

Short Communication

In Vitro Culture of the Tropical Sponge *Axinella corrugata* (Demospongiae): Effect of Food Cell Concentration on Growth, Clearance Rate, and Biosynthesis of Stevensine

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Abstract: In vitro culture is one possible method for supplying sponge metabolites for pharmaceutical applications, but appropriate feeding regimens that maximize both growth and metabolite biosynthesis are largely unknown. According to the natural concentration (NC) of cells 1 to 50 μm in size that are available to wild *Axinella corrugata*, we fed explants a multispecific diet of bacteria, microalgae, and yeast at 4 different concentrations: 1NC, 3NC, 5NC, and 5+1NC (the last consisted of 5 NC of bacteria and 1 NC of microalgae and yeast). Explants fed a 3NC diet had the best culture response, growing on average from 8.5 g to 10.3 g in 8 weeks, and showing a 110% increase in concentration (milligrams per gram of dry weight) of the antitumor compound stevensine. Stevensine production in 3NC explants, representing the total milligrams of metabolite per explant, increased by 157% over the study. Explants fed at 1NC had relatively stable weights, indicating that the diet met metabolic costs only. Explants fed at the two highest concentrations lost weight after 4 weeks, possibly because long-term high cell concentration blocked their aquiferous system, reducing their ability to feed efficiently. Stevensine production in explants fed the 1NC, 5NC, or 5+1NC diets were similar, and varied little from the initial amount. A separate experiment showed that the clearance rate for *A. corrugata* is similar between the examined food types and cell concentrations over 5 hours, averaging $766 \text{ ml h}^{-1} \text{ g DW}^{-1}$. Overall, this study demonstrates that relatively small changes in food abundance can greatly affect both sponge growth and metabolite biosynthesis. The good growth and increased production of the target metabolite stevensine for *A. corrugata* explants fed a 3NC diet suggests that in vitro culture is a viable method of supplying some sponge metabolites.

Keywords: *Axinella corrugata*, sponges, in vitro culture, cell concentration, growth.

INTRODUCTION

Of the entire marine fauna, sponges are the best sources of metabolites with biomedical potential (Ireland et al., 1993). Unfortunately, many sponges contain only trace amounts of

bioactive metabolites (Schmitz et al., 1993), and the need for large quantities for drug development and production has at times resulted in the near extinction of a population or a species (Anderson, 1995). It is therefore useful to develop methods to guarantee the supply of metabolites selected for development as pharmaceuticals. One supply method currently being developed is *in vitro* sponge culture, whereby sponges are grown under controlled environmental and nutritional conditions (Osinga et al., 1999b).

The nutritional and environmental conditions that promote sponge growth and metabolite biosynthesis are largely unknown (Osinga et al., 1999b); consequently, most studies examining *in vitro* sponge culture have had poor results (Osinga et al., 1997, 2001; Nickel et al., 2001). In their review of sponge culture, Osinga and colleagues (1999b) stated that determining suitable feeding regimens is the key to successful *in vitro* sponge culture. Sponges feed unselectively on bacteria and microalgae less than 50 μm in size (Reiswig, 1971; Pile et al., 1996). Growth of sponges generally increases as food concentration increases, but this relationship is not always linear (Poirrier et al., 1981; Huysecom et al., 1988). The effect of food concentration on the biosynthesis of sponge metabolites is unknown. However, considering that environmental conditions can greatly influence metabolite biosynthesis in cultured sponges (Thompson et al., 1987; Kreuter et al., 1992), it is likely that the diet will also affect production of the target metabolite.

In this study, we cultured the Caribbean Demospongiae *Axinella corrugata* (= *Teichaxinella morchella*), with explants being fed a multispecific diet at different cell concentrations. *Axinella corrugata* produces stevensine, an alkaloid metabolite with antitumor properties (Wright et al., 1991). Stevensine is a major secondary metabolite in *A. corrugata* and probably serves multiple defensive roles, having strong antipredatory properties (Wilson et al., 1999) and weak antimicrobial activity (Newbold et al., 1999). Studies have determined that *A. corrugata* retains the ability to produce stevensine in cell culture (Pomponi et al., 1997; Andrade et al., 1999), suggesting that stevensine is biosynthesized by *A. corrugata* and not a microbial symbiont.

MATERIALS AND METHODS

Sponge Collection and Cutting

Twenty individuals of *A. corrugata* were collected from a depth of 15 m off Long Key, Florida. To minimize har-

vesting impact, one third of each sponge was left intact and attached to rock. The collected sponges were placed in 20-L buckets containing aerated seawater and transported to Harbor Branch Oceanographic Institution (HBOI), Fort Pierce, Florida. In aerated seawater, the sponges were cut using sharp scalpels into approximately 60 cube-shaped explants. All explants had at least one side covered in pinacoderm and were, on average, 8.5 g (SE, 0.3 g) in weight. After cutting, explants were placed into three 80-L aquaria for 2 weeks to allow time for them to heal their cut surfaces and reorganize their canal system before being used. Each aquarium contained a mechanical and biological filter to maintain high water quality.

Feeding Experiment Setup

Twelve aquaria (25 \times 29 \times 41 cm) were used in this study, each containing 15 L of natural seawater. The seawater was first passed through a series of UV lights and filters (down to 1 μm pore size) to remove or kill any particles. Clean aerated water dripped at a rate of 2 (SE, 0.4) L h⁻¹ into each aquarium from large holding tanks situated above. Water flowed out from an outlet pipe on each aquarium so that volume was constant (15 L). In addition, the water in each aquarium was totally replaced with clean aerated seawater twice weekly. These procedures removed any uneaten food cells and detritus, and prevented ammonia levels from reaching high and possibly damaging levels. Dissolved ammonia was monitored using the salicylate-cyanurate method and a spectrophotometer (Hach 41800-02) and was, on average, 0.02 (SE, 0.003) mg L⁻¹ throughout the experiment. Ammonia levels were not significantly different between the feeding regimens (Kruskal-Wallis 1-way analysis of variance [ANOVA] on ranks: $H_{df(3)} = 4.47$, $P = 0.21$). Water temperature, salinity, and pH were kept constant throughout the experiment at 25°C, 35‰, and 8.1, respectively. Most days the explants were cultured on an 8-hour light, 16-hour dark regimen. Because silicon is an essential skeletal component for Demospongiae such as *A. corrugata* and may affect sponge growth, 125 μl of 227 mol L⁻¹ sodium metasilicate (SIGMA, S-4392) was added to each aquarium twice a week. No antibiotics were used at any stage during this study.

Into each aquarium, 3 explants were randomly chosen and placed onto a horizontal mesh support, more than 5 cm apart to prevent possible inter-explant interference. Explants remained in the same place throughout the experiment so their progress could be monitored individu-

ally. The mesh support in each aquarium was wedged 8 cm off the bottom, promoting water circulation around each explant and preventing possible smothering from detritus.

The seawater was aerated constantly from 2 air-stones located in opposite corners of each aquarium, and away from all explants to prevent direct exposure to the air bubbles. The seawater in each aquarium was aerated at a rate of $2 \times 20 \text{ ml s}^{-1}$ to provide sufficient O_2 for explant respiration and to promote good water circulation, keeping the added food particles in suspension.

Food Types and Concentrations

To determine appropriate cell concentrations to feed to *A. corrugata*, the natural concentration (NC) available to wild sponges was examined. Water samples were first collected next to sponges and then analyzed using a hemacytometer and a phase-contrast microscope. To better represent the sponges' natural diet, we divided cells into 3 size classes: 1–4 μm , concentration = $2 \times 10^5 \text{ cells ml}^{-1}$; 4–10 μm , $2 \times 10^4 \text{ cells ml}^{-1}$ and 10–50 μm , $3 \times 10^3 \text{ cells ml}^{-1}$. Bacteria dominated the smallest size class, while microalgae dominated the 2 larger size classes. These cell concentrations determined for the Florida Keys are comparable to concentrations reported elsewhere in similar habitats (e.g., Jamaica [Reiswig, 1971] and Bahamas [Pile, 1999]). Particles less than 1 μm could not be counted accurately using a hemacytometer.

Explants were fed a multispecific diet, with food types grouped into the 3 size classes. Cells 1–4 μm in size consisted of 5 bacterial strains: *Marinococcus halophilus*, *Vibrio alginolyticus*, *Escherichia coli*, *Bacillus subtilis*, and G365 (gram-negative motile rod isolated from *A. corrugata* by the Division of Biomedical Marine Research, HBOI). Cells 4–10 μm in size included the microalga *Isochrysis galbana* and the yeast *Saccharomyces cerevisiae*. Finally, the large cell size of 10–50 μm consisted solely of the diatom *Thalassiosira weissflogii*. The average size of each food type was 3 μm for the 5 bacterial strains, 5 μm for *I. galbana* and *S. cerevisiae* and, 12 μm for *T. weissflogii*.

The 5 bacterial strains were cultured individually in 200-ml Pyrex flasks containing 50 ml of marine broth (DIFCO, 0791-01-2) or nutrient broth (DIFCO, 0003-01-6). The first cultures were inoculated by adding 2 ml of cryopreserved stock culture. The subsequent cultures were inoculated using 2 ml from the previous culture. To reduce microbial contamination, new cultures were started from

cryopreserved stock every 2 weeks. The yeast *S. cerevisiae* was cultured in a separate 200-ml flask containing 50 ml of sugar-water; each culture was inoculated with 0.5 g of active dry yeast (Red Star) and 2 g of white sugar. All bacterial strains and *S. cerevisiae* were incubated in an environmental shaker (Innova 4900) at 210 rpm and 25°C before being used (final culture concentration of approx. $10^9 \text{ cells ml}^{-1}$ for the bacterial strains and $10^7 \text{ cells ml}^{-1}$ for the yeast). Because the microbial culture medium might also have interfered with sponge growth, it was removed by centrifugation prior to feeding the sponge explants. The bacteria and yeast cultures were poured into 2 separate vials and centrifuged at 1400 *g* for 20 minutes. The culture broth was removed, and the cell pellets of either bacteria or yeast were resuspended in large flasks containing 1000 ml and 100 ml of seawater, respectively.

Each week, *I. galbana* and *T. weissflogii* were cultured in separate 20-L vessels containing F/2-enriched seawater (Hoff and Snell, 1987). Silica was added to the diatom *T. weissflogii* culture to promote population growth. The microalgae were cultured for more than 5 days at 24°C on 24 hours of light before being used, thus allowing sufficient time for cell concentration to reach a high level (*I. galbana*, approx. $7 \times 10^6 \text{ cells ml}^{-1}$; *T. weissflogii*, approx. $4 \times 10^5 \text{ cells ml}^{-1}$). Each algal culture was used for 1 week before being replaced. Each feeding day, 400 ml of *I. galbana* was added to the yeast suspension, and 4 L of *T. weissflogii* was poured into a separate container. For each food solution, 4 subsamples were counted using a hemacytometer to determine their average cell concentrations.

Feeding Regimens

Four feeding regimens were examined: (1) 1NC (1–4 μm , $2 \times 10^5 \text{ cell ml}^{-1}$, 4–10 μm , $2 \times 10^4 \text{ cells ml}^{-1}$; and 10–50 μm , $3 \times 10^3 \text{ cells ml}^{-1}$); (2) 3NC; (3) 5NC; and (4) 5+1NC (5NC of 1–4 μm cells and 1NC of 4–10 and 10–50 μm cells). Feeding regimen 1, 1NC, acted as a feeding control. Feeding regimen 4, 5+1NC, was used to examine the response to high food concentration of small cells of 1 to 4 μm , while eliminating the possible smothering effect from larger food cells. The volume of each food solution required was calculated by dividing seawater volume (15 L) by the measured cell concentration of each solution. Explants of *A. corrugata* were fed in the morning, 5 times each week, for a total of 8 weeks. There were 3 aquaria, randomly distributed, for each feeding regimen. Thus, each regimen had 9 explants.

Examining Culture Response

Growth was examined weekly by measuring the weight of each explant (to 0.1 g) in a container of water resting on a scale. There was no significant difference in initial explant weight between the 4 feeding regimens (1-way ANOVA: $F_{df(3, 32)} = 0.53, P = 0.66$).

At the end of the experiment, 3 randomly chosen explants from each feeding regimen (1 explant per aquarium replicate) were chemically analyzed for stevensine content. Three explants frozen at the start of the experiment were also chemically analyzed to get an initial baseline of stevensine concentration. Explants were extracted by maceration in 100% ethanol. After steeping overnight, the extracts were filtered to remove tissue and the filtrate was dried by distillation under reduced pressure. The total weight of extract was measured, and 100 mg of each extract was subsampled and chromatographed on a Waters C-18 Sep-pak column (900 mg sorbent size). The column was eluted with a step gradient of CH₃CN in H₂O containing 0.1% trifluoroacetic acid (TFA). The fractions that eluted with H₂O-CH₃CN-TFA (60:40:0.1 and 20:80:0.1) were dried and analyzed by HPLC using UV detection at 230 nm. The HPLC method utilized a Vydac C-18 Large Pore Protein and Peptide Column (10 μ, 4.6 mm × 250 mm) eluted with H₂O-CH₃CN-TFA (90:10:0.1) at a flow rate of 1 ml min⁻¹. Stevensine was quantified by comparison to a standard curve (run each day of the analysis).

Clearance Rates

In a series of experiments, we examined the clearance rate of the 3 food sizes by *A. corrugata*. Each food size was examined separately, with each cell concentration (1NC, 3NC, and 5NC) using 3 replicate aquaria with 1 explant each and 3 aquaria with no sponges (controls). The 18 aquaria used in this experiment were each filled with 2 L of seawater, with temperature, salinity, and pH constant at 25°C, 35‰, and 8.1, respectively. Each experiment was done in the dark, and all treatments and replicates were distributed randomly.

Air was bubbled into each aquarium to provide O₂ for the sponges and to keep the food cells in suspension. At intervals of 0 (initial), 1, 2, 3, 4, and 5 hours after cell addition, a 1-ml water sample was taken from each flask and counted using a Coulter Multisizer 3 (Beckman Coulter). At the end of each experiment, the 9 explants were placed in an oven set at 150°C until their dry weights

had stabilized (approx. 12 hours). Using the average cell concentration per treatment, cell clearance was monitored over time. Clearance rate (*c*) of food cells per gram dry weight of sponge was determined by the formula:

$$c = [(V_w t^{-1}) \ln(C_0 C_t^{-1})] g DW^{-1},$$

where V_w represents the volume of water in the flasks (200 ml), and C_0 and C_t are the food cell concentrations at time 0 and time t respectively (Riisgård et al., 1993). Two-sample T tests determined that for the control treatments there was little difference between initial and final cell concentrations, and therefore any changes in concentration in the sponge treatments would result from filtration.

RESULTS

Growth and Survival

Final percentage of weight differed significantly between the 4 diets (1-way ANOVA: $F_{df(3, 32)} = 9.88, P = 0.0001$), being greatest overall for *A. corrugata* explants fed a 3NC diet (Figure 1). On average, 3NC explants grew from 8.5 g to 10.3 g, representing a 22% increase in weight. Growth of 3NC explants was not constant, however, as explants grew quickest during the first half of the study (Figure 1). The percentage weight of 1NC explants varied little during the experiment (Figure 1), with the mean final weight being 101.3% of initial weight. Growth of *A. corrugata* fed the 2 highest cell concentrations was poorest overall. The mean final weights of 5NC and 5+1NC explants were 81% and 87% of initial weights, respectively. *Axinella corrugata* explants fed 5NC and 5+1NC diets had relatively stable weight for the first few weeks of the study (Figure 1). Final percentage weight varied greatly between explants in the same treatment. At the end of the experiment, for example, the percentage weight of explants fed a 3NC diet ranged from 90% to 164%.

All 36 explants cultured in this study survived to the end of the experiment. Explants healed their cut surfaces in less than 2 weeks, and none attached to the horizontal mesh support.

Stevensine Concentration and Production

Stevensine concentration (mg g DW⁻¹ of explant) was highest for 3NC explants (Figure 2) and represented a 110% increase from the initial amount. The large variation

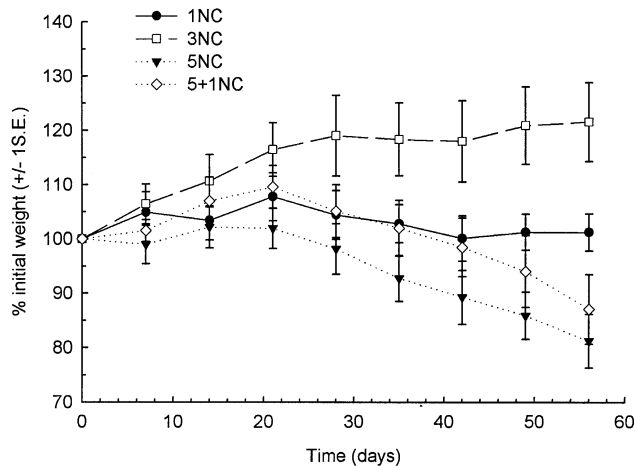


Figure 1. Percentage growth over time of *A. corrugata* explants fed a multispecific diet at different cell concentrations. NC, natural concentration. 1NC is 1–4 μm , 2×10^5 cells ml^{-1} ; 4–10 μm , 2×10^4 cells ml^{-1} ; and 10–50 μm , 3×10^3 cells ml^{-1} . 5+1NC is 5NC of 1–4 μm cells and 1NC of 4–10 and 10–50 μm cells. Error bars represent variation between explants.

in concentration between explants within each treatment (Figure 2), however, meant that there was no significant difference between treatments (1-way ANOVA: $F_{df(4, 10)} = 0.229$, $P = 0.13$). The average concentration of stevensine in the 3 wild sponges (initial treatment) was 193 (1 SE, 58) mg g DW^{-1} .

Stevensine production (total milligrams of stevensine per explant) differed significantly between the treatments (1-way ANOVA: $F_{df(3, 8)} = 6.07$, $P = 0.02$), greatest for the 3NC diet with explants containing an average 59 (SE, 8) mg of stevensine (Figure 3). This represented a 157% increase in stevensine weight per explant from the start of the experiment. Stevensine productions for explants fed the 1NC, 5NC, and 5+1NC diets were similar and varied little from the initial weight (Figure 3).

Clearance Rates

A 2-way ANOVA determined that clearance rates of *A. corrugata* were similar for the examined food types ($F_{df(2, 18)} = 2.63$, $P = 0.1$) and concentrations ($F_{df(2, 18)} = 1.38$, $P = 0.28$). The overall clearance rate of *A. corrugata*, averaged over 27 explants, was $766 \text{ ml h}^{-1} \text{ g DW}^{-1}$. However, individual explants showed great variation in pumping activity, ranging from 171 to $2353 \text{ ml h}^{-1} \text{ g DW}^{-1}$. Regression analysis determined that for *A. corrugata*, wet weight equaled 6.08 dry weight plus 1.03 ($r^2 = 0.662$, $n = 27$).

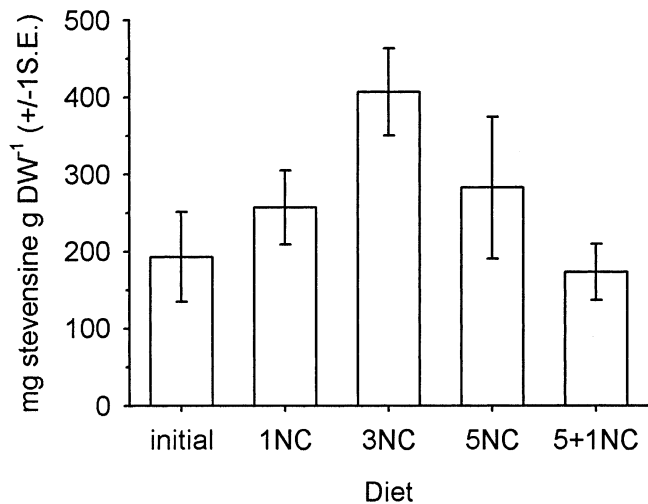


Figure 2. Concentration of stevensine (mg g DW^{-1}) in *A. corrugata* explants fed a multispecific diet at different cell concentrations. The initial or wild stevensine concentration is also shown. See Figure 1 for explanation of codes. Error bars represent variation between explants.

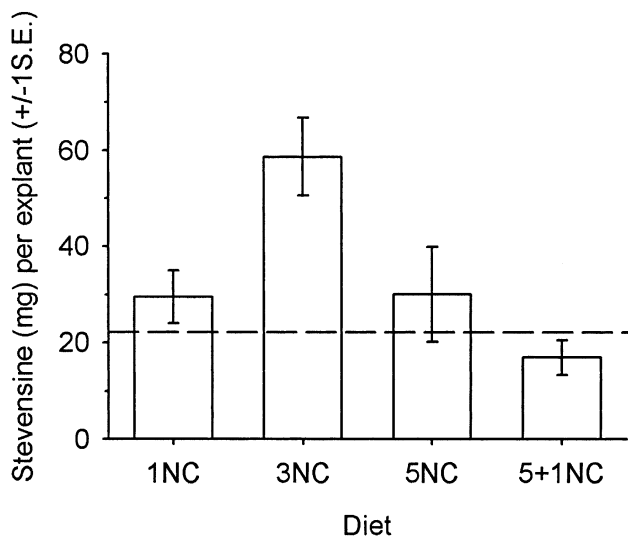


Figure 3. Production of stevensine (total milligrams) per *A. corrugata* explant fed a multispecific diet at different cell concentrations. The horizontal dashed line represents the initial amount of stevensine in explants. See Figure 1 for explanation of codes. Error bars represent variation between explants.

DISCUSSION

Explants of *A. corrugata* were fed a multispecific diet at 4 different concentrations based on the natural concentration of cells 1 to 50 μm in size available to wild *A. corrugata* in the Florida Keys. Food concentration greatly influenced the

culture response of *A. corrugata*, with explants fed a 3NC diet growing on average from 8.5 to 10.3 g in 8 weeks. In comparison, explants fed the 2 highest concentrations (5NC and 5+1NC) lost weight, while explants fed a 1NC diet had relatively constant weight over the study. This indicates that a 1NC diet supplied sufficient energy to meet metabolic costs only. Overall, these results suggest that growth of *A. corrugata* increases as food abundance increases until a threshold is reached at which high cell concentration has a negative impact. A similar response to increasing food concentration has been found for freshwater sponges (Poirrier et al., 1981; Huysecom et al., 1988), although high concentrations in these studies merely arrested sponge growth. High particle concentration can block the aquiferous system of sponges (Gerrodtt and Flechsig, 1979; Huysecom et al., 1988), which may reduce their ability to feed efficiently. In this study, similar clearance rates between cell concentrations and relatively stable weights for explants fed 5NC and 5+1NC diets for the first few weeks indicate that *A. corrugata* can withstand high cell concentrations for a short duration. This may be an ecological adaptation of *A. corrugata* to withstand short-term peaks in particle concentration resulting from plankton blooms or suspension of sediments following storms. The decrease in growth at high particle concentration was observed to be slightly less for 5+1NC explants than for 5NC explants. The 5+1NC diet consists of 5NC of bacteria but only 1NC of larger microalgae and yeasts and may therefore have slightly less of a blocking effect.

Sponges cultured in the laboratory often grow poorly or not at all (Osinga et al., 1997, 2001; Nickel et al., 2001). Considering that poor growth is one factor restricting the commercial development of in vitro sponge culture for the production of pharmaceuticals (Osinga et al., 1999b), the good growth rate of *A. corrugata* in one treatment is a promising result. A multispecific diet generally promotes the highest growth rates in cultured marine invertebrates (Mercer et al., 1993; Brown et al., 1998) because it provides essential vitamins and nutrients that may be deficient in a monospecific diet. Growth of the sponge *Halichondria melanadocia* is greater on a mixed diet of bacteria and microalgae than on a diet consisting solely of bacteria (A.R. Duckworth and S.A. Pomponi, unpublished data). The multispecific diet consisting of 5 bacterial strains, 2 microalgae species, and yeast fed to *A. corrugata* likely promoted growth of explants in the 3NC treatment. Although 3NC explants grew reasonably well in this study, sponges farmed in the sea generally grow faster. For ex-

ample, *Latrunculia wellingtonensis* and *Polymastia croceus* farmed in the sea grew by an average of 960% and 730% of their initial volume, respectively, in 6 months (Duckworth, 2001). In comparison, the estimated final weight of 3NC explants, if cultured for 6 months, would be approximately 180% of their initial weight. These differences probably result from interspecific variation in growth rates or indicate environmental or nutritional deficiencies with in vitro culture.

Explants in the same feeding regimen and thus experiencing similar culture conditions varied greatly in final weight. Variable growth among sponges grown in similar conditions is common to many species (Thompson et al., 1987; Duckworth et al., 1997), and it may result from differences in initial explant health (Thompson et al., 1987) or filtration rates. Explants fed at 3NC also showed variable growth over time, with growth rates greater in the first half than in the second half of the study. Osinga and colleagues (1999a) observed a similar growth pattern for the cultured Demospongiae *Pseudosuberites* aff. *andrewsi* and suggested that it may result from food limitation as the explants grew. Many sponges respond to tissue damage by growing rapidly (Ayling, 1981, 1983), and this may also explain the fast growth of some *A. corrugata* shortly after being cut into explants.

The increase in the concentration of stevensine, like growth, was highest for *A. corrugata* explants fed a 3NC diet. Increased production of both sponge biomass and target metabolite is a promising result for commercial in vitro sponge culture because it would lead to maximum metabolite yield. Because growth and metabolite biosynthesis both require energy, marine organisms may allocate energy to one process at a cost to the other (reviewed by Cronin, 2001). This study shows that under good conditions marine organisms can invest in both growth and metabolite biosynthesis. Stevensine concentrations in *A. corrugata* fed the other diets remained relatively unchanged after 8 weeks. Considering the 5NC and 5+1NC diets, this indicates that biosynthesis of stevensine was largely unaffected by the explants' losing weight. Many factors can affect the biosynthesis of metabolites in marine invertebrates, including light intensity (Thompson et al., 1987), individual size (Becerro et al., 1997), and season (Turon et al., 1996). This study shows that food concentration can also influence metabolite biosynthesis.

Stevensine concentration in 3NC explants increased by 110% after 2 months. Pomponi and colleagues (1997) also recorded a doubling in stevensine concentration in ar-

chaeocytes of *A. corrugata* (*Teichaxinella morchella*) after 8 days in cell culture, showing that *A. corrugata* can quickly increase production of stevensine. It is unknown whether stevensine concentration in 3NC explants in this study peaked within a few days and then stabilized, or increased gradually over the experiment. The average concentration of stevensine in wild *A. corrugata* was determined to be 193 mg g DW⁻¹ which equates to 32 mg g wet weight⁻¹. If we assume that 1 g wet weight equals 1 ml of sponge, then the natural concentration of stevensine found here is comparable to a study by Wilson and colleagues in 1991 (average, 19 mg ml⁻¹).

All of the 36 explants cultured in this study survived, and none showed signs of microbial infection problematic in some in vitro sponge studies (Ilan et al., 1996; Osinga et al., 1997; Nickel et al., 2001). Explants of *A. corrugata* were on average 8.5 g in size and generally larger than explants used in other studies (e.g., 1–3 ml, Barthel and Theede, 1986; 50 mm³, Nickel et al., 2001). Large explants would have greater energy reserves than small explants and thus possibly more ability to fight microbial infection, leading to higher survival. The greater energy reserves in large explants can also promote healing of the cut surfaces and a more rapid reorganization of their aquiferous system (Duckworth et al., 1997).

The clearance rate of *A. corrugata*, averaging 766 ml h⁻¹ g DW⁻¹, is comparatively low but still within the range found for other sponge species (Ribes et al., 1999: Table 5). Differences in clearance rates between species may result from differences in retention rates, sponge size, water temperature, and habitat (Reiswig, 1974; Frost, 1980; Risgård et al., 1993; Ribes et al., 1999). The clearance rates of *A. corrugata* were similar among the different cell sizes and concentrations. These results agree with other studies that have found that clearance rates of sponges are largely unaffected by cell size or concentration, within the ranges examined here (Reiswig, 1971; Frost, 1980; Francis and Poirrier, 1986; Ribes et al., 1999).

Determining suitable feeding regimens is one factor vital for the development of in vitro sponge culture to supply bioactive metabolites. This study shows clearly that relatively small changes in food concentration can greatly influence sponge growth and metabolite biosynthesis. The nutritional requirements to maximize production will undoubtedly vary between sponge species. Determining the natural cell concentration for each species, however, will provide important information when selecting appropriate cell concentrations. The good growth and increased pro-

duction of the target metabolite stevensine for *A. corrugata* explants fed a 3NC diet suggests that in vitro culture is a viable method of supplying some sponge metabolites.

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