

# Mycosporine-like Amino Acid Content in Four Species of Sea Anemones in the Genus *Anthopleura* Reflects Phylogenetic but Not Environmental or Symbiotic Relationships

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**Abstract.** We examine the occurrence of UV-absorbing, mycosporine-like amino acids (MAAs) in four sympatric species of sea anemones in the genus *Anthopleura*, all collected from intertidal habitats on the Pacific Coast of temperate North America. We compare patterns of MAAs in *A. elegantissima* of several types: specimens having predominately zooxanthellae (dinoflagellates comprising at least two species) or zoochlorellae as symbionts; those containing algal endosymbionts of both kinds, and naturally occurring aposymbiotic specimens that lack the endosymbionts typically found in most specimens. We also compare MAAs in zooxanthellate specimens of *A. sola* and *A. xanthogrammica*, and specimens from the asymbiotic species *A. artemisia*. Our findings indicate that the complements of the four major MAAs in these species of *Anthopleura* (mycosporine-aurine, shinorine, porphyra-334, and mycosporine-2 glycine) broadly reflect phylogenetic differences among the anemones rather than the taxon of endosymbionts, presence or absence of symbionts, or environmental factors. An exception, however, occurs in *A. elegantissima*, where mycosporine-2 glycine increases in concentration with the density of zooxanthellae. Our evidence also shows that *A. elegantissima* can accumulate MAAs from its food, which may explain the occasional occurrence of minor MAAs in some individuals.

## Introduction

Mycosporine-like amino acids (MAAs) are small (244–374 Da), ultraviolet-absorbing ( $\lambda_{\max} = 309\text{--}360$  nm) molecules that occur in virtually all taxa of marine and freshwater cyanobacteria, algae, and metazoans (reviewed by Bandaranayake, 1998; Dunlap and Shick, 1998; Karentz, 2001; Shick and Dunlap, 2002). Structural diversity among the 20-plus known MAAs and their precursors results from substitutions of various amines, amino acids, and amino alcohols at two conjugated positions of the UV-absorbing chromophore of the MAA base structure (see aforementioned reviews). The often-observed correlation between the exposure of organisms to solar or artificial ultraviolet radiation (UVR) and their concentration of MAAs (see reviews), plus optical considerations (Garcia-Pichel, 1994), suggest a role as UV sunscreens, a contention that has been verified experimentally (Adams and Shick, 1996, 2001; Neale *et al.*, 1998).

As products of the early shikimic acid pathway, MAAs (Shick *et al.*, 1999) and the closely related fungal mycosporines (Favre-Bonvin *et al.*, 1987) are expected to be synthesized by algae, cyanobacteria, fungi, and perhaps bacteria, but not by metazoans. This is because animals lack this biochemical pathway, as inferred from their inability to synthesize shikimate-derived aromatic amino acids (Kishore and Shah, 1988; Bentley, 1990; Haslam, 1993; Herrmann and Weaver, 1999), albeit apparently not from any demonstration that metazoans lack the enzymes of the shikimic acid pathway or the nucleotide sequences coding

Received 3 April 2002; accepted 7 October 2002.

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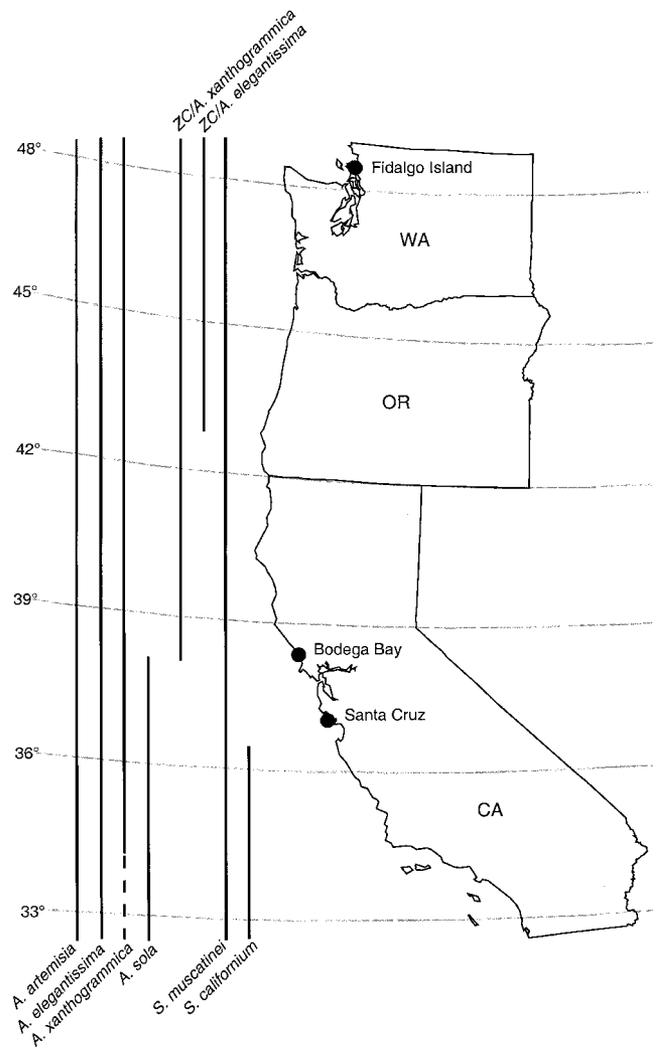
these enzymes (Shick and Dunlap, 2002). Therefore, the MAAs found in diverse marine microbial-invertebrate phototrophic symbioses were thought at first simply to originate in the algal or cyanobacterial partner (reviewed by Shick and Dunlap, 2002).

Carroll and Shick (1996) first confirmed experimentally the suggestion by Chalker *et al.* (1988) that non-symbiotic metazoans also containing MAAs obtain them from their diet. But the MAA complement in consumers does not always coincide with that in their diet, nor does the MAA complement in symbioses always match that produced by their microbial endosymbionts *in vitro*. Thus, MAAs in the diet may be modified by bacteria in the consumer's digestive tract, and MAAs synthesized by a symbiont could be modified after translocation to the host (see: Dunlap and Shick, 1998; Shick and Dunlap, 2002; and references therein).

Accounting for the MAAs found in a given organism or symbiotic consortium is therefore complicated and requires a detailed knowledge of the diet and the MAAs available therein; the MAAs that the symbionts can produce; and the capacity of the animal to absorb, retain, and modify dietary MAAs and those synthesized by its endosymbionts. These factors may vary spatially (Adams *et al.*, 2001), temporally (including ontogenetically [Adams and Shick, 1996, 2001] and during growth [Sebens, 1981]), and genotypically (Banaszak *et al.*, 2000).

Sea anemones in the genus *Anthopleura* contain taxonomically diverse microalgal endosymbionts that vary with latitude (LaJeunesse and Trench, 2000; Secord and Augustine, 2000), and four species of anemones in this genus overlap in intertidal and shallow subtidal habitats along the Pacific Coast of temperate North America (Fig. 1). From this array, we have selected sympatric populations to test the effects of the foregoing factors (especially the role of exposure to solar radiation, endosymbionts, and diet) on the number, profile, and concentration of MAAs.

We recognized early on that symbiotic and aposymbiotic individuals of *A. elegantissima* present exceptions to the empirically derived paradigms that concentrations of MAAs are determined by exposure to UVR, and that endosymbiotic dinoflagellates (zooxanthellae, *Symbiodinium* spp.) are the source of the MAAs. Exposing both symbiotic and aposymbiotic anemones to natural or artificial UVR does not affect the concentrations or complement of MAAs (Scelfo, 1988; Stochaj *et al.*, 1994; Banaszak and Trench, 1995), nor does *S. californium* isolated from *A. elegantissima* produce MAAs when cultured under UVR (Banaszak and Trench, 1995). A broader study of the MAAs in other species in this genus would provide a comparative context in which to examine these exceptions and clarify the contribution of the several factors involved in the accumulation of the observed complements of MAAs.



**Figure 1.** Collection sites for the sea anemones used in this study, and the reported ranges of *Anthopleura* species, *Symbiodinium* species, and zoochlorellae (ZC) symbiotic with *A. elegantissima* and *A. xanthogrammica* in Washington, Oregon, and California. Compiled from Hand (1955), Francis (1979), LaJeunesse and Trench (2000), Pearse and Francis (2000), and Secord and Augustine (2000).

Populations of *A. elegantissima* and *A. xanthogrammica* north of Central California host two taxa of phototrophic endosymbionts: zoochlorellae, which are green *Chlorella*-like chlorophytes (Muscatine, 1971), and the dinoflagellate *S. muscatinei* (LaJeunesse and Trench, 2000). An individual anemone may contain exclusively one or the other type of alga, or a combination of both (Muscatine, 1971). The balance between these algae may depend on their differential sensitivity to temperature and sunlight (O'Brien, 1980; O'Brien and Wyttbach, 1980; Saunders and Muller-Parker, 1997; Secord and Augustine, 2000). Moreover, zoochlorellae translocate, to the host, photosynthetic products that differ qualitatively from zooxanthella photosynthate (Minnick and McCloskey, cited by Verde and McCloskey,

1996), and zooxanthellae contribute more reduced carbon to the host's nutrition than do zoochlorellae (Verde and McCloskey, 1996). Such physiological differences in the algae, and the great evolutionary distance between these algal phyla, might be expected to yield rather different MAA complements in individual sea anemones harboring one or the other endosymbiont—if indeed zoochlorellae (like some other chlorophytes: Karentz, 2001) and *S. muscatinei* can produce MAAs. In addition, populations of *A. elegantissima* in Central and Southern California host only zooxanthellae (Secord and Augustine, 2000), in this case both *S. muscatinei* and *S. californium* (LaJeunesse and Trench, 2000).

In Central California, *A. sola* and *A. xanthogrammica* are sympatric with *A. elegantissima*, often cohabiting the same sites, where all three species harbor only zooxanthellae (Pearse and Francis, 2000). The species of *Symbiodinium* that occur in *A. sola* and *A. xanthogrammica* have not been determined, so these anemones may not host algae capable of synthesizing MAAs.

The asymbiotic species *A. artemisia* rarely (or, more likely, never: see discussion in Pearse and Francis, 2000) contains endosymbiotic algae. Therefore, it should contain only MAAs obtained or modified from its diet. The diet of *A. artemisia* is probably similar to that of *A. elegantissima*, because these two species co-occur at the same sites in Central California and have a similar body size (which would enable them to capture similar prey). Thus, any differences in the MAAs in these two species might arise from secondary modification of dietary MAAs and not from the presence or absence of symbionts. This is suggested by the finding that symbiotic and aposymbiotic specimens of *A. elegantissima* contain an identical complement of MAAs (Stochaj *et al.*, 1994; Banaszak and Trench, 1995). Size-dependent dietary differences between zooxanthellate *A. elegantissima* and *A. xanthogrammica* (Sebens, 1981), and, presumably, between *A. elegantissima* and the larger *A. sola*, may affect the MAAs they ingest in their prey, and result in different MAA complements among the anemones independently of those derived from their endosymbionts. We present the results of a simple experiment to show that *A. elegantissima* can indeed absorb and retain MAAs from its food.

Finally, a phylogeny based on mitochondrial DNA sequences is available for many species in the genus *Anthopleura* (Geller and Walton, 2001). This provides the opportunity to examine the provenance and presumed adaptive role of MAAs in a phylogenetic context (Mangum and Hochachka, 1998). Moreover, MAAs might be useful characters to help resolve the phylogenetic trichotomy of *A. elegantissima*, *A. sola*, and *A. xanthogrammica*, the interrelationships of which have not yet been clarified by DNA sequencing.

## Materials and Methods

### Collection of sea anemones

Specimens of four species of *Anthopleura* were collected in the summers of 1993 and 1996 in the States of Washington and California. Except as noted, all sea anemones were shipped alive to Orono, Maine, immediately after collection. The anemones were blotted, weighed, minced with a razor blade, and either analyzed immediately for MAAs, or lyophilized. If the material was lyophilized, its dry weight ( $dW$ ) was first measured, and it was then stored at  $-79^{\circ}\text{C}$  prior to biochemical analysis.

Green, brown, and mixed specimens of *Anthopleura elegantissima* (for descriptions, see Saunders and Muller-Parker, 1997) were taken from a single rock at about +0.6 m (MLLW) tidal height at Anaco Beach, Fidalgo Island, Washington ( $48^{\circ}29'\text{N}$ ,  $122^{\circ}42'\text{W}$ ). We chose this location (Fig. 1) because the anemones there harbor both zooxanthellae and zoochlorellae (Saunders and Muller-Parker, 1997). Differences in their oral disc pigmentation indicated that most of the anemones belonged to different clones, and we used this criterion to select the specimens for analysis, precluding clonemates.

Sea anemones from California were collected in 1996 from Santa Cruz County, a location that is south of the southern limits of zoochlorellae in *A. xanthogrammica* (Bodega Bay area) and *A. elegantissima* (central Oregon) (Fig. 1). Specimens of *A. artemisia*, *A. elegantissima*, and *A. sola*, and large specimens of *A. xanthogrammica*, were taken from various shore levels at Opal Cliffs near the seaward end of 41<sup>st</sup> Avenue, Capitola ( $36^{\circ}58'\text{N}$ ,  $121^{\circ}57'\text{W}$ ); small individuals of *A. xanthogrammica* were collected at Scott Creek Beach ( $37^{\circ}02'\text{N}$ ,  $122^{\circ}13'\text{W}$ ). Specimens of *A. elegantissima* came from distinct clonal aggregations of individuals differing in oral disc pattern, and one individual per clone was analyzed. The small specimens of *A. xanthogrammica* were collected in May and held in outdoor tanks without feeding until being shipped to Orono with the other sea anemones collected in August. Aposymbiotic individuals of *A. elegantissima* that had been collected locally and maintained in darkness in the laboratory in flowing seawater at seasonal temperatures for at least a year were also included. They were fed previously frozen adult brine shrimps every week or 2 weeks.

### Determination of algal complement

Several tentacles were snipped from each sea anemone before it was analyzed for MAAs. The tentacles were placed in a drop of artificial seawater on a microscope slide and crushed with a cover slip; the slurry was transferred to a hemacytometer, diluted if necessary, and 500–1000 algal cells were counted. Zoochlorellae and zooxanthellae were readily distinguishable by size and color (Secord and Au-

gustine, 2000). Because the proportions of zoochlorellae and zooxanthellae may vary between the tentacles and column (G. Muller-Parker, Western Washington University, pers. comm.), specimens of *A. elegantissima* from Washington that contained 100% of either algal type in their tentacles were examined further. A section of column wall of such specimens was excised, crushed, and examined for its algal complement to confirm that only one algal taxon was present. As noted above, zoochlorellae do not occur in *Anthopleura* spp. from Santa Cruz. Therefore, to confirm the singular presence of zooxanthellae in specimens from this location, or the lack of algal endosymbionts in *A. artemisia* and aposymbiotic specimens of *A. elegantissima*, only the tentacles were examined.

#### Feeding experiment

The distinctive complement of MAAs in the ocular lenses of fishes (Dunlap *et al.*, 1989) suggested the use of such lenses as sources of unambiguous dietary markers for a feeding study. The lenses were excised from the lumpfish, *Cyclopterus lumpus*, from Mt. Desert Island, Maine, and their wet weight ( $wW$ ) was measured. Preliminary analyses by D.S. Mason (University of Maine) indicated the presence of an unknown MAA. Based on its high absorbance at 357 nm and its co-elution with an identically absorbing compound from the red macroalga *Palmaria palmata*, this unknown MAA was tentatively identified as usujirene (Sekikawa *et al.*, 1980; Adams *et al.*, 2001). Because no quantitative standard was available for the presumed usujirene, its concentrations were approximated by matching its extinction coefficient to that of its *trans* isomer palythene (Adams *et al.*, 2001). Because the *cis* isomer will have a lower molar extinction coefficient than the *trans* isomer, this method will slightly overestimate the concentration of usujirene. Preliminary determinations indicated that both lenses from a single fish have the same complement and concentrations of MAAs, so that the amount of MAA available to an anemone ingesting one lens could be estimated from the MAA content of the contralateral lens from the same fish. One lens from each of two fish was frozen at  $-79^{\circ}\text{C}$  for later analysis of MAAs, and the other lens was fed to one of two clonemates of *A. elegantissima*, which were collected from the south jetty at the entrance to Bodega Bay, California ( $38^{\circ}19'\text{N}$ ,  $123^{\circ}02'\text{W}$ ) and held in the laboratory at Orono for 2 weeks at  $15^{\circ}\text{C}$ , without feeding, prior to the experiment. One anemone was frozen and lyophilized 4 days after feeding, and the second 8 days after feeding. The MAA concentrations in the fed anemones were compared with those in three additional clonemates (controls) that were not fed lenses.

#### Analyses of MAAs

Fresh, minced sea anemones or frozen lumpfish lenses were extracted in a refrigerator for 1 h in each of three changes of 100% HPLC-grade methanol; the material was sonicated for 30 s during the first extraction. Lyophilized tissues were similarly extracted, but in 80% aqueous methanol. To remove lipids and some lipophilic pigments, extracts of sea anemones were passed through a C18 Sep Pak Plus (Waters Corp., Milford, MA) before being injected onto the HPLC column.

Mycosporine-like amino acids were analyzed using the HPLC method described by Stochaj *et al.* (1994) and using quantitative standards prepared by W.C. Dunlap. The present analyses were carried out on a 250 mm  $\times$  4.6 mm Phenosphere 5- $\mu\text{m}$  C8 column (Phenomenex, Torrance, CA). The mobile phase for methanolic extracts of sea anemones consisted of 55% aqueous methanol with 0.1% acetic acid, this concentration of methanol providing the best separation of mycosporine-2 glycine from porphyra-334 (Stochaj *et al.*, 1994). MAAs from extracts of lumpfish lenses, and of anemones consuming them, were also separated using a mobile phase of 25% aqueous methanol and 0.1% acetic acid to resolve the less polar MAAs. Detection was at 334 and 310 nm for all specimens, and also at 357 nm for lenses and anemones used in the feeding experiment.

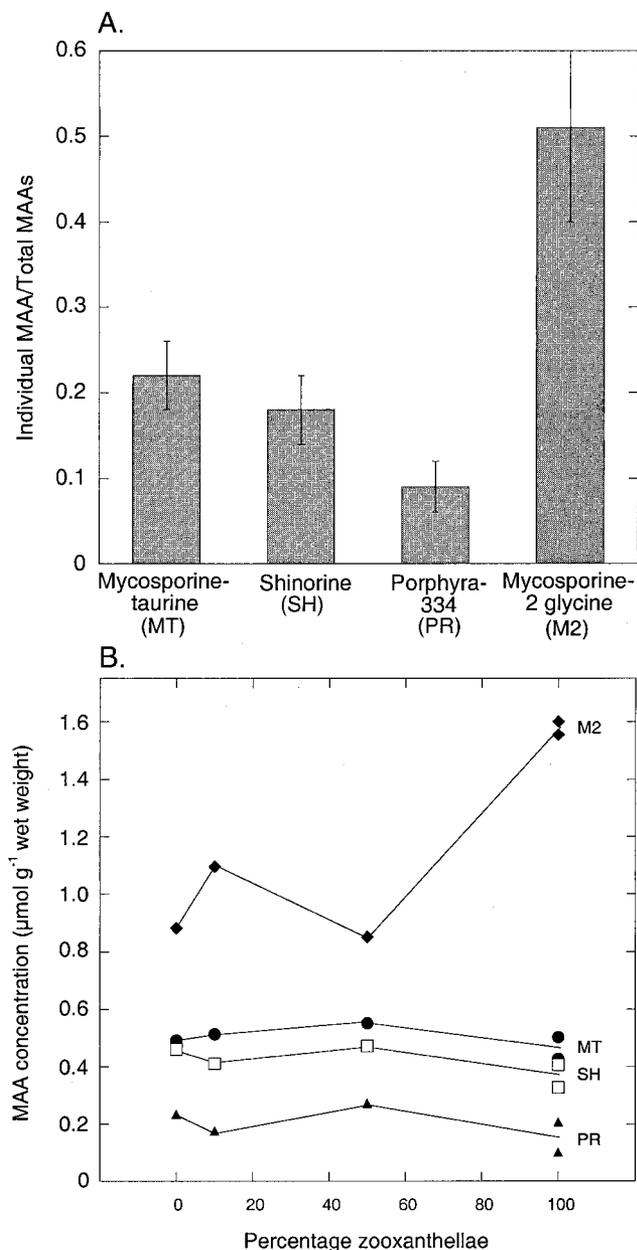
#### Taxonomic affinities of MAAs

Relationships among the four sympatric species of *Anthopleura* from Santa Cruz based on their MAA complements were examined by cluster analysis. Only the four MAAs that occurred in every specimen of all species studied were used in this analysis. Each of the four MAAs common to all species was expressed, for each specimen, as a molar fraction of the total MAA pool, and a matrix of distances was calculated using the average Euclidean distance (Sneath and Sokal, 1973). Distances were clustered using the unweighted pair group method algorithm (UP-GMA), and the phenogram was constructed with *Statistica*, release 5.1 (StatSoft, Inc., Tulsa, OK).

## Results

#### MAA and algal complements in *A. elegantissima*

In the 1993 collection of *A. elegantissima* from Washington, five MAAs were present in quantifiable amounts: mycosporine-aurine, shinorine, porphyra-334, mycosporine-2 glycine, and palythene, with mycosporine-2 glycine being the most concentrated. When the data for the four predominant MAAs were pooled for all specimens regardless of their algal complement, mycosporine-2 glycine accounted for half of the total concentration of MAAs (Fig. 2A). The highly variable level of palythene was always less than 10% of that of the next-most-concentrated MAA and is



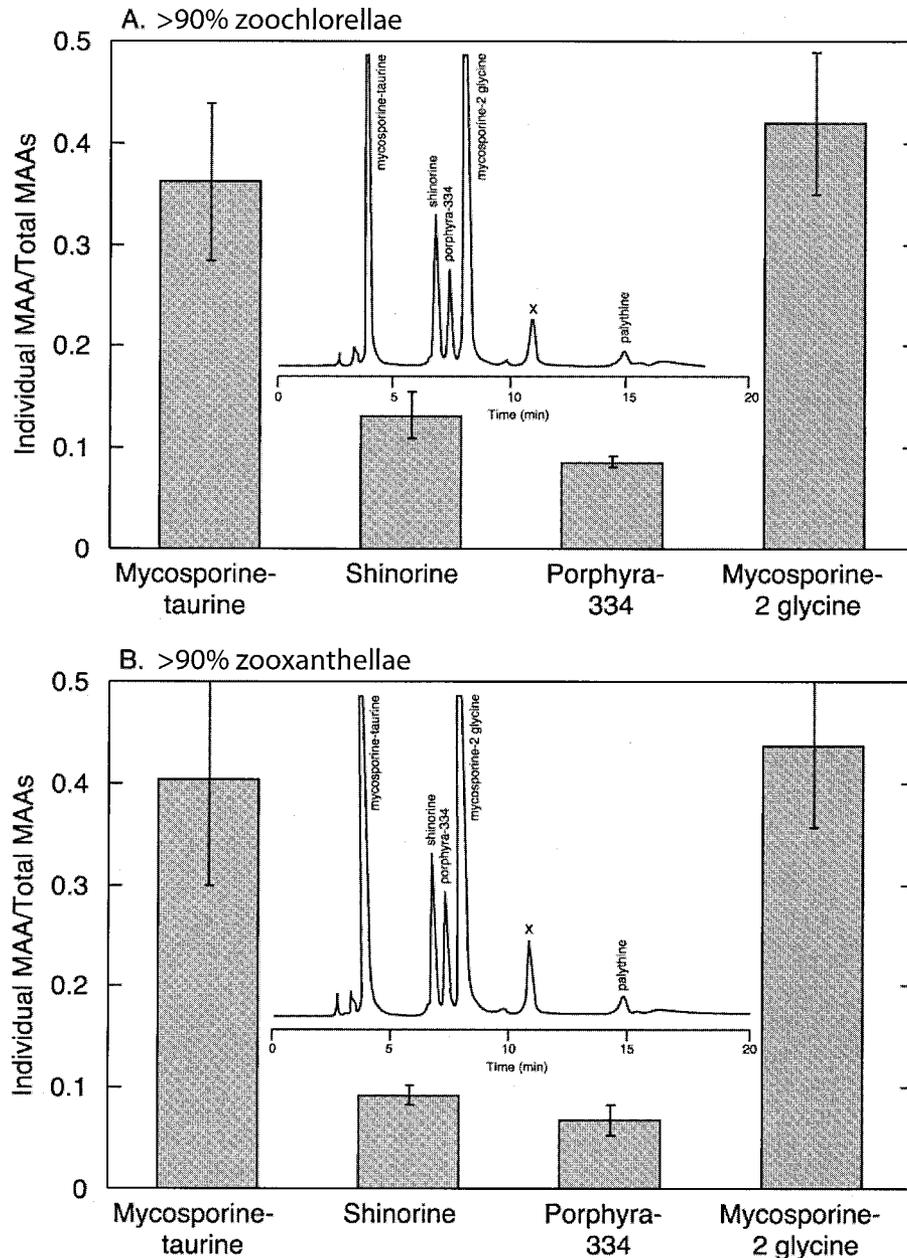
**Figure 2.** Myscosporine-like amino acids (MAAs) in specimens of *Anthopleura elegantissima* from Fidalgo Island, Washington, collected in 1993. (A) Proportion (molar fraction) of the total concentration of the four major MAAs represented by each. Data for 5 specimens were pooled regardless of their algal complement. Vertical bars represent  $\pm 1$  standard deviation (SD). (B) Concentration of individual MAAs in relation to the algal complement represented by zooxanthellae (percentage of total number of algae, zoochlorellae plus zooxanthellae). M2 = mycosporine-2 glycine; MT = mycosporine-taurine; SH = shinorine; PR = porphyra-334. Algal complement does not affect the relative concentrations of mycosporine-taurine, shinorine, or porphyra-334; the concentration of mycosporine-2 glycine is positively related to the proportion of zooxanthellae ( $r^2 = 0.816$ ,  $P = 0.036$ ,  $n = 5$ ). Assuming a fresh tissue water content of 80% (Shick, 1991), MAA concentrations shown in (B) can be multiplied by 5 to convert to dry weight for comparison with other data on MAA concentrations presented on that basis.

not shown here. There was no obvious trend in the concentration of individual MAAs in anemones having mixed algal complements up to about 50% zooxanthellae: 50% zoochlorellae. However, the concentration of mycosporine-2 glycine in anemones harboring zooxanthellae exclusively was substantially greater than in anemones having mixed symbionts (Fig. 2B). There was a significant, positive relation between the concentration of this MAA and the arcsine-transformed proportion of zooxanthellae in the anemones ( $r^2 = 0.816$ ,  $P = 0.036$ ,  $n = 5$ ). No other MAA concentration showed any significant relationship to the proportion of zooxanthellae ( $P > 0.05$  in all cases).

In the 1996 collection from the same site, the sea anemones had a more balanced distribution of mycosporine-taurine and mycosporine-2 glycine, and intermediate, similar proportions of shinorine and porphyra-334, regardless of whether they harbored >90% zoochlorellae or >90% zooxanthellae (Fig. 3A and 3B). Of these four MAAs, Student's *t* tests on arcsine-transformed data indicated that the molar proportions of three of them did not differ significantly between the predominantly zoochlorellate and predominantly zooxanthellate specimens; only shinorine showed a difference, its proportional concentration being marginally greater in the zoochlorellate anemones ( $t = 2.852$ ,  $d.f. = 4$ ,  $P = 0.046$ ).

#### Complements and concentrations of MAAs in sympatric species of *Anthopleura*

Microscopic examination of the sea anemones from Santa Cruz confirmed that *A. artemisia* and aposymbiotic specimens of *A. elegantissima* lacked algal endosymbionts, and that *A. elegantissima*, *A. sola*, and *A. xanthogrammica* harbored only zooxanthellae. Collectively, these four species (including zooxanthellate and aposymbiotic individuals of *A. elegantissima* and large and small specimens of *A. xanthogrammica*) contained seven MAAs in quantifiable amounts, but only four of these (mycosporine-taurine, shinorine, porphyra-334, and mycosporine-2 glycine) occurred in all individuals of all species (Fig. 4). Palythine and palythene occurred in some specimens of all species except for *A. artemisia*, which never contained these MAAs but uniquely contained mycosporine-glycine:valine. The total concentration of the four common MAAs (Fig. 5) varied interspecifically [ANOVA,  $F_{(5, 12)} = 11.923$ ,  $P < 0.001$ ] among the anemones from Santa Cruz. Both zoochlorellate and zooxanthellate specimens of *A. elegantissima* from Washington (1996 collection) had the same apparent concentration of these MAAs as their zooxanthellate conspecifics from Santa Cruz. (Data for the Washington specimens were included in Fig. 5 for heuristic reasons, but were excluded from the statistical analysis of the sympatric populations from California. Zooxanthellate and zoochlorellate specimens from Washington did not differ in their total

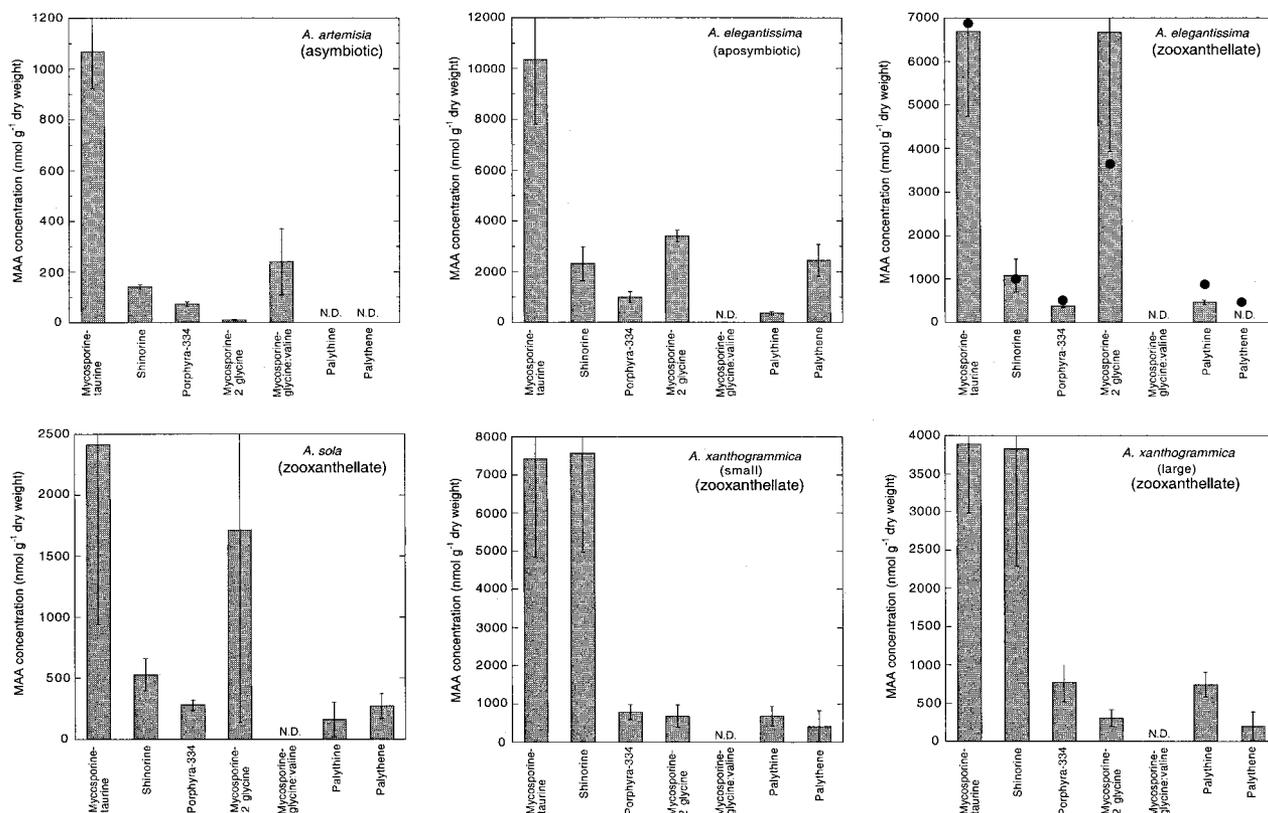


**Figure 3.** Proportions (molar fraction) of the major mycosporine-like amino acids (MAAs) in specimens of *Anthopleura elegantissima* collected at Fidalgo Island, Washington, in 1996. (A) Anemones in which the algal complement is >90% zoochlorellae. (B) Anemones in which the algal complement is >90% zooxanthellae. Only the proportion of shinorine differs significantly between predominantly zoochlorellate and predominantly zooxanthellate anemones ( $t = 2.852$ ,  $d.f. = 4$ ,  $P = 0.046$ ). Vertical bars represent  $\pm 1$  standard deviation (SD), where  $n = 3$  anemones in each group. Insets are HPLC chromatograms of the MAAs in a specimen that is 95% zoochlorellate (A) and in an entirely zooxanthellate individual (B); the peak indicated by an "X" is not an MAA.

concentration of MAAs [ $t$  test,  $P > 0.05$ ].) Student-Newmann-Keuls multiple comparison tests revealed the following relationships in the anemones from Santa Cruz. The asymbiotic species, *A. artemisia*, had the lowest absolute concentration of MAAs, overlapping with that in *A. sola*,

which in turn overlapped with that in large specimens of *A. xanthogrammica*. The highest concentration occurred in *A. elegantissima* (whether zooxanthellate or aposymbiotic) and small specimens of *A. xanthogrammica*.

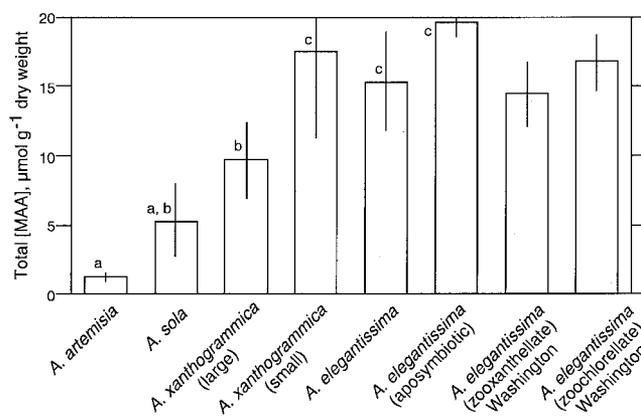
To facilitate interspecific comparisons, the molar propor-



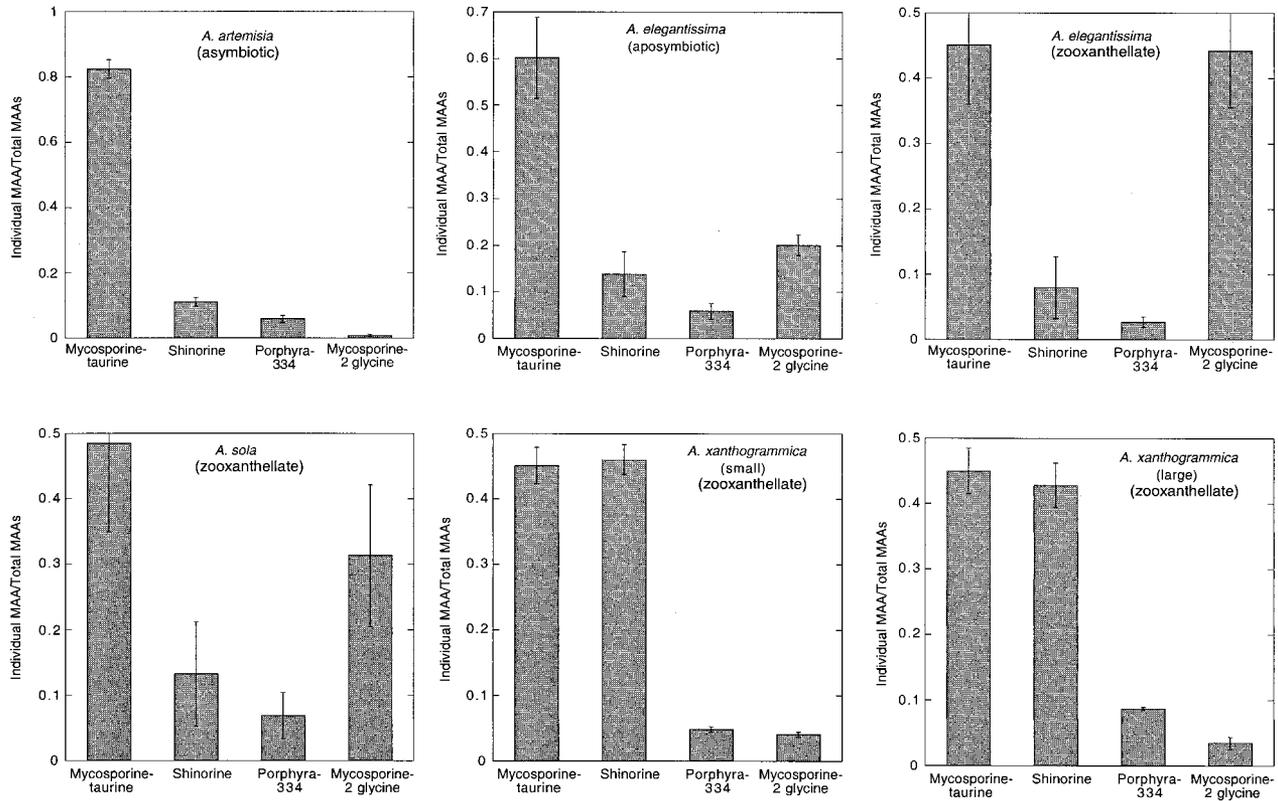
**Figure 4.** Concentrations of all quantifiable mycosporine-like amino acids (MAAs) in four species of *Anthopleura* collected in 1996 in Santa Cruz County, California, including aposymbiotic, laboratory-maintained specimens of *A. elegantissima*;  $n = 3$  for each species or group. Solid points (●) represent MAA concentrations in a partially buried, bleached specimen of *A. elegantissima* collected from one of the same three clones from which the zooxanthellate specimens were taken. Vertical bars represent  $\pm 1$  standard deviation (SD).

tions of the four common MAAs in the Santa Cruz specimens are shown in Figure 6, where several patterns are evident. *A. elegantissima* presented the same relative distribution of MAAs as seen in the 1996 collection in Washington: approximately equal proportions of mycosporine-taurine and mycosporine-2 glycine, and intermediate proportions ( $\approx 0.05$ – $0.15$  molar fraction) of shinorine and porphyrin-334. *A. xanthogrammica* was distinctly different, with mycosporine-taurine and shinorine each representing about 0.45 of the total MAAs. *A. artemisia* differed from both of the foregoing species in that mycosporine-taurine accounted for 0.8 of the total MAAs, and mycosporine-2 glycine was scarcely present. Aposymbiotic individuals of *A. elegantissima* showed an intermediate situation, having a proportion of mycosporine-2 glycine that was smaller than in zooxanthellate conspecifics but larger than in *A. artemisia* or *A. xanthogrammica*. *A. sola* evinced a proportion of mycosporine-2 glycine intermediate to that in *A. elegantissima* and the other species in the genus, amid much variation among individuals.

The phenogram (Fig. 7) based on the data in Figure 6 displays discrete clusters consistent with the foregoing qual-



**Figure 5.** Total concentration of the four major mycosporine-like amino acids (MAAs) in *Anthopleura* spp. from Santa Cruz County, California, and in zoochlorellate and zooxanthellate specimens from Fidalgo Island, Washington;  $n = 3$  for each group of California anemones, and  $n = 5$  for both groups of Washington anemones (1996 data). Vertical bars represent  $\pm 1$  standard deviation (SD). Letters above each bar for the Santa Cruz anemones give the results of Student-Newmann-Keuls multiple comparison tests; groups having different letters differ significantly at  $P = 0.05$ . Washington anemones were not included in the ANOVA for the California populations of anemones, which were sympatric.



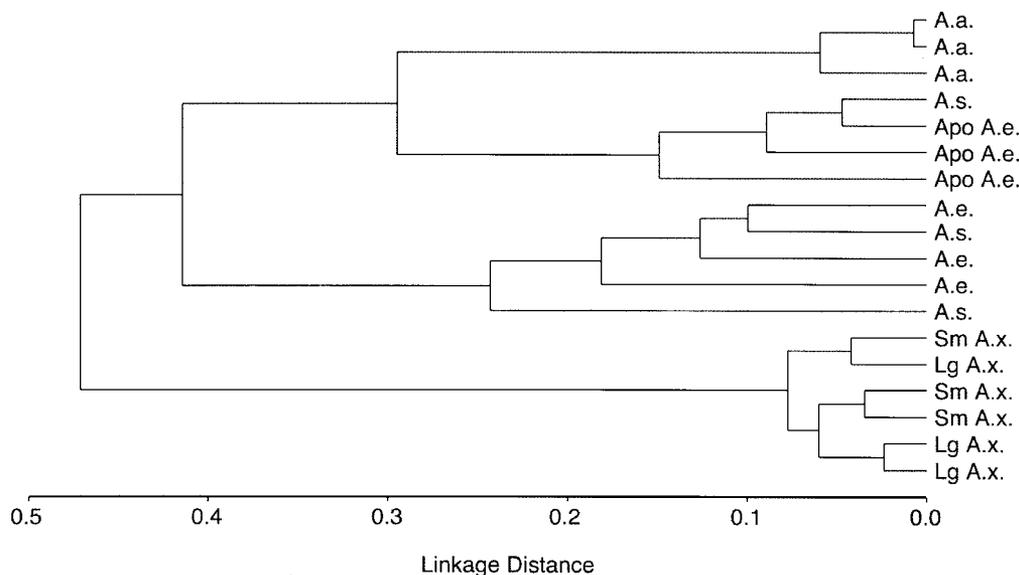
**Figure 6.** Proportional concentrations (molar fractions) of the four principal mycosporine-like amino acids (MAAs) common to all *Anthopleura* species from Santa Cruz County, California;  $n = 3$  for each group. Vertical bars represent  $\pm 1$  standard deviation (SD). These data were used to construct the phenogram shown in Figure 7.

itative description. Specimens of both *A. artemisia* and *A. xanthogrammica* formed distinct clades. *A. sola* was not clearly separated from *A. elegantissima*. Aposymbiotic individuals of *A. elegantissima* presented a separate clade, and the occurrence of one specimen of *A. sola* in this clade was owing to its exceptionally low concentration of mycosporine-2 glycine (less than half the mean value for the group), yielding an MAA profile distinctly like that of aposymbiotic specimens of *A. elegantissima*. Large and small specimens of *A. xanthogrammica* were not separated by their molar fractions of MAAs used to construct the phenogram, but were separable by their absolute concentrations of MAAs (Figs. 4 and 5).

#### Feeding experiment

Specimens of *A. elegantissima* readily ingested and retained the ocular lenses of *C. lumpus*. The fed anemones produced no visible fecal pellets or egesta, perhaps because the proteinaceous lenses (average  $w_w = 23$  mg, which was  $\approx 1\%$  of the anemones' average fresh weight) were completely absorbed (a 1% ration of brine shrimps would be at least 90% absorbed: Zamer, 1986). These lenses contained principally palythene and usujirene, with a smaller amount

of palythine and a trace of asterina-330 (not quantified). Unfed specimens of *A. elegantissima* from Bodega Bay contained the usual complement of MAAs seen in this species from the other locations, including palythene, and, exceptionally, usujirene, but no palythene. Clonemates of *A. elegantissima* given lumpfish lenses to eat did contain palythene 4 and 8 days after feeding (Fig. 8). Perhaps because of the relatively high endogenous concentrations of palythene in control anemones compared with the small amount available in a lens (Fig. 8A, B, top panel), the level of this MAA did not change significantly in fed anemones. The concentration of usujirene rose significantly by 4 days after feeding on a lens rich in this MAA, and declined to control levels by 8 days after a single meal of lumpfish lens. In those cases where ingesting a lumpfish lens led to a significant increase in the concentration of a particular MAA, the total change in that MAA was compared with the amount of that MAA available in the meal (Fig. 8B). For palythene, anemones eating a lens still contained 88% of this compound initially available in the lens (estimated from the MAA content of the second lens from the same fish) at 4 days, and 33% at 8 days after the meal. Perhaps because of the suboptimal chromatographic resolution of usujirene



**Figure 7.** Similarity phenogram for three individual specimens of each *Anthopleura* species collected in Santa Cruz County, California, based on the proportional concentrations (molar fraction) of the four mycosporine-like amino acids (MAAs) shown in Figure 6. Similarity was determined by cluster analysis and is expressed as linked Euclidean distance. *A. a.* = *A. artemisia*; *A. e.* = *A. elegantissima*; Apo *A. e.* = aposymbiotic *A. elegantissima*; *A. s.* = *A. sola*; Lg *A. x.* = large *A. xanthogrammica*; Sm *A. x.* = small *A. xanthogrammica*.

in the anemone extracts, the significant elevation of this MAA at 4 days after feeding was apparently 20% greater than the amount available in the meal.

### Discussion

Our data show clearly, and for the first time, that the complement of MAAs in the four North American species of *Anthopleura* broadly reflects the taxonomic and phylogenetic differences among these species. This conclusion is supported by our other findings: that the profiles and concentrations of the major MAAs are independent of the presence or absence of algal endosymbionts, the taxon of the symbionts that are present, and of several environmental factors. Nevertheless, our evidence does suggest that *A. elegantissima* can accumulate MAAs from its food, which may explain the temporal and local variations seen in the MAA profile, and the occurrence of minor MAAs in some individuals. These findings and others (Shick and Dunlap, 2002) lead us, finally, to consider the possibility that MAAs are synthesized *de novo* by the sea anemones.

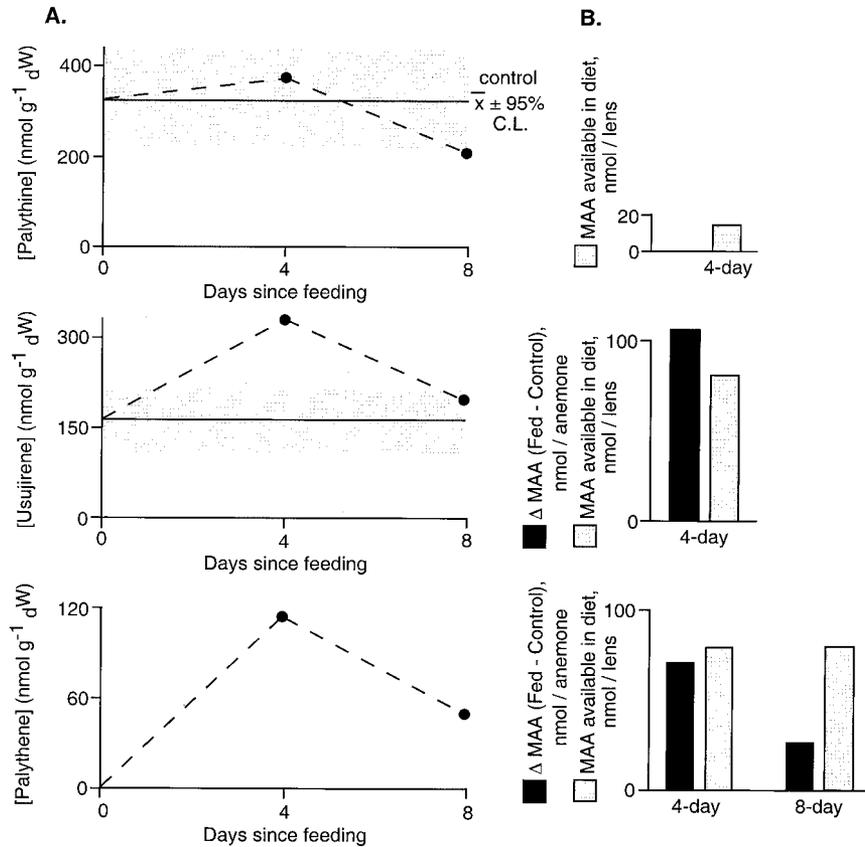
#### *Phylogeny of Anthopleura spp. as deduced from their complements of MAAs*

In the phenogram (Fig. 7) based on the proportional complements of the four major MAAs in *Anthopleura* spp. from California, specimens of *A. artemisia* and *A. xanthogrammica* form distinct clades. However, unlike the phylogenetic tree of Geller and Walton (2001) based on mito-

chondrial DNA sequences, in which the three species having algal endosymbionts form an unresolved trichotomy, the MAA complements indicate that *A. elegantissima* and *A. sola* are more closely related to each other than either is to *A. xanthogrammica*.

*A. elegantissima* and *A. sola* are difficult to distinguish morphologically (Francis, 1979), and Smith and Potts (1987) were unable to distinguish them by allozymic analysis. The reproductive isolation of the two species was confirmed only by a more thorough genetic study using allozymes (McFadden *et al.*, 1997), and by the analysis of Geller and Walton (2001), which was able to separate them by fixed differences in base pairs. Our finding that *A. elegantissima* and *A. sola* cluster together, distinct from *A. xanthogrammica*, supports the view of Pearse and Francis (2000) that they are sibling species and the view of McFadden *et al.* (1997) that the speciation was probably relatively recent.

Although it is gratifying, the finding that MAAs may contribute to phylogenetic understanding renders problematic any interpretations of the environmental factors affecting the MAA composition in a species of *Anthopleura*—that is, discriminating between the expression of this character as enforced by natural selection or as an ancestral legacy (Mangum and Hochachka, 1998). Until the biochemical steps involved in synthesizing and modifying individual MAAs *in vivo* have been elucidated and the factors controlling their expression have been determined, the issue will not be resolved.



**Figure 8.** (A) Concentrations of the major mycosporine-like amino acids (MAAs) from the lenses of lumpfish (*Cyclopterus lumpus*) present in two specimens of *Anthopleura elegantissima* 4 and 8 days after ingesting a lens. Shaded area represents 95% confidence interval for pre-feeding concentrations of MAAs based on analyses of three unfed clonemates (controls) of the experimental anemones. Palythene was not present in the control anemones. (B) Where significant, the increase in total MAA content ( $\Delta$ MAA, Fed minus Control) of an anemone eating a lens (solid bars) is compared with the amount of that MAA available in the contralateral lens (shaded bars) from the same fish.

#### Role of algal endosymbionts in determining the complements of MAAs in the anemones

The foregoing says nothing of the possible role of the algal endosymbionts—another layer of genotypic complexity to be considered—although the adoption of symbiosis itself is a trait that has been important in the evolution of the genus (Geller and Walton, 2001). Our data for the Washington specimens of *A. elegantissima* that harbored exclusively zooxanthellae or zoochlorellae (the extremes of the continuum in Fig. 2B) clearly demonstrate that the identity of the alga does not qualitatively affect the MAAs that are present in the consortium. This in turn strongly suggests that, in these anemones, the algal endosymbionts are not the source of the MAAs in the consortium, although the algae might provide a necessary precursor (such as deoxygadusol; Shick and Dunlap, 2002). That the algae do not determine the MAA complement is also indicated by our finding that the same proportional MAA complement occurs in anem-

ones harboring predominantly (>90%) one alga or the other (Fig. 3). It is conceivable that one type of alga produces MAAs and the other does not, so that the former, even if a minor proportion of the total algal complement, could produce the MAAs detected in the mixed symbiosis, but this would be inconsistent with our interpretation of the data for anemones harboring exclusively one alga or the other. Unfortunately, neither zoochlorellae nor *S. muscatinei* (the species of zooxanthella that occurs in Washington specimens of *A. elegantissima*: LaJeunesse and Trench, 2000) has been cultured successfully, so it is not known whether either can indeed produce MAAs. The placement of *S. muscatinei* in lineage B of the established phylotypes of *Symbiodinium* spp. (e.g., Rowan, 1998; LaJeunesse, 2001) suggests that it does not produce MAAs, because none of the other species in this clade do (Banaszak *et al.*, 2000; LaJeunesse, 2001).

Because MAAs that have accumulated in animal tissues

have a long residence time (Carroll and Shick, 1996; Adams and Shick, 1996, 2001; Newman *et al.*, 2000), it is also conceivable that the MAAs present in an anemone at a particular time are products not just of its population of symbionts at that time, but also of an earlier suite of symbionts. As has been shown in *A. elegantissima* (Saunders and Muller-Parker, 1997) and *A. xanthogrammica* (Bates, 2000), the symbiont population can change temporally, according to environmental factors. However, complete symbiont-switching has not been reported in *A. elegantissima*, and Weis and Levine (1996) found that naturally aposymbiotic specimens could not be experimentally infected with algae, and that anemones rendered aposymbiotic in the laboratory could be reinfected only with difficulty, and then only with the original algae. The most parsimonious explanation remains that zoochlorellae and *S. muscatinei* in Washington specimens of *A. elegantissima* are not the source of MAAs in those sea anemones.

In contrast to the foregoing conclusion, there are indications, at least in *A. elegantissima*, that the level of mycosporine-2 glycine in an anemone is associated with its density of zooxanthellae. Whereas the concentrations of MAAs did not differ among most Washington anemones having very different proportions of zoochlorellae and zooxanthellae, mycosporine-2 glycine was significantly elevated in individuals harboring *exclusively* zooxanthellae (Fig. 2B). Similarly, in the California anemones, aposymbiotic specimens of *A. elegantissima* had a lower proportion of mycosporine-2 glycine than zooxanthellate specimens (Fig. 6), which clearly separated the former from the latter, and, furthermore, placed the apozooxanthellate individuals nearer to the asymbiotic *A. artemisia* (Fig. 7), which scarcely contained any mycosporine-2 glycine. A lower level of mycosporine-2 glycine was also seen in a naturally bleached clonemate of one of the specimens of *A. elegantissima* from Santa Cruz (Fig. 4), as well as in naturally aposymbiotic individuals of *A. elegantissima* from Bodega Bay (Stochaj *et al.*, 1994) and Santa Barbara, California (Banaszak and Trench, 1995). The correspondence between the zooxanthellate condition and high levels of mycosporine-2 glycine was not absolute, however, because the levels of this MAA were relatively low in zooxanthellate specimens of *A. xanthogrammica*, and all specimens of all *Anthopleura* species contained some mycosporine-2 glycine (Fig. 2B and Fig. 4).

The situation is further complicated by the finding of Banaszak and Trench (1995) that *S. californium* isolated from *A. elegantissima* did not produce any MAAs in culture, nor indeed did these authors detect mycosporine-2 glycine in any species of zooxanthella cultured from diverse symbioses (Banaszak *et al.*, 2000). However, the mobile phase they used (25% aqueous, acidic methanol) would not have resolved mycosporine-2 glycine from porphyrin-334, even if it were present (Stochaj *et al.*, 1994); and we (J. M.

Shick and C. Ferrier-Pagès, unpubl. results) subsequently have found mycosporine-2 glycine in zooxanthellae cultured from the scleractinian coral *Galaxea fascicularis*.

The failure of *S. californium* to produce MAAs in culture is also strong evidence that zooxanthellae are not the source of the MAAs found in those symbiotic specimens of *A. elegantissima* from California that harbor this alga (Banaszak and Trench, 1995), nor perhaps in the sympatric *A. sola* and *A. xanthogrammica*, assuming that they also include *S. californium*. Because the anemones from Santa Cruz probably also harbor *S. muscatinei* (LaJeunesse and Trench, 2000), MAAs might originate with this species of zooxanthella; but as already mentioned, based on its phylogeny it is doubtful that *S. muscatinei* can produce MAAs. Consider also that zooxanthellae cultured from a symbiosis may not reflect the entire complement of algal genotypes present *in hospite* (Santos *et al.*, 2001); thus, although *S. californium* in culture does not produce MAAs (Banaszak *et al.*, 2000), the host anemones might contain other phylogenotypes of *Symbiodinium* that do. Zooxanthellae might also provide an essential precursor, such as deoxygadusol, that the anemones use to synthesize MAAs (Shick and Dunlap, 2002).

Even if the endosymbiotic algae are not the source of the MAAs in *Anthopleura* species, might their presence stimulate the accumulation of MAAs (from whatever source) in the host anemone? Aposymbiotic and zooxanthellate anemones differ qualitatively in their expression of proteins (Weis and Levine, 1996; Stochaj and Grossman, 1997) induced by the algae, and the presence of zooxanthellae enhances the synthesis of carbonic anhydrase by the host in *A. elegantissima* (Weis and Reynolds, 1999). Might the presence of algal endosymbionts also affect the expression of host enzymes involved in the biosynthesis or modification of MAAs (especially mycosporine-2 glycine)? The specific enzymes involved in the metabolism of MAAs have not been identified to test this possibility.

#### *Role of solar radiation in determining the concentrations of MAAs in Anthopleura spp.*

Are there environmental correlates that are consistent with a sunscreensing role of the compounds? Again, the evidence is equivocal, which is not surprising in light of previous experiments showing that varying the exposure of the anemones to UVR did not affect the concentrations of MAAs in zooxanthellate or aposymbiotic individuals of *A. elegantissima* (Scelfo, 1988; Stochaj *et al.*, 1994; Banaszak and Trench, 1995). Nevertheless, concentrations of MAAs are lowest in *A. artemisia* (Fig. 4), which typically inhabits sheltered microhabitats, such as deep holes in rocks and rocks buried in sand, so that only its oral disc is visible (Hand, 1955; Pearse and Francis, 2000). Might constitutively low concentrations of MAAs reflect the restriction of

this species to sheltered microhabitats? *A. sola* and large individuals of *A. xanthogrammica* exhibit the next-lowest concentrations of MAAs. These are low intertidal species that extend well into the subtidal or occur in tidepools and thus experience lower solar irradiances than does the characteristically intertidal *A. elegantissima*, which typically is exposed to direct sunlight during aerial exposure (Pearse, 1974; Shick and Dykens, 1984), and which has the highest concentrations of MAAs. Although low by comparison with the concentrations of MAAs in tropical corals ( $\approx 150\text{--}1000$  nmol MAA  $\text{mg}^{-1}$  protein), those in *Anthopleura* species (Fig. 5:  $10\text{--}20$   $\mu\text{mol}$  MAA  $\text{g}^{-1}$  dry weight, equivalent to about  $15\text{--}30$  nmol MAA  $\text{mg}^{-1}$  protein) are the same as in temperate red macroalgae ( $\approx 10$   $\mu\text{mol}$  MAA  $\text{g}^{-1}$  dry weight; Karsten *et al.*, 1998).

Several uncertainties temper this seemingly clear environmental correlation. Anemones from Fidalgo Island have concentrations of MAAs as high as those in specimens of *A. elegantissima* at Santa Cruz (Fig. 5). This is true even though the Fidalgo Island anemones live at a latitude  $11^\circ$  higher, and thus have lower daily and seasonal exposures to solar UVR (Madronich, 1993) than the Santa Cruz specimens (although the coastal fog in California in summer, and the early-morning timing of summer low tides, may diminish the dose of UVR there). Concentrations of MAAs in small specimens of *A. xanthogrammica* are just as high as those in *A. elegantissima*; however, small specimens of *A. xanthogrammica* settle in mussel beds, where ours were collected, and move to the low intertidal only as they grow in size. Thus, their relatively high levels of MAAs are not unexpected. We do not know whether concentrations in the former specimens changed while they were held in shallow outdoor aquaria for 3 months before analysis, although the aforementioned experiments with *A. elegantissima* suggest that they would not. Also, the concentration of MAAs in large specimens of *A. xanthogrammica* may be low compared with smaller specimens, because large individuals may have disproportionately greater amounts of extracellular mesoglea (Shick, 1991) relative to the mass of cells in which the MAAs are located, leading to a low mass-specific concentration of MAAs. The same may apply to large specimens of *A. sola*, as compared with small specimens of *A. elegantissima*. Finally, concentrations of MAAs in aposymbiotic individuals of *A. elegantissima* held in darkness in the laboratory for more than 1 year are just as high as or higher than in zooxanthellate specimens, as are those in a naturally bleached clonemate of the latter (Fig. 4; also see Stochaj *et al.*, 1994; and Banaszak and Trench, 1995). Thus, proximal environmental effects as determinants of MAA concentrations seem less important than these four species' evolutionary histories, including broad interspecific differences in habitat.

#### *Other possible physiological functions of MAAs*

MAAs function as osmolytes in cyanobacteria (Oren, 1997; Portwich and Garcia-Pichel, 1999), but this role does not seem important in sea anemones, because in *A. elegantissima*, the concentration of MAAs is only 3% of that of free amino acids (FAAs), the principal organic intracellular osmotic effectors here (Shick, 1991; Shick and Dunlap, 2002).

Next, FAAs have been implicated as "host factors" that induce the release of photosynthate from the endosymbiotic algae (Gates *et al.*, 1995), and taurine in particular has been studied as such by Wang and Douglas (1997). Might mycosporine-taurine or other MAAs also have such a regulatory role in these symbioses, as proposed by Gates *et al.* (1995)? The concentrations of MAAs are lowest in the asymbiotic *A. artemisia* (Fig. 4 and Fig. 5) and are higher in the symbiotic species, and mycosporine-2 glycine varies with the proportion of zooxanthellae in *A. elegantissima* (Fig. 2B); these facts are consistent with a role of MAAs as a host factor. A high concentration of MAAs in aposymbiotic individuals of *A. elegantissima* does not necessarily argue against such a role because aposymbiosis is a secondary, environmentally imposed condition that would not *per se* affect the inherent capacity of the host to accumulate MAAs from exogenous sources. Nevertheless, it remains to be established whether mycosporine-taurine, mycosporine-2 glycine, or any other MAA at physiologically relevant concentrations effects the release of photosynthate by zooxanthellae or zoochlorellae.

Finally, because specimens of *A. elegantissima* harboring these different algae have identical complements of MAAs, these compounds are unlikely to account for the differential palatability of zoochlorellate and zooxanthellate specimens to a predatory fish (Augustine and Muller-Parker, 1998), and both types of anemones are readily eaten by a predatory nudibranch (Seavy and Muller-Parker, 2002). In short, a clear physiological (osmolyte or host factor) or defensive (sunscreening or feeding deterrent) role for MAAs that would explain their patterns of occurrence among species of *Anthopleura* remains to be elucidated.

#### *The role of dietary MAAs*

Our experiments unequivocally show that *A. elegantissima* can absorb MAAs from its food and retain them for at least 8 days (Fig. 8). Not only does this result support a possible trophic origin of MAAs in sea anemones, but it also may explain local and temporal differences in the complement of minor and trace MAAs (*cf.* Stochaj *et al.*, 1994, and Banaszak and Trench, 1995), including the unusual and surprising occurrence of usujirene in specimens of *A. elegantissima* from Bodega Bay used in our feeding experiment. The possibility that unpredictable variations in diet can affect the complement of MAAs in a population must be

borne in mind when interpreting qualitative field surveys of MAAs in diverse animal taxa, particularly if the data are intended to delineate broad phylogenetic patterns (Karentz, 2001). This seems particularly relevant in polyphagous opportunists such as sea anemones (Shick, 1991). Indeed, such stochastic variability in the minor MAAs (*e.g.*, we had never seen usujirene in diverse seasonal collections of *A. elegantissima* from Bodega Bay dating back to 1988) prompted us to use only the four MAAs that occur in all specimens of all species in constructing the phylogeny (although this obscured the qualitative differences between *A. artemisia* and the other three species: Fig. 4). Thus, like these compounds in cyanobacteria (Karsten and Garcia-Pichel, 1996), dinoflagellates (Carreto *et al.*, 2001), and cultured zooxanthellae (Banaszak *et al.*, 2000; LaJeunesse, 2001), the predominant MAAs in *Anthopleura* spp. do appear to be useful phylogenetic characters.

Determining the diet and ration in sea anemones is tedious work, but it has been done in *A. elegantissima* (Sebens, 1981; Zamer, 1986). To establish the relative importance of various food items in affecting the MAAs present in the anemones in nature would require long-term studies of the MAAs in their natural diet for comparison with their own time-averaged complement of MAAs. A single sample of the phytoplankton, zooplankton, and particulate detritus >25  $\mu\text{m}$  diameter in the water column above the population of *A. elegantissima* at Bodega Bay (J.M. Shick, unpubl. data) revealed the presence of most major and minor MAAs found in the anemones (including usujirene). The exceptions were mycosporine-glycine:valine and mycosporine-aurine, the latter being the predominant MAA that apparently is unique to anemones in this genus. Thus, mycosporine-aurine may be produced by the anemones themselves, perhaps by modifying other MAAs, as seems to be the case for certain MAAs and their precursors in corals (Shick *et al.*, 1999), holothuroid echinoderms (Dunlap and Shick, 1998), fishes (Mason *et al.*, 1998), and red macroalgae (Franklin *et al.*, 1999).

As we have noted elsewhere (Stochaj *et al.*, 1994), taurine is by far the most concentrated component of the free amino acid pool in *Anthopleura* species. Although the biosynthetic origin of mycosporine-aurine remains enigmatic, it may result from the modification of a pre-existing (dietary) MAA or deoxygadusol by the substitution of taurine in the chromophore conjugation. Thus, the anemones may be exploiting the ready availability of this amino acid to synthesize mycosporine-aurine, which not only absorbs the shortest, most damaging UV wavelengths (309 nm) of any MAA, but which moreover is a moderate antioxidant (Shick *et al.*, 1996; Shick and Dunlap, 2002). This is an important consideration for a phototrophic symbiosis, in which the metazoan host experiences hyperoxia (Dykens and Shick, 1982), because this hyperoxia, together with exposure of the

animal to solar UVR, leads to an enhanced flux of reactive oxygen species (Dykens *et al.*, 1992).

We deliberately collected the specimens from Santa Cruz in a habitat where all four species of *Anthopleura* are present in close proximity, to ensure that all of them potentially have access to the same prey. Although Sebens (1981) documented size-related differences between *A. elegantissima* and *A. xanthogrammica* in the prey that they capture, he also found considerable overlap between these species in their major food items. Therefore, if the MAA complements of these anemones are affected by their diets, their disparate MAA spectra are surprising, unless the heavier reliance of *A. xanthogrammica* on mussels (*Mytilus* spp.), which are rich in shinorine (Chioccare *et al.*, 1979, 1985; J. M. Shick, unpubl. data), enhances the concentration of this MAA in *A. xanthogrammica*.

*A. artemisia* consistently contained an MAA that we never saw in any of the other species—mycosporine-glycine:valine. This MAA is common in Antarctic phytoplankton and the krill that eat them (Karentz *et al.*, 1991; Dunlap *et al.*, 1995; Newman *et al.*, 2000); this substance has also been found in a red-tide dinoflagellate (Vernet and Whitehead, 1996) and in particulate organic matter (Whitehead and Vernet, 2000) in California coastal waters. Thus, mycosporine-glycine:valine probably is present in the plankton available to all of these *Anthopleura* species (although it was not in the assemblage of particulate material sampled at Bodega Bay), and its restriction to *A. artemisia* underscores the biochemical distinction of this species from its sympatric congeners. This finding of a unique MAA, together with the absence of palythine and palythene from this species alone of the four (Fig. 4), reinforces the phylogenetic conclusion of Geller and Walton (2001) that *A. artemisia* is not closely related to the other three species, but belongs to a western Pacific clade of the genus *Anthopleura*.

Diet does not explain the MAA complement in aposymbiotic specimens of *A. elegantissima* maintained in the laboratory. Stochaj *et al.* (1994) suggested a dietary source of MAAs in laboratory-maintained aposymbiotic specimens, but the UV-absorbing compounds in the squid fed to the anemones in that study subsequently proved not to be MAAs, and the MAA complement after month-long feeding on this diet was the same as in freshly collected specimens (J.M. Shick, unpubl. data). Aposymbiotic individuals maintained in the laboratory in Santa Cruz for more than 1 year and occasionally fed previously frozen adult brine shrimps (*Artemia* sp.) had the same MAA complement, although in different proportions, as zooxanthellate individuals fresh from the field (Fig. 6). Those brine shrimps were not analyzed for MAAs, but results for other samples of *Artemia* indicate that they probably contained principally shinorine, along with some asterina-330 and porphyra-334 (Grant *et al.*, 1985), and possibly mycosporine-2 glycine (J.M. Shick, unpubl. data); *Artemia* tissues also contain gadusols (Grant

*et al.*, 1985), likely precursors of MAAs (Shick and Dunlap, 2002). The diet may provide the gadusols and certain MAAs that the sea anemones ultimately convert into the suites of MAAs that characterize the several species.

#### *De novo biosynthesis of MAAs by cnidarians?*

As we have noted here and elsewhere (Shick and Dunlap, 2002), the same major MAAs occur in aposymbiotic and zooxanthellate individuals of *A. elegantissima*, and controlled diets fail to modify this pattern; moreover, zooxanthellae freshly isolated from *A. elegantissima* lack MAAs, and cultured *S. californium* apparently cannot synthesize MAAs (Banaszak and Trench, 1995). These considerations all suggest that the anemones themselves make these compounds. The tentative finding that scleractinian corals can synthesize aromatic amino acids (Fitzgerald and Szmant, 1997) may also point to the unexpected presence of the shikimic acid pathway in these metazoans. But like Fitzgerald and Szmant (1997), we might equivocate, suggesting that like aromatic amino acids, the MAAs originate in bacteria associated with the anemones, although only one marine bacterium has ever been reported to produce MAAs (Arai *et al.*, 1992). Alternatively, the algal endosymbionts and the food might be sources of deoxygadusol, which the animal host might use to produce MAAs. Testing these possibilities, and looking more rigorously in cnidarians for, especially, the early steps of the shikimic acid pathway (including the postulated branchpoint leading to MAAs *via* deoxygadusol), might clarify the otherwise unexplained presence of certain MAAs in these metazoans.

In summary, the complements of the MAAs in four species of *Anthopleura* seem more closely associated with their phylogenetic position than with other factors. Several lines of evidence indicate that the algal endosymbionts do not contribute the MAAs seen in the symbiotic anemones, although in *A. elegantissima* the level of mycosporine-2 glycine does seem to be affected by the predominance of zooxanthellae. The successful culturing of zoochlorellae and of *Symbiodinium muscatinei* would facilitate the analysis of their MAA complements and do much to resolve the uncertainty about their contributions to the suite of MAAs in the symbiosis. Identifying the enzymes involved in the biosynthesis of primary MAAs, and in the secondary structural modification of these, remains a major unresolved problem. Its solution would allow us to study the phylogenetic distribution of the enzymes in concert with the occurrence of particular MAAs, and would also permit us to study the factors that affect the expression and regulation of these enzymes.

#### Acknowledgments

We thank Dr. Gisèle Muller-Parker for collecting the sea anemones in Washington, and Drs. James Clegg and Dennis

Hedgecock for facilities and facilitation at the Bodega Marine Laboratory. We are grateful to Donald Mason, who first identified the MAAs present in lumpfish lenses, where the rare presence of usujirene suggested their use as a source of this dietary marker, and to Amy Carroll, who assisted in the laboratory. We appreciate Dr. Irv Kornfield's advice regarding the cluster analysis and his comments on the manuscript, and Francis Wihbey's assistance with producing the map. We thank the editors and two anonymous reviewers for their heroic efforts. This research was supported by U.S. National Science Foundation grant IBN-9316426 to J.M. Shick and W.C. Dunlap, and in part by a grant from the National Geographic Society to J.M. Shick.

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