposed to full sunlight. Many capsules often have small apical holes as seen in this photograph; it is unknown if these holes are escape mechanisms for hatching offspring or the work of a predator.

Gelatinous egg masses consist of a jelly matrix in which many eggs are embedded (Figure 1.2). A microscopic vitelline capsule surrounds each egg or small group of eggs (Eyster 1986; Klussmann-Kolb & Wagele 2001) (Figure 1.2). Some species lay egg masses that are a variation on the typical gelatinous egg mass structure. Naticid sand collars, for example,

comprise adherent sand grains in addition to a gelatinous matrix in which microscopic capsules and embryos are embedded (Giglioli 1955). Other taxa, such as some Amphibolids, also incorporate relatively large amounts of sand into their egg masses (Benkendorff 1999; Pechenik *et al.* 2003). Unlike capsular masses, gelatinous masses ensure that individuals or small groups of embryos are isolated from other embryos within a mass by their surrounding vitelline capsule. It is likely that many embryos receive their nutrition from intracapsular fluid (Moran 1999), whereas others receive nutrition from yolk granules or sacs associated with the egg mass (Clark & Goetzfried 1978; Williams 1980; Boucher 1983).

Among gastropod egg masses, considerable variation can occur in the number of eggs per capsule (Grant 1983; Strathmann 1985), egg and embryo size (Strathmann 1977; Christiansen & Fenchel 1979) and colour (D'Asaro 1966; Switzer-Dunlap & Hadfield 1977), as well as the shape and size of the egg mass (Hurst 1967; Chambers & McQuaid 1994). Although at least some of this variation occurs among individuals of the same species (Eyster 1979), there are only a very few studies that examine how much of this variation is due to environmental effects (e.g. Thompson 1958; Hagerman 1970; Cheung 1997). Thus, it can be difficult to determine the difference between intrinsic variation and environmental effects on gastropod egg masses. The present review considers variation in embryonic development.

Terminology

For clarity and consistency, 'egg' will refer to anything before the blastomere stage, including unfertilised eggs. Developing young will be referred to as 'embryos' until they leave the capsule (Giese & Pearse 1974). As accepted in most previous research, 'capsule' will describe both the rigid layered wall in capsular masses, as well as the vitelline membrane surrounding embryos in gelatinous egg masses. 'Egg mass' will refer to the entire discrete gelatinous mass or capsule group in one site. In the case of species that lay among spawning aggregations (e.g. many anaspids or neogastropods), this definition could include ribbons or capsules from several individuals deposited in the same mass. 'Dead' and 'dying embryos' will include seriously deformed immobile embryos and embryos

showing tissue damage or loose debris within an internal capsule (Woods & DeSilets 1997) (Figure 1.1e).

Table 2 Orthogastropod classification system and corresponding egg mass type that occurs in at least some species within each group (Beesley *et al* 1998). First tier of classification is superorder followed by order, suborder, and infraorder/superfamily. Species listed are representative of some egg masses commonly found in southeast Australia (Benkendorff 1999, author's pers. obs).

Superorder, order, family	Egg mass type	SE NSW representative species
Vetigastropoda		
Trochoidea	gelatinous, capsules[1]	
Neritopsina		
Neritoidea	capsule	*Nerita atramentosa*
Caenogastropoda		
Sorbeoconchia		
Campaniloidea	gelatinous	
Cerithioidea	gelatinous, capsules[2]	
Hypogastropoda		
Littorinimorpha	gelatinous, capsule	*Bembicium nanum, Cabestana spengleri*
Neogastropoda	capsule	*Dicathais orbita, Mitra carbonaria*
Heterobranchia		
Architectonicoidea	gelatinous	
Pyramidelloidea	gelatinous	
Rissoelloidea	capsule	
Cephalaspidea	gelatinous	*Bullina lineata, Hydatina physis*
Sacoglossa	gelatinous	*Oxynoe viridis, Elysia australis*
Anaspidea	gelatinous	*Aplysia* sp., *Bursatella leachii*
Notaspidea	gelatinous	*Pleurobranchus* sp., *Berthellina citrina*
Thecosomata	gelatinous	
Gymnosomata	gelatinous	
Nudibranchia	gelatinous	
Doridina	gelatinous	*Dendrodoris nigra, Rostanga arbutus*
Dendronotina	gelatinous	*Melibe australis*
Arminina	gelatinous	
Aeolidina	gelatinous	*Austraeolis ornata, Spurilla macleayi*
Basommatophora	gelatinous	*Siphonaria* sp., *Salinator* sp.

[1] Trochoideans have various modes of reproduction; only a few species lay benthic egg masses (Hickman 1992).
[2] Some species of Cerithioidea are viviparous.

1.4.2 TEMPERATURE

Within tolerable temperature ranges for each species, the encapsulation period generally decreases as temperature increases for many invertebrates (Rothlisberg 1979; Boucher 1983; Rumrill 1990), including molluscs (Kress 1975; O'Dor *et al.* 1982; Palmer 1994;

Caveriviere *et al.* 1999) (Figure 1.6). For gastropod embryos within capsular egg masses, it has even been suggested that hatching time can be estimated knowing only the taxon and the temperature (Spight 1975; Palmer 1994). This, however, assumes that all other variables discussed in the present paper are static or do not significantly affect the embryonic developmental rate. In addition, predicting hatching time based solely on taxon and temperature may be problematic owing to potential geographic or temporal variation in temperature compensation. This has been observed, for example, in the muricid *Nucella emarginata*, with Alaskan populations hatching in significantly less time at temperatures 10°C lower than British Columbian populations (Palmer 1994).

Embryos become stressed and often die if exposed to extreme temperatures relative to their natural environment (Figure 1.6) and seem more vulnerable to temperature extremes than adults. Thompson (1958), for example, found that the adult nudibranch *Adalaria proxima* spawned and remained healthy at a relatively high 13°C, but this temperature was lethal to eggs. Despite vulnerability to both high and low temperature extremes (Figure 1.6), gastropod embryos may be more tolerant of lower temperatures within their range than higher temperatures. Struhsaker & Costlow Jr. (1969) found that planktotrophic larvae of *Littorina picta* had a high survival rate at temperatures lower than their established optimal developmental temperatures, but the larvae had lower survival rates at temperatures higher than optimal conditions. Similar observations have been made on encapsulated gastropod embryos. For example, Dehnel & Kong (1979) examined the effects of temperature on the egg masses of the nudibranch *Cadlinna luteomarginata* along the coast of British Columbia and found the hatching time was fourfold faster at 15°C than at 5°C, although there was no difference in overall hatching success. However, at 20°C, the average summer temperature, embryos degenerated by the fourth cleavage stage (Figure 1.6). Thus, the embryos of this species seemed much more tolerant of lower temperatures than higher temperatures within their natural thermal range, but because the egg masses used in the study were collected in winter, it is possible that they were better adapted to cold than those laid in the summer. Indeed, Dehnel & Kong (1979) do not specify whether this population even lays in the summer months or whether the egg masses persist into the spring and summer. Nevertheless, my own preliminary observations on south-eastern Australian gastropods are

consistent with the suggestion that embryos of both gelatinous and capsular egg masses are more tolerant of low than high temperature extremes.

Temperature tolerance of embryos and larvae may vary seasonally as suggested by studies on ascidians (Nomaguchi *et al*. 1997) and sea urchins (Fujisawa 1995). Seasonal acclimation to temperature extremes has yet to be empirically tested for intertidal encapsulated embryos, but such a possibility should be considered in future studies on embryonic responses to thermal stress.

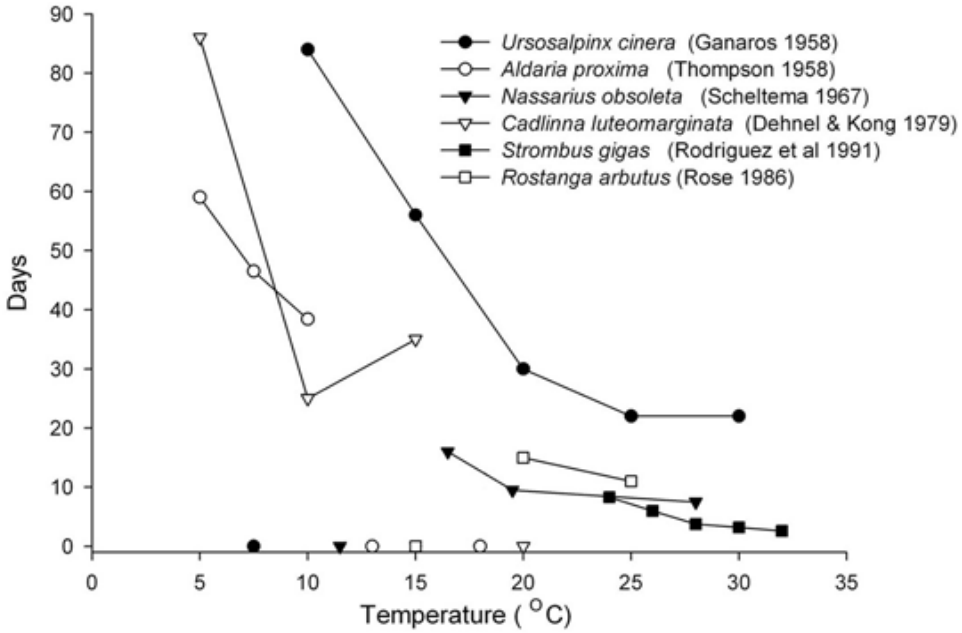


Figure 1.6 The effects of temperature on developmental rate in benthic gastropod egg masses according to available literature. Unconnected symbols resting on the x-axis represent egg masses that did not hatch at the specified temperature. At 10ºC, *Urosalpinx cinera* took longer than the experimental period of 84 days to hatch. The values for *Strombus gigas* were obtained using calculations performed by Rodriguez *et al* (1991) as they did not present raw data.

Although there is a lethal low and high temperature for gastropod embryos (Figure 1.6), some embryos are able to protect against high temperatures to a certain extent. Recent research has revealed the presence of heat shock proteins inside the gelatinous egg masses of the cephalaspid *Melanochlamys diomedea* (Podolsky & Hoffmann 1998; Podolsky 2000). These proteins allow embryos to withstand high temperatures, such as those reached during low tide on a summer day, by preventing the degradation of proteins during heat

stress and facilitating the refolding of proteins. These thermally protective proteins develop as the embryos mature (Podolsky & Hoffmann 1998). Thus, undeveloped embryos are especially vulnerable to high temperatures and they become less vulnerable to temperature extremes as they develop (Thorson 1950). It is presently unknown whether egg masses of other species contain thermally protective proteins.

Low temperatures can affect embryonic development by prolonging or halting it. Scheltema (1967) found that the embryonic development of the neogastropod *Ilyanassa obsoleta* slowed significantly as the temperature dropped (Figure 1.6). The embryos ceased development at the lower threshold of the species' temperature range. However, these embryos remained viable for up to 9 weeks of exposure and continued development when returned to their normal temperature. Similarly, Ganaros (1958) found that embryos of the muricid *Urosalpinx cinerea* remained viable after being subjected to sub-freezing temperatures and, if only exposed for a short period, they recovered and developed fully. However, the embryonic mortality rate increased as exposure time to cold water increased. No similar studies have been conducted on embryos of gelatinous egg masses.

Strathmann & Chaffee (1984) point out that lower temperatures decrease embryonic metabolic rates, thus resulting in slower development. In addition, they suggest that lower temperatures could increase intracapsular fluid viscosity and diffusion rates, thereby decreasing oxygen availability to the embryos (Figure 1.3). However, as metabolic rate slows, the demand for oxygen will also decrease; thus, the effects of less oxygen availability may be negligible in light of lower embryonic metabolism. Further research is needed to determine cause-and-effects of changes in oxygen availability, temperature, metabolic rates and developmental rates, because these are likely to be interdependent factors.

The available literature presents dissenting views about whether temperature is the primary developmental regulator in certain gastropod populations. It has been suggested that although temperature often influences the speed of development, oxygen availability is the primary factor controlling the encapsulated development of embryos (Cancino *et al.* 2003).

Furthermore, Clarke (1982) notes that many Antarctic marine invertebrates have very slow embryonic development, but suggests that this is not necessarily due to the extreme low temperatures. Rather, molluscs that have evolved in the Antarctic show temperature compensation and should not be subject to slow embryonic developmental rates due to low temperatures. Clarke (1982) suggests that, instead, it is the large egg size that results in the slow development of Antarctic invertebrate embryos. However, it is difficult to examine separately the effects of low temperature and large egg size in the field in polar regions because they often occur concurrently. In contrast, other studies suggest that temperature is the primary variable that controls the embryonic developmental rate (Spight 1975; Hoeghguldberg & Pearse 1995). It is unlikely that the relationship between temperature and egg size will be resolved until comparative experimental research is conducted, preferably on a species capable of producing eggs of different sizes (e.g. Jones *et al.* 1996) or a broad range of species with the same larval hatching type that produce eggs of different sizes (Clark & Goetzfried 1978).

Despite the obvious effect of temperature on gastropod developmental rate, some researchers fail to present adequate temperature data when reporting hatching times of egg masses (e.g. Govindan & Natarajan 1974; Pilkington 1974; Creese 1980). If the temperature is unknown, the developmental rate data are essentially useless. Therefore, it is imperative that all researchers reporting hatching times of gastropod egg masses monitor or control the temperature (e.g. Hurst 1967; Rose 1985; Chung *et al.* 2002).

Temperature is bound to several other abiotic and biotic factors that affect gastropod embryonic development (Figure 1.3). First, the seawater temperature of intertidal pools or other still water is higher in sunlight than in shade. Therefore, it can become difficult to separate the effects of UVR and temperature in the field. The effects of UVR and sunlight on temperature are easily controlled in an artificial seawater system where temperature can be kept independent of light. Furthermore, UVR and temperature significantly interact to cause coral zooxanthellae expulsion (Wissmann 2003), as well as to affect the growth of intertidal algae (Hoffman *et al.* 2003). No such studies examining the potentially synergistic effects of temperature and UVR have been conducted on gastropod egg masses.

The position of embryos within a mass may also influence their reaction to temperature stress. Embryos in the centre of a large mass are better protected from short-term environmental changes than embryos located peripherally (Strathmann & Hess 1999). In addition, temperature may directly affect predation (Sanford 1999) and microalgal fouling (Lee & Kim 2002). Furthermore, embryonic tolerance to temperature may be linked to salinity (Pechenik *et al.* 2003). Rose (1986) found that embryos of the nudibranch *Rostanga arbutus* show the widest temperature tolerance at an intermediate salinity of 34 ppt. Finally, higher temperatures have been associated with increased oxygen consumption within egg masses (Roller & Stickle 1989; Woods 1999), and recent research on biogeographic differences in size of brooding amphipods suggests that oxygen is more limiting at higher temperatures (Chapelle & Peck 2004). All these factors should be considered when examining the effects of temperature on gastropod embryonic development.

1.4.3 SALINITY

As with temperature, salinity extremes can affect embryonic mortality in egg masses. As salinity deviates from that within a species' normal habitat, the mortality of gastropod embryos increases (Struhsaker & Costlow Jr. 1969; Pechenik 1982; Woods & DeSilets 1997). However, as embryos develop they seem to become more tolerant to a wider range of salinities (Struhsaker & Costlow Jr. 1969; Pechenik 1983; Richmond & Woodin 1996). Thus, developing embryos vulnerable to salinity changes may require the protection of the egg capsule or associated gel matrix. Scheltema (1965) did not notice any significant differences between the levels of salinity that proved lethal to adults and hatched veligers of *Ilyanassa obsoleta*. It is possible that embryos of some species, particularly those in estuarine habitats, like *I. obsoleta*, use the protection of an egg capsule against salinity changes only during early development. Unfortunately, there is no comparable research among gelatinous egg masses and further research comparing the response of embryos, juveniles and adults is needed for both capsular and gelatinous egg masses.

Embryos within gelatinous egg masses do seem to be protected to some degree against salinity changes by both their vitelline capsules and the gelatinous matrix. In a detailed

study of nudibranch capsules, Eyster (1986) examined the vitelline capsule structure in relation to salinity. She found that the capsule walls inhibit the passage of large molecules, including salts (Figure 1.7a). She also noted that the capsules retain full structural integrity for the duration of encapsulation. However, embryos within the capsules were still vulnerable to high salinities owing to water efflux (Figure 1.7a). (Woods & DeSilets 1997) conducted similar experiments on the gelatinous matrix of *Melanochlamys diomedea* egg masses collected in areas subjected to periodic freshwater influx. They separated some embryonic capsules from the surrounding gel and exposed these to various test salinities. The gel improved survival only in very low salinity conditions by slowing the rate of salt efflux (Figure 1.7b). The embryos themselves were equally tolerant to high salinities, regardless of the presence of surrounding gel. Pechenik *et al.* (2003) similarly found that the embryos of the estuarine pulmonate *Amphibola crenata* were tolerant to extremely low salinities. The gelatinous egg collar itself showed no protective function to salinity changes, but tolerance was a property of the surrounding egg capsule or the embryos themselves. Further research examining both vitelline capsules and gelatinous matrices would likely reveal a combined protection of embryos against salinity changes that varied among species. These studies would be worthwhile on species that lay their egg masses in small intertidal pools, where salinity can reach high levels during low tide. Egg masses of such species may provide more effective protection against high salinities rather than low salinities. Indeed, a study comparing species that spawn in potentially high-salinity environments with estuarine species may reveal interesting adaptive differences to the extremes of low and high salinity.

The much larger leathery capsules of some neogastropods are permeable to both NaCl and water, although this permeability may vary among species within a genus (Pechenik 1982) (Figure 1.7c). Despite this apparent lack of protection against salinity changes, encapsulated embryos were found to have a significantly higher tolerance to salinity changes than embryos removed prematurely from the egg capsule (Pechenik 1983). Thus, the higher tolerance of encapsulated embryos to salinity stress is likely a result of capsule wall function. The capsules probably reduce the rate of salinity change, despite not protecting against the magnitude of change (Pechenik 1982, 1983); this function is similar

to that observed in gelatinous egg masses (Woods & DeSilets 1997). So far, these studies have been restricted to the genus *Nucella* (Table 1.2). Research on different species would provide a more general understanding of the role of neogastropod egg capsules in providing protection from salinity fluctuations. Furthermore, the potential function of intracapsular fluid and other capsular contents in protection against changes in salinity remains unexplored.

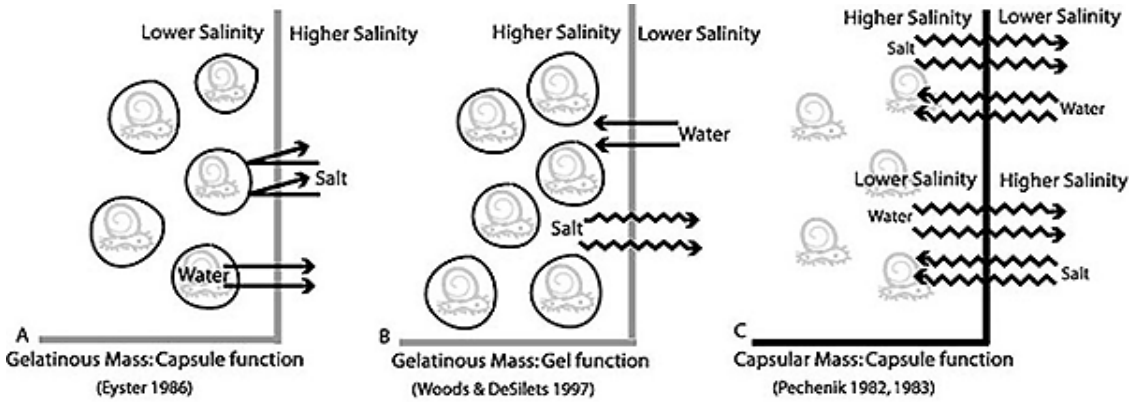


Figure 1.7 The embryonic protection afforded by capsular and gelatinous egg mass structures to changes in salinity. A zig-zag line means the rate of movement is decreased, but the overall magnitude of change is slight. Mechanisms illustrated are based on research from listed references. A) Function of vitelline capsules within gelatinous egg masses. B) Function of gelatinous matrix. C) Function of capsule walls in neogastropod egg masses.

In addition to embryonic mortality, salinity may also affect developmental rate. Rose (1986) observed that *Rostanga arbutus* embryos reared in 40 p.p.t. seawater developed more slowly than embryos reared in 34 p.p.t. seawater at the same temperature. Low salinities have also been shown to prolong embryonic development in other opisthobranchs, the sacoglossan *Elysia viridis* (Hagerman 1970) and three species of *Doto* (Kress 1975). Reasons for this are unknown, but may be due to the association between salinity and embryonic oxygen consumption rates, although this has only been documented on capsular egg masses (Roller & Stickle 1989).

Salinity is directly affected by temperature, with higher temperatures often leading to increased salinity through evapouration in small intertidal pools (pers. obs.). Salinity itself affects some biotic factors that can influence gastropod embryonic development (Figure

1.3). Salinity can influence the abundance and diversity of predators. As salinity deviates from normal conditions in a particular environment, the number of predators can decrease (McClanahan 1992). Similarly, salinity can affect the abundance and diversity of potential fouling microorganisms (Kocak & Kucuksezgin 2000; Lee & Kim 2002). Moreover, temperature may influence the effect of salinity changes on embryos. For example, Pechenik *et al.* (2003) found a significant interaction between salinity and temperature on the developmental rate and hatching success of *Amphibola crenata.*

1.4.4 ULTRAVIOLET RADIATION

Ultraviolet radiation, especially UV-B, is deleterious to many organisms (Karanas *et al.* 1981; Wood 1987; Bothwell *et al.* 1994). Among gastropod egg masses, exposure to UVR can stunt development, produce deformities and cause death (Biermann *et al.* 1992; Rawlings 1996; Carefoot *et al.* 1998). The severity of the effects of UVR may depend upon the age of the embryos. Biermann *et al.* (1992) exposed fresh and mature egg masses of the nudibranch *Archidoris montereyensis* to sunlight and found the rates of embryonic mortality and deformity were significantly less in the mature egg ribbons. However, the previous history of the mature egg masses was unknown. It is therefore possible other variables contributed to the mortality difference among fresh and mature egg masses. Although the developmental risk is probably greatest during cleavage and early embryo development, UVR still poses a serious risk to embryos for the duration of their encapsulation.

The most logical protection against UVR exposure is for the adults to lay egg masses under boulders or in other areas shielded from sunlight. Spawning under boulders may also protect egg masses against desiccation, predation and high temperatures. Benkendorff & Davis (2004) have found that over half the molluscs depositing egg masses on intertidal reefs around Wollongong (NSW, Australia) exclusively attached them to the undersides of boulders. Other taxa occasionally lay in areas exposed to UVR, such as *Aplysia* species Przeslawski *et al.* (2004). Indeed, certain molluscs, such as some *Siphonaria* species, lay exclusively in habitats exposed to full sunlight (Creese 1980; Benkendorff & Davis 2004). The vulnerability to and protection against UVR of certain amphibians is species specific

(Blaustein *et al.* 1994), and even population specific (Belden & Blaustein 2002). Recent work among gastropod egg masses has also revealed species-specific UVR vulnerability. (Przeslawski *et al.* 2004) have found that egg masses of species that lay exclusively in shaded habitats are very vulnerable to the harmful effects of UVR, whereas those that lay consistently in UVR-exposed habitats are not.

It has been suggested that species that regularly lay in UVR-exposed environments possess structural or chemical protection against UVR (Biermann *et al.* 1992). One possibility is that the egg masses are biochemically protected through UV-absorbing compounds. Ultraviolet-absorbing compounds, such as mycosporine-like amino acids (MAAs), have been identified recently in many marine organisms (reviewed by Shick & Dunlap 2002), including pelagic invertebrate eggs (Epel *et al.* 1999). It is generally accepted that marine animals obtain these MAAs from their diet or symbiosis with algae because animals lack the biochemical pathway to synthesise MAAs (Stochaj *et al.* 1994; Mason *et al.* 1998; Shick *et al.* 1999). With only a few exceptions discussed below, it is unknown whether gastropod egg masses contain UV-absorbing compounds.

To date, no MAAs or other UV-absorbing compounds have been found in capsular egg masses, although only those of the neogastoprods *Trophon* cf. *geversianus* and *Nucella emarginata* have been examined (Karentz *et al.* 1991; Rawlings 1996). Karentz *et al.* (1991) surveyed a broad range of marine Antarctic organisms, including a capsular egg mass from the neogastropod *Trophon* cf. *geversianus* and a gelatinous egg ribbon from the vetigastropod *Margarella antarctica*, and found that 90% of all organisms contained MAAs. Whereas most organisms contained a substantial concentration of a variety of MAAs, the two gastropod egg masses examined showed little or no sign of MAAs, despite their presence in the adults. Unfortunately, the authors do not specify whether the egg masses were collected from shaded or sunny habitats. Rawlings (1996) attempted to extract MAAs from capsules of the neogastropod Nucella emarginata, but without success. However, only the capsule walls were tested for MAAs, not the intracapsular fluid or embryos. Rawlings (1996) did, however, conclusively show that the outer capsule wall

absorbed UVR, particularly UV-B. Thus, embryos within the leathery capsule may have no need for the additional protection of MAAs.

In contrast with the experiments mentioned above, Carefoot *et al.* (1998) found that the gelatinous egg masses of *Aplysia dactylomela* were rich in MAAs, but adult diet determined their presence. *Aplysia* species lay their egg masses both under boulders and in areas exposed to sunlight, so the presence of MAAs may be an evolved protective mechanism rather than a coincidental dietary benefit. It would be worthwhile to conduct similar experiments on gastropods with different laying habitats, particularly those that lay exclusively in environments exposed to UVR, to determine whether the presence of MAA in egg masses is an evolved protection (Cockell & Knowland 1999).

The effects of UVR on gastropod egg masses are potentially confounded with several other factors (Figure 1.3), such as the placement of embryos within the egg mass. In gelatinous egg masses, where the embryos have a fixed position, the surrounding gel and embryos near the top can act as a shield for the inner embryos (Biermann *et al.* 1992). In addition, the water depth at which the egg masses occur could influence UVR penetration. Significant amounts of UV-B can be transmitted through 5–10 m water, whereas biologically harmful UVR can penetrate more than 20 m below the surface (Karentz & Lutze 1990; Booth & Morrow 1997). The penetration of UVR in seawater also depends on water clarity and, thus, UVR and the transmission of visible light can vary among regions at similar depths depending on the amount of sediment, phytoplankton and dissolved solutes (Shooter *et al.* 1998). Exposure to UVR can also be affected by surface fouling on the egg mass. Algal fouling is greater in sunlight and it has been suggested that such a covering over the egg mass could significantly shield the embryos within from UVR (Biermann *et al.* 1992). Like salinity and temperature, UVR and associated visible light directly affect microalgal fouling (Biermann *et al.* 1992) and predation (Williamson *et al.* 1999) (Figure 1.8). As mentioned previously, UVR can also interact significantly with temperature (Hoffman *et al.* 2003; Wissmann 2003) and the effects of this potential relationship need to be investigated on gastropod egg masses.

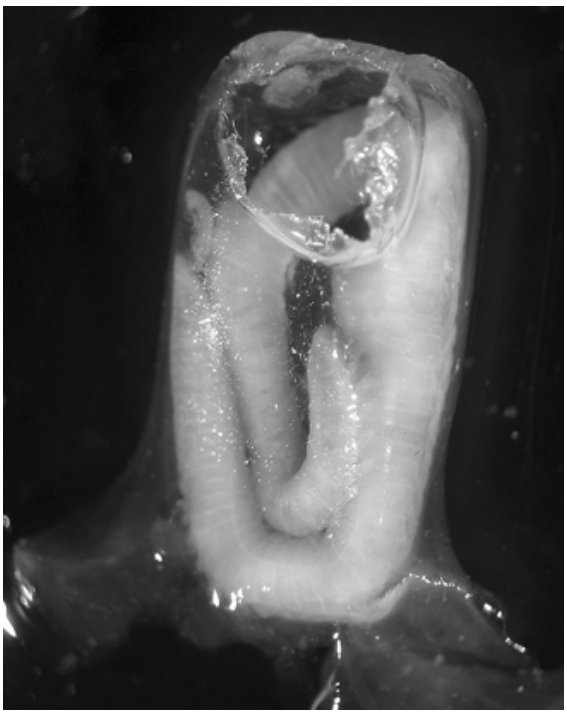


Figure 1.8 Empty egg capsule of *Ranella Australasia* with potentially predatory polychaete found under boulder at Bass Point, NSW. It is unknown if the hole observed in the capsules was made by the worm or the departing veligers.

1.4.5 OXYGEN AVAILABILITY

Oxygen availability can dramatically influence the development of embryos within egg masses. In a series of experiments, Strathmann & Strathmann (1995) demonstrated that oxygen limited embryonic development within gelatinous opisthobranch egg masses. They found that embryos of three species showed arrested development during hypoxia until they were returned to a normal oxygen level. Furthermore, lower oxygen availability throughout the development period reduced shell length at hatching. These observations have recently been supported in the capsular egg masses of *Chorus giganteus* (Cancino *et al.* 2003).

The direct effects of oxygen availability on gastropod embryo development are usually not studied independently but, rather, are used to explain the effects of other environmental factors. Oxygen availability within egg masses is inextricably linked to water flow, embryonic position within the egg mass and fouling (Figure 1.3).

Among gelatinous egg masses, flowing water accelerates the rate of embryonic development by decreasing hatching time and increasing embryo activity (Eyster 1986) because it most likely increases the overall oxygen supply to the mass through diffusion (Strathmann & Hess 1999). Chaffee & Strathmann (1984) found that flowing water decreases asynchronous development within a spherical gelatinous mass, whereas still water promotes relatively high developmental variation. The same study found no effect of still water on development among elongated gelatinous ribbons. Biermann *et al.* (1992) also found a significant interaction between water flow and egg mass thickness. There was no difference in embryonic developmental rates between egg ribbons maintained in still water and those exposed to a strong current; but, when the thin ribbons of the egg masses were layered, forming a thicker structure, there was a noticeable developmental retardation in still water. Therefore, it is likely that the shape of the egg mass determines, in part, the developmental effects of oxygen availability as controlled by water flow (Kranenbarg *et al.* 2001). There has been no similar research into the combined effects of oxygen availability, water flow and egg mass structure on the development of embryos within capsular egg masses.

The effects of desiccation on embryonic oxygen availability have not yet been studied in detail. Many intertidal gastropod egg masses can tolerate brief periods of desiccation (Strathmann 1987), and some species are desiccated daily in the intertidal zone during low tides (D'Asaro 1970; Benkendorff 1999). However, if the egg masses are allowed to dry out for more than several hours, the embryos within will die (Spight 1977; Creese 1980). The intertidal capsules of *Ilyanassa obsoleta* were no more effective protecting against desiccation than the capsules of a subtidal nassariid (Pechenik 1978). It is not known why certain egg masses are laid consistently in areas regularly subject to desiccation. Pechenik *et al.* (2003) found that pulmonate embryos in a sandy gelatinous egg mass hatched faster if they were exposed to air for a few hours a day and suggested that this reflected the greater oxygen availability in the air-exposed egg masses, although this has yet to be tested. If air exposure does, indeed, decrease the encapsulation period, then this may outweigh the potential risks associated with desiccation.

The placement of the embryos within the egg mass also influences embryonic oxygen availability. Many neogastropods deposit their egg capsules in a clump and the capsules near the periphery will generally develop faster than the central capsules (pers. obs.). This trend is even more accentuated in gelatinous masses (Figure 1.2a). Because embryos in a capsular mass are free to move within a relatively large chamber (D'Asaro 1986) (Figure 1.1), their position within the capsule changes constantly and oxygen supply is relatively uniform for most embryos within a capsule (Strathmann & Chaffee 1984). However, eggs and embryos of gelatinous masses are fixed in one location within the whole mass (Hurst 1967; Switzer-Dunlap & Hadfield 1977) (Figure 1.2). Embryos located within the central region of a gelatinous egg mass frequently show arrested or retarded development compared with embryos located peripherally (Chaffee & Strathmann 1984; Biermann et al. 1992; Lee & Strathmann 1998) (Figure 1.2a), which is associated with lower oxygen availability (Strathmann & Strathmann 1995). The egg masses of several species are normally hypoxic in central locations (Cohen & Strathmann 1996; Woods 1999). As a result, central embryos can be more developmentally dependent on environmental factors like water flow and algal fouling, whereas those at the periphery are more vulnerable to other environmental conditions, such as desiccation and extremes in temperature and salinity.

Although many selective pressures exist to reduce the period of encapsulation (Havenhand 1993), the benefits of the delayed development of centrally located embryos may outweigh the risks of slower development. A large egg mass with varying developmental rates can contribute to populations by releasing viable embryos from the mass over a period of days and even weeks (Gibson & Fu-Shiang 1994). With this bet-hedging strategy, veligers within one mass are exposed to a variety of conditions at hatching. Chances for optimal conditions for at least some veligers are maximised. An extreme example of centrally retarded development is the naticid mud snail *Conuber sordidus*, which lays its eggs in a large sausage-shaped jelly (Smith *et al.* 1989) (Figure 1.2d). Although the majority of the eggs are embedded near the outer surface, a large number of eggs exist throughout the entire mass. Because the egg mass is so large, the centrally located eggs are exposed to severe hypoxia and their development is arrested (Booth 1995). However, as the numerous

peripheral embryos hatch, the gel surrounding them degrades and the oxygen available to the central region increases, enabling continued development of the internal embryos. Thus, a single egg mass can release viable veligers over a period of weeks depending on temperature (Booth 1995; pers. obs.).

In addition to embryonic position, algal and microfaunal fouling can also have an effect on embryonic development by modifying oxygen availability. Algae alter the internal oxygen concentration of egg masses by producing oxygen in daylight and consuming oxygen at night (Strathmann 2000). By regulating the oxygen availability within egg masses, algal photosynthesis and metabolism may vary oxygen conditions in the egg mass, thereby affecting embryonic developmental rates (Cohen & Strathmann 1996). A study of amphibian eggs found that egg masses contained a green alga specific to amphibian eggs that increased oxygen availability to the embryos (Pinder & Friet 1994). Similarly, a symbiotic association has been found between diatom assemblages and polychaete egg masses. Algal fouling provided not only a food source to the encapsulated embryos, but it was linked to detachment and flotation of gelatinous egg masses which increased dispersal capability of larvae (Peyton *et al.* 2004). No similar studies on potential symbiotic associations between algae and gastropod egg masses have been conducted.

Despite potential benefits to gastropod embryonic development, algae also promote protist and bacterial growth (Fogg 1983). Unlike algae, these organisms do not produce oxygen; in fact, they deplete available oxygen through respiration (Cohen & Strathmann 1996). Bacteria and fungi have been shown to be deleterious to gastropod larval and embryonic development (Struhsaker & Costlow Jr. 1969; Biermann *et al.* 1992), although later developmental stages may be less susceptible (Struhsaker & Costlow Jr. 1969). It is not known whether the deleterious effects observed were due to hypoxia or to a byproduct of the fouling organisms. A more conclusive study was undertaken by Cancino *et al.* (2000), in which the oxygen availability to embryos of the muricid *Chorus giganteus* was reduced by sessile protozoa that fouled some egg capsules. The protozoa decreased oxygen tension and the embryos within fouled capsules not only had a much longer hatching time, but they also showed marked impairment of shell growth.

Certain egg masses are not vulnerable to heavy algal fouling owing to the laying behaviour of the adult. Because sunlight is necessary for algae to flourish, the risk of fouling is low when an egg mass is laid under boulders. Moreover, some egg masses hatch in a few days and are not heavily fouled owing to this short development period. It is not known whether gastropod egg masses contain any biochemical protection against algal fouling.

Similarly, gastropod egg masses may also provide protection against bacterial fouling and infection. Benkendorff et al. (2001) have reported antimicrobial properties in both capsular and gelatinous egg masses of 39 species of molluscs. In contrast, Pechenik *et al.* (1984) did not find any antibiotic properties in the intracapsular fluid of *Nucella lapillus* capsules, although this result should be interpreted cautiously because there may be limitations in the methods used to test for antimicrobial activity (Benkendorff *et al.* 2000). In addition, Pechenik *et al.* (1984) do not indicate the age of the capsules examined. Because chemical ripening has recently been found to occur in related species (Benkendorff & Bremner 2000), active compounds may have decomposed in mature or stressed egg capsules.

Oxygen availability and consumption may also be interdependent with several abiotic factors (Figure 1.3). Although oxygen availability is affected by temperature (Green & Carrit 1967), Cancino et al. (2003) found no significant interaction between temperature and oxygen availability on the encapsulated development of *Chorus giganteus.* Further research on capsular and gelatinous egg masses examining the potential relationship between oxygen availability and temperature would help clarify any interactions. Another study revealed a significant interaction between temperature and salinity on embryonic oxygen consumption (Roller & Stickle 1989). Temperature and salinity most likely affect oxygen consumption by affecting the health and metabolism of the embryos, as well as influencing the rates of oxygen diffusion.

1.4.6 DISCUSSION

Gastropod egg masses are often exposed to a wide variety of environmental conditions in the intertidal regions in which they are commonly laid. These environmental factors affect embryonic development and mortality, but they are not themselves independent of each other. The present paper has reviewed abiotic factors affecting gastropod embryonic development and mortality: temperature, salinity, oxygen availability and UVR. In addition, relationships among these factors and their interactions with biotic factors were explored (Figure 1.1).

Despite recent advances in our understanding of gastropod egg mass structure and development, there are still large gaps in our knowledge. First and foremost, fundamental structural and developmental data are still needed for the egg masses of many gastropods. Although North American and European species have been studied relatively frequently (e.g. Strathmann 1987) and several comprehensive studies have examined the egg mass structure of some Australasian taxa (Pilkington 1974; Rose 1985; Smith et al. 1989), details about the egg masses and embryos of many species remain unknown. In addition, possible egg mass or capsule changes over time have not been examined for most species. Without basic structural and developmental knowledge, there is no foundation for comparisons of the effects of environmental effects on egg mass development.

The majority of studies pertaining to environmental effects on gastropod egg mass development examine only one variable at a time (Table 1.1). There is, of course, nothing wrong with this approach and it can provide valuable information as long as other potential factors influencing development are controlled or are negligible. However, a multifactorial approach can reveal the relationships among factors that control embryonic development and mortality. This can be accomplished using various treatment combinations of two or more factors (e.g. Pechenik *et al.* 2003). Whenever possible, researchers should couple laboratory based experiments with field measurements of the factors examined and *in situ* developmental data of encapsulated gastropod embryos. This will ensure the relevance of laboratory studies and allow researchers to better interpret findings in the field.

Understanding the complex relationships among some of these factors will further our understanding of gastropod development in their natural environment. The present review has only discussed the potential effects of biotic variables on gastropod embryonic development in relation to their interactions with abiotic variables. There are still relatively few studies on the direct effects of biotic variables on gastropod egg mass development (Table 1.1) and future research should aim to clarify the interactions between abiotic and biotic factors that affect gastropod embryonic development (Figure 1.3).

Comparisons of the developmental effects of various environmental factors on both capsular and gelatinous masses are needed to better understand the differences among taxa regarding vulnerability and possible protection of egg masses against harmful environmental factors (e.g. Przeslawski *et al.* 2004). Such studies could have interesting implications for evolutionary divergences of certain groups of gastropods through comparative examinations of structural adaptations of egg masses. Within the context of the present review, capsular egg masses were represented exclusively by caenogastropods, whereas gelatinous egg masses, with one exception (Booth 1995), were heterobranchs. Although egg mass structure and phylogeny are closely related, there are enough exceptions (see Table 1.2) so that future research should attempt to distinguish the effects of phylogeny from egg mass structure. Further comparative research on the effects of environmental stresses on gelatinous caenogastropod, capsular caenogastropod, and heterobranch egg masses may help separate the effects of environmental stresses based on phylogeny or structure.

Finally, further research on a range of species is necessary in order to determine possible relationships among various gastropod groups and populations. Struhsaker & Costlow Jr. (1969) state that the tolerance of embryos to changes in conditions like salinity may be contingent on the stability of the environment in which the adults are normally found. Thus, larvae in a subtidal tropical region with stable conditions would be expected to be less tolerant of environmental changes compared with larvae in an intertidal temperate region experiencing abrupt salinity and temperature changes. This hypothesis remains largely untested because researchers have yet to study empirically the effects of environmental

stability on egg mass tolerance to environmental stresses. Such studies could provide valuable developmental information on a broad geographical range of species while identifying the vulnerabilities and tolerances of gastropod embryos.

Tolerance to environmental stress by encapsulated gastropod embryos may also depend on the zone of the shore in which they occur. Rawlings (1999) has suggested that intertidal egg masses may be no more effective in protecting against environmental stresses than subtidal egg masses, but this has yet to be tested. Studies comparing the developmental effects of environmental stresses between intertidal and subtidal egg masses may reveal habitat-specific adaptations important to intertidal ecological research. Regardless of the results, such studies would lead to examination of an evolutionary basis for intertidal spawning. For example, Spight (1977) found that *Thais lamellosa* showed no preference for intertidal spawning habitats, such as tidepools with reduced physical stresses, and that they often deposited their egg capsules in habitats where embryonic mortality was relatively high. Spight (1977) suggested that this may be due to higher site quality for other life stages, which outweighed the embryonic costs. Although several studies have explored the adaptations of egg masses to intertidal environmental stresses (reviewed by Pechenik 1978; Rawlings 1999), there is a lack of knowledge pertaining to the evolutionary advantages associated with egg mass deposition in this habitat. Species may deposit egg masses in the intertidal zone to minimise encapsulation period, reduce predation or fouling, or vary larval dispersal; however, this remains speculative. Survival advantages must exist to counteract the risks associated with spawning in such a physiologically hostile environment, but these are not necessarily the same for all taxa. With proper multifactorial experiments on a range of gastropod species, we can further understand the complex effects of intertidal environmental stresses on encapsulated gastropod development.

CHAPTER 2: Isolated Effects of Ultraviolet Radiation[1]

"If we knew what it was we were doing, it would not be called research, would it?"
Albert Einstein

[1] Entire chapter encompasses published manuscript:
Przeslawski R., Davis A.R. & Benkendorff K. (2004) Effects of ultraviolet radiation and visible light on the development of encapsulated molluscan embryos. *Marine Ecology Progress Series*, 268, 151-160

2.1 EFFECTS OF ULTRAVIOLET RADIATION AND VISIBLE LIGHT ON THE DEVELOPMENT OF ENCAPSULATED MOLLUSCAN EMBRYOS

Benthic egg masses laid in intertidal habitats are exposed to numerous environmental stresses including potentially damaging ultraviolet radiation (UVR). I sought to determine the developmental effects of UVR and visible light on molluscan embryos within egg masses from habitats with differential UVR exposure. Egg masses from 23 marine gastropod species were collected from three intertidal habitats, 1) full sun, 2) partial shade, and 3) full shade. Egg masses were then divided among four spectral treatments: full spectrum, no UV-B, no UV, and dark. Egg masses from full shade habitats showed significant vulnerability to UVR and visible light and had a higher overall mortality than other egg masses. Egg masses that were originally partially shaded did not show any significant mortality differences among spectral treatments, but highest mortalities occurred in full spectrum treatments while lowest mortalities occurred in dark treatments. Egg masses from full sun habitats showed no significant mortality differences between spectral treatments which is consistent with the possession of some sort of protection against the harmful effects of UVR. Overall, egg masses from full sun habitats showed a low mortality across spectral treatments. An ANOVA confirmed that a significant interaction between original habitat and spectral treatment affected mortality. In addition, egg masses hatched slower in the dark than the other three light treatments irrespective of habitat.

2.1.1 INTRODUCTION

Some intertidal organisms protect their offspring from the harsh conditions of their environment by enclosing them within benthic egg masses. Molluscs, in particular, often employ complex structures to contain their developing offspring. Gastropod egg masses can be divided into two general categories: capsular and gelatinous. Capsular egg masses have discrete capsules with rigid external walls that encase embryos within an intracapsular fluid. These egg masses can be further subdivided into those of neogastropods, which encase their eggs in a leathery walled capsule, and those of neritids, which encase their eggs in a rigid, partly calcareous capsule. In contrast, gelatinous egg masses are composed of an insoluble jelly matrix in which numerous eggs surrounded by vitelline capsules are embedded. Egg masses of some species may protect the embryos from predation (Grant 1983), bacterial infection (Benkendorff *et al.*

2001), desiccation (Strathmann & Hess 1999), temperature variations (Podolsky & Hoffmann 1998) and changes in salinity (Pechenik 1982) (reviewed by Pechenik 1979; Przeslawski 2004a). Molluscan embryos are also potentially vulnerable to ultraviolet radiation from sunlight, but research on the effects of UV radiation on molluscan egg masses is currently limited to a small number of species (e.g. Biermann *et al.* 1992; Rawlings 1996; Carefoot *et al.* 1998).

Ultraviolet radiation (UVR) at the earth's surface comprises UV-A (320-400 nm) and UV-B (280-320 nm). UV-B is particularly deleterious and can negatively affect reproduction, development, and behaviour in many organisms including marine invertebrates (Karanas *et al.* 1981; Rodriguez *et al.* 2000; Kuffner 2001). Previous studies indicate that in some molluscan egg masses, UVR exposure can cause stunted development, deformities, and death (Biermann *et al.* 1992; Rawlings 1996; Carefoot *et al.* 1998). A surge of recent interest in declining amphibian populations has revealed a striking species-specific difference in the vulnerability of eggs and embryos to the harmful effects of UVR (e.g. Blaustein *et al.* 1994). In fact, population-specific vulnerability has recently been documented in a species of salamander. Salamanders living in mountain regions where the UV-B levels are higher had a higher tolerance of UV-B than the same species living in valleys where the UV-B levels are lower (Belden & Blaustein 2002). my aim was to examine the response of organisms that lay in differentially UVR-exposed habitats in the marine environment.

Biologically harmful UVR can penetrate more than 20 metres below the ocean's surface (Karentz & Lutze 1990; Booth & Morrow 1997). Thus, even submerged egg masses in the intertidal and subtidal zones can be subjected to potentially harmful levels of UVR. Marine molluscs and other invertebrates may avoid exposure to UVR by depositing egg masses in shaded habitats. Many molluscs, particularly nudibranchs and neogastropods, always lay their egg masses in habitats shaded from all light including ultraviolet radiation (Biermann *et al.* 1992; Benkendorff & Davis 2004) (Table 1). Other species, particularly many *Aplysia* spp. lay in both shaded habitats and areas exposed to sunlight (pers. obs.). In turn, certain species consistently lay in areas exposed to full sunlight so their egg masses are barraged daily by UVR (Creese 1980; Benkendorff & Davis 2004). Given the thinning of the ozone layer and the potential negative effects of increased

Table 2.1 Egg masses used in this study with number of replicate egg masses (n) used in the analyses for mortality and encapsulation period. A dash indicates hatching analysis does not include that species because hatching in all spectral treatments did not occur for any replicate egg mass. An asterisk indicates egg masses that were collected from adults held in aquaria. Refer to text for classification definitions for habitat and type.

Superorder	Order/ Infraorder	Species	n (mortality)	n (hatching)	Habitat	Type
Neritopsina	Neritodoidea	*Nerita atramentosa*	3	n/a	Full Sun	Capsular
Caenogastropoda	Naticidea	*Conuber* species[1]	7	7	Full Sun	Gelatinous
		Naticid sand collar	5	5	Part Shade	Gelatinous
	Littorinimorpha	*Bembicium nanum*	7	7	Full Sun	Gelatinous
	Neogastropoda	*Agnewia tritoniformis*	5	-	Shade	Capsular
		Lepsiella reticulara	2	-	Shade	Capsular
		Mitra carbonaria	6	-	Shade	Capsular
Heterobranchia	Basommatophora	*Siphonaria denticulata*	7	7	Full Sun	Gelatinous
		Siphonaria zelandica	6	6	Full Sun	Gelatinous
	Cephalaspidea	*Bullina lineata*	5	5	Part Shade	Gelatinous
		Hydatina physis	7	5	Part Shade	Gelatinous
	Anaspidea	*Aplysia juliana*	3	2	Part Shade	Gelatinous
		Aplysia sydneyensis	3	2	Part Shade	Gelatinous
		*Bursatella leachii**	5	5	Part Shade	Gelatinous
		Dolabrifera brazieri	6	6	Shade	Gelatinous
		*Stylocheilus longicauda**	6	5	Part Shade	Gelatinous
	Notaspidea	*Pleurobranchus* sp1	1	-	Shade	Gelatinous
		Pleurobranchus sp 2	1	1	Shade	Gelatinous
	Sacoglossa	*Oxynoe viridis*	2	1	Part Shade	Gelatinous
	Nudibranchia	*Austraeolis ornata*	3	2	Shade	Gelatinous
		Dendrodoris fumata	3	-	Shade	Gelatinous
		*Hypselodoris obscura**	3	-	Shade	Gelatinous
		Platyodoris galbanni	3	-	Shade	Gelatinous

[1] These egg masses are from *Polinices sordidus, P. conicum,* or *P. melastomum* which are indistinguishable from each other.

UVR, I sought to assess the vulnerability of those species laying in habitats with elevated UVR. Here, cut-off filters are used to modify the quality of the incident radiation and to examine effects on the mortality of embryos and their period of encapsulation for a range of molluscan species drawn from habitats exposed to full sun, partial sun, and full shade.

2.1.2 METHODS

Egg Mass Collection

Egg masses from 23 species of marine gastropods were collected from intertidal habitats along the Illawarra coast, NSW, Australia during the low spring tides of September 2001- April 2002 (Table 1). All collection sites were rocky intertidal reefs with the exception of one estuarine mudflat (Figure 2.1). Most egg masses were identified according to previous research (Hurst 1967; Rose 1985; Smith *et al.* 1989); but with certain species, identification of a laying adult was the only way to identify the egg mass to species level. A few egg masses were obtained from adults that laid in laboratory aquaria within a week of captivity (Table 1).

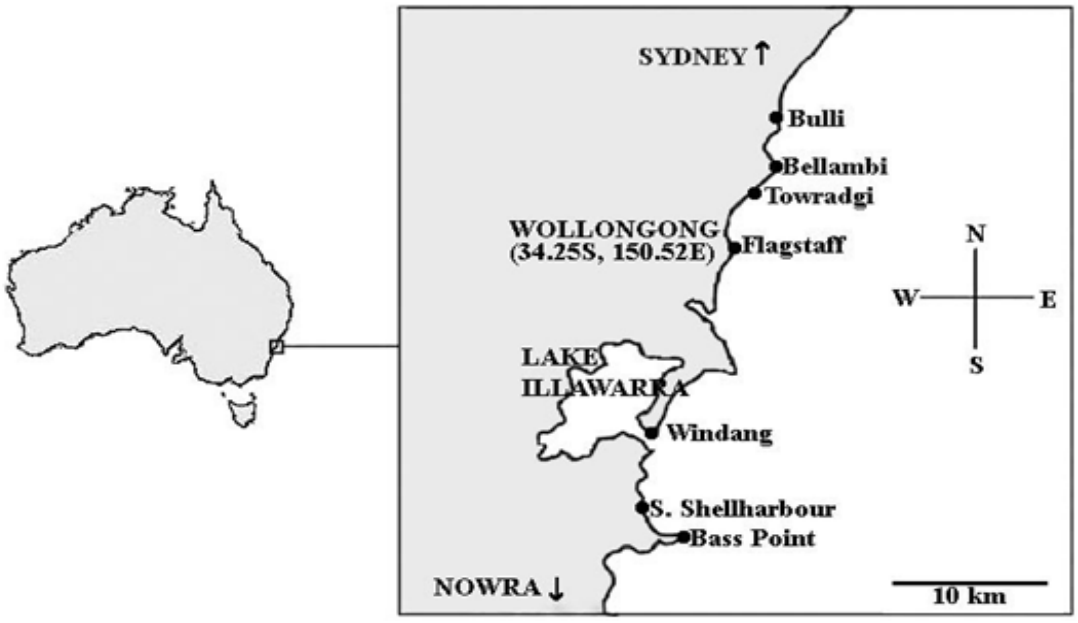


Figure 2.1 Map of sites used in this study along the Illawarra coast, NSW, Australia. All sites are rocky intertidal platforms with the exception of Windang, an estuarine mudflat.

Each species was classified according to the habitat in which they deposit egg masses (Table 1). Full sun species spawned only in areas consistently exposed to direct sunlight such as flat rock platforms. Shaded species laid eggs masses exclusively under boulders or in other areas completely blocked from sunlight. Partially shaded species deposited their spawn in habitats that varied in sunlight exposure such as algal beds or vertical rock faces. In addition, species such as *Aplysia juliana* that spawn in both full sun and shaded habitats were also classified as partially shaded species.

Experimental Design

I used a nested experimental design in which species were nested within habitats, and replicate egg masses were nested within species. Rather than discard or limit data, the design was unbalanced at the species and egg mass level because abundances of egg masses varied among species over time so a uniform number of egg masses collected for each species was unfeasible (Table 1). For example, it was relatively easy to find a large number of *Siphonaria* egg masses, but the egg masses of many nudibranch species were found rarely, sometimes only once. Furthermore, the number of species within each habitat varied because relatively few species typically deposit egg masses in full sun compared to shaded or partially shaded habitats (Benkendorff & Davis 2004) (Table 1).

Each egg mass was divided equally among the four spectral treatments in order to reduce potential variability attributed to different parental characteristics and developmental stages. Capsular masses were divided into four groups each containing at least two discrete capsules. Many capsules abruptly changed colour soon after collection. These capsules were not used because such colour change is indicative of stress and possible impending death (Pechenik 1982, pers. obs.). Gelatinous masses were cut into four pieces, and weights were recorded after gentle blotting. Preliminary studies revealed embryos at the edges of cut areas still developed normally. Each capsular group or gelatinous piece was then placed within one of four shallow plastic containers representing each spectral treatment by using spectral cutoff filters as lids (Figure 2.2): 1) full spectrum control with no filter, 2) UV-B cut-off filter with .175 mm thick mylar polyester film, 3) UV-A and UV-B cut-off filter with security film, and 4) dark control with opaque plastic. A diode array spectrophotometer was used to confirm the spectral transmission properties of the cut-off filters (Figure 2.2). Containers were

drilled with small holes to allow constant internal water circulation and submerged in an outdoor 300-litre recirculating seawater system exposed to natural sunlight. Temperature and water flow were recorded in all containers at random times for the duration of the experiment, and no discernible difference between treatments was detected.

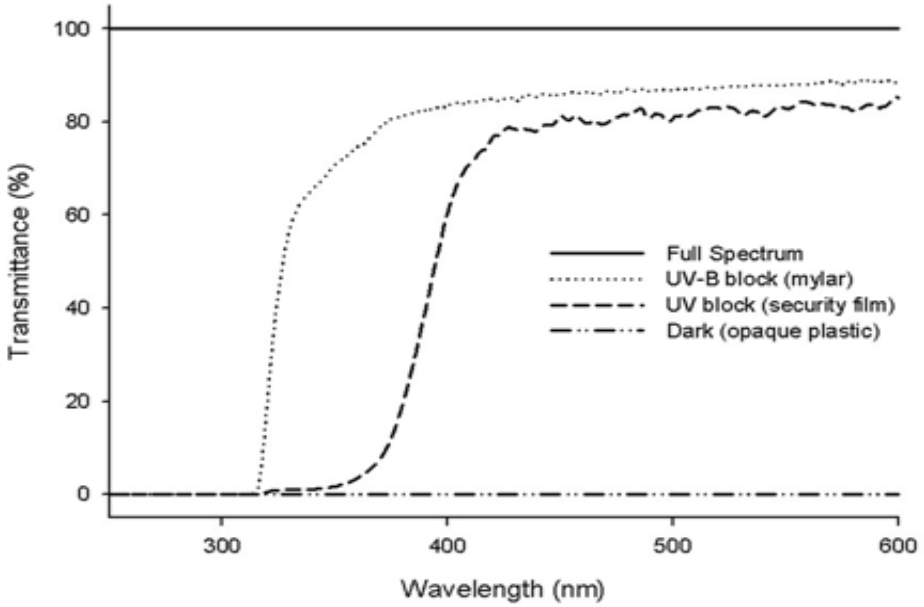


Figure 2.1 Spectrophotometric reading of cut-off filters used in this study. UV-B blocking filter (polyester Mylar) blocked below 320nm while UV blocking filter (acrylic window security film) blocked most wavelengths below 400nm. Full spectrum treatments had 100% transmission while dark treatments under opaque plastic had 0%.

Examination of Embryonic Development

After collection, each egg mass was examined under a microscope to verify fertilization and developmental stage. Only masses with developing eggs or immobile, unshelled embryos were used; unfertilised or mature egg masses were discarded. For each gelatinous mass, average number of eggs per mm^3 and wet weight of each mm^3 of egg mass was calculated. For each capsular mass, the number of eggs within each capsule was counted. In the case of *Mitra carbonaria,* the number of eggs was relatively high so ten subsamples were taken.

The date of collection and initial examination was denoted Day 1. The egg masses were then placed in the experimental tank in their respective spectral treatments and inspected every 1-2 days. After each inspection, they were returned to the experimental tank so that their positions and orientations were randomly changed every 1-2 days (e.g. Biermann *et al.* 1992). They were removed for examination when hatching was

imminent. For gelatinous egg masses, this was determined when the gel began to soften and break apart. For capsular masses, this was determined by colour change associated with the development of shelled veligers or inviability as defined below. In addition, if capsular masses showed any obvious change in turgidity, they were removed for examination.

Upon removal, egg mass pieces and capsules were examined under a dissecting microscope (25x magnification). The egg mass pieces or capsules were returned to the treatment container if embryos were still encapsulated and viable and, in the case of gelatinous masses, if the gel was still fairly firm and intact. If hatching had occurred as described below, mortality was quantified.

Mortality Quantification

If the entire egg mass piece or capsule was obviously inviable, mortality was recorded as 100%. In capsular masses, total inviability was often indicated by a dark black, brown, or purple colour change (Figure 2.3a). In both capsular and gelatinous masses, total inviability was also determined when all embryos degenerated into free granules or fused into large undifferentiated masses while still encapsulated (Figure 2.3a).

Due to different structures and hatching mechanisms among egg masses, it was necessary to develop different methods to quantify mortality of gelatinous egg masses, neogastropod capsules, and neritid capsules. Among gelatinous egg masses, each hatching or dissolving piece was first blotted and weighed. It was then placed in a small vial with 500µL filtered seawater and agitated by hand for approximately one minute until the egg mass was homogenized in the seawater. Homogenization ensured that embryos from the entire egg mass could be examined rather than only peripheral embryos which may not be representative of the entire egg mass. From each homogenized piece, ten 5µL aliquots examined microscopically. Dead embryos were counted within each sample, and the average was taken. This number was used to determine the estimated proportion of dead embryos (ß) within the 500µL mixture. I considered embryos to be dead if they were degenerating and encapsulated (Figure 2.3a) or if they were relatively undeveloped (Figure 2.3b). Embryos without a protoconch or with other deformities were not considered dead since these malformations could have occurred during the homogenization procedure. Thus, mortality was conservatively

estimated in this study. The following formula was used to quantify mortality (M) within the entire gelatinous mass piece:

$$M = ß / [W_a(n /W_b)] * 100\%$$

where ß= estimated proportion of dead embryos, W_a= blotted weight of the egg mass piece before homogenisation, W_b= average weight of a cubic millimetre of gel, and n = number of eggs per mm^3. Density of eggs was recorded after experimentation on egg masses containing veligers. M refers to percentage of dead embryos. A much simpler alternative would have been to score alive embryos and dead embryos. However, this method was rejected because it would have likely resulted in inaccurately high mortality rates because many of the viable embryos are released from the disintegrating mass by the time of examination. These embryos would not have been scored as they would be drifting freely within the entire recirculating seawater system.

In all neogastropod capsules, mortality was calculated by dividing the number of dead embryos remaining in the capsule after hatching by the initial number of eggs in the capsule. Preliminary developmental observations showed that none of the species examined in this study had nurse eggs so they were not considered in the mortality calculation. The embryos of *Nerita atramentosa* are difficult to observe due to their opaque calcareous capsules (Figure 1.5). In order to observe development and maturity of this species, it was necessary to puncture the apical capsule so that the shell could be removed and the embryos within examined. Consequently, no initial count of eggs could be made as this would have destroyed the capsule and prevented further development. Replicate capsules collected at the same time were opened approximately every week until the majority of capsules within that treatment showed signs of hatching. As hatching approached, the apical shell became more flexible and split off easily from the basal membrane leaving the embryos exposed. At this time, the apical shell of the remaining intact egg capsules was removed, and the proportion of dead to live embryos was determined.

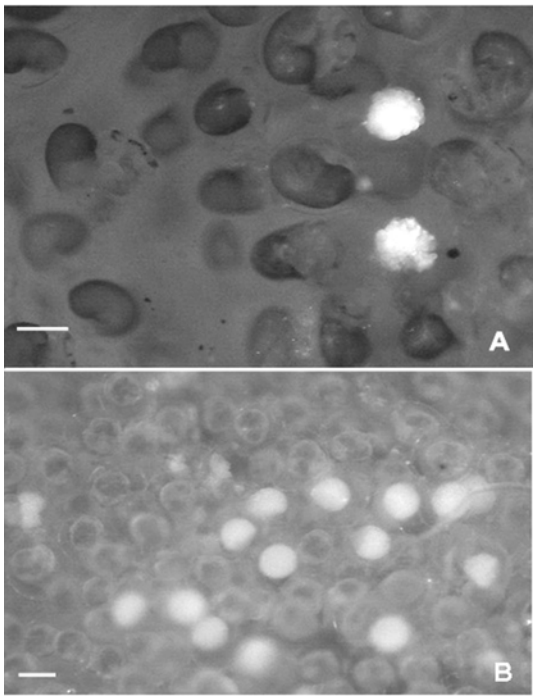


Figure 2.3 Inviable eggs and embryos within molluscan egg masses. Scale bar is 100 µm. A) Gelatinous egg mass of *Bembicium nanum*. Bright white eggs are very undeveloped relative to the rest of embryos. B) *Dolabrifera brazieri* embryos after exposure to UVR. All embryos have reached veliger stage with the exception of the white eggs in the centre which are relatively undeveloped or degenerating.

Determination of Encapsulation Period

Length of encapsulation was the time taken to hatch for each egg mass or capsule under each spectral treatment. Hatching was quite obvious in caenogastropod capsular egg masses because the apical aperture would open easily during examination, and the viable embryos would be released relatively quickly en masse. In gelatinous egg masses, hatching was often prolonged and less obvious. To standardize hatching time for gelatinous masses, hatching was recorded as the first day when more than 10% of the embryos had hatched or the gel dissolved. This value was chosen because preliminary observations revealed hatching to occur relatively quickly after this point. The hatching data from *Nerita atramentosa* was not used in the statistical analysis of hatching because determination of the length of encapsulation was too subjective and variable within this species. It was also likely to be affected by interference during examination. Furthermore, this species took up to three months longer to hatch than many other species, and the resulting variance hindered statistical analysis.

Statistical Analyses

Nested ANOVA tests were performed using restricted maximum likelihood (REML) technique in the statistical package JMP 4 with alpha = 0.05 unless otherwise noted. A plot of the residuals of mortality versus the predicted values showed a binomial pattern. Because the variance was a function of the predicted values in the raw data, a standard angular transformation of arcsine(√M) was used (Zar 1998). Tukey's HSD multiple comparison tests were performed *a posteriori* on all data yielding significant effects.

2.1.3 RESULTS

Species that laid gelatinous egg masses were found more frequently than those that laid capsular egg masses (Table 1). More egg masses were found in shaded or partially shaded habitats than in areas exposed to full sunlight (Table 1).

Mortality and Habitat

The effects of ultraviolet radiation or light on embryonic mortality were dependant upon the habitat in which the egg masses naturally occurred (Figure 2.4). ANOVA revealed a highly significant interaction between habitat and treatment ($F = 10.9645$, $p < 0.0001$) (Table 2.2a). Shaded egg masses were most vulnerable to UVR while egg masses from full sun habitats were the least vulnerable (Figure 2.4). Tukey's HSD revealed that egg masses from shaded spawning sites had significantly elevated mortality in open full spectrum treatments than UV-blocked and dark treatments. (Figure 2.4, Table 2.2b). Egg masses naturally laid in full sunlight or partial shade had no significant differences in embryonic mortality between spectral treatments (Figure 2.4, Table 2.2b). However, Figure 4 does indicate a trend of increasing mortality with exposure to UVR in egg masses from partial sun habitats.

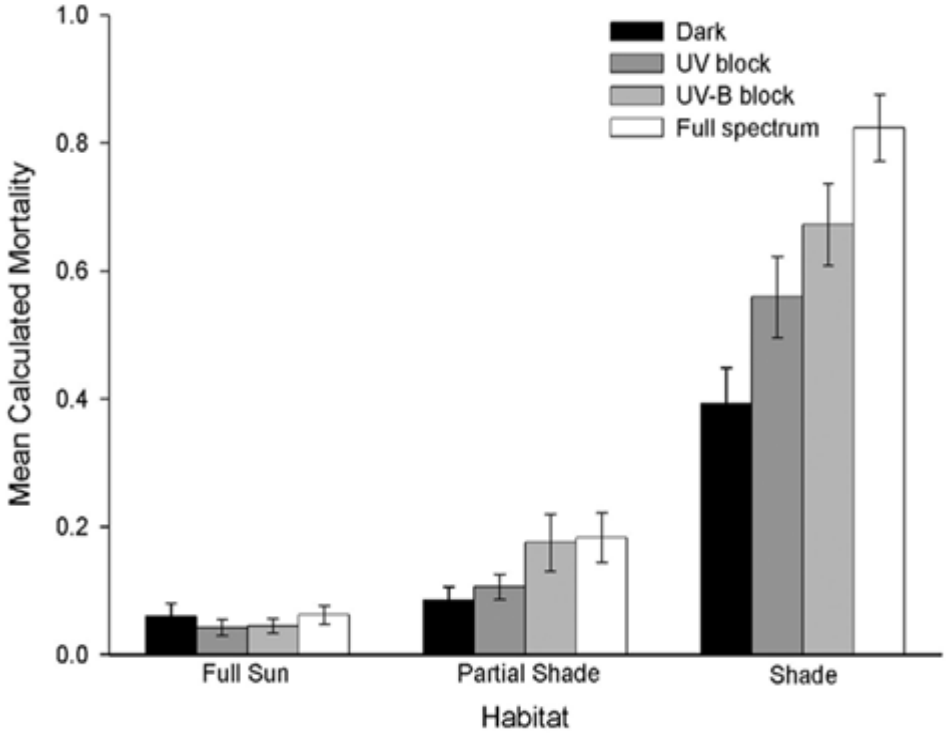


Figure 2.4 The mean embryonic mortality of molluscan egg masses collected from full sun, partial sun, and full shade habitats under four different spectral treatments: 1) Full spectrum (no block); 2) UV-B block (mylar); 3) UV block (security film); and 4) Dark (opaque plastic). Error bars are standard error of mean.

Mortality of embryos within egg masses was low for those laid in full sun habitats relative to those laid in other habitats (Figure 2.4, Table 2.2b). Tukey's HSD confirmed that egg masses from shaded habitats had significantly higher mortalities than the egg masses from other habitats at every spectral treatment (Table 2.2b). Although partially shaded egg masses showed higher mortality than full sun egg masses in all but the dark treatments, these differences were not significant (Figure 2.4, Table 2.2b).

Mortality and Egg Mass Type

Neogastropod capsular egg masses were found exclusively in fully shaded habitats (Table 2.1). Overall, embryos within these capsular egg masses exhibited a higher mortality than embryos within gelatinous masses (Figure 2.5). For example, neogastropod capsules showed almost 100% mortality in full spectrum treatments while in the same light treatment, gelatinous egg masses from shaded sites showed an estimated mortality of 73.5%.

Table 2.2 The effects of habitat, spectral treatment, species, and egg mass on embryonic mortality as determined by a) nested ANOVA with restricted maximum likelihood with mortality arcsine transformed and random factors italicized, and b) multiple comparisons of spectral treatments within and between each habitat with Tukey's HSD (alpha = 0.05). Mean mortalities are presented with the critical Q values in parentheses. Lines connect treatments that are not significantly different. S refers to shaded habitats; PS refers to partially shaded, and FS refers to full sun.

a)

Source	DF	MS	F	p
Habitat	2	1.8133	34.7033	< 0.0001
Treatment	3	1.2030	23.0443	< 0.0001
Habitat x Treatment	6	0.5724	10.9645	< 0.0001
Species [Habitat]	23	0.0921	2.0299	0.0153
Egg Mass [Species, Habitat]	96	0.1236	3.1140	< 0.0001
Residual	279	0.0522		
Corrected Total	383	0.2400		

b)

Within Habitats

	Full Spectrum	UV-B Block	UV Block	Dark
Full Sun (0.2000)	0.0619	0.0444	0.0419	0.0598
Partial Shade (0.1821)	0.1826	0.1749	0.1051	0.0847
Shade (0.1904)	0.818375	0.6719	0.5589	0.3918

Between Habitats

	Shade	Partial Sun	Full Sun
Full Spectrum	0.8184	0.1826	0.06190
UV-B Block	0.6719	0.1749	0.04438
UV-Block	0.5589	0.1051	0.04190
Dark	0.3918	0.0847	0.0598
	S x PS (0.1863)	PS X FS (0.1912)	FS x S (0.1953)

A nested ANOVA confirmed a significant interaction between spectral treatments and egg mass type (Table 3a). Tukey's HSD revealed a significant difference in mortality between the egg mass types in the UV-B treatments. (Table 3b). Nevertheless, both capsular and gelatinous egg masses did show a similar pattern in their response to the different UV spectral treatments. For example, both types of egg mass had significantly lower mortality in UV block and dark than in full spectrum treatments (Table 3b)

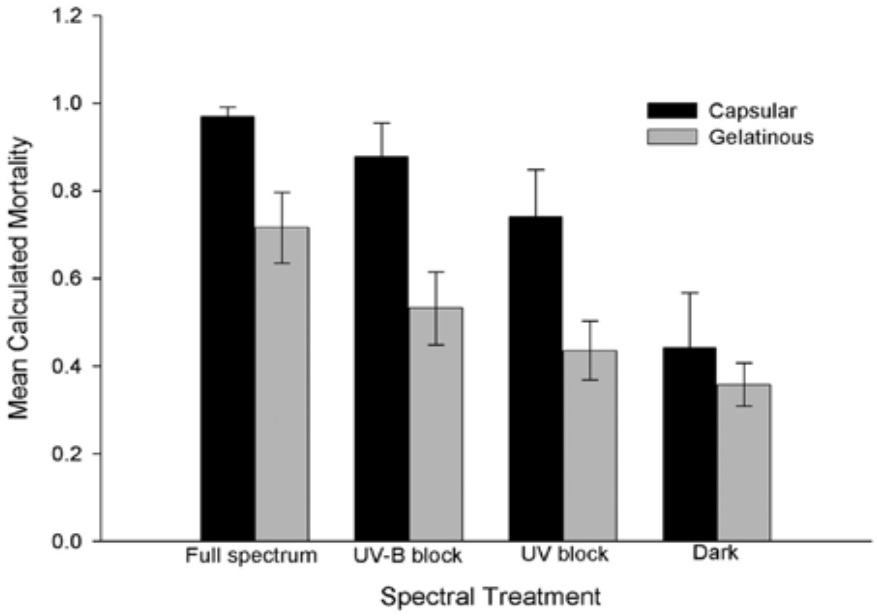


Figure 2.5 The mean embryonic mortality of gelatinous and capsular egg masses collected from shaded habitats under four spectral treatments: 1) Full spectrum (no block), 2) UV-B block (mylar), 3) UV block (security film), and 4) Dark (opaque plastic). Error bars are standard error of mean.

Mortality and Habitat (Gelatinous Egg Masses Only)

Due to the unequal distribution of egg mass types among habitats and their different mortalities, an ANOVA was performed on only gelatinous egg masses to determine if trends persisted. The results were consistent with the full data set, and my interpretation did not change. A significant interaction between habitat and spectral treatment was apparent ($F = 8.0232$, $p < 0.0001$), and Tukey's HSD revealed the same pattern as obtained for the full data set (data not shown).

Table 3 The effects of spectral treatment, egg mass type, species, and egg mass on embryonic mortality as determined by a) nested ANOVA with restricted maximum likelihood with random factors italicized and b) multiple comparisons with Tukey's HSD (alpha = 0.05). Mean mortalities of raw data are presented with the critical Q values in parentheses, and lines connect treatments that are not significantly different.

a)

Source	DF	MS	F	p
Treatment	3	2.3521	21.4146	< 0.0001
Type	1	0.4262	3.8801	0.0895
Type x Treatment	3	0.2850	2.5951	0.0574
Species [Type]	7	0.1182	1.0763	0.4096
Egg Mass [Species, Type]	23	0.3126	2.8458	0.0002
Residual	90	0.1098		
Corrected Total	127	0.2852		

b)

Within Habitats

	Full Spectrum	UV-B Block	UV Block	Dark
Capsular (0.3715)	0.968615	0.877231	0.739846	0.442
Gelatinous (0.3073)	0.715579	0.531421	0.435053	0.357474

Between Habitats

	Capsular	Gelatinous
Full Spectrum	0.968615	0.715579
UV-B Block	0.877231	0.531421
UV Block	0.739846	0.435053
Dark	0.4420	0.357474
(0.3409)		

Table 4 The effects of habitat, spectral treatment, species, and egg mass on the length of embryonic encapsulation as determined by a) nested ANOVA using restricted maximum likelihood with random factors italicized and b) multiple comparisons using Tukey's HSD (alpha = 0.05). Only gelatinous egg masses were used. Mean mortalities of raw data are presented with critical Q values in parentheses, and lines connect treatments that are not significantly different.

a)

Source	DF	MS	F	p
Habitat	2	1.4956	0.8569	0.4489
Treatment	3	41.0182	23.5034	< 0.0001
Habitat x Treatment	6	1.8541	1.0624	0.3865
Species [Habitat]	12	5.8914	3.3758	0.0010
Egg Mass [Species, Habitat]	55	39.2748	22.5044	< 0.0001
Residual	201	1.7879		
Corrected Total	279	18.9800		

b)

Treatment:			
Full Spectrum	UV-B Block	UV Block	Dark
13.5857	14.0000	13.8143	15.4143
(0.5889)			

Encapsulation Period

Egg masses from all habitats hatched significantly slower in the dark than in the light (Figure 2.6). Statistical analyses encompassed only gelatinous egg masses because no capsular egg masses hatched in full spectrum treatments (Table 1). ANOVA revealed no significant interaction between habitat and treatment on the length of the developmental period, but there was a significant difference between egg masses maintained in the dark and all three light treatments ($F = 18.0211$, $p < 0.0001$) (Table 4).

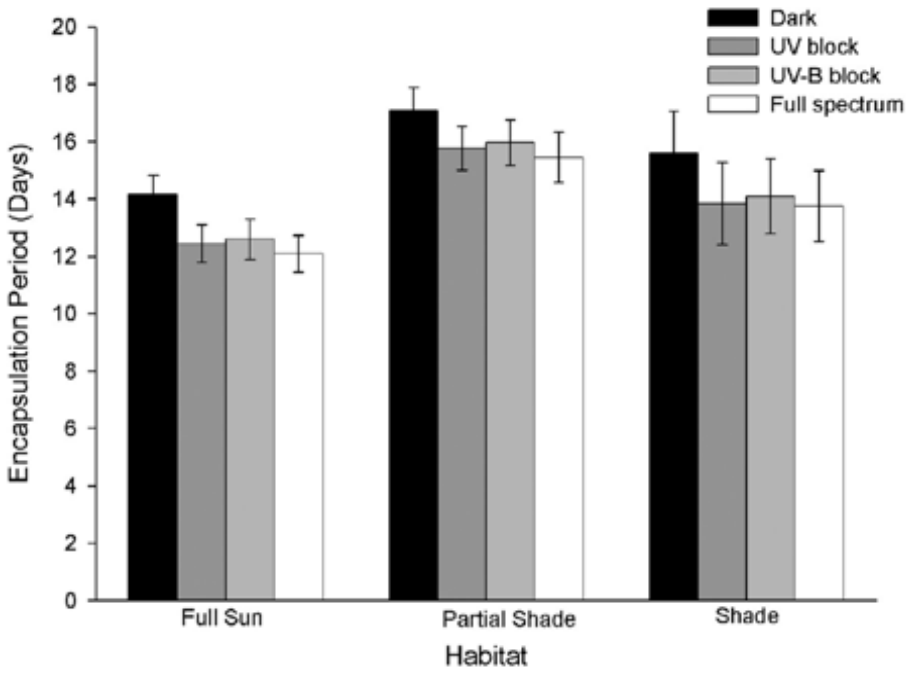


Figure 2.6 The mean hatching day of molluscan egg masses collected from full sun, partial sun, and full shade habitats under four different spectral treatments: 1) Full spectrum (no block); 2) UV-B block (mylar); 3) UV block (security film); and 4) Dark (opaque plastic). Only gelatinous egg masses were used. Error bars are standard error of mean.

2.1.4 DISCUSSION

This study has shown that embryonic responses to ultraviolet radiation and visible light are strongly influenced by the habitat in which the eggs are naturally deposited. To date, there has been no previous study examining the effects of an environmental stress on molluscan egg masses drawn from a broad range of taxa. Many studies have focused on one or two species and their responses to environmental variables (e.g. Scheltema 1967; Pechenik 1983; Biermann *et al.* 1992). Although representing valuable contributions to molluscan biology, research on single species is not sufficient to determine trends or to extrapolate to similar species or groups. This study has shown that a broad comparative approach for molluscan egg masses is feasible; indeed, the relatively small error bars (Figure 2.5) are remarkable considering the myriad species and egg mass structures examined and conservative mortality estimate. By using many species, I have been able to

identify a strong pattern in the embryonic responses of molluscan egg masses to ultraviolet radiation based on their natural habitat.

The encapsulated embryos of species that lay exclusively in shaded habitats are not only more vulnerable to UVR but also have a higher overall mortality (Figure 2.4). Such vulnerability is likely related to the protected habitat in which they are laid. Egg masses deposited on the undersides of boulders are sheltered from sunlight, and they are also likely to be more protected from physical disturbances such as buffeting by wave action and predator activity. Thus, some molluscan species appear to rely on parental site selection for egg deposition as the major means of protecting developing embryos from environmental stresses. Egg masses laid in partial sun habitats warrant further investigation as it is possible that egg masses within a species vary in their vulnerability to UVR according to the habitat in which they were laid (e.g. Belden & Blaustein 2002).

Caenogastropods in particular appear to select fully shaded habitats for attachment of their leathery egg capsules (Table 1). In UV-B blocking treatments, embryonic mortality was significantly higher in capsular egg masses than in gelatinous egg masses collected from the same habitat. The higher embryonic mortality in capsules is likely due to the embryos' lack of internal membranes. Since all embryos are freely integrated within a capsule, if one embryo becomes infected or dies, it could contaminate all the embryos in the capsule. Indeed, entire inviable capsules are quite common (Pechenik 1982). By comparison, molluscan embryos within gelatinous egg masses are separated individually or in small groups from the remaining embryos by a vitelline membrane (Figure 2.3). The relatively high susceptibility of encapsulated caenogastropod embryos to physical disturbance and UVR suggests that the selection of appropriate laying habitats is particularly important for these species.

Although caenogastropod capsules exhibited higher mortality, gelatinous egg masses collected from shaded habitats showed exactly the same pattern in response to the various UV treatments. This indicates that egg mass type was not a significant confounding factor

in this study. Thus, molluscan egg masses collected from shaded intertidal habitats are more vulnerable to the effects of UVR irrespective of their phylogenetic origin.

In contrast, those egg masses deposited in full sunlight are relatively resistant to UVR. This study examined only embryonic mortality, however, and UVR may cause sublethal effects such as embryonic deformities or small embryo sizes within these egg masses (Biermann *et al.* 1992; Pahkala *et al.* 2002). Furthermore, UVR exposure may also cause negative effects after metamorphosis (Pechenik *et al.* 1998). These effects could be synergistic with other environmental stresses such as temperature (Przeslawski 2004a). Laboratory experiments with controlled UVR intensity would clarify these issues.

Nevertheless, full sun egg masses most likely possess biochemical, structural, or cellular protection against potentially damaging UVR. In particular, it is likely that some of these egg masses contain UV-absorbing compounds such as mycosporine-like amino acids (MAAs). MAAs have been found in a variety of marine organisms including algae, cnidarians, echinoderms, and vertebrates; and they have been shown to protect against the damages of UVR (reviewed by Shick & Dunlap 2002). Carefoot *et al.* (1998) report that egg masses of some *Aplysia* were rich in MAAs although their presence was strictly diet-dependent and not a direct response to UV-exposure. Several *Aplysia* egg masses from partial shade habitats were used in this study (Table 1), and these did show some vulnerability to UV-B. If biochemical protection via MAAs does occur in molluscan egg masses, it will most likely be found in gelatinous egg masses laid in full sunlight. Two previous studies have been unable to identify significant amounts of MAAs or any other UV-absorbing compounds within caenogastropod egg capsules collected from intertidal and subtidal habitats (Karentz *et al.* 1991; Rawlings 1996). Unfortunately, these studies do not specify whether the egg masses were collected from habitats exposed to UVR. Rawlings (1996) found that the outer capsule of a neogastropod egg mass absorbed UV-B, but he was unable to identify the means of absorption. Given the fact that embryos enclosed within these types of capsules are vulnerable to UVR (Figure 2.5), the capsule wall may only be effective as a structural barrier against infrequent and low intensity UVR. In contrast, the egg capsules of *Nerita atramentosa* do occur in full sun habitats and appear to

be resistant to the effects of UVR (Figure 2.4), possibly due to the protection of the opaque calcareous capsule which may aborb UVR.

The evolution of UV-resistant egg masses may provide several advantages to species that live on intertidal reefs. First of all, competition for an appropriate egg deposition site may be lower in full sun habitats due to the fact that many species are vulnerable to UVR. Egg masses laid in direct sunlight might also be less prone to microbial infection because the microbes themselves could be harmed by ultraviolet radiation. Finally, as shown in this study, egg masses laid in sunlight will hatch faster than those laid in the shade irrespective of temperature (Figure 2.6). Therefore, direct sunlight might reduce the overall risks to embryos by minimizing the time spent in vulnerable life stages (Spight 1975; Strathmann *et al.* 2002).

The encapsulation period was shorter in egg masses that were exposed to light compared to those kept in the dark with hatching occurring on average 1-2 days earlier (Figure 2.6). This result was unexpected for egg masses collected from shaded habitats because it implies that their selected habitat is a compromise to minimize mortality. The faster hatching in light could be a direct response to light or related to other confounding effects such as surface algal fouling. Algal growth is dependent on light availability (e.g. Hernandez *et al.* 1997) and could alter the internal oxygen concentration of the egg mass during photosynthesis and cellular respiration. Oxygen availability has been shown to have a tremendous effect on the hatching rate within molluscan egg masses (Booth 1995; Cohen & Strathmann 1996).

Various degrees of algal and protist fouling were noted on many egg masses in the experimental tank used in this study. In addition to the potential positive effects on embryonic developmental rates by photosynthetic surface fouling organisms, fouling might also have detrimental effects. For example, byproducts of certain algae may be detrimental to the health of embryos (Fogg 1983; Tang & Dam 2001). Algal fouling can also provide a foundation for protist colonization and infestation. Protists have recently been shown to lower the oxygen availability of embryos in certain egg masses to dangerous levels (Cancino *et al.* 2000). Egg masses that were collected from shaded habitats showed significantly increased mortality in visible light treatments even when damaging UV

wavelengths were blocked (Figure 2.4). Approximately 10% of UV-B and 25% of UV-A passed through the UV-blocked filter in visible light treatments (Figure 2.2), and it is thus possible that these levels of UVR were significantly damaging to embryos from full shade egg masses. Another possibility is that the effect of visible light recorded here may be related to confounding factors such as surface fouling. Future research should consider the potential interaction among these variables in order to understand the potential synergistic effects of ultraviolet radiation and other factors such as algal fouling on encapsulated molluscan development.

CHAPTER 3: Interactions between ultraviolet radiation and other stressors[1,2,3]

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' but 'That's funny ...'"
Isaac Asimov

[1] Section 3.1 encompasses published manuscript
Przeslawski R, Davis AR, Benkendorff K (2005) Synergies, climate change and the development of rocky shore invertebrates. *Global Change Biology*, 11, 515-522
[2] Section 3.2 encompasses manuscript in press
Przeslawski R (2005). Combined effects of solar radiation and desiccation the mortality and development of encapsulated embryos of rocky shore gastropods. *Marine Ecology Progress Series*, 298, 169-177
[3] Section 3.3 includes published manuscript (except 3.3.1, 3.3.4.1)
Przeslawski R & Benkendorff K (2005). The role of surface fouling in the development of encapsulated gastropod embryos. *Journal of Molluscan Studies*, 71, 75-83

3.1 UV, TEMPERATURE & SALINITY: SYNERGIES, CLIMATE CHANGE AND THE DEVELOPMENT OF ROCKY SHORE INVERTEBRATES

Global climate change and ozone layer thinning will simultaneously expose organisms to increasingly stressful conditions. Early life stages of marine organisms, particularly eggs and embryos, are considered most vulnerable to environmental extremes. Here, I exposed encapsulated embryos of three common rocky shore gastropods to simultaneous combinations of ecologically realistic levels of ultraviolet radiation (UVR), water temperature stress and salinity stress to identify potential interactions and associated impacts of climate change. I detected synergistic effects with increases in mortality and retardation in development associated with the most physiologically stressful conditions. The effects of UVR were particularly marked, with mortality increasing up to 12-fold under stressful conditions. Importantly, the complex outcomes observed on applying multiple stressors could not have been predicted from examining environmental variables in isolation. Hence, we are probably dramatically underestimating the ecological impacts of climate change by failing to consider the complex interplay of combinations of environmental variables with organisms.

3.1.1 INTRODUCTION

In the face of stratospheric ozone loss, organisms are being exposed to elevated levels of ultraviolet radiation (UVR), particularly UV-B (280-320 nm) (Bjorn 1999). Global climate change will also herald other shifts in the abiotic variables to which organisms are exposed, including elevated temperatures and more variable precipitation (Helmuth *et al.* 2002; Karl & Trenberth 2003). Consequently, organisms may be simultaneously exposed to a variety of physiologically stressful conditions (Andrady *et al.* 2004), yet the impact of multiple stressors associated with climate change remains unclear.

Rocky intertidal environments are already amongst the most physiologically stressful (Gosselin & Chia 1995). In particular, intertidal biota must endure periods of emersion that can lead to severe desiccation, thermal stress and exposure to UV radiation (e.g. Dayton 1971; Sousa 1979; Dethier 1984). Abiotic stressors can be inextricably intertwined in the intertidal; UVR and associated sunlight can increase temperature, which can then increase evaporation rates in small pools and lead to increased salinity (Przeslawski 2004a). Intertidal organisms can adapt to severe physical stressors, so

long as they are frequent and predictable. However, stochastic events that alter the natural disturbance regimes are known to have devastating effects on intertidal biota (e.g. earthquakes in Castilla (1988) and flooding in Branch *et al.* (1990). Furthermore, tolerance to factors such as extreme salinity and temperature can dictate the distributional limits for some rocky shore invertebrates (reviewed by Menge & Branch 2000). There is already some evidence to suggest global warming can lead to a redistribution of species in intertidal rocky shore communities (Harley 2003).

While adult organisms in intertidal environments are often protected from physiological extremes (e.g. shells and operculi of molluscs); their eggs or larvae may be highly vulnerable (Altamirano *et al.* 2003; Kashenko & Korn 2003; Ushakova 2003). Exposure to UVR can cause death to encapsulated molluscan embryos (Carefoot *et al.* 1998; Przeslawski *et al.* 2004). High temperature can also increase embryonic mortality (Ganaros 1958; Dehnel & Kong 1979) and is one of the primary factors regulating developmental rate among marine invertebrates (Rodriguez *et al.* 1991; Palmer 1994). Salinity outside the normal range in which a species occurs can increase encapsulation period and embryonic mortality (reviewed by Przeslawski 2004a). Importantly, interactions among temperature and salinity have the potential to significantly affect development and productivity of marine invertebrates and algae (e.g. Pechenik 1987; Matta & Chapman 1995). Temperature and salinity can interact to affect the development of marine invertebrates (Kashenko & Korn 2003), but these effects can be species-specific (Ushakova 2003). Similarly, the interactions of temperature and UVR negatively affect the early life stages of some intertidal algae (Hoffman *et al.* 2003), supporting previous research on temperature mediation of UVR effects and vice-versa (Roos & Vincent 1998). Whilst a few studies have investigated interactions between temperature and salinity on gastropod development (e.g. Roller & Stickle 1989; Pechenik *et al.* 2003), studies on other abiotic factors and their interactive effects on molluscs are rare (reviewed by Przeslawski 2004a). Only a few studies examine more than two factors affecting molluscan embryos or larvae (e.g. Biermann *et al.* 1992; Salvato *et al.* 2001; Pechenik *et al.* 2003). With few exceptions (e.g. Roos & Vincent 1998; Hoffman *et al.* 2003; Macfadyen *et al.* 2004), the interaction of increasing UVR with other potentially physiologically stressful conditions has received scant attention (Przeslawski 2004a). Studying the effects of UVR in isolation may overlook potential synergistic interactions, and the effects of global climate change and ozone layer

thinning may thus be seriously underestimated. Here, I offer the first study to examine synergistic effects of UVR, temperature, and salinity on invertebrate development.

My preliminary field observations on rocky intertidal shores indicate that egg masses of the pulmonate *Siphonaria denticulata* and the littorinid *Bembicium nanum* are deposited in sun-exposed shallow pools that can reach salinities as high as 50 ppt with water temperatures up to 34°C. Embryonic mortality of mature intertidal egg masses collected from these habitats was extremely variable, but reached 100% for *S. denticulata* (n = 86) and exceeded 30% for *B. nanum* (n = 82) (unpublished data). By comparison, many intertidal molluscs such as the aplysiad *Dolabrifera brazieri*, spawn on the undersides of submerged boulders (Benkendorff & Davis 2004) where they should be buffered from these environmental extremes. Therefore, I predicted that embryos of *B. nanum* and *S. denticulata* would be more tolerant of abiotic stressors than *D. brazieri* embryos. Here, I elucidate the impacts of simultaneously varying key environmental variables (UVR, temperature and salinity in a total of 27 combinations) on the rates of molluscan development and levels of embryonic mortality for three common intertidal mollusc species. My experiments reveal striking interactions among these abiotic factors and caution against considering the impacts of environmental variables in isolation.

3.1.2 METHODS

For each species, six egg masses containing pre-trochophore embryos were collected during low tides from Bass Point (34°35’45”S, 150°53’20”E), New South Wales, Australia in December 2003 (Figure 2.1). *B. nanum* and *S. denticulata* egg masses were removed from separate random shallow pools on rocky platforms at mid- to high-shore, while egg masses of the low-shore *D. brazieri* were collected randomly from beneath boulders (Figure 3.1.1). *S. denticulata* egg masses are spiralled cylindrical gelatinous ribbons 2-11 cm in length containing 125 µm eggs at a density of approximately 37 eggs/mm^{-3} (Figure 3.1.1a). *D. brazieri* egg masses are flattened continuous gelatinous ribbons deposited in a characteristic zig-zag pattern containing 75 µm eggs at a density of approximately 66 eggs/mm^{-3} (Figure 3.1.1b). *B. nanum* egg masses are composed of several discrete gelatinous compartments containing 175 µm eggs at a density of approximately 20 eggs/mm^{-3} (Figure 3.1.1c). Each egg mass was divided into 27 pieces of approximately equal sizes between 2-4 mm in length. Experiments on two species

revealed that dividing egg masses into pieces this small did not significantly affect embryonic mortality (*S. denticulata*: df = 4, F = 0.167, p = 0.952; *B. nanum*: df = 6, F = 1.032, p = 0.428) as supported by previous research (Rose 1986). Segments of egg mass were placed in seawater in 120 mL bowls (18 cm diameter, 5 cm height) floating in one of three full 30-litre outdoor recirculating water baths filled from a common reservoir. All containers were opaque UV-blocking plastic. Water baths were completely full, and bowls had a flared edge so that egg masses were not shaded by containers during peak daylight hours (9am – 3pm). Seawater was changed every 24 hours to ensure adequate oxygen availability. Floating containers were randomly repositioned within the water baths during continuous recirculation of water and daily examination, and egg mass pieces within each bowl were randomly repositioned daily. In addition, the position of each water bath was changed twice randomly to counter spatial variation in environmental variables.

Twenty-seven treatment combinations were applied simultaneously in a factorial design. The salinity and temperature treatments were based on those commonly encountered in shallow pools on rocky reefs during summer (unpublished data). The three levels of salinity treatments (25 ppt, 35 ppt, and 45 ppt) were obtained by the evaporation of

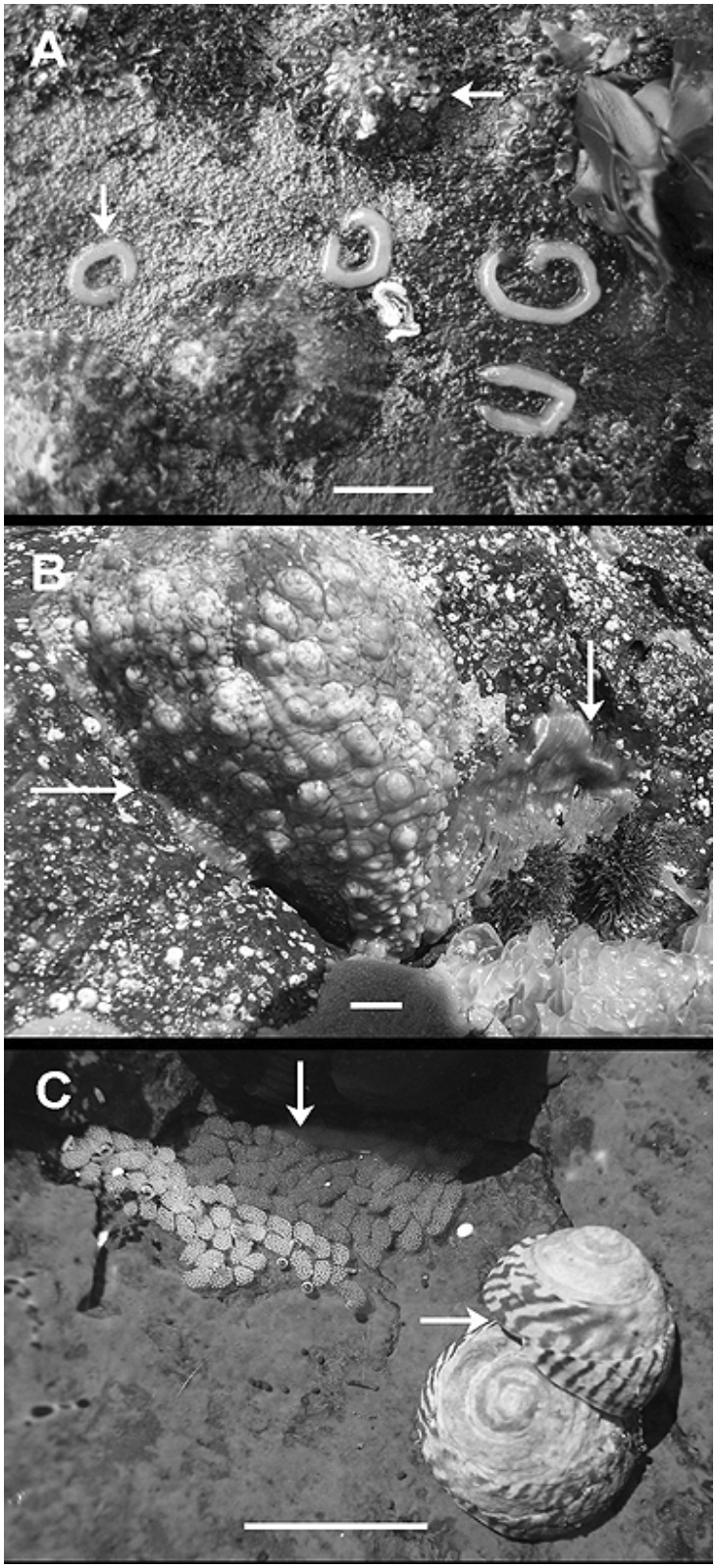


Figure 3.1.1 Adults and egg masses of a) *Siphonaria denticulata* on an emersed rock platform, b) *Dolabrifera brazieri* under a submerged boulder, and c) *Bembicium nanum* on a rock platform with undeveloped (white) and mature (brown) egg masses. Horizontal arrows indicate adults; vertical arrows indicate egg masses. Scale bars represent 2 cm.

seawater used in all treatments (by boiling) followed by the addition of Milli-Q filtered water to create the desired salinity. Salinity was measured and did not fluctuate within treatments during the study period. Temperature treatments within each water bath were maintained with submerged refrigeration coils and 100-watt aquarium heaters. Recirculating water in each bath ensured that temperatures were constant throughout each temperature treatment and this was confirmed with submerged thermal microchips (Thermochron I-Button). Periodic readings of various spectral treatments with a thermometer confirmed no thermal differences between shallow containers within the same water bath. Water baths were assigned to a temperature treatment at random, with three levels of this factor maintained for the 72-hour study period (17.8 ± 1.8°C, 21.3 ± 1.3°C, and 25.8 ± 1.8°C). Spectral treatments were achieved with natural sunlight and cut-off filters secured over each shallow container; (i) dark (black plastic), (ii) UV-blocked (polyethylene with UV absorption), and (iii) full spectrum (clear polyethylene cling wrap) (see Figure 2.2 for transmission properties). In addition, incident UV-A and UV-B were measured periodically during the study with a dosimeter (Grobel RM21). Mean UV-A irradiance during peak daylight hours (9am – 3pm) was 16.14 ± 6.21 W/m^2, and UV-B irradiance was 0.75 ± 0.37 W/m^2; skies were clear during peak daylight hours for the duration of the experiment.

After 72 hours in experimental treatments, all egg mass pieces were removed and examined with a dissecting microscope (40x magnification). This time period was short enough to inhibit potentially confounding microalgal fouling on the egg mass surface (Przeslawski & Benkendorff 2005). Mortality of peripheral embryos was estimated for each piece by scoring 50 embryos on each side of the egg mass as dead or alive. Due to the potential interaction between stress of being at the cut end of an egg mass and various experimental treatments, embryos near the cut ends of each piece were avoided. Embryos were considered dead if they were degenerating or relatively underdeveloped; underdeveloped embryos usually indicate arrested development associated with impending degeneration (pers. obs.). Developmental stage was defined for each egg mass piece as the stage in which >50% of embryos were found. If two stages were equally represented within one piece, the mean stage was recorded. Developmental

stages were classified as (1) non-ciliated (pre-trochophore), (2) ciliated with no shell (trochophore/early veliger), (3) partial shell (veliger), (4) full shell (late veliger).

I used restricted maximum likelihood (REML) ANOVAs to examine treatment effects for each species; the random factor was an individual egg mass from which segments were obtained. Significant interaction terms where variables increased in response to 2 or more factors were indicative of synergistic effects between the treatments. Tukey's HSD tests revealed significant *a posteriori* relationships (JMP, version 4.0). As mortality data were binomially distributed it was arcsin transformed for each species to satisfy ANOVA assumptions (Zar 1998).

3.1.3 RESULTS

When placed under physiologically stressful environmental conditions, embryos within molluscan egg masses showed elevated mortality and retarded development. Importantly, exposure to combinations of stressors (25 or 45 ppt, full spectrum, 26ºC) produced significant deleterious effects often many times higher than when egg masses were under environmentally moderate conditions such as those which occur during high tide (35 ppt, minimal UVR exposure, moderate temperatures) (Figure 3.1.2). When UV radiation was blocked or egg masses were held in the dark, mortality was generally low irrespective of the temperature or salinity combination to which each species was exposed (Figure 3.1.2). Dramatic increases in mortality became apparent once egg masses were exposed to full spectrum light, and these effects were clearest when conditions were the most physiologically demanding.

Embryos exposed to UVR showed an 8-12 fold increase in mortality when exposed to salinity different (25 or 45 ppt) to that of natural seawater (35 ppt), particularly at 26ºC (Figure 3.1.2), but these effects were species-specific and not uniform across treatments. ANOVA and *a posteriori* comparisons among means confirmed that temperature, salinity, and/or UVR effects were significant for each species (Appendix 1A). For the low-shore *Dolabrifera brazieri*, I detected a significant three-way interaction (salinity, temperature, and UV radiation) on embryonic mortality (Table 3.1.1a). At 18ºC and 26ºC, UVR exposure significantly increased embryonic mortality at 45 ppt; the same trend occurred at 21ºC and 25 ppt (Figure 3.1.2a). In contrast to full spectrum

treatments, blocking UV and keeping embryos in the dark for this species did not significantly alter mortality for each level of salinity or temperature (Figure 3.1.2a). The other two species showed significant two-way interactions between salinity and spectral treatments for embryonic mortality (Table 3.1.1a). UVR exposure significantly increased embryonic

Table 3.1.1 The effects of temperature, salinity, and spectral treatment on a) embryonic mortality (arcsin transformed), and b) developmental rate of three gastropod species as determined by ANOVAs with restricted maximum likelihood with random factors italicised (n = 6).

a)

		Dolabrifera brazieri			*Bembicium nanum*			*Siphonaria denticulata*		
Source	df	MS	F	p	MS	F	p	MS	F	p
Egg mass	5	0.255	8.916	<0.001	0.233	3.907	0.003	0.675	11.771	<0.001
Spectral (UV)	2	2.507	87.764	<0.001	1.035	17.392	<0.001	2.898	50.526	<0.001
Temperature (T)	2	0.053	1.844	0.162	0.122	2.047	0.133	0.006	0.102	0.903
Salinity (S)	2	0.231	8.087	0.0005	0.150	2.522	0.084	0.188	3.270	0.041
UV x S	4	0.286	10.012	<0.001	0.326	5.479	<0.001	0.185	3.230	0.015
UV x T	4	0.050	1.765	0.140	0.015	0.258	0.905	0.009	0.149	0.963
T x S	4	0.220	7.689	<0.001	0.116	1.949	0.106	0.159	2.778	0.030
UV x T x S	8	0.238	8.337	<0.001	0.067	1.125	0.351	0.104	1.814	0.080
Residual	130	0.029			0.060			0.057		
Corrected Total	161	0.092			0.088			0.121		

b)

		Dolabrifera brazieri			*Bembicium nanum*			*Siphonaria denticulata*		
Source	df	MS	F	p	MS	F	p	MS	F	p
Egg mass	8	12.758	51.293	<0.001	5.846	11.022	<0.001	9.971	77.388	<0.001
Spectral (UV)	2	1.847	7.793	<0.001	8.595	17.190	<0.001	3.782	29.356	<0.001
Salinity (S)	2	0.421	1.777	0.173	6.233	11.752	<0.001	2.264	17.571	<0.001
Temperature (T)	2	2.056	8.672	<0.001	12.418	23.413	<0.001	16.032	124.431	<0.001
UV x S	4	0.192	0.811	0.521	0.257	1.029	0.395	0.060	0.467	0.760
UV x T	4	0.174	0.732	0.571	0.383	0.723	0.578	1.044	8.103	<0.001
T x S	4	0.199	0.840	0.502	0.340	0.640	0.635	0.671	5.210	<0.001
UV x T x S	8	0.053	0.225	0.986	0.437	0.823	0.583	0.374	2.902	0.005
Residual	130	0.237			0.530			0.129		
Corrected Total	161	0.647			1.024			0.755		

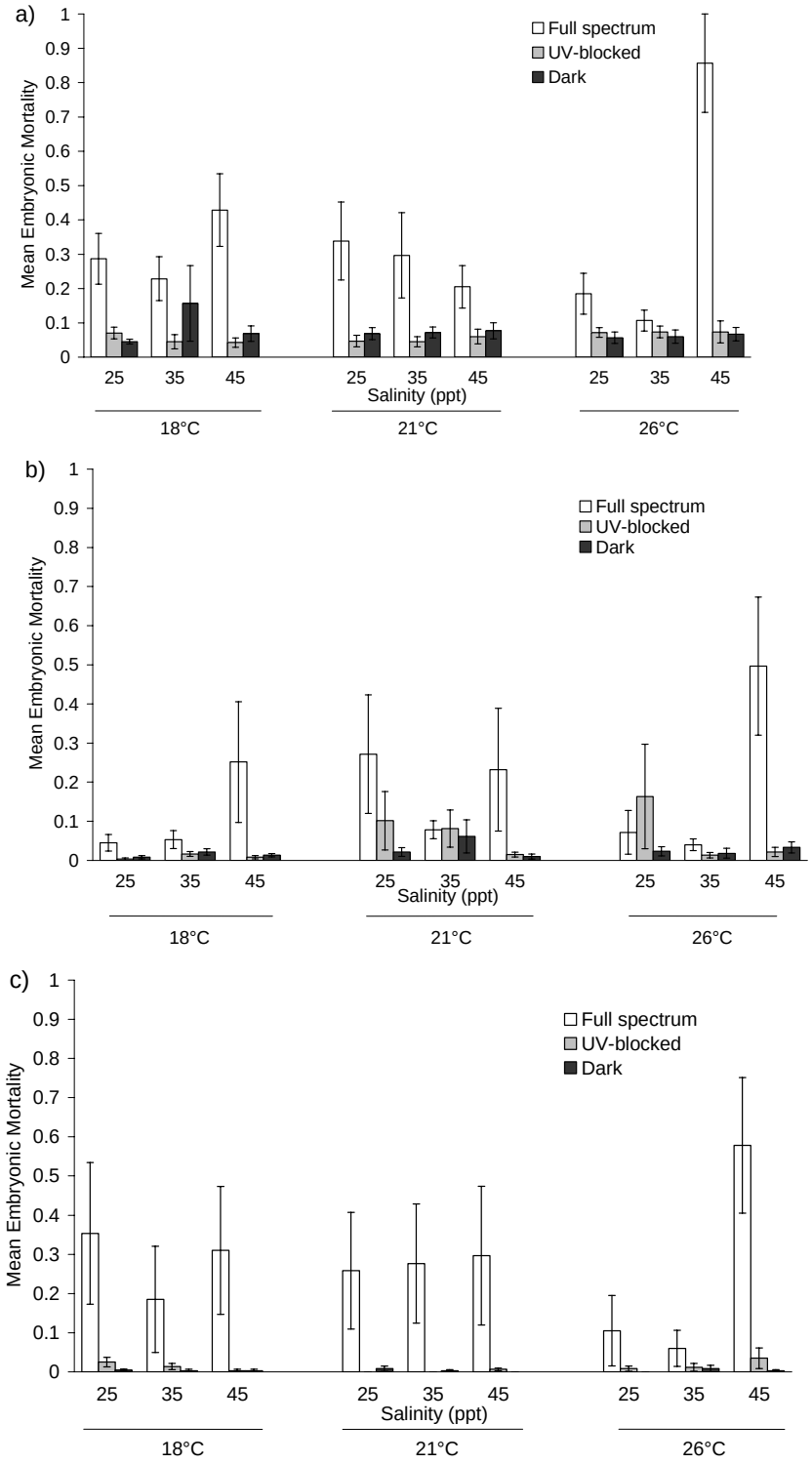


Figure 3.1.2 Effects of temperature, salinity, and spectral treatments on embryonic mortality of a) *Dolabrifera brazieri*, b) *Bembicium nanum* and c) *Siphonaria denticulata.* Error bars are standard error of mean, n = 6.

mortality for both *B. nanum* and *S. denticulata* at 45 ppt and also increased mortality at 25 ppt for *S. denticulata* (Figure 3.1.2b,c). In contrast, UVR exposure did not significantly affect embryonic mortality at 35 ppt as no significant differences were detected between spectral treatments at this salinity (Figure 3.1.2b,c). Furthermore, the embryos of both species were negatively affected by high salinity only when exposed to UVR (Figure 3.1.2b,c). I also detected a significant interaction between salinity and temperature on mortality of embryos of *S. denticulata* (Table 3.1.1a). When exposed to high temperatures, embryos showed significantly higher mortality at 45 ppt than at the lower salinity treatments (Figure 3.1.2c).

As anticipated, temperature had dramatic impacts on the developmental rates for all species (Table 3.1.1b), with lower temperatures slowing developmental rates (Figure 3.1.3, Appendix 1B). The other striking pattern was the depression in developmental rate in the presence of UVR. Embryos of all species developed more slowly when exposed to full spectrum sunlight relative to those exposed to UV-blocked sunlight (Figure 3.1.3). Developmental retardation in the presence of UVR was readily apparent at the highest temperatures to which I exposed egg masses, particularly for *B. nanum* and *S. denticulata*, (Figure 3.1.3b,c), although the significant three-way interaction complicated interpretation of the effects of UVR in isolation in the latter case (Table 3.1.1b). Salinity also affected the developmental rate of *B. nanum* (Table 3.1.1b), with embryos developing significantly more slowly in both extreme salinity treatments (Figure 3.1.3b), and *S. denticulata*, with development occurring significantly more slowly at 45 ppt in warm temperatures and UVR exposure (Figure 3.1.3c).

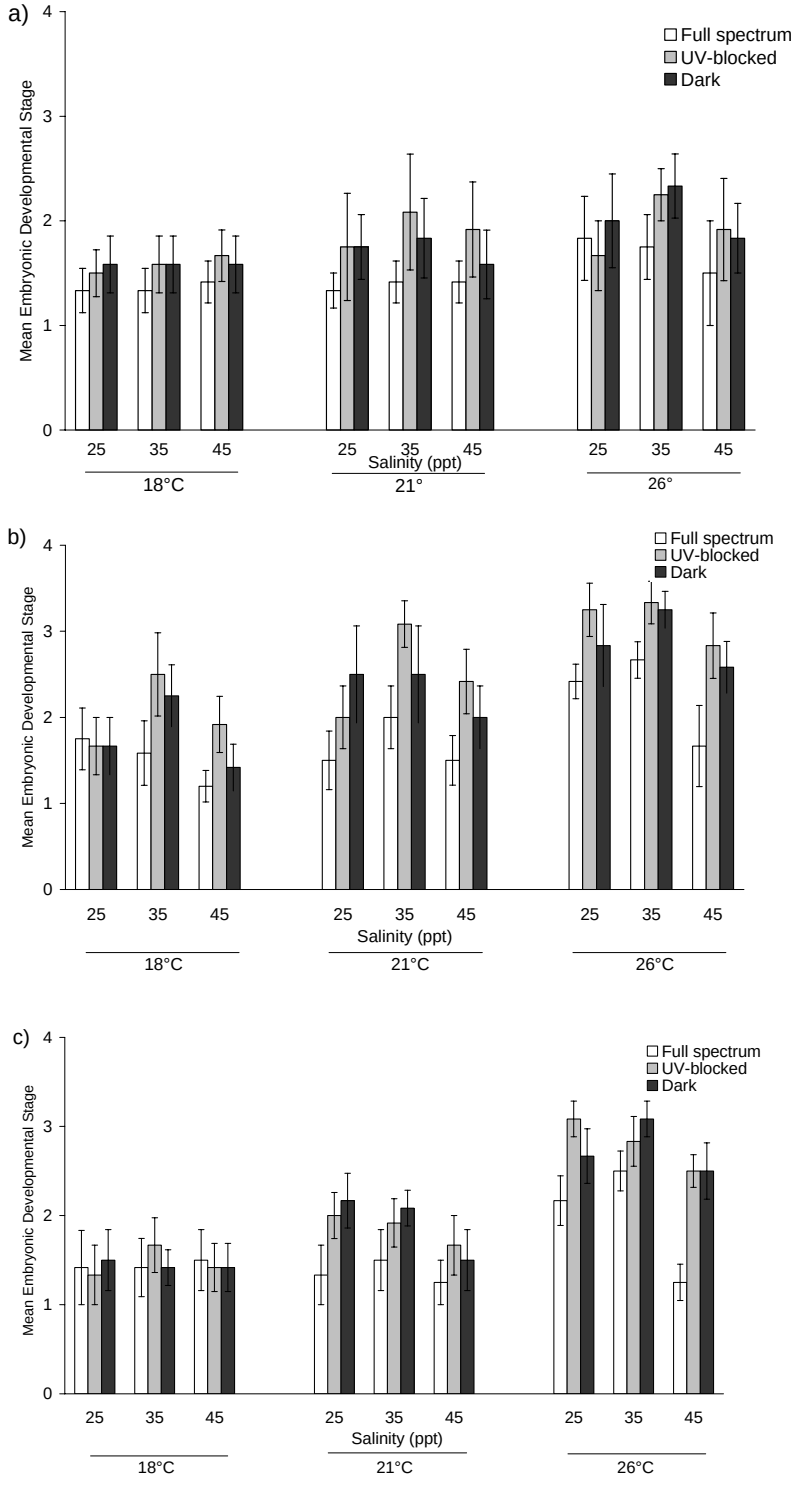


Figure 3.1.3 Effects of temperature, salinity, and spectral treatments on embryonic developmental rate of a) *Dolabrifera brazieri*, b) *Bembicium nanum* and c) *Siphonaria denticulata*. Developmental stages are classified as 1 = pre-trochophore, 2 = trochophore/early veliger, 3 = veliger, 4 = late veliger. Error bars are standard error of mean, n = 6.

3.1.4 DISCUSSION

Temperature, salinity, and UVR are potential stressors in the intertidal, and this study indeed revealed significant interactions of temperature, salinity, and UVR on embryonic mortality for all three gastropod species examined (Table 3.1.1a) as well as on developmental rates of two species (Table 3.1.1b). The magnitude of these effects could not have been predicted from manipulating these environmental variables in isolation. Indeed, for egg masses held in seawater (35 ppt) at 26^{O}C the effects of exposure to UVR were barely detectable, but at the highest salinity tested (45ppt), embryonic mortality soared (Figure 3.1.2). The importance of maintaining optimal environmental conditions for invertebrate development is well established (reviewed by Palmer 1994; Przeslawski 2004a). More recently, the focus on ecological impacts of the thinning ozone layer has demonstrated the hazards of UVR exposure to marine invertebrate embryos and larvae (e.g. Gleason & Wellington 1995; Adams & Shick 2001; Przeslawski *et al.* 2004). However, a typical focus on environmental factors in isolation may well underestimate their ecological effects. Indeed, studies on algae have demonstrated that the negative effects of UVR on some species can be exacerbated by ecologically realistic extreme salinities (Karsten *et al.* 2003) and increased temperature (Roos & Vincent 1998). Moreover, the interactive effects of temperature and salinity appear to be complex and species-specific (Hoffman *et al.* 2003). Altamirano *et al.* (2003) found that increased temperature worsened effects of UVR on one species of *Fucus*, but not the other two species examined. Furthermore, increasing temperature has been shown to decrease negative effects of UVR up to a certain temperature, after which rates of UVR-induced damage increase (Conkling & Drake 1984). Clearly, synergistic interactions among environmental variables can be extremely complex and have important implications for interpreting the effects of climate change on natural systems.

As I predicted, physiologically stressful conditions had a marked impact on the low shore species *Dolabrifera brazieri*, with embryonic mortality approaching 90% (Figure 3.1.2a). Under the same conditions, mortality among the two mid-high shore species was around 50% (Figure 3.1.2b,c), a surprising finding given that these species lay egg masses exposed to physiologically stressful conditions in the height of the austral summer and one may

expect them to be better adapted to associated stress (unpublished data). In addition, I observed that the rate of encapsulated development slowed, often markedly, when egg masses were exposed to environmental extremes (Figure 3.1.3). For example, UVR significantly reduced the mean embryonic developmental stage of *S. denticulata* when coupled with warm temperatures and extreme salinities (Figure 3.1.3c). Longer encapsulation periods are often associated with higher risks to developing intertidal embryos (e.g. Spight 1975; Havenhand 1993). Thus, species would be expected to spawn in sites that maximise their developmental rate while minimising exposure to potential stresses. However, *B. nanum* and *S. denticulata* spawn in habitats exposed to environmental stresses that prolong development and increase mortality. It has been suggested that spawning sites are not necessarily optimal for embryos, but are instead tied to adult life history or predator avoidance (Spight 1977).

It remains unclear by what mechanism(s) embryos are affected, although the direct action of UVR on nucleic acids or the production of oxygen free radicals likely contributes to the increased mortality and retarded development observed after UVR exposure (Epel *et al.* 1999). It is clear that a number of protective mechanisms have evolved as adaptations to cope with environmental stress. Heat-shock proteins may counteract the effects of elevated temperatures for intertidal organisms (Helmuth & Hoffman 2001), although to date the egg masses of only one mollusc species have been examined (Podolsky & Hoffmann 1998). Furthermore, the gelatinous matrix in some gastropod egg masses can buffer the rate and magnitude of salinity change (Woods & DeSilets 1997). Finally, the damaging effects of UVR can be reduced by modifying the exposure of eggs and larvae. UVR can be absorbed by the walls of some gastropod egg capsules (Rawlings 1996) or UVR screening compounds called mycosporine-like amino acids (MAAs) (Shick & Dunlap 2002). Concentrations of these compounds have recently been found in molluscan egg masses and vary dramatically among species (Przeslawski 2004b). A variety of organisms also use DNA repair mechanisms to protect embryos from negative effects of UVR (e.g. Blaustein *et al.* 1994). The rates of DNA repair are positively correlated with temperature so the net effect of UVR damage may be reduced with increasing temperatures (Macfadyen *et al.* 2004). All species examined in my study showed reduced mortality in full spectrum

treatments at 26˚C relative to 21˚C when high salinity treatments were excluded (Figure 3.1.2), a finding consistent with the presence of DNA repair mechanisms. As such, UVR-induced damage would be expected to be higher at lower temperatures; instead, all species in this study showed a slight decrease in mortality at 18°C compared to 21°C (Figure 3.1.2). This does not necessarily suggest that DNA repair mechanisms are not present in these egg masses or that they are not thermally dependent; rather, it could reflect slower developmental rates at lower temperatures which would also slow detection of UV-induced mortality. Levels of DNA repair enzymes have been correlated with the expected spectral exposure for amphibian eggs (Blaustein *et al.* 1994), but no similar research has been undertaken on eggs, embryos or larvae of marine invertebrates.

The effects of climate change are already apparent in natural systems with changes already occurring in aquatic trophic systems (Winder & Schindlera 2004) and the range and diversity of marine taxa (Riegl 2003; Hiscock *et al.* 2004). Increased UVR due to the thinning ozone layer may also increase stressful conditions to which intertidal organisms are exposed (Andrady *et al.* 2004). Although some studies indicate that the effects of UVR on natural marine systems are “weak and transitory” (e.g. Wahl *et al.* 2004), the present study underscores the risks associated with considering environmental variables in isolation. For example, previous research on the isolated effects of UVR on two species (*S. denticulata* and *B. nanum*) showed no significant differences in mortality based on spectral exposure (Przeslawski *et al.* 2004). In contrast, the present study reveals that these species are indeed significantly vulnerable to the negative effects of UVR when other abiotic stressors occur simultaneously. Clearly, as environmental variables interact, sometimes synergistically, there is a danger of underestimating the ecological impacts of global climate change (Hoffman *et al.* 2003). Such interactions are particularly important to dynamic habitats such as the intertidal because most environmental factors do not operate in isolation (Pechenik 1987 and references therein). Indeed, abiotic factors may also interact with biotic factors such as predation to affect the range and success of a species (Harley 2003). The use of multifactorial experiments to examine such effects at appropriate temporal and spatial scales represents a significant challenge to ecologists, but results from

this study indicate that potential interactions must be considered to properly assess potentially synergistic effects of increased UVR and global climate change.

3.2 UVR & DESICCATION: COMBINED EFFECTS OF SOLAR RADIATION AND DESICCATION ON THE ENCAPSULATED DEVELOPMENT OF ROCKY SHORE GASTROPODS

Intertidal encapsulated embryos may be synchronously exposed to many environmental stressors, but interactions between some of these factors remain poorly understood. Here, the effects of solar radiation and desiccation on embryonic mortality and developmental rates were assessed with laboratory and field experiments. Egg masses of three intertidal gastropod species were exposed for 72 hours to combinations of spectral (full spectrum, UV-blocked, dark) and daily emersion treatments (control, 15-, 30-, 60-minutes). Siphonaria denticulata *and* Bembicium nanum *embryos were expected to be tolerant to emersion and UVR as they are routinely deposited on exposed rock platforms. In contrast,* Dolabrifera brazieri *embryos were predicted to be vulnerable to these stressors as they are deposited in shaded, submerged habitats. Lab experiments revealed that light treatments and desiccation negatively affected the mortality and developmental rate of* D. brazieri. *The mortality of* B. nanum *did not significantly increase after UVR-exposure or emersion, and the developmental rate was significantly faster in light treatments than dark. Surprisingly, embryonic mortality of* S. denticulata *was significantly higher in UV-blocked treatments than full spectrum treatments after 60-minute emersion periods, but neither spectral treatments nor desiccation periods affected developmental rates. Field observations were also conducted to investigate the natural effects of desiccation on the embryonic mortality of* S. denticulata *and* B. nanum. *Despite the apparent resistance of these embryos to UVR and desiccation in the laboratory, mortality was significantly higher in desiccated habitats than in submerged habitats in the field, thus suggesting that selection of these spawning sites may not be optimal for embryos, particularly in light of global change.*

3.2.1 INTRODUCTION

Intertidal environments can be extremely harsh and complex, comprising dynamic microhabitats such as exposed shallow rock pools where temperature, salinity, spectral exposure and immersion vary daily. In contrast, other intertidal microhabitats are relatively

stable, such as the undersides of submerged boulders. Embryos and larvae are considered the most vulnerable life stage for most marine invertebrates (Thorson 1950; Jackson & Strathmann 1981) so environmental conditions of the microhabitats in which they develop are particularly important to their survival. Adults can reduce risk to their offspring from environmental stresses by spawning in relatively stable environments such as under boulders or in the subtidal. However, some species routinely deposit their egg masses in exposed habitats in which embryos are vulnerable to damaging ultraviolet radiation (UVR) and periods of desiccation for the duration of their encapsulation (Benkendorff & Davis 2004).

Surface UVR comprises UV-A (315-400 nm) and UV-B (280-315 nm) and can cause a range of deleterious effects on aquatic organisms including DNA damage, developmental abnormalities, and behavioural changes (reviewed by Haeder *et al.* 1998; Day & Neale 2002; Paul & Gwynn-Jones 2003). UVR exposure increases embryonic mortality of some corals (Gleason & Wellington 1995), sea urchins (Adams & Shick 1996), algae (Altamirano *et al.* 2003), and encapsulated molluscs (Biermann *et al.* 1992; Rawlings 1996). The effects of UVR on embryonic mortality of gastropods are species specific; encapsulated embryos of species that spawn in the sun are less vulnerable to the negative effects of UVR than embryos of those species that only spawn in the shade (Przeslawski *et al.* 2004). Recently, stratospheric ozone has decreased due to anthropogenic activity; UV-B flux at the Earth's surface has increased accordingly, thereby increasing exposure of organisms to these damaging wavelengths (WMO 1998). Importantly, UVR does not operate in isolation and has been shown to significantly interact with temperature and salinity to further increase embryonic mortality.

Desiccation is one of the most important factors contributing to embryonic mortality in the intertidal (Chambers & McQuaid 1994; Ocana & Emson 1999), but there is little empirical research on the effects of desiccation on encapsulated invertebrates (Rawlings 1999). The few existing studies have linked desiccation to decreased encapsulation periods (Pechenik *et al.* 2003) and increased mortality (Pechenik 1978; Creese 1980; Gosselin & Chia 1995; Yaroslavtseva *et al.* 2001). UVR exposure and desiccation often occur concurrently during

low tides, and research on bryophytes suggests that combined effects of UVR and desiccation exposure may be responsible for tolerance to these individual stressors (Takacs *et al.* 1999). Nevertheless, the potential synergistic effects on invertebrate development have surprisingly not yet been examined.

In this study, multifactorial laboratory experiments were conducted to determine the effects of UVR exposure and desiccation on the developmental rates and embryonic mortality of three common intertidal gastropods, the pulmonate *Siphonaria denticulata*, the opisthobranch *Dolabrifera brazieri*, and the littorinid *Bembicium nanum.* These species were chosen because they are abundant along the southeastern Australian coast, and they spawn year-round (see Chapter 4.1). Furthermore, their egg masses are deposited in habitats with differential exposure to environmental stressors such as UVR and desiccation. Encapsulated embryos are unable to seek shelter during periods of desiccation and UVR exposure associated with low tides, and species would thus be expected to spawn in habitats that are conducive to successful embryonic development (Pechenik 1978). Indeed, *D. brazieri* spawns underneath submerged boulders, but both *B. nanum* and *S. denticulata* spawn in sunny habitats prone to desiccation during low tides (Figure 3.1.1). Therefore, I predicted that embryos of *B. nanum* and *S. denticulata* would be less vulnerable to UVR and desiccation than *D. brazieri*, thus reflecting adaptation to stressors that occur frequently in their natural habitat. Complementary field observations were also conducted to determine natural environmental conditions and embryonic mortality of the two species that spawn in dynamic habitats, *S. denticulata* and *B. nanum.* The potential risks of exposure to UVR and desiccation may be outweighed by faster developmental rate (Pechenik *et al.* 2003; Przeslawski *et al.* 2004), and it is hoped that results from the present study will clarify this trend and highlight reasons for selection of apparently risky spawning sites.

3.2.2 METHODS

Laboratory Experiment

Undeveloped egg masses of *Siphonaria denticulata, Dolabrifera brazieri,* and *Bembicium nanum* were collected from the rocky shores of Bass Point (34^{o}35’45”S, 150^{o}53’20”E) in

southeastern New South Wales in April 2004 (Figure 2.1). An egg mass is defined as the entire discrete gelatinous mass of a given species; thus, for *B. nanum*, an egg mass comprises individual jelly capsules closely connected by a gelatinous matrix. *S. denticulata* egg masses are spiralled cylindrical gelatinous ribbons (Figure 3.1a); *D. brazieri* egg masses are flattened continuous gelatinous ribbons deposited in a characteristic zig-zag pattern containing (Figure 3.1b); and *B. nanum* egg masses are composed of several discrete gelatinous compartments (Figure 3.1c) (see Chapter 3.1 for egg mass sizes and egg densities). Six egg masses were collected from each species and were examined microscopically to confirm pre-trochophore stage (x 40 magnification).

Each egg mass was divided into 12 pieces of approximately equal size across species and placed in plastic containers (height 2.5 cm, diameter 6 cm) within a 300-litre outdoor recirculating seawater system. Previous research has revealed that cutting gelatinous egg masses does not deleteriously affect them (Rose 1986). Each container was drilled with 20 holes of 1mm diameter to allow water flow; the containers represented a combination of desiccation and spectral treatments. Spectral treatments were attained with cutoff filters whose transmission properties were confirmed with a spectrophotometer (Shimadzu UV1601): 1) dark attained by black plastic which allowed <2% PAR and 0% UVR, 2) UV-blocked achieved with polyethylene film with UV absorption which allowed 85% transmittance from 420-800 nm with a sharp decline at 420 nm to 0% at 350 nm, and 3) full spectrum attained with clear polyethylene cling wrap which allowed >95% transmittance from 250-800 nm (see Figure 2.2 for transmission properties). Desiccation treatments were conducted by removing egg masses from the main tank at noon and exposing them to air under their assigned cut-off filters for 15, 30 and 60 minutes. Egg masses were desiccated simultaneously and on the same plastic surface so that they were exposed to uniform conditions during emersion. Control egg masses were not exposed to air at all. In addition, all egg masses were randomly reoriented in containers each day. Water temperature was monitored with a thermal microchip (Thermochron I-Button) and was 19.90 ± 0.92ºC. Air temperature during desiccation was randomly monitored with a standard thermometer at approximately 22ºC, and no thermal difference was found between spectral treatments. Salinity was maintained at 35 ppt with a handheld refractometer and the addition of

distilled water. Egg masses were left in the experimental tank for 72 hours and were thus desiccated 3 times. This time period represented ecologically realistic exposure to stressors as evidenced by previous field observations (Przeslawski et al. 2005, per obs) and observations of encapsulation period of approximately 3-5 days for these species at water temperatures of 25°C (unpublished data). Furthermore, this time period allowed embryos to develop to a stage at which embryonic mortality could be detected.

After 72 hours in experimental treatments, all egg mass pieces were removed and examined with a dissection microscope (40x magnification). Mortality of peripheral embryos was estimated for each piece by randomly sampling 100 embryos on both sides of the egg mass and scoring alive and dead embryos. Thus, superficially, the sample size seems small, but the actual number of embryos examined in this study is over 21,000 (6 egg masses x 12 treatments x100 embryos per egg mass x 3 species). Embryos were considered dead if they were degenerating or relatively underdeveloped. Developmental stage was also recorded and defined as the stage in which > 50% of embryos within each egg mass piece were found. If two stages were equally represented within one piece, the mean stage was recorded. Developmental stages were categorized as: (1) Non ciliated (pre-trochophore), (2) Ciliated with no shell (trochophore/ early veliger), (3) Partial shell (veliger), (4) Full shell (late veliger). 2-factor ANOVAs were then performed on the data for each species with the restricted maximum likelihood (REML) technique in the statistical package JMP 4 where alpha = 0.05. Due to binomial distribution of residuals versus predicted values, all mortality data was transformed with a standard arcsin transformation such that ANOVA assumptions of heterogenous variances and normality were valid (Zar 1998). Tukey's HSD tests revealed significant relationships.

Field Observations

During the austral summer of 2003-2004, mature egg masses of *Siphonaria denticulata* and *Bembicium nanum* were randomly collected from mid-tidal zones on rocky shores along the Illawarra coast in southeastern New South Wales. Egg masses of *Dolabrifera brazieri* were not used in field observations as habitats with differential conditions did not exist; in a two-year survey, egg masses from this species were only found in submerged and shaded

habitats (Chapter 4.1). Collection occurred on the third day of low spring tide cycles (<.2 metres) where weather conditions had been similar and uniform across sites for all three days. Mature egg masses were identified by colour change associated with developed veliger shells or the relaxed gelatinous characteristic of mature or unviable egg masses of these species (Figure 3.1c). If an egg mass was submerged, water temperature was recorded, and a sample of the water was collected for salinity measurement in the laboratory at 20°C with a handheld refractometer.

Egg masses were examined in the laboratory under a dissecting microscope (40x magnification). Embryonic mortality was determined for each egg mass as described previously. In addition, surface algal fouling was recorded with visual quantification methods described in (Przeslawski & Benkendorff 2005). A two-way ANOVA was used to detect differences in embryonic mortality (arcsin transformed) and developmental rate between egg masses from static, moderate, extreme, and desiccated habitats. Static habitats had salinities of 35 ppt and temperatures below 22˚C, coinciding with conditions of open water environments during summer. Moderate habitats had salinities between 31-34 or 36-39 ppt and temperatures under 28˚C. Extreme habitats had salinities greater than 40 ppt or temperatures higher than 28˚C. Desiccated habitats were those in which water had evaporated leaving the egg mass exposed to the air. Tukey's tests were used to determine significant relationships.

Table 3.2.1 The effects of spectral treatment and desiccation on a) embryonic mortality (arcsin transformed), and b) developmental rate of three gastropod species as determined by ANOVAs with restricted maximum likelihood with random factors italicised (n = 6).

a)

		Siphonaria denticulata			*Dolabrifera brazieri*			*Bembicium nanum*		
Source	df	MS	F	p	MS	F	p	MS	F	p
Egg mass	5	0.4145	13.5890	<0.0001	0.0288	0.2925	0.9151	0.0005	0.4532	0.8091
Spectral (UV)	2	0.1982	6.4964	0.0029	1.7656	17.8994	<0.0001	0.0007	0.5920	0.5567
Desiccation (D)	3	0.0607	1.9907	0.1260	5.6643	57.4254	<0.0001	0.0023	1.9644	0.1300
UV x D	6	0.0792	2.5973	0.0274	0.2908	2.9478	0.0144	0.0024	2.1109	0.6666
Residual	55	0.0305			0.0986			0.0011		
Corrected Total	71	0.0698			0.3990			0.0012		

b)

		Siphonaria denticulata			*Dolabrifera brazieri*[1]				*Bembicium nanum*		
Source	df	MS	F	p	df	MS	F	p	df	MS	F	p
Egg mass	5	3.3424	34.4016	<0.0001	5	1.6300	4.2770	0.0060	5	0.7636	5.6000	0.0003
Spectral (UV)	2	0.1354	1.3938	0.2568	2	0.1736	0.4555	0.6393	2	0.6354	4.6597	0.0135
Desiccation (D)	3	0.1238	1.2746	0.2921	1	1.7778	4.6647	0.0406	3	0.0926	0.6790	0.5686
UV x D	4	0.2141	2.2038	0.0563	2	0.2986	0.7835	0.4677	6	0.0752	0.5517	0.7665
Residual	55	0.0972			25	0.3811			55	0.1364		
Corrected Total	71	0.3446			35	0.6344			71	0.1972		

[1] ANOVA was only conducted on egg masses in control and 15 minute emersion treatments due to high number of completely unviable egg masses in 30 and 60 minute emersion treatments.

3.2.3 RESULTS

Laboratory experiment

Spectral treatment and desiccation time interacted significantly to affect the mortality of *Siphonaria denticulata* ($p = 0.0274$, Table 3.2.1a) and *Dolabrifera brazieri* ($p = 0.0144$, Table 3.2.1a). Mortality of *S. denticulata* embryos was significantly higher in UV-blocked treatments than full spectrum treatments after 60 minutes desiccation as confirmed by Tukey's tests (see Appendix 3A). Furthermore, mortality of these UV-blocked embryos increased steadily and significantly between 0 and 60 minutes of desiccation (Figure 3.2.1a). In contrast, there were no significant effects of desiccation in dark treatments (Figure 3.2.1a). Mortality of *D. brazieri* embryos was significantly lower under dark conditions than either light treatment for all egg masses subject to desiccation; egg masses that were not desiccated showed no difference in embryonic mortality among spectral treatments (Figure3.2.1b, Appendix 3A). Moreover, embryonic mortality of *D. brazieri* significantly increased as desiccation time increased for egg masses in all spectral treatments as confirmed by Tukey's tests (Figure 3.2.1b). Embryonic mortality of *Bembicium nanum* was unaffected by either spectral treatment or desiccation (Table 3.2.1a, Figure 3.2.1c).

The developmental rate of *Bembicium nanum* was affected by spectral treatment ($p = 0.0135$, Table 3.2.1b, Figure 3.2.2c) with embryos in the dark developing significantly slower than embryos in both light treatments as revealed by Tukey's tests (Appendix 3B). Embryonic developmental rates of *Siphonaria denticulata* were unaffected by UVR or desiccation (Figure 3.2.2a, Table 3.2.1b). There were many *Dolabrifera brazieri* egg masses that were completely unviable after full spectrum treatments with 30 and 60 minutes emersion (Figure 3.2.1b), and developmental data was not available for these samples. Thus, the developmental rate of *D. brazieri* was only analysed for control samples and those subject to 15-minute emersion treatments. Desiccation significantly affected developmental rate ($p = 0.0406$, Table 3.2.1b) with more mature embryos occurring in control egg masses than those egg masses subject to 15 minutes emersion (Figure 3.2.2b).

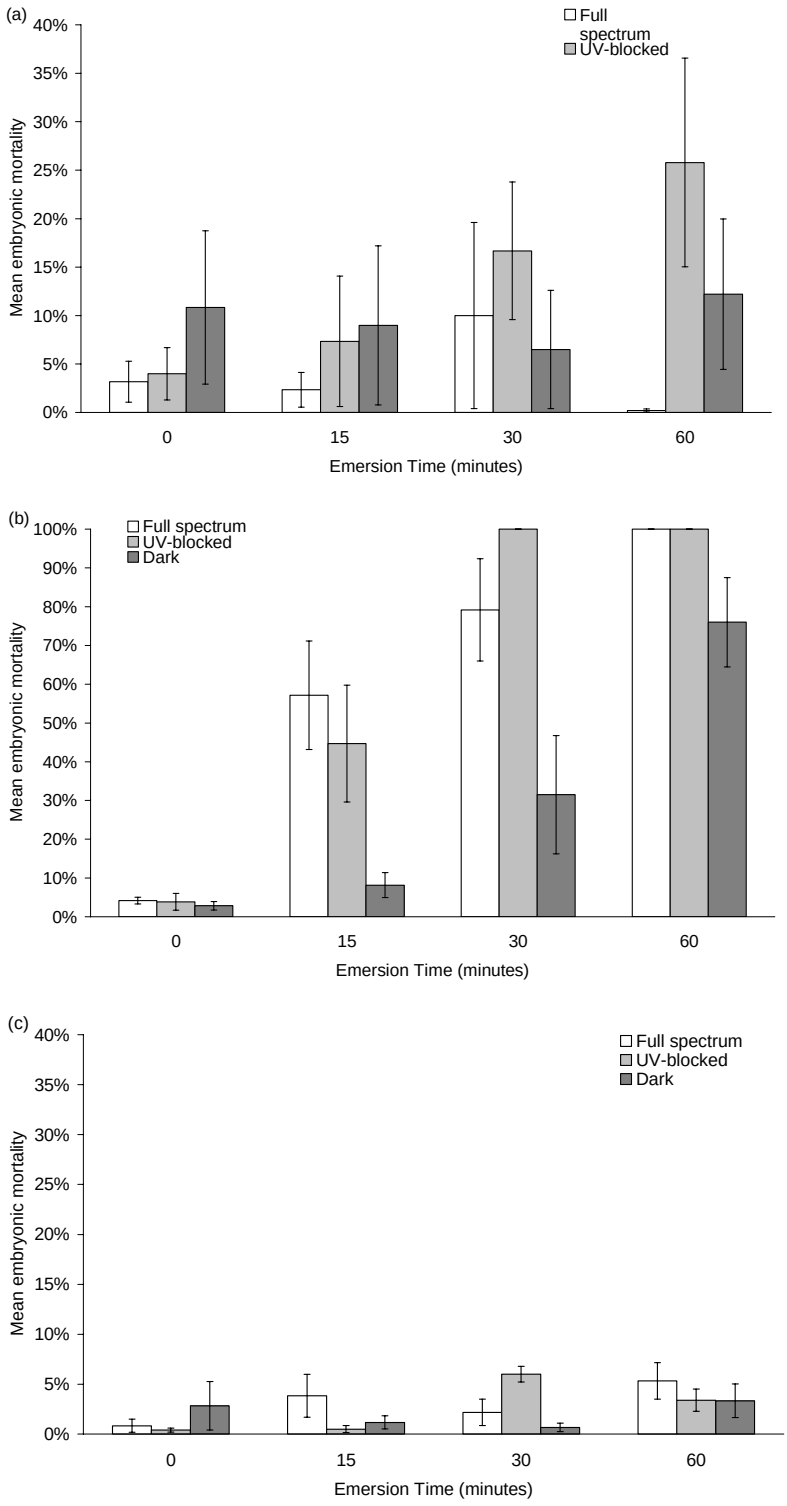


Figure 3.2.1 The effects of spectral treatments and emersion times on the embryonic mortality of a) *Siphonaria denticulata*, b) *Dolabrifera brazieri*, and c) *Bembicium nanum*. Error bars are standard error of mean; n = 6.

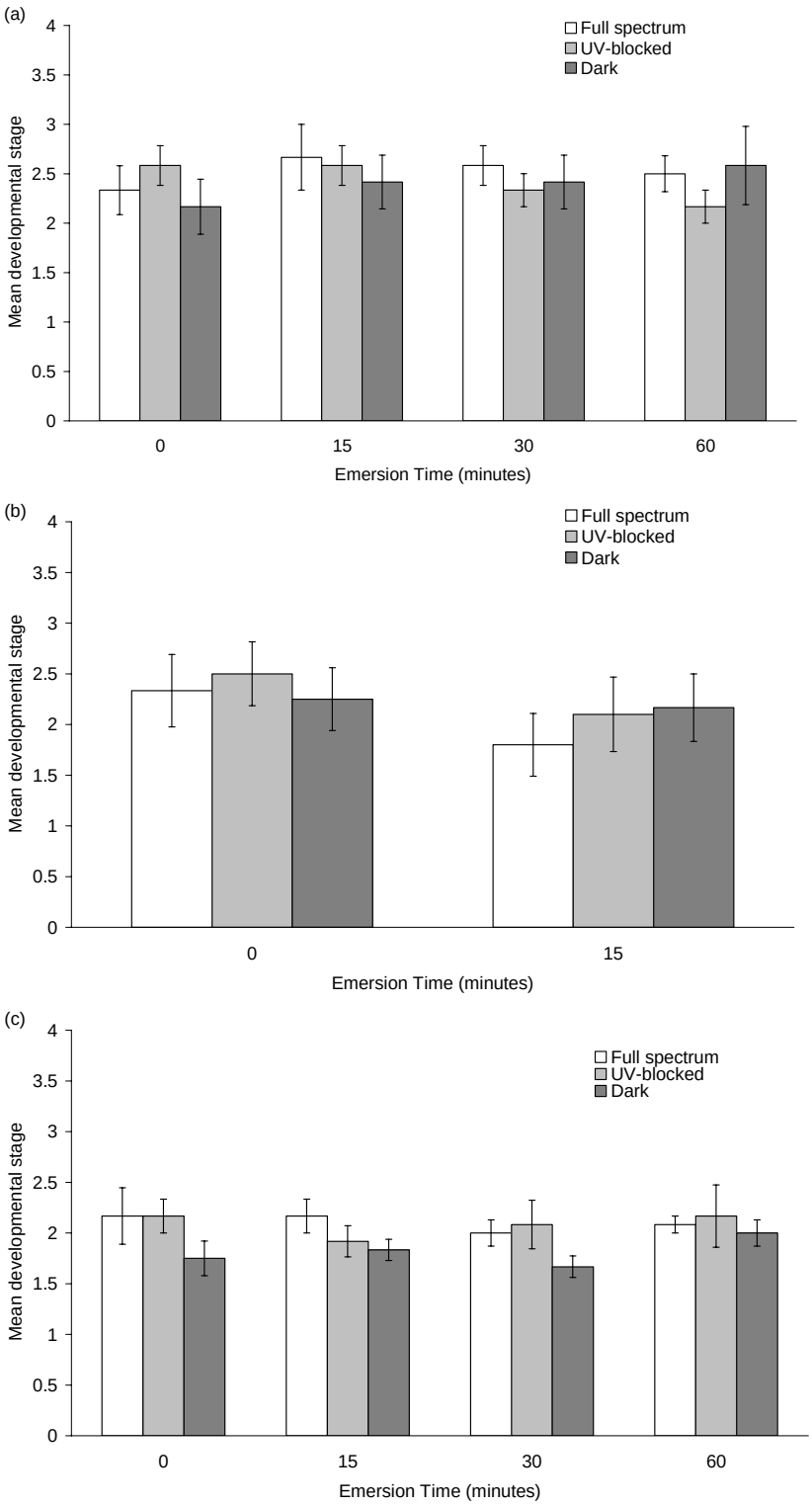


Figure 3.2.2 The effects of spectral treatments and emersion times on the developmental rate of a) *Siphonaria denticulata*, b) *Dolabrifera brazieri*, and c) *Bembicium nanum*. Error bars are standard error of mean; n = 6.

Field observations

A total of 82 *Bembicium nanum* and 86 *Siphonaria denticulata* egg masses were collected, and all egg masses were found in habitats exposed to sunlight. For both species, the least number of egg masses were found in static habitats (Figure 3.2.3), and over 70% of their egg masses were found in moderate or extreme habitats (Figure 3.2.3). Egg masses from desiccated habitats represented 18% of total *B. nanum* egg masses and 23% of total *S. denticulata* egg masses (Figure 3.2.3).

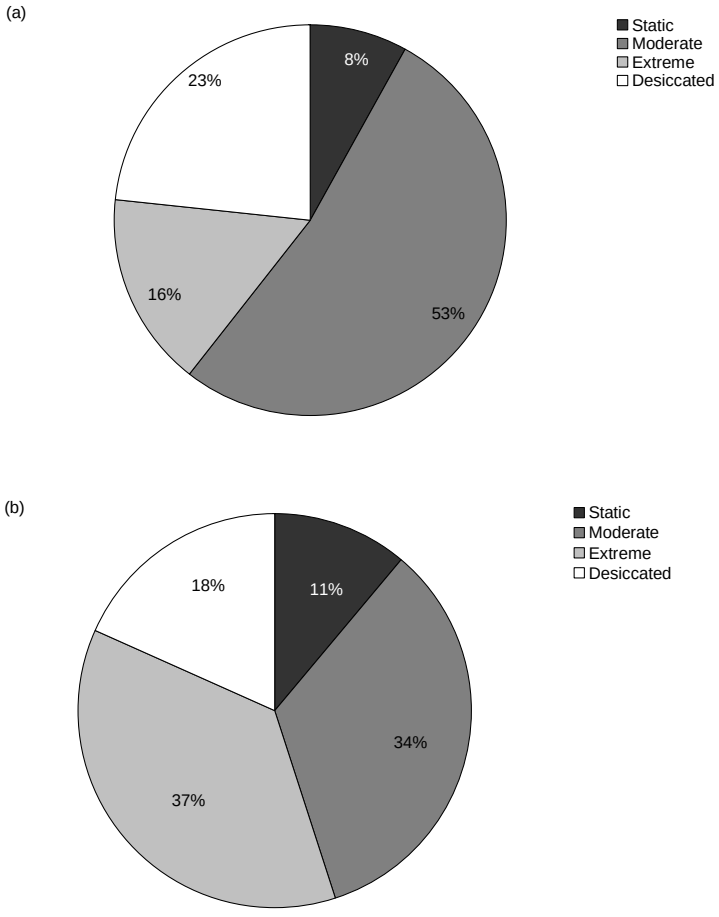


Figure 3.2.3 Egg mass abundance of a) *Siphonaria denticulata* (n = 86) and b) *Bembicium nanum* (n = 82) according to conditions during low tide of the microhabitat in which they were deposited. Static habitats had salinities of 35 ppt and temperatures below 22˚C, coinciding with conditions of open water environments during summer. Moderate habitats had salinities between 31-34 or 36-39 ppt and temperatures under 28˚C. Extreme habitats had salinities greater than 40 ppt or temperatures higher than 28˚C. Desiccated habitats were those in which water had evaporated leaving the egg mass exposed to the air.

Spawning habitat significantly affected embryonic mortality of both species as confirmed by a 2-way ANOVA ($p < 0.0001$, Table 3.2.2). Tukey's tests confirmed that embryonic mortality of *Siphonaria denticulata* and *Bembicium nanum* from desiccated habitats was significantly higher than those from habitats in which they were still submerged during low tides (Figure 3.2.4). Spawning habitat had no significant effect on algal fouling ($F = 1.2557$, $p = 0.2929$), but the above dataset was analysed again with the exclusion of egg masses with over 20% algal fouling to remove this potentially confounding factor (Przeslawski & Benkendorff 2005). Significant relationships were unchanged (data not shown).

Table 2 The effects of spawning microhabitat on embryonic mortality (arcsin transformed) of *Siphonaria denticulata* (n = 86) and *Bembicium nanum* (n = 82) as determined by a 2-factor ANOVA (see text in 'Methods' for habitat class definitions).

	df	MS	F	p
Species	1	0.0292	0.2663	0.6065
Habitat class	3	3.8014	34.7192	<0.0001
Species x Habitat class	3	0.1853	1.69250	0.1708
Residual	160	0.1095		
Corrected total	167	0.1758		

3.2.4 DISCUSSION

This study revealed that ultraviolet radiation and desiccation time significantly affect both embryonic mortality and developmental rate of certain gastropods and that these effects may be synergistic. Moreover, field observations reveal that egg masses of *Siphonaria denticulata* and *Bembicium nanum* are frequently deposited in areas in which they are subject to desiccation (Figure 3.2.3). As such, embryos of *B. nanum* and *S. denticulata* may be better protected against the negative effects of UVR and desiccation than embryos of *Dolabrifera brazieri*. Indeed, the effects of spectral treatment and desiccation time are species specific (Table 3.2.1). As expected, *D. brazieri* had the highest overall mortality which increased under light and longer periods of emersion (Figure 3.2.1b). Surprisingly, these effects do not seem to be caused by UVR. Rather, visible light is detrimental to this species as the UV-blocking light treatment significantly increased embryonic mortality (Figure 3.2.1b). Previous studies have found that *D. brazieri* embryos are generally no

more vulnerable to synergistic effects of UVR, temperature, and salinity than the other two species (Przeslawski et al. 2005), but the present study indicates that *D. brazieri* is vulnerable to light and possibly UVR when subject to desiccation. Although not synergistic (i.e. additive), the interactions of these two factors may thus play a key role in limiting the spawning habitat of this species.

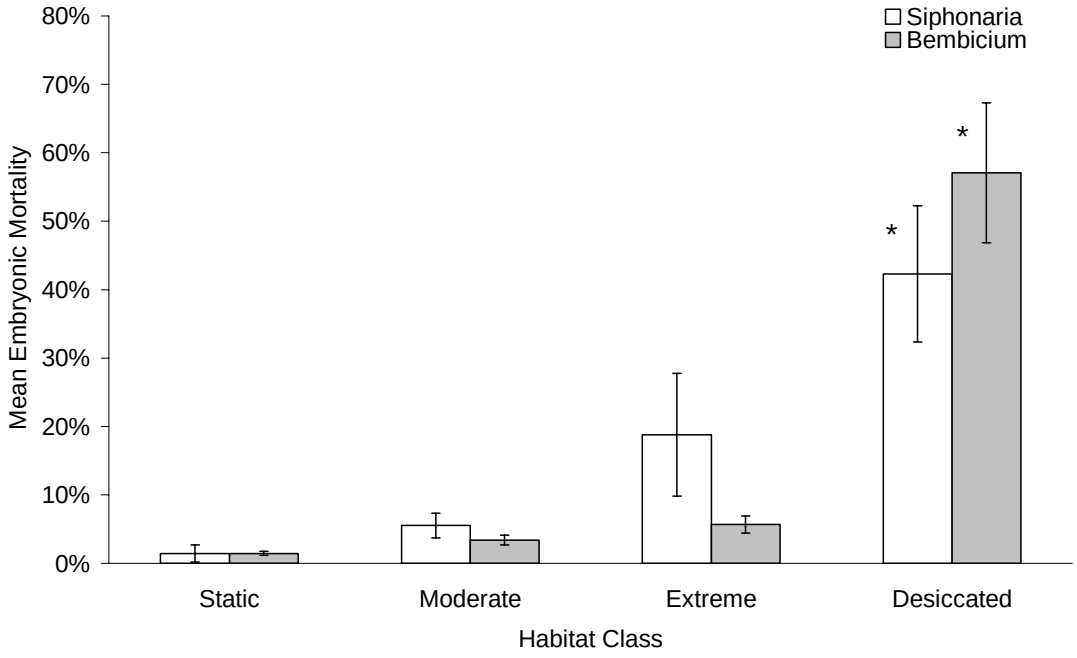


Figure 3.2.4 The effects of microhabitat on the embryonic mortality of *Siphonaria denticulata* and *Bembicium nanum* (see Figure 3.2.3 caption for microhabitat classifications). * indicates a significant differences at p = 0.0001

As predicted, embryos of *Siphonaria denticulata* and *Bembicium nanum* appear to be well adapted to UVR exposure and periodic desiccation. The overall mortality of *B. nanum* in the laboratory was relatively low and not increased by either stressor (Table 3.2.1a, Figure 3.2.1c). Furthermore, developmental rate of this species was faster in light treatments than in the dark (Figure 3.2.2c), thereby reducing time spent in vulnerable encapsulated embryonic stages (Spight 1975; Havenhand 1993). Similarly, previous research examining the isolated effects of UVR and light on embryonic development of 22 gastropod species

found that light exposure decreased encapsulation period (Przeslawski *et al.* 2004). These trends may reflect a currently unknown, direct response to light or UVR. For example, UVR exposure can stimulate production of mycosporine-like amino acids in some organisms, and these compounds may help regulate development (Shick & Dunlap 2002). Alternatively, rising temperatures associated with sunlight may have increased developmental rate (Palmer 1994). Although air and water temperatures showed no significant differences between treatments in this study, internal temperature of egg masses was not monitored and may have been slightly higher for *B. nanum* in light treatments.

In contrast to spectral treatments, desiccation did not positively affect developmental rate of any species examined in this study. Faster development rates after desiccation have previously been observed in encapsulated gastropods (Pechenik *et al.* 2003) and may reflect increased oxygen availability to the embryos (Strathmann & Hess 1999). The lack of faster development after desiccation does not necessarily suggest no increase in oxygen availability; rather, it may reflect other factors that retard development such as salinity fluctuations or photodamage (Przeslawski 2004a).

Unlike *Bembicium nanum*, the developmental rate of *Siphonaria denticulata* was not significantly faster in the light (Figure 3.2.2a), but embryos showed a similar resistance against the negative effects of UVR (Figure 3.2.1a). Surprisingly, embryos of this species showed lower mortality in full spectrum treatments than UV-blocked treatments irrespective of desiccation time. This trend was particularly striking after an hour of emersion where embryonic mortality under full spectrum treatments was negligible, but over 25% of embryos died under UV-blocked treatments (Figure 3.2.1a). These results may be explained by the presence of UV-A inducible repair mechanisms in encapsulated embryos of *S. denticulata*. Such mechanisms have not been investigated in marine invertebrates, but they have been found in other marine organisms. Previous research has shown that UV-A induces the production of the antioxidant coenzyme Q in marine bacteria (Dunlap et al. 2002) and carotenoids in marine microalgae (Jahnke 1999). UV-A can also stimulate the production of DNA polymerase alpha in diatoms, a necessary enzyme for eukaryotic cell replication and repair (Wei et al. 2004). In the present study, UV-blocking

treatments may have eliminated cues for the initiation of similar repair processes in *S. denticulata;* and damage from long emersion periods accumulated, thereby resulting in the death of many embryos. Further investigations on *S. denticulata* should incorporate UV-B blocking treatments to isolate the effects of UV-A. In addition, examining the rates of DNA and protein damage and repair may reveal why more embryos die without UVR exposure when synchronously desiccated for relatively long periods.

Despite the apparent tolerance of *Siphonaria denticulata* and *Bembicium nanum* embryos to UVR and desiccation in the laboratory, field results indicate that they are indeed vulnerable to desiccation and potential synergistic effects with UVR. The leathery egg capsules of neogastropods do not seem to provide sufficient embryonic protection against the stresses associated with emersion (Rawlings 1999), and results from the present study indicate this may also apply to species that deposit structurally different gelatinous egg masses. Mature embryos from desiccated habitats had significantly higher mortality than those from all submerged habitats (Figure 3.2.4). This suggests that periods of emersion in natural environments can exceed an hour, thus resulting in the high mortalities observed in the field. Moreover, the effects of splash are likely negligible for many egg masses, and the effects of wind may increase desiccation rates in the field. The laboratory study here only examined emersion times up to an hour, and longer desiccation periods may have revealed negative effects similar to those observed in *Dolabrifera brazieri* (Figure 3.2.1b). Indeed, my field results support previous observations by Creese (1980) in which more *S. denticulata* embryos died in high shore habitats than low-shore habitats, presumably due to longer emersion periods.

The vulnerability of these embryos to desiccation and UVR in the field suggests that they may be negatively impacted by global change, including stratospheric ozone depletion and climate change. Stratospheric ozone depletion results in elevated levels of incident UV-B (WMO 1998), and global climate change may herald shifts in temperature, precipitation, and even sea level (Karl & Trenberth 2003). As such, embryos of *B. nanum* and *S. denticulata* may be synchronously exposed to elevated UVR and temperature and changes in salinity and emersion periods. Intertidal organisms are vulnerable to the negative effects

of interactions between abiotic stressors associated with global change (Hoffman *et al.* 2003; Przeslawski *et al.* 2005), and the present study corroborates these findings in relation to UVR and desiccation.

The potential protective mechanisms of these embryos are unknown, but it is likely the capsule wall and / or gel matrix reduce some water loss. Multifactorial studies on both encapsulated embryos and pelagic larvae may clarify the role of encapsulating structures in protection against desiccation. Previous studies have found that water loss was also affected by egg mass size (Bayne 1968b; Strathmann & Hess 1999) and egg mass shape (Chambers & McQuaid 1994; Strathmann & Hess 1999). Indeed, a study on Australian *Siphonaria* sp. suggested that coiled egg ribbons similar in shape to *S. denticulata* have the highest rates of water loss (Chambers & McQuaid 1994). Results from this study indicate that *S. denticulata* is in fact tolerant of emersion periods up to an hour, and it may be that water loss rates are not necessarily indicative of negative embryonic responses (e.g. Meyer & Santarius 1998).

Protection against UVR may be provided by specialised compounds called mycosporine-like amino acids (MAAs) that absorb UVR and act as chemical sunscreens (reviewed by Shick & Dunlap 2002). MAAs occur in egg masses of all three species examined in this study (Przeslawski 2004b), but have been found to significantly decrease mortality of only *Bembicium nanum* embryos in the presence of full UVR (unpublished data). Furthermore, DNA repair mechanisms and/or antioxidants may help mitigate UVR damages, and there is some evidence that *Siphonaria denticulata* may possess such photoprotective mechanisms based on extremely high survivorship of egg masses with low MAA concentrations after UVR exposure (Wraith *et al.* in prep).

Laboratory and field observations from this study reveal that interactions between desiccation and UVR have similar negative effects on intertidal gastropod development to those observed between UVR, temperature and salinity (Przeslawski *et al.* 2005). This suggests that effects of abiotic factors in the intertidal and other dynamic habitats may be underestimated if potential interactions are not considered. Furthermore, interactions

between these factors are species-specific as confirmed by the present study and (Przeslawski *et al.* 2005). Thus, generalisations concerning interactive effects of abiotic factors should be made cautiously and only when numerous species have been examined lest oversimplification occurs.

The upper vertical limit of gastropods on rocky shores often depends on adult tolerances to desiccation (Underwood 1979), but this study has shown that the same trend does not necessarily apply to distribution of encapsulated offspring. Despite their vulnerability to desiccation, both *S. denticulata* and *B. nanum* spawn in potentially risky habitats, suggesting that embryonic welfare may not be a driving force in spawning site selection for these species. Instead, spawning site preference may be tied to adult behaviour (Spight 1977). Alternatively, other benefits such as reduction of predation may outweigh the potential risks associated with emersion and UVR-exposure. Based on the abundances of these species on many southeastern Australian rock platforms, their spawning habitats are obviously conducive to successful development and recruitment of offspring despite the potential risks of desiccation and UVR.

3.3 UVR & FOULING: THE ROLE OF SURFACE FOULING IN THE DEVELOPMENT OF ENCAPSULATED GASTROPOD EMBRYOS

Surface fouling and UVR are potential environmental stressors for intertidal egg masses and may be detrimental to encapsulated molluscan development. This study attempted to synchronously examine the effects of fouling and UVR on molluscan development through the use of an algicide, but such a method was ultimately unsuccessful. As a result, the effects of fouling and UVR are analysed separately. I examine the influence of UVR, egg mass structure (capsular vs. gelatinous) and spawning habitat (shaded, partially shaded & full sun) on the amount of surface algal fouling on gastropod egg masses collected along the southeastern Australian coast. In addition, I examine the effects of surface fouling on embryonic mortality and encapsulation period. Egg masses from 18 species were collected and subjected to standardised aquaria conditions under cutoff filters: (i) dark, (ii) UV-blocked, and (iii) full spectrum. Algal fouling levels were calculated visually (F_v) and biochemically through chlorophyll content (F_c), but only visual quantification was deemed appropriate. The presence or absence of protists was also recorded. Algal fouling levels were higher in the light treatments than the dark, and UVR significantly inhibited algal growth and protist presence.

For the remaining analyses, only egg masses under UV-blocking filters were included to eliminate any confounding effects of UVR on molluscan development. The level of fouling on the egg masses was not related to the habitat in which they were spawned. Gelatinous egg masses were fouled by both algae and protists at a significantly higher rate than leathery capsules. Egg masses colonised by protists had a higher level of algal fouling, and overall these egg masses had a significantly higher incidence of embryonic mortality. No effects of protists were observed on the period of encapsulation. When the effect of algal fouling on embryonic mortality was analysed for each species separately, significant positive relationships were found for several species. As F_v increased, the embryonic mortality of Conuber (Polinices) *sp.,* Austraelois ornata, Bembicium nanum *and* Hoplodoris nodulosa *increased. Algal fouling was significantly correlated with encapsulation period in only two species. As fouling increased, encapsulation period decreased for* Aplysia

sydneyenis *and* Dendrodoris fumata. *Overall, this study reveals that both surface colonisation and the effects of fouling on gastropod encapsulated development are species-specific. Furthermore, UVR indirectly affects molluscan development through its effects on egg mass fouling.*

3.3.1 INTRODUCTION

Embryonic and early larval development is the most sensitive time during molluscan development (Rumrill 1990), particularly in the intertidal zone where environmental stresses can be frequent and harsh (Gosselin & Chia 1995). Many molluscs enclose their eggs within benthic egg masses that may reduce embryonic mortality. Among gastropods, these egg masses can be grouped into two main structures, capsular and gelatinous. Capsular egg masses include leathery egg capsules of neogastropods in which the embryos are surrounded by a tough capsule wall. Gelatinous egg masses are deposited by heterobranchs and some other caenogastropods, with the embryos embedded in a gelatinous matrix and surrounded by a microscopic vitelline membrane. The egg masses of some species appear to provide protection against predation, extremes in temperature and salinity, desiccation, and microbial infection (reviewed by Rawlings 1999; Przeslawski 2004a). Despite such protection, embryos within egg masses are still vulnerable to environmental stresses, including surface fouling and ultraviolet radiation (UVR) (Biermann *et al.* 1992).

Every surface in the ocean is potentially a substratum for other micro- or macroscopic organisms, with colonisation occurring from the settlement of water borne adults, spores or larvae (Davis *et al.* 1989). Egg masses are ephemeral, and the short period before decomposition could make them unsuitable for colonisation by most fouling organisms. Nevertheless, the surfaces of all molluscan egg masses seem to provide appropriate substrates for the settlement of epiphytes since they are primarily composed of polysaccharides and proteins (Hunt 1966; Bayne 1968a; Flower *et al.* 1969). These types of biological matrices can facilitate settlement and attachment (refer to Davis *et al.* 1989). Previous studies on molluscan egg masses do indicate that these are fouled by a range of

surface fouling organisms, including macroscopic filamentous and encrusting algae, some marine invertebrates, diatoms and protists (Biermann *et al.* 1992; Benkendorff 1999; Cancino *et al.* 2000; Steer *et al.* 2002; pers. obs.).

Photosynthetically active radiation (PAR) is necessary for photosynthesis, and thus positively affects the growth of microalgae. UVR and high levels of PAR, however, can inhibit photosynthesis (reviewed by Cullen & Neale 1994). Exposure to both UV-A and UV-B has been shown to decrease primary productivity of phytoplankton (Smith *et al.* 1992). UVR and PAR are inextricably linked in sunlight so the potential positive effects of PAR on microalgae growth may be countered by the potential negative effects of UVR and high levels of PAR. Indeed, a recent study found significant interactive effects between PAR and UV-B on marine microalgae (Shelly *et al.* 2002). Biermann *et al.* (1992) analysed the separate effects of UVR and algal fouling on embryonic development, but they did not examine the potential relationships between the two factors. No other studies have focused on the effects of spectral exposure and surface algal growth on egg masses.

The rate of surface fouling on egg masses appears to vary between species (Biermann *et al.* 1992; pers. obs.), and this could be in part influenced by the substrate (Marszalek *et al.* 1979; Devi 1995). In particular, gastropod egg capsules and gelatinous egg masses have different surface textures that might influence the rate of settlement and attachment. The spawning habitat could also influence colonisation according to patterns of water flow and sunlight availability. Many marine gastropods only deposit egg masses on the undersides of boulders (Benkendorff & Davis 2004), where the number of potential water borne colonisers may be reduced and the availability of sunlight for photosynthesis by algal foulers would be limited. On the other hand, a number of intertidal gastropods deposit egg masses in habitats that are exposed to full or partial sunlight (Benkendorff & Davis 2004; Przeslawski *et al.* 2004). These egg masses are likely to be more exposed to potential foulers and therefore may have evolved mechanisms to reduce surface colonisation. Potential relationships between surface fouling and species, in conjunction with their associated egg mass structure or spawning habitat, have yet to be explored.

Surface fouling could lead to a complex suite of potentially harmful or beneficial effects on encapsulated embryos (see Fogg 1983; Bachman *et al.* 1986; Biermann *et al.* 1992; Cohen & Strathmann 1996; Turner & Tester 1997), yet few studies have investigated the effects of surface colonisation on molluscan development. Microalgal fouling has been shown to increase embryonic mortality in the nudibranch *Archidoris montereyensis* (Biermann *et al.* 1992). Similarly, protists on the surface of *Chorus giganteus* egg masses resulted in prolonged development and higher mortality (Cancino *et al.* 2000). In contrast, Steer *et al.* (2002) found that egg masses of *Sepioteuthis australis* fouled by microalgae developed more synchronously and had a lower embryonic mortality than unfouled egg masses. The net effect of microalgal fouling on a broader range of species needs to be studied under standard conditions to determine species-specific differences.

Our aim was to identify the potential effects of UVR on surface fouling as well as the effects of fouling on development of encapsulated gastropod embryos. I also test the hypotheses that fouling loads differ significantly according to egg mass structure and spawning habitat. This study addresses a fundamental gap in our understanding of how UVR and microalgal fouling may affect the development of a broad range of molluscan species.

3.3.2 MATERIALS AND METHODS

Pilot Study on the Use of Algicide

This study attempted to synchronously examine the effects of fouling and UVR on molluscan development through the use of an appropriate algicide (refer to Appendix 3), but such a method was ultimately unsuccessful. The study described here was initially conducted with an algicidal treatment (.25mL/L Simazine) and a control in order to investigate potentially synergistic effects of UVR and fouling on molluscan development. Unfortunately, the algicide was ineffective at preventing surface fouling on the egg masses. In fact, significantly more algae was recorded on egg masses subjected to algicide treatments than the control ($df = 1$, $F = 11.6124$, $p = 0.0007$), possibly due to an effectively

low concentration of algicide which may actually stimulate algae growth (Ibrahim 1984). Thus, effects of fouling and UVR could only be analysed separately.

Egg Mass Collection

Undeveloped egg masses were collected from the Illawarra intertidal coast of NSW, Australia during four occasions in the austral summer of 2002-03 (Dec – Jan) encompassing a period of 29 days (Table 3.3.1). Egg masses were mainly identified from previous research (Creese 1980; Rose 1985; Loch 1989; Smith *et al.* 1989). However, in some cases identification of a laying adult was the only way to identify the egg mass to genus or species level. Several egg masses were deposited in aquaria by adults within three days of captivity (Table 3.3.1). All egg masses were examined with a dissecting microscope (40x magnification) to verify fertilised and pre-trochophore states. The date of collection and initial examination was denoted Day 1.

Experimental Design

A total of 18 species were used in this experiment (Table 3.3.1). Species were chosen based on previous research (Przeslawski *et al.* 2004) to represent a range of families, egg mass structures, and deposition habitats (Table 3.3.1). The number of replicate egg masses collected from each species varied according to availability and local abundance. Some capsules abruptly changed colour soon after collection. These capsules were not used because such colour change is indicative of stress and possible impending death (Pechenik 1982; pers. obs.).

The experiments were run with identical aquarium conditions during four overlapping time periods (Table 3.3.1). Replicate egg masses from some species were used in all

Table 3.3.1 List of species and spawn characteristics used in this study. The microhabitat used for the deposition of egg masses by each species is provided in terms of the relative exposure to sunlight. The number of replicate egg masses (n) used to assess embryonic mortality (Mort) and encapsulation period (EP) are also given. Egg masses were randomly assigned to four overlapping experimental trials commencing; 1) 4 Dec 2002; 2) 8 Dec 2002; 3) 17 Dec 2002; 4) 2 Jan 2003. References include representative literature describing and / or illustrating the spawn.

Species	Sub/ Infraorder	Trial	Structure	Habitat	n		Reference
					Mort	EP	
Littorinimorpha	*Bembicium nanum*	1,2,3,4	Gel	Full Sun	11	10	Smith *et al.* 1989
Littorinimorpha	*Polinices (Conuber)* sp.	4	Gel	Full Sun	7	7	Smith *et al.* 1989
Neogastropoda	*Mitra badia*	4	Capsule	Shade	5	n/a	Loch 1989
Neogastropoda	*Mitra carbonaria*	1,2	Capsule	Shade	7	n/a	Loch 1989
Neogastropoda	*Agnewia tritoniformis*	1,2,4	Capsule	Shade	8	n/a	Benkendorff 1999
Neogastropoda	*Dicathais orbita*	1	Capsule	Shade	4	n/a	Smith *et al.* 1989
Cephalaspidea	*Bullina lineata*	1,3	Gel	Partial	9	8	Rose 1985
Anaspidea	*Aplysia juliana*	1,2	Gel	Partial	4	<3	Switzer-Dunlap & Hadfield 1977
Anaspidea	*Aplysia sydneyensis*	2,4	Gel	Partial	7	5	pers. obs.
Anaspidea	*Dolabella auricularia*	3,4	Gel	Partial	7	7	Switzer-Dunlap & Hadfield 1977
Anaspidea	*Dolabrifera brazieri*	1,2,3,4	Gel	Shade	14	10	pers. obs.
Anaspidea	*Stylocheilus longicauda*	2,4	Gel	Partial	4	4	Switzer-Dunlap & Hadfield 1977
Sacoglossa	*Oxynoe viridis*	4	Gel	Partial	6	4	Rose 1985
Aeolidia	*Austraeolis ornata*	2,4	Gel	Shade	6	6	Smith *et al.* 1989
Nudibranchia	*Dendrodoris fumata**	3,4	Gel	Shade	4	4	Przeslawski 2003
Nudibranchia	*Hoplodoris nodulosa**	4	Gel	Shade	4	3	Rose 1985
Basommatophora	*Siphonaria denticulata*	1,2,3,4	Gel	Full Sun	12	12	Creese 1980
Basommatophora	*Siphonaria zelandica*	1,4	Gel	Full Sun	8	8	Smith *et al.* 1989

* Egg masses deposited in aquaria.

four periods (Table 3.3.1). No overall differences in fouling levels on these egg masses were detected between the time periods (2-Way ANOVA, $F = 1.157$, $p = 0.345$). Furthermore, no significant intraspecific differences were detected for any of the species used in replicate trials. Consequently, data have been pooled across experimental trials for subsequent analyses.

Each egg mass was cut into three pieces and placed in an outdoor 300-litre recirculating seawater tank within a shallow 1 litre submerged plastic container perforated by 20 1 cm diameter holes to allow uniform water flow and temperature. Seawater was collected from Bellambi Point, one of the rock platforms where egg masses were collected. The water was filtered with mesh netting to remove large particles, but no further conditioning was applied. Salinity was monitored with a handheld refractometer and kept constant at 35 ppt with the regular addition of small amounts of distilled water throughout the experimental period. This experiment was conducted outdoors because variation and intensity of sunlight more closely paralleled natural conditions than artificial lighting. To promote algal growth without incorporating the confounding effects of ultraviolet radiation, a UV-blocking clear polyethylene film was placed over each container (Figure 2.1). Cutoff filters blocking all light and transmitting all light were also used to determine effects of PAR and UVR on algal fouling. Temperature was maintained with a refrigeration unit at 20.6 ± 3.0°C and recorded at 3-hour intervals with a thermal microchip (Thermochron I-Button). Water flow of 2.6 ± 0.6 cm/sec was monitored at random intervals during the experiment with neutrally buoyant beads. Near sunset, all egg masses were removed from the outdoor tank and moved to an aerated laboratory tank also filled with natural seawater (20°C, water flow 1.5 ± 0.25 cm/sec) to avoid potential interference from predators or vandals. In the morning, egg masses were replaced in the outdoor tank.

Among gelatinous egg masses, mortality and fouling for each egg mass were assessed when at least one replicate showed signs of hatching. This occurred when the gel began to soften and break apart, gradually releasing veligers. Egg masses that were not yet hatching were returned to the main tank after mortality and fouling calculation to determine duration of encapsulation. In contrast, the hatching day of capsular egg masses was not examined due

to some species' relatively prolonged development and often abrupt hatching mechanisms (Przeslawski *et al.* 2004). Instead all capsular egg masses were removed at Day 10.

In addition to the laboratory experiment, field observations were made on egg masses from *Bembicium nanum* and *Siphonaria denticulata*. The egg masses of these two species are extremely common on rock platforms and can be found all year. Both species deposit their egg masses consistently in habitats exposed to full sunlight, and thus may be vulnerable to high levels of algal fouling in their natural habitats. Mature egg masses containing shelled veligers were randomly collected from Bass Point and Bellambi in southeastern NSW, Australia. Egg masses were examined microscopically (40x magnification), and visual fouling levels and embryonic mortality were recorded as described below.

Quantification of Algal Fouling

Surface algal fouling was measured visually (F_v) and biochemically (F_c) to establish reliable quantification methods to determine fouling on egg masses. F_v was recorded under microscopic examination (40x magnification) by randomly sampling five sections of each egg mass piece and recording 100 point counts with an ocular ruler spanning 2.5mm (e.g. Biermann *et al.* 1992). In addition, protists were recorded as present or absent for each sample and were defined as ciliated and/or mobile microorganisms. Samples with more than 1% fouling were then stored at -80°C to be used in chlorophyll extractions to estimate microalgal fouling (e.g. Marrs *et al.* 1999). Egg masses were weighed, and chlorophyll was extracted in 90% acetone using a manual tissue grinder and a probe sonicator to disrupt cells. Supernatant was pooled, and solvent added to form 3mL extract, which was then analysed in a diode array spectrophotometer. Total chlorophyll content (F_c) was determined with the extinction coefficients of (Jeffrey & Humphrey 1975), and the sum of chlorophylls a, b, and c was calculated as recommended for mixed algal communities (Jeffrey *et al.* 1997). Regression analysis after removal of an outlier (Chaterjee & Price 1977) revealed that F_v and F_c were not significantly correlated (Figure 3.3.1; $R^2 = 0.010$; $p = 0.273$); relationship remained insignificant with inclusion of outlier. It is likely that chlorophyll extractions did not provide an accurate estimate of algal biomass due to interfering pigments and by-products from egg mass or associated microfauna that interfered with

calculations (Jeffrey *et al.* 1997). For example, the aplysiads consume relatively large amounts of different types of algae (Carefoot 1987) and incorporate algal pigments into their eggs (Carefoot *et al.* 1998). Thus, I believe that visual quantification provides a more reliable measure of surface algal fouling on molluscan egg masses and therefore is the only fouling method considered in the remainder of this paper.

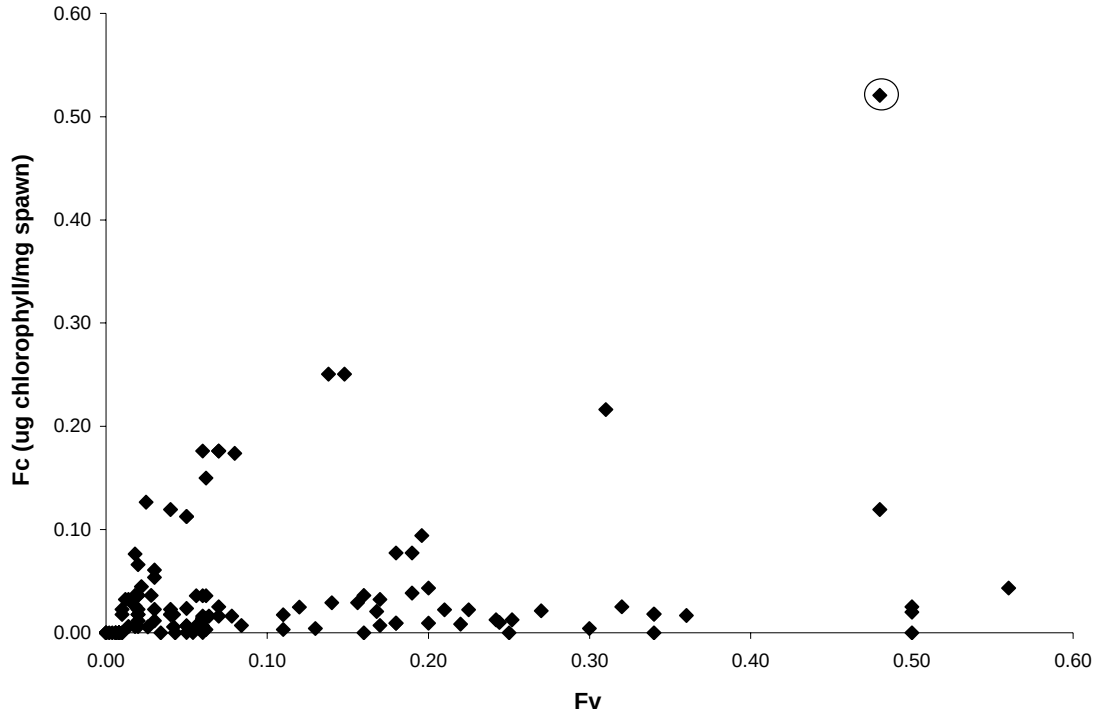


Figure 3.3.1 The relationship between visual algal fouling (F_v) and chlorophyll content (F_c) on the surface of molluscan egg masses. No significant correlation was found. An outlier is circled and was omitted from correlation analysis (n = 126).

Quantification of Embryonic Mortality

For both gelatinous and capsular egg masses, mortality was calculated by counting dead embryos in ten subsamples of ten embryos. Subsamples incorporated both sides of the egg mass. Quantification of mortality only encompassed peripheral embryos because it was easier to see them without destroying the integrity of the egg mass. *Mitra badia* was the only species with nurse eggs. In this species, only 1-2 eggs develop to the veliger stage, and all other eggs remain relatively undeveloped with the embryos eventually hatching from the capsule as crawling juveniles (pers. obs). Subsamples were not used to count *M. badia*

embryos, and the total number of dead and alive veligers was recorded. I considered embryos to be dead if they were degenerating and encapsulated or, with the exception of *M. badia,* if they were relatively underdeveloped. Thus, mortality was conservatively estimated because it did not account for deformed or recently deceased embryos. If the entire egg mass piece or capsule was obviously non viable, mortality was recorded as 100%.

Period of Encapsulation

For gelatinous egg masses, length of encapsulation was the time taken to hatch for each replicate egg mass. To standardize hatching time, hatching was recorded as the first day when more than 10% of the embryos had hatched or the gel dissolved (e.g. Przeslawski *et al*. 2004).

Statistical analyses

All error measurements given are standard errors of means. Effects of spectral treatment on algal fouling were analysed with nested 2-factor ANOVAs with the restricted maximum likelihood technique. Spectral effects on protist presence were analysed with individual chi-square tests for each species showing both presence and absence of protists in JMP v. 4. Tukey's HSD revealed significant relationships.

Species differences in fouling levels on egg masses under UV-blocking treatments were compared with a nonparametric Kruskal-Wallis test since Levene's test revealed that data had unequal variances (not correctable by transformation) between species. Furthermore, due to variation in abundances, the design was unbalanced at the egg mass level (n= 4-14) (Table 3.3.1). Consequently, the results of the post-hoc analyses have been interpreted cautiously for potential Type 1 errors. Post-hoc analyses were performed with Tukey's HSD, with alpha ≤ 0.01 indicating reliable significant differences. For *Bembicium nanum* and *Siphonaria denticulata,* differences in fouling and mortality were investigated with a nonparametric Kruskal-Wallis test for egg masses collected mature from the field compared with those held in aquaria during development.

The effects of habitat and type of encapsulating structure on the level of fouling observed under UV-blocking treatments were examined with two factor nested ANOVAs, with species nested within habitat or type. To examine these factors independently, analysis of habitat included only gelatinous egg masses, while analysis of encapsulating structure included only egg masses from species that spawn exclusively in shaded habitats. Tukey's HSD tests were applied post hoc, with alpha = 0.01 to reduce type 1 error due to unequal variances. The effects of habitat and type of encapsulating structure on the proportion of egg masses containing protists were examined with Kruskal-Wallis nonparametric tests on split data files. The level of fouling was also compared in egg masses with and without protists with Kruskal-Wallis nonparametric tests.

Independent t-tests or Kruskal-Wallis tests were applied to examine the effect of protist presence on embryonic mortality and encapsulation period. The effects of fouling on embryonic mortality and encapsulation period in both laboratory and field samples were examined with Pearson's correlations. Since the above analyses revealed differences in the level of fouling and mortality between species, the correlations were performed on each individual species rather than the pooled data set. For these analyses a significance level of alpha = 0.10 was used due to the low number of replicates in most of the species. Statistical analyses were performed on SPSS version 11 with alpha = 0.05 unless otherwise stated.

3.3.3 RESULTS

3.3.3.1 *Effects of solar radiation on fouling*

Spectral treatment affected surface algal fouling ($p = 0.0062$, Table 3.3.2). Significantly more algae were found on egg masses under UV-blocked treatments than either dark or full spectrum treatments as confirmed by Tukeys HSD (Figure 3.3.2a).

Table 3.3.2 Effects of spectral treatment and species on surface algal fouling as revealed by an ANOVA (restricted maximum likelihood). Italics denote a random factor.

Effect	df	MS	F	p
Spectral	2	0.0330	5.2117	0.0062
Species	17	0.0288	4.5477	<0.0001
Spectral x Species	34	0.0079	1.2452	0.1778
Egg mass (species)	108	0.0064	1.0059	0.4785
Residual	216	0.0063		
Corrected Total	377	0.0108		

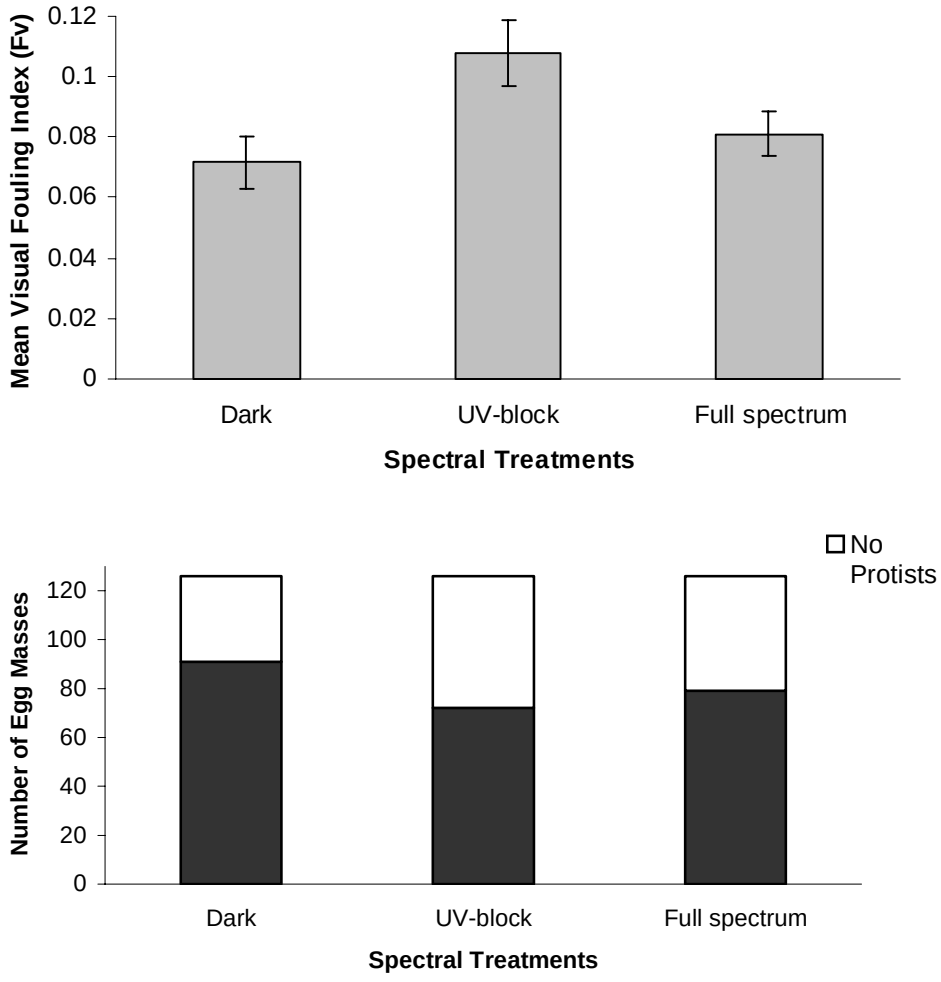


Figure 3.3.2 The effects of spectral treatment on egg mass a) algal fouling and b) protist presence. Error bars represent standard error of mean.

The highest proportion of egg masses with protists were found under dark treatments, and the lowest proportions of egg masses with protists occurred in light treatments (Figure 3.3.2b). Despite these trends, individual chi-square tests did not reveal any significant effect of spectral treatment on protist presence in any species except *B. nanum* (6.243, p = 0.0441). Nevertheless, results should be interpreted cautiously due to low replicates within many species.

3.3.3.2 *Effects of fouling on molluscan development*

Quantification of algal fouling

Almost all of the algae observed on the egg masses in aquaria and in the field were diatoms or green filamentous colonies. The level of visually obvious fouling of surface area (algal cover) observed ranged from 0.0 – 0.56 (Figure 3.3.1).

In standardised conditions in aquaria, there was a large variation in fouling levels both within and between species (Figure 3.3.3), and the differences between species were found to be significant with a Kruskal-Wallis test ($p < 0.0005$). Post-hoc analysis with Tukey's HSD revealed only a few significant differences between species at alpha = 0.01 (Figure 3.3.3). In particular, the level of fouling on the gelatinous egg masses of *Hoplodoris nodulosa* was found to be significantly higher than that recorded on the leathery egg capsules of two neogastropods (*Agnewia tritoniformis* and *Mitra carbonaria*; Figure 3.3.3). A further two species, *Mitra badia* and *Conuber (Polinices)* sp., were significantly different from *H. nodulosa* at the 5% error level, and these appear unlikely to represent Type 1 errors (Figure 3.3.3).

Comparison of fouling levels on mature eggs collected from the field and those deposited in the laboratory revealed species-specific effects. No significant differences were recorded for *Siphonaria denticulata* ($p = 0.343$), whereas egg masses of *Bembicium nanum* cultured in aquaria had significantly higher levels of algal fouling at 0.195 ± 0.053 than mature egg masses collected in the field at 0.030 ± 0.005 ($p < 0.0005$) (Figure 3.3.4).

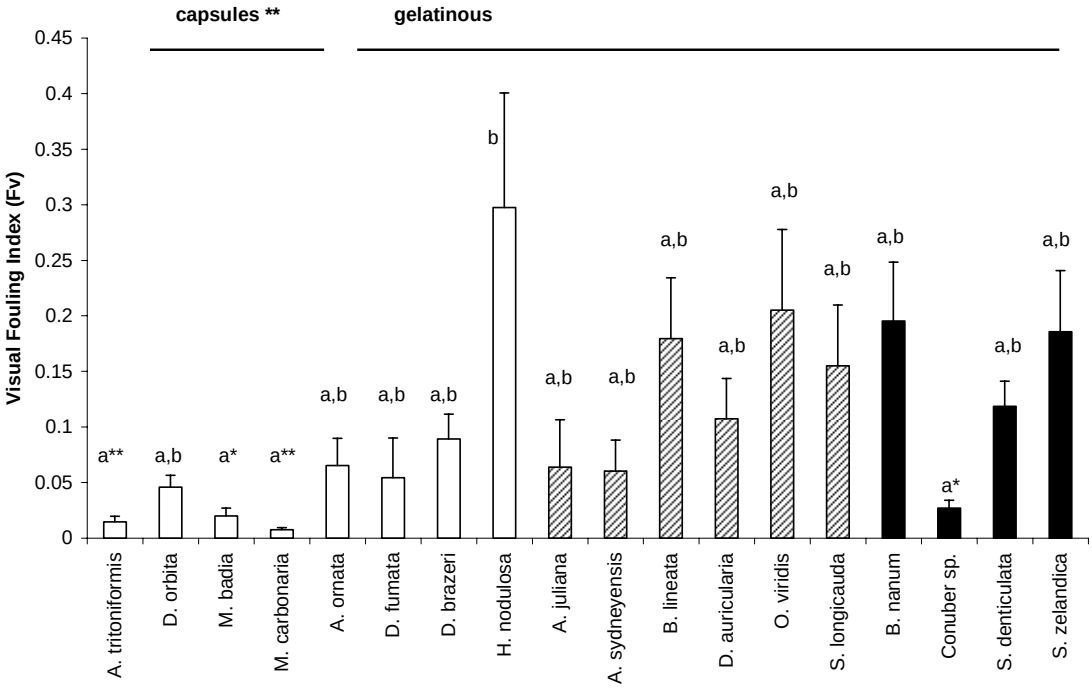


Figure 3.3.3 Comparison of the level of algal fouling (F_v) recorded on the surface of 18 species of intertidal molluscs under standardised laboratory conditions. The egg masses have been collected from a range of differentially light exposed habitats; shaded = white bars; partially shaded = hatched bars; fully sun exposed = black bars. Within the shaded habitat two different types of egg masses were collected; leathery capsules and gelatinous egg masses. Egg masses from the other two habitats were all gelatinous. The influence of the type of egg mass on the level of fouling was tested for egg masses collected from shaded habitats only, whereas the influence of habitat was tested for gelatinous egg masses only. The letters above the bars are used to indicate homogeneous subsets; * significant at alpha = 0.05; ** significant at alpha = 0.01.

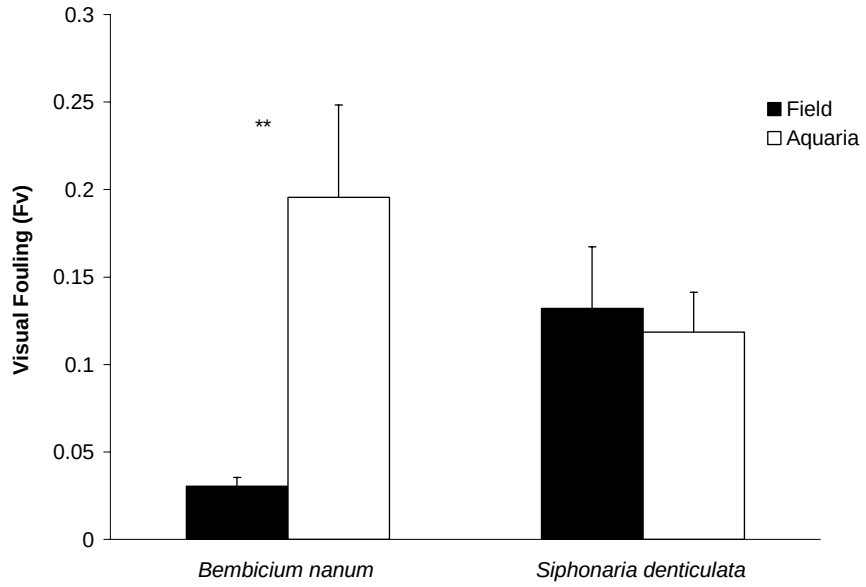


Figure 3.3.4 The level of visual fouling (Fv) on egg masses collected mature from the field compared to those held in aquaria to develop under a UV blocking filter for two species of intertidal molluscs; ** significant at alpha = 0.01.

Frequency of protist colonisation

The proportion of egg masses that were colonised by protists ranged from zero in one species (*Dicathais orbita*) to 1.0 in four species (*Dendrodoris fumata*, *Hoplodoris nodulosa*, *Oxynoe viridis* and *Stylocheilus longicauda*; Figure 3.3.10). Protists were observed on *Bembicium nanum* and *Siphonaria denticulata* egg masses held aquaria, whereas none were observed on any of the egg masses collected mature from the field. Egg masses that were colonised by protists in the aquaria were found to have significantly higher levels of algal fouling (0.13 ± 0.02) than those without protists (0.08 ± 0.01) ($p = 0.007$).

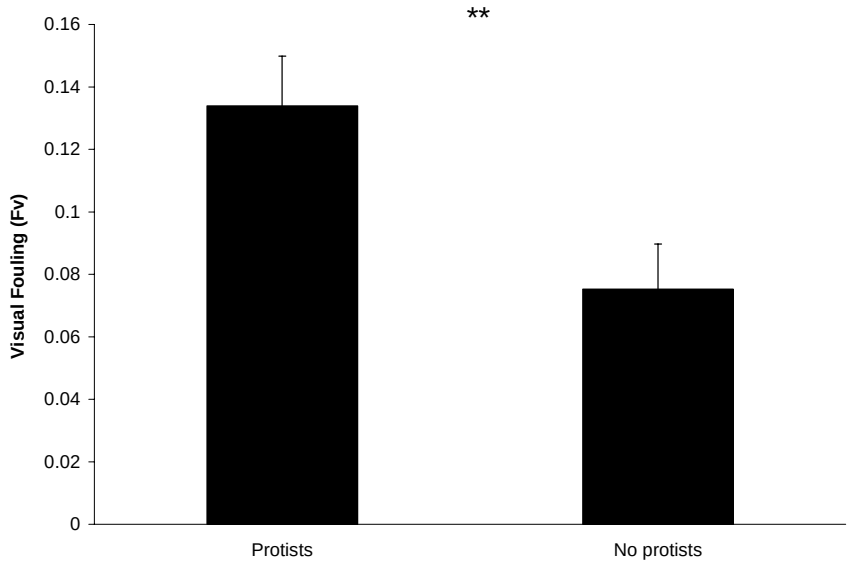


Figure 3.3.5 The relationship between protist colonisation and visual algal fouling (Fv) on egg masses from 18 species. ** significant at alpha = 0.01.

The effect of habitat and encapsulating structure on surface fouling

For those egg masses collected from shaded habitats, the level of surface algal fouling of egg masses in the outdoor aquaria varied according to egg mass structure. Gelatinous egg masses had a significantly higher algal fouling level than leathery egg capsules ($p < 0.0005$; Figure 3.3.3). Furthermore, the proportion of leathery capsules found to be colonised by protists was significantly lower than that observed for gelatinous egg masses ($p = 0.02$; Figure 3.3.6).

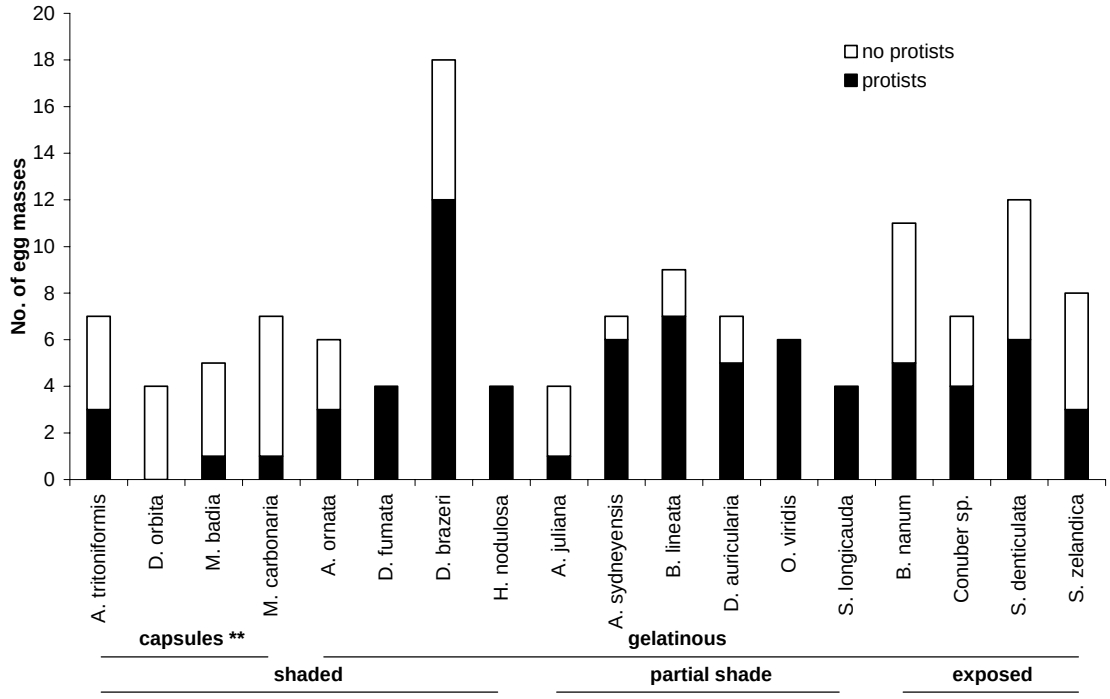


Figure 3.3.6 The proportion of egg masses that were colonised by protists under standardised laboratory conditions. Leathery egg capsules from four species and gelatinous egg masses from 14 species of molluscs were collected from a range of differentially light exposed intertidal habitats. The influence of the type of egg mass on the proportion colonised was tested for egg masses collected from shaded habitats only, whereas the influence of habitat was tested for gelatinous egg masses only; ** significant at alpha = 0.01.

For gelatinous egg masses, the deposition habitat of egg masses had no significant effect on algal fouling for egg masses held in outdoor aquaria (Figure 3.3.3). Similarly, there was no significant difference in the proportion of gelatinous egg masses that were colonised by protists, according to their deposition habitat (Figure 3.3.6). The relatively large error bars for gelatinous egg masses suggest that there is great variation in the level of surface fouling between species, irrespective of the habitat from which these egg masses are collected (Figure 3.3.3).

The effect of fouling on embryonic mortality

Overall, egg masses that were colonised by protists had significantly higher mortalities (0.24 ± 0.04) than those without protists (0.128 ±0.035) (df = 124, nonparametric t = 2.063, p = 0.025) (Figure 3.3.7). Notably, these egg masses also had significantly higher algal

fouling levels (Figure 3.3.5). The association between protist colonisation and embryonic mortality was also analysed for individual species with three or more replicates both with and without protists (Table 3.3.1 for n values). No significant differences in mortality were found at the 5% confidence level for *Agnewia tritoniformis* (nonparametric t = 1.732; p = 0.182), *Austraeolis ornata* (t = 1.162, p = 0.310), *Bembicium nanum* (nonparametric t = 1.377, p = 0.420), *Conuber* sp. (nonparametric t = 2.303, p = 0.093), *Dolabrifera brazeri* (t = 0.333, p = 0.745), *Siphonaria denticulata* (t = -0.629, t = 0.543) or *Siphonaria zelandica* (t = -0.380, p = 0.717) (Figure 3.3.7).

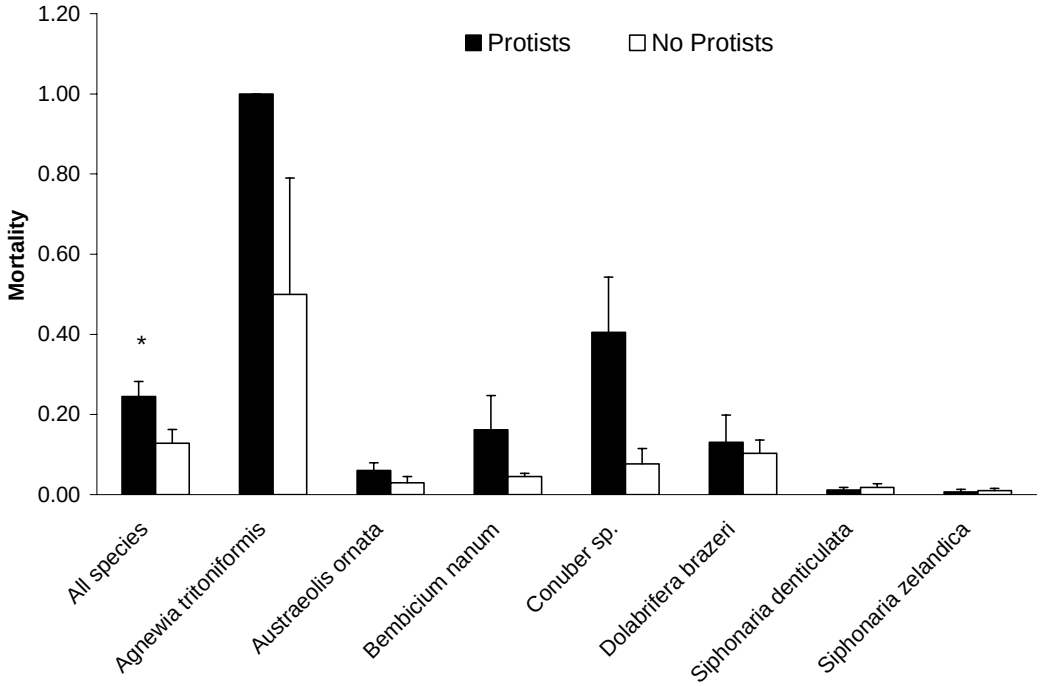


Figure 3.3.7 The relationship between protist colonisation and embryonic mortality on the egg masses of 18 species of marine molluscs combined (all species), as well as seven individual species with > 3 replicates both with and without protists; * significant at alpha = 0.05.

Due to the significant differences in fouling between species (Figure 3.3.3), the effects of algal fouling on embryonic mortality were tested with separate correlation analyses (Figure 3.3.3), revealing that the effects of fouling on embryonic mortality are species-specific. Embryonic mortality significantly increased as fouling levels increased among the naticid *Conuber (Polinces)* sp. (Figure 3.3.3; Figure 3.3.8). Three further species show significant positive relationships at the 10% level (*Austraeolis ornata*, *Bembicium nanum* and

Hoplodoris nodulosa; Figure 3.3.3; Figure 3.3.8). The low number of replicates for several other species would provide low power for these correlation analyses. However, for at least *Dolabrifera brazieri* and *Siphonaria denticulata* there appear to be no effects of fouling on embryonic mortality (Figure 3.3.3). Analysis of mature egg masses from the field revealed no significant relationships between algal fouling levels and embryonic mortality among *S. denticulata* or *B. nanum* (Figure 3.3.3).

Table 3.3.3 Results of linear regression analyses on the effects of fouling (F_v) on embryonic development (embryonic mortality or encapsulation period). Significant effects at alpha = 0.10 are in bold. Asterisks mark mature egg masses collected in the field.

Species	Embryonic mortality			Encapsulation period		
	d.f.	R^2	p	d.f.	R^2	p
A. tritoniformis	6	0.345	0.165	n/a		
D. orbita	n/a	100% survival		n/a		
M. badia	4	0.500	0.182	n/a		
M. carbonaria	6	0.032	0.700	n/a		
A. juliana	3	0.042	0.795	n/a		
A. sydneyensis	6	0.002	0.921	5	**0.782**	**0.047**
A. ornata	5	**0.593**	**0.073**	4	0.462	0.207
B. nanum	10	**0.337**	**0.061**	9	0.013	0.754
B. lineata	8	0.126	0.348	6	0.240	0.218
Conuber sp.	6	**0.966**	**0.000**	6	0.325	0.182
D. brazieri	13	0.041	0.486	9	0.281	0.115
D. fumata	6	0.111	0.666	3	**0.982**	**0.086**
D. auricularia	6	0.006	0.871	6	0.220	0.289
H. nodulosa	3	**0.861**	**0.072**	2	0.884	0.222
O. viridis	5	0.528	0.102	3	0.797	0.107
S. denticulata	11	0.127	0.255	11	0.029	0.597
S. zelandica	7	0.021	0.733	7	0.016	0.767
S. longicauda	3	0.361	0.399	3	0.254	0.496
*B. nanum**	39	0.016	0.437	n/a		
*S. denticulata**	28	0.001	0.905	n/a		

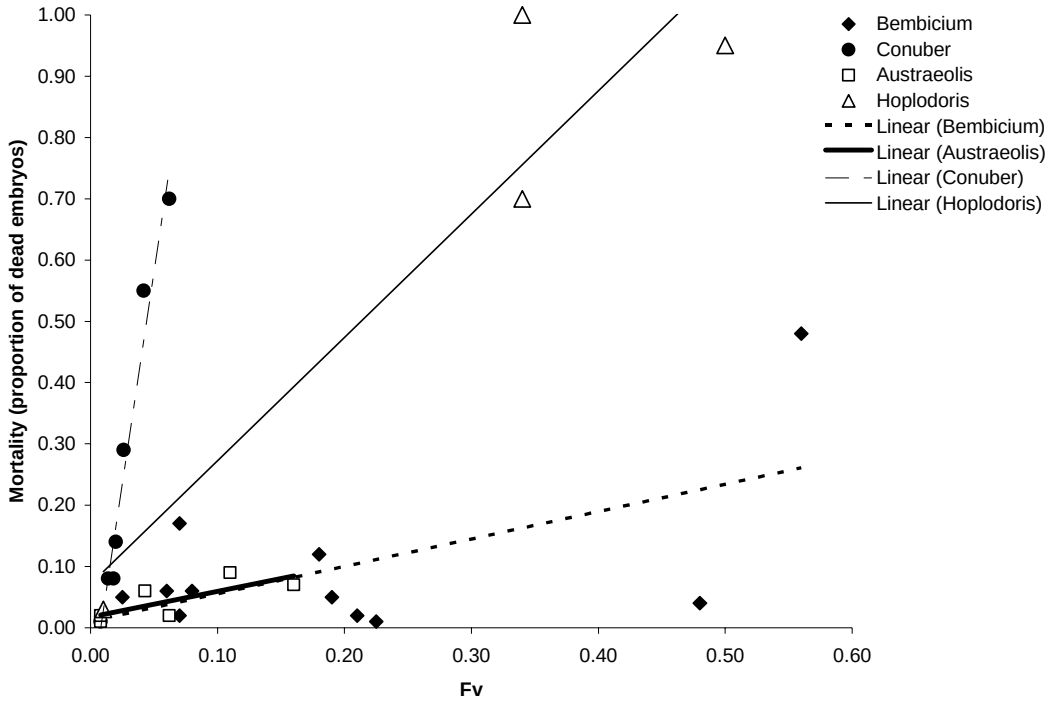


Figure 3.3.8 The relationship between fouling (F_v) and embryonic mortality in the egg masses of four species of marine molluscs: *Conuber* sp. (n = 7), *Austraeolis ornata* (n = 6), *Bembicium nanum* (n = 11), *Hoplodoris nodulosa* (n = 4). Lines represent linear best fits.

The effect of fouling on encapsulation period

Only gelatinous egg masses that hatched in all treatments were considered in analysis of encapsulation period. Overall, there were no significant differences in the encapsulation period for gelatinous egg masses that were colonised by protists ($t = 0.891$, $p = 0.376$) (Figure 3.3.9). In addition, the encapsulation periods of species that included three or more replicate egg masses representing both presence and absence of protists were analysed individually (Table 3.3.1 for n values). No significant differences were observed for *Bembicium nanum* ($t = 0.851$, $p = 4.19$), *Conuber* sp. ($t = -0.524$, $p = 0.623$), *Siphonaria denticulata* ($t = 0.000$, $p = 1.00$), or *Siphonaria zelandica* ($t = -1.369$, $p = 0.220$) (Figure 3.3.9).

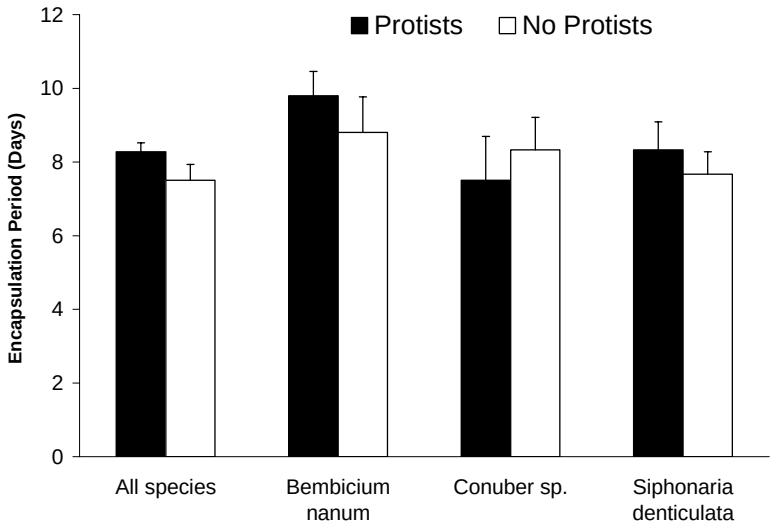


Figure 3.3.9 The relationship between protist colonisation and encapsulation period for the gelatinous egg masses of 14 species of marine molluscs combined (all species), as well as four individual species with > 3 replicates both with and without protists.

Encapsulation period was significantly correlated with algal fouling in only two species (Table 3.3.2). As fouling levels increased on the egg masses of *Aplysia sydneyensis*, encapsulation period decreased (Table 3.3.2; Figure 3.3.10). Similarly, encapsulation period decreased with increasing fouling on *Dendrodoris fumata* egg masses (Figure 3.3.10), although this was only significant at the 10% level (Figure 3.3.3), probably due to the low number of replicates.

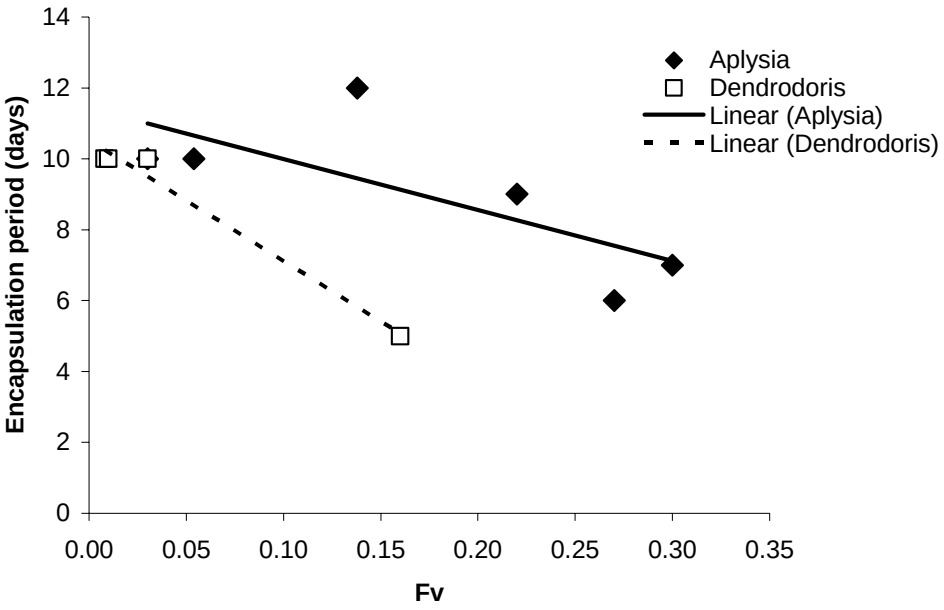


Figure 3.3.10 The relationship between fouling (F_v) and encapsulation period in the egg masses of two species of marine molluscs: *Aplysia sydneyensis* (n = 6) and *Dendrodoris fumata* (n = 4). Lines represent linear best fits.

3.3.4 DISCUSSION

Unsurprisingly, PAR promoted algal growth on egg mass surfaces as evidenced by significantly more algae on egg masses under UV-blocked treatments than dark treatments (Figure 3.3.2a). This finding is consistent with previous research (e.g. Biermann *et al.* 1992) and basic plant biology. Therefore, egg masses deposited under boulders and other shaded habitats may be less vulnerable to algal fouling than those deposited in habitats exposed to sunlight. However, UVR significantly inhibited algal growth as shown by less algae in full spectrum than UV-blocked treatments (Figure 3.3.2a). UVR may have directly harmed the growth of the microalgae (e.g. Davidson *et al.* 1994) or it may have caused photoinhibition resulting in a slower growth rate (Cullen & Neale 1994). Based on the detrimental effects of UVR on microalgal growth observed in this study, egg masses in full sunlight may be slightly less fouled than egg masses in habitats with temporal or spatial variation in sunlight exposure such as vertical rock faces or algal beds. Field studies quantifying fouling levels on egg masses from a variety of habitats would confirm the validity of this suggestion. In contrast, spectral treatment did not significantly affect protist

presence. However, these results should be interpreted cautiously as this study used only a crude measurement of protist colonisation, and differences in protist abundances between treatments may not have been detected. Furthermore, protist colonization is associated with algal fouling (Figure 3.3.2b), and so UVR may indeed affect protist fouling of egg masses indirectly through its effects on algal fouling.

There was a large variation in the level of surface fouling observed on gastropod egg masses under UV-blocking treatments (Figure 3.3.3). Differences in fouling levels have been previously reported from the egg masses of two opisthobranchs collected in the field (Biermann *et al.* 1992). However, small-scale environmental differences in the field may influence the availability of potential colonisers and suitability for settlement and growth. Consequently, controlled laboratory experiments such as those used in this study are required to compare the level of fouling on a range of different species under equivalent environmental conditions. The interspecies variation observed here could be due to differences in the suitability of surface texture and/or settlement cues for microalgal attachment. Chemical antifouling agents could also explain some of the variation in fouling levels observed between species (Figure 3.3.3). Previous research has revealed that antifouling compounds are species-specific among some marine invertebrates (Tsoukatou *et al.* 2002), and molluscan egg masses may be no exception.

Overall, the level of fouling observed on gastropod egg mass surfaces was significantly lower on leathery capsules and varied substantially among species with gelatinous egg masses (Figure 3.3.3). Thus, gelatinous masses appear to provide a more favourable substrate for biofilm formation, which promotes early algal colonization (Marszalek *et al.* 1979). Indeed, Biermann *et al.* (1992) found that gelatinous nudibranch egg masses had significantly more fouling microalgae than glass slides, as would be expected from a biological matrix composed of proteins and polysaccharides. Leathery egg capsules provide a proteinaceous matrix (Hunt 1966; Flower *et al.* 1969) with little or no glycosylation, which may explain why they are less suitable as a substrate for the settlement of fouling organisms.

Embryos within leathery egg capsules often have a much longer encapsulation period than those in gelatinous egg masses (Palmer 1994; per. obs.), and thus the potential for colonisation by fouling organisms could generally be greater on leathery capsules in the field. The experimental periods required in this study were relatively short (10 days), although those for gelatinous egg masses were on average shorter at 8.1 ± 2.0 days. Consequently, the different development times are not responsible for the various levels of fouling observed on leathery capsules and gelatinous egg masses in this study.

Selective pressure for the evolution of mechanisms to control surface fouling on gastropod egg masses could stem from both the concentration of potential foulers in the environment and the severity of associated detrimental impacts. The relative exposure of benthic egg masses to colonising surface foulers is expected to vary according to the microhabitat used for egg deposition. However, I found no significant difference in algal fouling levels of egg masses collected from different deposition habitats when held under controlled experimental conditions (Figure 3.3.3). Thus it seems unlikely that antifouling mechanisms have evolved in accordance with specific egg deposition habitats. Nevertheless, further studies are required to quantify the fouling loads in the different habitats used for egg mass deposition at the time of spawning. This could be done with an artificial control substrate across all habitats, as well as a range of egg masses selected from the different habitats.

The standardised conditions in the laboratory may not have adequately reflected the dynamic conditions of the intertidal habitat where these egg masses are found. Interestingly, higher levels of fouling were observed on *Bembicium nanum* egg masses held in aquaria compared with those collected mature from the field. UVR and wave force may inhibit algal fouling on egg masses of species that spawn in exposed intertidal habitats (e.g. Montero *et al.* 2002) (Figure 3.3.2a). However, no significant differences between field and aquaria-held egg masses were found for *Siphonaria denticulata*, which also deposits eggs on exposed intertidal rock platforms. An alternative possibility is that desiccation at low tide reduces fouling on *B. nanum* egg masses in the field, since this species lays eggs high on the rock platform, whereas *S. denticulata* typically deposits eggs in shallow pools at mid-low intertidal level (Benkendorff & Davis 2004). Nevertheless, these results suggest

that conditions in the laboratory were not equally representative of natural spawning habitats between species, and thus further studies should be undertaken on egg masses in the field.

Compared with the biochemical method for assessing surface algal fouling (Jeffrey & Humphrey 1975; Jeffrey *et al.* 1997), the visual method (F_v) provides an opportunity to determine the presence (and abundance) of all fouling organisms irrespective of whether or not they photosynthesise. In this study, protists were observed on the surface of a high proportion the egg masses in the laboratory (Figure 3.3.6). Algal fouling levels were found to be significantly higher on egg masses that had protists compared with those that didn't (Figure 3.3.5). This suggests that protists will only settle on the surface of egg masses after a reasonable amount of algal colonisation has occurred. Alternatively, the protists may be attracted to dead or dying embryos already affected by high algal fouling. In this study the mean fouling level for the detection of protists equated to about 13% of the egg mass surface visually covered by algae. Protists were not observed on any of the egg masses collected from the field, for which the level of algal fouling was generally lower than 13%. These egg masses developed in habitats exposed to UVR that could also affect protist colonisation and/or survival. In light of the potential relationship between algal fouling and protist colonization, the effects of algal fouling on embryonic mortality cannot be considered independent of the whole microfouling community, including protists.

In general, significantly higher mortality rates were found in egg masses with high algal fouling levels and protists present (Figures 3.3.7, 3.3.8). I have also been able to demonstrate significant relationships between algal surface fouling and embryonic mortality in several species of gastropods (Figure 3.3.3). A highly significant relationship was observed for *Conuber (Polinices)* sp., whereby embryonic mortality increased with remarkably small increases in surface algae (Figure 3.3.8). Similarly, embryonic mortality increased with increasing fouling in *Austraeolis ornata, Bembicium nanum* and *Hoplodoris nodulosa* (Figure 3.3.8). The low number of replicates and limited range in fouling levels on some other species would have reduced the power of detecting a relationship with

embryonic mortality, and thus the potential for harmful effects on other species should not be excluded at this stage.

Encapsulated embryos are often assumed to be at risk from protists and other fouling organisms, although the exact mechanisms of this risk are unknown. Protists may consume intracapsular fluid, eggs or embryos if they can penetrate beyond the surface (pers obs). Furthermore, deleterious effects of surface foulers could be attributed to the addition of toxic wastes (Fogg 1983; Turner & Tester 1997), and/or the depletion of oxygen (Bachman *et al.* 1986; Pinder & Friet 1994; Cohen & Strathmann 1996). Protists and other fouling organisms have been shown to alter the internal oxygen levels of egg masses through photosynthesis and cellular respiration (Cohen & Strathmann 1996; Cancino *et al.* 2000). Microalgal fouling has been shown to affect the supply of oxygen to internal eggs in amphibian (Bachman *et al.* 1986; Pinder & Friet 1994), nudibranch and polychaete egg masses (Cohen & Strathmann 1996).

Oxygen availability plays a tremendous role in both embryonic development and hatching rate (Strathmann & Strathmann 1995), with many encapsulated embryos believed to be near the limit for adequate oxygen supply (Lee & Strathmann 1998). Hypoxia can lead to arrested development or a prolonged time to hatching in gastropod egg masses (Strathmann & Strathmann 1995; Cancino *et al.* 2000). Interestingly, the encapsulation period of *Aplysia sydneyensis* and *Dendrodoris fumata* were found to decrease with increased fouling (Figure 3.3.10). A previous study by Pinder & Friet (1994) revealed that green algae specific to amphibian eggs increased oxygen availability to the embryos. This suggests that the photosynthetic activity of fouling microalgae by day can outweigh depletion by cellular respiration at night. Nevertheless, the remaining 16 species used in this study showed no significant effects of fouling on encapsulation period (Figure 3.3.3). Furthermore, the present study revealed no effects of protists on encapsulation periods (Figure 3.3.9), in contrast to previous research (e.g. Cancino *et al.* 2000). Since protists were also associated with higher levels of algal fouling in this study, photosynthesis and metabolism by fouling organisms may result in no net effects on oxygen availability to the embryos. Alternatively, the lack of any apparent relationship between encapsulation period and algal fouling in

some species could be due to relatively short encapsulation periods and low overall levels of fouling levels, again providing low power for the regression analyses. Further research to investigate the effects of higher fouling levels and longer periods of encapsulation on a greater range of species is recommended. This may be undertaken with lower temperatures, which are known to prolong the developmental period (Palmer 1994), or by targeting species with longer encapsulation periods. Quantification of protist numbers in addition to visual assessment of algal cover could also be used in a multiple regression to help tease out the relative contribution of each factor to embryonic mortality. In general, egg masses colonized by protists warrant further investigation as they may reveal the mechanisms by which protists affect embryonic development.

Overall, this study has shown that surface microfouling can play a significant role in the development of encapsulated molluscan embryos. Fouling levels and effects on encapsulated molluscan embryos vary between species and egg mass structures. Based on the high levels of variation in this study, future generalisations of fouling effects based on one species should be avoided. Instead, I recommend incorporating multiple species or narrowing the hypothesis to a particular species. Furthermore, UVR and PAR can indirectly affect embryonic development by affecting algal growth and protist presence. Thus, relationships between abiotic and biotic stressors should always be considered as they can play an important role in development of intertidal embryos and larvae, particularly in relation to the species and egg mass type being examined.

CHAPTER 4:
Protection against UVR-induced damage [1,2]

"Nothing shocks me. I'm a scientist."
Harrison Ford (Indiana Jones)

[1] Section 4.1 encompasses manuscript in preparation:
Przeslawski R, Davis AR. Does intertidal spawning behaviour minimise exposure of encapsulated invertebrate larvae to environmental stressors? To be submitted to *Marine Biology*

[2] Section 4.2 encompasses published manuscript:
Przeslawski R, Benkendorff K, Davis AR. 2005. A quantitative survey of mycosporine-like amino acids (MAAs) in intertidal egg masses from temperate rocky shores. *Journal of Chemical Ecology,* 31, 2419-2440

4.1 BEHAVIOURAL PROTECTION: DOES INTERTIDAL SPAWNING BEHAVIOUR MINIMISE EXPOSURE OF ENCAPSULATED INVERTEBRATE LARVAE TO ENVIRONMENTAL STRESSORS?

*Intertidal environments are extremely dynamic, and organisms within them are often exposed to a range of potential stressors. Whereas adults are able to seek shelter to avoid stress associated with low tides, embryos within egg masses are essentially sessile for the duration of their encapsulation. Here, I conducted surveys of intertidal egg masses at two sites in southeastern Australia over two years to determine if spatial and temporal variations in spawning behaviour reduce potential environmental stress to encapsulated intertidal invertebrates. I predicted that egg masses will be predominantly deposited in shaded habitats not prone to environmental extremes. Furthermore, I anticipated that egg masses deposited in habitats exposed to UVR, desiccation, or extremes in temperature and salinity will be smaller and occur less frequently in these habitats during seasons of high environmental stress. Egg masses from 34 taxa were unambiguously identified, and only four of these spawned on rock platforms in full sun (*Bembicium nanum, Nerita morio, Siphonaria zelandica *and* S. denticulata*). Summer had the highest UVR index, water temperature, and air temperature as well as the lowest tides during daylight; but egg mass assemblages and abundances of target species were highest during this season with no change in egg mass sizes. I conclude that those species spawning on the upper surfaces of rocky reefs do not protect their encapsulated offspring by avoidance of physiologically stressful conditions. This study suggests that spatial and temporal variation in spawning behaviour reflect evolutionary adaptations and physiological responses to a variety of abiotic and biotic factors.*

4.1.1 INTRODUCTION

Intertidal environments are extremely dynamic, and organisms within them are often exposed to a range of potential stressors including desiccation, wave force, solar radiation, and fluctuations in temperature and salinity. These abiotic factors do not operate in isolation; rather, each is often related to other environmental parameters (Przeslawski 2004a). For example, solar radiation may increase water temperature in a rock pool which may in turn increase evaporation rate and therefore salinity

(Przeslawski et al. 2005). Thus, organisms in such environments may be synchronously exposed to a multitude of stressors during low tides.

Larvae are considered the most vulnerable life stage of most organisms (Thorson 1950; Jackson & Strathmann 1981), and exposure to environmental stress associated with low tides may thus be critical to survival (reviewed by Moran 1999). Previous research has found that embryonic mortality and abnormality rates of marine invertebrates increased after exposure to ecologically realistic levels of ultraviolet radiation (UVR) (Adams & Shick 2001), desiccation (Pechenik 1978), fouling (Biermann *et al.* 1992), and extremes in salinity (Yaroslavtseva *et al.* 2001) and temperature (Ushakova 2003; reviewed by Przeslawski 2004a). Many intertidal organisms circumvent most of these stressors by releasing gametes or larvae directly into the water column or brooding their offspring, but some species enclose their embryos in benthic egg masses where they remain for at least the early stages of their development (Thorson 1950). Whereas adults are able to seek shelter to avoid stress associated with low tides, embryos within these egg masses are essentially sessile for the duration of their encapsulation.

Encapsulated intertidal embryos must therefore rely on other protection against potentially harmful environmental factors. Encapsulating structures themselves may reduce desiccation (Pechenik *et al.* 2003) and the rate of salinity change (Eyster 1986; Pechenik 1982, 1983) in some species. Certain compounds may also guard against environmental stress; heat shock proteins protect embryos of the cephalaspid *Melanochlamys diomedea* from thermal stress (Podolsky & Hoffmann 1998), and chemical sunscreens have been identified in egg masses from a range of gastropods (Carefoot *et al.* 1998; Przeslawski 2004b).

Alternatively, spawning behaviour can minimize exposure of encapsulated offspring to potentially harmful environmental factors. Marine gastropods of southeastern Australia deposit egg masses under boulders where they are shielded from UVR, desiccation, and other extreme conditions (Benkendorff & Davis 2004). Some intertidal species may also spawn seasonally or tidally to maximise favourable conditions for developing larvae (Hodgson 1999; Ocana & Emson 1999), but such data are limited for encapsulated larvae. Furthermore, egg mass size may vary during spawning periods (pers obs), with smaller egg masses containing fewer embryos (Hoagland 1986; Kasugai & Ikeda 2003).

Thus, depositing smaller and fewer egg masses during stressful periods may reduce the number of encapsulated embryos exposed to potentially harmful conditions. Alternatively, larger egg masses may protect embryos against desiccation and thermal stress better than smaller egg masses. It is presently unknown if embryonic exposure to intertidal stresses is passively avoided through spawning behaviour for a range of species.

Here, I conducted surveys of intertidal egg masses at two sites in southeastern Australia over two years to determine if spatial and temporal variation in spawning behaviour has the potential to reduce environmental stress on encapsulated intertidal invertebrates. I predicted that egg masses will be predominantly deposited in shaded habitats not prone to environmental extremes. Furthermore, I anticipated that species that consistently spawn on rock platforms where they are exposed to full sun and associated stressors will be smaller and occur less frequently during seasons of high environmental stress.

4.1.2 METHODS

Rocky intertidal surveys were conducted at Bellambi (34°37'08"S, 150°92'03"E) and Bass Point (34°35'45"S, 150°53'20"E) in southeastern Australia (Figure 2.1). These sites were chosen because they have a high molluscan species diversity (Benkendorff & Davis 2002), and numerous egg masses have been found consistently at both of them (pers obs). Egg masses at each site were recorded five times during each season for two years. Visits to each site were chosen randomly from days with low tides during daylight. Visits to a site were separated by at least a week so as to minimise repeated counting of the same egg masses and to reduce disturbance, particularly for egg masses under boulders (see Chapman & Underwood 1996).

Three surveys were undertaken at each site on each visit. Counts within quadrats of two sizes were made on the reef surface at random positions along a 100 metre transect line running parallel to the shore along the mid-intertidal zone where egg masses are known to occur. The number and species of all egg masses except *Nerita morio* (formerly *N. atramentosa.* See Waters *et al.* 2005) were recorded in a 0.5 m^2 quadrat (n = 25). As egg masses of *N. morio* were so numerous, the egg capsules of this species were quantified within 0.25 m^2 quadrats (n = 25) thrown haphazardly on the same transect. The third

survey involved examination of the undersides of random boulders (n = 25) in the mid-intertidal zone in which all egg masses were identified and recorded. Boulder volume was 12600 ± 1100 cm^3 (mean ± SEM, n = 75) and did not significantly vary between sites or times. Preliminary observations suggest that these quadrat sizes and number of replicates are sufficient to detect differences between low egg mass occurrence and high egg mass occurrence within a species. An egg mass was defined as a ribbon, group of capsules, or gelatinous structure deposited by a single adult; this was determined by the orientation of the encapsulating structure, similar developmental stage or the presence of a connecting basal membrane. For surveys of *N. morio,* individual egg capsules were counted as it was not possible to determine which groups of capsules a particular adult had deposited. In addition, sizes of random egg masses were recorded for the following species which were sufficiently abundant: *Siphonaria denticulata, Bembicium nanum, Dolabrifera brazieri,* and *Mitra carbonaria*. For gelatinous egg masses, length and width were recorded; and for capsular egg masses, number of capsules was counted.

Egg masses were identified according to previous research (Rose 1985; Smith *et al.* 1989; Benkendorff 1999) or identification of spawning adults (Table 4.1.1). Certain species deposit egg masses which are indistinguishable in the field from other species (e.g. *Dendrodoris fumata* and *D. nigra, Agnewia tritoniformis* and *Cronia contracta*), and I was thus unable to identify these to species level (Table 4.1.1).

Seasonal variation in potential environmental stressors was explored with ANOVA tests. Air temperature, water temperature and tidal data were acquired from the National Tidal Facility, Bureau of Meterology; and UVR data were obtained as monthly averages of standard erythema doses (SED) from the Australian Radiation Protection and Nuclear Safety Agency. For seasonal effects, eight levels were examined in order to account for potential variation across years. Variances for low tides and temperatures were heterogeneous, and these data were log transformed to comply with assumptions of ANOVA. Tukey's HSD tests were used to identify significant relationships *a posteriori*.

Table 4.1.1 List of species with egg masses found in this study and spatial and temporal characteristics in which they were recorded. UB = under boulders, RP = rock platform surface. BP = Bass Point, Bell = Bellambi. 1 = Winter, 2 = Spring, 3 = Summer, 4 = Autumn. References include descriptions of egg mass structure or embryos.

Species	**(Infra)Order**	**Habitat**	**Site**	**Seasons**	**Reference**
Aplysia spp.	Anaspidea	UB	BP, Bell	1,2,3,4	(Switzer-Dunlap & Hadfield 1977)
Dolabrifera brazieri	Anaspidea	UB	BP, Bell	1,2,3,4	pers. obs.
Siphonaria denticulata	Basommatophora	RP[1]	BP, Bell	1,2,3,4	(Anderson 1965)
Siphonaria zelandica	Basommatophora	RP[2]	BP, Bell	1,2,3,4	(Smith *et al.* 1989)
Bembicium nanum	Littorinimorpha	RP[3]	BP, Bell	1,2,3,4	(Smith *et al.* 1989)
Cypraea erosa[4]	Littorinimorpha	UB	BP	4	pers. obs.
Ranella australasia	Littorinimorpha	UB	BP	1,4	(Laxton 1969)
Agnewia tritoniformis/ Cronia contracta	Neogastropoda	UB	BP, Bell	1,2,3,4	(Benkendorff 1999)
Bedeva sp.	Neogastropoda	UB	BP	3,4	(Anderson 1965)
Cominella eburnea[6]	Neogastropoda	UB	BP, Bell	1,2,3	pers. obs. [5]
Conus papilliferus[6]	Neogastropoda	UB	BP, Bell	2,3,4	pers. obs.
Dicathais orbita	Neogastropoda	UB	BP, Bell	2,3	(Smith *et al.* 1989)
Mitra badia[6]	Neogastropoda	UB	BP	1,2,3,4	(Loch 1989)
Mitra carbonaria	Neogastropoda	UB	BP, Bell	2,3	(Loch 1989)
Morula marginalba	Neogastropoda	UB	BP	3,4	(Anderson 1965)
Nerita morio	Neritopsidea	RP	BP, Bell	1,2,3,4	(Smith et al. 1989)
Berthellina citrina	Notaspidea	UB	BP	3	(Hutton 2003)
Pleurobranchus sp.	Notaspidea	UB	BP, Bell	3,4	pers. obs.
Aeolidiella foulisi	Nudibranchia	UB	BP, Bell	3,4	pers. obs.
Austraeolis ornata	Nudibranchia	UB	BP, Bell	1,2,3,4	(Smith *et al.* 1989)

Dendrodoris carneola	Nudibranchia	UB	Bell	2,3,4	(Smith et al. 1989)
Dendrodoris fumata/ Dendrodoris nigra	Nudibranchia	UB	BP	2,4	(Rose 1985; Przeslawski 2003a)
Doriopsilla miniata[4]	Nudibranchia	UB	Bell	2	(Rose 1985)
Doriopsis granulosa[4]	Nudibranchia	UB	BP	3	(Przeslawski 2003b)
Goniodoris meracula	Nudibranchia	UB	Bell	2,3,4	(Przeslawski 2003c)
Goniodoris sp.[4]	Nudibranchia	UB	Bell	2	
Hoplodoris nodulosa	Nudibranchia	UB	BP, Bell	4	(Rose 1985)
Platydoris galbanus[4]	Nudibranchia	UB	Bell	1	(Benkendorff 1998)
Rostanga arbutus[6]	Nudibranchia	UB	Bell	1,2,3,4	(Przeslawski 2003b)
Spurilla macleayi	Nudibranchia	UB	BP, Bell	3,4	pers. obs.
Unknown heterobranch spp.	Unknown	UB	BP, Bell	1,2,3,4	n/a
Unknown mollusc	Unknown	UB	BP, Bell	4	n/a

[1] Found under boulders on nine occasions
[2] Found under boulders on five occasions
[3] Found under boulder once
[4] Egg masses from this species found on only one occasion
[5] Preliminary species identification based on crawling juveniles hatched from egg capsules
[6] Hatch as crawling juveniles

I also examined spatial and temporal variation in egg mass complements between sites, years, and seasons with non-metric multidimensional scaling plots and permutational MANOVAs (Anderson 2001). Egg mass complements were defined as the overall number and species of egg masses. The effects of season on egg mass abundance were determined with 2-way ANOVAs on all egg masses and egg masses of target species. Target species were defined as those for which more than five egg masses were found on more than five occasions or those species which contributed >1% of dissimilarity in egg mass composition between sites or seasons as determined by the SIMPER function in PRIMER v.5: *Bembicium nanum, Nerita morio, Siphonaria denticulata,* and *Siphonaria zelandica* which spawn on rock platform surfaces; and *Agnewia tritoniformis/Cronia contracta, Cominella eburnea, Dolabrifera brazieri,* and *Mitra carbonaria* which spawn under boulders (pers. obs). Variances for some species were heterogeneous, and these data were log transformed to comply with assumptions of ANOVA.

I used JMP v.4 for ANOVAs and PERMANOVA v.6 for permutational MANOVA tests. PRIMER v.5 was used for all other multivariate functions. $\alpha = 0.05$ in all statistical tests.

4.1.3 RESULTS

4.1.3.1 *Spatial Variation in Egg Mass Deposition*

Egg masses from 34 species or taxonomic groups were found (Table 4.1.1). Only four species spawned on rock platforms in full sun (*Bembicium nanum, Nerita morio, Siphonaria zelandica* and *S. denticulata*); egg masses of 88% of species examined in this study were found only under boulders (Table 4.1.1).

Egg masses of each species were generally found exclusively under boulders or exclusively in full sun habitats. Indeed, the only species with egg masses found in both habitats were those that predominantly spawn in full sun. This occurred very sporadically and only during peak spawning seasons (i.e. summer and early autumn). These events did not occur with sufficient frequency to allow analysis of intraspecific differences in spawning site between seasons (Table 4.1.1) (e.g. Spight 1977).

4.1.3.2 *Temporal Variations in Egg Mass Deposition*

Seasonal Patterns in Environmental Stress

As anticipated, I detected compelling evidence for seasonal variation in environmental stress. UVR levels varied significantly between seasons ($df = 7$, $F = 10.18$, $p < 0.001$). Summer had significantly higher UVR levels than autumn or winter across both years (Figure 4.1.1) as confirmed by Tukey's HSD tests. Lowest daylight tides were also significantly affected by season ($df = 7$, $F = 40.73$, $p < 0.001$). Winter had the highest low tides while spring and summer had the lowest (Figure 4.1.1). Tukey's HSD tests confirmed significant differences between all seasons except spring and summer and, during the 2nd year, winter and autumn. Significant seasonal differences were also detected in air ($df = 7$, $F = 128.85$, $p < 0.0001$) and water ($df = 7$, $F = 309.27$, $p < 0.0001$) temperatures. Air temperature varied significantly between all seasons with summer showing the highest temperatures and winter the lowest (Figure 4.1.1). Water temperature varied significantly between all seasons except summer and autumn, these seasons had the highest water temperatures while winter had the lowest (Figure 4.1.1).

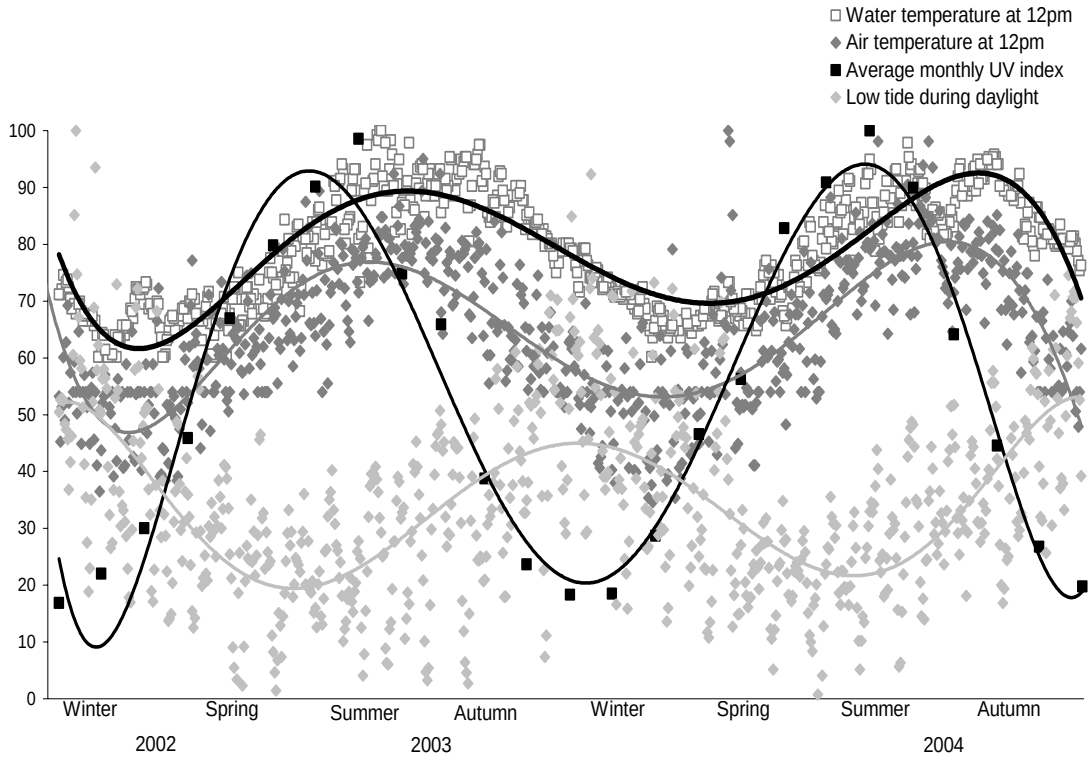


Figure 4.1.1 Seasonal fluctuations in intertidal environmental stressors over two years at Wollongong, NSW, Australia. Data is expressed as percentage of maximum value. Max air temperature = 26.3˚C, max water temperature = 23.6˚C, max UVR = 50.17 standard erythemal dose (SED), and max daylight low tide = 1293 mm. Lines represent 6th order polynomial best fits: bold black line = water temperature; dark gray line = air temperature; black line = UV index; and light gray line = low tide. Sources of data are indicated in text.

Seasonal Variation in Egg Mass Complements

Complements of egg masses varied across seasons according to site. This was confirmed by a permutational MANOVA which revealed a significant interaction between site and season on egg masses (df = 7, F – 1.7812, p = 0.0017). There was a significant different in egg mass complements across years only at Bellambi during spring 2002 and spring 2003 as revealed by pairwise comparisons. Egg mass complements were significantly different at both sites between winter and spring, winter and summer, and spring and autumn across all years. Pairwise comparisons also confirmed significant differences in egg mass complements between spring and summer, winter and autumn, and summer and autumn; but these differences were site-specific and only occurred during seasons in one year.

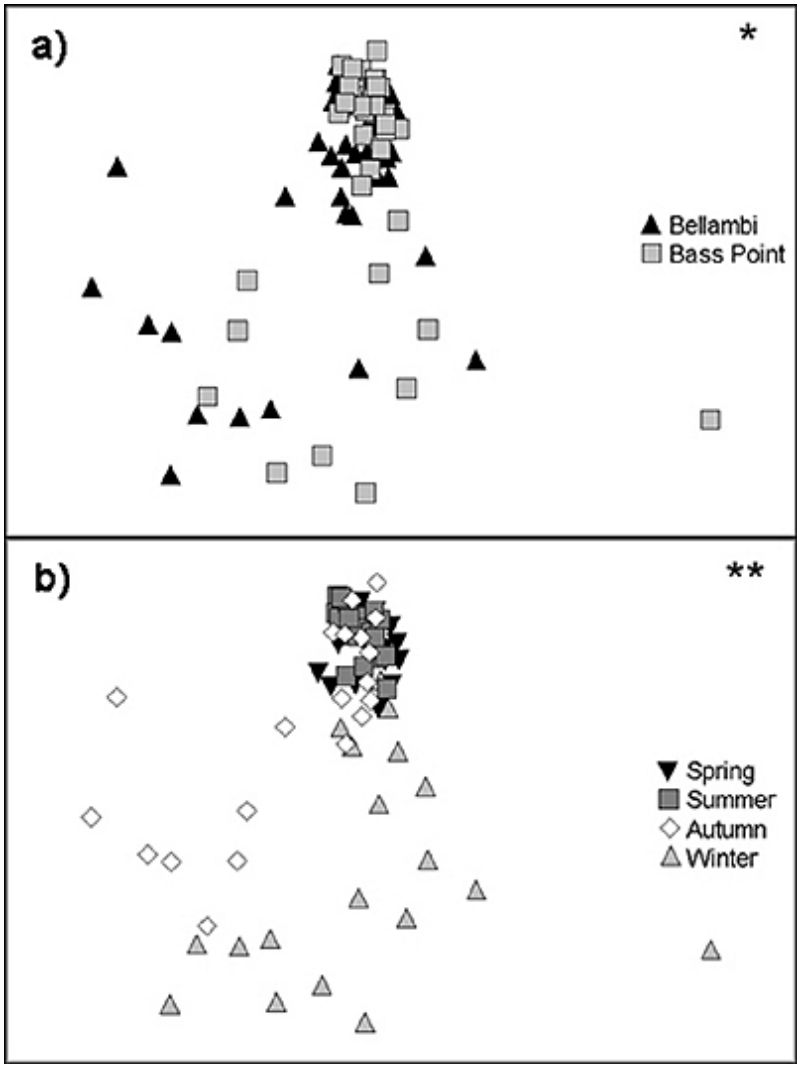


Figure 4.1.2 Similarities of egg mass composition as revealed by nMDS plots according to a) site (n = 40) and b) season (n = 20). Stress = 0.10. * denotes significance at alpha = 0.01, and ** at alpha = 0.001.

Seasonal Variation in Egg Mass Abundance

Total abundance of egg masses showed significant seasonal variation on the undersides of boulders ($p < 0.0001$) (Table 4.1.2, Figure 4.1.3b). Significantly fewer egg masses were found in winter than in any other season as revealed by a Tukey's HSD test (Figure 4.1.3b). In contrast, total number of egg masses showed no significant seasonal differences on rock platform surfaces ($p = 0.07$) (Table 4.1.2, Figure 4.1.3a). *Nerita morio* was excluded from this analysis because egg capsules of this species were incomparable with those from other species as capsules from a single adult are indistinguishable from other capsules.

Table 4.1.2 Effects of season and site on mean number of all egg masses on a) rock platform surfaces and b) undersides of boulders[1]. All factors are fixed.

	Effect	df	MS	F	p
a)	Season	7	9.1680	1.8170	0.9880
	Site	1	0.1240	0.0246	0.8758
	Season x Site	7	2.3119	0.4586	0.8607
	Residual	64	5.0408		
	Corrected Total	79	5.1025		
b)	Season	7	5.8517	13.8873	<0.0001
	Site	1	0.4218	1.0010	0.3208
	Season x Site	7	0.1477	1.0338	0.4167
	Residual	64	0.4214		
	Corrected Total	79	10.2002		

[1] Data log transformed to correct heterogeneous variances

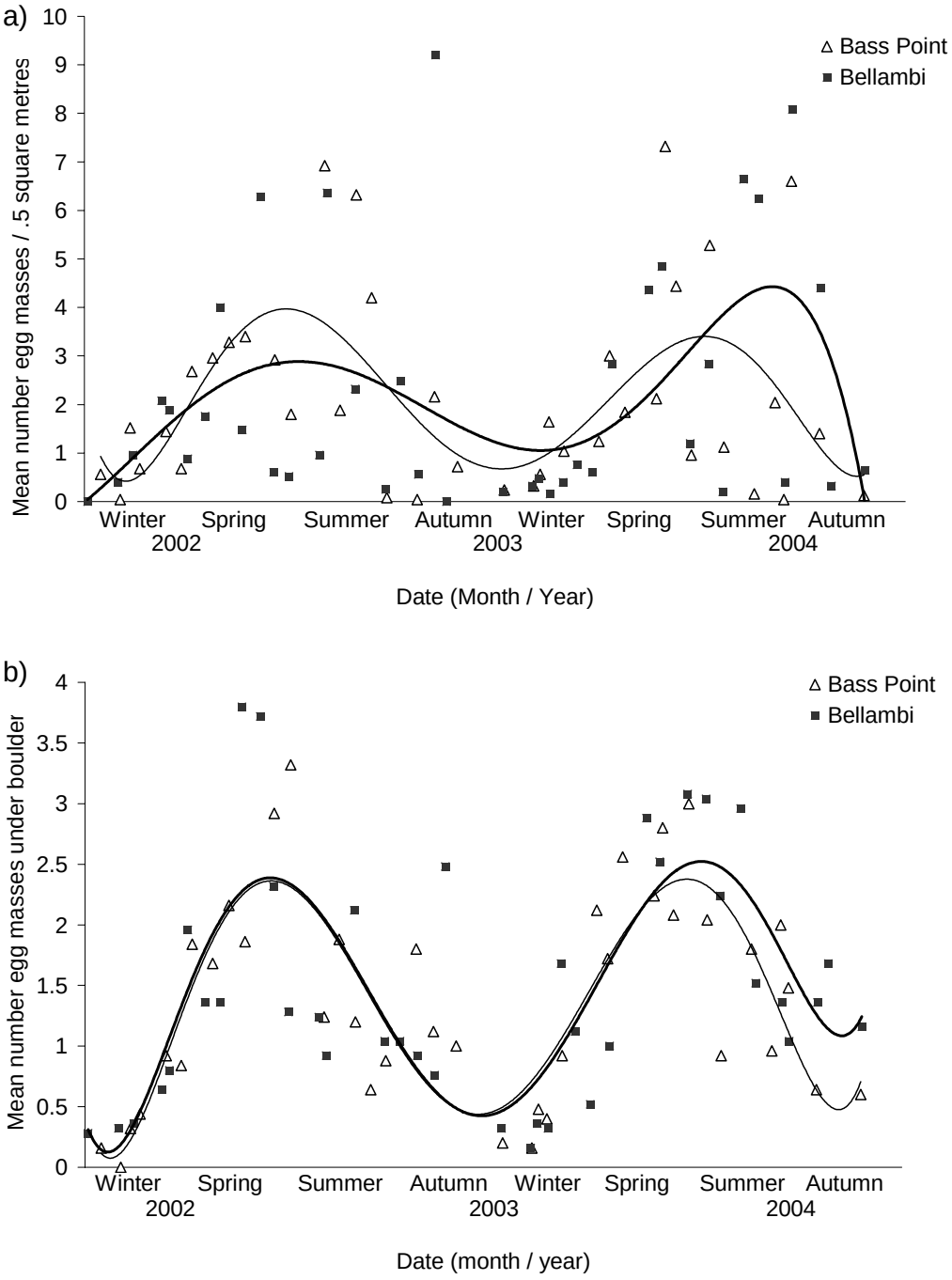


Figure 4.1.3 Seasonal variation in total assemblages of egg masses in two microhabitats: a) Rock platform surfaces, and b) Under boulders. All species found are included except *Nerita morio* because egg masses of this species were not comparable to other egg masses as individual capsules were counted, rather than entire egg masses (see text). Lines represent 6th order polynomial best fits: black line = Bass Point and bold black line = Bellambi.

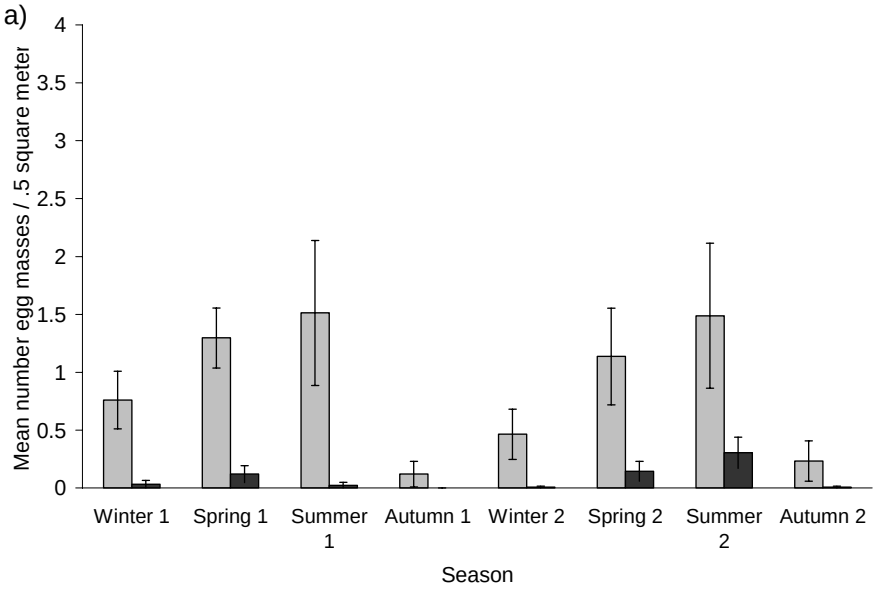
Egg mass abundance of target species varied between seasons for most target species, and these effects were species specific. We detected significant seasonal variation in egg mass abundance for all target species with the exception of *S. zelandica* and *S. denticulata* (Table 4.1.3, Figure 4.1.4c,d). During both years, more *B. nanum* egg

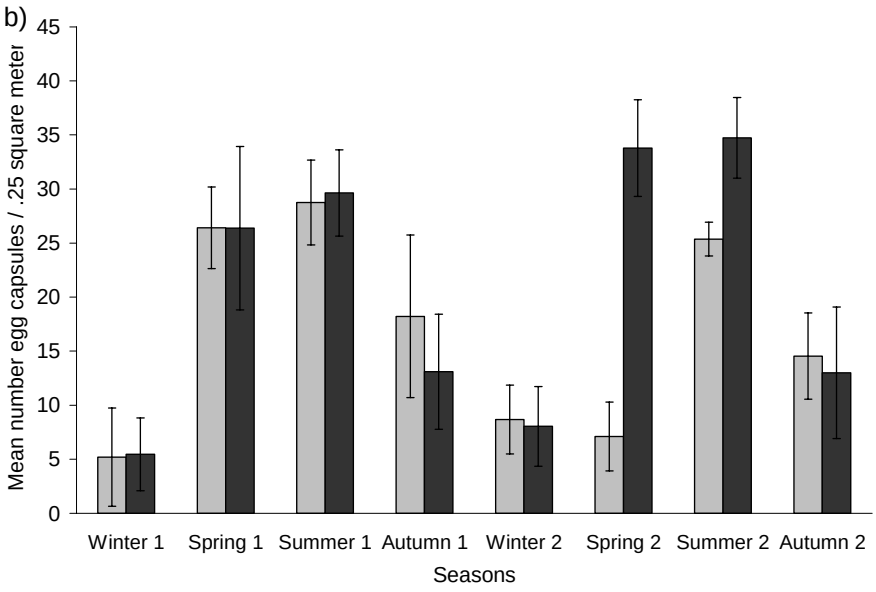
masses were found in spring and summer than autumn ($p = 0.0002$) (Figure 4.1.4a). Similarly, more N. morio egg capsules were found in spring and summer than in winter of the first year surveyed ($p < 0.0001$) (Figure 4.1.4b). During the 2nd year, significantly more *D. brazieri* egg masses were found in summer than winter ($p = 0.0023$); this same trend existed in the first year but was not significant (Figure 4.1.4g). *M. carbonaria* egg masses were found only once at Bellambi, and we were thus able to analyse seasonal effects only at Bass Point. Here, significantly more egg masses were found during spring of the 2nd year than autumn or winter of either year. ($p = 0.0002$) (Figure 4.1.4h). Season and site interacted to affect the abundance of *A. tritoniformis/C. contracta* ($p = 0.0026$) and *Cominella eburnea* ($p = 0.0002$). Significantly more egg masses of *A. tritoniformis/C. contracta* were found at Bass Point during spring and summer than autumn and winter, but there were no seasonal differences in egg mass abundance at Bellambi, likely due to low egg mass abundance of this species here (Figure 4.1.4e). More egg masses of *Cominella eburnea* were found at Bellambi during spring months of the first year surveyed than any other season. This same pattern was detected at Bass Point, but there were no significant seasonal differences in egg mass abundance at this site (Figure 4.1.4f). Furthermore, site significantly affected egg mass abundance of *B. nanum* ($p < 0.0001$, Table 4.1.3a) and *S. zelandica* ($p = 0.0002$, Table4.1. 3d) with more *B. nanum* egg masses found at Bass Point (Figure 4.1.4a) and more *S. zelandica* egg ribbons found at Bellambi (Figure 4.1.4d).

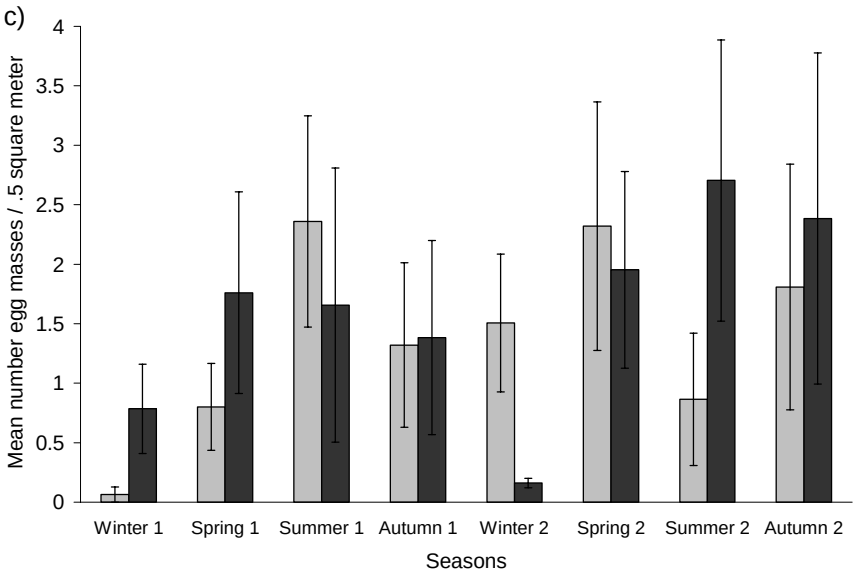
Table 4.1.3 Effects of season and site on egg mass abundance of target species or groups, a) *Bembicium nanum*[1], b) *Nerita morio*[1], c) *Siphonaria denticulata*, d) *Siphonaria zelandica,* e) *Agnewia tritoniformis / Cronia contracta*[1], f) *Cominella eburnea* g) *Dolabrifera brazieri*[1], h) *Mitra carbonaria*[2], and. All factors are fixed.

	Effect	df	MS	F	p
a)	Season	7	65.5056	4.9478	0.0002
	Site	1	842.9996	63.6745	<0.0001
	Season x Site	7	16.9993	1.2840	0.2725
	Residual	64	13.2392		
	Corrected Total	79	28.7069		
b)	Season	7	34.1227	7.9232	<0.0001
	Site	1	7.0476	1.6364	0.2054
	Season x Site	7	2.2541	0.5234	0.8136
	Residual	64	4.3067		
	Corrected Total	79	6.8014		
c)	Season	7	5.5767	1.6595	0.1351
	Site	1	2.7528	0.8192	0.3688
	Season x Site	7	1.6806	0.5001	0.8310
	Residual	64	3.36050		
	Corrected Total	79	38.3751		
d)	Season	7	0.4439	1.1955	0.3182
	Site	1	6.0280	16.2340	0.0002
	Season x Site	7	0.4328	1.1655	0.3350
	Residual	64	0.3713		
	Corrected Total	79	5.1328		
e)	Season	7	17.8837	7.9905	<0.0001
	Site	1	350.4787	156.5948	<0.0001
	Season x Site	7	8.0191	3.5830	0.0026
	Residual	64	2.2381		
	Corrected Total	79	8.5448		
f)	Season	7	10.0119	3.6297	0.0023
	Site	1	0.0255	0.0092	0.9237
	Season x Site	7	2.6595	0.9642	0.4649
	Residual	64	2.7583		
	Corrected Total	79	3.3577		
g)	Season	7	0.3512	5.5932	0.0002
	Residual	32	0.0591		
	Corrected Total	39	0.1115		
h)	Season	7	0.4976	4.8658	0.0002
	Site	1	1.8313	17.9082	<0.0001
	Season x Site	7	0.4893	4.7846	0.0002
	Residual	64	0.1023		
	Corrected Total	79	0.1935		

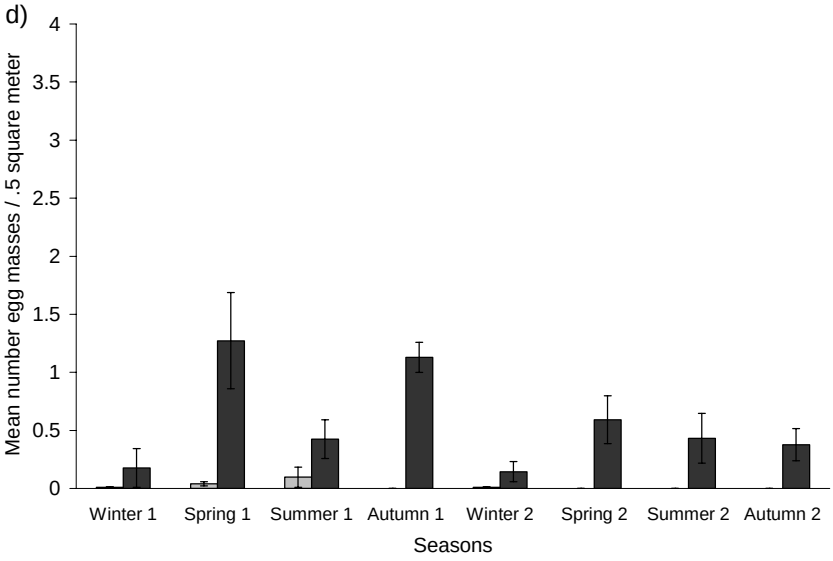
[1] Data log transformed to correct heterogeneous variances
[2] Only data from Bass Point analysed


a)
Mean number egg masses / .5 square meter
0
0.5
1
1.5
2
2.5
3
3.5
4
Winter 1
Spring 1
Summer 1
Autumn 1
Winter 2
Spring 2
Summer 2
Autumn 2
Season

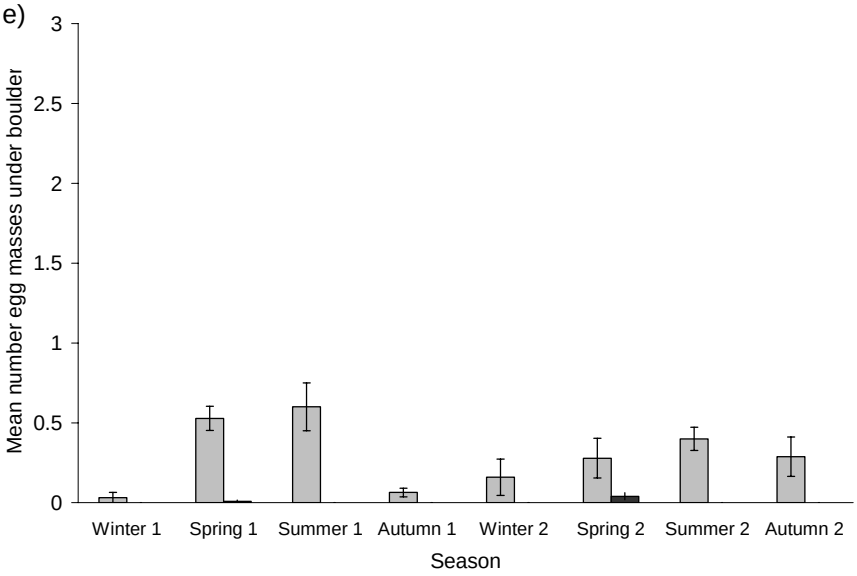

b)
Mean number egg capsules / .25 square meter
0
5
10
15
20
25
30
35
40
45
Winter 1
Spring 1
Summer 1
Autumn 1
Winter 2
Spring 2
Summer 2
Autumn 2
Seasons


c)
Mean number egg masses / .5 square meter
0
0.5
1
1.5
2
2.5
3
3.5
4
Winter 1
Spring 1
Summer 1
Autumn 1
Winter 2
Spring 2
Summer 2
Autumn 2
Seasons

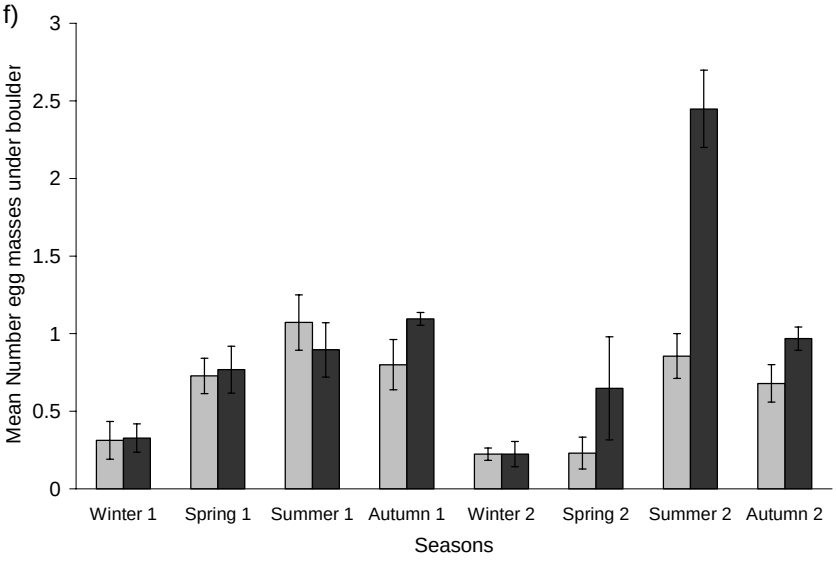
d)


Mean number egg masses / .5 square meter
4
3.5
3
2.5
2
1.5
1
0.5
0
Winter 1
Spring 1
Summer 1
Autumn 1
Winter 2
Spring 2
Summer 2
Autumn 2
Seasons

e)


Mean number egg masses under boulder
3
2.5
2
1.5
1
0.5
0
Winter 1
Spring 1
Summer 1
Autumn 1
Winter 2
Spring 2
Summer 2
Autumn 2
Season

f)


Mean Number egg masses under boulder
3
2.5
2
1.5
1
0.5
0
Winter 1
Spring 1
Summer 1
Autumn 1
Winter 2
Spring 2
Summer 2
Autumn 2
Seasons

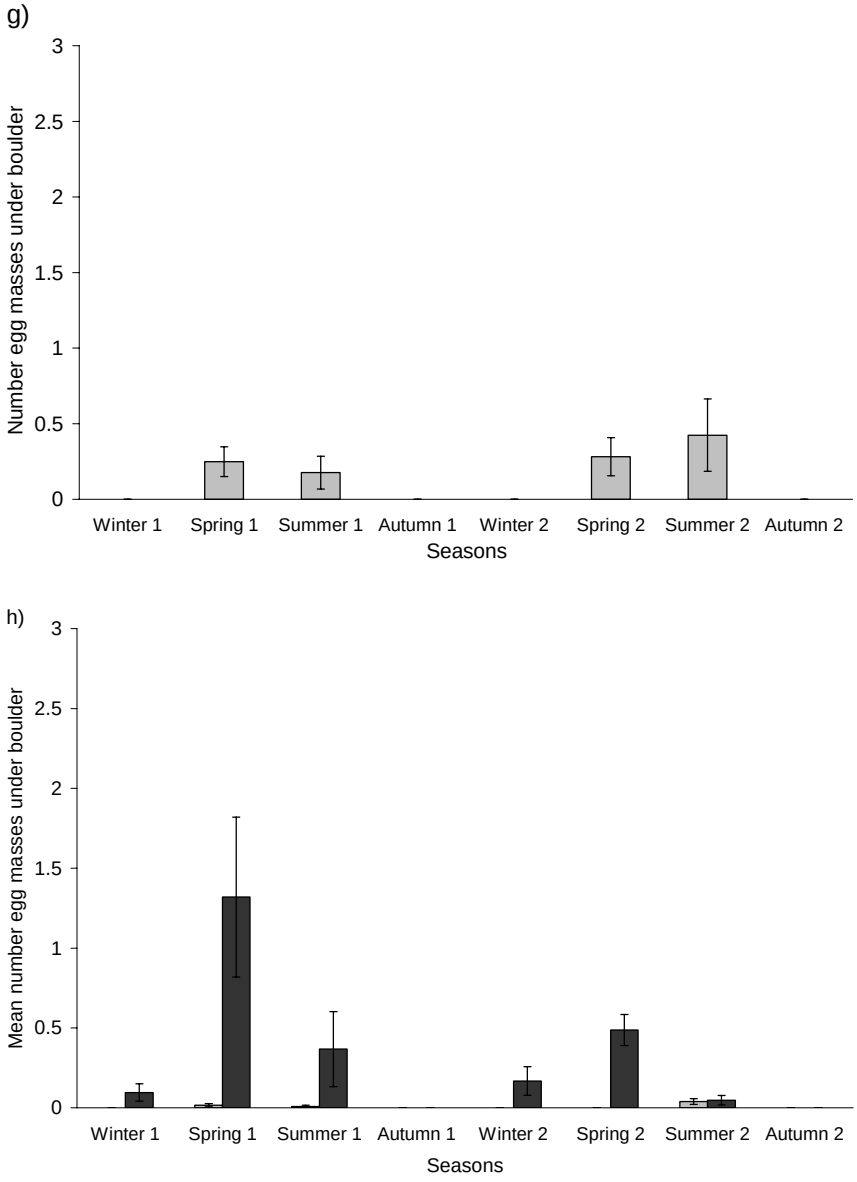


Figure 4.1.4 Effects of season and site on egg mass abundance of target species. a) Bembicium nanum, b) Nerita morio, c) Siphonaria denticulata, d) Siphonaria zelandica, e) Agnewia tritoniformis / Cronia contracta, f) Cominella eburnea, g) Dolabrifera brazieri, and h) Mitra carbonaria, and. Light gray bars indicate data from Bellambi, and dark gray bars indicate data from Bass Point. Error bars are standard error of mean.

Seasonal Variation in Egg Mass Size

Sizes of egg masses in all seasons at both sites were obtained for *S. denticulata* (n = 34) and *D. brazieri* (n = 26). Sufficient numbers of *B. nanum* (n = 44) and *M. carbonaria* (n = 14) in all seasons were obtained only at Bass Point. Significant seasonal variation occurred in egg mass size of *D. brazieri* (p = 0.0026); larger egg masses were found in spring than the other seasons as confirmed by a Tukey's test. Egg mass size of other species examined did not vary seasonally (Table 4.1.4); but site significantly affected egg mass size of *S. denticulata* (p = 0.0018, Table 4.1.4b), with larger egg ribbons occurring at Bellambi. Egg mass size of the other species examined did not vary significantly between sites (Table 4.1.4).

Table 4.1.4 Effects of season and site on egg mass size of a) *Bembicium nanum*[1], b) *Siphonaria denticulata*, c) *Dolabrifera brazieri*, and d) *Mitra carbonaria*[1]. All factors are fixed.

	Effect	df	MS	F	p
a)	Season	3	1.4065	2.2271	0.0868
	Residual	172	0.6315		
	Corrected Total	175	0.6448		
b)	Season	3	1.5827	1.6745	0.1728
	Site	1	9.3523	0.0018	0.0018
	Season x Site	3	2.2983	0.0654	0.0654
	Residual	274	0.9450		
	Corrected Total	281	0.9948		
c)	Season	3	225.8866	4.9107	0.0026
	Site	1	60.5718	1.3168	0.2525
	Season x Site	3	99.2941	2.1586	0.0941
	Residual	200	45.9990		
	Corrected Total	207	49.4489		
d)	Season	1	0.7424	1.4799	0.2347
	Residual	26	0.5017		
	Corrected Total	27	0.5106		

[1] Data log transformed to correct heterogeneous variances

4.1.4 DISCUSSION

Species composition of gastropod egg masses and egg mass abundance of target species show tremendous spatial and temporal variation, but results from this study also indicate that it is not spawning behaviour alone that minimises exposure of encapsulated intertidal larvae to stresses associated with low tides. Contrary to my prediction, egg masses of many species, particularly those that spawn consistently on surfaces of rock platforms, are deposited most frequently during seasons of high environmental stress.

The potential stressors examined here peaked during summer months. During this time, water temperature, air temperature and UVR index were highest while tides were lowest compared to other seasons (Figure 4.1.1). Thus, intertidal organisms are exposed not just to isolated seasonal peaks of stressors; rather, they may be synchronously exposed to extremes of a multitude of stressors during summer. Moreover, there are abiotic factors not examined in this study which may also contribute to cumulative stress. For example, higher temperatures during summer may encourage algal fouling on egg masses (Beardall & Raven 2004) and increase water evaporation rate thereby raising salinity in small rock pools.

Egg masses from four species found in this study occurred routinely on rock platforms where they were exposed to full sun and associated higher temperatures, salinity, and desiccation rates (Przeslawski *et al.* 2004; Przeslawski 2005)(Table 4.1.1). Although overall composition of egg masses varied between seasons (Figure 4.1.2b), egg masses from species that spawn on rock platform surfaces did not show any temporal variation that may confer protection (Figures 4.1.3a, 4.1.4a-d). Indeed, peak abundance of egg masses from these exposed habitats occurred during summer and spring (Figure 4.1.3a, 4.1.4a-d) when many physical stressors are at highest levels (Figure 4.1.1). This is surprising considering the greater risk to embryos of these species of exposure to cumulative summer environmental stress than egg masses spawned under boulders. Previous studies have suggested that spawning behaviour of intertidal gastropods may be timed to minimise thermal stress (Podolsky 2003), but results from the present study indicate this trend does not apply on a seasonal scale. In fact, seasonal spawning behaviour in some gastropods does not minimise exposure to physical stresses associated with summer, but rather maximises such exposure.

In contrast, egg masses of most species found in this study occurred under boulders (Table 4.1.1). Embryos of these species may avoid extremes of environmental stressors by being deposited in protected microhabitats. In fact, more species than detected likely spawn in protected microhabitats as the present study did not account for egg masses in many other sheltered intertidal habitats such as algal beds, overhangs, or caves. Previous studies have found that most spawning sites for rocky shore gastropods similarly encompass sheltered microhabitats (Smith 1972; Benkendorff & Davis 2004).

Thus, spatial patterns in spawning behaviour of many species likely protect encapsulated embryos from environmental stressors, particularly UVR and desiccation.

Another way for adults to minimise exposure of offspring to abiotic stress is to reduce sizes of egg masses, and therefore number of embryos, during seasons of highest stress. Alternatively, adults may increase the size of egg masses during seasons of highest stress as larger gelatinous egg masses may reduce stress associated with desiccation to internal embryos. However, neither adaptation existed among the four species examined here. Only *D. brazieri* egg masses showed significant seasonal variation in size, but this pattern did not indicate protection against physical stress as the largest egg ribbons were found during spring when potential stressors were relatively high (Figure 4.1.1). Furthermore, this species spawned in sheltered environments where stressors examined in this study would be reduced (i.e. water temperature) or absent (i.e. UVR). Egg masses can show extraordinary inter- and intra-species variation in shape, colour, ultrastructure, and size (e.g. Pilkington 1974), and it is assumed that many of these variations reflect evolutionary adaptations to maximise hatching success and minimise energetic costs amongst potentially stressful environments (Clark & Goetzfried 1978). However, egg masses of the four species examined here, particularly *B. nanum* and *S. denticulata* that spawn on rock platforms, do not show any obvious seasonal reduction in egg mass size that could minimise numbers of embryos exposed to peak stressful conditions.

Although overall egg mass composition varied significantly between sites (Figure 4.1.2a), the general spawning trends observed in this study were consistent across sites. The spawning periods of target species peaked during the same season at both sites (Figure 4.1.4), and egg mass assemblages on both rock platform surfaces and undersides of boulders showed similar temporal variation at both sites (Figure 4.1.3). Indeed, temporal variation in egg mass assemblages under boulders showed almost identical patterns across sites as evidenced by remarkably similar trendlines (Figure 4.1.3b). Based on these similarities between sites, peak spawning periods generally occur in spring or summer, regardless of spawning habitat.

Other studies examining single species have also reported that temporal and spatial spawning patterns do not maximise encapsulated embryonic survival (Spight 1977;

Yamahira 1996), and this trend may be explained by several reasons. First of all, encapsulated embryos may possess direct protection against some environmental stressors, making protection associated with spawning behaviour unnecessary. Compounds such as mycosporine-like amino acids (MAAs) and heat-shock proteins have been identified in intertidal egg masses of some gastropods and may afford chemical protection against UVR and high temperatures (Carefoot *et al.* 1998; Podolsky & Hoffmann 1998; Przeslawski 2004b). Furthermore, the encapsulating structure may buffer against UVR (Rawlings 1996), desiccation, and changes in salinity and temperature (Pechenik 1979, 1982). Nevertheless, these mechanisms do not provide complete protection; field observations on *Bembicium nanum* and *Siphonaria denticulata* suggest that rock platform surface are not conducive to embryonic survival (Przeslawski et al. 2005; Przeslawski 2005). Second, spawning behaviour may simply reflect adult reproductive physiology. Spawning of many gastropods is thermally regulated (Underwood 1979), and it may be that reproductive output is generally higher in warmer seasons despite increased potential for larval exposure to environmental stress. Third, seasonal variation in egg mass abundance and size may not reflect conditions that maximise embryonic survival, but instead maximise developmental rate. Highest temperatures occur in summer months (Figure 4.1.1), and rock pools in which *S. denticulata, B. nanum* and *N. morio* spawn can reach 35ºC in summer during low tides (pers. obs.). Temperature is directly related to developmental rate up to a threshold (reviewed by Palmer 1994). A faster developmental rate reduces time spent in vulnerable larval stages (Havenhand 1993) and hastens development of some protective mechanisms that may further increase survival (Podolsky 2003). These benefits of increased developmental rates may counteract the negative effects of increased UVR and desiccation during summer months. Finally, spawning behaviour may reflect optimal conditions for later larval stages, rather than encapsulated embryos. Summer coincides with peak period of dispersal capability and phytoplankton (Underwood 1979), and optimal conditions for larval recruitment and /or food availability may thus outweigh benefits associated with spawning in autumn or winter.

Adaptations in spawning behaviour may exist that were not examined here. Spatial variation in egg mass abundance or size may occur on a smaller scale than that examined in the present study. Previous studies have found that extremely small microhabitats such as damp crevices significantly protect encapsulated embryos from

environmental stress (Pechenik 1978; Ocana & Emson 1999). Here, I differentiated habitats into only two groups, and it may be that this was too broad to detect potential protection conferred by smaller microhabitats on rock platform surfaces. Casual observations indicate that egg masses from *B. nanum, Siphonaria* spp., and *N. morio* do indeed show a preference for depositing their egg masses in damp crevices or shallow pools on the rock platform surface (pers. obs.). Similarly, egg mass abundance or size may minimise embryonic exposure to environmental stress by variation on a smaller temporal scale. For example, some species, including many siphonarids, spawn in tidal cycles (Creese 1980; Ocana & Emson 1999) which may reduce exposure to thermal stress (Podolsky 2003). Indeed, egg mass abundance of species such as *S. denticulata* and *S. zelandica* showed relatively large variation within seasons as evidenced by the large error bars (Figure 4c,d). This supports previous research (Creese 1980; Ocana & Emson 1999) and suggests that variations in spawning for these species may be regulated by smaller temporal scales such as lunar cycles rather than seasonal trends. The present study quantified egg mass abundance and size relatively infrequently and focused on seasonal variations. Future studies incorporating daily surveys may help identify adaptations in spawning behaviour in response to physical stress that may exist over shorter time periods.

Nonetheless, many gastropods consistently spawn in the intertidal where they are may be exposed to full sunlight. Although there has been much speculation as to the reasons behind this risky spawning habitat, there is no conclusive evidence to date. It may be that competition for appropriate space is reduced because the habitat is inhospitable to most species. Alternatively, some species may spawn in potentially risky habitats to reduce predation as the predators themselves may be less tolerant to the negative effects of UVR (Spight 1977; Ocana & Emson 1999). It is currently unknown if this applies to the species in this study. Recent research shows that the egg masses of many gastropods, including *B. nanum, S. denticulata,* and *D. brazieri,* are unpalatable to two common intertidal predators (Watson 2002); but more predatory species must be tested before generalisations can be made. As mentioned before, some gastropods may spawn in sunlight to increase developmental rate. In the field, UVR and increased temperature are inseparable. Therefore, direct sunlight might reduce the general risk to embryos by reducing the time spent in vulnerable larval stages (Spight 1975; Strathmann et al. 2002). Finally, it has also been suggested that spawning site selection depends more on

juvenile/adult welfare than embryonic welfare (Spight 1977), but this is difficult to empirically investigate. Regardless of the reasons for spawning in risky intertidal habitats, this study suggests that intertidal spawning patterns do not reflect avoidance of environmental stress and may instead reveal species-specific evolutionary adaptations and physiological responses to a variety of abiotic and biotic factors.

4.2 BIOCHEMICAL PROTECTION: A QUANTITATIVE SURVEY OF MYCOSPORINE-LIKE AMINO ACIDS (MAAS) IN INTERTIDAL EGG MASSES FROM TEMPERATE ROCKY SHORES

Mycosporine-like amino acids (MAAs) have been reported as functional chemical sunscreens in a variety of marine organisms, but their role in development of marine larvae remains largely unexplored. In this study, I quantified MAAs from intertidal egg masses of 46 species of mollusc, two species of polychaete, and one species of fish from southeastern Australia. I sought to elucidate potential patterns of occurrence and variation based on egg mass maturity, adult diet, spawning habitat, phylogeny, and viability. my analyses revealed that maturity and spawning habitat did not significantly affect MAA composition within egg masses. In contrast, adult diet, phylogeny, and viability significantly affected MAA composition. Herbivores had significantly higher levels of certain MAAs than carnivores; similarly, viable egg masses had higher levels of some MAAs than inviable egg masses. MAA composition varied according to taxonomic group, with nudibranchs and anaspids showing different MAA composition to that of neogastropods, sacoglossans, and polychaetes. Basommatophoran egg masses had significantly more porphyra-334 than the other groups, and anaspids had more mycosporine-2-glycine than the other groups. MAAs occurred in relatively high concentrations in intertidal molluscan egg masses when compared to adult molluscs and other common intertidal organisms. Despite the complexity of factors affecting MAA composition, the prevalence of MAAs in some species is consistent with protection afforded to offspring against negative effects of UVR.

4.2.1 INTRODUCTION

Surface ultraviolet radiation (UVR) has been shown to deleteriously affect reproduction, development, growth, and behaviour of many organisms including marine invertebrates (reviewed by Haeder *et al.* 1998). Biologically significant UVR comprises UV- B (280-315nm) and UV-A (315-400nm) (Cockell & Knowland 1999), with UV-B absorbed strongly by the ozone layer (Paul & Gwynn-Jones 2003). Although UVR is attenuated in the water column, biologically significant levels of UV-B may still penetrate to 20 metres or more (Booth & Morrow 1997). Hence, organisms in shallow water or intertidal habitats are expected to be especially vulnerable. Recent research confirms

that encapsulated larvae of intertidal molluscs are at high risk of UVR damage (Przeslawski *et al.* 2004).

Larvae may reduce or eliminate exposure to UVR in several ways. Larvae may simply avoid UVR as a result of adult spawning behaviour. Many free-spawning invertebrates, for example, release gametes nocturnally so that fertilised eggs undergo early developmental stages in darkness (e.g. Pagano *et al.* 2004). Other organisms, particularly gastropods, enclose offspring within leathery capsules (Figure 1.1) or gelatinous egg masses (Figure 1.2). The embryos are essentially sessile for the duration of their encapsulation and reliant on adult spawning habitat for protection against UVR. Many gastropods spawn only under boulders or in other fully shaded microhabitats, but some species consistently deposit their egg masses in habitats exposed to sunlight (Benkendorff & Davis 2004). The developing embryos within these egg masses are potentially exposed to high intensities of UVR with no visible protection. Nevertheless, gastropod embryos of species that consistently spawn in full sunlight are less vulnerable to the negative effects of UVR than embryos from species that spawn only in shaded habitats (Przeslawski *et al.* 2004).

Alternatively, some encapsulated embryos may be protected from UVR through chemical sunscreens, such as mycosporine-like amino acids (MAAs). MAAs are a suite of 19 compounds with absorption maxima at 310 – 360nm. They are produced *de novo* via the shikimate pathway in algae, fungi, and bacteria (Bentley 1990), and animals are assumed to acquire MAAs through diet or symbioses with these organisms (Shick & Dunlap 2002). MAAs have been found in adults from a variety of organisms including algae, fish, cnidarians, molluscs, and echinoderms (reviewed by Shick & Dunlap 2002). The concentration and composition of MAAs can vary in organisms according to their latitude (Bosch *et al.* 1994), altitude (Tartarotti *et al.* 2001), season (Michalek-Wagner 2001), diel fluctuation (Yakovleva & Hidaka 2004), depth (Gleason & Wellington 1995), sex (Michalek-Wagner 2001), and species (Xiong *et al.* 1999). MAAs have also been reported from the eggs and larvae of several invertebrates including urchins, corals, ascidians, and gastropods (reviewed by Karentz 2001). Only two molluscan egg masses were covered in this review, so Przeslawski (in press b) conducted a preliminary investigation on a further 46 gastropod egg masses. This revealed that the total MAA content varies significantly according to taxonomic group and adult diet but it is still not

clear how MAA composition varies with these and other factors. Larvae are generally considered the most vulnerable life stage and so MAA composition in such species may be especially important to the overall success of a population.

In this study, I have collected benthic egg masses from a range of intertidal invertebrates and quantified their MAA composition. I aimed to identify common MAAs in intertidal egg masses and to determine which factors account for variation in MAA composition and abundance. Here I investigate the effects of egg mass maturity, adult diet, spawning habitat, taxonomic group (order), and embryo viability. I predicted that herbivores would have a richer complement of MAAs at higher concentrations in their spawn than carnivores as they have a more direct link to *de novo* MAA sources. I also anticipated that egg masses of species that routinely spawn in habitats exposed to full sun would contain a larger number of MAAs at higher concentrations than those spawned in shaded habitats. In addition, I collected adults of several shelled species and predicted that adults would show fewer MAAs at lower concentrations than spawn due to the need for photoprotection of vulnerable developmental stages.

4.2.2 METHODS AND MATERIALS

I undertook a quantitative survey of MAA composition and concentration in egg masses from 49 intertidal organisms representing 46 gastropod species, two unidentified polychaete species, and one gobiesocid fish (Table 4.2.1). Most egg masses were collected from intertidal habitats along the Illawarra coast November 2001- February 2004. Some samples were collected from adults held in laboratory aquaria, and egg capsules of *Nucella lapillus* were collected from Cornwall, England. Egg masses were identified to species level where possible based on observations of laying adult or previous research (Rose 1985; Smith *et al.* 1989; Benkendorff 1999). Potential spatial and temporal variation in MAA composition was examined on a subset of the egg masses. I restricted these analyses to species with three or more replicates. Spatial differences in MAA composition were analysed for *Dolabrifera brazieri, Dicathais orbita, Placida* cf. *dendritica,* and *Siphonaria denticulata.* Temporal differences were examined in egg masses of *Agnewia tritoniformis, Conus papilliferus, Dendrodoris fumata, D. brazieri, P.* cf. *dendritica,* and *S. denticulata*. Nested ANOSIMs revealed no significant spatial ($R = 0.049$, $p = 0.273$) or temporal ($R = 0.17$, $p = 0.114$) variations in

MAA concentrations for any of the species examined. Therefore, egg mass data for all species collected from different sites and times were pooled in the remaining analyses.

Both capsular and gelatinous egg masses were collected. Egg masses from herbivores represented 16 species and carnivores represented 30 species; the diet of three species was unknown (Table 4.2.1). I collected egg masses of 6 species from full sun habitats (exposed rock platforms), 13 species from partial sun (algae beds, sand or vertical rock faces), and 30 species from shaded habitats (under boulders) (Table 4.2.1). Egg masses were examined under a dissecting microscope (40x magnification) to determine development, and they were classed accordingly into one of four developmental stages: 1) Undeveloped egg masses contained eggs that had not yet developed to the trochophore stage; 2) Egg masses with intermediate development contained eggs with trochophores or early veligers; 3) Mature egg masses had late stage veligers, crawling juveniles or showed signs of hatching; 4) Inviable egg masses contained no viable eggs and often showed colouration changes associated with damage or stress (Pechenik 1983; Przeslawski *et al.* 2004). After examination, egg masses were cleaned by agitation in filtered seawater for 30 seconds followed by gentle blotting to remove excess water. Egg masses were then lyophilised, and dry weight was recorded. Samples were stored at -80ºC until extractions were performed. No degradation of MAAs was detected in an 18-month storage period, as also noted by previous studies (Dunlap & Chalker 1986; Karsten *et al.* 1998).

Preliminary tests were performed to determine the ideal extraction conditions for these samples as recommended by (Tartarotti & Saommaruga 2002). Samples were extracted with various temperatures (4˚C, 20˚C, and 40˚C), sample weights (2 mg, 10 mg, 20 mg, 50 mg) and solvent concentrations (60%, 80% MeOH); and optimal extractions occurred at 20˚C with 20 mg sample in 80% methanol). Based on the preliminary extraction test, three serial extractions were performed on 20 mg dry weight of each egg mass in 0.5 mL 80% HPLC-grade MeOH for one hour at room temperature (≈ 20˚C). Following extraction of each sample, the supernatant was pooled, and the absorption spectra from 250-450 nm of the extract was taken in a quartz cuvette by a scanning spectrophotometer (Shimadzu UV-1601). Extraction efficiency was tested on egg masses of *Bembicium nanum, Bursatella leachii,* and *Siphonaria denticulata* and; for each species, I recovered over 95% of total MAAs after three of six extractions.

MAAs were separated by reverse-phase high performance liquid chromatography (RP-HPLC) on a Phenosphere C8 column (5μ 4.6 internal diameter x 250 mm) with guard (Phenomenex) at a flow rate of 0.8mL/ minute. The aqueous mobile phase was 39.9:0.1:60 water:acetic acid:methanol. Nine MAAs were identified and quantified with maximum wavelength absorption and co-chromotography with prepared standards representing the most common MAAs (Karentz 2001; Shick & Dunlap 2002): Standards for mycosporine-glycine and palythinol were prepared from *Palythoa tuberculosa*; mycosporine-2-glycine and mycosporine taurine from *Anthopleura elegantissima;* shinorine and porphyra-334 from *Porphyra tenera*; palythine from *Mastocarpus stellatus;* and palythene and asterina-330 from the ocular lens of *Plecropomus leopardus.* Concentration was calculated based on standards and primary calibration of equipment at the Australian Institute of Marine Science and expressed in nmol/mg sample dry weight.

In order to provide a standardised comparison between egg masses and other organisms, I collected several intertidal organisms from full sun habitats (n = 1): *Actinia tenebrosa* (anemone), *Haliclona* sp. (sponge), *Hormosira banksii* (phaeophyte), and *Ulva lactuca* (chlorophyte). Adult gastropods from the following species were also collected (n = 4): *B. nanum, S. denticulata,* and *S. zelandica.* These specimens were removed from their shells and prepared and analysed as mentioned above with the exception that >20 mg dry weight of the whole adult (including gonads and *in vivo* eggs) was used in extractions.

Non metric multi-dimensional scaling (nMDS) plots of the data were used to clarify the distribution of MAAs in egg masses based on maturity, spawning habitat, adult diet, phylogeny (order), and viability. Nested ANOSIMs were conducted on data subsets encompassing all species where $n \geq 3$ (Table 4.2.1). Samples with no MAAs were given a nominal value of 0.0001 nmol/mg for mycosporine-glycine, to enable calculation of Bray-Curtis similarity index for MDS plots and ANOSIMs. I used PRIMER v. 5 (Plymouth Routines in Multivariate Ecological Research) for all multivariate analyses. In addition, 2-way or nested ANOVAs were conducted on all species in JMP v. 4. These were done separately for each MAA examined with egg mass maturity, adult diet, spawning habitat, order, and viability as factors in the analysis. Each species was

considered independent and was appropriately nested or crossed with these factors (see Westoby *et al.* 1995b, a). Most data was skewed or had unequal variances, and data was log transformed (Zar 1998) to ensure that the assumptions of ANOVA were met. Tukey's HSD tests were used post hoc to reveal significant relationships. alpha = 0.05 for all statistical analyses unless otherwise specified.

Table 4.2.1 List of species used in this study and their associated egg mass characteristics. n = number of viable egg masses quantified.

Order	Species	n	Habitat	Diet	Structure
PHYLUM MOLLUSCA: CLASS GASTROPODA					
Neritidae	*Nerita morio*	2	Full sun	Herbivore	Capsule
Littorinimorpha	*Bembicium nanum* [1, 2]	18	Full sun	Herbivore	Gel
Littorinimorpha	*Cabestana spenglerii* [1, 2,]	5	Shade	Carnivore	Capsule
Littorinimorpha	*Conuber* sp. [1]	10	Full sun	Carnivore	Gel
Littorinimorpha	*Cypraea erosa* [3]	1	Shade	Herbivore	Capsule
Littorinimorpha	*Ranella australasia*	1	Shade	Carnivore	Capsule
Neogastropoda	*Agnewia tritoniformis*	5	Shade	Carnivore	Capsule
Neogastropoda	*Bedeva sp.*	1	Shade	Carnivore	Capsule
Neogastropoda	*Conus papilliferus* [1]	6	Shade	Carnivore	Capsule
Neogastropoda	*Dicathais orbita* [1, 2]	8	Shade	Carnivore	Capsule
Neogastropoda	*Lepsiella reticulata* [2]	2	Shade	Carnivore	Capsule
Neogastropoda	*Morula marginalba*	2	Shade	Carnivore	Capsule
Neogastropoda	*Mitra badia*	1	Shade	Carnivore	Capsule
Neogastropoda	*Mitra carbonaria* [1]	13	Shade	Carnivore	Capsule
Neogastropoda	*Nucella lapillus* [2]	2	Partial sun	Carnivore	Capsule
Neogastropoda	*Cominella eburnea* [1, 2,4]	5	Shade	Carnivore	Capsule
Cephalaspidea	*Bulla quoyii*	1	Partial sun	Herbivore	Gel
Cephalaspidea	*Bullina lineata*	5	Partial sun	Carnivore	Gel
Cephalaspidea	*Hydatina physis* [1]	8	Partial sun	Carnivore	Gel
Sacoglossa	*Aplysiopsis formosa*	2	Partial sun	Herbivore	Gel
Sacoglossa	*Oxynoe viridis* [1]	6	Partial sun	Herbivore	Gel
Sacoglossa	*Placida* cf. *dendritica* [1]	9	Partial sun	Herbivore	Gel
Notaspidea	*Berthellina citrina*	1	Shade	Carnivore	Gel
Notaspidea	*Pleurobranchus peronii*	2	Shade	Carnivore	Gel
Notaspidea	*Pleurobranchus* sp.	2	Shade	Carnivore	Gel
Anaspidea	*Aplysia juliana* [2]	9	Partial sun	Herbivore	Gel
Anaspidea	*Aplysia sydneyensis* [1]	14	Partial sun	Herbivore	Gel
Anaspidea	*Aplysia parvula*	2	Partial sun	Herbivore	Gel

Anaspidea	*Bursatella leachii* [1, 2]	6	Partial sun	Herbivore	Gel
Anaspidea	*Dolabella auricularia* [2]	2	Partial sun	Herbivore	Gel
Anaspidea	*Dolabrifera brazieri* [1, 2]	16	Shade	Herbivore	Gel
Anaspidea	*Stylocheilus striatus* [1]	8	Partial sun	Herbivore	Gel
Nudibranchia	*Aeolidiella foulisi*	1	Shade	Carnivore	Gel
Nudibranchia	*Austraeolis ornata*	5	Shade	Carnivore	Gel
Nudibranchia	*Dendrodoris carneola*	1	Shade	Carnivore	Gel
Nudibranchia	*Dendrodoris fumata* [1]	7	Shade	Carnivore	Gel
Nudibranchia	*Dendrodoris nigra*	1	Shade	Carnivore	Gel
Nudibranchia	*Doriopsilla miniata*	1	Shade	Carnivore	Gel
Nudibranchia	*Goniodoris meracula*	3	Shade	Carnivore	Gel
Nudibranchia	*Hoplodoris nodulosa*	4	Shade	Carnivore	Gel
Nudibranchia	*Hypselodoris obscura*	3	Shade	Carnivore	Gel
Nudibranchia	*Platydoris galbanus*	3	Shade	Carnivore	Gel
Nudibranchia	*Plocampherus imperialis*	1	Shade	Carnivore	Gel
Nudibranchia	*Rostanga arbutus*	3	Shade	Carnivore	Gel
Basommatophora	*Siphonaria denticulata* [1]	14	Full sun	Herbivore	Gel
Basommatophora	*Siphonaria zelandica*	6	Full sun	Herbivore	Gel
PHYLUM ANNELIDA: CLASS POLYCHEATA					
Polychaete	Unknown polychaete 1	3	Shade	Unknown	Gel
Polychaete	Unknown polychaete 2	4	Full sun	Unknown	Gel
PHYLUM CHORDATA: CLASS OSTEICTHYES					
Gobiosocid	*Aspasmogaster costatus*[5]	2	Shade	Carnivore	Gel

[1] Species used in analysis of maturity

[2] Inviable egg masses of these species also collected

[3] This species is either an omnivore or herbivore (Beesley et al., 1998). For statistical analyses, I have classified it as an herbivore since it likely ingests some algae.

[4] This species is tentatively identified based on crawling juveniles

5 This species is tentatively identified based on repeated sighting of adults on egg masses

4.2.3 RESULTS

Spectrophotometry indicated the presence of UVR-absorbing compounds in many species as evidenced by maximal absorbance at UVR wavelengths (Figure 4.2.1). Of the 49 species tested, I detected and quantified MAAs in 43 viable egg masses (Table 4.2.2). I did not detect MAAs in the egg masses of the polychaetes or in egg masses from four species of mollusc (*Ranella australasia*, *Mitra carbonaria*, *Cominella eburnea,* and *Aplysiopsis formosa*) (Table 4.2.2). The mean total concentration of MAAs was found to be over 20nmol/mg in the egg masses of *Austraeolis ornata* and was greater than 15nmol/mg in the gelatinous masses from two other species of opisthobranch (*Stylocheilus striatus* and *Plocampherus imperialis*). The other intertidal organisms examined showed a similar range in total MAA concentration to the molluscs, with the brown algae *Hormosira banksii* showing no detectable levels of MAAs, while low levels were found in the green algae *Ulva lactuca* (1.24 nmol / mg d.w.) and the sponge *Haliclona* sp. (2.18 nmol / mg d.w.). The anemone *Actinia tenebrosa* had moderately high levels of total MAAs (9.05 nmol / mg d.w.).

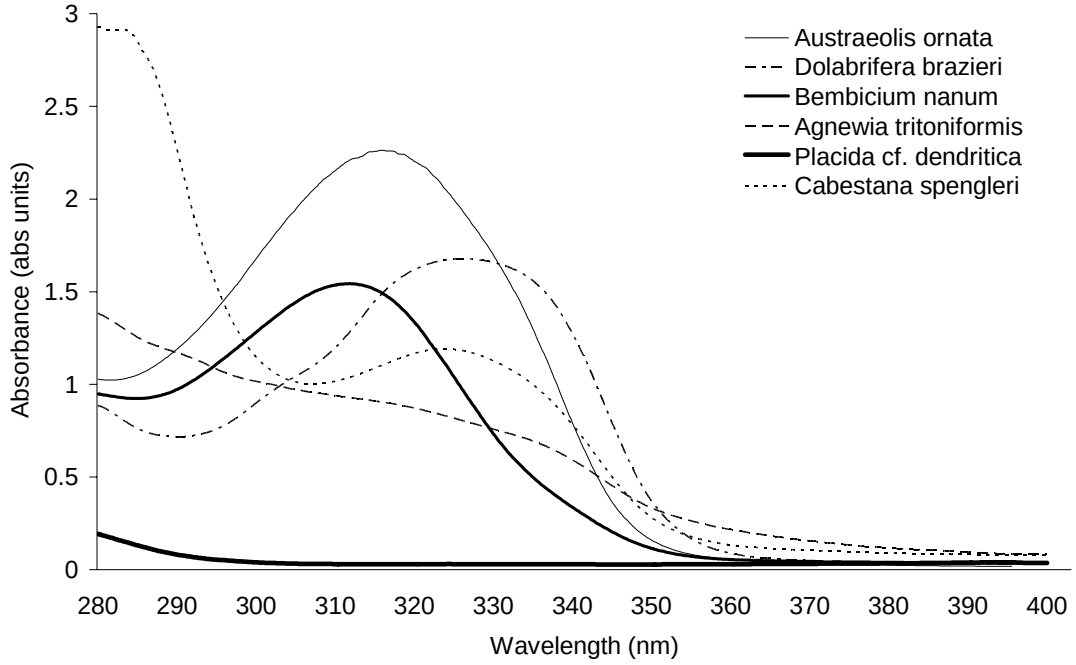
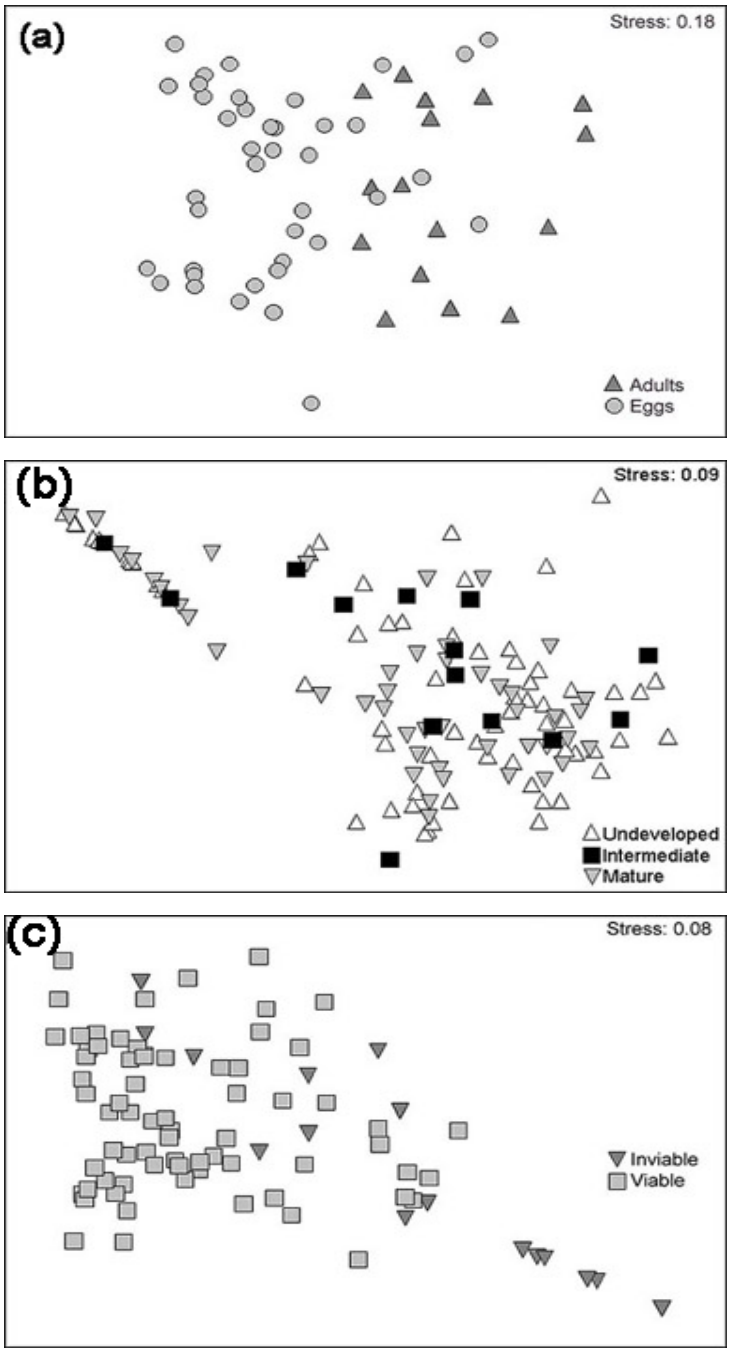


Figure 4.2.1 UVR-absorption of methanol extracts of egg masses from various species. All extracts are diluted 1:1 in 80% aqueous methanol except *Cabestana spengleri* and *Placida* cf. *dendritica* which are undiluted.

Table 4.2.2 MAA concentrations of viable egg masses used in this study (Mean ± SEM nmol/mg dry weight). See Table 4.2.1 for samples sizes. An asterisk indicates an unidentified MAA was detected in egg masses from this species. Structures for these compounds may be found in (Shick & Dunlap 2002).

Species	Myc-gly	Shinorine	Porphyra	Myc-2-gly	Palythene	Palythine	Asterina	Palythinol	Total
N. morio	0.68 ± 0.02	0.21 ± 0.03	0.05 ± 0.05	0.01 ± 0.01	0.00	0.00	0.00	0.00	0.95 ± 0.14
B. nanum	4.41 ± 0.59	0.68 ± 0.08	0.90 ± 0.16	0.15 ± 0.05	0.02 ± 0.02	0.36 ± 0.29	0.03 ± 0.01	0.01 ± 0.01	6.57 ± 2.28
C. spenglerii	0.61 ± 0.18	0.31 ± 0.13	0.46 ± 0.11	0.13 ± 0.04	0.00	1.98 ± 0.20	0.10 ± 0.03	0.00	3.58 ± 0.90
Conuber sp.	0.24 ± 0.13	0.08 ± 0.07	0.00	0.00	0.00	0.00	0.00	0.00	0.29 ± 0.63
C. erosa	2.20	0.77	0.32	0.07	0.00	0.06	0.00	0.00	3.41
R. australasia	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
*A. tritoniformis**	2.78 ± 0.86	1.02 ± 0.13	0.44 ± 0.05	0.09 ± 0.03	0.00	0.43 ± 0.39	0.00	0.15 ± 0.08	4.91 ± 2.56
Bedeva sp.	1.91	0.47	0.10	0.00	0.00	0.48	0.09	0.08	3.13
Cominella eburnea	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C. papilliferus	0.16 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.17 ± 0.03
*D. orbita**	2.72 ± 0.61	1.48 ± 0.36	0.30 ± 0.07	0.12 ± 0.01	0.00	0.03 ± 0.03	0.00	0.00	4.65 ± 2.16
*L. reticulata **	1.08 ± 0.36	0.33 ± 0.33	0.00	0.00	0.00	0.00	0.00	0.00	1.41 ± 0.97
M. marginalba	2.45 ± 0.85	3.51 ± 1.09	0.21 ± 0.01	0.00	0.00	0.18 ± 0.03	0.00	0.21 ± 0.04	6.56 ± 2.65
M. badia	0.00	0.00	0.00	0.00	0.00	1.05	0.00	0.00	1.05
M. carbonaria	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
N. lapillus	2.96 ± 0.23	0.40 ± 0.17	0.12 ± 0.01	0.04 ± 0.04	0.00	0.78 ± 0.01	0.00	0.00	4.31 ± 0.48
B. quoyii	1.10	2.89	1.00	0.18	0.00	1.35	0.16	0.00	6.68
B. lineata	1.20 ± 0.19	1.77 ± 0.21	0.41 ± 0.10	0.06 ± 0.01	0.00	1.45 ± 0.39	0.29 ± 0.26	0.00	5.17 ± 1.60
H. physis	0.78 ± 0.18	0.68 ±0.11	0.20 ±0.13	0.01 ± 0.00	0.00	0.91 ± 0.16	0.02 ±0.01	0.00	2.60 ± 1.49
A. formosa	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
O. viridis	0.45 ± 0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.45 ± 0.21
P. cf. *dendritica*	0.30 ± 0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.30 ± 0.17
B. citrina	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.00	0.23
P. peronii	2.84 ± 2.12	0.21 ± 0.03	0.09 ± 0.03	0.11 ± 0.02	0.00	1.78 ± 1.54	0.46 ± 0.46	0.00	5.49 ± 5.94
Pleurobranchus sp.	0.64 ± 0.30	0.06 ± 0.06	0.03 ± 0.03	0.03 ± 0.03	0.00	0.28 ± 0.28	0.00	0.13 ± 0.13	1.15 ± 0.30
A. juliana	0.53 ±0.18	0.72 ± 0.42	0.48 ± 0.31	0.09 ± 0.08	0.00	0.08 ± 0.07	0.04 ± 0.02	0.08 ± 0.05	2.02 ± 2.89
A. sydneyensis	2.43 ± 0.47	3.77 ± 0.73	1.38 ± 0.29	0.45 ± 0.17	0.28 ± 0.22	2.76 ± 0.66	0.03 ± 0.01	0.42 ± 0.11	11.50 ± 7.05
A. parvula	0.60 ± 0.01	1.41 ± 0.87	4.41 ± 1.45	0.14 ± 0.06	0.00	0.98 ± 0.73	0.12 ± 0.05	0.00	7.65 ± 4.44
*B. leachii**	2.65 ± 0.66	1.76 ± 0.42	0.63 ± 0.11	0.55 ± 0.14	0.33 ± 0.09	0.63 ± 0.12	0.39 ± 0.12	0.00	6.94 ± 3.69
D. auricularia	0.62 ± 0.35	1.90 ± 0.18	1.71 ± 0.73	0.11 ± 0.06	1.39 ± 0.08	3.62 ± 0.01	0.00	0.95 ± 0.69	10.30 ± 2.72
*D. brazieri**	2.19 ± 0.16	2.52 ± 0.21	3.91 ± 0.54	0.30 ± 0.05	1.19 ± 0.31	2.16 ± 0.35	0.57 ± 0.09	0.14 ± 0.10	12.97 ± 4.83
S. striatus	2.17 ± 0.44	2.27 ± 0.55	0.67 ± 0.12	0.47 ± 0.08	2.48 ± 0.50	2.95 ± 1.25	0.00	3.88 ± 0.89	16.78 ± 2.39

A. foulisi	0.06	0.02	0.05	0.00	0.00	0.06	0.00	0.08	0.28
A. ornata	2.15 ± 0.47	5.80 ± 1.93	1.87 ± 0.50	0.35 ± 0.11	0.00	9.67 ± 2.49	0.60 ± 0.20	0.12 ± 0.12	20.56 ± 11.52
D. carneola	0.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.25
D. fumata	1.24 ± 0.31	0.67 ± 0.17	0.31 ± 0.07	0.05 ± 0.01	0.00	2.64 ± 0.76	0.00	0.26 ± 0.10	5.16 ± 3.62
D. miniata	0.17	0.00	0.00	0.00	0.00	0.08	0.00	0.00	0.56
D. nigra	0.29	0.06	0.03	0.01	0.00	0.17	0.00	0.00	0.25
G. meracula	0.00	0.10 ± 0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.10 ± .03
*H. nodulosa**	0.55 ± 0.35	0.38 ± 0.24	0.29 ± 0.20	0.03 ± 0.03	0.00	0.70 ± 0.42	0.00	0.11 ± 0.04	2.06 ± 2.51
*H. obscura**	0.54 ± 0.08	0.22 ± 0.08	0.10 ± 0.05	0.02 ± 0.01	0.00	0.53 ± 0.30	0.00	0.09 ± 0.05	1.50 ± 0.68
P. galbanus	3.07 ± 0.39	2.46 ± 0.92	0.96 ± 0.20	0.14 ± 0.04	0.00	6.08 ± 0.97	0.00	1.64 ± 0.49	14.35 ± 4.35
P. imperialis	4.72	4.78	1.53	0.29	0.00	7.06	0.50	0.00	18.89
*R. arbutus**	0.00	0.19 ± 0.19	0.00	0.00	0.00	0.00	0.32 ± 0.32	0.00	0.51 ± 0.69
*S. denticulata**	2.39 ± 0.45	1.73 ± 0.27	3.44 ± 0.89	0.02 ± 0.01	0.50 ± 0.32	1.79 ± 0.47	0.45 ± 0.17	0.00	10.32 ± 6.90
*S. zelandica**	0.97 ± 0.21	1.04 ± 0.26	4.95 ± 1.38	0.01 ± 0.00	0.00	0.60 ± 0.26	0.05 ±0.02	0.00	7.62 ± 4.76
Unkn polychaete 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unkn polychaete 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
A. costatus	0.00	0.00	0.00	0.00	0.50 ± 0.03	0.00	0.00	0.00	0.50 ± 0.03


(a)
Stress: 0.18
Adults
Eggs
(b)
Stress: 0.09
Undeveloped
Intermediate
Mature
(c)
Stress: 0.08
Inviable
Viable

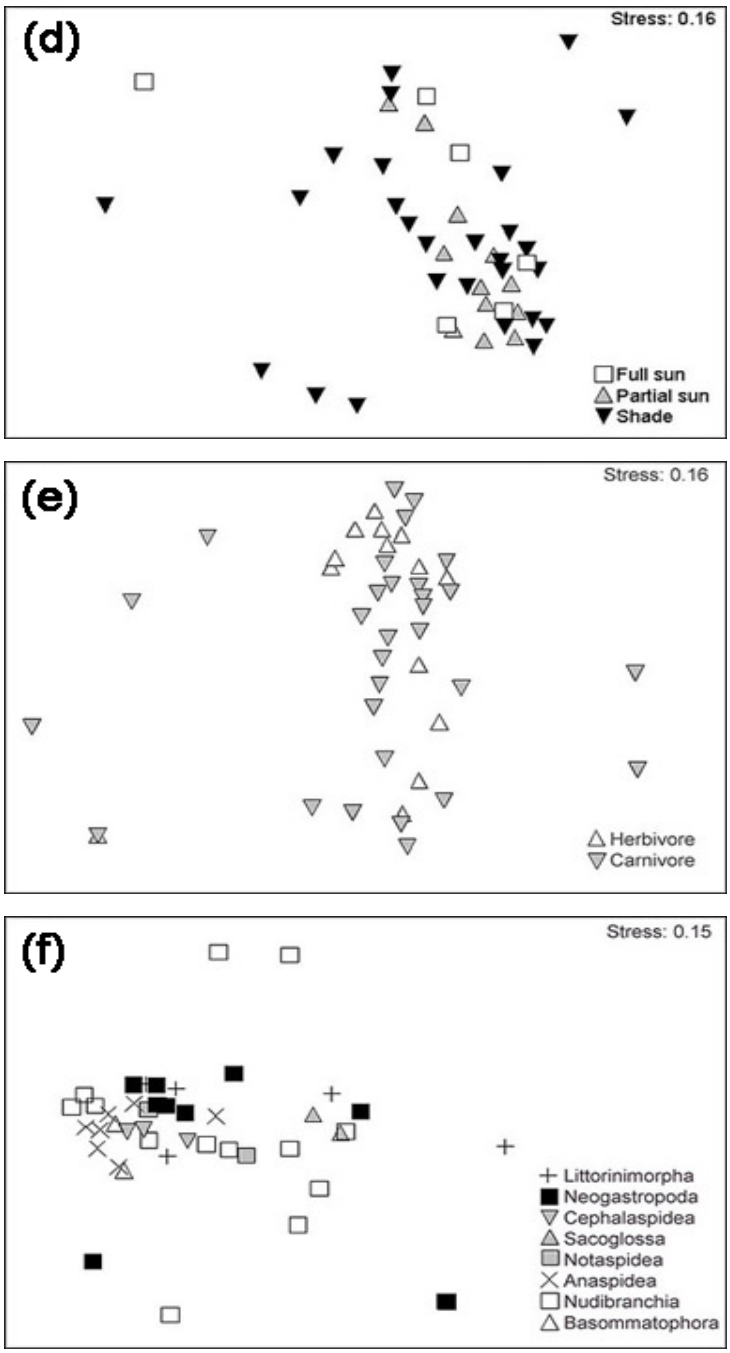


Figure 4.2.2 Differences in the composition and relative abundance of MAAs in the egg masses of selected species as revealed by nMDS plots contrasting: a) *Bembicium nanum, Siphonaria denticulata* and *Siphonaria zelandica* adults and their egg masses, b) effects of maturity on viable egg masses from 16 species, c) effects of viability on egg masses from 10 species, d) effects of spawning habitat on viable egg masses from 49 species (means shown), e) effects adult diet on viable egg masses from 45 species with known diet (means shown), and f) gastropod phylogeny on viable egg masses from 45 species (means shown).

Eight of the nine MAAs analysed in this study were detected in the egg masses examined. Overall, mycosporine glycine was the most common MAA, occurring in egg masses from 38 species (Table 4.2.2). Palythene was the least common, occurring in just eight species (Table 4.2.2). Palythine occurred at the highest concentration; one egg mass of the nudibranch *Austraeolis ornata* contained 16.833 nmol/ mg palythine. Mycosporine-taurine was not found in any sample collected.

Unidentified HPLC peaks were detected in viable egg masses of the following species (approximate retention time in minutes, max absorbance): *Agnewia tritoniformis, Lepsiella reticulata,* and *Dicathais orbita* (4.5, 307nm); *Rostanga arbutus, Hoplodoris nodulosa,* and *Hypselodoris obscura* (6.3, 296nm); *Bursatella leachii* (12.2, 346nm); *Siphonaria denticulata* (7.1, 334nm), *Siphonaria zelandica* (2.9, 319nm); and *Aspasmogaster costatus* (4.2, 325nm). These peaks did not match with any of the MAA standards used and therefore could not be positively identified or quantified for analysis.

4.2.3.1 *Comparison with Adults*

An nMDS plot of viable egg masses and adults of species for which I had replicates revealed distinct clusters (Figure 4.2.2a), suggesting MAA composition of egg masses was different from that of adults. ANOSIMs on each species confirmed significant differences between egg masses and adults of *B. nanum* ($R = 0.556$, $p = 0.02$), *S. denticulata* ($R = 0.343$, $p = 0.016$), and *S. zelandica* ($R = 0.504$, $p = 0.019$). The egg masses of these species contained significantly higher concentrations of mycosporine-glycine ($F = 23.5012$, $p < 0.001$), shinorine ($F = 21.214$, $p = 0.04$), mycosporine-2-glycine ($F = 11.6937$, $p = 0.001$), and palythine ($F = 8.385$, $p = 0.006$) than the adults as revealed by a 2-factor ANOVA (Figure 4.2.3). In contrast, adults had significantly more palythene than egg masses ($F = 40.873$, $p <0.001$). A significant interaction between species and life stage was detected for palythinol concentrations ($F =51.336$, $p < 0.001$), and Tukey's HSD tests revealed that *B. nanum* adults had significantly more palythinol than the egg masses (Figure 4.2.3).

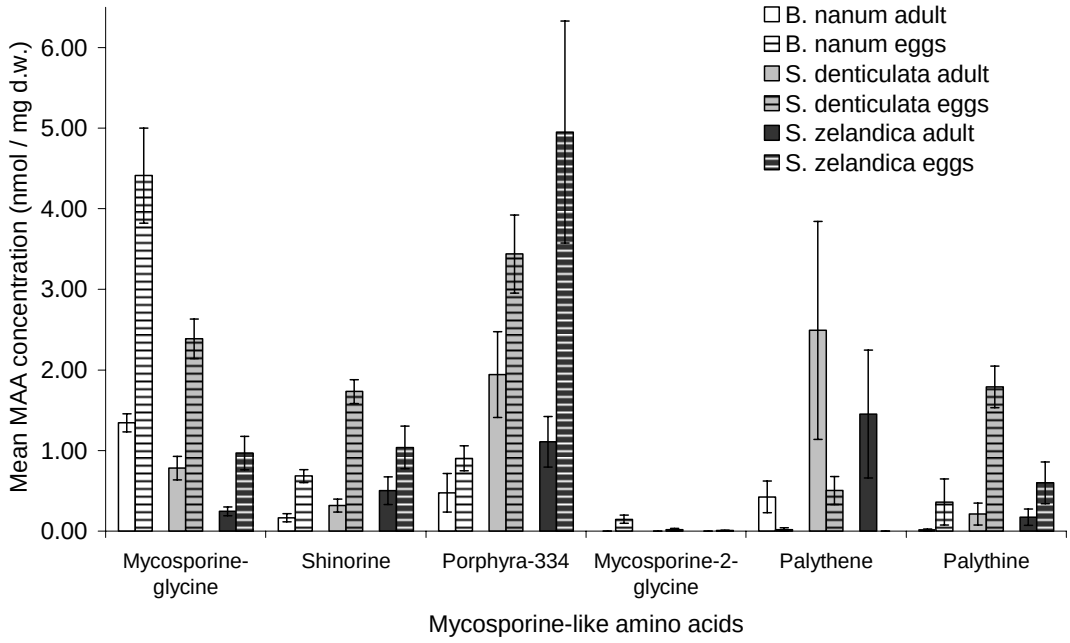


Figure 4.2.3 MAA compositions of adults and egg masses of four species. Asterina-330 and palythinol are not included because only trace amounts were detected in these species (<0.05 nmol / mg), and no significant relationships were observed. Error bars are standard error of mean.

4.2.3.2 ***Egg Mass Maturity and Viability***

MAA composition did not vary as egg masses matured for all 16 species of molluscs tested. There was no apparent difference in MAA content between undeveloped, intermediate, and mature egg masses as revealed by an nMDS plot (Figure 4.2.2b) and a nested ANOSIM on data subsets including species where $n \geq 3$ ($R = 0.079$, $p = 0.072$) (refer to Table 4.2.1 for species tested). Thus, viable egg masses at all stages of development were pooled for each species in remaining analyses.

An nMDS ordination comparing MAA composition in viable and inviable egg masses of 10 species in which MAAs were present revealed minimal segregation of the samples (Figure 4.2.2c, see Table 4.2.1 for species tested). ANOSIMs were conducted separately on the five species where $n \geq 3$ for both viable and inviable egg masses, and these revealed species-specific effects. MAA concentration was significantly different between viable and inviable egg masses for two species: *B. nanum* ($R = 0.927$, $p = 0.001$) and *L. reticulata* ($R = 0.274$, $p = 0.001$). Similar trends were seen for *D. orbita* ($R = 0.914$, $p = 0.06$) and *D. brazieri* ($R = 0.393$, $p = 0.054$) although they were not significant at alpha = 0.05. No apparent differences were observed for *C. papilliferus* (R

= 0.074, p = 0.274). Two factor ANOVAs on the transformed data for these species revealed significant interactions between viability and species on concentrations of mycosporine-glycine (F =10.03, p < 0.001), shinorine (F =5.470, p = 0.001), porphyra-334 (F =5, p < 0.001), mycosporine-2-glycine (F =5.8418, p = 0.0005) and palythene (F =3.9071, p = 0.007). Tukey's HSD tests revealed that viable egg masses of *B. nanum* and *D. orbita* had significantly more mycosporine-glycine, shinorine, porphyra-334, and mycosporine-2-glycine than inviable egg masses; and viable *D. brazieri* egg masses had significantly more mycosporine-2-glycine and palythene than inviable egg masses (Figure 4.2.4).

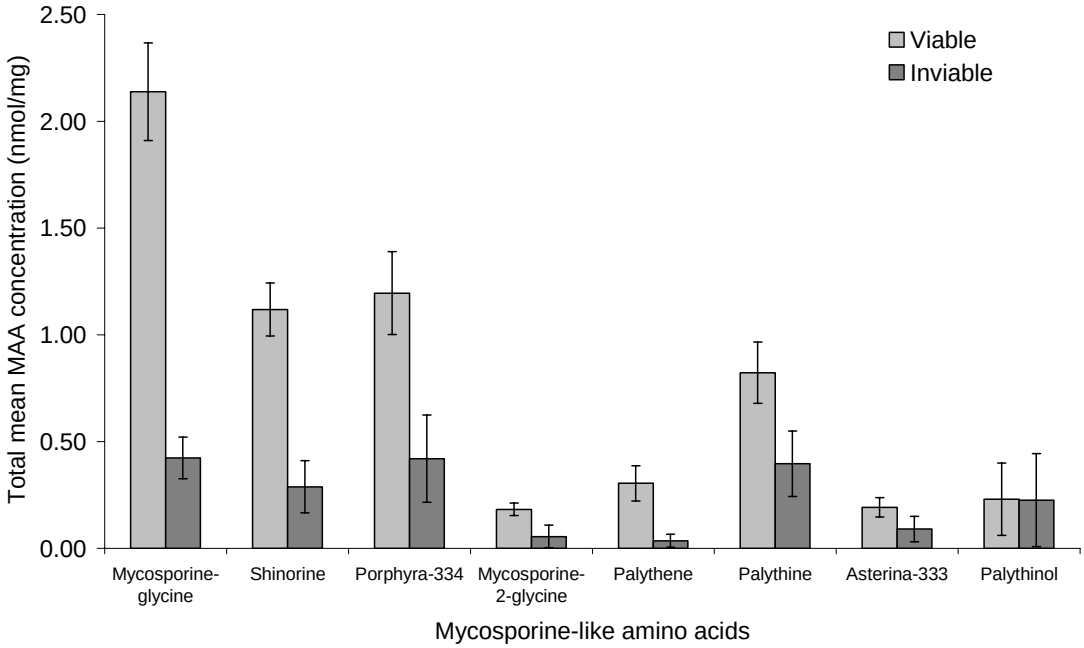


Figure 4.2.4 MAA concentration in viable (n = 81) and inviable (n =22) egg masses of *Bembicium nanum, Cabestana spengleri, Ranella australasia, Conus papiilliferus, Dicathais orbita, Lepsiella reticularis, Nucella lapillus, Cominella eburnea*, *Aplysia juliana, Bursatella leachii, Dolabella auricularia,* and *Dolabrifera brazieri.* Error bars are standard error of mean.

4.2.3.3 *Spawning Habitat and Adult Diet*

An nMDS plot of MAA composition revealed no clear separation between egg masses deposited in full sun habitats compared to those deposited in shaded habitats (Figure 4.2.2d). Similarly, a nested ANOSIM failed to detect effects based on spawning habitat (R = -0.048, p = 1). Overall, the concentrations of mycosporine-glycine and porphyra-334 were highest in egg masses from species that spawn in full sun, while palythine, palythinol and shinorine concentrations were lowest in egg masses from these species

(Figure 4.2.5). However, nested ANOVAs on the concentration of each MAA supported the multivariate results, showing no significant differences between spawning habitats.

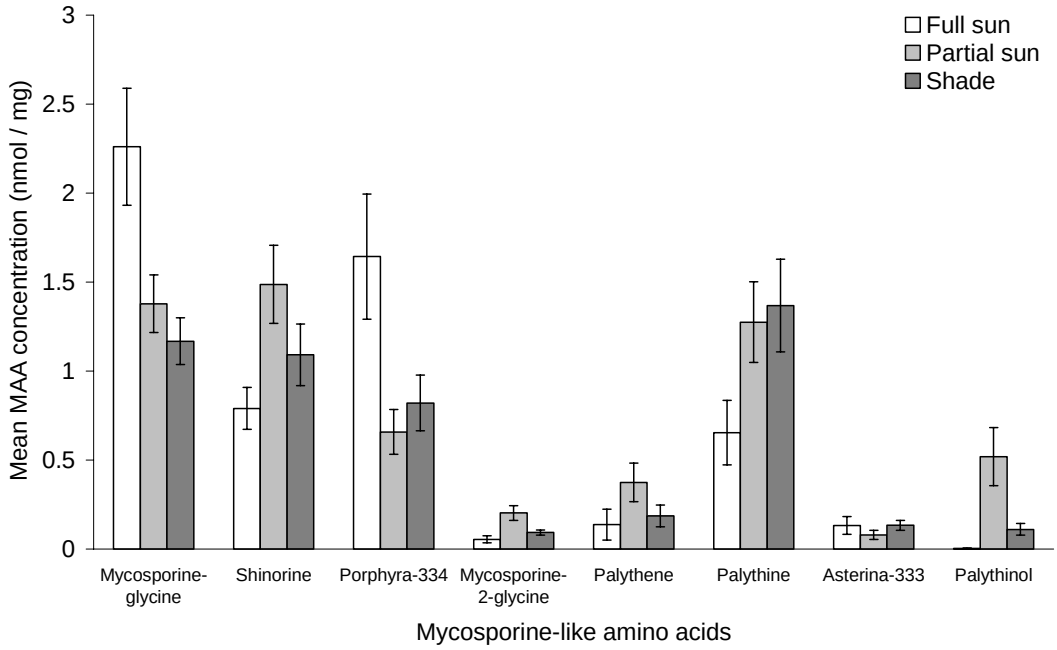


Figure 4.2.5 Effects of spawning habitats on MAA concentration in all viable egg masses collected in this study. Habitats varied in spectral exposure and encompassed full sun (n = 54), partial sun (n = 76), and shade (n = 107). Error bars are standard error of mean.

A multivariate analysis of MAA composition in egg masses showed no overall effect of adult diet. The nMDS plot reveals no distinct separation of samples according to diet (Figure 4.2.2e), and a nested ANOSIM confirmed no significant differences between herbivores and carnivores (R = -0.285, p = 1). However, nested ANOVAs on individual MAAs did reveal some significant differences according to diet. Herbivores had significantly higher levels of porphyra-334 (F = 5.24, p = 0.027) and palythene (F = 11.95, p = 0.001), with no palythene recorded in any egg masses from carnivores (Figure 6). Although not statistically significant, similar trends were also detected for mycosporine-glycine (*F* = 3.860, p = 0.056) (Figure 4.2.6). Carnivores showed higher levels of shinorine and palythine, but these differences were not significant.

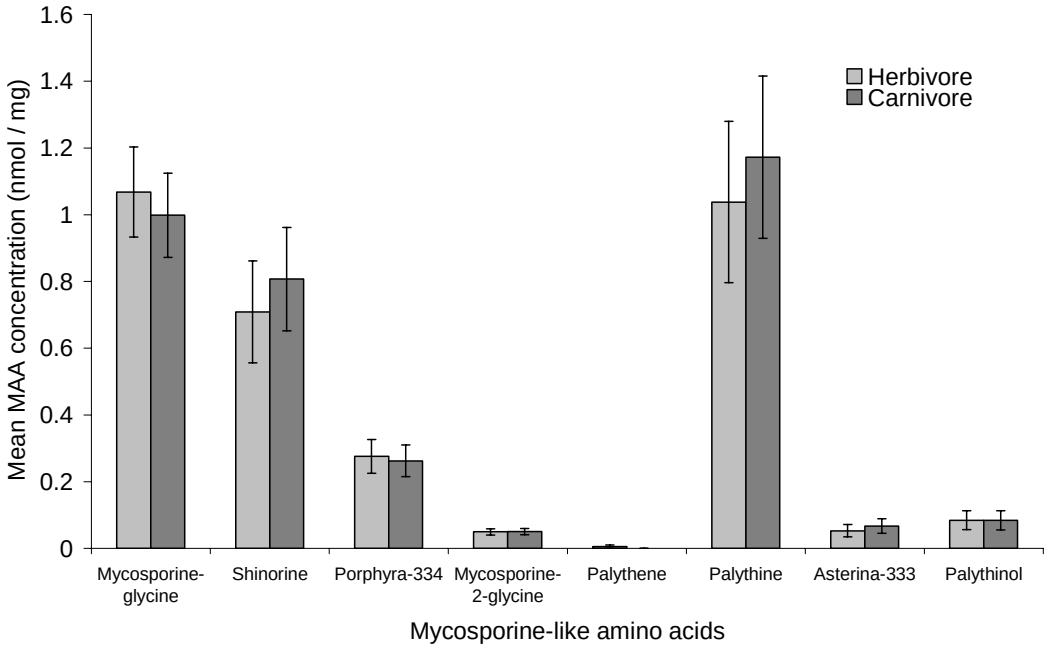


Figure 4.2.6 Effects of adult diet on MAA concentration for all viable egg masses in which adult diet is known to be either herbivore (n = 114) or carnivore (n = 112) (refer to Table 1). Error bars are standard error of mean.

4.2.3.4 *Gastropod Phylogeny*

The taxonomic grouping (order) of gastropods strongly affected MAA content in egg masses. An nMDS plot showed some taxonomic clusters, particularly for anaspids (Figure 4.2.2f). Considerable variation was observed within many orders (e.g. Nudibranchia and Neogastropoda), as evidenced by the large spread of points on the nMDS (Figure 4.2.2f) and relatively large error bars for individual MAA concentrations (Figure 4.2.7). A nested ANOSIM on all orders with two or more species indicated less variation within than between taxonomic groups in their MAA composition ($R = 0.082$, $p = 0.098$). Pair-wise tests showed that the MAA content of anaspid egg masses was different from that of sacoglossans ($R = 0.818$, $p = 0.048$), and possibly neogastropods ($R = 0.248$, $p = 0.063$). The MAA composition of nudibranch egg masses appeared different from that of neogastropods ($R = 0.244$, $p = 0.057$) and sacoglossans ($R = 0.396$, $p = 0.056$). Nested ANOVAs of individual compounds confirmed significant phylogenetic differences in the quantity of shinorine ($F = 3.9103$, $p = 0.0028$), porphyra-334 ($F = 3.3023$, $p = 0.0079$), mycosporine-2-glycine ($F = 3.3023$, $p = 0.0079$), palythene ($F = 5.4158$, $p = 0.0002$), and palythine ($F = 2.9569$, $p = 0.0145$). Sacoglossans had significantly less shinorine than anaspids, cephalaspids, nudibranchs, and basommatophorans; and similarly, they had less palythine than anaspids (Figure

4.2.7). Anaspids had significantly more porphyra-334 and mycosporine-2-glycine than sacoglossans and neogastropods; and they had more palythene than all other orders except the basommatophorans (Figure 4.2.7). Basommataphorans had significantly more porphyra-344 than sacoglossans (Figure 4.2.7).

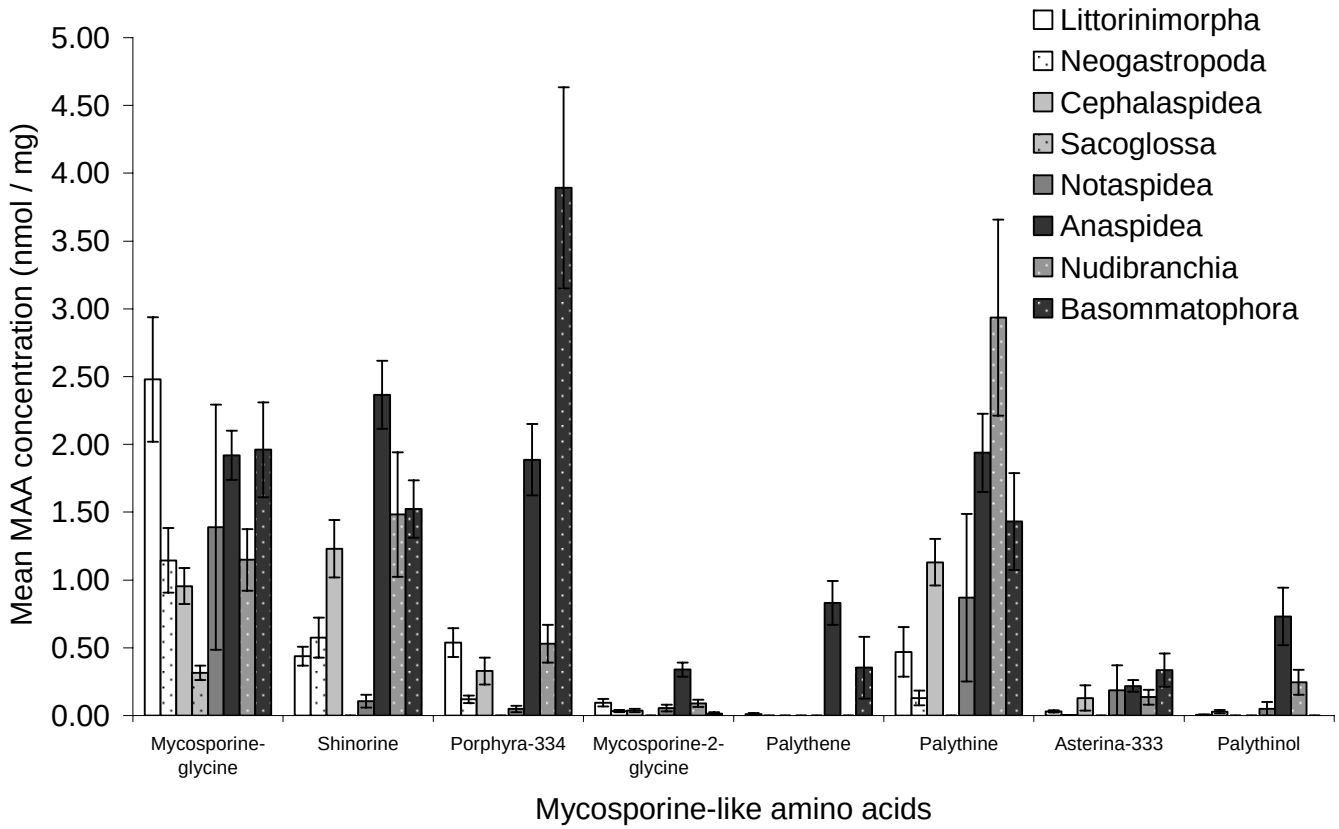


Figure 4.2.7 Effects of gastropod order on MAA concentration in viable egg masses collected in this study: Littorinimorpha (n = 4), Neogastropoda (n = 10), Cephalaspidea (n = 3), Sacoglossa (n = 3), Notaspidea (n = 3), Anaspidea (n = 7), Nudibranchia (n = 12) and Basommatophora (n = 2) where n indicates number of species. Error bars are standard error of mean.

4.2.4 DISCUSSION

This study is the first quantitative survey of mycosporine-like amino acid composition in eggs or extra-embryonic structures. It confirms that MAAs are prevalent in the intertidal egg masses of many gastropods on temperate rocky shores. Unfortunately, it is difficult to directly compare MAA concentration of egg masses used here with organisms from many other studies due to the persistent use of several different measures of concentration across the literature (Karentz 2001). However, those studies that reported MAA concentration in the units used here (nmol/mg) show that non-symbiotic animals have similar ranges of MAA concentrations as those exhibited in this study (McClintock & Karentz 1997; Banaszak *et al.* 1998). For example, larvae of the urchin *Strongylocentrotus droebachiensis* contain 4-8 nmol mg^{-1} total MAAs (Adams & Shick 2001) which represents the middle range of the concentrations I detected (Table 4.2.2). Indeed, direct comparisons with other intertidal organisms in the present study such as *Hormosira banksii, Ulva lactuca* and *Haliclona* sp. confirm that MAA concentrations in egg masses of some species are relatively high.

Egg masses of *B. nanum, S. denticulata,* and *S. zelandica* are laid in habitats where they are directly exposed to UVR. They contained more MAAs than whole adults examined (Figure 4.2.3) which is consistent with the suggestion that MAAs have a photo-protective role in these species. Other intertidal invertebrates have been found to sequester MAAs in spawn to minimise UV-induced damage to offspring in a potentially hostile environment; this trend has been recorded in the sea urchin *Strongylocentrotus droebachiensis* (Adams *et al.* 2001) and the sea hare *Aplysia dactylomela* (Carefoot *et al.* 1998; Carefoot *et al.* 2000). It remains unknown if species that spawn exclusively in shade sequester MAAs in eggs.

The relatively high concentrations of MAAs in some egg masses suggest that embryos of certain species are protected from the damaging effects of UVR. This is consistent with previous research in which embryos of species that consistently spawn in habitats exposed to full sun were more resistant to the damaging effects of UVR than embryos of species that only spawn in shaded habitats (Przeslawski *et al.* 2004). However, contrary to my prediction, I have found no clear pattern in MAA composition relating to spawning habitat (Figure 4.2.2d, 4.2.5). Furthermore, a direct comparison of total MAA

concentration in egg masses of species used in both the present study and Przeslawski *et al.* (2004) revealed no significant correlation between MAA concentration and the difference in embryonic mortality between full spectrum and UV-blocked treatments ($R = 0.004$, $p = 0.795$). This suggests that MAAs are not the sole source of protection afforded to encapsulated intertidal embryos.

There are many other ways for marine organisms to mitigate the deleterious effects of UVR (see Bandaranayake 1998). Antioxidants may play an important role in minimising UVR damage in marine organisms (reviewed by Dunlap *et al.* 1999). Indeed, the most common MAA observed in this study, mycosporine-glycine, is also a moderate antioxidant (Dunlap & Yamamoto 1995). The potential importance of this compound is supported by comparisons with adults and inviable egg masses. Eggs had significantly higher concentrations of mycosporine-glycine than adults (Figure 4.2.3); and viable egg masses had higher concentrations than inviable egg masses (Figure 4.2.4). Furthermore, egg masses from species that spawn in full sun contained the highest levels of mycosporine-glycine relative to species that spawned in partial or full shade (Figure 4.2.3). Mycosporine-glycine is the only MAA found in this study that absorbs maximally at 310 nm (Figure 4.2.1), the range of UVR (UV-B) that is most biologically damaging (Paul & Gwynn-Jones 2003). Thus, embryos encapsulated in environments exposed to UVR may use mycosporine-glycine in a dual protective role as a UV-B sunscreen and an antioxidant. Egg masses routinely deposited in habitats exposed to full sun may also possess other protection against damage caused by UVR. Other metabolites such as carotenoids may provide photo-protective antioxidant functions; such compounds have already been found in holothurian eggs (Bandaranayake 1998) and warrant further investigation. In addition, high levels of the DNA repair enzyme photolyase have been found in several adult molluscs, including *Bursatella leachii* (Carlini & Regan 1995), a species used in this study. The capability of encapsulated intertidal embryos to repair UVR-induced DNA damage is currently unknown.

MAA composition in gastropod egg masses shows enormous phylogenetic variation (Figure 4.2.7), and phylogeny may well overwhelm the influence of all other factors examined in this study. Even unknown compounds detected in this study elicited phylogenetic patterns. For example, the same unknown peak was seen only in egg

masses of three species of neogastropods; and a different unknown peak, possibly deoxygadusol (see Shick & Dunlap 2002) was found only in three species of nudibranchs. Previous research on sea anemones has similarly revealed that differences in MAA concentration primarily reflect phylogeny rather than environmental factors (Shick *et al.* 2002). Alternatively, phylogeny may be related to an ecological factor not considered in the present study; and indeed, variation based on phylogenetic and ecological factors is not often mutually exclusive (Westoby *et al.* 1995a).

Phylogeny and diet can often be confounded, but the effects of phylogeny are unlikely to be influenced by diet in the present survey. In general, egg masses from herbivores had higher levels of some MAAs than those from carnivores (Figure 4.2.6); but when analysed according to order, egg masses of carnivorous nudibranchs had significantly more MAAs than egg masses from most herbivorous orders (Figure 4.2.7). Nevertheless, MAAs in eggs or larvae are likely dependent on adult diet, and studies on single species of molluscs and echinoderms reveal that eggs have higher MAA content if they are deposited by adults that consumed food rich in MAAs compared to adults that ate food containing few or no MAAs (Carefoot *et al.* 1998; Carefoot *et al.* 2000; Adams *et al.* 2001). The present study indicates that this diet-dependence may extend to differences in MAAs between trophic levels (Figure 4.2.6).

Nevertheless, analysis of MAA composition based on adult diet was not as definitive as previous analyses of total MAA concentration in which herbivores unilaterally showed higher concentrations of MAAs than carnivores (Przeslawski, in press b). In the present study, I found minimal differences in MAA composition between trophic levels (Figure 4.2.2e) and significant differences in only two of the eight MAAs detected (Figure 4.2.6). These results underscore the value of MAA composition analysis compared to the potentially overly simplistic interpretations arising from univariate analysis of total MAA concentration. For example, in the previous univariate analyses, it was suggested that herbivores have more direct links to sources of MAAs, and bioaccumulation does not occur in a broad range of species (Przeslawski 2004b, Appendix 4). However, the potential impact of MAA bioaccumulation cannot be ignored and has previously been recorded in a trophic chain including phytoplankton, herbivorous pteropods, and carnivorous pteropods (Whitehead *et al.* 2001). Indeed, the high concentrations of some MAAs in certain nudibranch egg masses are consistent with MAA bioaccumulation

from their prey and associated zooxanthellae (Table 4.2.2). For example, egg masses from the aeolid *Austraeolis ornata* contained the highest concentrations of MAAs in this study, and this species consumes zooxanthellate anemones capable of synthesising MAAs. Furthermore, analyses of total MAA concentration cannot account for different strategies employed by organisms based on MAA composition. Some species, such as *Siphonaria denticulata* in this study, may incorporate several compounds at low or moderate concentrations (Table 4.2.2). Other species, such as *Bembicium nanum*, may use higher concentrations of a single MAA to confer protection (Table 4.2.2).

Viable egg masses had a richer complement of MAAs and had higher concentrations of individual compounds than inviable egg masses (Figure 4.2.4). These results support previous univariate analysis of total MAA concentration in these egg masses (Przeslawski 2004b, Appendix 4). No previous study has investigated MAA composition in inviable eggs, and the mechanisms behind the relationship here are unclear. It is unknown if lower MAA concentration in these species resulted in the lack of viable embryos or if it was a consequence of inviability. The egg masses in this study were collected *in situ* after spawning so the history of the spawning adult and egg mass prior to collection was not known.

MAA concentration of egg masses varied tremendously within species (Table 4.2.2, Figure 4.2.2). Indeed, previous research has shown that unique MAA compositions can be used to identify clones within a species of coral (Diamond 1986). The intraspecific variation in the present study may have limited the detection of significant differences according to habitat, diet and taxonomic order. Notably, however, the present survey has generally incorporated more replicates than most other surveys of MAAs. Indeed previous surveys have reported MAA concentration based on single samples for each organism (e.g. Karentz *et al.* 1991; McClintock & Karentz 1997) or a very low number of replicates (e.g. Bosch *et al.* 1994; Büdel *et al.* 1997; Teai *et al.* 1997; Xiong *et al.* 1999). This lack of replication may yield biased estimates of MAA concentrations; and results should be treated with caution, particularly in cases where MAA composition shows high intraspecific variation.

Although the magnitude of their effectiveness as sunscreens for organisms in this study remains unclear, MAAs are likely important to developing encapsulated embryos.

MAAs may play a role in osmotic regulation and developmental regulation (reviewed by Shick & Dunlap 2002), and such functions would certainly be vital to encapsulated embryos developing in the intertidal. Unfortunately, there is a paucity of empirical research on alternate functions of MAAs to photo-protection in marine invertebrates. In contrast, there is a wealth of evidence that MAAs minimise UV-induced abnormality and mortality (reviewed by Karentz 2001). Previous research shows MAA concentration is logarithmically correlated to developmental success in sea urchin embryos due to reduction in UVR exposure (Adams & Shick 1996). No similar studies have been conducted on embryos of the species used in this study, but it seems likely that MAAs confer protection to embryos from species that consistently spawn in full sun habitats. However, MAA composition does not seem to be simply based on exposure to UVR, and instead likely represents complex and potentially confounding influences from both phylogenetic and ecological factors.

CHAPTER 5: Summary & General Discussion

"The outcome of any serious research can only be to make two questions grow where only one grew before."
Thorstein Veblen

On the rock platform, the water has receded with the low tide and left only small, rapidly disappearing pools. The sun is high in the sky, baking everything underneath with its intense rays. The rock is hot and sparkles with tiny salt crystals from evaporated sea water. Most animals have sought shelter in subtidal regions or underneath dark and damp boulders. A few tough species of algae defiantly poke out of rock pools, but they still look wilted and stressed. Despite their protective shells, even most snails have stopped foraging to crowd together in moist crevices until the water returns. Next to the snails are a few strands of translucent jelly, loosely coiled into spirals glued to the drying rock platform. Embedded in these strands are thousands of tiny snail embryos without shells, each spinning slowly in a clear capsule, seemingly unaware of the harsh surrounding environment. Every day, these developing embryos are exposed to the extreme sun, heat, and desiccation that have driven most other organisms under cover.

--R.P. (written after too much sun on one of the final days of writing this thesis)

5.1 SUMMARY

In the studies comprising this work, I sought to determine the effects of UVR and associated stressors on encapsulated embryos of temperate rocky shores, particularly embryos from those species that routinely deposit egg masses in the environments described above. I found that UVR exposure generally increases molluscan embryonic mortality and decreases encapsulation period (Chapter 2) while retarding development (Chapter 3). An extensive range of previous work has found that UVR increases larval mortality among other marine invertebrates, fish, and amphibians (Adams & Shick 2001; Tietge *et al.* 2001; Pahkala *et al.* 2002; Altamirano *et al.* 2003; Hakkinen *et al.* 2004); but no other work has examined effects of UVR on encapsulation period. As predicted, these effects were species specific, with embryos from species that consistently spawn in habitats exposed to full sun showing significantly more tolerance to the negative effects of UVR than embryos from those species that spawn only in shaded habitats. However, subsequent experiments revealed that the effects of UVR on encapsulated embryos are not as simple as results from the initial single-factor experiment in Chapter 2 would suggest. Indeed, UVR significantly interacts with

temperature, salinity, and desiccation stress to further increase embryonic mortality, and these effects vary across species (Chapter 3). I also determined that UVR significantly affects both algal and microbial fouling of egg masses which in turn can further increase embryonic mortality of some species (Chapter 3.3). The effects of algal fouling on encapsulated embryos were also species-specific and did not seem to be as great as those observed by Biermann *et al.* (1992) on nudibranch embryos.

In this study, I also characterised some of the potential photo-protective mechanisms of encapsulated embryos and identified factors that influence such protection (Chapter 4). As found previously by Benkendorff & Davis (2004), the majority of species examined in the present study spawned under boulders, thus minimizing exposure of vulnerable embryos to extremes of environmental stress associated with low tides and UVR exposure. However, spawning behaviour of species that deposit egg masses on rock platform surfaces does not seem to confer protection to developing embryos as spawning peaked during seasons of the highest environmental stress (Chapter 4.1). Embryos of these species are likely protected against UV-induced damage at least in part by MAAs which were prevalent in egg masses from a large range of species (Chapter 4.2). Among species that spawn on rock platform surfaces exposed to solar radiation, MAAs occurred in higher concentrations in egg masses than adults, suggesting a photo-protective role of these compounds to developing embryos. However, this role was confounded by other factors that affect MAA composition such as adult diet and phylogeny.

5.2 INTERSPECIFIC VARIATION IN UVR EFFECTS AND PROTECTION

Effects of UVR varied tremendously across species, with both spawning habitat and egg mass type affecting embryonic vulnerability (Chapter 2). After exposure to UVR, however, significant interspecific variation in embryonic mortality rates (Chapter 2) and fouling levels (Chapter 3.3) also occurred between species within particular habitats and egg mass types. Inter- and intra-specific variation in embryonic responses to UVR in the presence of associated stressors was also detected in gelatinous egg masses from two species with similar spawning habitats (*S. denticulata* and *B. nanum*, Chapter 3). These

species specific responses imply some variation in the genetic adaptation against environmental stressors within gastropods that deposit egg masses on intertidal reefs. Indeed, examination of MAA composition of egg masses from 46 species indicate that there is generally a very high level of variation in photo-protection across gastropod taxa. Caratenoid concentration in echinoderm larvae also varies across species (Lamare & Hoffman 2004), suggesting that photo-protective mechanisms in general may have high levels of interspecific variation.

Previous studies examining the effects of environmental stressors on egg masses often only consider one or very few species (Table 1.1), and generalisation from such studies should be made cautiously. The present experiments highlight the importance of examining numerous species when quantifying responses to environmental stress; this ensures accurate assessment of species-specific effects and avoids false generalisations.

5.3 INTERACTIONS BETWEEN UVR AND OTHER STRESSORS

This study confirms that consideration of potential interactions is vital, particularly in dynamic habitats where multiple stressors can simultaneously affect organisms (see Figure 1.3). The typical focus on studying environmental factors in isolation may well underestimate realistic ecological effects, and this is of the utmost concern in intertidal habitats where conditions change daily (Hoffman *et al.* 2003; Przeslawski *et al.* in press). Results from the single-factor study on UVR-induced mortality (Chapter 2) suggest that embryos of species that spawn in full sun habitats are not negatively affected by UVR. However, when potential interactions with other abiotic stressors are examined (Chapter 3.1, 3.2); embryos of *B. nanum* and *S. denticulata* are vulnerable to UVR under synchronous exposure to temperature, salinity, or desiccation stress. These deleterious effects could not have been predicted from single-factor experiments. In fact, these two species actually exhibited higher mortality in the field than in the lab, indicating that UVR and other stressors related to low tides may impact encapsulated embryonic development even more than suggested by my lab-based experiments, particularly the initial single-factor experiment conducted in Chapter 2.

Biotic factors may also interact with abiotic factors to further affect development of encapsulated embryos. For example, UVR can inhibit algal fouling on egg masses while associated PAR can encourage algal growth (Chapter 3.3). This complex interplay between abiotic and biotic factors further strengthens the importance of multifactorial experiments or consideration of potentially significant interactions. The experiments here thus provide an ecologically realistic insight into the impacts of UVR and other stressors associated with low tides on benthic encapsulated larval development.

5.4 MULTIPLE PHOTO-PROTECTIVE MECHANISMS

Organisms can mitigate UV-induced damage in three ways: avoid, reduce or repair (Cockell & Knowland 1999). These protective mechanisms are by no means exclusive, and organisms may possess a combination of them to protect against damage associated with UVR.

Most species examined in this study spawn under boulders or in other shaded habitats (Table 4.1.1), and their offspring thus passively avoid UVR altogether. However, egg masses of many of these species also have relatively high levels of MAAs (e.g. *Dolabrifera brazieri, Austraeolis ornata*), suggesting that embryos of these species are protected against UV-induced damage both biochemically and through adult spawning behaviour. MAAs in the egg masses of species that only spawn under boulders do not likely now serve as functional photo-protection to encapsulated embryos since embryos of these species are simply not exposed to UVR. Rather, MAA presence in these species may be beneficial to hatched veligers which can spend weeks or even months in the water column prior to metamorphosis and benthic settlement (Strathmann 1985; Strathmann 1987; Havenhand 1993; Pechenik 1999). During this time, they are potentially exposed to UVR and may have need of photo-protection. Indeed, MAAs in neogastropod egg masses are not located in the capsule walls, and may instead occur in the embryos themselves, thus providing protection during planktotrophic or lecithotrophic development (Przeslawski in press b). Alternatively, the presence of MAAs in these species may reflect ancestral reproductive patterns of molluscs in which all species were free spawning with pelagic larvae potentially exposed to UVR (Thorson

1950). Indeed, MAAs occur almost ubiquitously among marine organisms, and they may have evolved from a common ancestral origin (Karentz 2001), perhaps occurring even prior to the formation of the ozone layer when the earth was barraged by not only UV-A and UV-B, but also biologically damaging UV-C (Cockell & Knowland 1999). This supports the idea that modern MAA presence in egg masses of many species that spawn under boulders may simply be coincidence of diet (Adams & Shick 1996; Carefoot *et al.* 1998; Carefoot *et al.* 2000; Whitehead *et al.* 2001).

In contrast, embryos within egg masses deposited in habitats exposed to full sun definitely require protection against UVR-induced damage. The 2-year surveys in this study reveal that spawning of these species peak during summer months, thus maximising, not reducing, embryonic exposure to UVR (Figure 4.1.3). Therefore, embryos of these species need biochemical or cellular protection against UVR. Egg masses of these species do indeed have significantly higher concentrations of numerous MAAs compared to adults (Figure 4.2.4), implying a photo-protective role for these compounds. In fact, results from some experiments here suggest that at least some of these species possess multiple effective protective mechanisms against UVR-induced damage. First of all, MAAs may not be the sole source of photo-protection for embryos of species that spawn in full sun habitats as there was no clear pattern in MAA composition based on spawning habitat (Chapter 4.2.3.3). Second, embryos of *S. denticulata* and *B. nanum* showed less UV-induced damage in warmer temperatures, consistent with the presence of thermally regulated DNA repair enzymes (Chapter 3.1) (Rozema et al. 2002; Kim et al. 2004; Macfadyen *et al.* 2004), which may act in tandem with MAAs to mitigate negative effects of UVR. Finally, embryos of *S. denticulata* may possess UV-A inducible repair mechanisms as evidenced by significantly higher embryonic mortality in UV-blocked treatments than full spectrum treatments after extreme desiccation (Chapter 3.2). The exact mechanisms are unknown but may include DNA repair enzymes or antioxidant potential, both of which may act with MAAs to protect developing embryos. Further research investigating the presence and impact of these underlying photo-protective mechanisms in embryos and larvae of these species may help reveal how these embryos are protected against UVR and associated stressors. The presence of a variety of photo-protective mechanisms in a single species would

confirm frequent assumptions that some intertidal organisms use multiple defences against UVR-induced damage, and it would have important implications for the interpretation of future studies focusing on only a single mechanism of protection.

The effects of UVR on marine embryos have been comprehensively explored in the present study as well as some previous studies (Biermann *et al.* 1992; Gleason & Wellington 1995; Adams & Shick 2001; Kuffner 2001); but surprisingly, there has been no concentrated effort to fully examine the entire arsenal of any organism against UVR-induced damage. I found MAAs in the benthic egg masses from numerous species (Chapter 4.2), but their photo-protective role can only be speculated based on comparisons with adults. Studies on UVR phototaxis, DNA repair ability, and antioxidant activity in pelagic marine larvae are limited to only a few species (Dunlap *et al.* 1999; Epel *et al.* 1999; Persaud *et al.* 2003), and similar research on encapsulated embryos and the synchronous examination of multiple photo-protective functions has yet to be conducted. In particular, investigations on potential inter- and intra-specific variation in these photo-protective mechanisms are warranted. As with MAAs, recent research has shown that antioxidant potential in some organisms may vary according to depth (Camus & Gulliksen 2005), latitude (Abele & Puntarulo 2004), season (Lau *et al.* 2004; Regoli *et al.* 2004), and diurnal cycles (Dupont *et al.* 2004). Antioxidant potential may also be affected by environmental conditions such as oxygen availability (Wilhelm *et al.* 2005). Thus, the responses of many organisms to UVR and other stressors may be governed by a variety of mechanisms, each controlled by complex relationships between abiotic and biotic factors.

5.5 RISKY SPAWNING SITES & EVOLUTION OF SPAWNING BEHAVIOUR

This study has supported previous work of Benkendorff & Davis (2004) in which the majority of species that deposit benthic egg masses were found to spawn under boulders or in other habitats sheltered from environmental stress, including UVR. Other species, however, consistently deposit their egg masses in environments exposed to wind, sun, and emersion during low tides. The encapsulated embryos are thus vulnerable to negative effects associated with UVR, desiccation, fouling, and extremes in temperature

and salinity. Indeed, field observations in this study revealed that *S. denticulata* and *B. nanum* deposit their egg masses in habitats that increase embryonic mortality and retard development, thereby increasing time spent in vulnerable larval stages (Spight 1975) (Chapter 3). Rather than spawning in seasons that minimise embryonic exposure to environmental stress, these species spawn most frequently during seasons of peak environmental stress (Chapter 4.1).

Previous studies suggest that many species spawn in microhabitats to optimise embryonic survival (Pechenik 1978; D'Asaro 1986), but results from the studies here indicate that *S. denticulata, B. nanum* and other species consistently spawn in microhabitats in which they are vulnerable to environmental stress. Nevertheless, during field collection and surveys conducted in the work presented here, I frequently observed egg masses of these species in damp crevices or shallow puddles where their exposure to stressors may have been slightly reduced. Future studies should quantify these microhabitats within habitats exposed to full sun, and this may reveal protective spawning patterns on very small spatial scales.

Although embryos of these species likely possess some photo-protective functions against environmental stressors on rock platform surfaces, these mechanisms do not provide complete protection as evidenced by mortality observed in both the laboratory and the field (Chapter 3). As mentioned previously in Chapter 4.1.4, there are several reasons why species may spawn in habitats with conditions that are potentially damaging to their offspring. These reasons are outlined below in relation to the present studies:

Some species may spawn in potentially risky habitats to reduce predation or fouling of egg masses (Spight 1977; Menge 1978b, a; Ocana & Emson 1999). If this were the case for molluscan egg masses, the risk of these biotic factors should be greater than the risk associated with exposure to environmental stressors. Only neogastropod capsules showed any signs of predation during the course of this study; sporadic single capsules within a large groups of capsules were occasionally observed with chewed and opened capsular walls, emptied of most embryos (Figure 1.8). Gelatinous egg masses, on the

other hand, were observed intact with no interruption to the overall structure, such as that which may be observed with predation (Benkendorff 1998b). All neogastropod embryos were deposited under boulders so a comparison between spawning habitat and predation frequency of these egg masses was not possible. Nevertheless, no gelatinous egg masses from either shaded or exposed habitats exhibited any evidence of predation during this study. Previous studies have found that many molluscs and their egg masses are unpalatable to predators (Ocana & Emson 1999; Watson 2002). It thus seems unlikely that the molluscs examined in the present studies spawn on rock platform surfaces to reduce the risk of macroscopic predation. However, their spawning site may reflect avoidance of microscopic predation or fouling. UVR inhibited algal fouling and associated protist colonisation of egg masses (Chapter 3.3). However, algal fouling did not negatively affect embryos of most species, and it is therefore unlikely to be a significant selective pressure for species to spawn in otherwise risky habitats. The risk of protist colonisation could, however, dictate spawning site selection in some species. Protists may not only consume embryos; they may also compete for valuable resources such as oxygen which may retard development and increase embryonic mortality (Booth 1995; Strathmann & Strathmann 1995; Cohen & Strathmann 1996; Cancino *et al.* 2000). Further studies examining individual species of protists and their potential role as predators and resource competitors of encapsulated embryos are warranted.

Another reason for risky spawning site selection may be to maximise developmental rate. If maximisation of developmental rate applied to *S. denticulata*, *B. nanum* and other species examined here, the risk associated with stressful conditions on flat rock platforms would outweigh the benefits associated with increased developmental rates. A faster developmental rate may decrease larval exposure to environmental stressors (Havenhand 1993) and accelerate development of protective mechanisms that may increase survivorship (Podolsky 2003). Developmental rate is directly related to temperature (Palmer 1994) (Chapter 1), and elevated temperatures are linked to sunlight (Figure 1.3). Thus, rock platform surfaces which are exposed to direct sunlight can be expected to be warmer than habitats that are not, such as the undersides of boulders. Indeed, encapsulated embryos of *S. denticulata* and *B. nanum* develop faster in warm temperatures (Chapter 3.1). However, the negative effects of these stressors are also

exacerbated by elevated temperatures (Chapter 3.1). It may be that these species use a ‘bet-hedging’ strategy; they are very abundant and lay egg masses almost year-round (Chapter 4.1). Over their lifespan, there is a good chance that they will lay at least some egg masses during periods of suitable environmental conditions. Faster embryonic development means the duration of these periods shorten, and the hatching success of embryos increases. Future research on developmental rate in the field using continuous thermal data loggers would help determine if developmental rate and temperature conditions regulate spawning site selection of species that deposit egg masses on rock platform surfaces.

Finally, some species may spawn on rock platform surfaces so as to optimise conditions for other life stages, rather than encapsulated embryos (Spight 1977). The wave action on rock platform surfaces may be more conducive to dispersal of hatched veligers than beneath boulders; and optimal conditions for larval dispersal and / or recruitment may outweigh benefits of spawning in more sheltered habitats. The present study did not address this possibility, and although speculative, I mention it in the hope that it will foster research on the effects of various environmental conditions on encapsulated embryos, hatched veligers, and juveniles, as well as knowledge of effects of intertidal wave action on larval dispersal.

5.6 IMPLICATIONS OF GLOBAL CHANGE

Results from the studies detailed here may help predict the impacts of events related to anthropogenic activity, namely the thinning of the ozone layer and climate change, by identifying potential interactions between environmental stressors that may further increase the negative effects of these phenomena. In the work here, I identified species with early life stages that may be affected by factors associated with global change (i.e. UVR and temperature). Previous research on the effects of global change has been limited by single-factor studies. Initially, I found that embryos of some species seemed invulnerable to natural intensities of UVR (Chapter 2). In single factor studies such as this, impact assessments of local, regional, and global changes may falsely suggest that increased UVR will not significantly affect survival or development. Subsequent

multifactorial experiments revealed that these 'invulnerable' embryos are in fact susceptible to the negative effects of UVR when exposed to synchronous stressors (Chapter 3). Such knowledge may lead to improved estimates of larval survival, recruitment success, and impacts of local and global change on populations and overall biodiversity. Accurate assessments of the impact of climate change are essential if we are not only to develop effective policies to curb further ecological harm, but to even convince some policy-makers of the necessity for such actions in the first place (Ascher 2004; Nilsson *et al.* 2004; Baker 2005; Leggett 2005).

As evidenced by the work here, molluscs represent ideal model organisms to better understand and predict the effects of climate change. They represent one of the largest phyla of multicellular organisms and occur in marine, terrestrial, and freshwater environments (Beesley *et al.* 1998). Thus, a broad range of species in a variety of habitats can be examined, increasing the overall potential for accurate generalisation about anthropogenic impacts. Moreover, the relative abundance of molluscs, short generation time, high fecundity, and variety of reproductive and development strategies make them suitable for both laboratory and field-based studies, thus minimising time and cost and increasing the overall scope of associated studies.

Future studies should focus on the effects of potential interactions of some abiotic stressors on biological functions, thus investigating the processes underpinning the observed effects of mortality and development rates in the studies here. This research would represent a fundamental contribution to global change biology as it would reveal the means by which organisms are protected against UVR and associated stressors. In addition, it may also help explain how photo-protective functions are affected by changing variables related to global climate change and stratospheric ozone loss. There is already some evidence that antioxidant potential is negatively affected by reduced oxygen (Wilhelm *et al.* 2005), and DNA repair ability is undoubtedly thermally regulated (Kim *et al.* 2004; Macfadyen *et al.* 2004). Therefore, it seems likely that climate change will have some effect on organisms' ability to prevent and repair stress-induced damage, which in itself may increase due to climate change. Furthermore, some photo-protective mechanisms may also have other biological functions crucial to the

success of an organism. For example, antioxidants may be linked to sexual signals in animals (Neill & Gould 2003) and light attenuation in plants (von Schantz *et al.* 1999). Investigation of the direct impact of stressors on these functions may also reveal indirect impact on organisms through associated functions. Finally, research on potential combinations of protective functions is warranted because they may provide suitable indicators of overall climate change effects in a particular region. Antioxidant activity, DNA damage and repair rates, and heat-shock proteins are regularly used as bioindicators of pollution in aquatic environments (e.g. Mukhopadhyay et al. 2003; Agell et al. 2004; Company et al. 2004; Lah et al. 2004), and they may also be useful as a gauge of stress associated with changing environmental conditions.

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“If you steal from one author, it's plagiarism; if you steal from many, it's research.”
--Wilson Mizner

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APPENDICES

APPENDIX 1: TUKEY'S HSD TESTS FOR CHAPTER 3.1 (UVR, TEMPERATURE & SALINITY)

APPENDIX 1A: Effects of spectral treatment, temperature, and salinity on embryonic mortality of a) *Dolabrifera brazieri* , b) *Bembicium. nanum* and c) *Siphonaria denticulata* as determined by multiple comparisons using Tukey's HSD test (ANOVAs in Table 3.3.1a). Mean mortalities of raw data are presented with critical Q values in parentheses; lines connect treatments that are not significantly different.

a) *D. brazieri* (5.327)

Between spectral treatments	Full spectrum	UV-blocked	Dark
18˚C, 25ppt	0.2867	0.0700	0.0450
18˚C, 35ppt	0.2283	0.0450	0.1567
18˚C, 45ppt	0.4283	0.0425	0.0683
21˚C, 25ppt	0.3383	0.0467	0.0683
21˚C, 35ppt	0.2967	0.0450	0.0717
21˚C, 45ppt	0.2050	0.0600	0.0767
26˚C, 25ppt	0.1850	0.0717	0.0567
26˚C, 35ppt	0.1067	0.0733	0.0600
26˚C, 45ppt	0.8567	0.0733	0.0667
Between salinity treatments	25ppt	35ppt	45ppt
18˚C, Full spectrum	0.2867	0.2283	0.4283
18˚C, UV-blocked	0.0700	0.0450	0.0425
18˚C, Dark	0.0450	0.1567	0.0683
21˚C, Full spectrum	0.3393	0.2967	0.2050
21˚C, UV-blocked	0.0467	0.0450	0.0600
21˚C, Dark	0.0683	0.0717	0.0767
26˚C, Full spectrum	0.1850	0.1067	0.8567
26˚C, UV-blocked	0.0717	0.0733	0.0733
26˚C, Dark	0.0567	0.0733	0.0667

Between temperature treatments	18˚C	21˚C	26˚C
25ppt, Full spectrum	0.2867	0.3393	0.1850
25ppt, UV-blocked	0.0070	0.0467	0.0683
25ppt, Dark	0.0450	0.0683	0.0567
35ppt, Full spectrum	0.2283	0.2967	0.1067
35ppt, UV-blocked	0.0450	0.0450	0.0733
35ppt, Dark	0.1567	0.0717	0.0733
45ppt, Full spectrum	0.4283	0.2050	0.8567
45ppt, UV-blocked	0.0425	0.0600	0.0733
45ppt, Dark	0.0683	0.0767	0.0667

b) *B. nanum* (4.468)

Between spectral treatments	Full spectrum	UV-blocked	Dark
25 ppt	0.1294	0.0894	0.0178
35 ppt	0.0572	0.0372	0.0339
45 ppt	0.3267	0.0150	0.0189

Between salinity treatments	25ppt	35ppt	45ppt
Full spectrum	0.1294	0.0572	0.3267
UV-blocked	0.0894	0.0372	0.0150
Dark	0.0178	0.0339	0.0189

c) *S. denticulata* (4.468)

Between spectral treatments	Full spectrum	UV-blocked	Dark
25ppt	0.2389	0.0111	0.0044
35ppt	0.1739	0.0083	0.0050
45ppt	0.3950	0.0150	0.0022

Between salinity treatments	25ppt	35ppt	45ppt
Full spectrum	0.2389	0.1739	0.3950
UV-blocked	0.0111	0.0083	0.0150
Dark	0.0044	0.0050	0.0022
18˚C	0.1278	0.0672	0.1056
21˚C	0.0889	0.0933	0.1011
26˚C	0.0378	0.0267	0.2908

Between temperature treatments	18˚C	21˚C	26˚C
25ppt	0.1278	0.0889	0.0378
35ppt	0.0672	0.0933	0.0267
45ppt	0.1056	0.1011	0.2908

APPENDIX 1B: Effects of spectral treatment, temperature, and salinity on embryonic developmental stage of a) *Dolabrifera brazieri* , b) *Bembicium. nanum* and c) *Siphonaria denticulata* as determined by multiple comparisons using Tukey's HSD test (ANOVAs in Table 3.3.1b). Mean developmental stage of raw data are presented with critical Q values in parentheses; lines connect treatments that are not significantly different.

a) *D. brazieri* (3.356)

Between spectral treatments	Full spectrum	UV-blocked	Dark
	1.4815	1.8148	1.7870
Between temperature treatments	18˚C	21˚C	26˚C
	1.5093	1.6759	1.8981

b) *B. nanum* (3.356)

Between spectral treatments	Full spectrum	UV-blocked	Dark
	1.8093	2.5556	2.3333
Between salinity treatments	45ppt	25ppt	35ppt
	1.9481	2.1759	2.5741
Between temperature treatments	18˚C	21˚C	26˚C
	1.7722	2.1667	2.7593

c) *S. denticulata* (5.327)

Between spectral treatments	Full spectrum	UV-blocked	Dark
18˚C, 25ppt	1.4167	1.3333	1.5000
18˚C, 35ppt	1.4167	1.6667	1.4167
18˚C, 45ppt	1.5000	1.4167	1.4167
21˚C, 25ppt	1.3333	2.0000	2.1667
21˚C, 35ppt	1.5000	1.9167	2.0833
21˚C, 45ppt	1.2500	1.6667	1.5000
26˚C, 25ppt	2.1667	3.0833	2.6667
26˚C, 35ppt	2.5000	2.8333	3.0833
26˚C, 45ppt	1.2500	2.5000	2.5000

Between salinity treatments	25ppt	35ppt	45ppt
18˚C, Full spectrum	1.4167	1.4167	1.5000
18˚C, UV-blocked	1.3333	1.6667	1.4167
18˚C, Dark	1.5000	1.4167	1.4167
21˚C, Full spectrum	1.3333	1.5000	1.2500
21˚C, UV-blocked	2.0000	1.9167	1.6667
21˚C, Dark	2.1667	2.0833	1.5000
26˚C, Full spectrum	2.1667	2.5000	1.2500
26˚C, UV-blocked	3.0833	2.8333	2.5000
26˚C, Dark	2.6667	3.0833	2.5000
Between temperature treatments	18˚C	21˚C	26˚C
25ppt, Full spectrum	1.4167	1.3333	2.1667
25ppt, UV-blocked	1.3333	2.0000	3.0833
25ppt, Dark	1.5000	2.1667	2.6667
35ppt, Full spectrum	1.4167	1.5000	2.5000
35ppt, UV-blocked	1.6667	1.9167	2.8333
35ppt, Dark	1.4167	2.0833	3.0833
45ppt, Full spectrum	1.5000	1.2500	1.2500
45ppt, UV-blocked	1.4167	1.6667	2.5000
45ppt, Dark	1.4167	1.5000	2.5000

APPENDIX 2: TUKEY'S HSD TESTS FOR CHAPTER 3.2 (UVR & DESICCATION)

APPENDIX 1A: Effects of interaction of spectral treatment and desiccation on embryonic mortality of a) *Siphonaria denticulate* and b) *Dolabrifera brazieri* as determined by multiple comparisons using Tukey's HSD test (ANOVAs in Table 3.2.1a). Mean mortalities of raw data are presented with critical Q values in parentheses; lines connect treatments that are not significantly different.

a) *S. denticulata* (6.789)

Between spectral treatments:	Full spectrum	Dark	UV-blocked
0 minutes	0.0317	0.1083	0.0400
15 minutes	0.0233	0.0900	0.0733
30 minutes	0.1000	0.0650	0.1667
60 minutes	0.0020	0.1220	0.2580

Between desiccation treatments:	0 minutes	15 minutes	30 minutes	60 minutes
Full spectrum	0.0317	0.0233	0.1000	0.0020
UV-blocked	0.0400	0.0733	0.1667	0.2580
Dark	0.1083	0.0900	0.0650	0.1220

b) *D. brazieri* (6.789)

Between spectral treatments:	Full spectrum	UV-blocked	Dark
0 minutes	0.0417	0.0383	0.0283
15 minutes	0.5717	0.4467	0.0817
30 minutes	0.7917	1.0000	0.3150
60 minutes	1.0000	1.0000	0.7600

Between desiccation treatments:	0 minutes	15 minutes	30 minutes	60 minutes
Full spectrum	0.0417	0.5717	0.7917	1.0000
UV-blocked	0.0383	0.4467	1.0000	1.0000
Dark	0.0283	0.0817	0.3150	0.7600

APPENDIX 2B: Effects of spectral treatment and desiccation time on embryonic developmental stage of a) *Dolabrifera brazieri* and b) *Bembicium. nanum* as determined by multiple comparisons using Tukey's HSD test (ANOVAs in Table 3.2.1b). Mean developmental stage of raw data are presented with critical Q values in parentheses; lines connect treatments that are not significantly different.

d) *D. brazieri* (51.96)

	0 minutes	15 minutes
30 minutes	60 minutes	
	2.3611	2.0294
n/a	n/a	

e) *B. nanum* (14.75)

	Full spectrum	UV-blocked
Dark		
	2.1042	2.0833
1.8125		

APPENDIX 3: A METHOD TO INHIBIT ALGAL FOULING ON EGG MASSES

Introduction

Algicides provide a novel way to examine the synergistic effects of UVR and algal fouling on development of encapsulated embryos. Several pesticides and herbicides have been shown to kill marine microalgae (Walsh 1972; Hollister & Walsh 1973). The effectiveness of algicides depends not only on the chemical used, but also on the target species (Kusk & Nyholm 1992), environmental parameters (Mayasich *et al.* 1986), and algicide concentration (Ibrahim 1984). Indeed, one study found that a relatively low algicide concentration may actually stimulate algal growth (Ibrahim 1983). In addition, the algicide might detrimentally affect non-target organisms. I hoped to identify an appropriate control algicide for use in experimental research on the effects of algal fouling and UVR on molluscan egg masses. The appropriate algicide and concentration would not significantly affect embryonic mortality of molluscan egg masses.

Methods

Undeveloped molluscan egg masses were collected on the same day in late September 2002 from the intertidal rock platforms of Bass Point, NSW (Figure 2.1). Species were randomly chosen based on the abundance found (Table A.1). Among each species, discrete egg masses or capsules were divided into seven equal groups (see Table A.2). Each group was submerged in a tub containing 1 litre of seawater; tubs were repositioned randomly throughout the experiment. Each tub had a particular algicide and concentration (see Table A.2) with the exception of the control which only contained seawater. Water was aerated and tubs were covered so that the samples were not exposed to direct sunlight. Egg masses were checked every day and removed for examination if they showed any signs of hatching. In addition, egg masses were removed when they showed obvious signs of complete inviability. The embryonic mortality rate of each egg mass was estimated by methods described in Chapter 3.3.3.

Table A.1 Species and number of egg masses or capsules used in each treatment within this experiment

Species	Structure	n
Bembicium nanum	Gelatinous	4
Dolabrifera dolabrifera	Gelatinous	3
Siphonaria denticulata	Gelatinous	4
Agnewia tritoniformil	Capsular	7
Mitra carbonaria	Capsular	10

Table A.2 Algicide treatments used in this experiment

Treatment	Algicide	Active chemical	Concentration
A1	Algitrol	benzalkonium chloride	.050ml/L
A2	Algitrol	benzalkonium chloride	.025ml/L
S1	Simazine	2-chloro-4-,6-bis(ehtylamino)-s-triazine	.250ml/L
S2	Simazine	2-chloro-4-,6-bis(ehtylamino)-s-triazine	.100ml/L
R1	Biactive Roundup	glyophosphate	10ml/L
R2	Biactive Roundup	glyophosphate	5ml/L
Control	--	--	--

Results

Surprisingly, the control treatment for *M. carbonaria* had an average mortality of almost 100% (Figure A.1). Microscopic observation revealed dense protist colonies settled on most egg capsules. The protist infection likely increased mortality within the control group so the data for *M. carbonaria* was not included in statistical analysis.

The effect of various algicides on embryonic mortality varied among species as confirmed by a significant interaction between algicidal treatment and species ($F=2.5179$, $p=.0020$). A Tukey's HSD multiple comparison was performed to determine significant relationships. Both concentrations of Biactive Roundup significantly affected all species resulting in 100% embryonic mortality in all cases (Figure A.1). Algitrol (.050ml/L) also resulted in 100% mortality in all species with the exception of *S. denticulata* (Figure A.1). The egg masses of *S. denticulata* were not significantly affected by this algicidal treatment despite its toxicity to the other species. Neither the lower concentration of Algitrol (.025ml/L) nor either concentration of Simazine significantly affected the embryonic mortality rate of any species of egg mass (Figure A.1).

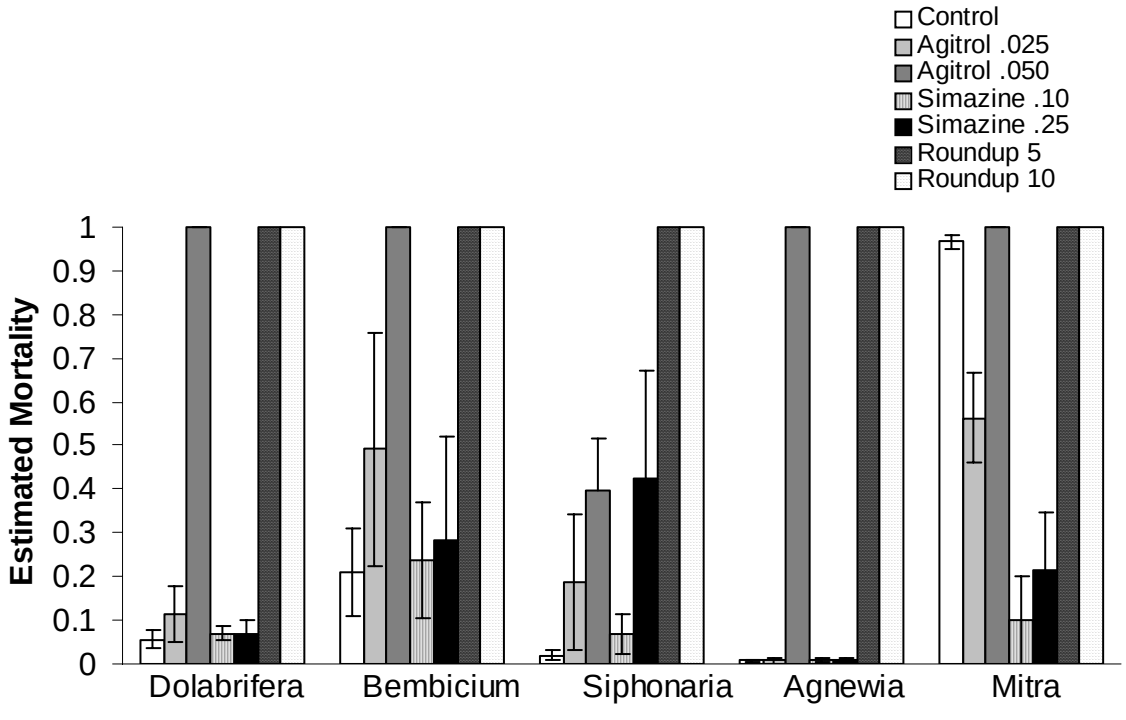

Figure A.1 Effects of various algicides and concentrations on embryonic mortality of five species of encapsulated molluscs. Error bars are standard error mean.

APPENDIX 4

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Chemical sunscreens in intertidal gastropod egg masses

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Summary

Many marine organisms contain mycosporine-like amino acids (MAAs), a suite of chemical sunscreens that absorb potentially damaging ultraviolet radiation. No previous study has quantified MAAs in invertebrate larvae from a broad range of species. In this study, intertidal egg masses of varying developmental stages from 44 gastropods were collected from the southeastern Australian coast to determine potential patterns of MAA occurrence. Total MAA concentration did not change as egg masses matured. MAA concentration differed significantly according to gastropod order. Herbivores generally deposited egg masses with more MAAs than carnivores, but spawning habitat and egg mass structure had no effect on total MAA concentration. Furthermore, fewer MAAs occurred in inviable egg masses than viable ones. In capsular egg masses, MAAs existed only in embryos or intracapsular fluid.

Key words: MAAs, gastropod, egg mass, intertidal

Introduction

Marine invertebrate larvae are vulnerable to a multitude of environmental stressors, particularly in the intertidal where conditions can change abruptly and frequently (Spight, 1977; Gosselin and Chia, 1995). Some gastropods encase their offspring in benthic egg masses in the intertidal where they may be exposed to extreme temperatures, salinity changes, desiccation, predation and ultraviolet radiation (UVR) for the duration of their encapsulation (reviewed by Przeslawski, 2004). UVR can negatively affect reproduction, development, and behaviour in marine invertebrates (Rawlings, 1996; Carefoot et al., 1998; Haeder et al., 1998). Previous studies indicate that in some encapsulated molluscan embryos, UVR exposure can cause stunted development, deformities, and death (Biermann et al., 1992). However, UVR effects on encapsulated molluscan embryos are species-specific; embryos of species that regularly spawn in full sun habitats are less vulnerable to the deleterious effects of UVR than species that only spawn under boulders in full shade (Przeslawski et al., 2004). The method of protection against UVR is unknown.

One possibility is that they are protected through chemical sunscreens called mycosporine-like amino acids (MAAs). MAAs encompass 19 known compounds that have UV absorption maxima between 310–360 nm (Bandaranayake, 1998; Shick and Dunlap, 2002). Although the exact mechanisms are not known, it is likely that MAAs are synthesised through the shikimate pathway (Bandaranayake, 1998), a branched metabolic pathway in which aromatic compounds are formed (Bentley, 1990). It is assumed that animals lack this pathway although its absence has

only been shown to be widespread in vertebrates (Shick and Dunlap, 2002). It is therefore widely held that animals must obtain MAAs through diet or symbioses.

MAAs have been found in a variety of marine organisms including algae, cnidarians, echinoderms, and vertebrates, and they have been shown to protect against the damages of UVR (reviewed by Shick and Dunlap, 2002). Previous research reveals that there are few taxonomic boundaries to MAA presence (Bandaranayake, 1998), but there may be taxonomic patterns to MAA concentration. MAAs exist in the eggs and embryos of several invertebrates including urchins, corals, ascidians, and gastropods (reviewed by Karentz, 2001). However, each of these studies focused on offspring of only one species, respectively; and there has been no previous survey of MAAs in marine embryos or extraembryonic structures from a broad range of species. Thus, there is a fundamental gap in our knowledge of the importance of MAAs in the protection of invertebrate embryos. This study aims to identify potential patterns of MAA distribution among intertidal gastropod egg masses and examine factors that may affect MAA concentration: egg mass maturity and viability, spawning habitat, egg mass structure, adult diet, and molluscan order.

Methods

Most egg masses were collected from intertidal habitats along the Illawarra coast from November 2001 to February 2004. Some samples were collected from adults held in laboratory aquaria, and one species was collected from Cornwall, England. Egg masses were identified to species level where possible based on observations of the laying adult or previous research (Rose, 1985; Smith et al., 1989; Benkendorff, 1999). Egg masses were examined under a dissecting microscope (40× magnification) to determine development, and they were classed accordingly into one of five stages: (1) undeveloped egg masses containing eggs that had not yet devel-oped to the trochophore stage; (2) intermediate developed egg masses containing trochophores or early veligers; (3) mature egg masses containing late stage veligers, crawling juveniles and/or showing signs of hatching; (4) empty egg masses containing very few or no embryos and/or intracapsular fluid; (5) inviable egg masses containing no viable eggs as evidenced by degenerating eggs or colour change associated with inviability (e.g., see Przeslawski et al., 2004). After examination, egg masses were cleaned by agitation in filtered seawater for 30 s followed by gentle blotting to remove excess water. Egg masses were then lyophilised, dry weight was recorded, and samples were stored at −80°C until extractions were performed.

MAAs were separated by reverse-phase high performance liquid chromatography (HPLC) on a Phenosphere C8 (5 μm 4.6 i.d.×250 mm) column with guard (Phenomenex) at a flow rate of 0.8 mL/ min. The aqueous mobile phase was 39.9:0.1:60 water:acetic acid:methanol. MAAs were identified using maximum wavelength absorption and co-chromatography with prepared standards (e.g., see Adams and Shick, 1996). Total MAA concentration was determined by the sum of individual MAAs detected (mycosporine-glycine, shinorine, porphyra-334, mycosporine-2-glycine, palythine, palythene, asterina-333, and palythinol). Concentration was calculated in nmol/mg sample dry weight.

Results

Egg mass maturity

To determine if total MAA concentration varied with egg mass development, developmental stage data from the following species were analysed using individual one-way ANOVAs: *Bembicium nanum* (Lamarck, 1822), *Aplysia sydneyensis* (Sowerby, 1869), *Bursatella leachii* (de Blainville, 1817), *Cabestana spengleri* (Perry, 1811), *Polinices* (*Conuber*) sp., *Conus papilliferus* (Sowerby, 1834), *Dicathais orbita* (Gmelin, 1791), *Dolabrifera brazierii* (Sowerby, 1870), *Hydatina physis* (Linnaeus, 1758), *Mitra carbonaria* (Swainson, 1822), *Oxynoe viridis* (Pease, 1861), *Placida* cf. *dendritica* (Alder and Hancock, 1843), *Siphonaria denticulata* (Quoy and Gaimard, 1833), *Stylocheilus striatus* (Quoy and Gaimard, 1832), and an unknown neogastropod. These species were chosen because they had more than three replicates in each of the stages examined. There were no significant differences between total MAA concentration in undeveloped, intermediate, and mature egg masses of any of these species ($\alpha = 0.05$). Because there was no difference in total MAA concentration based on egg mass maturity, all viable egg masses were pooled in remaining analyses of MAAs.

Egg mass viability

To determine if total MAA concentration varied among viable and inviable egg masses, one-way ANOVAs were used on the following species: *Lepsiella reticulata* (de Blainville, 1832), *C. papilliferus*, *D. orbita*, *B. nanum* and *D. brazieri*. These encompass three leathery egg capsules and two gelatinous egg masses with more than three replicates in each species

Table 1. List of species used in this study and the factors examined. All egg masses were collected from SE Australia in 2001–2004. Total MAA concentrations derived from viable egg masses only

Species	Order	Habitat	Diet[a]	Structure	*n* (viable, inviable, empty)	Total MAAs ± SD (nmol/mg d.w.)
Bembicium nanum	Littorinimorpha	FS	H	Gel	18, 3, 0	6.57 ± 2.28
Cabestana spenglerii	Littorinimorpha	SH	C	Capsule	5, 0, 3	3.58 ± 0.90
Polinices (Conuber) sp.[b]	Littorinimorpha	FS	C	Gel	10, 0, 0	0.29 ± 0.63
Cypraea erosa	Littorinimorpha	SH	U	Capsule	1, 0, 0	3.41
Agnewia tritoniformis	Neogastropoda	SH	C	Capsule	5, 0, 0	4.91 ± 1.15
Bedeva sp.	Neogastropoda	SH	C	Capsule	1, 0, 0	3.13
Conus papilliferus	Neogastropoda	SH	C	Capsule	6, 3, 4	0.17 ± 0.03
Dicathais orbita	Neogastropoda	SH	C	Capsule	8, 3, 3	4.65 ± 2.16
Lepsiella reticulata	Neogastropoda	SH	C	Capsule	2, 3, 0	1.41 ± 0.97
Morula marginalba	Neogastropoda	SH	C	Capsule	2, 0, 0	6.56 ± 2.65
Mitra badia	Neogastropoda	SH	C	Capsule	1, 0, 0	1.05
Mitra carbonaria	Neogastropoda	SH	C	Capsule	13, 0, 0	0.00
Nucella lapillus[c]	Neogastropoda	SH	C	Capsule	2,0,0	4.31 ± 0.48
Unknown neogastropod[d]	Neogastropoda	SH	C	Capsule	5, 0, 0	0.00
Bulla quoyii	Cephalaspidea	PS	H	Gel	1, 0, 0	6.68
Bullina lineata	Cephalaspidea	PS	C	Gel	5, 0, 0	5.17 ± 1.60
Hydatina physis	Cephalaspidea	PS	C	Gel	8, 0, 0	2.60 ± 1.49
Aplysiopsis formosa	Sacoglossa	PS	H	Gel	2, 0, 0	0.00
Oxynoe viridis	Sacoglossa	PS	H	Gel	6, 0, 0	0.45 ± 0.21
Placida cf. *dendritica*	Sacoglossa	PS	H	Gel	9, 0, 0	0.30 ± 0.17
Berthellina citrina	Notaspidea	SH	C	Gel	1, 0, 0	0.23
Pleurobranchus peronii	Notaspidea	SH	C	Gel	2, 0, 0	5.49 ± 5.94
Pleurobranchus sp.	Notaspidea	SH	C	Gel	2, 0, 0	1.15 ± 0.30
Aplysia Juliana	Anaspidea	PS	H	Gel	9, 0, 0	2.02 ± 2.89
Aplysia sydneyensis	Anaspidea	PS	H	Gel	14, 0, 0	11.50 ± 7.05
Aplysia parvula	Anaspidea	PS	H	Gel	2, 0, 0	7.65 ± 4.44
Bursatella leachii	Anaspidea	PS	H	Gel	6, 0, 0	6.94 ± 3.69
Dolabella auricularia	Anaspidea	PS	H	Gel	2, 0, 0	10.30 ± 2.72
Dolabrifera brazieri	Anaspidea	SH	H	Gel	16, 3, 0	12.97 ± 4.83
Stylocheilus striatus	Anaspidea	PS	H	Gel	8, 0, 0	16.78 ± 2.39
Aeolidiella foulisi	Nudibranchia	SH	C	Gel	1, 0, 0	0.28
Austraeolis ornata	Nudibranchia	SH	C	Gel	5, 0, 0	20.56 ± 11.52
Dendrodoris carneola	Nudibranchia	SH	C	Gel	1, 0, 0	0.25
Dendrodoris fumata	Nudibranchia	SH	C	Gel	7, 0, 0	5.16 ± 3.62
Dendrodoris nigra	Nudibranchia	SH	C	Gel	1, 0, 0	0.56
Doriopsilla miniata	Nudibranchia	SH	C	Gel	1, 0, 0	0.25
Goniodoris meracula	Nudibranchia	SH	C	Gel	3, 0, 0	0.10 ± .03
Hoplodoris nodulosa	Nudibranchia	SH	C	Gel	4, 0, 0	2.06 ± 2.51
Hypselodoris obscura	Nudibranchia	SH	C	Gel	3, 0, 0	1.50 ± 0.68
Platydoris galbanus	Nudibranchia	SH	C	Gel	3, 0, 0	14.35 ± 4.35
Plocampherus imperialis	Nudibranchia	SH	C	Gel	1, 0, 0	18.89
Rostanga arbutus	Nudibranchia	SH	C	Gel	3, 0, 0	0.51 ± 0.69
Siphonaria denticulata	Basommatophora	FS	H	Gel	14, 0, 0	10.32 ± 1.84
Siphonaria zelandica	Basommatophora	FS	H	Gel	6, 0, 0	7.62 ± 4.76

FS, full sun; PS, partial sun; SH, shade; H, herbivore; C, carnivore; U, unknown.
[a]Adult diet based on family and genus descriptions from Beesley et al. (1998) and Rudman (2004).
[b]Egg masses are *Polinices* (*Conuber*) *sordidus*, *P. conicum* and/or *P. melastomum*, which are indistinguishable from each other.
[c]Collected from Cornwall, England.
[d]Corresponds with unidentified egg mass sp. 2 in Table 2.3 of Benkendorff (1999). Tentatively identified as *Cominella eburnea* based on crawling juvenile.

(Table 1). Inviable egg masses had a much lower total concentration of MAAs than viable egg masses in *B. nanum* ($F = 20.50$, $p = 0.0002$) and *D. orbita* ($F = 12.54$, $p = 0.0063$). Inviable egg masses of *D. brazieri* ($F = 3.54$, $p = 0.0783$) and *L. reticulata* ($F = 6.09$, $p = 0.0903$) had slightly less MAAs than viable egg masses at $\alpha = 0.10$. In contrast, MAA concentration of egg masses from *C. papilliferus* did not significantly vary between viable and inviable egg masses, but the MAA concentration in viable egg masses of this species was very low relative to the other species examined (Table 1).

Egg mass structure, spawning habitat, adult diet, order

Univariate analyses were conducted on viable egg masses from all 44 species with four nested ANOVAs in the statistical software package JMP (v. 4) (Table 1 for *n* values). Species were nested within one of four factors: spawning habitat, egg mass structure, adult diet, and order (Table 1). Analysis of spawning habitat included only viable egg masses from gelatinous egg masses, and analysis of egg mass structure included only those from shaded habitats; thus, the potential confounding effects of these two factors were separated. Neither spawning habitats nor egg mass structure significantly affected total MAA concentration. However, total MAA concentration did differ significantly according to adult diet ($F = 6.48$, $p = 0.0142$). Egg masses from herbivores contained a significantly higher quantity of MAAs than those from carnivores (Fig. 1a). MAA concentration within egg masses also varied significantly according to gastropod order ($F = 14.20$, $p < 0.0001$). Tukey's HSD tests were conducted to determine significantly different relationships. Anaspidean egg masses had significantly higher amounts of MAAs than egg masses from littorinimorphs, neogastropods, cephalaspideans, notaspideans, nudibranchs, and sacoglossans (Fig. 1b). Similarly, basommatophoran egg masses had more MAAs than those from littorinimorphs, neogastropods, nudibranchs and sacoglossans (Fig. 1b). Sacoglossans had less MAAs than nudibranchs (Fig. 1b).

MAA concentration in capsule walls

To determine if MAAs were contained in the capsule walls, MAA content of intact viable capsules was compared with empty capsules for the following species: *C. spenglerii*, *C. papilliferus*, and *D. orbita* (Table 1). Empty capsules had a much lower total concentration of MAAs than intact capsules in *C. spenglerii* ($F = 28.23$, $p = 0.0032$) and *D. orbita* ($F = 8.53$, $p = 0.0193$). In contrast, MAA concentration of egg masses from *C. papilliferus* did not significantly vary between empty and intact capsules.

Discussion

This study has shown that MAAs are prevalent in intertidal gastropod egg masses, and certain species have high concentrations of these compounds relative to other marine invertebrate eggs (e.g., see Adams and Shick, 1996). Viable egg masses had higher concentrations of MAAs than inviable egg masses, but it is unknown if lower MAA concentrations detrimentally affect embryonic development or if they were a consequence of an already inviable egg mass. No differences in total MAA concentration were found between various developmental stages, suggesting

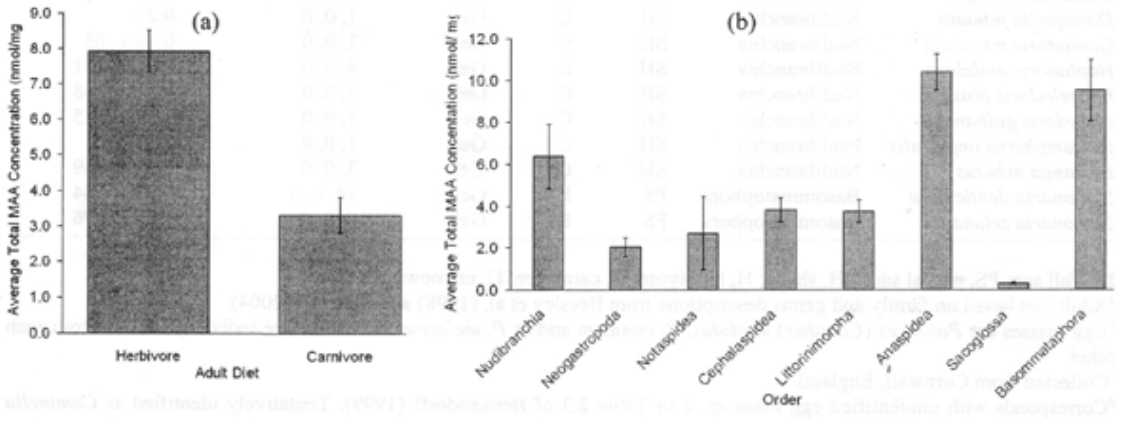

Fig. 1. Effects of (a) adult diet (excluding *Cypraea erosa* with unknown diet) and (b) molluscan order on total MAA content of viable egg masses from 44 molluscan species. Error bars are standard error of mean.

MAA content within molluscan egg masses remains constant throughout embryonic encapsulation.

Egg masses from herbivores had more total MAAs than those from carnivores (Fig. 1a). Previous research has revealed some evidence of MAA bioaccumulation in higher trophic levels (Whitehead et al., 2001), but the present study suggests that bioaccumulation of MAAs in carnivores does not generally occur in molluscan egg masses. Rather, herbivores may have a more direct link to MAA sources than carnivores since MAAs can only be synthesised by plants, and this is reflected in their spawn. Previous research has shown that MAA content in the egg ribbons of *Aplysia dactolomela* (Rang, 1828) is highly diet-dependent; adults fed red algae rich in MAAs incorporated higher concentrations of MAAs in their spawn than those adults fed green algae poor in MAAs (Carefoot, 1998). The present study supports these findings for a broader range of gastropod species in a more general trophic context (herbivore vs. carnivore).

MAA content of spawn is linked to adult diet, but phylogenetic variation may potentially confound effects since trophic level is often similar within a molluscan order (Table 1). In this study, the greatest amounts of MAAs were found in the spawn of anaspids and basommatophorans and the least amounts in sacoglossan egg masses (Fig. 1b). Species in all of these gastropod orders are herbivorous (Table 1), thus suggesting that factors other than diet also affect MAA content.

There were no differences in total MAA content between gelatinous and capsular egg masses. Results from this study indicate that MAAs in capsular egg masses are located within the intracapsular fluid or embryos. Rawlings (1996) also found that the capsule walls of *Nucella emarginata* (Deshayes, 1839) did not contain MAAs, but that they nonetheless absorb UVR. Capsular egg masses from some neogastropods may thus have the combined protection of UVR absorption through the capsular wall coupled with protection from intracapsular MAAs. The location of MAAs in gelatinous egg masses was not examined in this study and therefore remains unknown.

If MAAs were an evolved protection against UVR damages, egg masses from species that consistently spawn in full sun would be expected to have more MAAs than egg masses from species that only spawn in shaded habitats. Surprisingly, spawning habitat did not affect MAA concentration in this study. However, this result could be confounded by other factors such as adult diet and phylogeny. It remains unknown if MAAs in molluscan egg masses are an evolved protective mechanism, a coincidental dietary benefit or both. This survey represents a preliminary analysis of these data by examining total MAA concentration with univariate analyses. Further analyses will include multi-variate statistics that may reveal more subtle and com-plex relationships between the factors examined here and the MAA distributions of individual compounds. Nonetheless, the present study has provided a foundation for future studies examining the ecological role of MAAs in marine invertebrate development.

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