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Distribution of mycosporine-like amino acids in the sea hare *Aplysia dactylomela*: effect of diet on amounts and types sequestered over time in tissues and spawn

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Abstract

We investigated the interaction of diet and accumulation of UV-absorbing mycosporine-like amino acids (MAAs) in body tissues and spawn of the sea hare *Aplysia dactylomela* to determine if MAA accumulation reflects type and level of dietary intake. Food sources were the red algae *Acanthophora spicifera*, *Centroceras clavulatum*, and *Laurencia* sp., and the green alga, *Ulva lactuca*. Adults were maintained on these foods for 40 days, after which feces were collected and tissues separated by dissection. Field animals were similarly sampled at this time. All spawn from experimental and field animals was collected over the study period. Samples, including seaweed foods, were analysed for six MAAs. Overnight consumption experiments using a variety of common seaweeds and one seagrass from *A. dactylomela*'s habitat showed that the four seaweeds selected as foods were among those best-eaten by *Aplysia*. After 40 days levels of specific MAAs in the tissues of experimental animals showed excellent correlation with those in their diets, suggesting that the MAAs were dietarily-derived. Relative MAA contents in spawn from all diet groups correlated well with those in spawn from field animals. Commonest MAAs in spawn were porphyra-334, shinorine, and palythine, in this order. Concentrations of these MAAs were maintained at constant levels over time in spawn from all diet groups eating red algae and from field animals. Spawn from the *Ulva* dietary group showed an initial significant decline in MAA concentrations, but levels stabilized after the first 2 weeks. Skin was rich in porphyra-334 and shinorine, and levels of these in experimental animals correlated well with comparable levels in the skin of field animals. Digestive glands contained high levels of asterina-330, particularly those of the *Centroceras* dietary group, where concentrations reached a maximum of 21 mg· dry g^{-1}.

Keywords: MAA accumulation; Mycosporine-like amino acids; UV protection; Sea hare, *Aplysia dactylomela*

1. Introduction

Mycosporine-like amino acids (MAAs) strongly absorb UV radiation in the 310–360 nm range and are widely believed to act as sunscreens against biologically damaging ultraviolet B (UVB, 280–320 nm) and ultraviolet A (320–400 nm)

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wavelengths. Support for this notion comes from studies showing greater concentrations of MAAs in organisms in shallow ocean habitats and lower MAA levels in organisms inhabiting greater depths (Dunlap et al., 1986; Karentz et al., 1991, 1997; Gleason, 1993; Shick et al., 1995). Although these observations point strongly to a functional role of MAAs as sunscreens, direct evidence supporting this idea is often lacking or equivocal (e.g. Wood, 1989; Shick et al., 1991, 1995; Gleason, 1993; Dionisio-Sese et al., 1997; Dunlap and Shick, 1998; Carefoot et al., 1998). Exceptions are the observations of Garcia-Pichel and Castenholz (1993) and Garcia-Pichel et al. (1993) that intracellular MAA concentrations in several strains of cyanobacteria are directly related to intensity of UV irradiation received and this can be correlated with increased resistance to UV radiation. Adams and Shick (1996) also report that fertilized sea urchin eggs with more MAAs show less of a delay in cleavage when exposed to UV than do eggs with less MAAs and Neale et al. (1998) have demonstrated in marine phytoplankton that MAAs can protect against inhibition of photosynthesis by UV radiation.

Eighteen MAAs have been identified in marine organisms (for review see Karentz et al., 1991; Bandaranayake, 1996; Dunlap and Shick, 1998; Sinha et al., 1998). Direct evidence of a dietary origin of MAAs is known for sea urchins (Adams and Shick, 1996; Carroll and Shick, 1996) and fish (Mason et al., 1998). Alternative origins in animals as from from de novo synthesis or gut symbionts have been considered (Shick et al., 1992), but the necessary experiments to show these have not been done. Recently, Shick et al. (1999) have shown UV-induction of MAAs in the coral *Stylophora pistillata* and, by use of specific shikimic acid-pathway blockers, have identified this as the route for their synthesis (presumably in the algal symbionts of the coral). An increasingly evident feature of MAAs is the variability in their distribution both within and among different invertebrate taxa (Karentz et al., 1991, 1997; McClintock and Karentz, 1997; Banaszak et al., 1998). As a result, it has been virtually impossible to identify patterns in the distribution of types or amounts of MAAs as a guide to their possible function or phylogenetic affinities. To date, research has principally focused on the UV-screening function of MAAs. Consideration of alternative but related functions, such as an antioxidant role (Dunlap and Yamamoto, 1995), or more active metabolic functions other than an optical filter, such as reproductive (Bandaranayake et al., 1996), has been virtually neglected.

The sea hare *Aplysia dactylomela* represents an ideal model for investigation of the biological role of MAAs. It lives in shallow tropical areas and is exposed regularly to strong sunlight through its development from eggs attached to shallow rocks and seaweeds, to feeding adult. The eggs are enclosed in clear capsules embedded within a transparent gelatinous strand through which light penetrates easily. A unique feature of sea-hare spawn is that the eggs accumulate pigments that are specific to the types of seaweeds eaten by the adults. These pigments range from greenish-yellow to mauves, purples, and pinks, and so characteristic are they that single-algal diets can be identified within a day or two from changes in the colour of the spawn produced (Carefoot, 1987). The function of these pigments is not known. Adult *A. dactylomela* primarily eat red algae which are rich in MAAs (Karentz et al., 1991; Carefoot et al., 1998; Mason et al., 1998). However, based both on the identity of secondary metabolites that have been isolated from their digestive glands (Faulkner, 1992) and on behavioural observations (Carefoot, 1987), it is known that adults do occasionally feed on green and brown macroalgae.

In a previous 2 week-long study (Carefoot et al., 1998) we tested for interactive effects of UV exposure, diet, MAA concentrations, and vitality of spawn in *A. dactylomela*. We observed that while MAAs were abundant in spawn and concentrations highly dependent on diet, MAAs had no apparent role in protecting the eggs from UV.

The present study extends our investigation on MAAs in *Aplysia dactylomela* by examining over a longer time-period the interaction of diet and accumulation of MAAs in tissues such as skin and digestive gland, and in spawn. Of particular interest was whether the overall concentration and proportion of different MAAs in skin and spawn would be maintained at steady levels with varying dietary intake of MAAs. This type of differential sequestration of MAAs has been shown by Mason et al. (1998) to occur between eyes and skin in medaka fish. If also true for sea hares, it would suggest that MAAs were playing a functional role in survival of both adults and their eggs. We were additionally interested in whether or not different MAA compounds were functional analogues.

2. Materials and methods

2.1. Collection and maintenance of specimens

Twenty-four sea hares of 400–600 g live mass were collected from shallow (1–2 m depth) areas of Discovery Bay, Jamaica, near the Discovery Bay Marine Laboratory, during May 1997. This area is rich in algae, especially rhodophytes (Chapman, 1961, lists over 150 species of red algae in the Discovery Bay region). The sea hares were housed in plastic-mesh baskets buoyed to float just beneath the sea surface. Fresh algae were provided ad libitum daily, with uneaten remnants from the previous day's feeding being discarded. The baskets were brushed daily to remove diatom growth. The baskets received full sunlight from 07:00 to 17:00 h.

Animals were fed on single algal species over the 40-day experiment. Three red algae: *Acanthophora spicifera*; *Laurencia* sp.; and *Centroceras clavulatum*, and one green alga: *Ulva lactuca*, comprised the four diets. The diets were selected on the bases of known palatability to sea hares (*Acanthophora* and *Laurencia*), and known richness (*Acanthophora* and *Centroceras*) or paucity (*Ulva*) of MAAs (Carefoot et al., 1998). To confirm that the four species selected for the study were readily eaten by *Aplysia dactylomela*, we conducted a series of overnight feeding experiments.

2.2. Laboratory food-consumption experiments

To examine food consumption quantitatively for a wide variety of plants found commonly in the *Aplysia* habitat, we allowed a fresh set of animals to feed on twelve species of macroalgae and a seagrass in a single-choice experimental design. Animals of known mass were housed individually in flow-through cages in a seawater system and offered a single food species in the late afternoon. The mass of food offered was weighed, as was the amount remaining the following morning. The change in mass of each diet was adjusted by the change in mass of paired no-herbivore replicates, which accounted for plant growth or disintegration; however, mass change in all no-herbivore replicates was minor. We ran six to eight replicates each of the following: Rhodophyta, *Acanthophora spicifera*, *Centroceras clavulatum*, *Ceramium* sp., *Galaxaura oblongata*, *Laurencia* sp., *Gelidiella acerosa*; Chlorophyta, *Chaetomorpha linum*, *Codium isthmocladum*, *Ulva lactuca*; Phaeophyta, *Dictyota cervicornis*, *Padina jamaicensis*, *Sargassum polyceratium*; Anthophyta (turtle grass), *Thallassia testudinum*. Sea-hare mass (191 ± 11 SE live g) did not differ significantly among diets.

2.3. Collection of tissues for MAA analysis

A 5-g subsample of every spawn mass laid over the 40-day study period was taken, placed in a glass vial, and frozen at -20°C. Numbers of spawn masses sampled for respective diets were: 22 for *Acanthophora*; 19 for *Laurencia*; 22 for *Centroceras*; and 19 for *Ulva*. During the same period all field spawn was collected ($N = 7$) and similarly prepared. At intervals, a sample of each food plant was taken from the field ($N = 6$ for each species). Samples were lyophilized within 1 month and MAA analyses completed within 2 months of sampling.

On Day 40, 5-g samples of digestive gland and skin (taken from the parapodial region) were dissected from each specimen and frozen in glass vials for MAA analysis. At the same time, six specimens were collected from the field and similarly sampled.

One day prior to the above tissue sampling, feces (≈ 2 g fresh mass) were collected from each animal (held for 12 h daylight in individual containers), and similarly bottled and frozen for MAA analysis. Fecal samples from the six field animals were collected at the same time.

2.4. MAA analysis

Samples were dried in a rotary evaporator, ground to a powder, and MAAs identified and quantified by high-performance liquid chromatography (HPLC: for specific details on methodology see Karentz et al., 1991; McClintock and Karentz, 1997). Briefly, samples were sequentially extracted in 80% methanol and individual MAAs separated by reverse-phase HPLC (Brownlee RP-8 column). Most samples were quantified with an aqueous mobile phase of 55% methanol containing 0.1% acetic acid; however, a variety of methanol concentrations were required (10, 25, 55 and 75%) to optimize the separation of peaks in the diverse tissue types which were analyzed. Flow rate was 0.8 ml min^{-1}. MAAs were identified and

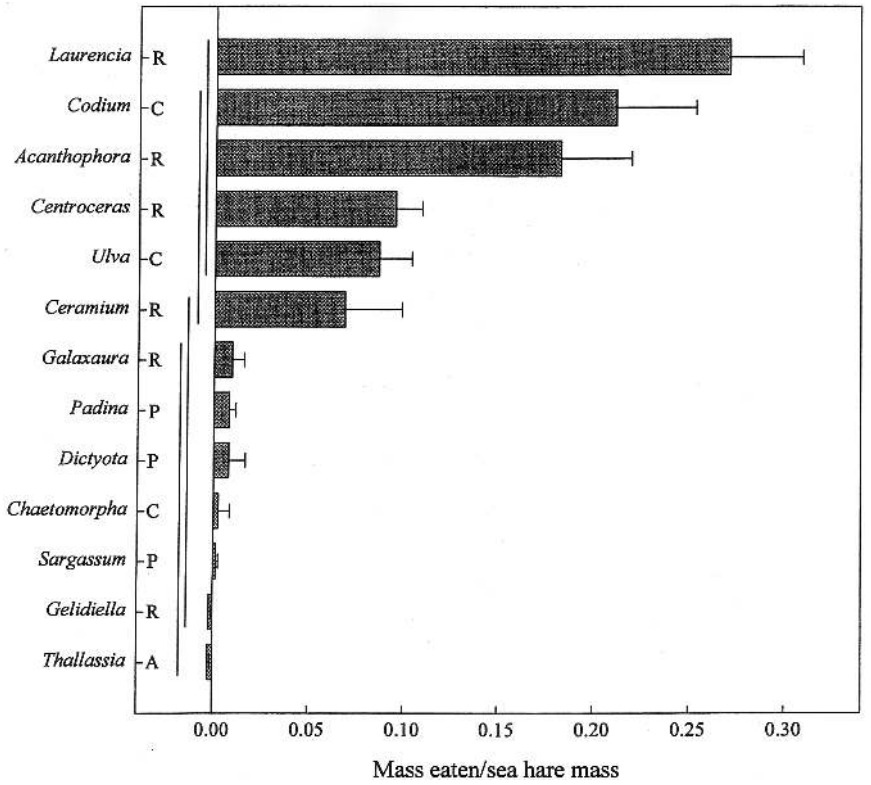

Fig. 1. Consumption of different diets in overnight feeding experiments by *Aplysia dactylomela*. Data (live mass eaten/sea hare live mass) are means ± S.E.; vertical bars on the left join means that are not significantly different (ANOVA, $F_{12,69} = 16.63$, $P < 0.0001$, with Ryan–Einot–Gabriel–Welsch multiple range test, $P < 0.05$). R, Rhodophyta; C, Chlorophyta; P, Phaeophyta; A, Anthophyta.

quantified by wavelength ratios of absorbance (313:340 nm) and co-chromatography with samples of known MAA content: *Curdiea racovitzae* (Karentz et al., 1991); *Limacina helicina* (Karentz et al., 1991); *Anthopleura elegantissima* (Stochaj et al., 1994); and *Aplysia dactylomela* (Carefoot et al., 1998).

2.5. Statistical analyses

Comparisons of total MAA contents in body tissues or feces among diets were made using ANOVA or, where assumptions of normality or homogeneity of variances were violated, using the non-parametric equivalent Kruskal–Wallis one-way ANOVA (K-WANOVA). Multiple-comparisons tests were applied where ANOVA rejected a multisample hypothesis of equal means. Time-based comparisons of spawn MAAs were made using repeated-measures ANOVA (R-MANOVA), followed by Tukey tests. Where data were converted to percentages, arc-sine transformations were first applied before analysis. Correlations between relative amounts of MAAs in feces and tissues of field animals versus those in the experimental groups were done using Spearman-rank tests.

3. Results

3.1. Food consumption

Overnight consumption differed strongly between diets (Fig. 1, $F_{12,69} = 16.63$, $P < 0.0001$). The sea hares ate large amounts (ca. 7–30% of their body mass) of *Laurencia*, *Codium*, *Acanthophora*, *Centroceras*, *Ulva*, and *Ceramium*. Other plants were eaten in trivial amounts or ignored. The four seaweeds chosen for the present study were amongst those readily eaten by *Aplysia dactylomela*.

3.2. MAA analyses

Six MAAs were identified in the *Aplysia* and algal samples: mycosporine-glycine, shinorine, porphyra-334, palythine, asterina-330, and palythinol. In addition, four other consistent peaks appeared on the chromatograms. However, it is not known if any of these were MAA compounds. One peak matched the characteristics of mycosporine-taurine based on co-chromatography with *Anthopleura elegantissima* extracts and analysis of wavelength ratios. However, since we were uncertain about peaks absorbing at wavelengths less than 313 nm and since mycosporine-taurine has only been reported in sea anemones *Anthopleura* spp. (Stochaj et al., 1994; Banaszak and Trench, 1995; Shick et al., 1996), confident identification and quantification was impossible. Until further chemical analyses can be completed, this peak is designated as Unknown 1. Unknowns 2 and 3 had retention times of ≈ 7.4 and 7.6 min, respectively and both had higher absorbance at 340 nm than at 313 nm. Unknown 4 had a retention time of 9.5 min and greater absorbance at 313 nm than at 340 nm. The four unknown peaks are noted here but not included in the considerations to follow.

3.3. Total MAA contents of foods, feces and body tissues

As expected, the seaweed foods differed significantly in total amounts of MAAs (Fig. 2a–d; $H = 17.11$, $P = 0.001$, K-WANOVA), with the three red algae having over an order of magnitude higher concentrations of MAAs than the green alga *Ulva* (2600–3700 vs. 50 μg· dry g^{-1}, respectively; $P < 0.05$,Tukey test). That *Ulva* had any MAAs at all may have been due to microscopic growth of epiphytic diatoms or other micro-algae on the thalli, as our earlier study showed MAAs to be absent from this seaweed (Carefoot et al., 1998).

On analysis, MAAs of feces segregated into two statistically homogenous groups: one represented by the *Acanthophora*, *Ulva*, and *Laurencia* dietary groups, and the field group (40–140 μg· dry g^{-1}); the other, by the *Centroceras* dietary groups (1000 μg· dry g^{-1}, respectively) (Fig. 2a–e; $H = 31.64$, $P < 0.001$, K-WANOVA; $P < 0.05$, Tukey test). Without knowing feeding rates on the different algae and extent of leaching of MAAs from the feces, it was not possible to determine how much of each MAA was absorbed.

Digestive-gland MAAs were an order of magnitude higher in the *Centroceras* dietary group than in the other dietary groups and field animals (13 600 vs. 800–1700 μg· dry g^{-1}, respectively; Fig. 2a–e; $H = 18.80$, $P < 0.001$, K-WANOVA; $P < 0.05$,Tukey test).

Skin MAAs were generally highest in *Acanthophora*-fed animals, lowest in *Ulva*-fed ones, and intermediate in the remaining dietary groups including field animals (Fig. 2a–e; $H = 18.12$, $P < 0.001$, K-WANOVA), but with considerable statistical overlap amongst them.

Spawn MAAs were significantly lower in *Ulva*-fed animals, intermediate in *Centroceras*- and *Laurencia*-fed ones, and highest in *Acanthophora-fed* ones and in field animals (1200, 1600–1700, and 2200 μg· dry g^{-1}, respectively; Fig. 2a–e; $F_{4,77} = 38.47$, $P < 0.001$, ANOVA), but with statistical overlap between the intermediate and high groups ($P > 0.05$, Tukey test).

Time-effects on spawn MAAs were significant (Fig. 3, $F_{3,77} = 2.95$, $P = 0.04$, ANOVA), but only for the *Ulva*-fed dietary group where values dipped significantly after the 1st week from about 1800 μg· dry g^{-1} to a level which ranged around 1000 μg· dry g^{-1} for the remaining 3 weeks of the study ($P < 0.05$, Tukey test).

3.4. Specific MAA contents of foods and tissues

In order to determine if certain MAAs were being preferentially taken up and sequestered in specific tissues, the data were transformed to percentages of total MAAs and plotted for the food, tissues, spawn, and feces from each group of animals (Fig. 4a–e). The data arrays are complex but some general trends are evident.

First, the most common MAAs in skin and spawn regardless of diet were porphyra-334, shinorine, and palythine. Digestive glands in the caged animals had a preponderance of asterina-330 with lesser proportions of porphyra-334. In digestive glands of field animals these MAAs also predominated, but in reverse order. Digestive glands in the *Centroceras* dietary group were unusual in their possession of only asterina-330 (other peaks were present, but these comprised $< 1\%$ of asterina-330 peak areas and were impossible to separate from the main peak), and concentration of this MAA was an order of

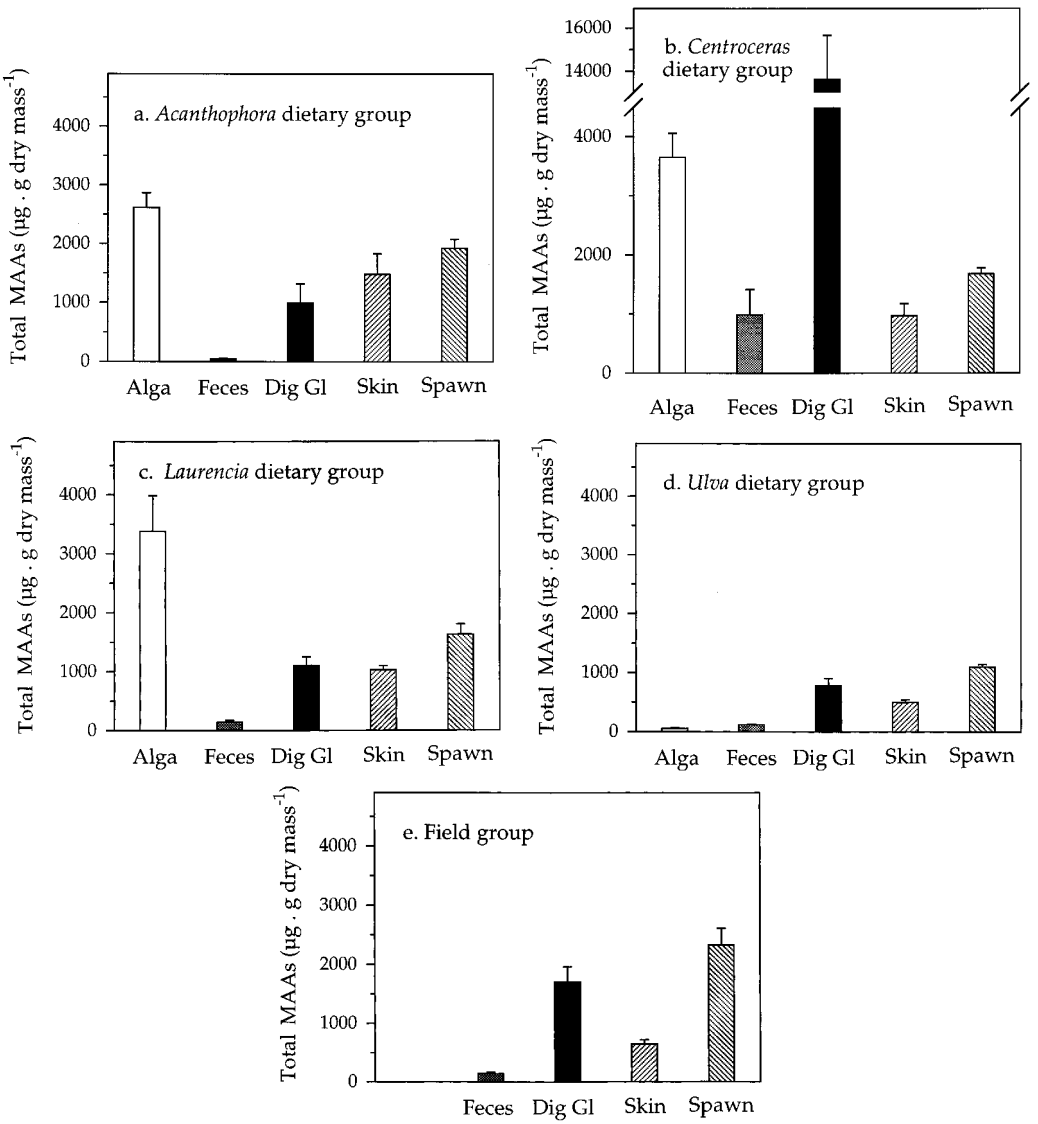

Fig. 2. Total MAA contents of food, feces, tissues, and spawn of *Aplysia dactylomela* feeding on different algal diets under caged and field conditions: (a) *Acanthophora spicifera*; (b) *Centroceras clavulatum*; (c) *Laurencia* sp.; (d) *Ulva lactuca*; (e) Field. Values are mean $\pm$ S.E. µg MAAs· dry g^{-1} for $N = 6$.

magnitude greater than in other digestive glands, including those of field animals.

With the idea that field animals could serve as a 'model' for disposition of MAAs (that is, field animals would have a variety of food sources available and might be expected to optimise the types and also amounts of essential MAAs in their tissues), we compared the relative (%) MAA contents of each tissue and feces from the caged animals with those in field animals in a series of correlation tests. With respect to spawn, for example, we tested for correlation of percentage composition of the six MAAs in field spawn with percentage composition of the same MAAs in spawn of each dietary group (four tests in total). This was repeated for skin, digestive gland, and feces. Results showed that: (1) spawn from all treatment groups correlated significantly in terms of percentage MAAs with spawn from field animals (all $r > 0.82$, all $P < 0.05$); (2) the pattern of MAAs in skin of the treatment groups generally matched that in field animals for the *Laurencia* and *Acanthophora* diet-groups (both $r > 0.90$, both $P < 0.04$), less so for the *Ulva* group ($r =$

0.75, $P = 0.13$), and not at all for the *Centroceras* group ($r = 0.09$, $P > 0.50$); (3) percentage MAAs in digestive glands and feces of the treatment animals correlated poorly with comparable values in field animals (all $r < 0.65$, all $P > 0.10$), with the exception of feces of the *Laurencia*, *Acanthophora*, and *Ulva* dietary groups (all $r = 0.83$, $P > 0.05$). Overall, the *Laurencia* and *Acanthophora* dietary groups showed closest match with field animals, each with three out of four significant correlations.

A second feature noted in Fig. 4 was the generally good correlation between MAAs predominant in the diets and those sequestered in the tissues. Thus, tissues rich in porphyra-334, palythine, and asterina-330 in the *Acanthophora* dietary group were produced from a diet containing mainly porphyra-334 and palythine[1]. In the *Centroceras* dietary group, tissues rich in shinorine, palythine, and asterina-330 came from a diet containing predominantly these MAAs. Tissues from

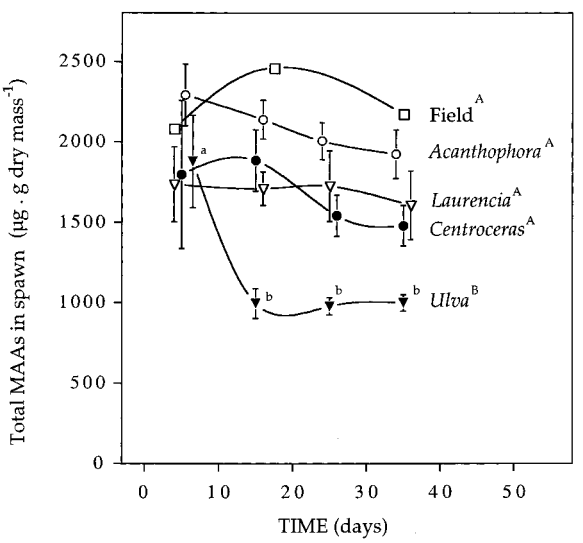

Fig. 3. Total MAA contents of spawn from *Aplysia dactylomela* feeding on different seaweeds over a 40-day period and from the field (the food is indicated by the genus label for each curve save for 'Field', where the precise diet was unknown). Each point for algal diets represents the mean ± S.E. µg MAAs· dry g^{-1} for all spawn masses deposited in a given 10-day period ($N = 3$–8 masses per period). Field spawn is represented by only two samples per point; hence, error bars are omitted. Capital letters to the side of each genus (and 'Field') name indicate significant differences in overall total MAAs in the spawn; small letters by each *Ulva* point indicate significant differences in MAA content over time in spawn from this dietary group ($F_{3,17} = 41.12$, $P < 0.001$, ANOVA; $P < 0.05$, Tukey test).

the *Laurencia* dietary group contained mainly porphyra-334 and asterina-330 (most notably in the digestive gland), and the alga contained a preponderance of porphyra-334. Finally, tissues of the *Ulva*-fed group contained predominantly mycosporine-glycine, shinorine, porphyra-334, and asterina-330 (mainly digestive gland), while the diet contained mostly the first three MAAs plus some palythine.

Fig. 4 also shows that the digestive glands in all dietary groups tended to sequester large proportions of asterina-330. That this was true for the *Ulva*-eating animals, despite the diet not containing any asterina-330, must be explained by retention of this MAA from when the animals were eating a mixed field diet prior to being collected.

Comparison of seaweed MAAs with tissue MAAs of field animals showed best match with *Laurencia* (all tissue MAAs correlated well with plant MAAs, with $r > 0.64$), second-best match with *Acanthophora* (all tissues with $r > 0.61$), and no match with *Centroceras* or *Ulva* (all $r < 0.41$ and $P > 0.30$).

A third point arising from Fig. 4 was that relative MAA contents of feces showed no apparent correlation with either food or tissue MAAs.

In order to determine if MAAs were being differentially allocated to spawn over time in a common pattern throughout the treatment groups, the three most abundant MAAs in the spawn of a given dietary group and field animals were plotted for10-day intervals as done for total MAAs in Fig. 3 (Fig. 5a–e). We knew from Fig. 4 that spawn of the different dietary groups and field animals differed in types and amounts of MAAs, but this last question addressed the issue of whether concentrations of the predominant MAAs would be regulated over time. The three MAAs selected accounted for 83–88% of all MAAs present in the spawn; hence, were likely to be playing the most important functional roles. However, only spawn from the *Ulva* dietary group showed significant time effects ($F_{3,15} = 8.4$, $P < 0.01$) in that the most abundant MAA, porphyra-334, was maintained at a fairly consistent level

[1] During the 40-day experiment, as well as producing large amounts of spawn, animals in all treatments grew prodigiously; minimum increase in live mass was 50%, while some individuals in the *Acanthophora* and *Laurencia* dietary groups more than doubled in size.

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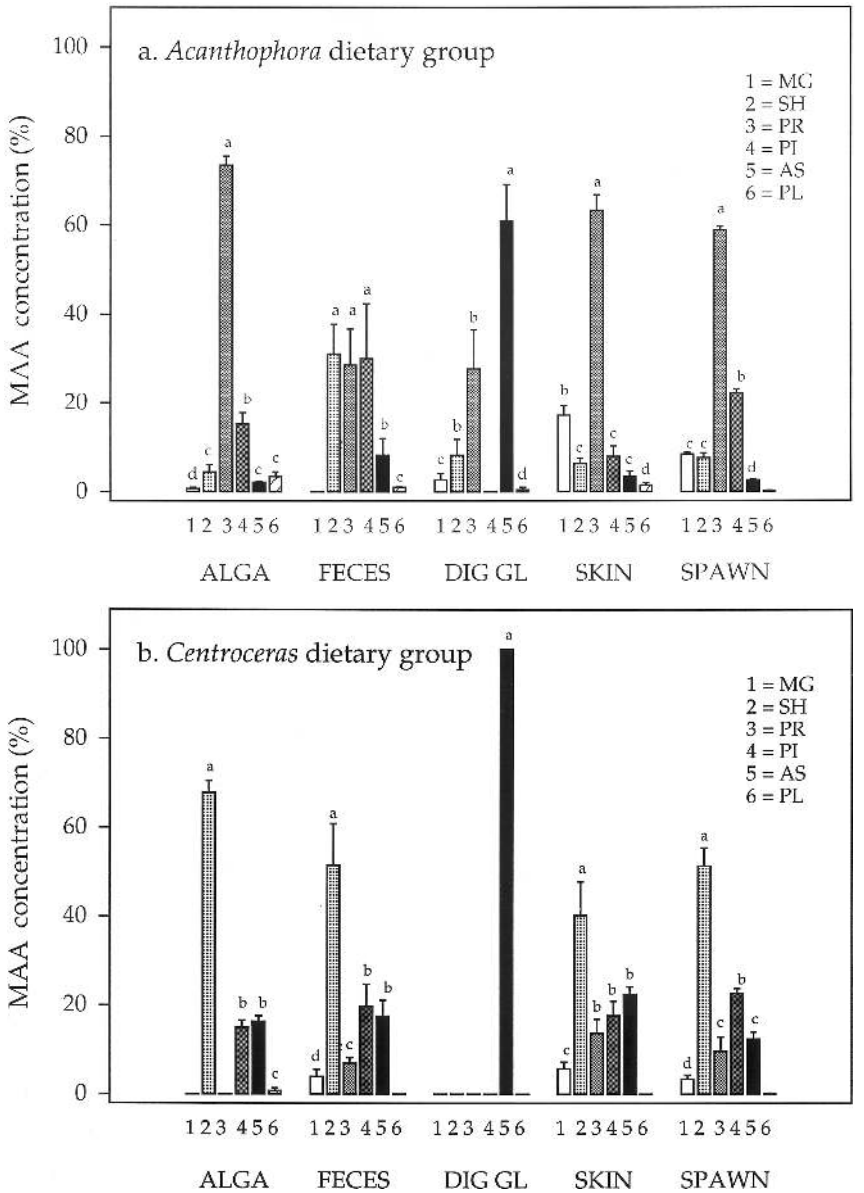

Fig. 4. Specific MAA contents of food, feces, tissues, and spawn (representing all spawn deposited over the study period) laid by *Aplysia dactylomela* feeding on different algal diets under caged and field conditions: (a) *Acanthophora spicifera*; (b) *Centroceras clavulatum*; (c) *Laurencia* sp.; (d) *Ulva lactuca*; (e) Field. Values are mean ± S.E. % dry-mass MAAs for $N = 6$. MG, mycosporine-glycine; SH, shinorine; PR, porphyra-334; PI, palythine; AS, asterina-330; and PL, palythinol.

over the latter part of the study after experiencing a significant drop in concentration during the initial 2 weeks (Fig. 5d,Tukey's test, $P < 0.05$).

4. Discussion

The low concentration of MAAs in the animals fed *Ulva* and the different MAA complements in different dietary treatments strongly supports a dietary origin of these compounds in *Aplysia* (see also Dunlap et al., 1991; Adams and Shick, 1996; Carroll and Shick, 1996; Carefoot et al., 1998). Results from the time-series experiments with spawn similarly suggest that incorporation of MAAs into spawn depends heavily upon current diet, as well as on selective uptake. The first 10-day period of samples from the *Ulva*-fed animals had high concentrations of MAAs, but some of these eggs, at least, were being made before

treatment was imposed. MAA concentrations then dropped significantly, but were maintained at constant level, perhaps by reallocating MAAs from other tissues; however, this reallocation was insufficient to maintain spawn MAAs at levels similar to those found in animals fed MAA-rich diets.

The internal distribution of MAAs contrasts strikingly with the internal distribution of sequestered algal secondary metabolites such as terpenes found commonly in *Aplysia* species (for review see Carefoot, 1987). In most cases, concentrations of MAAs were highest in spawn, then skin, then digestive gland. This pattern of distribution is consistent with an adaptationist argument (as proposed by Shick et al., 1992) that sea hares selectively locate MAAs where they can function most effectively as sunscreens. In contrast, diet-derived secondary metabolites such as terpenes are an order of magnitude higher in sea-hare digestive glands than skin, and are completely absent in spawn (Faulkner, 1992; Pennings and Paul, 1993; De Nys et al., 1996). The deposition of sequestered secondary metabolites into

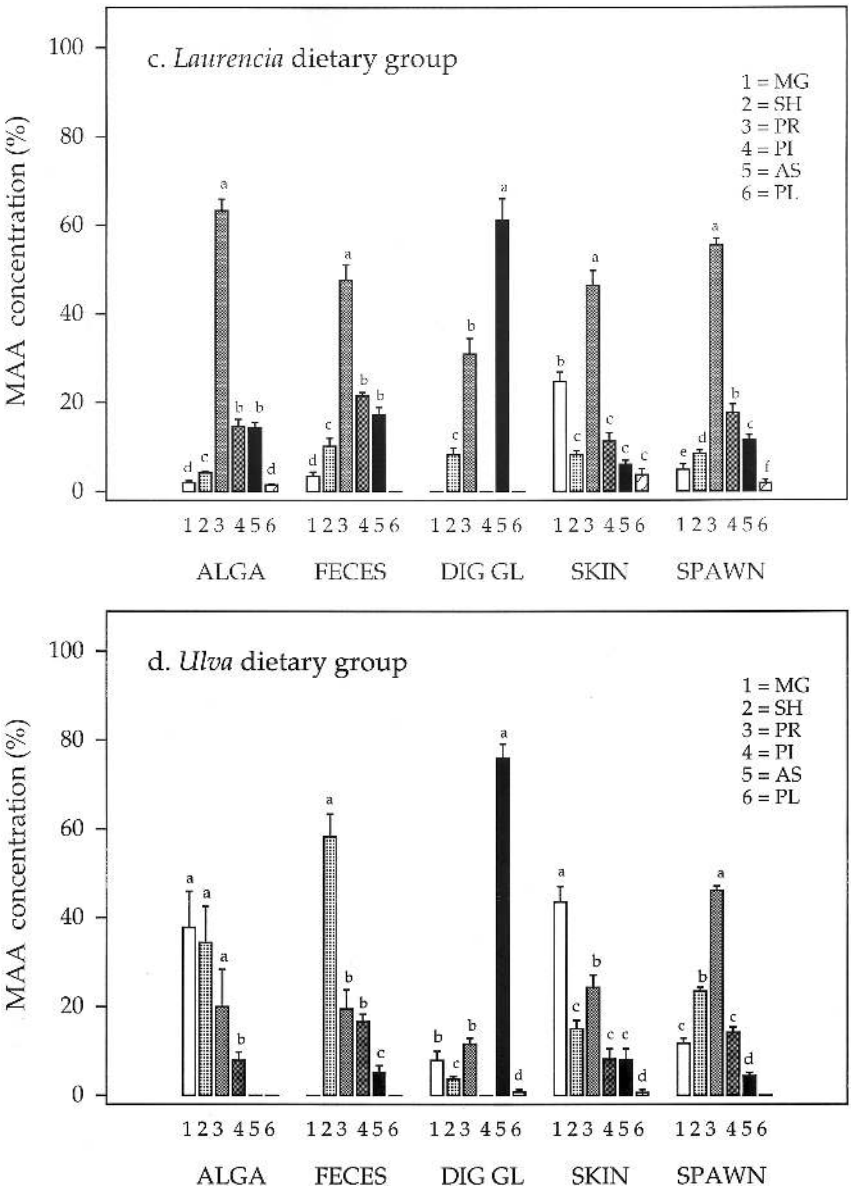

Fig. 4. (*Continued*)

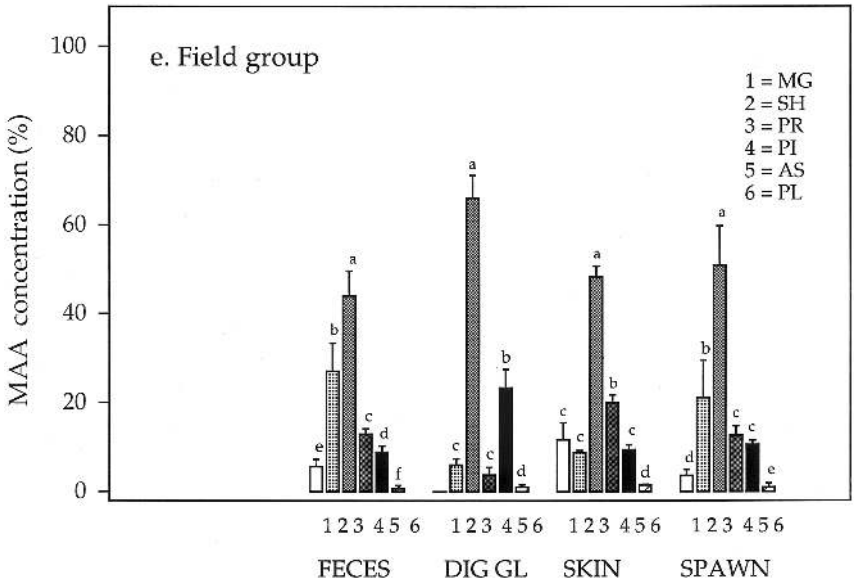

Fig. 4. (*Continued*)

internal organs rather than skin and eggs would seem strikingly maladaptive if these compounds were serving a defensive role, and is one factor that has led to criticism of the conventional wisdom that sea hares sequester algal metabolites for defense (Pennings and Paul, 1993; Pennings, 1994; Nolen et al., 1995). It might be argued that unknown physiological constraints allow only small amounts of secondary metabolites to be translocated to the skin and spawn, but the fact that MAAs were readily incorporated into skin and spawn suggests that this is not the case. Although the animal's ability to transport MAAs and terpenes from the digestive gland to skin or eggs may well differ, the contrast between the distributions of these different kinds of compounds suggests that MAAs are adaptively transported to function as sunscreens, but that secondary metabolites like terpenses are not adaptively transported to function in defense from predators.

The significant correlations of relative MAA contents of spawn laid by all diet groups with that of spawn from field animals suggests that certain MAAs are required in the spawn. Based on relative concentration, the important MAAs in *Aplysia dactylomela* spawn are porphyra-334, shinorine, and palythine, in this approximate order (present data on field spawn combined with that from Carefoot et al., 1998). Adams and Shick (1996) also noted abundant quantities of porphyra-334 and shinorine in eggs of sea urchins eating the red alga *Mastocarpus stellatus* (noted by the authors to be rich in shinorine), suggesting that the MAAs were diet-derived. The common occurrence of these two MAAs in the eggs of these herbivores suggests a similar but, as yet, unknown reproductive function. Karentz et al. (1992, 1997) also recorded high levels of shinorine in the ovaries of an Antarctic limpet, *Nacella concinna*, and high levels of porphyra-334 in the ovaries of an Antarctic sea urchin, *Sterechinus neumayeri*. Both MAAs are common in tissues of Antarctic invertebrates (Karentz et al., 1991), and shinorine is comparatively abundant in the gonads and other organs of shallow water-inhabiting coral-reef holothuroids (Shick et al., 1992) and temperate-dwelling echinoderms (Banaszak et al., 1998). Interestingly, in instances where one or other of porphyra-334 or shinorine were absent or in low concentration in the diet of *Aplysia*, palythine was substituted in the spawn, suggesting that it is either a functional analogue to the others or simply substituted when one of the others is lacking or in low concentration in the diet.

That a minimum level of MAAs in the spawn is likely functionally important is shown by the constancy of MAA concentrations over time in all but the *Ulva* diet-group. Even here, the decline in MAA concentrations associated with a switch from an MAA-rich field diet to an MAA-poor *Ulva* diet stabilized after about two weeks to a level of about 900 μg· dry g^{-1}. Based on the

comparatively low hatching success exhibited by spawn from *Aplysia dactylomela* eating an *Ulva* diet (Carefoot et al., 1998), this level might be considered minimal for good health of the spawn. Other laboratory experiments have shown that *Aplysia* spp. normally subsisting on red algae, but restricted to a diet of *Ulva*, could produce viable spawn for several months (for review see Carefoot, 1987), indicating that nutrients essential for development were being provided by the diet. The intent in the present study, of course, was to maintain animals on an *Ulva* diet for sufficient time to exhaust all supplies of MAAs in their tissues, but even 40 days was not long enough (see also Carroll and Shick, 1996). Spawn MAA levels maintained by the red algae-eating experimental groups and field animals ranged between 1500–2400 μg· dry g^{-1} but, since the spawn of *Laurencia*-eating animals exhibited most consistent and highest levels of hatching (89–92%: comparable to that of field animals: Carefoot unpub. data) we are inclined to think that the lower part of this

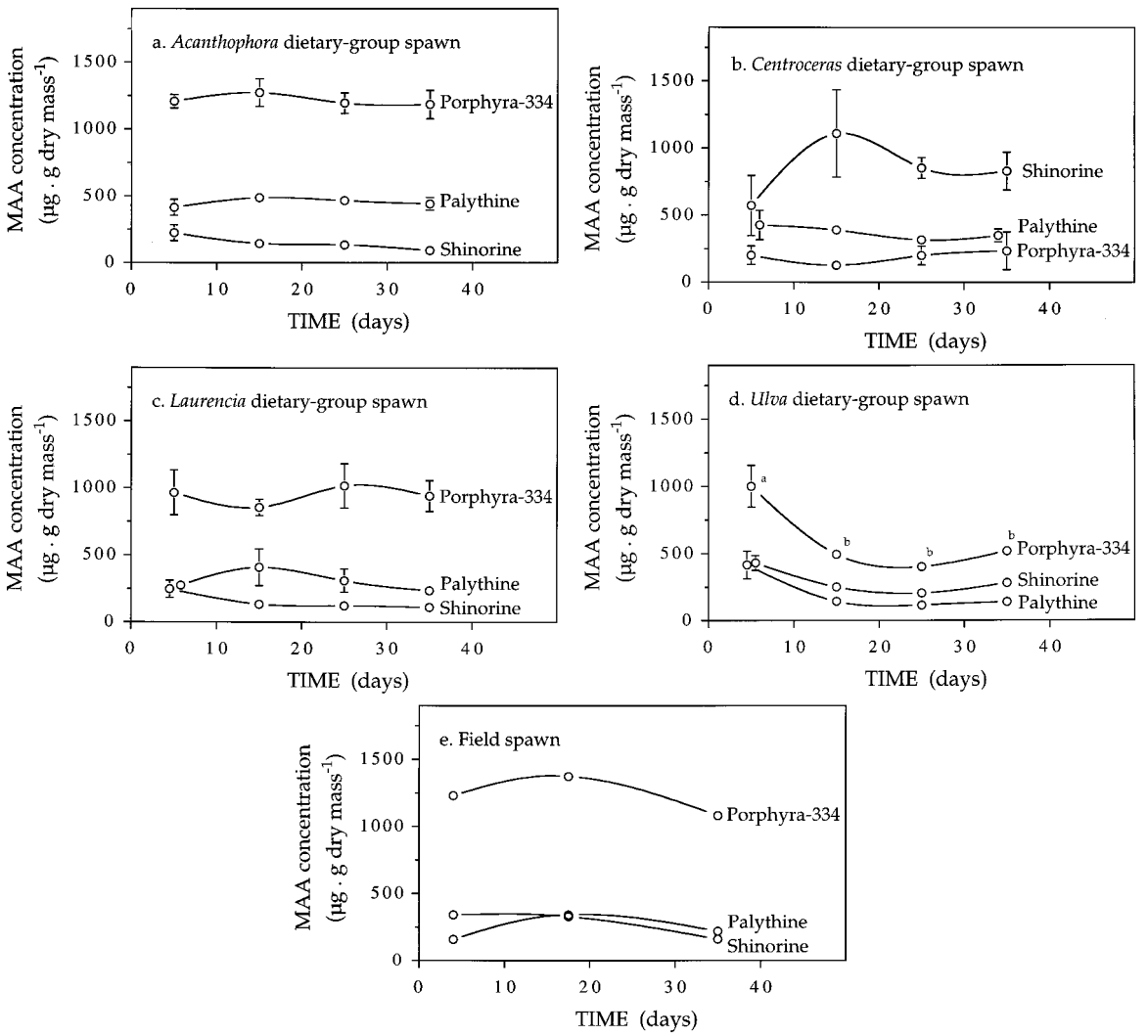

Fig. 5. Change in contents of selected MAAs in spawn from each diet group of *Aplysia dactylomela* over a 40-day period. Each point for algal diets (a–d) represents the mean ± S.E. μg MAAs· dry g^{-1} for all spawn masses deposited in a given 10-day period ($N = 3–8$ masses per period). In several instances error bars are contained within the dimensions of the points. Field spawn (e) is represented by only two to three samples per point; hence, error bars are omitted. Small letters by each porphyra-334 point in graph (d) indicate significant differences in contentration over time ($F_{3,15} = 8.4$, $P < 0.01$, ANOVA; $P < 0.05$, Tukey test). Dietary groups: (a) *Acanthophora spicifera*; (b) *Centroceras clavulatum*; (c) *Laurencia* sp.; (d) *Ulva lactuca*; and (e) field group.

range (ca. 1700 μg· dry g^{-1}; see Fig. 3: *Laurencia*) is likely optimal for best health of spawn and/or is an upper physiological limit.

The second-best match of relative levels of tissue-MAAs between caged and field animals was with skin and spawn in complements of shinorine and porphyra-334. In a survey of MAAs in tissues of shallow-water holothuroids on the Great Barrier Reef, Shick et al. (1992) noted that four MAAs predominated in skin, including mycosporine-glycine, shinorine, palythine, and asterina-330. Highest overall MAA concentration was found in the skin of *Pearsonothuria graeffei*, a species noted by the authors to inhabit shallow water and to be active in daylight. Ishikura et al. (1997) similarly noted in clams that highest MAA concentrations, most notably mycosporine-glycine, porphyra-334, shinorine, and palythine, were in the outer layers of mantle tissues. Based on the assumption that highest concentrations of UV-protective compounds would be expected in tissues most exposed to sunlight, the authors concluded that the MAAs were serving a UV-protective function. If this also explains the high concentration of MAAs in the skin of *Aplysia dactylomela*, it is interesting that so many different types of MAAs can fulfill the same role in the same and different invertebrate species. The variety suggests that several MAAs are functional analogues with a variety of origins, which in turn supports the idea that tissue-sequestered MAAs are diet-derived. The presence of multiple MAAs in a tissue is commonly thought to be because their different maximum wavelengths of absorbance increases the 'bandwidth' of UV absorbed. However, the wavelengths of maximum absorbance are so close in palythine (320 nm), asterina-330 (330 nm), shinorine (334 nm), and porphyra-334 (334 nm), that it is hard to say if this is true in *Aplysia*.

Digestive glands of experimental and field *Aplysia dactylomela* contained high levels of asterina-330. This was particularly true for the *Centroceras* diet group, in which not only was asterina-330 the only digestive-gland MAA identified, but it was present in extremely high concentration (up to 21 mg· dry g^{-1}). Furthermore, in all dietary groups asterina-330 was selectively sequestered from foods containing unremarkable concentrations of this MAA or, in the case of the *Ulva* diet group, from a food entirely lacking in it. Possible explanations for this include biochemical transformation from precursor substances (perhaps mycosporine-glycine as proposed by Shick et al., 1991) or from the synthetic activities of symbiotic intestinal micro-flora. However, in the absence of evidence in support of either of these ideas, it could simply be that asterina-330 was remnant from previous field diets or, alternatively, represented accretions from the food in however small concentrations contained therein. In our previous study (Carefoot et al., 1998) we showed that even though a certain MAA were present in only minute concentration in a food, the vast amounts eaten daily by a sea hare (up to 30% of its live body mass; Fig. 1) would provide to the animal ample amounts of a given MAA. The presence of asterina-330 in the digestive glands of the *Ulva* dietary group supports the 'remnant-from-field-diet' hypothesis because, although trace amounts of MAAs were recorded in this alga (possibly from epiphytic red algae or diatoms), asterina-330 was absent. Also, there was a trend towards lower concentrations of MAAs in digestive glands of the *Ulva* diet group, suggesting a steady shunting of MAAs to other bodily destinations. Because of its active role in metabolism and digestive processes the digestive gland is probably acting both as repository and clearing-house for MAAs required in other parts of the body. However, this could not explain the extraordinarily high levels of asterina-330 in digestive glands of the *Centroceras* dietary group, especially in view of the rich concentrations of other MAAs, most notably shinorine and palythine, which would be expected to be similarly sequestered in the digestive gland based on their relative abundance in other tissues.

The strikingly different pattern of distribution of different MAAs in the tissues of *Aplysia dactylomela*, especially that of asterina-330, could also reflect chemical differences among the compounds that affect how readily they are transported from the gut, presumably via the digestive gland, to other locations. Alternatively, the differences may be adaptive. If so, we would expect asterina-330 to be much poorer in fulfilling the skin and spawn functions of, say, porphyra-334 or palythine. These patterns suggest that studies investigating differences in UV-absorption, gut absorption/transport, and perhaps toxicity of different MAAs in *Aplysia* would be of value (see Mason et al., 1998, for related studies on mice and human carcinoma cells). If asterina-330 were found to be

playing a defensive anti-predator role in *Aplysia*, it would join a host of over 30 secondary-metabolites already described from the digestive glands of *Aplysia* spp., most or all of which have been considered as playing some sort of defensive role (Carefoot, 1987; Faulkner, 1992).

The strong correlation of relative MAA contents of spawn and skin of animals eating *Laurencia* and *Acanthophora* with the same tissues of field animals may simply relate to the common foods being eaten. Based on our feeding study where both seaweeds were readily consumed (see Fig. 1), and from previous analyses of gut contents of field *Aplysia dactylomela* from the same location where both seaweeds made up the main crop components (Carefoot unpublished data), these two red algae may represent the main supply of MAAs for field animals. Our inability to demonstrate a sunscreen role for MAAs in *Aplysia* spawn (Carefoot et al., 1998) does not preclude a similar function in juveniles or adults. The preference by most species of *Aplysia* for red seaweeds has previously been attributed to their ability to sequester secondary metabolites from these chemically-rich plants for defensive purposes (Faulkner, 1992, but see Pennings and Paul, 1993 and Pennings, 1994). An alternative hypothesis is that sea hares in shallow waters exposed to UV radiation may preferentially eat seaweeds rich in MAAs in order to obtain sunscreens for themselves, not for protection of their eggs. Serious consideration of this hypothesis will require comparative studies of the shallow water-inhabiting tropical and subtropical *Aplysia* species (most notably *A. juliana*, *A. fasciata*, *A. depilans* and *A. vaccaria*) that subsist on green and brown algae that completely or mostly lack MAAs.

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