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Physiology and cryosensitivity of coral endosymbiotic algae (*Symbiodinium*) ☆

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ABSTRACT

Coral throughout the world are under threat. To save coral via cryopreservation methods, the *Symbiodinium* algae that live within many coral cells must also be considered. Coral juvenile must often take up these important cells from their surrounding water and when adult coral bleach, they lose their endosymbiotic algae and will die if they are not regained. The focus of this paper was to understand some of the cryo-physiology of the endosymbiotic algae, *Symbiodinium*, living within three species of Hawaiian coral, *Fungia scutaria, Porites compressa* and *Pocillopora damicornis* in Kaneohe Bay, Hawaii. Although cryopreservation of algae is common, the successful cryopreservation of these important coral endosymbionts is not common, and these species are often maintained in live serial cultures within stock centers worldwide. Freshly-extracted *Symbiodinium* were exposed to cryobiologically appropriate physiological stresses and their viability assessed with a Pulse Amplitude Fluorometer. Stresses included sensitivity to chilling temperatures, osmotic stress, and toxic effects of various concentrations and types of cryoprotectants (i.e., dimethyl sulfoxide, propylene glycol, glycerol and methanol). To determine the water and cryoprotectant permeabilities of *Symbiodinium*, uptake of radio-labeled glycerol and heavy water (D_2O) were measured. The three different *Symbiodinium* subtypes studied demonstrated remarkable similarities in their morphology, sensitivity to cryoprotectants and permeability characteristics; however, they differed greatly in their sensitivity to hypo- and hyposmotic challenges and sensitivity to chilling, suggesting that standard slow freezing cryopreservation may not work well for all *Symbiodinium*. An appendix describes our H_2O:D_2O water exchange experiments and compares the diffusionally determined permeability with the two parameter model osmotic permeability.

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Introduction

Coral reefs are some of the oldest and most diverse ecosystems on our planet. They are one of the ocean's main nurseries and feeding grounds for fish and invertebrates, provide natural storm barriers for coastlines, and are a potential source for novel pharmaceuticals. Throughout their range, coral reefs are dying due to human influences. Even in the most remote marine bioreserves, such as the northwestern Hawaiian Islands [33], human activities are damaging fragile coral ecosystems [4]. As greenhouse gasses increase, atmospheric and sea-surface temperatures are also expected to increase [15,14] coupled with anthropogenic stresses, reefs will remain in crisis, threatening their existence worldwide [22,16,23].

In situ conservation practices, such as habitat preservation, are an important way to conserve coral reefs. However, reefs now face global rather than just local threats. Therefore it is critical that *ex situ* conservation practices are incorporated into conservation solutions for coral reefs. Novel *ex situ* conservation techniques, such as genetic banks using frozen samples, hold strong promise for rapid improvements in preserving species and genetic diversity within ecosystems. These frozen banks reflect a new and major type of preservation that can be added to conventional archives, but in this case, the living biomaterials go beyond dried materials to include gametes, embryos, somatic and stem cells, blood, and DNA.

The cryo-physiology of coral larvae is fairly well-known and coral sperm has been successfully cryopreserved [18,19]. Three genome repositories worldwide now hold endangered cryopreserved coral sperm from the endangered coral, *Acropora palmata* (Hagedorn et al., unpublished data). Our long-term goal is to create a genetic bank for all types of coral cells and their endosymbiotic cells, such as zooxanthellae. Genome repositories have important passive and active functions. First, genetic material can remain

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frozen but alive for hundreds of years in liquid nitrogen, allowing the time necessary to mitigate and restore habitats. Second, large samples of a gene pool can be maintained, preventing species extinction. Third, the banks can be used actively to increase genetic diversity within an ecosystem through the use of thawed samples to 'seed' shrinking populations [2,51,52].

The focus of this paper was to understand some of the cryophysiology of the endosymbiotic algae, *Symbiodinium*, living within three species of Hawaiian coral, *Fungia scutaria, Porites compressa* and *Pocillopora damicornis* in Kaneohe Bay, Hawaii. The algae in the genus *Symbiodinium* (often referred to as symbionts, endosymbionts or zooxanthellae) live within some coral cells and produce energy-rich compounds in exchange for the carbon substrates needed for photosynthesis (Fig. 1). When coral bleach, they lose their zooxanthellae and often die. Many adult coral directly transfer these endosymbionts to offspring, whereas other larvae must assimilate the algae from surrounding water into their cells during development. Throughout the world's oceans, there are many types of *Symbiodinium* divided into eight genetically distinct clades further subdivided into numerous subtypes [1,46]. LaJeunesse et al. [28] used molecular tools to analyze the diversity of the *Symbiodinium* inhabiting corals in Kaneohe Bay. Although coral can sometimes harbor one or more types of *Symbiodinium* [46], presumably in our samples, all of the endosymbionts were from different subtypes of clade C [28].

There are several algal culture collections around the world, including the Provasoli-Guillard National Center for Culture of Marine Phytoplankton and the Hawaii Culture Collection, and these centers routinely cryopreserve many marine and freshwater algae [41,10,11,36,12,32,20]. However, there are limited reports on successful cryopreservation of *Symbiodinium* [45], yielding relatively low post-thaw results. Because of this, *Symbiodinium* are generally maintained in live serial cultures in national collections (J. Sexton, Provasoli-Guillard National Center for Culture of Marine Phytoplankton, pers. comm.). A major problem in cryopreserving *Symbiodinium* is having a clear post-thaw measure of their health and viability. To address this challenge, we used a Pulse Amplitude Fluorometer (PAM). This instrument measures reflected energy conversion efficiency in Photosystem II reaction centers of algal chloroplasts [26]. The efficiency with which light energy is utilized is a function of cell 'health'. When cells are under stress, the system becomes saturated more easily and light is not efficiently utilized. This method yielded a reliable physiological index of how specific cryopreservation methods damaged zooxanthellae.

The fundamental cryobiological information resulting from this study may provide key elements for preserving *Symbiodinium* algae. Cryopreserved zooxanthellae are a critical element to help restore wild populations; especially for the two coral species currently listed under the Endangered Species Act, *Acropora palmata* and *Acropora cervicornis*. Restoration may require these larvae to be settled and grown in semi-captive environments far from coral reefs where they must be inoculated with their species-appropriate *Symbiodinium* [38]. Such restoration efforts are already underway by SECORE (www.secore.org). Having the correct zooxanthellae for the larvae to assimilate at these remote sites, where adult coral of the right species may be absent, will be critical for their ability to be returned to the wild. Our work may help provide a solution for this important conservation process. Additionally, oil rich marine photosynthetic algae are the focus of many bio-prospecting teams searching for better biofuels for alternate energy production. As important strains are found, the algae must be frozen in a repository or face the same issues of genetic drift and contamination affecting serial culture. Although cryopreservation is fairly well established for many marine algae [7,8,41], the oil-rich algae may be as difficult to cryopreserve as coral symbionts and other oil-rich plants [37].

To understand how these cells might withstand the rigors of cryopreservation, freshly-extracted *Symbiodinium* from three coral species were exposed to the following physiological challenges and viability assessed, including sensitivity to chilling temperatures, osmotic stress, and toxic effects of various cryoprotectants (i.e., dimethyl sulfoxide, propylene glycol, glycerol and methanol). To determine the water and cryoprotectant permeabilities of *Symbiodinium* uptake of radio-labeled glycerol and heavy water (D_2O) were measured.

Fig. 1. Zooxanthellae, *Symbiodinium* sp., are intracellular symbionts living within coral tissue with dark brown-green pigments. The tissue whole-mount compressed the larval coral tissue making the symbionts more apparent. These symbionts are evident as the brown spots observed throughout the preparation. Bar = 50 μm.

Methods

Morphology

Digital images of the zooxanthellae from all three species were captured with an Olympus BX41 microscope with an attached digital camera Sony DFWV300 and the major and minor axes measured with NIH Image (V1.62) software. Higher resolution images were captured with a Nikon Cool-Pix 900.

Collection and extraction of zooxanthellae

Using the PAM technique, the cryosensitivity of the zooxanthellae from three coral species were examined, including *F. scutaria, P. compressa* and *P. damicornis*. Coral fragments were collected for extraction of zooxanthellae. *P. compressa* and *P. damicornis* fragments were collected from the reef surrounding Coconut Island in Kaneohe Bay, Hawaii (Latitude:21° 26.2′ N; Longitude: 157° 47.6′ W). *F. scutaria* fragments were collected in the Bay or from captive colonies housed in outdoor tanks at the Hawaii Institute of Marine Biology on Coconut Island, Oahu. Five cm square fragments were broken off using a chisel, leaving the remaining coral intact. Fragments were placed into a container of seawater and transported to the laboratory and zooxanthellae extracted, as adapted from the methods of Schwarz et al. [42].

Briefly, coral tissue was dissociated from the skeleton using a WaterPik filled with filtered seawater. Tissue was separated from the skeleton into plastic bags, moved to 50 ml plastic centrifuge tubes, and then concentrated with a Beckman Allegra 6R centrifuge at 4070g for 15 min to remove excess water. Tissue was homogenized using a glass homogenizer, and re-suspended into filtered

seawater. Samples were then reconcentrated with an Eppendorf 5415 centrifuge at 8100g for 10 min, causing zooxanthellae to pellet at the bottom of tube so that coral tissue and seawater could be removed. The resulting pellet was re-suspended, homogenized and spun until zooxanthellae appeared clean under microscope. All solutions were made up in filtered seawater (FSW, ~1000 mOsm natural seawater filtered through a 0.5 μm filter), unless specified otherwise.

Pulse amplitude modulated (PAM) fluorometry

Zooxanthellae from each species of coral were assessed physiologically using an Underwater Fluorometer Diving-PAM (Walz GmbH, Germany) to examine effects of chilling, changes in osmolality, and exposure to cryoprotectants. Coral fragments of ~5 cm^2 were harvested from each species and their resulting zooxanthellae concentrated to ~2×10^6 cells/ml. This concentration yielded consistent readings with the PAM. Zooxanthellae counts were made with a microscope using a hemocytometer. The PAM determined the maximum quantum yield of Photosystem II measured as the ratio of variable to maximum fluorescence (F_v/F_m) [26]. This measurement was used as an indication of the health of Photosystem II. For all trials, 1 ml total volume samples of zooxanthellae (2×10^6 cells/ml) and treatment solution, when applicable, were placed into 5 ml conical plastic tubes. At each time point 5 readings per sample were taken with the PAM's main optical fiber immersed completely into the sample at 1 cm from the bottom of the tube to assure that the reading were consistent. However, only the first measurement was used for statistical analysis, because Photosystem II slowly saturated and the measurements went down gradually. All readings were taken under the same light conditions in a small confined room, using the same tube holder in the same position for consistency of light intensity and temperature. As positive controls, untreated zooxanthellae were examined using the PAM. As a negative control, a sample of zooxanthellae was boiled and examined with the PAM. Procedures specific to each of the treatment trials are continued below. Results are reported as the photosynthetic yield.

To determine the dynamic range of the PAM, live and dead zooxanthellae were mixed in known amounts. Zooxanthellae (2×10^6 cells/ml) were flash frozen and thawed three times in liquid nitrogen to destroy the cell membranes. These zooxanthellae were mixed with viable zooxanthellae at the same concentration in 10% increments and PAM readings made. These measurements were repeated 2 times for the three *Symbiodinium*. The yield was measured, the data normalized to the first reading (100% viable), then averaged and plotted. It is possible to get a false reading with the PAM, but we took multiple readings over time. So, if a cell was dead, but its chloroplast was still intact, these readings over time would reveal a decline in PAM activity. Additionally, a lower PAM reading after a metabolic stress could be transient, and the algae could recover. Therefore, we monitored the cells in many cases for hours to determine whether the stability of the PAM readings.

Cell solids by Boyle van't Hoff

To determine the non-osmotic portion of the zooxanthellae volume (V_b), we used the Boyle van't Hoff (BVH) analysis in which the volume is plotted versus the reciprocal osmolality of the test solutions (500 mOsm FSW, FSW 1000 and 1500 mOsm FSW). Zooxanthellae (~2×10^6 cells/ml) were diluted 1:1 (v/v) either with DI to make the 500 mOsm FSW solution, or a 2× FSW (FSW supplemented with NaCl), yielding a 1500 mOsm hyperosmotic solution. In these plots, the *y*-axis intercept is V_b. Briefly, cells were mixed into the test medium and the volumes of 10 cells/solution/species were measured (as described above) after a 30-min exposure at 23–25 °C.

Cell solids by wet-dry weight

To determine the actual dry weight of the algal cells, all water was driven off from the cells. Eppendorf tubes were weighed, filled with cells (~2×10^6/ml), centrifuged at 8100g, the excess water decanted, the pellet dried by blotting with tissues, the tube reweighed to determine wet weight, placed in a 60 °C oven for 14 days, reweighed, and the dry weight calculated. Five samples were processed for each species and a mean dry weight calculated. The cell solids fraction, Vsb, were calculated as (Dry Weight/Wet Weight). No carry-over of surface water on the blotted pellet was assumed in this calculation. However, if we assumed that 5% by mass of carry-over water, this would have raised the measured cell solids volume by less than the error of the measurement. Note that the Boyle van't Hoff V_b includes osmotically bound water while Vsb does not. Thus these measures of cell solids are similar, but not necessarily identical.

Physiological responses to osmotic changes

To determine the physiological sensitivities of the zooxanthellae to osmotic stress, the algae from the three coral species were moved into hyper- and hyposmotic conditions, left in these solutes, and then their response to the stress was followed over time (i.e., 5 min, 1, 2, 3 and 20 h) with the PAM. Zooxanthellae (~2×10^6 cells/ml) were diluted 1:1 (v/v) either with DI to make the 500 mOsm FSW solution, or 2 × FSW (FSW supplemented with 1000 mOsm NaCl) to make hyperosmotic (1500 mOsm) solution. PAM readings were taken 5 min after mixing, and every hour after mixing for 3 h, and for some samples overnight, at 23–25 °C. This was repeated three times for the zooxanthellae from each of the three coral species.

Cryoprotectant toxicity

To determine a potential candidate cryoprotectant or cryoprotectants for successful cryopreservation of *Symbiodinium*, the toxic effects of various cryoprotectants was assessed on the function of algal Photosystem II. Zooxanthellae were exposed to each of these cryoprotectants at 2.5%, 5%, and 10% concentrations (V/V) in FSW (i.e., methanol, dimethyl sulfoxide, glycerol and propylene glycol). The molar concentration conversions for the 2.5%, 5% and 10% cryoprotectant solutions were as follows: methanol, 0.62, 1.24, and 2.47 M; dimethyl sulfoxide, 0.352, 0.705 and 1.41 M; glycerol, 0.34, 0.69, and 1.37 M; and propylene glycol, 0.34, 0.70, and 1.36 M. Exposures were conducted by adding zooxanthellae (~2×10^6 cells/ml) in FSW to the test cryoprotectant. Specifically, 25, 50, or 100 μl of the test cryoprotectant solution was placed in an Eppendorf tube and cooled on ice to ~18–20 °C for 1 min to compensate for the exothermic reaction during mixing, then zooxanthellae in FSW were added to equal 1 ml. After mixing, each sample was examined with the PAM at 5, 10, 15, and 20 min at 23–25 °C. Trials were repeated four times for zooxanthellae from each of the three coral species.

Zooxanthellae density

The density of the zooxanthellae was needed for some of the permeability measurements discussed below. *F. scutaria* zooxanthellae were extracted as described above, and to estimate their density, 10 μl drops of zooxanthellae (2×10^7 cells/ml in 0.2 μm filtered seawater) were introduced into a series of sucrose solutions made up in Instant Ocean® (0–0.4 M). This range was deter-

mined by reasonable estimates of the cell densities (g/ml), knowing the other biophysical properties of the cell (i.e., volume, dry weight etc.). A single 10-μl drop of zooxanthellae was introduced mid-solution into 3 ml of a specific sucrose solution in a conical plastic tube, and the movement of the cells observed. The zooxanthellae were easy to observe because of their dark brown/green pigments (Fig. 1). If the compact cloud of cells was denser than the sucrose solution, it sank within 5 s, if the cloud was less dense than the sucrose solution it floated to the surface within 5 s. The sucrose solution that produced neutral buoyancy was identified when the cloud hovered mid-water for more than 20 s.

The densities of the sucrose test-solutions were determined, using data from the CRC Solution Tables for seawater and sucrose [53]. We assumed water and Instant Ocean® to be comparable solvents for sucrose since Instant Ocean™ is 98.8% water (V/V, based on the properties of seawater). Next the incremental increase in density was computed for a water solution to which a given quantity of sucrose was added. This was found to be: dq (g/ml) = 0.1327 M–0.00222 M^2, where M is the molarity of the added sucrose. Finally, the density of a given molarity of sucrose in Instant Ocean® was computed as q (Instant Ocean®) + dq.

The density of Instant Ocean® was measured directly: One ml samples (N = 7) of Instant Ocean™ (made up according to the package, ~1000 mOsm) were measured on a Metler AE 240 analytical balance (Columbus OH), and the mean computed. The micropipette was calibrated by measuring and comparing to the known density of deionized water.

Mathematically, the density of the zooxanthellae is given by:

$$q_z = (q_w \cdot V_w + q_s \cdot V_s)/(V_w + V_s); \quad (1)$$

where q = density, V = volume, and the subscripts z, w, and s represent zooxanthellae, water, and solids, respectively. From this we can solve for the density of the solids:

$$q_s = (q_z \cdot (V_w + V_s) - q_w \cdot V_w)/V_s: \quad (2)$$

This provides a useful cross check of the data, as we know that q_s should be a little greater than 1 since the cells solids are cellulose, proteins, nucleic acids, and lipids.

Cryoprotectant and water permeability

Uptake of radio-labeled glycerol

Radio-labeled cryoprotectant experiments provided a means to measure cryoprotectant permeability; however they only poorly constrain the value of the water permeability. Instead of using individual algal cells (which are very small), small algal pellets (~4 mm in diameter) were made and tested in these solutions (see below for details). These pellets were placed into a 1.54 molal glycerol solution consisting of radio-labeled 14 C-glycerol (Sigma Aldrich, St Louis, MO) and cold glycerol made up in FSW. The hot glycerol was purchased mixed in water at a sub-milli-molar concentration and thus, for the purpose of calculating solution concentrations, can be consider as pure water. The activity of the hot glycerol in counts per min (CPM) was determined via a series of dilution curves (1:1 to 1:1000) in FSW. The larval test solution contained a small amount of labeled glycerol, FSW, and cold glycerol at the desired concentration (1.54 molal). Thus, we were able to prepare any desired concentration of test glycerol for the larvae. Knowing the CPM of the labeled glycerol and proportions of labeled and cold glycerol in the final solution, we can convert CPM in the algae to moles of total glycerol that crossed the membrane of the algae.

To produce the algal pellets, a cleaned zooxanthellae (~2×10^6 cells/ml) sample was divided into appropriate number of Eppendorf tubes. The algal solution was concentrated (described above), the FSW discarded, the pellets removed from the tube and then run through a series of 'hot' and 'cold' 10% glycerol solutions (1.54 M) in well slides. For hot trials, the pellet was rinsed in one well of cold glycerol to remove the excess FSW from the outside of the pellet, then allowed to sit in hot glycerol for 30 s, 1 min, 2.5 min, 5 min, or 15 min at 23–25 °C, then rinsed through five cold wells, and placed into glass scintillation vials immediately with as little liquid as possible. For control trials, the pellet was treated similarly, except the timed rinse was 10% unlabeled glycerol. A sample from the last rinse was taken to serve as a background count and this was subtracted from the total counts. Each vial per time point (cold pellet, hot pellet, and background) was filled with 4 ml scintillation fluid (ScintSafe Econo 1, Fisher Scientific) and 10 μL of 0.1% Triton-X, mixed, and allowed to sit for at least 8 h to ensure that the algal cell walls were disrupted. Afterward, the samples were placed in the Beckman LS 3801 Scintillation Counter for counting. Trials were repeated at least 4 times for zooxanthellae from each of the three species of coral. Data were modeled as in previous studies [25] by numerical integration and least squares parameter fitting of the two coupled transport equations for Lp and P_s. Dimensional data and fitting parameters are shown in Table 1. The algal surface area (Ao) was considered fixed and given by the area of the initial volume.

H_2O:D_2O exchange experiments

To determine the water permeability (Lp) of the zooxanthellae using classical volumetric analysis was impossible because of the presence of the outer cell wall that prevented responsive osmotic changes from being measured. Instead, experiments similar to H_2O:D_2O exchange experiments described by Harvey and Chamberlain [21] were performed. In these experiments, cells were placed into a test solution containing some D_2O in which they initially floated at the surface. As the cells exchange H_2O for D_2O, their density increased. If the density of the D_2O test solution was carefully chosen, the increase in cell density was sufficient to cause them to sink. The length of time it took the cells to sink was a measure of the H_2O:D_2O exchange time, i.e., the water permeability, Lp, of the cell (assuming that no water channels are present and therefore the diffusional water permeability P_d equals the water filtration coefficient P_f [13]. The details of this method, including the choice of proper density of the D_2O test solution and the determination of Lp from the time to sink data are detailed in Appendix 1.

Experimentally, Instant Ocean® was prepared in regular H_2O with various percentages (2.5%, 3%, and 3.5%) of D_2O (Aldrich, #151882, St. Louis, MO), substituting for the H_2O (V/V). These test

Table 1
Physiological parameters of zooxanthellae from various coral species.

Parameter	*F. scutaria*	*P. damicornis*	*P. compressa*
Vo: algal volume (μm^3)[C]	1030 ± 80	1100 ± 82	820 ± 120
A: algal area (μm^2)[A]	493	515	424
Vsb: fractional volume of solids[B]	0.164 ± 0.008[a,b]	0.236 ± 0.006[a]	0.143 ± 0.004[b]
Internal glycerol concentration mmol/kg wet mass[C]	1.65 ± 0.65	1.52 ± 0.35	0.60 ± 0.13
Cell density (gm/ml)	1.0436	—	—
Lp (μm/min/atm)[D]	$2.0 \pm 0.2 \times 10^{-3}$	—	—
P_s (Gly) (cm/min)[E]	5×10^{-5}	5×10^{-5}	7×10^{-5}
Lp (Gly) (μm/min/atm)[F]	0.01–10	0.01–10	0.01–10

[A] Assuming a spherical volume.
[B] Vsb is comparable to the Boyle van't Hoff V_b, assuming a negligible amount of osmotically inactive water in the algae.
[C] Data from [54].
[D] Measurements made at 22 °C.
[E] Measurements made at 23–25 °C.
[F] Lp in the presence of glycerol. Data not well constrained.
[a,b] Values with the unshared superscripts denote significant differences ($P < 0.05$).

solutions were placed into 5 ml conical plastic tubes to a height of approximately 3 cm. Then 10 µl of zooxanthellae (2×10^7 cells/ml) suspended in H_2O Instant Ocean were gently injected with a pipette near the middle of the solution column and observed for approximately 30 s. Initially the 'cloud' of zooxanthellae cells was observed to rise toward the surface and then as D_2O was taken up, the bottom of the cloud began to sink. We measured the time till clear sinking was evident (10% of the cloud) which generally occurred a few seconds after the first hints of sinking. The entire cloud of cells did not act together in concert due in part to turbulence and cell-to-cell variations. These experiments were repeated 7, 14, and 7 times for the 2.5%, 3%, and 3.5% D_2O solutions, respectively, with individual sinking times ranging from 7 to 23 s.

Chilling

Samples of zooxanthellae in FSW (1 ml at $\sim 2 \times 10^6$ cells/ml) were placed into an Eppendorf with a thermocouple (Omega J-K-T Model HH23, Stamford, CT) and cooled on ice until they reached at least 4 °C. Samples were left on ice for 15, 30 or 60 min. Upon removal, samples were examined using the PAM at 0, 5, 15, 60 and 120 min and overnight at 20 h. Trials were repeated 3 times for zooxanthellae from each of the three species of coral.

Statistics

The determine whether there were significant differences among the treatment means in the test groups, ANOVA and non-parametric test, such as Mann–Whitney *U*-test or Krusgal–Wallis, were conducted using GraphPad Instat 3.0 B software for the Macintosh (San Diego, CA).

Results

Overall, the three species of zooxanthellae were more similar in many of their biological and biophysical attributes than they were different. However, there were major differences in how they responded to various stressors, such as chilling, osmotic and cryoprotectant stresses.

Morphology, osmometric behavior, dry weight, density

The zooxanthellae from the three species did not differ in their external volume when measured in FSW ($P > 0.05$) and appeared similar in their overall appearance (Fig. 2A–C). When each species was stressed osmotically with hyper- or hyposmotic solutions, the cells did not change in size as a result of external solutes ($P > 0.05$). Boyle van't Hoff measurements yielded inaccurate values of V_b of 0.86–0.94 for the three species. Therefore, V_b was estimated by desiccation to determine how much of the cell was dry weight, yielding Vsb—a fractional volume of the solids (Table 1) for the three species, specifically *F. scutaria* (0.164 ± 0.008 SEM), *P. damicornis* (0.236 ± 0.006 SEM), and *P. compressa* (0.143 ± 0.004 SEM). Only *P. damicornis* and *P. compressa* differ in their dry weights ($P < 0.05$). So although these algal cells did not appear to be different in their external appearance and volume, there are clear differences in V_s, *P. damicornis* having the highest percent solids. Measurements of the *F. scutaria* zooxanthellae using sucrose solutions yielded a density of 1.044 g/ml that in turn yielded an estimated cell solids density of 1.28 g/ml (Table 1).

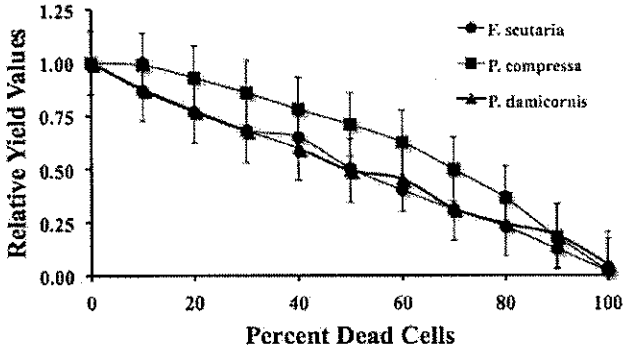

Fig. 3. The dynamic range of PAM, which measures the viability of Photosystem, was determined for each species using a known mixture of live and dead cells. The relative yields are similar for each species studied, and the relationship is fairly linear indicating that the relative quantum yield numbers of the PAM determined the live/dead ratio of cells fairly well.

Physiological responses to osmotic changes

The PAM measured maximum quantum yield of Photosystem II, and in order to determine what that meant in terms of numbers of live cells, we mixed live and dead algal cells in known percentages and measured their quantum yields (Fig. 3). The relationship for all three species is linear and basically the same for all three ($y = -0.0091x + 0.9683$). Thus, a percent yield decrease of 50% from the original value corresponded to a decrease in cell viability of 50%.

The three species of zooxanthellae responded differently when exposed to changing osmotic conditions (Fig. 4). Under isotonic conditions (FSW), the quantum yield measurements for *P. compressa* and *F. scutaria* remained stable over a 20 h period, whereas those for *P. damicornis* remained stable for the first 3 h, then decreased 58% by 20 h. Under either hyper- or hyposmotic conditions, *P. compressa* zooxanthellae were remarkable tolerant, showing a uniform decrease in quantum yield of ~ 8% after 20 h. In contrast, *P. damicornis* zooxanthellae had the greatest sensitivity of the three species tested to changes in osmolality. They exhibited decreasing quantum yields as the hyper- and hyposmotic stresses continued with almost 100% of the cells being dead after a 20 h

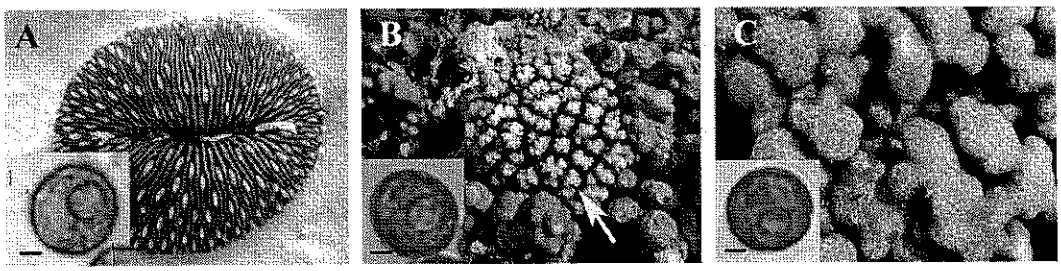

Fig. 2. Morphology of the *Symbiodinium* (insets) extracted from each species of coral, specifically (A) *Fungia scutaria*, (B) *Pocillopora damicornis*, (C) *Porites compressa*. Morphologically, these species are very similar. Bar = 3 µm.

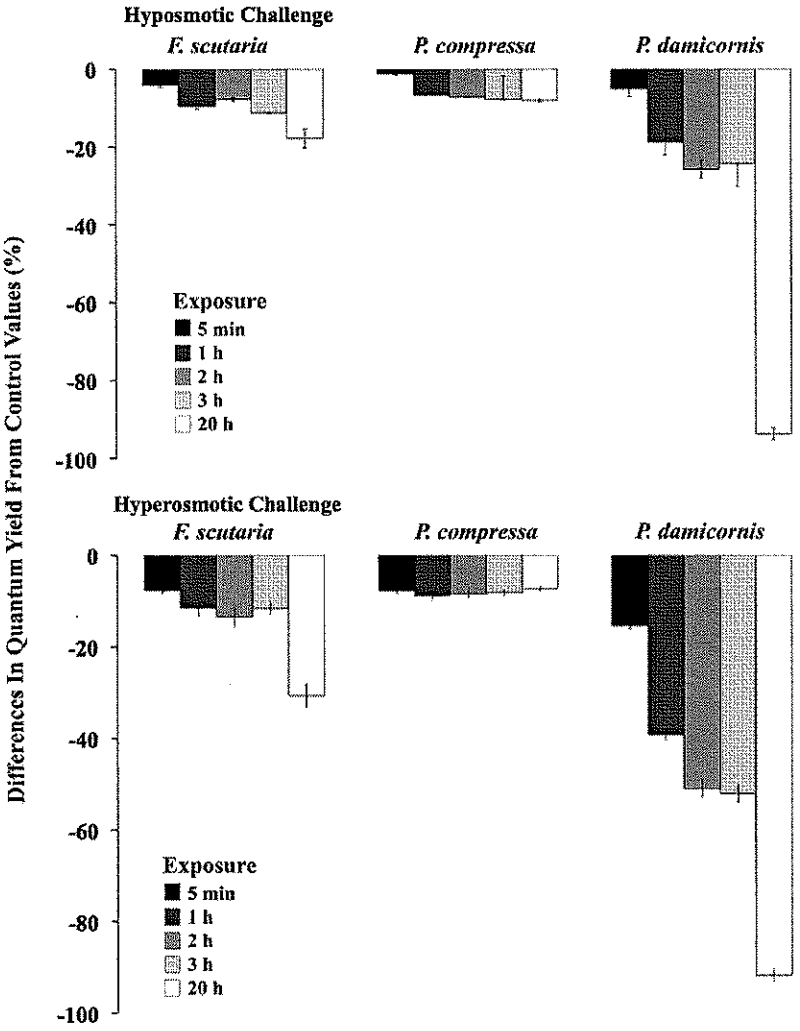

Fig. 4. Osmotic response: these two histograms show the response of the *Symbiodinium* from the three test coral species were exposed to hyposmotic (500 mOsm) or hyperosmotic (1500 mOsm) conditions. Left in these solutes, and then their responses to these conditions were followed over time (e.g., 5 min, 1 h, 2 h etc.) with PAM which measures the viability of Photosystem II. The *Symbiodinium* from *P. compressa* was the most resistant to these osmotic challenges, showing very little changes from initial conditions.

exposure. *P. compressa* zooxanthellae responded to hyper- or hyposmotic conditions somewhat in between the previous two species.

Cryoprotectant and water permeability

The calculated fits to the radio-labeled glycerol uptake data suggested that all of the zooxanthellae from the three species had rapid and similar glycerol uptake characteristics (P_s ~5 × 10^{-5} cm/min; Table 1; Fig. 5). This analysis yielded values for cryoprotectant permeability (P_s), but the water permeability, *Lp*, was only poorly constrained. Instead, H_2O:D_2O exchange experiments were used to determine *Lp*, yielding a value of 2.0 ± 0.2 × 10^{-3} μm/min/atm for species the *F. scutaria Symbiodinium* (see Table 1 and Appendix 1 for details).

Cryoprotectant toxicity

Four different cryoprotectants, dimethyl sulfoxide, propylene glycol, methanol and glycerol, were tested at three concentrations, 2.5%, 5.0% and 10% (V/V) in FSW over time, but only the final 20 min exposures were shown in Fig. 6. While no one cryoprotectant stood out as particularly toxic for the zooxanthellae (Fig. 6),

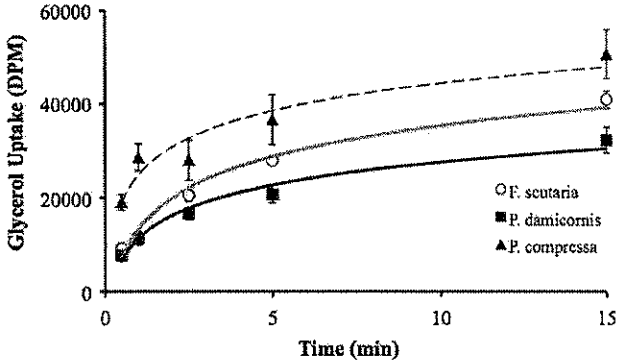

Fig. 5. Cryoprotectant permeabilities of *Symbiodinium* at 23–25 °C from the three test coral species were determined by the responses of the algal cells in 10% radio-labeled glycerol in FSW for up to 15 min. Data is denoted with symbols and lines indicate the fits to the data. The algal cells showed similar responses characteristics with glycerol permeabilities of 5–7 × 10^{-5} cm/min (see Table 1).

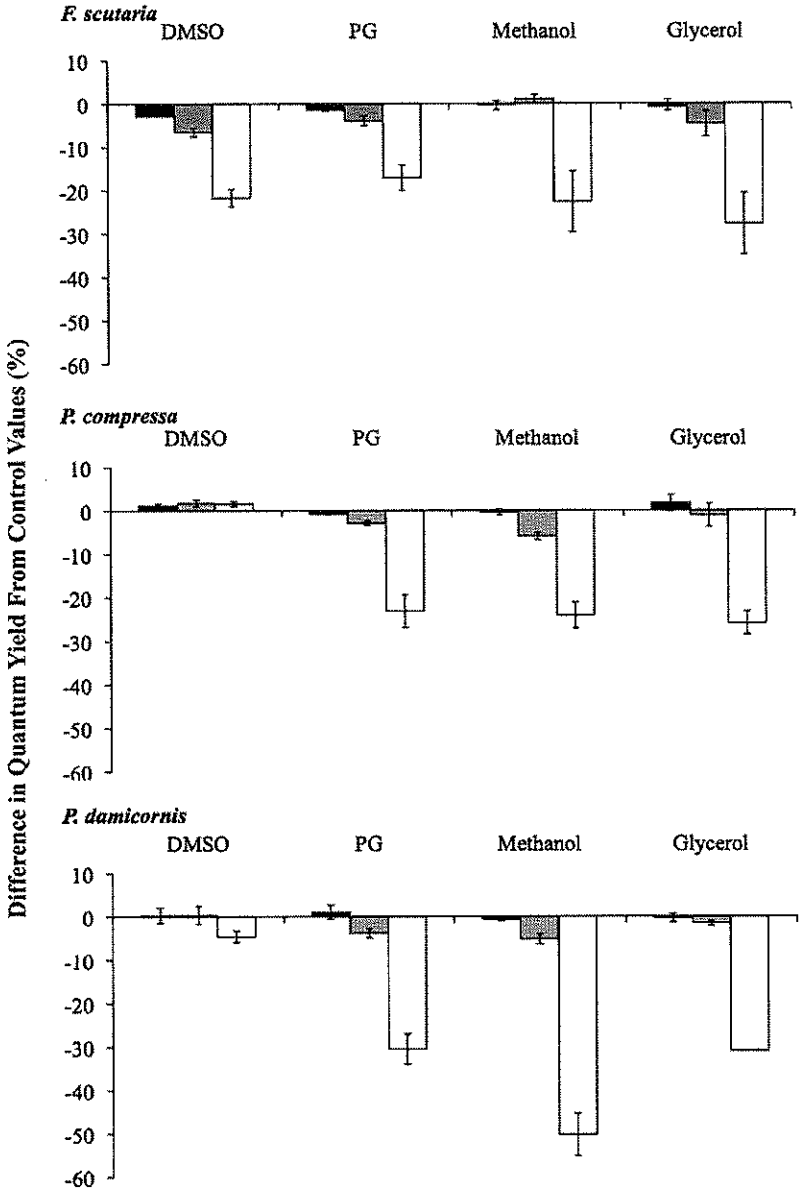

Fig. 6. Toxicity studies: these three histograms show the toxicity response of *Symbiodinium* from the three test coral species exposed to four cryoprotectants (DMSO, dimethyl sulfoxide; PG, propylene glycol, methanol and glycerol) concentrations (black bars = 2.5%, gray bars = 5% and white bars = 10%) after 20 min of exposure. The *y*-axis indicated the change in quantum yield of Photosystem II from control values. Concentrations at 5% or lower do not impact the viability of the *Symbiodinium*, however, the 10% was more detrimental causing reductions in yields after 20 min. Black bars = 2.5%.

the highest concentration of the cryoprotectants tested (10%) caused a decrement in the quantum yield of the zooxanthellae after 20 min ($P < 0.05$).

Chilling sensitivity

There was a large difference in the physiological chilling sensitivity for the three species of zooxanthellae tested. *F. scutaria* was not sensitive to chilling, whereas the other two species were very sensitive to chilling (Fig. 7). When *F. scutaria*'s zooxanthellae were chilled for 15, 30 or 60 min on ice, there was no difference in their post-exposure responses and only a small impact on their quantum yield ($P > 0.05$). In fact, when measured immediately after being taken out of the ice bath (and presumable still at 0 °C), the quantum yield was lower and improved post-chill. The other two species were consistent in their post-exposure responses regardless of the time spent at chilling temperatures. Specifically, they showed post-exposure losses in quantum yield of 40–45% up to 60 min and 100% loss at 20 h.

Discussion

Symbiodinium have been endosymbionts for over 200 million years [44], and those studied here demonstrated remarkable similarities in their morphology, sensitivity to cryoprotectants and permeability characteristics, they differed greatly in their sensitivity to hypo- and hyposmotic challenges and sensitivity to chilling, suggesting that standard slow freezing cryopreservation may not work well for all *Symbiodinium* species. Using various freezing methods on marine algae, Ben-Amotz and Gilboa [3] observed that

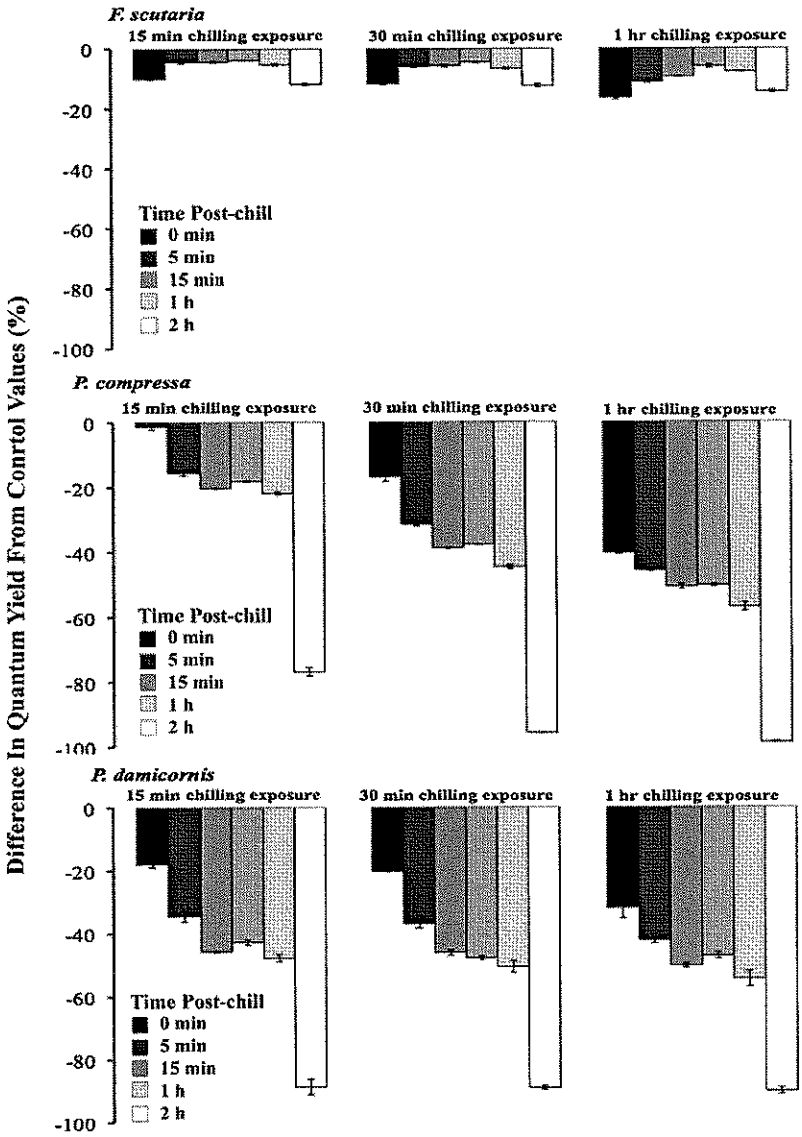

Fig. 7. Chilling sensitivity: these three histograms show the responses of *Symbiodinium* from the three test coral species exposed to chilling temperature of 0 °C for 15, 30 and 60 min. The *Symbiodinium* were followed for up to 2 h after the chilling exposure to determine whether they had delayed responses to the chilling. The y-axis indicated the change in quantum yield of Photosystem II from control values to three chilling exposures, 15, 30 and 60 min. Then, once the algae were removed from the 0 °C bath, their post-chill response was monitored over 2 h. Even with a modest chilling exposure of 15 min, the *Symbiodinium* for *P. compressa* and *P. damicornis* demonstrated a loss of viability, however the *Symbiodinium* from *F. scutaria* was not particularly chill-sensitive even after 60 min of chilling exposure.

post-thaw survival was species dependent. This complexity is supported by the lack of large numbers of *Symbiodinium* samples successfully stored and revived in marine algal stock centers worldwide.

The cells used in these experiments were freshly extracted from host tissue, and this extraction alone may have damaged the cells and only have shown up when they were stressed by the various cryophysiological tests. One solution is to culture the cells for a few days, because some algal cells can change their physiological properties in response to culture conditions improving their tolerance to cryopreservation [35], but it is not clear what else may change. Yancey et al. [54] have described a suite of intracellular organic osmolytes for *Symbiodinium* that match those of their host species—none are exactly alike. Would culture alter these osmolytes in some way, making the algae less compatible for symbiosis?

There are several other options available besides standard cryopreservation, such as vitrification and drying. However there are two elements working against vitrification, the need to load a high concentration of cryoprotectant in a short amount of time. The cryotoxicity experiments revealed that exposure to 10% cryoprotectant solution over 20 min caused damage. This could be mitigated if the cells were cooled, but there is clear sensitivity to chilling, except for the zooxanthellae from *F. scutaria*. If cells can be loaded with sugars they can be dried. However, when zooxanthellae were exposed to a 0.5 M trehalose solution for over 18 h, none was taken up (G.D. Elliot, pers. comm.), so dehydration may not be optimal for these cells.

There is a long history of using of deuterated or tritiated water to measure the diffusional permeability of cell membranes. In the early work, particularly, the flux of D_2O or T_2O was generally detected by a change in cell weight or buoyancy (floating to sinking), [27,39,40,31,21,24]. However, more recent work has used fluorescent markers to examine water movement [29,55] and NMR [5,43]. The older-styled H_2O:D_2O exchange experiments using buoyancy

proved very useful for the zooxanthellae investigated here, because the traditional volumetric methods did not work well, and the floating or sinking of the concentrated suspension of zooxanthellae could be monitored with the naked eye.

As mentioned earlier, the relatively inflexible algal cell wall inhibits the study of osmotic fluxes across the inner plasma membrane. However, a wide variety of very creative techniques have been employed to understand water and solute movement across algal membranes [6,50,9,56]. Many of these studies used large macro-algal cells that can tolerate larger scale techniques, such as external and internal pressure probes to monitor these fluxes [47]. However, Tanaka et al. [48] and Walsh et al. [49] developed optical methods to monitor the water, dimethyl sulfoxide, and methanol transport in fresh water micro-algal cells of relatively small-sized *C. texanum* algae (of ~24 μm in diameter). We compared our results to those for *Chlorococcum texanum*, because they relatively similar diameters to *Symbiodinium* (~12 to14 μm in diameter) than the larger macroalgae. *C. texanum*'s water permeability (34×10^{-3} μm/min/atm at 21 °C)) was considerably higher, 15×, than that found for *Symbiodinium*, i.e., $2.0 \pm 0.2 \times 10^{-3}$ μm/min/atm at 22 °C [48,49]. The low water permeability of *Symbiodinium* suggests an absence of water channels in these algae. Radiolabeled glycerol yielded consistent permeability measures, 5 to 7×10^{-5} cm/min at 24 °C, for all three *Symbiodinium* subtypes, similar to *C. texanum*'s dimethyl sulfoxide permeability of 10×10^{-5} cm/min at 23 °C. On the other hand, C. texanum's permeability to the solute methanol is much higher, $P_s = 4.7 \times 10^{-2}$ cm/min at 25 °C, but this is not unexpected as methanol often has a much higher membrane permeability than other solutes [49]. Finally, *C. texanum* had a higher cell solids volume, $V_b = 0.38$, than *Symbiodinium's* smaller values of $V_b = 0.143$–0.236.

Stat et al. [46] observed a correlation between coral health and *Symbiodinium* clades, noting that Acroporids with clade A were more susceptible to disease than clade C. Currently, clade A is being used by many zoos and aquariums to inoculate their *ex situ* rearing of *A. palmata* juveniles (M. Carl, pers. comm.). If clade C were superior, management decisions would have to be made to consider the use of this clade for future restoration processes.

In some of the key cryobiological factors the *Symbiodinium* subtypes differed, specifically osmotic tolerance and chilling sensitivity. The osmotic tolerance parallels the species intertidal robustness. For example, *P. compressa* is exposed to large tidal cycles and exposure many times throughout the year in Kaneohe Bay, whereas the other two species do not. Perhaps, one of the biggest obstacles to ease of slow freezing cryopreservation is their chilling sensitivity. Santiago-Vazquez et al. [45] were able to outrun some of this chilling (if this was an issue for *Symbiodinium* from a sea fan) by dropping the cells immediately to −20 °C, but other subtypes may be more sensitive. With our studies outlining the cryosensitivities of some types of *Symbiodinium*, we can move forward with an approach that may yield higher post-thaw results, but it might entail tailoring a cryopreservation protocol for each particular type.

Acknowledgments

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Appendix 1. H_2O:D_2O exchange experiments

The essential concept of D_2O for H_2O exchange is simple. A cell is placed in a solution containing D_2O in which it initially floats. After exchanging some D_2O for H_2O, the cell becomes denser and sinks. The length of time it takes to begin sinking is a measure of the rate at which D_2O enters and H_2O leaves the cell; namely the water diffusional permeability. The first consideration is to carefully choose the D_2O suspending medium. If it is too dense, the cells will never sink. If not dense enough, they will sink immediately. Previously, in Eq. (1), we gave a formula for the density of the zooxanthellae. Generalizing that formula to the case where there was both regular and deuterated water within the cell yielded:

$$q_z = (q_d \cdot V_d + q_h \cdot V_h + q_s \cdot V_s)/(V_T); \quad (3)$$

where q and V are density and volume, respectively, and the subscripts z, d, h, s, and T stand for zooxanthellae, deuterium, light (H_2O) water, cell solids, and total, respectively. Using this notation, the total cell volume was:

$$V_T = (V_d + V_h + V_s): \quad (4)$$

Now if we assumed simple diffusion (no osmotic driving forces), one molecule of H_2O left the cell for ever molecule of D_2O entering the cell and, noting that the molar volumes of H_2O and D_2O differ by less than one percent, we set the total cell volume at any time to the value of the initial cell volume, i.e., a constant:

$$V_T = V_o: \quad (5)$$

We called the density of the deuterated test medium in which the cells were suspended q_{dtm}. As the cells took up D_2O they reach a point in time, t, when they became neutrally buoyant:

$$q_z(t) = q_{dtm}: \quad (6)$$

This is the time at which the cells began to sink. At time zero; we required $q_z(0) < q_{dtm}$, so that the cells initially floated. To determine the conditions at the point of neutral buoyancy, we set q_z equal to q_{dtm} in Eq. (3) and then solved the simultaneous Eqs. (3) and (4), for V_d to yield:

$$V_d = [q_{dtm} \cdot V_T - q_h(V_T - V_s) - q_s \cdot V_s]/(q_d - q_h): \quad (7)$$

It was useful to re-express this as the percentage of internal water, by volume, which was deuterated:

$$V_d\% = 100\% \cdot V_d/(V_d + V_h): \quad (8)$$

We needed to mix the deuterated test medium, Instant Ocean®, to a known density by varying the percentage of D_2O substituted for H_2O. Assumed a simple linear relationship:

$$q_{dtm} = (d\%/100)q_d + (100 - d\%)q_h/100 + q_{ios}: \quad (9)$$

$d\%$ was the percentage of deuterated water by volume, relative to total water, in the Instant Ocean® and q_{ios} was the contribution of the Instant Ocean® salts to the total density. The latter was computed as the density of H_2O:Instant Ocean® minus the density of H_2O, all at 22 °C, and equals 0.0435 g/ml. The density of H_2O and D_2O water at 22° C were assumed to be 0.9978 and 1.1048 g/ml, respectively [30]. The density of the deuterated Instant Ocean® as a function of the% D_2O by volume was plotted in Fig. 8; gray curve, left axis.

It is useful now to re-express Eq. (8) in terms of the relative percent of external D_2O which enters the cell; namely:

$$R\% = 100\% \cdot V_d\%/d\%: \quad (10)$$

This parameter can vary between 0% and 100% and is the percentage of outside D_2O which has to enter the cell to reach the point of neutral cell buoyancy. For example, if the outside D_2O concentra-

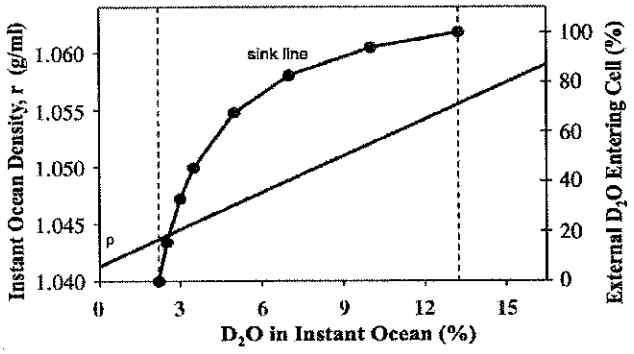

Fig. 8. Uptake of D_2O water in *F. scutaria Symbiodinium*. Only a narrow range of D_2O concentrations in Instant Ocean® yielded a test solution in which zooxanthellae, $q_z(0) = 1.0436$ g/ml, initially floated, then sank as D_2O entered (see text). The upward sloping straight line in the plot, labeled q, depicted the density (left axis) of Instant Ocean® as a function of the percentage of D_2O replacing regular water, by volume (*X* axis). The upward curving sink-line depicted the percentage (right axis) of external D_2O which had to enter the zooxanthellae to make them neutrally buoyant (as a precursor to sinking). Below 2.2% D_2O in Instant Ocean®, the zooxanthellae sank immediately (left dotted line). Above 13.2% D_2O in Instant Ocean®, the zooxanthellae floated regardless of the length of time they were held in the D_2O-Instant Ocean (right dotted line). Thus the D_2O-Instant Ocean® concentration was chosen carefully to yield a useful 'float-sink' experiment.

tion, *d*%, is 10% and the intracellular D_2O concentration for neutral buoyancy, V_d%, is 9.4%, then the concentration inside, relative to outside, *R*%, is 94%. This parameter is also plotted in Fig. 8; black curve, right axis. It shows that, for $q_z = 1.044$ g/ml, the deuterated Instant Ocean must have a D_2O content of 2.2–13.2% to yield a test medium in which the zooxanthellae cells initially float and then sink upon H_2O:D_2O exchange.

Typically, the H_2O:D_2O exchange data are analyzed in terms of diffusion and a water diffusional permeability coefficient, P_d, is determined from the time to sink data [24]. Alternatively, the D_2O may be considered a solute in the external medium and it enters the cell because of the concentration gradient of D_2O. This latter system can be analyzed using the standard two parameter (2*P*) formalism which is used to describe water and solute flux across a membrane [34,25]. Both viewpoints are thermodynamically valid and should yield the same result for water permeability (assuming there are no water channels, as discussed later). We consider both approaches here. Table 2 summarizes the experimental conditions used and the results for both approaches, as well as some illustrative conditions, not used.

First we consider an overview of the experimental conditions listed in Table 2. If the density of the external test medium is too low (Case 1, 0% D_2O), any cells placed in it will immediately sink, without an initial period of floating. If the density of the test medium matches that of the cells, then the cells will remain neutrally buoyant, neither floating nor sinking. This occurs for $d\% = 2.2\%$ (not shown). At intermediate solution densities, say Case 3, the test solution is initially denser than the cells, they float; but over time exchange D2O for H2O, increase in density and sink. At high solution densities, $d\% > 13.2\%$, the solution is sufficiently dense that the cells float and are never able to take on sufficient D_2O to sink, even when the inside D_2O concentration has equilibrated with the outside.

Now we consider the two approaches for determining the water permeability from the time to sink data (t_{sink}), starting with the diffusional exchange approach [24]. Expressed in the notation of this paper, the water diffusion coefficient, P_d, is given by:

$$P_d = \ln[1=(1 - V_d\%=d\%)] \cdot V_o=(A \cdot t_{sink}): \quad (11)$$

These P_d are easily computed from the data in Table 2 for the three cases in which t_{sink} was measured. Assuming there are no water channels, P_d equals the water filtration coefficient, P_f, and this can be converted to a hydraulic conductivity, *Lp* (see footnotes to Table 2). Before discussing these results, we continue to the two parameter method for determining *Lp*.

The two-parameter (2*P*) membrane permeability formalism is widely used in cryobiology and membrane biophysics to determine the water and solute permeability of a membrane in the presence of a solute gradient [34,25]. With a few simple modifications, it can be applied to the permeability problem considered here. The deuterated water was considered to be a solute dissolved in the H_2O:Instant Ocean®. The driving force for solute permeation was the solute activity that was approximated as molality, here D_2O molality, and this is computed in Table 2 for the cases of interest. Second, the solute permeability of D_2O, P_s, was set equal to the water permeability. Thus $P_s = P_f = RT * Lp=\bar{V}_w$ and the 2*P* Model reduces to a one parameter model, variable *Lp*, assuming that the membrane permeability to D_2O and H2O were the same. Note, P_f and *Lp* were the same quantity expressed in a dimensionally differ-

Table 2
Experimental conditions for and results of the zooxanthellae 'float/sink' experiments.

Case[a,b,c] (#)	*d*% (ext %)	r_{dtm}[d] (g/ml)	Molarity (mol/l)	Molality (mol/kg)	*R*% (%)	V_d% (%)	t_{sink}[e] (s)	P_d (cm/min)	$Lp(P_d)$ (μm/min/atm)	$Lp(2P)$ (μm/min/atm)
1	0	1.0413	0	0	—	—	0	—	—	—
2	2.5	1.0440	1.378	1.414	15.7	0.39	7.9 ± 0.6	2.7E−04	2.0E−03	1.6E−03
3	3.0	1.0445	1.654	1.705	33.1	0.99	16.5 ± 0.7	3.1E−04	2.3E−03	1.8E−03
4	3.5	1.0450	1.930	2.000	45.5	1.59	19.4 ± 1.0	3.9E−04	3.0E−03	2.4E−03
5	10.0	1.0520	5.514	6.127	94.0	9.40	—	—	—	—
6	13.2	1.0555	7.278	8.386	100.0	13.20	∞	—	—	—
								Average:	2.4E−3	2.0E−3

[a] Column meanings: *d*% is the percentage of D_2O, by volume, which has replaced H_2O in the external test medium (Instant Ocean®); r_{dtm} is the density of the external test medium; molarity and molality are the molarity and molality, respectively, of the D_2O in the external medium; *R*% is the percentage of external D_2O which has entered the cell at the time when neutral buoyancy is attained; V_d% is the percentage by volume of D_2O which has replaced H_2O inside the cell at the point of neutral buoyancy ($V_d\% = R\% \times d\%/100\%$); t_{sink} is the observed time for cells, initially floating, to begin sinking in the test medium (see Methods for details), the errors are standard errors of the mean; P_d is the water diffusional permeability of the cell membrane based on an H_2O:D_2O diffusional exchange analysis; $Lp(P_d)$ is just a units conversion of P_d to μm/min/atm, $Lp = P_d * \bar{V}_w=RT$ where *R* is the universal gas constant, *T* the Kelvin temperature, and $\bar{V}_w$ is the partial molar volume of water, taken to be 18.1 g/ml (see [25]); $Lp(2P)$ is the hydraulic water conductivity of the cell membrane derived from a two parameter analysis assuming a flux of water (H_2O) and a flux of solute (D_2O) with identical membrane permeabilities [25].

[b] A diverse collection of units are found in Tables 1 and 2; typically the units commonly used for the quantity in question. When substituting values into the formulae found in this paper, it is essential to convert to a dimensionally consistent set of units appropriate to the particular formula.

[c] Measurements made at 22 °C.

[d] For comparison, the initial density of zooxanthellae of *F. scutaria* in H_2O:Instant Ocean® is 1.0436 g/ml.

[e] The values of 0 and ∞ seconds are theoretical values, not measured values. See Methods for details of the experimental t_{sink} values.

ent fashion; see footnotes to Table 2 and [13]. The numerical solution of the coupled partial differential equations of the 2*P* model has been detailed elsewhere [25,17]. Basically we adjusted *Lp* until the desired intracellular molality of D_2O, (col. 5, Table 2) was achieved in the time observed to reach neutral buoyancy, t_{sink} (col. 8, Table 2). The resultant two-parameter *Lp* (2*P*) values were listed in the last column, Table 2.

In principle, the diffusional approach and the 2*P* solute gradient approach should yield the same value for *Lp*. What we find, Table 2, is that the 2*P* permeability is consistently 20% less than the diffusionally determined permeability. Considering the substantially different nature of the two approaches, we considered this remarkably good agreement. As previously mentioned, one of the assumptions, required for comparison of the two methods was that no water channels were present in the cell membrane. The presence of water channels were unlikely in as much as the permeability values obtained here are two to three orders of magnitude less than those found in cells with water channels (0.1–10 μm/min/atm). Also, in both methods, there was a clear trend of increasing permeability as the density of the test solution increased. This is unexpected and may well be due to the somewhat subjective visual determination of when cell sinking began after injection of cells into the deuterated test medium. Nevertheless, with all the uncertainties taken into account, we believe that we have determined *Lp* to within a factor of two or better. We take the average of the 2*P* values, $2.0 \pm 0.2 \times 10^{-3}$ μm/min/atm, as our best estimate of *Lp*. The 2*P* value was chosen rather than the diffusional value as we are more familiar with the 2*P* approach and for consistency with our other published permeability data for coral.

Pragmatically, the determination of water permeability by the diffusional approach, Eq. (11), is substantially simpler than that by the 2*P* approach which requires numerical integration of a pair of coupled differential equations. For future experiments we would recommend the diffusional approach. Nevertheless, it was a useful measure of our understanding of this system that we obtained such similar results using such different approaches.

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