Analysis of Internal Osmolality in Developing Coral Larvae, *Fungia scutaria*

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ABSTRACT

Coral species throughout the world are facing severe local and global environmental pressures. Because of the pressing conservation need, we are studying the reproduction, physiology, and cryobiology of coral larvae with the future goal of cryopreserving and maintaining these organisms in a genome resource bank. Effective cryopreservation involves several steps, including the loading and unloading of cells with cryoprotectant and the avoidance of osmotic shock. In this study, during the time course of coral larvae development of the mushroom coral *Fungia scutaria*, we examined several physiologic factors, including internal osmolality, percent osmotically active water, formation of mucus cells, and intracellular organic osmolytes. The osmotically inactive components of the cell, V_b, declined 33% during development from the oocyte to day 5. In contrast, measurements of the internal osmolality of coral larvae indicated that the internal osmolality was increasing from day 1 to day 5, probably as a result of the development of mucus cells that bind ions. Because of this, we conclude that coral larvae are osmoconformers with an internal osmolality of about 1,000 mOsm. Glycine betaine, comprising more than 90% of the organic osmolytes, was found to be the major organic osmolyte in the larvae. Glycerol was found in only small quantities in larvae that had been infected with zooxanthellae, suggesting that this solute did not play a significant role in the osmotic balance of this larval coral. We were interested in changes in cellular characteristics and osmolytes that might suggest solutes to test as cryoprotectants in order to assist in the successful cryopreservation of the larvae. More importantly, these data begin to reveal the basic physiological events that underlie the move from autonomous living to symbiosis.

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Introduction

Coral reefs cover approximately 0.2% of the ocean floor but provide homes to approximately 25% of all marine species (Cesar et al. 2003). This may encompass millions of organisms that are associated with coral reefs at some point in their life cycle (Cesar et al. 2003). Reefs protect our coastlines, are the foundation for successful fishing and tourism, and provide livelihoods for approximately 100 million people. In total, coral reefs produce an annual net benefit of $30 billion to the world's diverse economies (Cesar et al. 2003).

Unfortunately, coral species throughout the world are facing severe local and global environmental pressures (Bellwood et al. 2004). Near-shore local pressures—such as increased sedimentation from building sites and deforested slopes, elevated nutrients and pollutants from rivers and sewage pipes, and physical abuse from swimmers—have local sources and potentially local solutions. Globally, coral also are experiencing stress because of increased water temperature that can lead to bleaching and death. For example, during the 1998 worldwide bleaching event, 75%–90% of living coral died in the Indian Ocean, with a greater than 90% loss in the Seychelles Islands (Linden et al. 2002; Sheppard 2003). As a result of bleaching, Graham et al. (2006) observed severe reductions in species richness and loss of species within key functional groups of reef fish. In 2005, a massive bleaching event followed by disease, such as "white pox" and other unidentified diseases, swept through the Caribbean, causing a 90% incidence of bleaching and nearly 40% mortality on monitored reefs in the U.S. Virgin Islands (McCreedy et al. 2006). Even in the most remote marine bioreserves, such as the northwestern Hawaiian Islands (Maragos et al. 2004), human activities are damaging fragile coral ecosystems (Bellwood et al. 2004), and losses in genetic diversity make coral more vulnerable to subsequent stressful events such as bleaching (Graham et al. 2006).

The demise of reefs not only may have severe economic consequences but also may worsen our already warming earth (Kleypas et al. 2006). As more carbon dioxide is dissolved in

the oceans, pH is lowered, decreasing the availability of carbonate ions and reducing the calcium carbonate uptake of shellfish and coral. Decreased carbonate concentrations and further loss of reefs and shellfish will significantly reduce the ocean's buffering capacity and ability to accept carbon dioxide from the atmosphere (Kleypas et al. 2006). As greenhouse gasses increase, atmospheric and sea surface temperatures are increasing. This, coupled with anthropogenic stresses, threatens the existence of reefs worldwide (Hoegh-Guldberg 1999; Goreau et al. 2000; Hughes et al. 2003). In fact, the dual effect of ocean acidification, where lower pH will cause a reduction in calcium carbonate uptake for coral, and warming oceans may make coral rare by the middle of this century (Hoegh-Guldberg et al. 2007). Thus, although there remain many unknowns, it is clear that ocean ecosystems are vulnerable to destabilization, and thus there is a need for more basic research.

Because of the pressing conservation needs of coral, we are studying the reproduction, physiology, and cryobiology of coral larvae, with the future goal of cryopreserving and maintaining these organisms in a genome resource bank. In this bank, the larvae would remain frozen but alive for many decades in liquid nitrogen. Potentially, these frozen populations could be thawed and released back into the reef environment once the ecological conditions for their survival had improved. Thus, it is feasible that these genome resource banks may prevent the extinction of many coral species.

At present, no genome bank for coral species exists. However, the technology is now available to begin developing such a repository for coral species. Hagedorn et al. (2006*a*, 2006*b*) have examined some of the important cryobiological features of coral larvae and have successfully cryopreserved coral sperm. Although early studies have been encouraging, there is still a great need for more extensive investigations.

Effective cryopreservation involves several steps, including the loading and unloading of cells with cryoprotectant and the avoidance of osmotic shock. Glycerol is a common cryoprotectant used on many types of cells. In coral, glycerol is a byproduct of respiration by symbiotic coral algae (Muscatine 1967; Gates et al. 1995; Gates and Edmunds 1999); it crosses coral cell membranes, and a 10% solution or less is nontoxic to coral (Hagedorn et al. 2006*b*). Glycerol may be an ideal cryoprotectant for coral because they are tolerant to it and can metabolize it.

However, coral larvae have not been successfully cryopreserved, and the physiological mechanisms blocking this are only partially understood (Hagedorn et al. 2006*b*). A more thorough understanding of the internal osmolality is an important starting parameter to help identify some of the obstacles to cryopreservation of coral larvae. Therefore, a detailed analysis of coral larvae internal osmolality and glycerol content was undertaken.

Corals, like almost all marine invertebrates, are thought to be osmoconformers; that is, their internal osmolality should be about the same as that of seawater, about 1,000 mOsm. In general, marine osmoconformers have extracellular fluids very similar to seawater, with NaCl dominating as the major solute (i.e., osmolyte). However, intracellular fluids have relatively low levels of inorganic ions, with certain organic osmolytes making up as much as half or more of cellular osmotic pressure. Organic osmolytes are used instead of NaCl or KCl because the latter disrupt proteins and DNA at high concentrations while the former do not. Thus, organic osmolytes are sometimes called compatible solutes. In marine invertebrates that have been well analyzed for organic osmolytes, these are predominately free amino acids, such as glycine and taurine, and methylamines, such as glycine betaine, also known as trimethyl glycine (Yancey 2005). Surprisingly, coral anthozoans have been analyzed for organic osmolytes only recently (Andrell et al. 2007), despite the fact that these solutes are typically the most concentrated organic compounds in marine invertebrates and are thus important in understanding energy budgets and, in this case, host-symbiont relationships.

Many types of coral have symbiotic algae, called zooxanthellae, that live within their cells. These zooxanthellae contribute organic metabolites to the energy budget of their hosts. It has been postulated that free amino acids, common osmolytes in other marine invertebrates, and glycerol, a major product of symbiotic zooxanthellae, might be major coral osmolytes; however, no studies have been done on osmotic contributions of these solutes (Mayfield and Gates 2007). The only coral solute that has been clearly identified as an osmolyte is dimethylsulfonopropionate (DMSP), also a product of zooxanthellae (Broadbent et al. 2002).

In this study, we examined the time course during coral larvae development of several physiologic factors. These included the internal osmolality, the percent osmotically active water, the formation of mucus cells (whose polyglycans can bind ions; Chahine et al. 2005), and potential intracellular organic osmolytes, such as glycerol.

Material and Methods

Animal Collection

These methods focused on the developmental changes in internal osmolality for the coral larvae *Fungia scutaria* up to day 6, when they begin to metamorphose. Larvae from in vitro fertilization were raised June–October from breeding *F. scutaria* adults collected from various shallow reef flats in Kaneohe Bay (21°26′N, 157°46′W) at the Hawaii Institute for Marine Biology, University of Hawaii. These adults have been maintained in breeding condition at the marine station for many years, and the methods for obtaining gametes are well known (Krupp 1983; Jokiel et al. 1985; Weis et al. 2001). All the larvae for this article were raised in bowls filled with filtered seawater (FSW) that were temperature regulated by an open-air, flowing seawater system.

Most groups were not exposed to zooxanthellae except those used for high-performance liquid chromatography (HPLC) analysis. When needed, day 3 or 4 larvae were infected with symbiotic zooxanthellae from a single adult *F. scutaria* that was collected according to modified methods of Weis et al. (2001). It takes at least 48 h for the infection to be considered per-

manent, so the larvae are not tested until day 6. Briefly, to collect the zooxanthellae, a pressurized air jet (Northern Industrial Air Blow Gun with a 1/4-in inlet, model ATBG092AVA with a scuba tank) was used to remove tissue from the coral skeleton. The adult was surrounded in a plastic bag with a hole in the bottom, and the sloughed tissue was filtered through a 335-μm mesh to help remove the excess mucus and skeletal debris. The residual was collected into 50-mL plastic tubes. The tubes were spun at 3,200 *g* for 10 min to pellet the zooxanthellae. The supernatant was removed, and 10 mL of fresh FSW was added to each tube for resuspension of the zooxanthellae. All FSW used for solutions and larval rearing was filtered by passing it through a 0.45-μm Millipore filter.

Morphometric Analysis

A major component of the larval analyses in these experiments hinged on natural developmental changes in volume as well as changes in volume in response to external solutes. To calculate the volume of each larva, its cross-sectional area was measured in its test solution using computer-aided microscopy. A 20-μL drop of solution with tens of larvae was placed onto a depression slide with a coverslip to prevent drying. Digital images of a single larva were captured using a microscope (Olympus BX41) with an attached digital camera (Sony DFWV300). The major and minor axes were determined using a G4 Macintosh computer and NIH Image software (ver. 1.63), and the volume was calculated assuming a prolate spheroid ($V = 4/3\pi(a/2) \times (b/2)^2$, where a and b are the major and minor axes, respectively).

Developmental Changes in Larval Osmotically Inactive Volume Fraction

These data were needed for calculation of the larval internal osmolality. Although an average osmotically inactive volume fraction, V_b, of *F. scutaria* from days 3–5 was approximately 0.15 from pooled samples (Hagedorn et al. 2006*b*), it was not known whether this value changed significantly during development. It made sense that V_b might change significantly because the larvae metabolize much of their fat stores during development. To obtain wet weights, larval samples from days 1, 2, 3, and 5 (N = thousands of larvae/basket for a total of six baskets at each development day) were transferred into preweighed Falcon cell-sorting baskets (40 μm mesh size). The baskets with larvae were blotted with Kimwipes to remove the residual liquid, reweighed, placed in a 50°C oven to dry for 12–24 h, and then weighed again. The mean larval wet/dry weight ratio was calculated as

$$\frac{\text{mean dry weight} - \text{mean basket weight}}{\text{mean wet weight} - \text{mean basket weight}}.$$

These experiments were repeated two to three times for each development period and averaged. Twenty milligrams of external surface water carryover was assumed for the wet measurements.

As in the study by Hagedorn et al. (2006*b*), we used the following formula and assumptions to find the osmotically inactive volume fraction, that is, the fractional solids volume (V_b). We assumed that the gastrovascular cavity had a negligible volume during these developmental periods (ca. less than 10% for *Fungia*; M. Hagedorn, V. Carter, and R. Steyn, unpublished data) and take no account of it in these formulas:

$$V_b = \frac{W_b}{W_b + (\rho_s/\rho_w)(1 - W_b)}, \quad (1)$$

where

$$W_b = \text{weight fraction of solids} = \frac{W_s}{W_s + W_w},$$

W = weight, V = volume, ρ = density, s = solids, and w = water. The densities of the solids and water were assumed to be 1.15 and 1.0 g/cm^3, respectively. These data were corrected assuming a 20-mg water carryover in the wet larva baskets. We used a microbalance with 0.0001 g accuracy to determine the larval wet/dry weight ratio, and every cell basket or basket and larval tissue were weighed five times and the mean value used.

Developmental Osmotic Measurements

The internal osmolality is one of the most important variables to obtain for biophysical modeling. To determine the internal osmolality of the coral larvae throughout development in FSW, a vapor pressure osmometer (Model 5520, Wescor, Logan, UT) was used. Because we expected the osmolality to be high (~1,000 mOsm), and because crushed cells are viscous, the cell preparations were diluted with water by a factor of five before measurement in the osmometer.

The fourfold mass dilution with water does not decrease the osmolality fourfold. The procedure for calculating the osmolality is covered in "Calculating the Internal Osmolality." Thousands of larvae from days 1–6 (N = 10 baskets for each developmental day) were concentrated into Falcon cell-sorting baskets (Fisher, 40 μm mesh size) and exposed to FSW for 3 min. The baskets with larvae were blotted on Kimwipes to remove residual liquid, and the larval tissue was scraped into preweighed microcentrifuge tubes. The sample was flash frozen in liquid nitrogen to destroy cell integrity, weighed, and then diluted with four times the weight of the tissue (weight of tube and tissue − weight of the preweighed tube = weight of tissue) with deionized water. Finally, 2 μL of 0.1% Triton-X was added to each sample to further solubilize the cells, and the solution was vortexed. The osmolality of each sample was determined by testing three 10-μL samples from each tube and averaging their values. The mean value from each developmental day was recorded. These experiments were repeated in four different breeding months, and their values were averaged. More than likely, the developmental time points may not be exactly the

same because of the change in air and water temperature from earlier summer months to later summer months.

Control experiments were done to assess the effect of the amount of time the samples were exposed to the air during processing and to determine whether it would affect the water content of the tissue. These baskets with larvae were exposed to FSW for 3 min; blotted on Kimwipes to remove residual liquid; and left on the bench for 1, 3, or 10 min before the larval tissue was scraped into preweighed microcentrifuge tubes and processed as above.

We used a microbalance (Mettler AB104 or AE240) with 0.0001 g accuracy to determine the weights of microcentrifuge tubes alone or tubes with larval tissue. Because of the possible error, everything was weighed five times, and the average value was used.

Calculating the Internal Osmolality

Because the cell preparations were diluted with deionized water by a factor of five before measurement, it is necessary to back calculate the original osmolality of the coral cells. A trivial but incorrect back calculation would be to simply multiply the osmolality of the diluted solution by the dilution factor ($\times 5$ here) to determine the osmolality of the original solution. This is wrong for three reasons. First, the original solution is not pure water but contains solids, and thus the dilution of the water component of the original solution is greater than $\times 5$. Because we know the V_b of the larvae for each day (0.25–0.16), this can easily be accounted for. Second, there is carryover FSW on the outside of the larvae. We make an estimate of 20% (by volume) of the carryover of FSW coating the outside of the larvae. The results are insensitive to the exact number because the external FSW carryover is comparable in osmolality to the larvae cytoplasm. The third factor to be considered for an accurate back calculation is the nature of osmolality. Osmolality does not scale linearly with dilution of the water component of the sample. Osmolality differs from molality in this respect. The osmolality of real (nonideal) solutions cannot be theoretically computed but must be determined from actual solution measurements. Because NaCl is a major osmolyte in this system, we used its osmotic properties as a simplification or proxy for the entire system (CRC Handbook of Chemistry and Physics solution tables, 1974). In brief, NaCl was assumed to be the only osmolyte in the system, and the CRC solution tables were used to determine what the original osmolality of the larvae must have been to yield the actual measured osmolality for the known degree of water dilution of the ground-up larvae samples. Specifically, the measured osmolality was converted to the equivalent NaCl molality. This in turn was "undiluted" to the original test solution molality using the known dilution factor (because molality scales directly with water mass), and finally the original osmolality was read from the tables. Cubic interpolation was used to fill in the table values, and all calculations were done using Microsoft Excel 2002. In practice, the osmolality of NaCl is fairly linear in this range (0.1–1.5 osmolal), and thus the corrections for nonlinearity amount to a few percent at most. The biggest correction comes from keeping proper track of the nonosmotic fraction of the solutions and therefore of the water content. All of the above calculations were done in Microsoft Excel 2002. To verify this method of back calculation for the internal osmolality, test solutions of sodium chloride of known osmotic strength (500–2,000 mOsm) were constructed and processed in a similar fashion to the coral larvae.

Multiple Larval Compartments and Osmolality

As detailed in "Discussion," our osmolality measurements gave some unexpected results for an osmoconformer. This led us to develop a three-compartment model of the larva to understand and analyze the osmolality data. These three compartments are the primary, nonmucus part of the larvae, the mucus layer surrounding the larvae, and the mucus cells in the larvae. The mucus cells and mucus layer contain bound osmolytes, which we assumed were released during the grinding and Triton-X treatment preparatory to the osmolality measurements. When discussing the osmolalities of the mucus compartments, we mean the osmolalities the compartments would have if all the bound osmolytes were released. For simplicity, we assumed that the osmolytes of the three compartments produced a simple additive effect on osmolality. This is described by the following equation:

$$\mathrm{Osm}_f = \frac{\mathrm{Osm}_1 \times V_{w_1} + \mathrm{Osm}_2 \times V_{w_2} + \mathrm{Osm}_3 \times V_{w_3}}{V_{w_1} + V_{w_2} + V_{w_3}}, \quad (2)$$

where Osm_f refers to the final osmolality of the ground-up larva, Osm means osmolality, V_w means osmotic water volume, and 1, 2, and 3 refer to the larvae nonmucus compartment, the mucus layer, and the mucus cells, respectively. The osmotic water volumes of the compartments are the microscopically determined total volumes of the compartments minus the nonosmotic portion, given by V_b. The volume of the mucus cells was determined from their cross-sectional areas in the micrographs. The volume of the mucus layer was estimated by multiplying its thickness by the surface area of the larvae.

Internal Organic Osmolytes

To determine what organic osmolytes might be playing a role in the osmotic balance of coral, we analyzed osmolytes in whole *F. scutaria* larvae. Developing larvae (days 1–6) were collected into Falcon cell-sorting baskets (Fisher, 40 μm mesh size), excess seawater was removed, and the larvae were moved into Eppendorf tubes, weighed on a scale (Mettler AB104 or AE204 that read to 0.0001 g), flash frozen in liquid nitrogen, and stored at -80°C. Two types of larvae were analyzed: those that had not been infected with zooxanthellae (days 1–6) and those that had been infected on days 3 or 4 for measurement on day 6 (designated day 6i). The larval tissue samples were then homogenized in nine to 19 volumes (relative to tissue mass) of 7% perchloric acid. The homogenates were centrifuged for 20

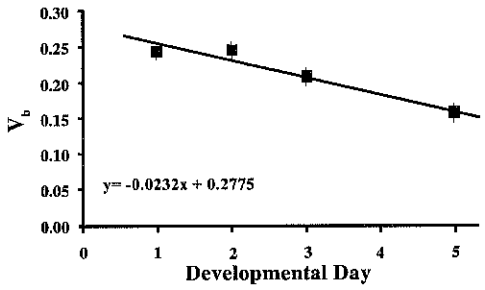

Figure 1. V_b values obtained from wet/dry measurements of the larvae during development. Squares represent an average V_b for 2–3 mo (10 samples/mo) for days 1, 2, 3, and 5. The line represents the best fit to the data, indicating a reduction in V_b with developmental age.

min at 4°C at 15,000 *g*; the supernatants were neutralized with KOH and then passed through C18 BondElut cartridges (Varian) and 0.45-μm filters, as previously described (Wolff et al. 1989). The final extracts were analyzed by HPLC as previously described (Wolff et al. 1989; Yin et al. 2000). The primary method used a SugarPak column (Waters, Milford, MA) that separates sugars, polyols, neutral amino acids, and methylamines, which are then detected by refractive index.

Although the HPLC method detects glycerol, it does not detect phosphorylated forms. Therefore, we used a glycerol test kit from Cayman Chemical (Ann Arbor, MI) that measures free glycerol by conversion to glycerol 3-phosphate and then dihydroxyacetone phosphate.

Mucosecretory Cell Development

The development of the mucosecretory cells was analyzed because the glycoproteins present in mucus cells can bind ions. This can potentially change the measured osmolality of the coral larvae when the mucus cells are crushed, as was done to analyze the internal osmolality of the larvae. Specific staining was done to examine the presence of mucosecretory cells. Larval tissues were fixed in fresh 4% paraformaldehyde solution in FSW in glass scintillation vials. Larvae were removed from the vials with chilled FSW and placed onto lens paper that was then folded like an envelope and inserted into a microcassette for processing. This held the larva in place without loss or damage while the microcassettes were moving from solution to solution. The single piece of lens paper (ca. 4 cm × 6 cm) did not inhibit the infiltration of solutions into the larvae; however, we did increase the processing time to allow for complete infusion through the paper. The microcassettes were placed into 70% EtOH over night and then dehydrated through a series of alcohols and xylenes (i.e., 95% × 3, 100% × 3, xylenes × 3, all at 40 min/solution), infiltrated with paraffin (liquid Paraplast × 3 for 40 min/solution), and embedded in fresh paraffin in small peel-away molds. Between the final 95% and first 100% EtOH solutions, the larvae were stained with Eosin Y (Fisher Scientific) for 1 min to increase visibility in the paraffin blocks. To transfer the larva from the lens paper envelopes into the final paraffin blocks, the envelopes were opened using warm forceps, then inverted into warm paraffin until all the larvae were collected from the paper into a warmed metal dish. These collected larvae were poured into molds, cooled, and rewarmed to liquid to ensure that all the larvae had sunk to the bottom of the mold (i.e., the front of the block).

Blocks were sectioned using a rotary microtome at 6 μm. Sections were placed onto the surface of a warm-water bath to flatten; collected with single untreated, clean glass slides; dried overnight on a slide warming tray (45°C); stained with Harris's hematoxylin; dehydrated; and then coverslipped with Permount.

To determine when mucopolysaccharides and glycoproteins began developing in mucus cells, serial sections were examined on days 1 and 4 (N = 10/d) using an Olympus BX41 microscope and × 100 oil immersion objective for the appearance of dark blue staining indicating the presence of these sugars. Mucosecretory cells were counted in each section, the cross-sectional area was measured in the section where the cell had the largest profile to determine the size of the cells, and an average cell size was determined assuming a prolate spheroid shape. In addition, the mucus layer thickness was measured in the day 1 and day 4 larvae.

Statistical Analyses

A Kruskal-Wallis nonparametric ANOVA test using GraphPad Instat 3.0 B software for Macintosh (San Diego, CA) was used to analyze whether the means of the samples from the dehydration tests were significantly different.

Results

Developmental Changes in Larval Osmotically Inactive Volume Fraction

V_b represents the osmotically inactive fraction of the larvae. During *Fungia scutaria* development, a decrease in V_b was ob-

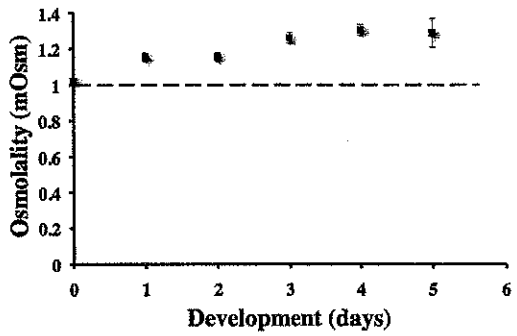

Figure 2. Developmental changes in the internal osmolality of coral larvae over time. Squares represent the internal osmolality of coral larvae over time. The line represents 1,000 mOsm of seawater. These data suggest that the internal osmolality of coral larva was not isosmotic with their environment.

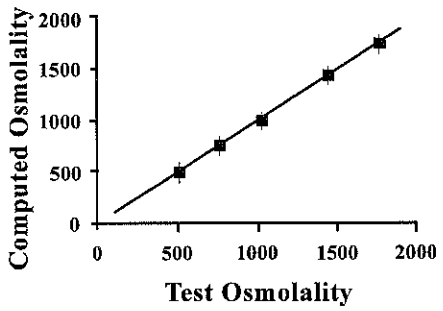

Figure 3. To test the osmotic back calculation method used for measuring the osmolality of the coral larvae, a range of filtered seawater and NaCl solutions with osmolalites of 500–2,000 mOsm were prepared and measured with a vapor pressure osmometer. The line is an aid showing the ideal case where the output measured omolality agrees exactly with the input starting osmolality. The squares show the amount by which the actual computed osmolalities miss the ideal case (*line*).

served (Fig. 1). Specifically, days 1 and 2 remained similar at 0.240 and 0.245, respectively, and then V_b declined in the following developmental stages from 0.208 at day 3 to 0.167 at day 5. These data were fit with a straight line, and the fit was used to determine V_b for each day as needed in the calculations below. From the fit, we estimated a $V_b = 0.278$ for day 0 for the unfertilized oocytes, indicating a 40% decrease in V_b from the oocyte to day 5.

Developmental Osmotic Measurements

The internal osmolality of the larvae demonstrated an increase from isotonic values around 1,000 mOsm on day 0 increasing by almost 27% at day 4 (Fig. 2). The control solutions used to test the method for calculating the osmolality demonstrated good linearity, suggesting that the calculation method was accurate (Fig. 3). We considered the possibility that during the processing for the osmolality measurements, the larvae might have dehydrated, thus reducing the amount of water in the cells and raising their osmolality. However, the osmolality of larvae left on the bench for 1, 3, or 10 min showed no difference among the groups ($P > 0.05$), suggesting that this upward shift in osmolality observed during development was not due to dehydration during processing. The apparent increase in internal osmolality observed in Figure 2 was unexpected. We will consider this further, but first we will examine the nature of the osmolytes in the larvae.

Internal Organic Osmolytes

The dimensions of the larvae and V_b appear to be relatively similar throughout development, suggesting that the cell increases its osmolality by increasing its osmolytes. Generally, about 300–400 mOsm of a marine invertebrate's cells' osmolytes are thought to be accounted for by ions; therefore, the intracellular organic osmolytes were analyzed at all stages of larval development (Fig. 4) to determine what these were and whether they might account for the increasing internal osmolality observed in Figure 2. Glycine betaine accounted for more than 90% of the identified organic osmolyte-type solutes (Fig. 4), reaching as high as 215 mmol/kg wet mass. This was also the case for larvae analyzed at day 6 that had been infected with zooxanthellae at day 4 (column 6i in Fig. 4). Of the other amino acids, taurine and glycine were detected at moderate levels while other amino acids were less than 1 mmol/kg each (not shown). Myo-inositol was also found at 1.5–2.9 mmol/kg. Glucose was detected only at trace amounts (<0.2 mmol/kg wet mass) in all stages. The total quantity of these osmolyte-type solutes increased between day 1 and day 2 by approximately 30% (Fig. 4). However, the increase in the amount of intracellular organic osmolytes was not sufficient to explain the upward shift in intracellular osmolality observed in Figure 2 during development. At day 6, we examined larvae infected (day 6i) and uninfected (day 6u) with zooxanthellae and found no difference in the type or amounts of organic osmolytes except for glycerol. Glycerol was not detected in any uninfected samples (days 1–5 and 6u) but was found at very low amounts (i.e., 0.86 + 0.17 mmol/kg wet mass) in the infected samples using HPLC. The glycerol test kit yielded a value of 8.58 + 1.87 mmol/kg wet mass in the day 6i larvae. This value represents the sum of free glycerol, glycerol 3-phosphate, and dihydroxyacetone phosphate.

Mucosecretory Cell Development

It is known that mucopolysaccharides and glycoproteins can bind ions. Therefore, we examined the development of the mucus layer and cells. On day 1, glycoproteins were evident in the epithelia of the pharynx (Fig. 5), and by day 4, in addition

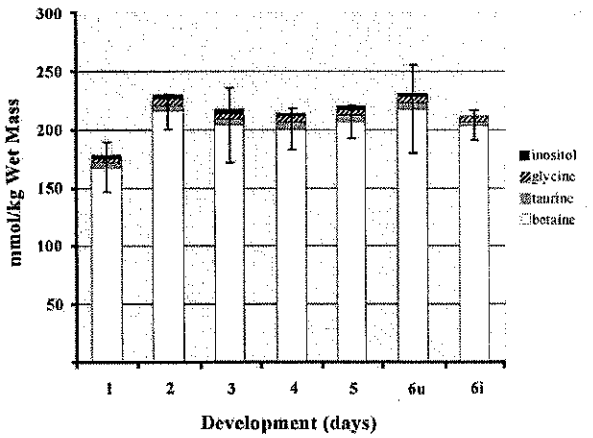

Figure 4. Major organic osmolytes present in developing *Fungia scutaria* larvae. All of the larvae were azooxanthellate except for day 6. On day 6, both infected (6i) and uninfected (6u) were analyzed. A small amount of glycerol (i.e., 0.86 + 0.17 mmol/kg wet mass) was detected in the 6i samples, but it was not large enough to show up on the graph compared with the other osmolytes.

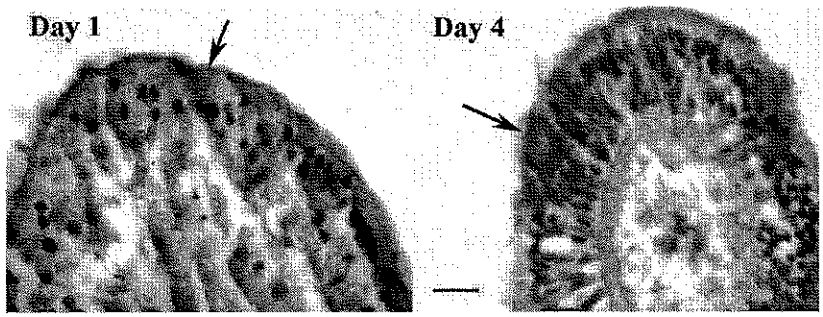

Figure 5. Development of mucus cells in *Fungia scutaria*. Day 1 larvae had no mucus cells, only a blue-staining mucus layer that is about 2 μm thick (*arrow*). In contrast, day 4 larvae had well-developed, dark-staining mucus cells (*arrow*) but a less distinct mucus layer. Bar = 20 μm.

to the epithelial layer, there were numerous dark-staining cells abundant throughout the larvae (Fig. 5). The light micrographs of the larvae show a mucus layer of ~2 μm thickness on day 1 becoming less distinct on day 4. The average number of mucus cells on day 1 (N = 10 larvae/d) is 0.1 ± 0.0 SEM, and by day 4, the larvae have a mean number of 69.2 ± 0.6 mucus cells of volume 929 μm^3 each (Fig. 5). Thus, only one mucus cell was found in all larvae examined on day 0, but on average there were 69 mucus cells in each day 4 larva. Ions are bound to the glycoproteins in the epithelia and mucus cells. These were probably released during the grinding and other preparative steps, and that release gave rise to the apparent increase in intracellular osmolality

To test this hypothesis, we calculated the necessary osmolality (in the form of bound ions; eq. [2]) in the mucus layer and mucus cells to yield the apparent increase in larvae osmolality with development (Table 1). We assumed that no mucus existed on day 0 and that the measured day 0 osmolality of 1,021 mOsm was the osmolality of the nonmucus compartment. On day 1, we assumed that the nonmucus portions of the larvae remained at 1,021 mOsm and that the mucus layer contributed enough to raise the measured osmolality to 1,154 mOsm. On day 4, we assumed that an additional contribution from developing mucus cells was sufficient to raise the measured osmolality to 1,304 mOsm. With these assumptions, we determined an osmolality of 2.62 and 4.31 Osm for the mucus layer and the mucus cells, respectively. Often on day 4, the mucus layer is slightly thinner than on day 1; if this is the case, then the mucus cell would have to have a slightly higher osmolality to compensate for this.

Discussion

Given all of the data presented here, we concluded that in coral larvae (1) V_b decreased with development, (2) the internal osmolality remained constant with development, and (3) there were no differences between infected larvae and uninfected larvae in their organic osmolytes within the initial onset of symbiosis. Moreover, the most reasonable conclusion is that *Fungia scutaria* larvae are isosmotic with seawater, acting as osmoconformers. Thus, we need an explanation of the unexpected data (Fig. 2) that shows a general increase in osmolality with development day. The following considerations might help explain these results.

There were only a few variables that went into the determination of the internal osmolality. The larvae were measured in FSW, so there was no solute flux or volume change to consider in these calculations. Thus, only two parameters were required for the calculation of internal osmolality: (1) the fraction of water surrounding the cells and (2) the osmotically active portion of water within the cells ($1 - V_b$). For the former, we assumed a volume carryover of approximately 20% FSW surrounding the cells on the basis of estimates of the amount of FSW that had to be added back to oven-dried larvae to reproduce their initial appearance of wetness. For the latter, we note the following. Had V_b increased by 60% from day 0 to day 4, then this shift would have given rise to the upward shift in internal osmolality observed on day 4. However, the measured V_b's in Figure 1 decreased with development, and these changes in V_b were taken into account in the internal osmolality calculation. Thus, we can think of no error source that would have led to these results.

Instead, we propose that the glycoproteins present in the mucus and the mucus cells bound many ions that increased the apparent osmolality of the coral larva. This is not a new notion for coral cells. For example, Lubbock et al. (1981) demonstrated that nematocysts from sea anemones have calcium bound to proteins that, when released, reach a concentration of 5,000–6,000 mOsm. Additionally, Clode and Marshall (2002) have also shown through x-ray microanalysis that scleractinian mucus binds high concentrations of Ca^{+2} that help facilitate calcium deposition in the skeleton. In addition, high concentrations of K^+, Sr^{+2}, Na^+, and Cl^- have been found in adult coral mucus (Marshall and Wright 1991, 1995). Clearly, this ability to bind ions starts very early in development, day 1 for *F. scutaria*, and readies the larvae to begin depositing calcium carbonate for its exoskeleton once it has completed metamorphosis and begins to settle.

Coral osmolytes have received little study (Mayfield and

Table 1: Mucus osmolality calculations

Variable	Developmental Day 0	1	4[a]	Units
Larvae osmolality:				
Measured (total)	1.021	1.154	1.304	Osm
Assumed (nonmucus)	1.021	1.021	1.021	Osm
Larvae volume (total)[b]	1.09×10^{-3}	1.09×10^{-3}	1.09×10^{-3}	mm^3
Larvae V_b[c]	.278	.254	.185	...
Mucus layer volume[d]	0	1.03×10^{-4}	1.03×10^{-4}	mm^3
Mucus layer V_b[e]	...	.35	.35	...
Mucus cell volume[f]	0	0	6.41×10^{-5}	mm^3
Mucus cell V_b[e]	...	...	.35	...
Mucus layer osmolality[g]	...	2.62	2.62	Osm
Mucus cell osmolality[h]	...	...	4.31	Osm

Note. Measured larvae osmolality is presumed to include contributions from both the nonmucus and the mucus compartments. On day 0, there is no mucus compartment, and the measured larvae osmolality is 1.021 (~filtered seawater). We assume this same value for the nonmucus compartment on subsequent days. Then, using equation (2), we compute and report here the contributions required of the developing mucus compartments to yield the measured osmolalities on days 1 and 4 as outlined.

[a] On day 4, we assume that the mucus layer contribution from day 1 applies, and we compute the additional contribution required of the mucus cells. Also, the mucus layer is slightly thinner than on day 1; if this is the case, then the mucus cells would have to have slightly higher osmolality to compensate for that.

[b] Determined from light micrographs of fixed-stained larvae on day 4 and held fixed for other days.

[c] Determined from wet/dry measurements (Fig. 1).

[d] Mucus layer volume = thickness × area = 2×10^{-3} mm × 5.13×10^{-2} mm^2 (see footnote c). Thickness determined from light micrographs.

[e] Estimated from data from Ducklow and Mitchell (1979; i.e., 380 μg solids/mL mucus).

[f] Determined from light micrographs.

[g] Calculated for day 1 using equation (2) and assuming the mucus cell volume was 0 on day 1.

[h] Calculated using equation (2), the known volumes, and the osmolalities of the larvae and mucus layer.

Gates 2007), and this is the first full report of their detailed analysis for larval coral, although Andrell et al. reported preliminary results in 2007 (Andrell et al. 2007). It has been speculated that glycerol (Muscatine 1967; Gates et al. 1995; Gates and Edmunds 1999) and DMSP (Broadbent et al. 2002; Jones and Trevena 2005) are likely candidates for osmolytes produced mostly by symbiotic zooxanthellae. The developing *F. scutaria* larvae in this article were azooxanthelate except for the samples analyzed on day 6 that had been infected 2 d previously. Regardless of whether the larvae were infected, the basic pattern for organic osmolytes did not vary. Glycine betaine concentration dominated all days of development regardless of infection with algal symbionts. Glycine betaine can be an algal byproduct (Blunden et al. 1992), but this analysis indicated that these corals do not obtain betaine from their symbionts. Total organic osmolyte content at days 2 and 6 were significantly higher than at day 1 (Fig. 4).

According to Gates and Edmunds (1999, p. 38), "Glycerol is generally considered to be a substrate for host respiration," and they speculated that "glycerol may play a fundamental role in the osmoregulation of these symbioses as has been demonstrated in other systems." However, in our report, free glycerol was found (by HPLC) only in small amounts (0.86 mmol/kg wet mass) in the larvae infected with zooxanthellae (day 6i samples). The low level of glycerol in the infected day 6 larvae may indict that it is metabolized as soon as it is produced. This is supported by the findings of the glycerol test kit, which gave a value for free glycerol, glycerol 3-phosphate, and dihydroxyacetone phosphate about 10-fold higher than the HPLC value for free glycerol. These results suggest that glycerol does not play a significant role in the osmoregulation of the larval coral.

Glycine betaine could be a good candidate as a cryoprotectant for coral because it is a main osmolyte. Betaine has not been used to cryopreserve any animals, but Cleland et al. (2004) found it to be a successful cryoprotectant for prokaryotes.

The goal of these experiments was to develop a fundamental and more comprehensive understanding of the internal osmotic factors of the developing coral larvae because so little is known about them. Ultimately, we are interested in cryopreserving coral gametes and/or larvae. This requires knowledge of the osmotic characteristics of cells, the permeability of the membranes to water and cryoprotectants, and the selection of nontoxic cryoprotectants. As a by-product of our studies, we have learned more about the basic physiological events that underline the move from autonomous living to symbiosis for coral. Additionally, some of these factors may potentially affect the

transport of water and solutes to either help or hinder the cryopreservation process.

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