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

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Abstract- 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, we quantified MAAs from intertidal egg masses of 46 species of mollusc, two species of polychaete, and one species of fish from southeastern Australia. We sought to elucidate potential patterns of occurrence and variation based on egg mass maturity, adult diet, spawning habitat, phylogeny, and viability. Our 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.

Key Words- Gastropod, egg mass, larvae, marine invertebrate, chemical sunscreen, intertidal, mycosporine-glycine

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 and Knowland, 1999), with UV-B absorbed strongly by the ozone layer (Paul and 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 and 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 or gelatinous egg masses (see Przeslawski, 2004). The embryos are essentially sessile for the duration of their encapsulation and reliant on adult spawning habitat for protection against UVR. Many gastropods only spawn under boulders or in other fully shaded microhabitats, but some species consistently deposit their egg masses in habitats exposed to sunlight (Benkendorff and 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 only spawn 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 and Dunlap, 2002). MAAs have been found in adults from a variety of organisms including algae, fish, cnidarians, molluscs, and echinoderms (reviewed by Shick and Dunlap, 2002)). The concentration and composition of MAAs can vary in organisms according to latitude (Bosch et al., 1994), altitude (Tartarotti et al., 2001), depth (Gleason and 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), 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, we have collected benthic egg masses from a range of intertidal invertebrates and quantified their MAA composition. We aimed to identify common MAAs in intertidal egg masses and to determine which factors account for variation in MAA composition and abundance. Here we investigate the effects of egg mass maturity, adult diet, spawning habitat, taxonomic group (order), and embryo viability. We 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. We 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, we 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.

METHODS AND MATERIALS

We 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 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 (Benkendorff, 1999; Rose, 1985; Smith et al., 1989). Potential spatial and temporal variation in MAA composition was examined on a subset of the egg masses. We 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 difference 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 1). We 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, overhangs, or caves) (Table 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 and/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 (data not presented), as also noted by previous studies (Dunlap and Chalker, 1986; Karsten et al., 1998).

Preliminary tests were performed to determine the ideal extraction conditions for these samples as recommended by (Tartarotti and Saommaruga, 2002). Samples were extracted using 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, we recovered over 95% of total MAAs after three of six extractions for each species.

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 using maximum wavelength absorption and co-chromotography with prepared standards representing the most common MAAs (Karentz, 2001; Shick and 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, we 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 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. We 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 using 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., 1995a, b). Most data was skewed and/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.

RESULTS

Spectrophotometry indicated the presence of UVR-absorbing compounds in many species as evidenced by maximal absorbance at UVR wavelengths (Figure 1). Of the 49 species tested, we detected and quantified MAAs in 43 viable egg masses (Table 2). We did not detect MAAs in the egg masses of the polychaetes or in egg masses from four species of mollusc (*Ranella australasia, Cominella eburnea, Mitra carbonaria,* and *Aplysiopsis formosa*) (Table 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.).

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 2). Palythene was the least common, occurring in just eight species (Table 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, λ_{max}): *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 *A. 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.

Comparison with Adults. An nMDS plot of viable egg masses and adults of species for which we had replicates revealed distinct clusters (Figure 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 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 3).

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 2b) and a nested ANOSIM on data subsets including species where $n \geq 3$ ($R = 0.079$, $p = 0.072$) (refer to Table 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 2c, see Table 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).

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

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 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 6).

Gastropod Phylogeny. The taxonomic grouping (order) of gastropods strongly affected MAA content in egg masses. An nMDS plot showed some taxonomic clusters, particularly within anaspids (Figure 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 2f) and relatively large error bars for individual MAA concentrations (Figure 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). Although not significant at $\alpha = 0.05$, non-significant results here should be interpreted cautiously due to the large intra- and inter-specific variation in MAA complements (Figure 2f).

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 6). 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 7). Basommataphorans had significantly more porphyra-344 than sacoglossans (Figure 7).

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 (Banaszak et al., 1998; McClintock and Karentz, 1997). For example, larvae of the urchin *Strongylocentrotus droebachiensis* contain 4-8 nmol mg^{-1} total MAAs (Adams and Shick, 2001) which represents the middle range of the concentrations we detected (Table 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 3) which is consistent with the suggestion that MAAs have a photoprotective 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 our prediction, we have found no clear pattern in MAA composition relating to spawning habitat (Figure 2d, 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 and 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 3); and viable egg masses had higher concentrations than inviable egg masses (Figure 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 3). Mycosporine-glycine is the only MAA found in this study that absorbs maximally at 310 nm (Figure 1), the range of UVR (UVB) that is most biologically damaging (Paul and 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 photoprotective 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 and 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 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 and 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., 1995b).

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 6); but when analysed according to order, egg masses of carnivorous nudibranchs had significantly more MAAs than egg masses from most herbivorous orders (Figure 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 (Adams et al., 2001; Carefoot et al., 1998; Carefoot et al., 2000). The present study indicates that this diet-dependence may extend to differences in MAAs between trophic levels (Figure 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). In the present study, we found minimal differences in MAA composition between trophic levels (Figure

2e) and significant differences in only two of the eight MAAs detected (Figure 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, in press). 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 2). 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 2). Other species, such as *Bembicium nanum*, may use higher concentrations of a single MAA to confer protection (Table 2).

Viable egg masses had a richer complement of MAAs and had higher concentrations of individual compounds than inviable egg masses (Figure 4). These results support previous univariate analysis of total MAA concentration in these egg masses (Przeslawski, in press). 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 2, Figure 1). 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 and 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 and 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 photoprotection 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 and Shick, 1996). No similar studies have been conducted on embryos of the species used in this study, but MAAs may indeed 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.

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TABLE 1: List of species and associated egg mass characteristics used in this study. n refers to the number of viable egg masses collected. Taxa refer to superfamily (SF), infraorder (IO), or order (O).

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

Anaspidea (O)	*Aplysia juliana* [2]	9	Partial sun	Herbivore	Gel
Anaspidea (O)	*Aplysia sydneyensis* [1]	14	Partial sun	Herbivore	Gel
Anaspidea (O)	*Aplysia parvula*	2	Partial sun	Herbivore	Gel
Anaspidea (O)	*Bursatella leachii* [1, 2]	6	Partial sun	Herbivore	Gel
Anaspidea (O)	*Dolabella auricularia* [2]	2	Partial sun	Herbivore	Gel
Anaspidea (O)	*Dolabrifera brazieri* [1, 2]	16	Shade	Herbivore	Gel
Anaspidea (O)	*Stylocheilus striatus* [1]	8	Partial sun	Herbivore	Gel
Nudibranchia (O)	*Aeolidiella foulisi*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Austraeolis ornata*	5	Shade	Carnivore	Gel
Nudibranchia (O)	*Dendrodoris carneola*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Dendrodoris fumata* [1]	7	Shade	Carnivore	Gel
Nudibranchia (O)	*Dendrodoris nigra*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Doriopsilla miniata*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Goniodoris meracula*	3	Shade	Carnivore	Gel
Nudibranchia (O)	*Hoplodoris nodulosa*	4	Shade	Carnivore	Gel
Nudibranchia (O)	*Hypselodoris obscura*	3	Shade	Carnivore	Gel
Nudibranchia (O)	*Platydoris galbanus*	3	Shade	Carnivore	Gel
Nudibranchia (O)	*Plocampherus imperialis*	1	Shade	Carnivore	Gel
Nudibranchia (O)	*Rostanga arbutus*	3	Shade	Carnivore	Gel
Basommatophora (O)	*Siphonaria denticulata* [1]	14	Full sun	Herbivore	Gel
Basommatophora (O)	*Siphonaria zelandica*	6	Full sun	Herbivore	Gel
PHYLUM ANNELIDA: CLASS POLYCHEATA					
Unknown order	Unknown polychaete 1	3	Shade	Unknown	Gel
Unknown order	Unknown polychaete 2	4	Full sun	Unknown	Gel
PHYLUM CHORDATA: CLASS OSTEICTHYES					
Gobiesociformes (O)	*Aspasmogaster costatus*[5]	2	Shade	Unknown	Gel

[1] Species used in analysis of maturity

[2] Inviable egg masses of this species also collected

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

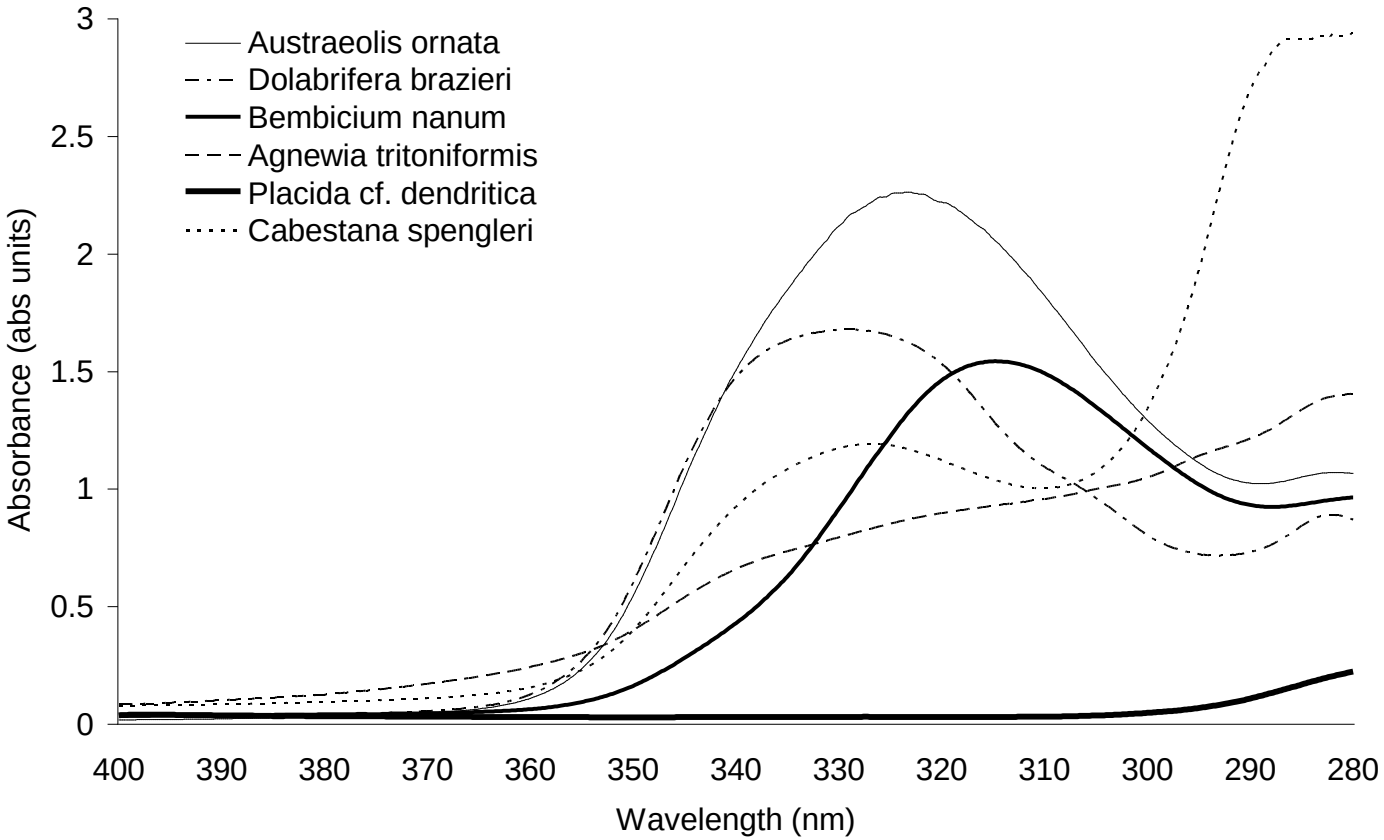
[4] This species is tentatively identified based on crawling juveniles that emerged from capsules

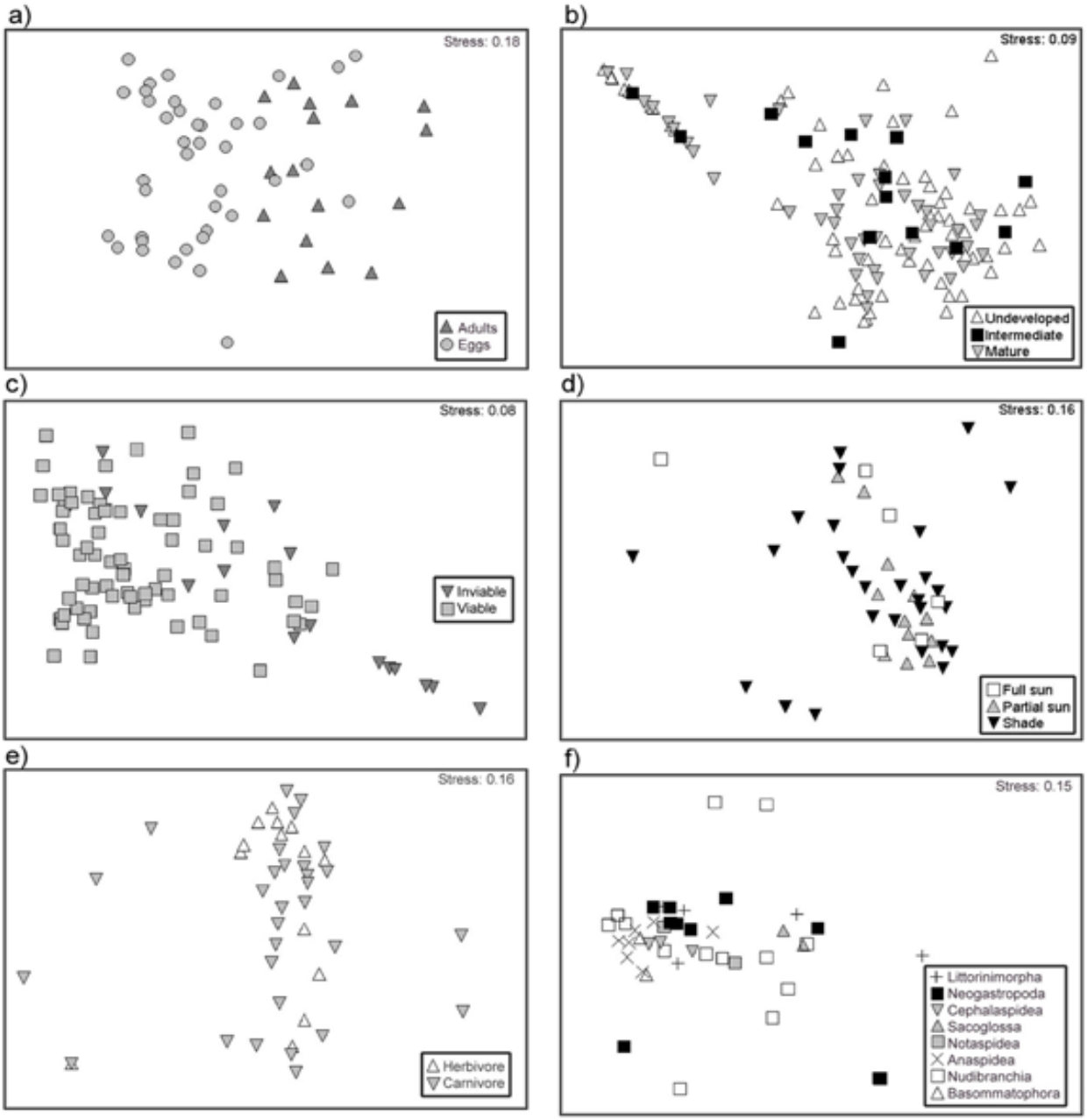
[5] Tentative identification based repeated sighting of adults on and near egg masses

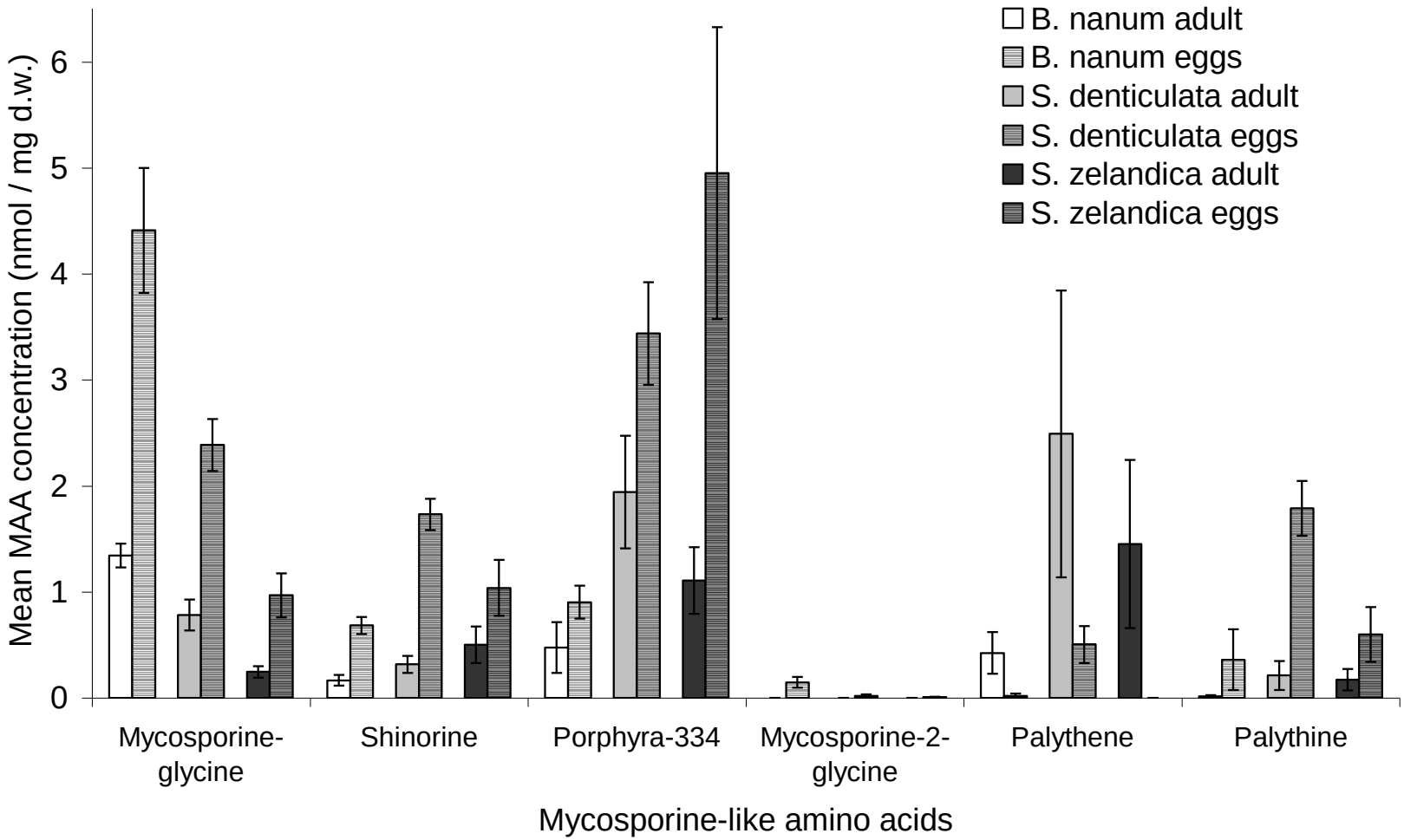
TABLE 2: MAA concentrations of viable egg masses used in this study (Mean ± SEM nmol / mg dry weight). See table 1 for sample sizes. * indicates an unknown peak was detected in at least one egg mass from this species

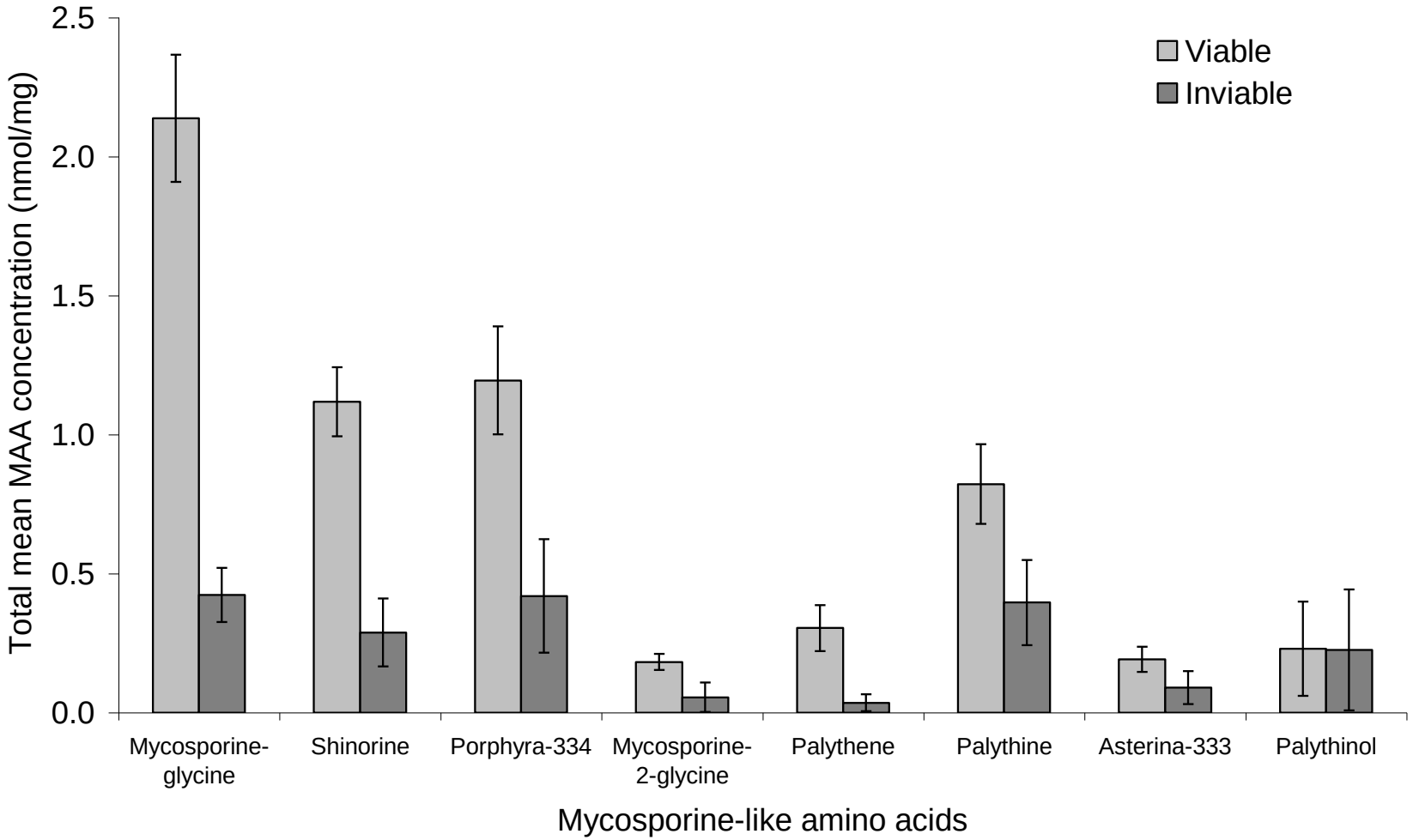
Species	Myc-gly	Shinorine	Porphyra	Myc-2-gly	Palythene	Palythine	Asterina	Palythinol	Total
PHYLUM MOLLUSCA: CLASS GASTROPODA									
SUPERORDER NERITOPSINA									
N. atramentosa	0.68 ± 0.02	0.21 ± 0.03	0.05 ± 0.05	0.01 ± 0.01	0.00	0.00	0.00	0.00	0.95 ± 0.14
SUPERORDER CAENOGASTROPODA									
B. nanum	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
C. 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
SUPERORDER HETEROBRANCHIA									
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

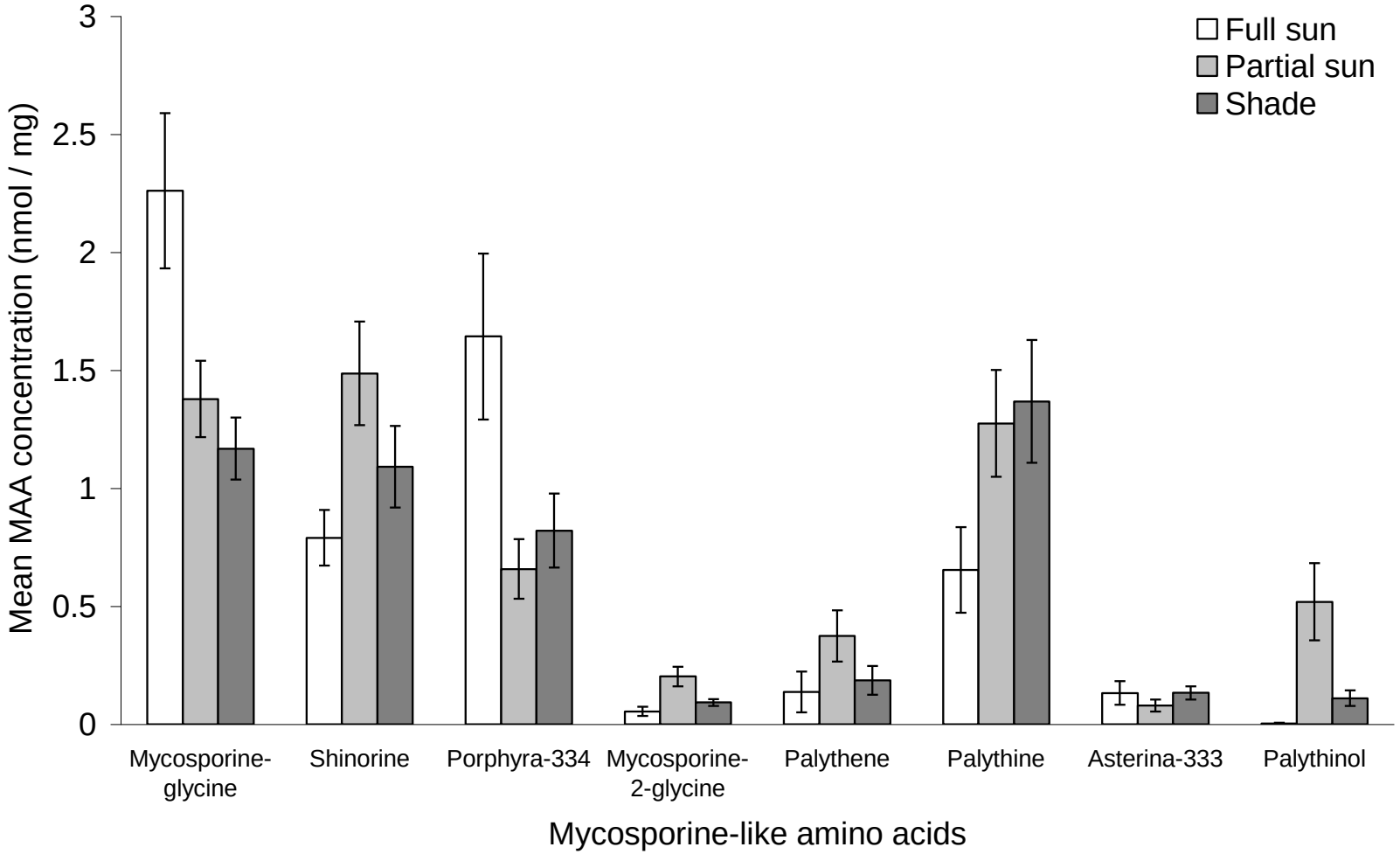
*H. nodulosa**	0.55 ± 0.35	0.38 ± 0.24	0.29 ± 0.20	0.03 ± 0.03	0.00	0.70 ± 0.42	0.00	0.11 ± 0.04	2.06 ± 2.51
*H. obscura**	0.54 ± 0.08	0.22 ± 0.08	0.10 ± 0.05	0.02 ± 0.01	0.00	0.53 ± 0.30	0.00	0.09 ± 0.05	1.50 ± 0.68
P. galbanus	3.07 ± 0.39	2.46 ± 0.92	0.96 ± 0.20	0.14 ± 0.04	0.00	6.08 ± 0.97	0.00	1.64 ± 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
PHYLUM ANNELIDA: CLASS POLYCHEATA									
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
PHYLUM CHORDATA: CLASS OSTEICTHYES									
A. costatus *	0.00	0.00	0.00	0.00	0.50 ± 0.03	0.00	0.00	0.00	0.50 ± 0.03

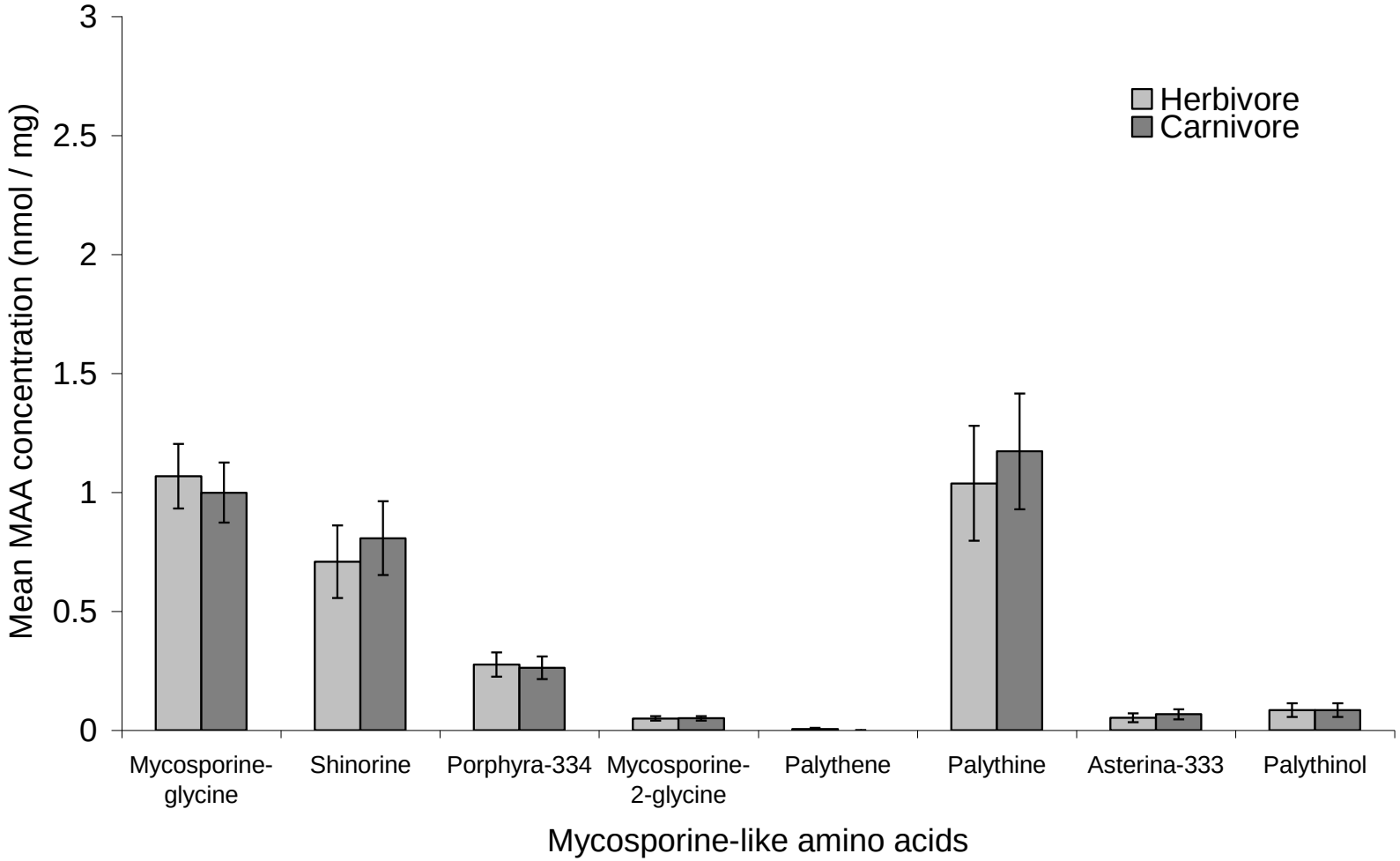

Austraeolis ornata
Dolabrifera brazieri
Bembicium nanum
Agnewia tritoniformis
Placida cf. dendritica
Cabestana spengleri
Absorbance (abs units)
3
2.5
2
1.5
1
0.5
0
400
390
380
370
360
350
340
330
320
310
300
290
280
Wavelength (nm)


a)
Stress: 0.18
Adults
Eggs
b)
Stress: 0.09
Undeveloped
Intermediate
Mature
c)
Stress: 0.08
Inviable
Viable
d)
Stress: 0.16
Full sun
Partial sun
Shade
e)
Stress: 0.16
Herbivore
Carnivore
f)
Stress: 0.15
Littorinimorpha
Neogastropoda
Cephalaspidea
Sacoglossa
Notaspidea
Anaspidea
Nudibranchia
Basommatophora


B. nanum adult
B. nanum eggs
S. denticulata adult
S. denticulata eggs
S. zelandica adult
S. zelandica eggs
Mean MAA concentration (nmol / mg d.w.)
0
1
2
3
4
5
6
Mycosporine-glycine
Shinorine
Porphyra-334
Mycosporine-2-glycine
Palythene
Palythine
Mycosporine-like amino acids


Total mean MAA concentration (nmol/mg)
2.5
2.0
1.5
1.0
0.5
0.0
Viable
Inviable
Mycosporine-glycine
Shinorine
Porphyra-334
Mycosporine-2-glycine
Palythene
Palythine
Asterina-333
Palythinol
Mycosporine-like amino acids


Full sun
Partial sun
Shade
Mean MAA concentration (nmol / mg)
3
2.5
2
1.5
1
0.5
0
Mycosporine-glycine
Shinorine
Porphyra-334
Mycosporine-2-glycine
Palythene
Palythine
Asterina-333
Palythinol
Mycosporine-like amino acids


Herbivore
Carnivore
Mean MAA concentration (nmol / mg)
3
2.5
2
1.5
1
0.5
0
Mycosporine-glycine
Shinorine
Porphyra-334
Mycosporine-2-glycine
Palythene
Palythine
Asterina-333
Palythinol
Mycosporine-like amino acids

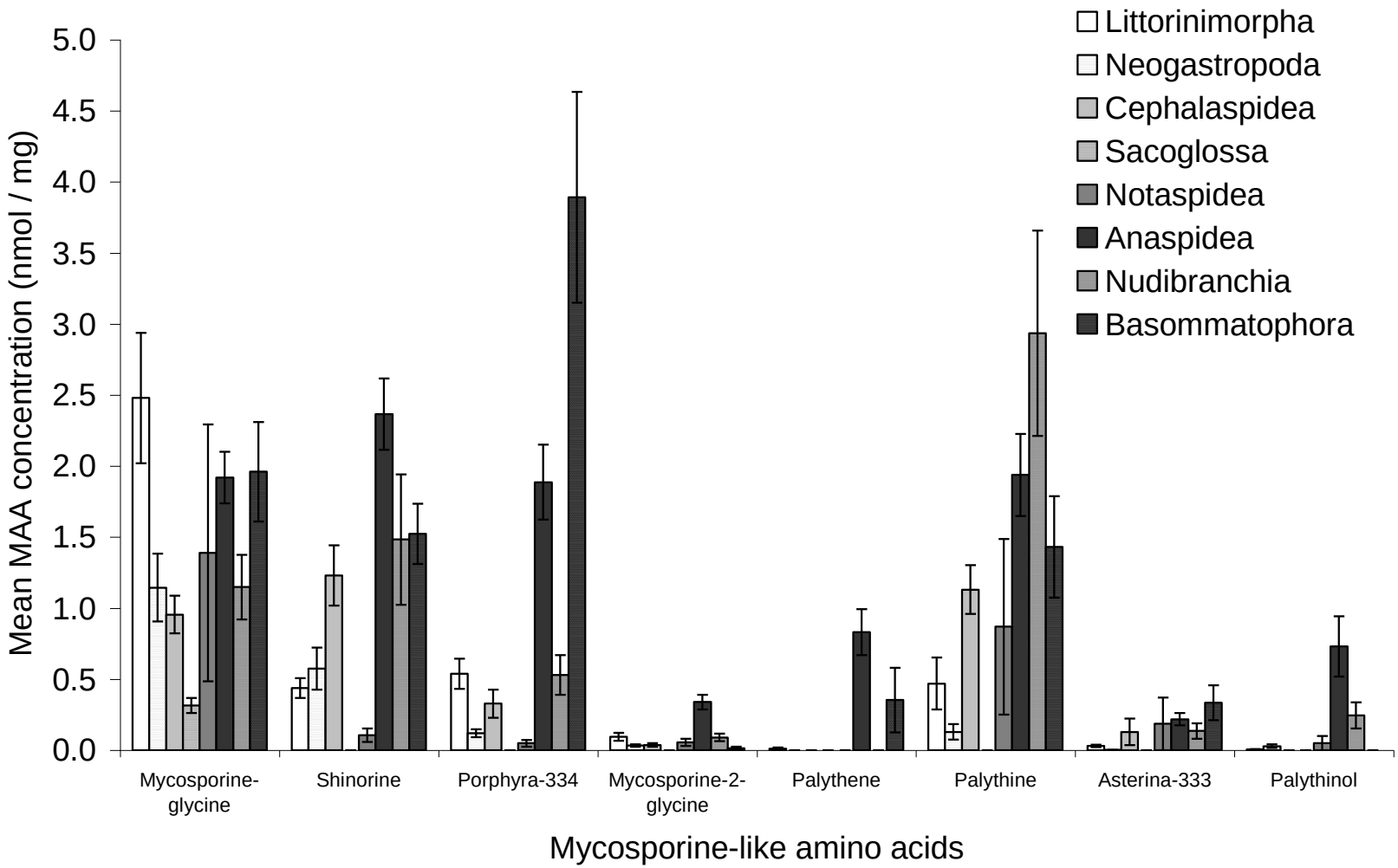

Littorinimorpha
Neogastropoda
Cephalaspidea
Sacoglossa
Notaspidea
Anaspidea
Nudibranchia
Basommatophora
Mean MAA concentration (nmol / mg)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Mycosporine-glycine
Shinorine
Porphyra-334
Mycosporine-2-glycine
Palythene
Palythine
Asterina-333
Palythinol
Mycosporine-like amino acids

FIGURE CAPTIONS:

FIG. 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.

FIG. 2: Differences in the composition and relative abundance of MAAs in the egg masses of selected species as revealed by nMDS plots constrasting: 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).

FIG. 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.

FIG. 4: MAA concentration in viable (n = 81) and inviable (n =22) egg masses of *Bembicium nanum, Cabestana spengleri, Mayena australis, Cominella eburnea, Conus papiilliferus, Dicathais orbita, Lepsiella reticularis, Nucella lapillus, Aplysia juliana, Bursatella leachii, Dolabella auricularia,* and *Dolabrifera brazieri.* Error bars are standard error of mean.

FIG. 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.

FIG. 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.

FIG. 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.