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Assessing the impacts of cryopreservation on the mitochondria of a thermotolerant *Symbiodinium* lineage: Implications for reef coral conservation

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ABSTRACT

Earth's coral reefs are threatened by a barrage of anthropogenic insults, and cryopreservation-based conservation measures are warranted. Successfully cryopreserved corals could then thawed and out-planted on reefs when ocean temperatures stabilize. In such efforts, it will be necessary to also cryopreserve the photosynthetic dinoflagellates (genus *Symbiodinium*) that reside within the corals' gastrodermal cells. Given this need, *Symbiodinium* (clade D) cells were cryopreserved in 2 M propylene glycol by a two-step freezing protocol herein and then cultured for 42 days post-thaw. To gauge the effect of cryopreservation, mitochondrial DNA content and intracellular ATP concentration were assessed, and the former parameter was nearly 2-fold higher in freeze-thawed cells compared to controls after 14 days of post-thaw culture. In contrast, intracellular ATP concentration was relatively lower in freeze-thawed cells after seven days of post-thaw culture, though returned to control levels in samples cultured for 42 days post-thaw.

Cryopreservation is a promising, cost-effective technique for long-term preservation of cells, tissues, and organs especially, germ cells and embryos [1,2]. The establishment of *Symbiodinium* cryobanks would be especially beneficial because they would allow for the future infection of cryopreserved coral such that the resulting adult colony could be out-planted onto a reef when, for instance, sea surface temperatures stabilize. Such efforts would be especially desirable if the one of the most high-temperature thermotolerant lineage of *Symbiodinium* (clade D) could be cryopreserved. However, to date only three studies have reported the successful cryopreservation of *Symbiodinium* [2–4]; in the former work; clade B *Symbiodinium* isolated from the octocoral *Pseudopterogorgia elisabethae* was successfully cryopreserved using methanol and ethanol as cryoprotectants (CPAs). Alternatively, Chong et al. [2] used a two-step freezing protocol, which resulted in a high post-thaw survival rate.

Mitochondria play crucial roles in cellular energetics, and it is within the inner mitochondrial membrane that adenosine triphosphate (ATP), the energetic currency of all living organisms, is generated via the electron transport chain (ETC). Since the protein components of the ETC are encoded by both nuclear and mitochondrial genes, transcription of mitochondrial DNA (mtDNA) is essential for efficient production of ATP [5]. Given the importance of mitochondria and ATP, it will be critical to show that cryopreserved *Symbiodinium* maintain the ability to both transcribe mitochondrial genes and generate ATP upon thawing. Therefore, the purpose of the present study was to investigate the effects of cryopreservation on mitochondrial DNA copy numbers (CN) and ATP levels in post-thaw samples of the one of the most high-temperature tolerant linear of *Symbiodinium* (clade D) in order to make conjectures about the health of these ecologically important dinoflagellates.

Symbiodinium clade D (CCMP2556) was cultured at National museum of Marine Biology & Aquarium, Taiwan. Prior to cryopreservation experiments, *Symbiodinium* cells were cultured in f/2 medium (Sigma-Aldrich, USA) at 25 ± 1 °C and 100 μmol m^{-2} s^{-1} of photosynthetically active radiation under a 12-hr light:dark cycle in a Kansin Instruments incubator (Taiwan) at a density of 10^5 cells·mL^{-1}. The culture media was replenished every 10 d with fresh f/2 media containing 10 μg mL^{-1} of streptomycin and 10 unit·mL^{-1} of penicillin

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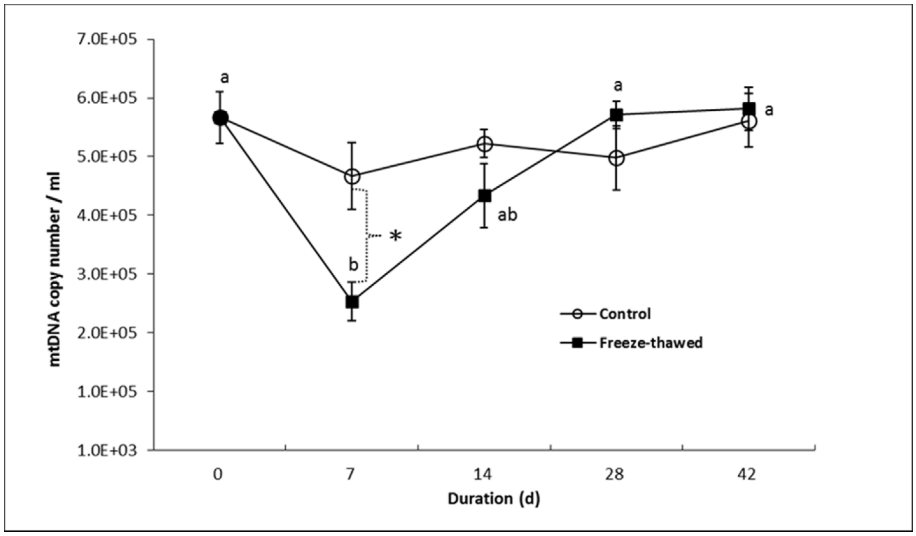

Fig. 1. mtDNA CN of control and freeze-thawed *Symbiodinium* cells (clade D). Error bars represent standard error of the mean. Data points with different letters are significantly different (Tukey's *post-hoc* test, $p < 0.05$).

(Invitrogen, USA).

The cryopreservation experiment was conducted three times (i.e., in three different weeks). In each experiment, three cell culture replicates were chosen for each of the two treatments (control and freeze-thaw [described below]), and cell aliquots were taken after 0, 7, 14, 28, and 42 days of post-thaw culture. Therefore, 30 samples were analyzed for the response variables described below (*Symbiodinium* density, mtDNA content, and ATP concentration) in each of the three experiments. Polyethylene glycol (PG) was used as the CPA, and it was prepared as a 2 M solution in filtered seawater (FSW). The *Symbiodinium* culture medium was replaced with 2 M PG to a final cell density of 1.5×10^6 cells/mL. The cells were cryopreserved by a two-step freezing protocol [6]. In brief, after equilibration, the cryostraws were placed on top of a freezing device (Taiwan patent No.: M394447) at a distance of 5 cm from liquid nitrogen (LN2) and cooled with the LN2 for 20 min. The straws (0.25 ml) were then immediately plunged into LN2 and stored for 2 h. Thawing was conducted by retrieving the straws and introducing them into a warm water bath at 37 °C for 10 s. Control and freeze-thawed cells were then cultured in f/2 media as above, though with the culture media replenished every two days. Cell aliquots were then removed for cell density, mtDNA CN, and ATP concentration analyses at the following time points: 7, 14, 28, and 42 days.

Aliquots of 10 µL of *Symbiodinium* samples (n = 9/treatment) were pipetted into Neubauer counting chambers for determination of post-thaw algae cell density. It is worth repeating that the cell density of each sample was ~1.5 x 10^6 cells/mL before being cryopreserved. The intracellular ATP concentrations of *Symbiodinium* samples were measured with the ApoSENSOR™ Cell Viability Assay Kit (BioVision, USA) [2]. The ATP assy utilizes luciferase to catalyze the formation of light from ATP and luciferin and the light was measured using a luminometer (Lumat 9507, Berthold Technologies, Germany).

DNA extraction from *Symbiodinium* was performed with the genomic DNA extraction kit from Favorgen Biotech (Taiwan) following the manufacturer's protocol. Forward ('5-AATGCCTGGCTTGTTTGGAG-3') and reverse ('5-CGTCCATCCTGTACCACCTC-3') primers were used to PCR amplify a target mtDNA fragment of 172 bp. The PCR conditions were as follows: initial denaturation and hot-start enzyme activation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 10 s, followed by a final extension at 72 °C for 5 min. The PCR products were checked by 1.5% agarose gels electrophoresis, and the gels were stained with safe dye (DNA VIEW). The PCR product bands were visualized under a Digimage Gel Documentation System equipped with a UV transilluminator (Major Science, Taipei, Taiwan), excised from the gel, and purified with FavorPrep TM Gel Purification Mini Kit (FAVORGEN, Biotech Corp., Taiwan) according to the manufacturer's recommendations. These purified PCR products were quantified with a NanoDrop spectrophotometer (Thermo Scientific) and diluted to 2 ng μL^{-1} before being serially diluted (10-fold) to serve as standards for real-time PCR (qPCR).

qPCR-based determination of *Symbiodinium* mtDNA CN was conducted using an Applied Biosystems 7500 real-time PCR device with SYBR® Green I chemistry (QuantiNova® SYBR® Green mastermix with ROX® passive reference dye, Qiagen, Germany). A standard curve with the aforementioned, serially diluted standards (1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg) was analyzed alongside the samples (2 µL). *Symbiodinium* mtDNA was analyzed in triplicate in 18 µL reactions with 10 µL of Syber green, 0.4 µL of ROX, 0.4 µL of each primer, and 6.8 µL of H_2O. PCRs were performed as follows: initial denaturation and hot-start enzyme activation at 95 °C for 2 min, followed by 40 cycles of 90 °C for 5 s, 60 °C for 32 s, and 72 °C for 15 s. At the end of each reaction, a dissociation curve was analyzed from 60 to 95 °C in increments of 0.5 °C for 15 s to screen for the presence of unexpected products. The mtDNA CN of *Symbiodinium* samples and amplification efficiency were calculated by comparing against the co-run standards.

In order to determine the effects of cryopreservation on clade D *Symbiodinium*, their mtDNA CN was first tracked over time and between treatments (Fig. 1). There was no statistically significant effect of control group on mtDNA CN. The mtDNA CN of treated *Symbiodinium* decreased on day 7 with statistically significant differences (Tukey's post-hoc test $p < 0.05$). Then mtDNA CN was gradually increased and reached to the same amount of control on day 14. There was no statistically significant difference of mtDNA CN after 14 days of post-thaw culture as compared with the control group.

The effect of freezing on density of mtDNA CN during 42 days of post-thaw cultivation is shown in Fig. 2. There was no significant difference in mtDNA CN/cell between control and freeze-thawed *Symbiodinium* cells on day 7 (Fig. 2). However, there were both treatment and treatment × time interaction effects in the repeated measures ANOVA. In contrast to our hypothesis, freeze-thawed *Symbiodinium* cells possessed more mtDNA than controls on days 14 and 28 (Tukey's *post-hoc* test, $p > 0.05$). After 28 days of post-thaw culture, the production of mtDNA CN was statistically similar between treatments.

The normalized ATP concentration between control and freeze-thawed *Symbiodinium* cells was significantly different on day 7 (Fig. 3). Specifically, cellular ATP content was lower in freeze-thawed cells at this time. However, by day-14, concentrations had returned to

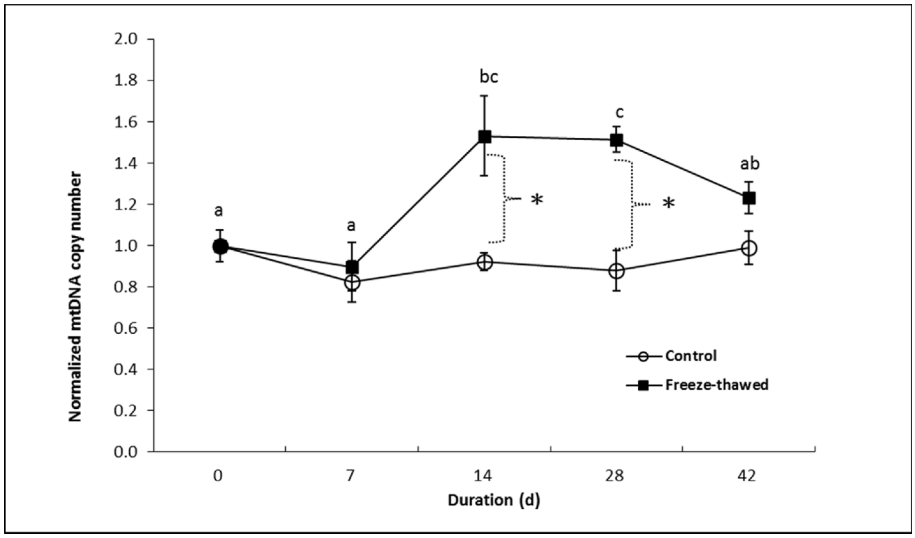

Fig. 2. mtDNA CN per control and freeze-thawed *Symbiodinium* cell (clade D). Error bars represent standard error of the mean. Data points with different letters are significantly different (Tukey's *post-hoc* test, $p < 0.05$).

statistically similar levels as the controls, and this null difference was perpetuated throughout the remainder of the experiment.

Cryopreservation is based on dehydration of the cells and prevention of ice crystal formation using CPAs. Although chosen because of their relatively inert nature, some CPAs may have an impact on biological and physiochemical properties of the cells; what is the best CPA for one organism may not be the best for another. In cnidarian-*Symbiodinium* cryopreservation studies, a variety of CPAs have been utilized, including glycerol (Gly), dimethyl sulfoxide (Me_2SO), and ethylene glycol (EG). The former was found to be adequate [6], whereas the latter two were relatively toxic to hard coral oocytes, at least when compared to methanol (MeOH; [7]. In algae, MeOH (for freshwater microalgae), Me_2SO (for marine microalgae), and Gly (for green algae) are the most commonly used CPAs for cryopreservation [8]. PG had also been successfully used as a CPA in the cryopreservation of marine microalgae and *Symbiodinium*, and we corroborated these findings herein. Previously, we found that after freezing clade D *Symbiodinium* for 10 days with 2 M EG and 2 M PG in liquid nitrogen, an increase in ATP concentration was exhibited, and the survival rate was high [6]. Therefore, in future works, it may be worthwhile to attempt to use both PG and EG as CPAs with the two-step freezing protocol of employed herein in attempt to boost *Symbiodinium* cell survival.

The production of both mtDNA and ATP reflect the ability of a cell to repair itself after exposure to low temperatures. It was evident herein that freezing injury did not occur immediately; a mtDNA CN decrease was not observed until several days of post-thaw culture. In contrast, a decrease in ATP concentration was observed at the first post-thaw sampling time (7 d). Despite this drop, ATP levels eventually returned to control levels by the final sampling time, indicating that the cells had recovered from cryo-trauma.

In many animal cells, a minimal level of mtDNA is required for proper fertilization and subsequence development [9]. While the minimum amount of mtDNA necessary for survival is still unknown for most invertebrates, it was estimated to be about 3×10^7 copies/oocyte in the hard coral *Echinopora* sp. [10]. Based on the observation in this experiment, we estimated that the critical value of mtDNA CN in *Symbiodinium* is lower than 2.5×10^5 CN/mL. Given the fact that cells of both treatments continued to divide throughout the experiment, mtDNA content was deemed to have been sufficient, even in temperature-stress samples. Although mtDNA content can affect the ATP

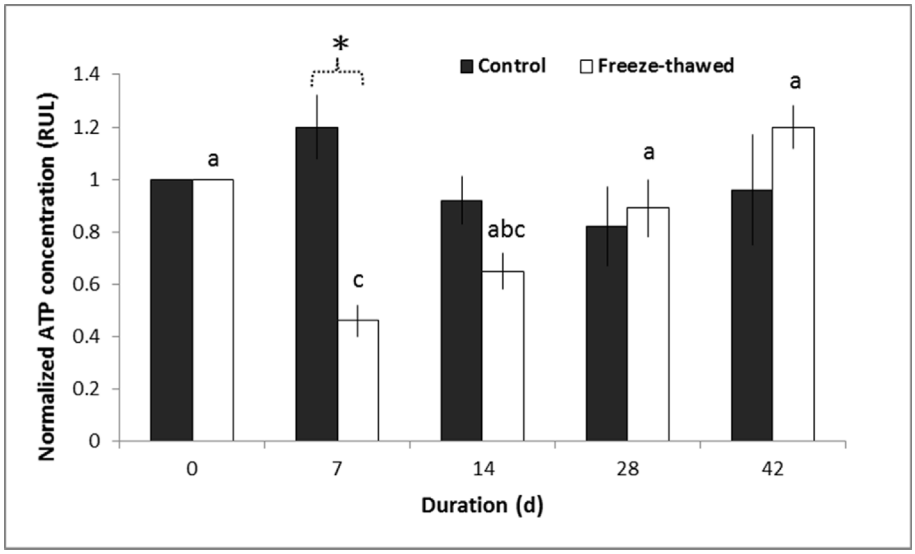

Fig. 3. Normalized ATP concentration of control and freeze-thawed *Symbiodinium* cells (n = 9/treatment). Lowercase letters denote significance between individual means, as determined by Tukey's *post-hoc* tests ($p < 0.05$).

content of cells, *Symbiodinium* also produce energy via photosynthesis. Therefore, there may be relatively less mtDNA found in algae like *Symbiodinium* compared to other organisms, and, furthermore, they may be less likely to become physiologically compromised upon experiencing decreases in cellular mtDNA content.

Symbiodinium cell mtDNA CN dropped on sample day-7. However, those cells that survived may have been producing more mitochondria in order to maintain sufficient energy production for survival. Nevertheless, ATP production dropped in post-thaw cells at this time, though levels did recover by day-14. It is possible that when the treated cells succumbed to freezing injuries, some of the mitochondria malfunctioned; this could explain the lack of correlation between mtDNA content and ATP production [5]. Additionally, decreases in ATP production was potentially caused by freezing injury and other stress stemming from the cryopreservation process (e.g., freeze-thawing and CPA toxicity; [6]. The mtDNA peak on day 14, in turn, may have been a defense mechanism to compensate for the loss of mitochondrial function and the declining ATP production (on day 7). This behavior also has been documented in hard coral oocytes (*Echinopora* spp.; [5]. In the end, though, all response variables assessed returned to control levels after 42 days of post-thaw culture, indicating that cryopreservation of clade D *Symbiodinium* is readily achievable. Furthermore, given their role in cell energy budgets, we believe that the two response variables assessed herein, mtDNA and ATP content, could be useful for others interested in gauging the potential effects of cryo-trauma on algal cell biology.

Conflicts of interest

None.

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