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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.

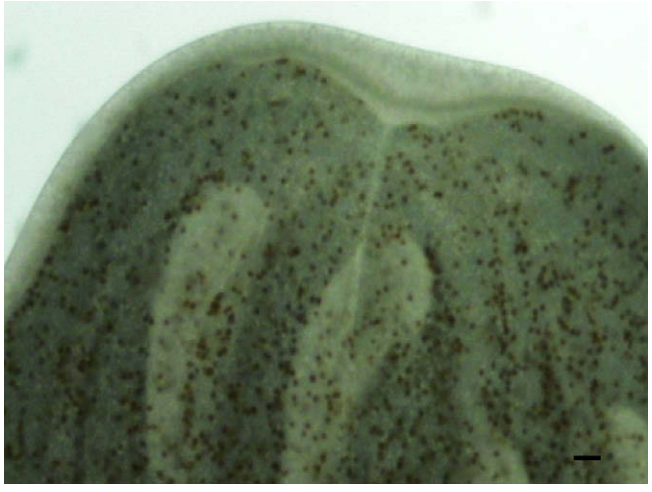

Fig. 1. Zooxanthellae, *Symbiodinium* sp., are intracellular symbionts living within coral tissue with dark brown-green pigments. The tissue whole-mount compressed the larval coral tissue making the symbionts more apparent. These symbionts are evident as the brown spots observed throughout the preparation. 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 4070*g* for 15 min to remove excess water. Tissue was homogenized using a glass homogenizer, and re-suspended into filtered