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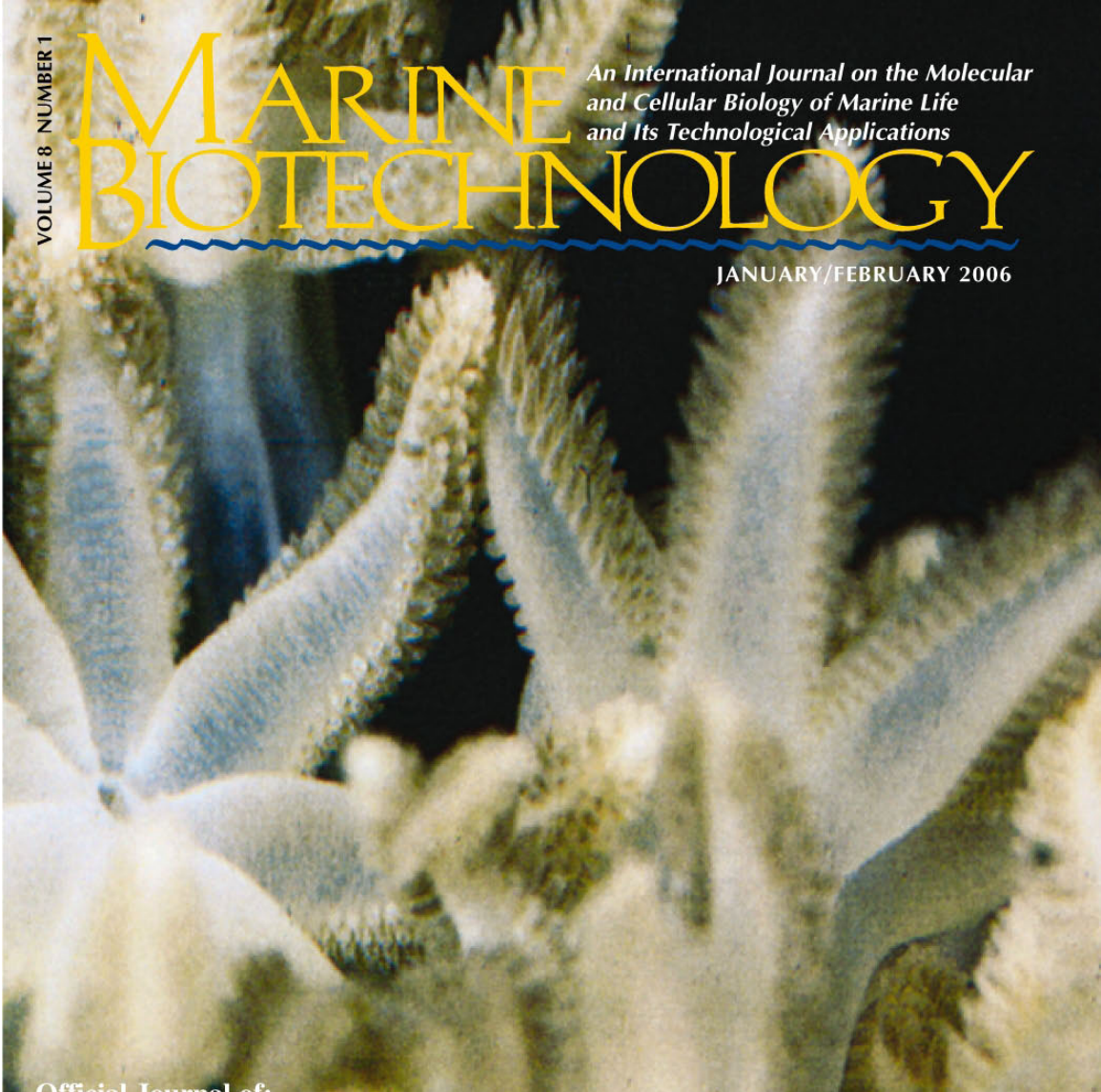

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Short Communication

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Comparative Proteomics of Symbiotic and Aposymbiotic Juvenile Soft Corals

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Abstract

The symbiotic association between corals and photosynthetic unicellular algae is of great importance in coral reef ecosystems. The study of symbiotic relationships is multidisciplinary and involves research in phylogeny, physiology, biochemistry, and ecology. An intriguing phase in each symbiotic relationship is its initiation, in which the partners interact for the first time. The examination of this phase in coral–algae symbiosis from a molecular point of view is still at an early stage. In the present study we used 2-dimensional polyacrylamide gel electrophoresis to compare patterns of proteins synthesized in symbiotic and aposymbiotic primary polyps of the Red Sea soft coral *Heteroxenia fuscescens*. This is the first work to search for symbiosis-specific proteins during the natural onset of symbiosis in early host ontogeny. The protein profiles reveal changes in the host soft coral proteome through development, but surprisingly virtually no changes in the host proteome as a function of symbiotic state.

Keywords: zooxanthellae — symbiosis-related proteins — 2D-PAGE — symbiosis — soft corals

Introduction

Symbiotic interactions are widespread in marine and terrestrial environments and include a great diversity of partners. One of the most conspicuous symbioses in the marine environment is that between cnidarian hosts, such as stony corals, soft corals, and anemones, and their dinoflagellate phototrophic symbionts *Symbiodinium* spp. This symbiosis forms the trophic and structural foundation of the entire coral reef ecosystem (Trench, 1993). Most cnidarian hosts acquire their algal symbionts from the environment (known as horizontal transmission) at an early developmental stage such as planula or primary polyp (Trench, 1987). The initiation of a symbiotic relationship is often accompanied by morphologic, physiologic, biochemical, and molecular changes in both partners (Taylor, 1973; McFall-Ngai and Ruby, 1991; Douglas, 1994; Montgomery and McFall-Ngai, 1994). In certain symbioses, such as plant-microbe endosymbioses, this biochemical and molecular interplay between the partners has been extensively studied (e.g., Bestel-Corre et al., 2004). In others, however, such as cnidarian-algal symbioses, these interactions remain largely undescribed. Although numerous studies have focused on the breakdown of the symbiotic association, namely, bleaching (e.g., Brown, 1997; Lesser, 1997; Hoegh-Guldberg, 1999; Toller et al., 2001; Brown et al., 2002; Diaz-Pulido and McCook, 2002; Franklin et al., 2004), examination of the initiation of the symbiotic relationship between corals and symbiotic algae from a molecular point of view is still at an early stage (Weis and Levine, 1996; Kuo et al., 2004). In choosing a system to investigate the initial stages of coral–algal symbiosis, the best strategy is to study an association with horizontal transmission, in which there are both aposymbiotic and symbiotic early host ontogenetic stages.

To date all studies that have examined symbiosis-specific genes and expressed proteins have been on adult sea anemones. The temperate sea anemone *Anthopleura elegantissima* occurs naturally in both the symbiotic and aposymbiotic state and has been used for the identification of symbiosis-related proteins (Weis and Levine, 1996; Weis and Reynolds, 1999; Reynolds et al., 2000). Similarly the tropical sea anemone *Aiptasia pulchella*, which can be grown in a symbiotic state, or rendered aposymbiotic following treatment with 3-(3,4-dichloro-Phenyl)-1,1-dimeth-

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yl-urea (DCMU) or prolonged incubation in darkness, has been used recently in two studies aimed at the characterization of symbiosis-related genes (Chen et al., 2004; Kuo et al., 2004).

In this study we compared patterns of proteins synthesized in symbiotic and aposymbiotic primary polyps of the soft coral *Heteroxenia fuscescens*.We used 2-dimensional polyacrylamide gel electrophoresis (2D PAGE) and silver stain, which is considered the gold standard in the biotechnology industry for detection of minor proteins (Nishihara and Champion, 2002). Silver staining can result in high sensitivity, with as little as 0.5 ng of protein detected (Nishihara and Champion, 2002); however, the linear dynamic range of this technique is reported to be less than that of other protein detection techniques (Smales et al., 2003). This is the first work to search for symbiosis-specific proteins during the natural onset of symbiosis in early host ontogeny. *H. fuscescens* is a common zooxanthellate soft coral occurring on the reefs of the northern Red Sea (Benayahu, 1985). It is hermaphroditic and broods planulae (Benayahu, 1991) that are released from the parent nearly year-round (Ben-David-Zaslow et al., 1999). Planulae lack zooxanthellae, and algal acquisition occurs at an early primary polyp stage (Yacobovitch et al., 2003). Populations of primary polyps of identical age can be maintained in both the symbiotic and the aposymbiotic states for up to 2 months (Yacobovitch et al., 2003), and there are no differences in the timing and sequence of morphogenetic events during metamorphosis between symbiotic and aposymbiotic primary polyps in the laboratory (Yacobovitch et al., 2003). Therefore, *H. fuscescens* provides an ideal system for the study of protein and gene expression during the onset of symbiosis.

Materials and Methods

Maintenance of Animals. Planulae from *Heteroxenia fuscescens* were collected in Eilat (Red Sea) following the methodology described in Yacobovitch et al. (2003). Planulae released from each adult colony (a batch) were counted and divided into groups of 50 planulae. Each group was placed in a 50-ml plastic container with 0.45 μm of filtered seawater (FSW). Containers were placed in an incubator (Yihdern, LE509) set to the temperature of ambient seawater during the same time period in Eilat. The light regimen was 12 hours of light (30 μmol quanta ms and 12 hours of dark. Half of the water in each container was changed every other day. Planulae underwent metamorphosis after 10 to 20 days. Soon after metamorphosis occurred, one half of the batch was infected with freshly isolated zooxanthellae obtained from an adult colony (see Yacobovitch et al., 2003). Polyps were inspected under a light microscope to verify symbiotic state. Aposymbiotic and symbiotic primary polyps were frozen in liquid nitrogen at different time intervals after infection (3 days, 1, 2, 3, 4, and 6 weeks). To process animals for freezing, polyps were detached from the plastic containers using a glass pipette fitted with a syringe needle, washed in 0.22 μm FSW, transferred to microfuge tubes (50 polyps per tube), placed in liquid nitrogen, and stored at −80°C prior to further analysis. Polyp age was calculated from the day of planula release. A total of 3031 primary polyps were raised, belonging to 6 different mother colonies. In addition, samples containing 50 planulae and 2 to 3 polyps from several mature coral colonies were frozen.

Protein Extraction from Animal Tissue. Thawed samples were homogenized at 4°C in a glass tissue grinder in 100 μl of extraction buffer (EB: 40 mM Tris, 10 mM EDTA, pH 7.4) with a protease inhibitor mix (Sigma). The homogenate was transferred to a microfuge tube, and the grinder was washed with an additional 50 μl of EB. Extracts were centrifuged for 12 minutes at 12000 g at 4°C. The supernatant fluid, containing the soluble fraction of coral proteins, was removed, and protein concentration was determined spectrophotometrically using a Coomassie assay (Pierce). It is important to note that algal cells were removed unbroken during centrifugation.

2D-PAGE. The Multiphor II flatbed system (Amersham, Pharmacia) was used according to the manufacturer's instructions. Immobiline Dry Strip IEG gels (nonlinear pH gradient of 3–10, 18 cm) were used to resolve proteins in the first dimension. The strips were first soaked overnight in rehydration solution (8 M urea, 2% ampholytes, 0.5% Triton-X, dithiothreitol [DTT], and a few grains of bromophenol blue) containing 40 μg of protein. The first dimension was run at 20°C for 19.5 hours (0.01 hour at 500 V, 5 hours of gradient to 3500 V, followed by 14.5 hours at 3500 V). Isoelectric focusing gels not immediately used for the second dimension were frozen at −80°C immediately prior to the second-dimension run, IEF gels were incubated successively in 2 equilibration solutions for 10 minutes each. The solutions contained (I) 50 mM Tris-HCl buffer (pH 6.8) containing 6 M urea, 30% glycerol, 1% sodium dodecylsulfate (SDS), and 0.8% DTT; and (II) 50 mM Tris-HCl buffer (pH 6.8) containing 6 M urea, 30% glycerol, 1% SDS, and 7.2% Iodoacetamide and a few grains of bromophenol blue. Precast gels Excel-Gel SDS 12% to 14% gradient, 245 × 180 × 0.5 mm, were used to resolve proteins in the second dimen-

Table 1. Tallied Results from 2D-PAGE of Soluble Proteins from *Heteroxenia fuscescens*

Age of polyps (weeks)	*Year collected*	*Days or weeks after infection*	*No. of samples*
Planulae	2000	–	1
3	2001	3 d	1 sym, 1 apo
3	2000	1 wk	1 apo
3	2001	1 wk	1 sym, 1 apo
4	2003	2 wk	1 sym, 1 apo
5	2001	2 wk	1 sym, 1 apo
5	2003	3 wk	1 sym
6	2000	2 wk	1 sym
6	2000	3 wk	1 sym, 1 apo
6	2001, 2003	4 wk	3 sym, 3 apo
Adult	2003	–	2

sion. The second dimension was run at 15°C for a total of 3.5 hours at 1000 V. Low molecular weight protein standards (Pharmacia) were run on each gel alongside each sample. Gels were silver-stained (methods modified from Heukeshoven and Dernick, 1985), covered with Mylar, soaked in 1% glycerol, and air-dried overnight at room temperature.

Analysis of 2D Gels. For each developmental stage of *H. fuscescens*, 2 to 3 replicate samples were resolved on gels. Each sample was compared with the other replicates for the presence or absence of proteins. No marked differences were detected between replicates. Gels were analyzed pairwise by eye for differences in their protein patterns by overlaying the gels on a light table.

Control for Possible Algal Protein Contamination in Host Gels. Despite procedures to remove symbiotic algae unbroken from symbiotic host tissues, it is possible that some algae broke and that algal protein contaminated host protein extracts. If this were true, then algal proteins would mistakenly be identified as symbiosis-enhanced host proteins. To avoid this a protein extract from freshly isolated algae was resolved using 2D-PAGE and compared with all the symbiotic host protein gels.

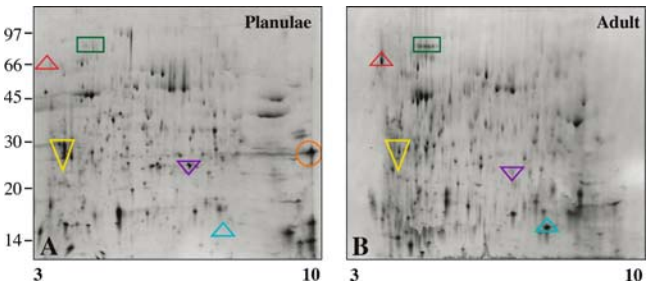

Fig. 1. Silver-stained 2D gels of soluble proteins from planulae (**A**) and an adult colony (**B**) of *Heteroxenia fuscescens*. Triangles surround spots that intensify with age; inverted triangles, those that decrease in expression with age; rectangles, those that intensify only in the polyp stage; and circles, those that are unique to the planulae stage. The horizontal dimension represents pH, and the vertical dimension represents relative molecular mass.

Results

We obtained 22 protein profiles of *Heteroxenia fuscescens* across a range of ontogenetic stages and symbi-

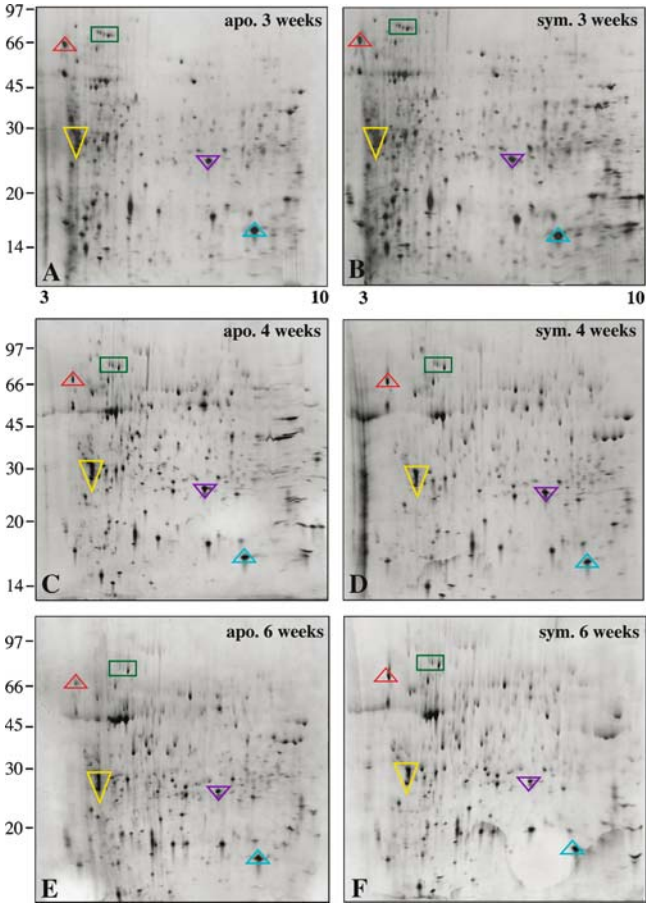

Fig. 2. Silver-stained 2D gels of soluble proteins from primary polyps of *Heteroxenia fuscescens*, differing in their ages and symbiotic states: 3-week-old aposymbiotic polyps (**A**); 3-week-old symbiotic polyps, 1 week after infection with zooxanthellae (**B**); 4-week-old aposymbiotic polyps (**C**); 4-week-old symbiotic polyps. 2 weeks after infection with zooxanthellae (**D**); 6-week-old aposymbiotic polyps (**E**); 6-week-old symbiotic polyps, 4 weeks after infection with zooxanthellae (**F**). Triangles surround spots that intensify with age; inverted triangles, those that decrease in expression with age; rectangles, those that intensify only in the polyp stage. The horizontal dimension represents pH, and the vertical dimension represents relative molecular mass.

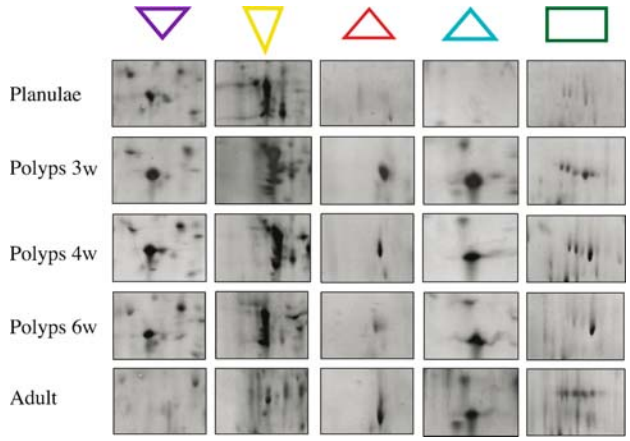

Fig. 3. Summary of changes in spot intensity through host ontogeny. Enlargement of the spots marked on Figure 1.

otic states (Table 1). Figure 1 shows representative gels of aposymbiotic planulae and symbiotic adult colony. Profiles of primary polyps contained approximately 170 spots. Figure 2 shows 3 pairs of protein profiles obtained from primary polyps at ages 3, 4, and 6 weeks. Each pair of gels shows the profiles of aposymbiotic (apo) and symbiotic (sym) polyps derived from the same batch of planulae. The profiles of the symbiotic polyps represent 4 different ages of polyps as well as 3 different times after infection with algae, 1, 2, and 4 weeks. Comparisons of protein profiles of aposymbiotic and symbiotic primary polyps revealed no consistent differences regardless of polyp age or time after infection.

Overall we found changes in protein expression that corresponded only to the developmental stage of the samples. Highlighted spots in Figures 1 and 2 indicate examples of proteins that are differentially abundant as a function of host age. Each differential protein is marked with a colored geometric form. Triangles surround spots that intensify with age; inverted triangles, those that decrease in expression with age; and rectangles, those that intensify only in the polyp stage (see Figures 1, 2). Spots that are unique to a certain developmental stage are marked with a circle. The circled spot in Figure 1(A) is unique to the planula stage. This 26-kDa protein is likely a yolk protein, similar to that described in larval *Fungia scutaria* (Schwarz et al., 1999). Changes in expression through time of the other marked spots are summarized in Figure 3.

Discussion

The present comparative proteomic study revealed changes in the host soft coral proteome through development but surprisingly virtually no changes in the host proteome as a function of symbiotic state. This suggests that during the first days and weeks of symbiosis between *Heteroxenia fuscescens* and *Symbiodinium* sp., translational and posttranslational changes specific to the symbiotic state may not be occurring in the host. These findings are remarkably similar to a recent comparative proteomic study of symbiotic and aposymbiotic larvae of the stony coral *Fungia scutaria* (deBoer, 2004; M. deBoer and V.M. Weis, manuscript in preparation). The 2D-PAGE patterns of host proteins revealed only one consistent difference out of approximately 450 proteins between symbiotic and aposymbiotic larvae of the same age.

Our findings are, however, in stark contrast to those obtained from other comparative proteomic studies of symbioses. In work comparing symbiotic and aposymbiotic specimens of the temperate sea anemone *Anthopleura elegantissima*, numerous proteins were shown to be specific to or enhanced in the symbiotic state (Weis and Levine, 1996). Two abundant proteins were further characterized and identified as carbonic anhydrase, an enzyme known to function in inorganic carbon transport in cnidarian-algal symbioses (Weis, 1991; Al-Moghrabi et al., 1996; Weis and Reynolds, 1999; Furla et al., 2000), and sym32, a fasciclin I homologue that may function in hosts-symbiont communication (Reynolds et al., 2000; Schwarz and Weis, 2003). A 2D protein profile study of the squid *Euprymna scolopes* compared the changes in the soluble proteome of the symbiotic bacterial light organ during the first 96 hours of symbiosis in symbiont-colonized and uncolonized organs (Doino-Lemus and McFall-Ngai, 2000). Numerous symbiosis-related differences were found at 48 and 96 hours after the onset of symbiosis, and these changes were more abundant than age-related changes. Multiple studies using 2D-PAGE and other techniques on the symbiosis of leguminous plants and nitrogen-fixing bacteria have resulted in the characterization of a suite of plant genes specifically expressed during onset of symbiosis (Govers et al., 1985; Gloudemans and Bisseling, 1989; Natera et al., 2000; Saalbach et al., 2002). In addition, the onset of pathogenic associations has also been shown to cause changes in patterns of host proteins (e.g., Abshire and Neidhardt, 1993; Kwaik, 1994).

There are several other explanations for the uniformity of proteome patterns between symbiotic and aposymbiotic hosts. It is possible that changes are occurring but they are not being detected. This could be due to a variety of factors including slow protein turnover with age of the host proteome, transient expression of symbiosis-specific or enhanced proteins, and expression of these proteins in very low

quantities below detection levels. If symbiosis-specific protein expression is indeed limited to those cells housing the symbionts, then these host cells number only in the hundreds to thousands in the early weeks of symbiosis (Yacobovitch, 2001). In a background of many thousands of cells comprising a juvenile polyp, any symbiosis-specific protein signal might be lost. An example that further demonstrates this possibility is in *E. scolopes*, in which a differential result was obtained only in its symbiosis-specific organs that had been dissected away from the rest of the animal (Doino-Lemus and McFall-Ngai, 2000). Since in Cnidaria algal symbionts are not housed in such a specific location, detecting these symbiosis-specific changes is made difficult by a high non symbiosis cell background.

In conclusion, the onset of symbiosis between the soft coral *H. fuscescens* and *Symbiodinium* sp. is not accompanied by gross changes in the host proteome in the first weeks of the association. Further studies using more sensitive techniques, such as complementary DNA microarrays, are needed to identify proteins involved in the initial interactions between the partners.

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