MESOFaUNA ASSOCIATED WITH THE SUBTROPICAL/TROPICAL MARINE SPONGE *AMPHIMEDON VIRIDIS*: ARE FAUNAL ASSOCIATIONS LIKELY TO PROVIDE REFUGE FROM FISH PREDATION DUE TO PHYSICAL OR CHEMICAL FEEDING DETERRENT PROPERTIES OF THIS SPONGE?

by

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A THESIS

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MESOFAUNA ASSOCIATED WITH THE SUBTROPICAL/TROPICAL MARINE SPONGE *AMPHIMEDON VIRIDIS*: ARE FAUNAL ASSOCIATIONS LIKELY TO PROVIDE REFUGE FROM FISH PREDATION DUE TO PHYSICAL OR CHEMICAL FEEDING DETERRENT PROPERTIES OF THIS SPONGE?

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ABSTRACT

Sessile benthic marine organisms such as sponges are frequently colonized by a wide diversity of invertebrates. The present study focused on a quantitative analysis of the mesofauna associated with the common subtropical/tropical sponge *Amphimedon viridis* and explored several criteria for the basis for this mesofauna-sponge relationship. Specimens of *Amphimedon viridis* were hand-collected individually in bags from both Saint Joseph Bay, Florida Panhandle, and Sugar Loaf Key, Florida Keys between April - November 2006. Quantitative analyses of sponge surfaces and interiors revealed that the most common sponge-associated group was comprised of amphipods, followed by polychaetes and tenaid crustaceans. 35 percent of sponge-associated individuals were recovered from the interstices of the sponge. A total of 707 individuals per 1 cm$^3$ of sponge material were found within 15 sponge samples of *A. viridis*. Shannon diversity and evenness indices indicated that *A. viridis* supports a diverse community of sponge-associated invertebrates including amphipods, polychaetes, tenaids, decapods, and isopods. These sponge-associated invertebrate groups may occupy sponges for a number of reasons. For example, physical or chemical protection from fish predation may impose a strong selective pressure that sustains these relationships. Moreover, nutritional
needs, reproductive behaviors, and social interactions could also contribute to these sponge associations.

In order to evaluate whether *Amphimedon viridis* provides a refuge from predation for sponge-associated mesofauna, laboratory feeding experiments were conducted to assess the palatability of *A. viridis* to the generalistic sympatric pinfish *Lagodon rhomboides*. In feeding assays employing small pieces of sponge tissue and control squid tissue, pinfish displayed a strong significant rejection for sponge tissues when compared with control squid tissues. Alginate food pellets loaded with ecologically relevant concentrations of spicules isolated from *A. viridis* caused a weak but significant deterrent response in pinfish when compared to food pellets lacking spicules. However, alginate food pellets containing tissue-level concentrations of lipophilic and hydrophilic sponge extracts were highly deterrent to pinfish when compared to control food pellets. It is concluded that while both physical and chemical characteristics of *A. viridis* may contribute to its quality as a habitat resource, potent secondary metabolites, likely halotoxins and amphitoxins, play a particularly important role in providing a chemical refuge for associated mesofauna.
ACKNOWLEDGEMENTS

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INTRODUCTION

Symbiosis is characterized generally as a phenomenon whereby two or more species exhibit an intimate and prolonged relationship. Such symbiotic relationships can be categorized into three basic types: mutualistic (both organisms benefit), parasitic (one organism does harm to the other), and commensalistic (one organism benefits, the other is neither harmed nor benefited). Understanding these relationships is significant because they have important ramifications in understanding processes that determine the distribution and abundance of individuals comprising communities.

Symbiotic associations are well documented in a variety of sessile marine invertebrates. Perhaps the most well known symbiotic relationship is exemplified by the scleractinian hard corals and the associated zooxanthellae that reside in the endoderm of each polyp. The associated zooxanthellae are photosynthetic and thus able to provide fixed carbon to the coral polyps (Swart, 2005). Remarkably, a significant percentage of the energy budget of the coral may be derived from this symbiotic relationship. The coral, in turn, provides a source of nitrogen (from its nitrogenous wastes) and a protective habitat for the associated zooxanthellae.

Both freshwater and especially marine sponges also have symbiotic relationships with micro- and macro-organisms including algae, bacteria, cyanobacteria, polychaete worms, amphipods, isopods, brittlestars, and even other sponge species (e.g., Pawlik 1982, Oshel & Steele 1985, Rai 1990, Frost et al. 1997, Althoff et al. 1998, Friedrich et al. 1999, Bieler 2004, Thakur & Muller 2005, Henkel & Pawlik 2005). Similar to scleractinian corals with their symbiotic zooxanthellae,
sponges and cyanobacteria have similar symbiotic relationships (Rai 1990, Usher et al. 2005). Moreover, cyanobacteria not only produce photosynthetic products exploited by sponges, but they have also been shown to produce defensive secondary metabolites that defend their host sponge (Rai 1990, Thakur & Muller 2005). In turn, sponges provide nutrients (Saller 1989) and a secure habitat for their associated cyanobacteria. Through this mutualistic relationship, sponges with cyanobacterial symbionts are likely to allocate more energy to growth and, presumably, reproduction (Usher et al. 2005). The two examples given above illustrate the adaptive significance of symbiotic relationships in sessile marine organisms. Given their significance, it is likely that such relationships flourish between sessile macro-invertebrates and a variety of bacterial and invertebrate symbionts. Much remains to be learned about these interactions and their role in marine ecosystems.

Many benthic sessile organisms employ defensive strategies including chemical and structural defenses, physical defenses such as tissue toughness or skeletal elements, or a reduction in nutritional value. Marine sponges are particularly intriguing because they provide a combination of defensive strategies including both chemical (secondary metabolites), and structural defenses (spicules) (Chanas and Pawlik 1995, Jones et al. 2005, Clavico et al. 2007). Sponges are generally soft bodied organisms that are reasonably nutritious as prospective prey (mean of 20.7 mg soluble protein per cubic ml sponge tissue, N = 71 sponge species; Chanas and Pawlik 1995). Known sponge predators include vertebrates such as fish (e.g. Randall and Hartman 1968, Dunlap and Pawlik 1996, Jones et al. 2005, Wulff 2006) and turtles (Meylan 1988, 1990), and primarily, invertebrates including nudibranchs.

Demonsponges may possess a variety of structural elements including siliceous and calcium carbonate spicules (Chanas and Pawlik 1995, Hooper and Van Soest 2002, Jones et al. 2005, Clavico et al. 2007) as well as proteinaceous spongin fibers (Bergquist 1978). Chanas and Pawlik (1995) proposed that sponge spicules may be an effective structural defense against some generalist predators, but are ineffective against specialist predators (1995, Randall and Hartman 1968, Meylan 1998). However, the effectiveness of spicules or sclerites as structural defenses against predators is not clear cut. For example, Harvell et al. (1988) found that the calcitic sclerites from the gorgonian soft coral *Pseudopterogorgia acerosa* inhibited feeding in many predatory fishes (i.e., blue headed wrasse *Thalassoma bifasciatum*, yellow head wrasse *Halichoeres garnoti*, clown wrasse *Halichoeress maculipinna* etc) while Chanas and Pawlik (1995) found that food pellets containing spicules of eight demosponges were palatable to the Caribbean generalist wrasse *Thalassoma bifasciatum*. Moreover, Burns et al. (2003) reported that food pellets containing natural concentrations of spicules of four Caribbean sponges and two Red Sea sponges all deterred feeding in the generalist Red Sea wrasse, *Thalassoma klunzingeri*. Interestingly, Burns et al. (2003) conducted fish feeding experiments by offering food pellets containing both natural concentrations of sponge crude organic extract and spicules (*Crella cyatophora*) and demonstrated that these food pellets were significantly more deterrent to *T. bifasciatum* than either food pellets containing
only extract or spicules. Thus, in this study chemical and structural factors acted in a
synergistic rather than an additive fashion. Clearly, additional studies are needed to
evaluate the ability of sponge spicules to deter feeding in grazing predators such as
generalist fish.

The prevailing theory for the role of sponge secondary metabolites (whether
produced by the sponge itself or its microbial symbionts) is that they act as chemical
defenses to deter predation (e.g., Pawlik et al. 1995, Assmann et al. 2000, McClintock
and Baker 2001, Burns et al. 2003). There are, though, several other potential roles
for sponge chemical defenses including alleopathy (spatial competition) (e.g., Engel
and Pawlik 2000), inhibition of bacterial colonization and fouling by algae and
invertebrates (e.g., Kelman et al. 2001, Kelly et al. 2003), and protection from
of chemical anti-predatory defenses and found that 49 out of 71 Caribbean sponge
species (69%) were rejected by a generalist fish (the Caribbean blue headed wrasse
*Thalassoma bifasciatum*) when presented to fish as food pellets containing a feeding
stimulant and organic extracts of sponges. Interestingly, small pieces of the same 71
sponges when offered to the same fish were all rejected, suggested that fish may have
been deterred by factors other than chemistry for those sponges with palatable
extracts.

Crustacean mesograzers are commonly found in association with benthic
seaweeds and marine seagrasses (Duffy et al. 2000, Dauby et al. 2001). However,
mesograzers have also been found to associate with and graze on sessile marine
invertebrates including sponges (e.g., Pawlik 1982). Cruz-Rivera and Hay (2000)
conducted a study to evaluate food choice and fitness in marine mesograzers. They found that while certain crustacean mesograzers do not prefer to graze on sponges, they will do so when their preferred diet of algae was removed. In a study conducted by Rios and Duffy (1999), the shrimp *Synalpheus williamsi* was found to reside in and also graze upon its chemically defended host sponge, *Hymeniacidon caerulea*. This behavior has also been displayed similarly in other crustacean mesograzers but on different hosts (Hay et al. 1988, Duffy and Hay 1994, Wulff 2006). In contrast, some host sponges may not directly provide nutritional resources to meso-crustacean commensals. For example, Shuster (1989) found that the isopod *Paracerceis sculpta* resides within the bread crumb sponge *Leucetta losangelensis* yet does not appear to feed on the sponge; rather it exploits it as a site for courtship and possibly a physical refuge from predators.

The shallow benthic environment of Saint Joseph Bay, Florida provides an opportunity to investigate a model sponge-meso- and macroinvertebrate associated system. Here, in both near and offshore waters the marine sponge *Amphimedon viridis* occurs in seasonal abundance (Y. Huang, personal observation). *Amphimedon viridis* has a broad biogeographic range including a distribution predominately throughout the Caribbean, but it also occurs in regions of the Red Sea and Micronesia (Burns et al. 2003). It is known to possess potent defensive compounds including a purified mixture of halitoxin and amphitoxin, both highly bioactive pyridinium alkaloids (Kelman et al. 2001). Burns et al. (2003) demonstrated that chemical extracts of *A. virdis* are highly deterrent to two species of sympatric wrasse.
Within soft bottom habitats of Saint Joseph Bay there are a variety of crustacean mesograzers that have been qualitatively observed to occur in association with the sponge *A. viridis* (Y. Huang, unpublished observation). Moreover, there are a number of prospective generalist fish consumers that include small crustaceans and possibly sponges in their diets, especially the common pinfish, *Lagodon rhomboides* (Heck et al. 2000).

**Study Objectives**

The present study broadly evaluates the prospective role of the common tropical/sub-tropical sponge *Amphimedon viridis* to serve as a habitat for associated mesofauna, with a primary focus on mesocrustaceans. The study also examines the question of whether sponge-associations are possibly related to the provision of structural or chemical refugia from fish predators. The common sympatric omnivorous pinfish *Lagodon rhomboides* is used as a model predator.

In order to address this broad study objective the following were determined: 1) the abundance, species evenness, species richness and species diversity (and their respective intraspecific variability) of sponge-associated meso- and macroinvertebrate fauna within a population of *A. viridis* collected from Port Saint Joseph Bay, Florida, and 2) the potential role of *A. viridis* in providing structural and/or chemical refuge from fish predation to its associated meso- and macroinvertebrate fauna through an evaluation of the ability of this sponge to deter fish predation by physical (spicule) and/or chemical (lipophilic or hydrophilic secondary metabolite) defenses.
MATERIALS AND METHODS

Sponge Collections and Processing

Fifteen individuals of the common demosponge *Amphimedon viridis* were collected from shallow seagrass habitats of Saint Joseph Bay, Florida (N29°36.032 W085°24.264) in April 2006. In addition, ten individuals of *A. viridis* were collected in October 2006 from a shallow (1-2 m depth) soft bottom sea grass habitat near Sugarloaf Key, Florida (N24°38.255 W°81 34.779 and N24 39.639 W81 26.194). This collection was necessary due to the seasonal ephemeral nature of the population in Port Saint Joseph Bay making collections impossible during fall and winter months. Each individual sponge was collected by hand while snorkeling. Sponges were gently removed from the substrate and placed immediately into a sealed zip-lock baggie in order to ensure that macroinvertebrate sponge-symbionts associated with each individual sponge were retained. Sponges were transported on ice in a cooler to the laboratory at the University of Alabama at Birmingham where they were each weighed (wet wt) and their volume determined by volumetric displacement of seawater in a graduated cylinder. Sponges from Port Saint Joseph Bay were dissected while fresh (see below). Sponges from the Florida Keys were frozen at -20°C until used for organic extract preparations and used to isolate spicules for feeding deterrent bioassays (see below).

Fish Procurement and Maintenance
Twenty pinfish (*Lagodon rhomboides*) (mean fish size = 10 cm length) were procured from the Gulf Specimen Marine Lab (Panacea, FL 32346, phone (850) 984-5297). Fish were maintained in the laboratory at 25° C at densities of 2 fish per 9 gallon aquarium, each equipped with recirculating artificial seawater (33 ppt) and a charcoal filter system (Tetra Whisper® Biobags). Fish were fed a standard maintenance diet consisting of approximately 2 dry g commercial fish food flakes (Tetramarine) per fish per week prior to feeding experiments.

**Sponge dissection and associated symbiont enumeration and identification.**

The fifteen sponges collected from Saint Joseph Bay, Florida were removed from zip-lock bags and dissected while fresh using a dissection microscope (Nikon Model 77041). All meso- and macroinvertebrate sponge associates found on the surface and within the interstices of each sponge were recorded and placed into 70% ethanol for enumeration and identification. Vouchers of meso-crustaceans were shipped to Dr. Sara E. LeCroy, University of Southern Mississippi, Gulf Coast Research Laboratory, for taxonomic identification or verification. Mean numbers of meso- and macroinvertebrate sponge associates (total associate load) and the mean percent composition of each associated species were determined for the Port Saint Joseph sponge population. Species diversity (Shannon diversity index), species evenness (Shannon evenness index – higher values reflect less variation between numbers of associated species), and species richness (total numbers of associated faunal species per sponge) values for sponge associates within the sampled sponge population were also determined. The Shannon diversity index (Shannon and Weaver
1949) was defined as \( H' = \sum_{i=1}^{s} (p_i \ln p_i) \) where \( s \) is the number of species in the sample and \( p_i \) is the proportion of the organisms in the sample represented by the \( i \)th species. The Shannon evenness index was calculated as \( E_H = H'/H'_{\text{max}} = H'/\ln S \) where \( S \) is the number of species in the sample, \( H' \) is the Shannon Diversity Index, and \( H'_{\text{max}} \) is the maximum value of \( H' \).

**Fish feeding deterrent assays using frozen sponge material**

Small, bite-size, spherical pieces (2 mm diameter) of frozen whole sponge tissue were presented to individual pinfish. The outcome of the feeding trial was scored as either 1) accepted (pinfish ingested and swallowed the item), or rejected (pinfish mouthed the item and then spit it out). Immediately following the presentation of each small piece of sponge tissue, each fish was similarly presented a control food item (piece of fresh squid tissue similar in shape and size to the sponge tissue). If fish rejected the control food then this fish was considered satiated and the trial was not included in the data base subjected to statistical analysis. At least ten replicate trials were conducted for each experimental treatment using a different pinfish for each trial. A Fisher’s Exact Test was used to evaluate whether there was significant rejection of fresh sponge tissues when compared with control pieces of squid tissue.

**Preparation of sponge spicules**

A small piece from six individual sponges was haphazardly selected from sponges collected from the Florida Keys and Port Saint Joseph Bay. Based on
preliminary quantitative determinations of the variation in the spicule content (mass)
between individual sponges (mean 48.3 mg/ml; range 42 mg/ml to 55 mg/ml; n=6),
this sample size was deemed representative. A volume of 10 cubic cm of sponge
tissue (taken consistently from within 1-cm of the sponge surface) was determined by
seawater displacement. Each 10 ml volume of sponge was then placed into a separate
glass test tube and filled with 20 ml of standard chlorine bleach (sodium hypochlorite,
5.25%). Once the bleach solution had stopped bubbling (approximately 5 h), the
suspension was centrifuged, the supernatant was discarded and fresh bleach was
added to the spicule preparation. This process was repeated three times until the
addition of bleach yielded no more bubbles, yielding a pure pellet of spicules at the
bottom of each test tube. The spicule pellet was then washed 3 times with distilled
water in order to remove any remaining bleach. The pellet was then transferred to a
25 ml glass scintillation vial. The natural concentration of the spicules in the sponge
tissue was calculated by dividing the dry weight of the spicule mass by the wet
weight of the sponge tissue dissolved in bleach. This ratio was then used to prepare
alginate feeding pellets containing ecologically relevant concentrations of spicules for
fish feeding deterrent assays.

**Sponge extraction and preparation of crude organic extracts.**

The ten sponges collected from the Florida Keys population were frozen,
weighed and then freeze dried using a lyophilizer. Dry weights of each freeze dried
sponge was then determined and the sponge broken into small pieces by hand (∼1
cm³). The pieces of sponges from all 10 individuals were then combined for the
extraction. Sponge pieces were placed into a 1000 ml glass beaker and subject to three exchanges of a 1:1 mixture of dichloromethane (DCM) and methanol yielding lipophilic extract. Each exchange lasted 24 h and was conducted at room temperature (25°C). The lipophilic extracts were then filtered (Whatman Qualitative #1, 125 mm diameter pore size filter paper), evaporated to dryness using a rotary evaporator, and combined. To ensure the complete removal of the 1:1 mixture of dichloromethane:methanol, the combined extracts were placed in speed vacuum and spun for 24 h. The dry weight of the final combined extract was determined.

Following the preparation of the lipophilic extract, a hydrophilic extract was prepared by subjecting the extracted sponge material to three exchanges of 1:1 methanol:water. The hydrophilic extracts filtered, evaporated to dryness on the rotary evaporator, combined and then weighed. Both crude organic extracts were stored at -20°C in a glass scintillation vial until further use. The natural concentration of the crude organic metabolites extracted from the sponge tissues was calculated by dividing the dry weight of the crude organic extract by the total wet weight of the sponges from which it was derived. This ratio was used to prepare alginate feeding pellets containing ecologically relevant concentrations of secondary metabolites for the fish feeding deterrent assays.

**Preparation of alginate food pellets containing sponge spicules or extracts**

Alginate food pellets containing spicules or organic extracts were prepared using a modification of the procedures of Burns et al. (2003). Control alginate pellets were prepared by combining 0.3 dry g of alginic acid with 0.5 g freeze-dried
powdered squid mantle tissue as a feeding stimulant and adding distilled water to bring the final volume to 10 ml. This solution was then taken up in a 3 ml syringe, the tip submerged into a 0.25 M solution of calcium chloride solution, and then ejected to form a long string of hardened alginate. This string was then cut with a razor into small pellets each measuring 3 mm in length. Experimental pellets containing either spicules or organic extracts were prepared using the same procedure but with the addition of natural concentrations of sponge spicules or extracts.

**Fish feeding assays with alginate food pellets**

Control and experimental food pellets were presented to individual pinfish. In each trial, fish (each first isolated in the tank) were first presented with a control food pellet to ensure they were sufficiently hungry to feed. If a fish did not consume the control pellet, the fish was not used in the experiment. Following consumption of the control pellet, the fish was presented an experimental food pellet containing either spicules or an organic extract. If this pellet was rejected, then a subsequent control pellet was presented to the fish as a satiation control. If this final control pellet was not consumed then this entire feeding trial was not included in the experimental treatment. In addition, a second solvent control was conducted for only the chemical feedings deterrent bioassays. Here fish were presented alginate pellets containing squid mantle homogenate that had been treated with the same amount of solvents used in the preparation of either lipophilic or hydrophilic extracts. The solvent controls were then used to statistically compare acceptance rates between experimental and control pellets. Alginate pellets containing lipophilic and
hydrophilic crude organic sponge extracts were tested on different days. No fish were presented any given treatment more than once and at least 10 fish were tested in each treatment. Differences in rejection of experimental and control pellets were evaluated using a Fisher’s Exact Test.

RESULTS

Sponge associates

Analysis of the sponge-associate loads from the 15 individual sponges collected from Port Saint Joseph Bay, Florida revealed a meso- and macroinvertebrate faunal community comprised, from most to least abundant in terms of total numbers, amphipods, tenaids, polychaetes, decapod crabs, isopods and tunicates (Table 1). A total of 707 sponge-associated individuals were found living within or on the surface of the 15 individual *Amphimedon viridis*. Furthermore, 35 percent of sponge-associated individuals were found within the sponge while 65 percent of sponge-associated individuals were recovered from the surface of the sponge (Table 2). When standardized to numbers of sponge-associates based on a volumetric measure of sponge tissue, the abundance (total associate load) values ranged from 19 to 121 individuals per 100 cm$^3$ of wet sponge tissue. The mean number ± 1 SD of individuals was 53 ± 36 per 100 cm$^3$ of wet sponge tissue. The most common faunal sponge associated groups were amphipods (78% of total fauna). The amphipod *Laticorophium baconi* (family Corophiidae) comprised the greatest fraction of the amphipods (62% of total amphipods). Similarly, this amphipod species also comprised the largest fraction of the sponge-associated invertebrate fauna (52% of
total fauna) and was the numerically dominant sponge associate in all but two of the fifteen individual sponges. Other amphipods that occurred in association with the sponges included *Elasmopus levis*, (Family Melitidae), *Ericthonius brasiliensis*, and *Bemlos setosus* (Family Aoridia). Next most common were three representatives of the Polychaeta (*Diopatra cuprea* and one unknown tube building serpulid and one errant species) (14% of total fauna). The next most common sponge associate group was the tenaid crustaceans (3% of the total fauna) comprised of *Hargeria rapax* (all females keyed out to this species) and possibly the tenaid *Leptochelia dubia* in the case of males. The crab, *Libinia dubia* was also widespread across the sponge population with 66% of the 15 sponges having a single crab present. Surprisingly, very few species of isopods occurred in sponges. The only isopod identified was *Paracerceis caudata* (Family Sphaeromatidae) and it occurred in low numbers.
Table 1: Abundances of meso- and macroinvertebrates associated with a population of the sponge *Amphimedon viridis* collected from Saint Joseph Bay, Florida in October 2006. Abundances for the 15 sponges presented in the table have been standardized to numbers of associates per 100 cm\(^3\) wet sponge mass.

<table>
<thead>
<tr>
<th>Sponge #</th>
<th>Amphipod</th>
<th>Isopod</th>
<th>Tenaid</th>
<th>Crab</th>
<th>Polychaete</th>
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<td></td>
<td><em>Laticorophium baconi</em></td>
<td><em>Elasmopus levis</em></td>
<td><em>Ericthonius brasiliensis</em></td>
<td><em>Bemlos setosus</em></td>
<td><em>Paracerceis caudate</em></td>
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<tr>
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Table 1 (continued): Abundances of meso- and macroinvertebrates associated with a population of the sponge *Amphimedon viridis* collected from Saint Joseph Bay, Florida in October 2006. Abundances for the 15 sponges presented in the table have been standardized to numbers of associates per 100 cm³ wet sponge mass.

<table>
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<tr>
<th>Sponge #</th>
<th><em>Laticorophium baconi</em></th>
<th><em>Elasmopus levis</em></th>
<th><em>Eriochonius brasiliensis</em></th>
<th><em>Bemlos setosus</em></th>
<th><em>Paracerceis caudate</em></th>
<th><em>Hargeria or Leptochelia</em></th>
<th><em>Libinia dubia</em></th>
<th><em>Copepod</em></th>
<th>Unknown Errant Polychaete</th>
<th>Unknown Serpulid</th>
<th><em>Diopatra cuprea</em></th>
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<td>44</td>
</tr>
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<td>19</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>12</td>
<td>28</td>
<td>0</td>
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<td>4</td>
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<td>52</td>
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<td>12</td>
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<td>26</td>
<td>3</td>
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<td>2</td>
<td>0</td>
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<tr>
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<td>26</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>4</td>
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<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>45</td>
</tr>
</tbody>
</table>


**Table 1 (continued):** Abundances of meso- and macroinvertebrates associated with a population of the sponge *Amphimedon viridis* collected from Saint Joseph Bay, Florida in October 2006. Abundances for the 15 sponges presented in the table have been standardized to numbers of associates per 100 cm$^3$ wet sponge mass.

<table>
<thead>
<tr>
<th>Sponge #</th>
<th>Laticorophium baconi</th>
<th>Elasmopus levis</th>
<th>Ericthonius brasiliensis</th>
<th>Bemlos setosus</th>
<th>Paracerceis caudate</th>
<th>Hargeria or Leptochelia</th>
<th>Libinia dubia</th>
<th>Copepod</th>
<th>Unknown Errant Polychaete</th>
<th>Unknown Serpulid</th>
<th>Diopatra cuprea</th>
<th>Unknown Tunicate</th>
<th>Total Individuaals</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>19</td>
<td>14</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>41</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>49</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
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<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>61</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>26±26</td>
<td>10±14</td>
<td>2±4</td>
<td>0.7±3</td>
<td>0.8±2</td>
<td>2±2</td>
<td>0.2±0.6</td>
<td>2±2</td>
<td>0.2±2</td>
<td>3±4</td>
<td>0.6±2</td>
<td>0.1±0.3</td>
<td>53±36</td>
</tr>
</tbody>
</table>
Table 2: Numbers and percentages of total amphipods found occurring on the surfaces versus the interiors of ten individuals of the sponge *Amphimedon viridis*. Data are presented as total sponge associates per 100 cm³ wet sponge weight and only for those individual sponges with >10 amphipods (as initial raw data).

<table>
<thead>
<tr>
<th>Sponge #</th>
<th>Percent within the sponge</th>
<th>Percent on the surface of the sponge</th>
<th>Total number of amphipods</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32</td>
<td>68</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>65</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>60</td>
<td>72</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>62</td>
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</tr>
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<td>32</td>
<td>68</td>
<td>103</td>
</tr>
<tr>
<td>6</td>
<td>30</td>
<td>70</td>
<td>33</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>50</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>38</td>
<td>62</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>78</td>
<td>36</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>65</td>
<td>52</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>35±7</td>
<td>65±7</td>
<td>51±26</td>
</tr>
</tbody>
</table>

Shannon diversity indices calculated for the fifteen individual sponges collected from the population at Port Saint Joseph Bay indicated that values ranged from a low of 0.65 to a high of 1.39 (mean = 1.08±0.23) (Table 3). Shannon evenness indices ranged from 0.40 to 0.96 (mean = 70±0.17). Sponge-associated species richness values ranged from 3 to 9 species with a mean of 5.1±1.5 species across the 15 sponges.
Table 3: Shannon diversity index, Shannon evenness index and species richness calculated for 15 sponge samples of *Amphimedon viridis*.

<table>
<thead>
<tr>
<th>Sponge #</th>
<th>Shannon Diversity Index</th>
<th>Shannon’s Evenness Index</th>
<th>Species Richness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.04</td>
<td>0.95</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>1.33</td>
<td>0.96</td>
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</tr>
<tr>
<td>3</td>
<td>1.17</td>
<td>0.84</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>1.39</td>
<td>0.87</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>1.13</td>
<td>0.63</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>0.65</td>
<td>0.40</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>0.81</td>
<td>0.59</td>
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<td>8</td>
<td>1.19</td>
<td>0.54</td>
<td>9</td>
</tr>
<tr>
<td>9</td>
<td>1.07</td>
<td>0.77</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>0.85</td>
<td>0.77</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>1.26</td>
<td>0.79</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>0.98</td>
<td>0.55</td>
<td>6</td>
</tr>
<tr>
<td>13</td>
<td>1.26</td>
<td>0.70</td>
<td>6</td>
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<tr>
<td>14</td>
<td>1.33</td>
<td>0.74</td>
<td>6</td>
</tr>
<tr>
<td>15</td>
<td>0.78</td>
<td>0.43</td>
<td>6</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>1.08±0.23</td>
<td>0.70±0.17</td>
<td>5.1±1.5</td>
</tr>
</tbody>
</table>

**Fish feeding deterrent properties of sponges**

Small pieces of fresh frozen tissues excised from each of ten individuals of *Amphimedon viridis* were highly deterrent (P = 6 x 10^-9) to the omnivorous pinfish *Lagodon rhomboides* (Figure 1). In no instances were any of the sponge pieces presented to fish ingested. Instead, fish would generally approach the introduced piece of sponge, place it within their mouth, and then rapidly spit it out. In a given feeding trial, individuals did not mouth the sponge piece more than a single time. Controls consisting of similar sized pieces of squid tissue were always ingested.

In contrast, pinfish presented alginate food pellets containing natural volumetric concentrations of monaxon spicules (mean length and standard deviation = 4.2 ± 0.2 µm, n = 5) isolated from *Amphimedon viridis* displayed a weak but
statistically significant ($P = 0.045$) deterrent response (Figure 1). Thirty percent of the alginate spicule pellets were rejected by fish, while all control alginate pellets containing a feeding stimulant only (homogenized squid mantle) were readily ingested. Fish presented alginate control food pellets responded to them in a fashion similar to small pieces of squid mantle. In contrast, alginate pellets containing spicules were often mouthed by individual fish, chewed slightly, and then spit out. Fish did not return to mouth pellets a second time, primarily because the pellets disintegrated after the initial mouthing.

Alginate food pellets containing both hydrophilic and lipophilic extracts of *Amphimedon viridis* were strongly deterrent to pinfish ($P = 0.00001$ and $P = .00082$, respectively). Fish consumed all alginate food and solvent control pellets in both sponge extract feeding bioassays. In contrast, pellets containing chemical sponge extracts were mouthed and generally spit out. In some cases, upon pellet rejection it was apparent that the fish had broken up the pellet in their mouth prior to it being spit out. In no instances did the fish return to try and consume intact or pieces of pellets after initial tasting.
Figure 1: Histogram showing the percentages of pieces of fresh sponge (*Amphimedon viridis*) tissue or control food (squid mantle tissue) consumed by the pinfish *Lagodon rhomboides*. Also shown are the percentages of alginate food pellets containing natural concentrations of sponge spicules versus control alginate food pellets lacking spicules consumed by pinfish. (n=13). Percentages of alginate food pellets containing natural concentrations of lipophilic and hydrophilic extracts from the sponge *Amphimedon viridis* and solvent control pellets lacking sponge extracts consumed by pinfish are presented on the right of the histogram. (n=15) Single and double asterisks indicate statistical significance at the 0.05 and the 0.01 levels, respectively (Fisher’s exact test). All experimental treatments are depicted in white bars, and all control treatments in black bars.
DISCUSSION

The population of *Amphimedon viridis* sampled from the shallow subtropical waters of Port Saint Joseph Bay, Florida was found to possess a moderately diverse assemblage of sponge-associated mesofauna. A total of 12 meso- or macroinvertebrate taxa with a mean species richness value across all fifteen sponges of $5.1 \pm 1.5$ were found living either on the surfaces or the internal interstices of the fifteen individual sponges examined. When standardized to abundances of sponge-associates per unit volume sponge, the range of densities (19 to 121 individuals per 100 cm³ wet sponge tissue) is an order of magnitude higher than densities of endofauna found to occur in association with two species of the tropical sponge *Haliclona* sp. (0.004 to 4.4 individuals per 100 cm³ wet sponge; Abdo 2007). Even if only the endofauna found in association with *A. viridis* are considered (4.4 to 39 individuals per liter sponge), these abundances are still an order of magnitude higher when compared with that of *Haliclona* sp. Shannon diversity values of sponge associates ranged two fold (0.65 to 1.39) across the sponge population while species richness varied three fold, both indicating that *A. viridis* supports a diverse community of sponge associated fauna that vary in composition from sponge to sponge, likely reflecting opportunistic host exploitation. Nonetheless, species evenness values also reflected a community of sponge associated species that shared a degree of commonality across the suite of sponges. That is, amongst the 12 species of sponge associates there are some species that often occur widely across individual sponges.
While the collection technique did not allow for a rigorous discrimination of whether meso- or macrofauna occurred on the surfaces or interiors of individual sponges, it was quantitatively determined that a total of 35% of the sponge associates recovered from sponges were dissected from the interior tissues of the sponges (Table 2). This is a conservative estimate as some sponge inhabitants were likely to have escaped from the interstices of sponge tissues due to disturbance from handling during collection. Thus, sponge-associated fauna are not only exploiting the outer pinacocytic surfaces of *A. viridis* as substrate, but living within the interstices of the sponge. There is the possibility that a small portion of the sponge-associated fauna were incidental “by catch” associated with the base of the sponge in contact with the sediment, and collected when the sponges were placed into bags *in situ*. While sponge-associated fauna consisted of representatives of tenaids, decapod, crabs, isopods, ascidians and polychaetes, by far the most common representatives were members of the Order Amphipoda. Indeed, 78% of the total sponge-associated fauna were amphipods, and one of these species, *Laticorophium baconi*, dominated the amphipods collected.

Amphipods are common inhabitants and associates of marine sponges (Biernbaum 1981, Duffy and Paul 1992, Spanier et al. 1993, Seger and Moran 1996, Crowe 2002, Ribeiro 2003, Richards 2007). Interestingly, none of the amphipods found to occur in association with *Amphimedon viridis* belong to taxa known to be truly sponge endocommensals, that is, primarily representatives of the taxa *Colomastix* and *Leucothoe* (S. LeCroy, personal communication). While the taxa of amphipods that were detected in association with *A. viridis* are also common in seagrass and rubble habitats in moderate to high salinity environments, and may occur in sponges more or
less in passing (LeCroy, S. 2002, 2004, S. LeCroy, personal communication), the fact that one species, *Laticorophium baconi*, was found to occur in comparatively high abundance suggests that this species may have some commensalistic tendencies with this sponge. Nonetheless, *L. baconi* is globally distributed with a biogeographic range that includes the Gulf of Mexico, Caribbean, Atlantic, Pacific, and the South China Sea. It has also been reported to occur in association with a broad spectrum of habitats including rock jetties, seawalls, offshore platforms, buoys, other hard substrates, seagrass beds, floating *Sargassum* rafts, and sand and mud bottoms (LeCroy, 2004).

Thus, *L. baconi* certainly does not show specificity to sponges as requisite habitat.

Regardless of whether they are obligate commensals or simply incidentally exploiting sponges as habitat, amphipods and other sponge-associated fauna may derive benefits from their associations with sponges. These benefits may include using sponges as a direct or indirect source of nutrition (Oshel and Steele 1985, Duffy and Paul 1992, Crowe and Thomas 2002, Becerro et al. 2006, Crawley and Hyndes 2007). As all amphipods brood their young (Crowe 2002, Richards 2007, Munguia, 2007, Sotka 2007) it is possible that sponges may provide a source of nutrients during brooding as well as for juvenile recruits. Interestingly, Munguia (2007) reported that certain species of adult amphipods that reside in the pen shell (*Atrina rigida*), display a dramatic decline in population numbers just after releasing their brooded offspring. They suggest there may be a limited food supply, and adults sacrifice feeding and ultimately their lives, in order to ensure sufficient nutrient resources for their offspring (Munguia 2007).
Amphipods that reside in sponges may also display indirect modes of nutrient exploitation via filter feeding of organic water particles that pass through sponge channels by currents generated by sponge choanocytes. Amphipods that do so are competing for nutrient resources with sponges. Amphipods that exploit sponges either directly or indirectly for food enjoy the added advantage of being able to feed with little energy invested in prey search activities, while residing within an environment that likely provides structural and/or chemical protection from predators (Duffy 1992, Crowe 2002, Thomas and Klebba 2006, Richards et al. 2007). Those species that are associated with the surfaces of sponges are more vulnerable to predators, but it is possible that sponges that possess chemical defenses provide a chemical refuge even here. For example, some species of chemically defended marine macroalgae confer chemical protection to amphipods that associate with the stipes and blades (Hay et al. 1990).

An additional reason that amphipods may associate in high frequency with sponges is that sponges may provide refuge for reproductive or social behaviors. In the present study amphipods (L. baconi and E. levis) were often found within the tissues of Amphimedon viridis carrying broods (20-30% of total amphipods). Moreover, juveniles of several amphipod species were evident suggesting that sponges may provide a suitable habitat for juvenile recruits. Other mesocrustaceans utilize sponges as “reproductive centers”. For example, the isopod Paracerceis sculpta utilizes the bread crumb sponge, Leucetta losangelensis, for reproduction and social interactions (Shuster 1989). Specifically, alpha-males defend the sponge, preventing the entrance of other males so as to protect their harem of females. Interestingly, some species of
shrimp in the genus *Synalpheus* exploit sponges as a refuge for complex eusocial behaviors that include reproductive interactions (Duffy 1993, Spanier et al. 1993). Moreover, Seger and Crowe (1996) suggest that commensals living within sponges may display cooperative brood care, reproductive divisions of labor, and overlapping generations.

Avoidance of predation is likely one of the primary selective factors driving sponge associations in mesofauna. Vectors of predation may include those predators that can move into and within the interstices of the sponge tissue to capture sponge-associated mesofauna. For example in the present study, errant predaceous polychaetes and *Libinia dubia* occurred within some of individual sponges. Both are capable of capturing and consuming mesocrustaceans. Moreover, a tenaid crustacean (*Hargeria rapax* or *Leptochelia dubia*) was found with a half consumed amphipod in its jaws. Collectively these observations suggest that there would be some level of predation pressure even within sponge tissues. Mesofauna are likely to be even more susceptible to predators that remove these individuals from the sponge surface than those predators that bite into sponge tissue to either directly or indirectly consume associated individuals. For example, the sparid pinfish *Lagodon rhomboides*, the model fish predator employed in the present study, is a well established omnivore with carnivorous tendencies that as juveniles especially target small crustaceans such as amphipods (Livingston 1982, Strong and Livingston 1984). Pinfish are capable of either plucking mesocrustaceans from the surfaces of sponges or, since they have the necessary dentition, biting sponges to attempt to ingest mesocrustaceans taking refuge within sponge tissues. A related sparid, *Diplodus holbrooki*, that occurs in sympathy with
pinfish in seagrass meadows, is even known to include sponges in its diet as adults (Strong and Livingston 1984).

It is important to note that the population *Amphimedon viridis* in Port Saint Joseph Bay, Florida appears to have a seasonal cycle with individuals absent during fall and winter months (Y. Huang and J. Huang, personal observations). Sponge populations reappear in the spring (April), and begin to dissipate by mid-summer (July). It is likely that the temporal periodicity of *A. viridis* in this region of the northern Gulf of Mexico is at its northern biogeographic extreme and that low winter temperatures limit its survival during winter months. While this ephemeral population cycle appears to be unique to its general geographic distribution, it does raise the issue of what the impact on sponge-associates during the time period sponges are absent. As amphipods are the primary sponge associates, the fact that none of the taxa are obligate sponge commensals means that they likely move to seagrass beds or other hard substrata during such time periods, perhaps to recolonize *A. viridis* in the spring.

Fresh tissues of the common demosponge *Amphimedon viridis* were clearly highly deterrent to the omnivorous pinfish *Lagodon rhomboides*. In no instance was a piece of sponge ingested by pinfish, but rather sponge pieces were immediately spit out following mouthing and in no case did fish return to attempt to swallow the piece of sponge a second time. The basis of this feeding rejection by pinfish could be related to a number of potential factors. These include a low and unattractive nutritional value of the food item, or the presence of some physical or chemical deterrent. The former hypothesis is unlikely as sponges are well known prey for a number of marine invertebrate and grazing fish species (Dayton et al. 1974, Randall and Hartman 1978,
McClintock 1987), and are also known to possess sufficient levels of protein, carbohydrate and lipid that should make them attractive foods (e.g., McClintock, 1987). Thus, it is more likely that the rejection responses observed in pinfish to this sponge species are related to the presence of structural or chemical defenses.

Sponge spicules are thought to have evolved for a variety of purposes including, importantly, the provision of structural integrity (Bergquist 1978, Clavico et al. 2007), as well as playing roles in prey capture (in specialized carnivorous sponges only; Vacelet and Duport 2004) and physical defenses against prospective predators (e.g. Chanas & Pawlik 1995, Hooper and Van Soest 2002, Burns et al. 2003, Hill et al. 2005, Jones et al. 2005, Penney 2006, Wulff 2006, Clavico et al. 2007). In the present study, monaxon spicules isolated from _Amphimedon viridis_ and imbedded in alginate food pellets, weakly, but statistically significantly, deterred feeding by pinfish. As the pinfish _Lagodon rhomboides_ is a well known generalist, these results support the prediction of Chanas and Pawlik (1995) that sponge spicules may be an effective structural defense against some generalist predators. Other studies have examined the question of whether sponge spicules or soft coral sclerites deter generalist fish predators. Harvell et al. (1988) demonstrated that calcitic sclerites from the soft coral _Pseudopterogorgia acerosa_ inhibited feeding in the wrasses _Thalassoma bifasciatum_, _Halicloeres garnoti_, and _Halicloeres maculipinna_. In contrast, Chanas and Pawlik (1995), after predicting that some generalist fish should be deterred by sponge spicules, reported that artificial food pellets containing natural concentrations of the spicules of eight common Caribbean demosponges were all palatable to the generalist wrasse _Thalassoma bifasciatum_. Moreover, Burns et al. (2003) reported that artificial food
pellets containing natural concentrations of spicules from four Caribbean sponges and two Red Sea sponges all deterred feeding in the generalist Red Sea wrasse, *Thalassoma klunzingeri*. Interestingly, Burns et al. (2003) conducted feeding experiments using the generalist wrasse *T. bifasciatum* by offering fish artificial food pellets containing both natural concentrations of crude organic extract and/or spicules of the sponge *Crella cyatophora*. They demonstrated that food pellets containing both extract and spicules were significantly more deterrent to wrasse than either food pellets containing extract or spicules alone. Thus, in their study, chemical and structural factors acted in a synergistic fashion. Although synergies between spicules and chemical defenses were not investigated in the present study, as indicated below, both spicules and, especially, secondary metabolites, appear to play a role in defense against fish predation in the sponge *A. viridis*.

In contrast to the weak feeding deterrent activity displayed by pinfish when presented alginate food pellets containing spicules isolated from *Amphimedon viridis*, both lipophilic and hydrophilic extracts imbedded in alginate pellets at natural concentrations caused strong feeding deterrent responses in pinfish when compared with solvent control food pellets. This complements and supports the observations of Burns et al. (2003) who found that combined lipophilic and hydrophilic extracts of *Amphimedon viridis* from the Red Sea and the congenor *Amphimedon compressus* from the Caribbean were highly deterrent to the wrasses *Thalassoma bifasciatum* and *T. klunzingeri*. Berlinck et al. (1996) isolated and described a halitoxin from the tissues of *A. viridis* collected in the southeastern Brazilian coast. Moreover, a mixture of halitoxin and amphitoxin were identified from methanol extracts of *A. viridis* from the
Red Sea (Kelman et al. 2001). While no bioassays were conducted to evaluate whether these cyto- and neurotoxic alkyl pyridine alkaloids (salts) from *A. viridis* caused feeding deterrence in sympatric fish, this is certainly a possibility that needs further exploring. It has been demonstrated that amphitoxin isolated from a congenor, *Amphimedon compressa* is a deterrent against sympatric generalist fish (Albrizio et al. 1995).

As both lipophilic and hydrophilic extracts of *Amphimedon viridis* were deterrent to pinfish in the present study, additional deterrent compounds are likely present as halitoxin and amphitoxin are both generally considered lipophilic compounds. Regardless of the identify of the fish antifeedants, the fact that both hydrophilic and lipophilic extracts were active indicates that sympatric generalist pinfish may be deterred from pursuing mesocrustaceans and other mesofauna associated with *A. viridis* through both chemodetection of feeding deterrents leaching from the sponge surface [hydrophilic deterrent compound(s)] or by taste perception through incidental or direct biting of sponge tissues [lipophilic deterrent compound(s)].
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Swart PK, Saied A, Lamb K (2005) Temporal and spatial variation in the $\delta^{15}N$ and $\delta^{13}C$ of coral tissue and zooxanthellae in *Montastraea faveolata* collected from the Florida reef tract. Limnol Oceanogr 50:1049-1058


NOTICE OF APPROVAL

DATE: October 25, 2006

TO: James B. McClintock, Ph.D.
    CH-368 1170
    FAX: 975-6097

FROM: Judith A. Kapp, Ph.D., Chair
       Institutional Animal Care and Use Committee

SUBJECT: Title: Food or Shelter? Factors Influencing Isopod and Amphipod Mesograzers
          Symbionts in the Common Subtropical Marine Sponge Amphipod Vintis
          (Jonathan Huang)
          Sponsor: Internal
          Animal Project Number: 061007842

On October 25, 2006, the University of Alabama at Birmingham Institutional Animal Care and
Use Committee (IACUC) reviewed the animal use proposed in the above referenced application.
It approved the use of the following species and numbers of animals:

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number In Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>A</td>
<td>20</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from October 2006. Approval from the IACUC
must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate
granting agency.

Refer to Animal Protocol Number (APN) 061007842 when ordering animals or in any
 correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this
 study. If you have concerns or questions regarding this notice, please call the IACUC office at
 934-7692.