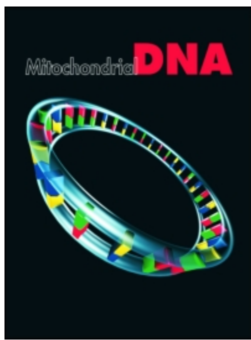

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SHORT COMMUNICATION

Impacts of low temperature preservation on mitochondrial DNA copy number in oocytes of the hard coral *Echinopora* sp.

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Abstract

Given the current threats to coral reefs worldwide, there is an urgent need to develop protocols for the cryopreservation of reef-building corals. However, chilling may alter coral mitochondrial distribution and membrane potential, resulting in reduced ATP production. The aim of this study was to investigate the impacts of chilling on mitochondrial DNA copy number (CN) in oocytes of the hard coral *Echinopora* sp. Oocytes were exposed to 0.5 M, 1 M or 2 M methanol at 5, 0 or −5 °C for 2, 4, 8 and 16 h. When oocytes were chilled with no cryoprotectant (CPT) or 1 M methanol at 5 or 0 °C, the mtDNA CNs initially increased at hour 2 of incubation, although it decreased significantly over the 16 h of incubation in chilled oocytes at −5 °C. The mtDNA CN increased and picked in 0.5 M methanol at 5 °C and 0 °C at hour 8 of incubation in chilled oocytes indicating that the high mtDNA CN of these oocytes is probably responsible for withstanding high chilling sensitivity. We currently propose that 0.5 M methanol is the optimal CPT for oocytes of *Echinopora* sp., and potentially other reef corals.

Keywords

Chilling injury, coral, cryopreservation, mitochondrial DNA, oocyte

History

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Introduction

Coral reefs provide a valuable habitat for many economically important fish and invertebrate species (Hagedorn & Spindler, 2014; Lin et al., 2012) and harbor a disproportionally large proportion of the overall marine biodiversity. However, these calcium carbonate-based ecosystems are threatened on a global scale due to anthropogenic impacts, most notably global climate change. Specifically, both rising ocean temperatures and acidity stand to lead to large-scale loss of corals and overall reef accretion in the coming decades (Lin et al., 2013; Tsai et al., 2010).

The development of suitable cryopreservation techniques for coral oocytes and larvae requires an understanding of their responses to chilling; however, limited studies have been carried out on this topic (Hagedorn & Spindler, 2014; Lin et al., 2011, 2012; Lin & Tsai, 2012). Although the health of the embryos of many aquatic species can be assessed by monitoring their development or by employing vital staining dyes, coral oocyte viability assessment is more difficult, as methods are limited. Only an ATP assay was able to uncover a differential response between control and low-temperature treatments (Tsai et al., 2010).

High levels of ATP output from the electron transport chain are contingent upon high levels of transcription and translation of both nuclear and mitochondrial genes (Dumollard et al., 2007; Tsai et al., 2014). As mtDNA copy number (CN) plays a crucial role in the capacity for ATP synthesis in both the oocytes and early embryos, detection of mtDNA content has been found to be valuable in assessing oocyte performance and viability (Tsai et al., 2014). This could be a particularly important parameter to monitor in organisms, such as reef corals, in which direct measurements of *in vitro* fertilization and developmental capacity have not yet been developed. Therefore, mtDNA CN was measured in oocytes of the reef-building coral *Echinopora* sp. that had been subjected to cryopreservation in order to determine the effects of chilling on this parameter.

Material and methods

Collection of coral oocytes in the field was performed according to Tsai et al. (2014) (Kenting National Park Permit 101-0411). After one step replacement of the filter seawater with cryoprotectant (CPT)-supplemented medium, oocytes in 1.5 ml of eppendorf tubes containing CPT solutions were placed in a dry bath (CB-1502, Medclub Scientific CO., LTD., Taiwan) at 5, 0, or −5 °C, where the oocytes were allowed to chill for 2, 4, 8, or 16 h. Control oocytes were kept in filtered natural seawater at 25 (RT control), 5, 0, or −5 °C. The selected temperature conditions and chilling duration caused thermal injury to oocytes under hypothermic conditions from our previous study (Lin & Tsai, 2012). The DNA extraction and quantitative real-time PCR of mtDNA CNs were conducted as previous description by Tsai et al. (2014). In brief, groups of 10 oocytes in each extraction for each of the 36 groups were used to isolate DNA. Generation of

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Table 1. PCR reactions and thermocycling conditions.

Component	Taq 1x	10 μl
	Primer	0.5 μM
	DNA	2 μl
Protocol	95 °C, 10 min; then 40 cycles of 95 °C, 30 s, 59 °C, 30 s and 72 °C, 30 s; 72 °C, 5 min	

PCR was performed for a total volume of 20 μl.

Table 2. Real-time qPCR reactions and thermocycling conditions.

Component	SYBR® Green Premix 1x	0.3 μM
	Primer	0.3 μM
	DNA	2 μl
Protocol	95 °C, 10 min; then 50 cycles of 95 °C, 15 s, 59 °C, 15 s and 72 °C, 15 s; 60 to 90 °C increments of 0.5 °C for 10 s	

Real-time qPCR was performed for a total volume of 25 μl.

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standards for real-time PCR was done by conventional PCR using primers specific for *Echinopora gemmacea* (forward primer: 5′-GGTTTGGAAACTGGCTAGTGC-3′; reverse primer: 5′-ATA AACCGTTCATCCCGTTCCTGC-3′; amplicon size: 158 bp). PCR reactions and conditions are shown in Table 1. Quantitative real-time PCR using SYBR Green chemistry (SYBR® Green with ROX passive reference dye, Yeastern Biotech, Taiwan) was performed on an Applied Biosystems 7500 real-time PCR machine in order to measure the number of mtDNA molecules. Real-time PCRs and the temperature parameters for amplification are shown in Table 2. The PCR efficiency was determined from serial dilutions of the PCR product standards.

Statistical analyses

A total of 30 oocytes per time point were used to obtain three biological replicates, each of which was analyzed three times. The t-test and a 2-way, repeated measures ANOVA with Tukey's post hoc test were carried out to determine the effects of temperature, CPT concentration, and time on mtDNA. The mtDNA CN for each sample was calculated as described by Tsai et al. (2014).

Results

Control oocytes demonstrated an average of $4.7 \pm 0.46 \times 10^6$ copies per oocyte after a16-h incubation (Figure 1a–l). When oocytes were chilled without the addition of cryoprotectants at 5 or 0 °C for 16 h, a decrease in mtDNA CN was observed (Figure 1a–c). The mtDNA CNs initially increased after 2 h of incubation ($p > 0.05$), although decreased significantly over the entire 16-h incubation period in chilled oocytes without addition of CPT at −5 °C ($p < 0.05$). Similar results were obtained when oocytes were exposed to 1 M methanol at all three tested temperature for 16 h.

The mtDNA CN increased and peaked in oocytes incubated for 8 hr in 0.5 M methanol at 5 °C and 0 °C. A small decrease in mtDNA CN was found after 16 h of incubation, resulting in $5.9 \pm 0.59 \times 10^6$ and $4.6 \pm 0.56 \times 10^6$ copies per oocyte, respectively (Figure 1d and e). However, this trend was not observed in oocytes chilled at −5 °C, in which mtDNA CN decreased after 4 h of incubation ($p < 0.05$). In contrast, low mtDNA CNs were measured in oocytes incubated in 2 M methanol (Figure 1j–l); specifically, the mtDNA CN rapidly declined at all three tested temperatures after 4 h of incubation ($p < 0.05$). The mtDNA CNs were generally below 2×10^6 per oocyte after 8 h of incubation for chilled oocytes. The deleterious effects of temperature on chilled oocytes were more notable at −5 °C (Figure 1c, f, i, and l) at all different concentrations of methanol ($p < 0.05$). The mtDNA CNs were generally below 3×10^6 per oocyte after 4 h of incubation.

Discussion

Sensitivity to chilling is dependent upon cell type, temperature, and exposure time, amongst other parameters (Lin et al., 2013; Valdez Jr. et al., 2005). In the present study, the mtDNA CN peaked in oocytes incubated in 0.5 M methanol at 5 °C and 0 °C for 8 h, and we propose that the high mtDNA CN of these oocytes is partially responsible for their ability to withstand chilling exposure, possibly due to their ability to sustain high levels of ATP production. Our previous results (Tsai et al., 2010) suggested that methanol was the best cryoprotectant because it was lesser toxic to coral oocytes than DMSO, EG, and PG. Methanol has been found to be the most effective cryoprotectant for oocytes and embryos of other species as a result of its low biochemical effect and high permeability (leading to reduced osmotic damage) (Guan et al., 2008; Liu et al., 2003). On the contrary, it is likely that, despite the use of this CPT, ice crystallization may have nevertheless occurred at high enough rates *in vivo* that cellular function was compromised, since mtDNA CN significantly decreased at all concentrations tested at −5°C.

Cells may be able to recover from exposure to low temperatures. For example, the highest number of mtDNA copies was measured in oocytes incubated in 0.5 M methanol at 5 and 0 °C for 8 h, as well as those incubated in 2 M methanol at 5 and 0° for 2 h; such as increase in mitochondria might be necessary to maintain sufficient ATP levels for survival, as the function of each individual mitochondrion has been shown to be impaired by such low temperatures (Tsai et al., 2014). However, it is possible that such a compensation mechanism does not impede a certain degree of irreversible damage. Indeed, a very low mtDNA CN was found in oocytes exposed to −5° for 8 h, and it is unlikely that the mitochondria of respective cells were able to produce sufficient quantities of ATP necessary for cell survival.

When the mtDNA CN is lower than a certain level (e.g. $4–5 \times 10^5$ for mouse embryos), fertilization cannot take place (Sturmey & Leese, 2003). Although no such data exist for invertebrates, we estimate that 3,000,000 mtDNA copies are required for coral embryo development; this is based on the observation that oocytes were no longer viable when mtDNA CNs fell below 2,000,000 (Lin & Tsai, 2012; Tsai et al., 2014). In this study, relatively low mtDNA CNs (less than 2,000,000) were found in cells treated with 2 M methanol at −5 °C. So far, the minimum mtDNA CN required for successful fertilization and embryonic development is unknown for corals. However, abnormally low mtDNA CNs may be associated with a lower capacity for energy metabolism, which directly affects the fertilization and post-fertilization processes. Furthermore, after fertilization mtDNA replication begins at different stages for different species. Indeed, verifying the point at which mtDNA replication first occurs in coral embryos is an active topic of research in our laboratory.

This study is the first to address the effects of low temperature preservation on the molecular biology of a coral species. We currently propose that 0.5 M methanol is the optimal CPT for oocytes of *Echinopora* sp., and potentially other reef corals. Future work will seek to determine the viability and fertilization capacity of oocytes that have been frozen for prolonged periods

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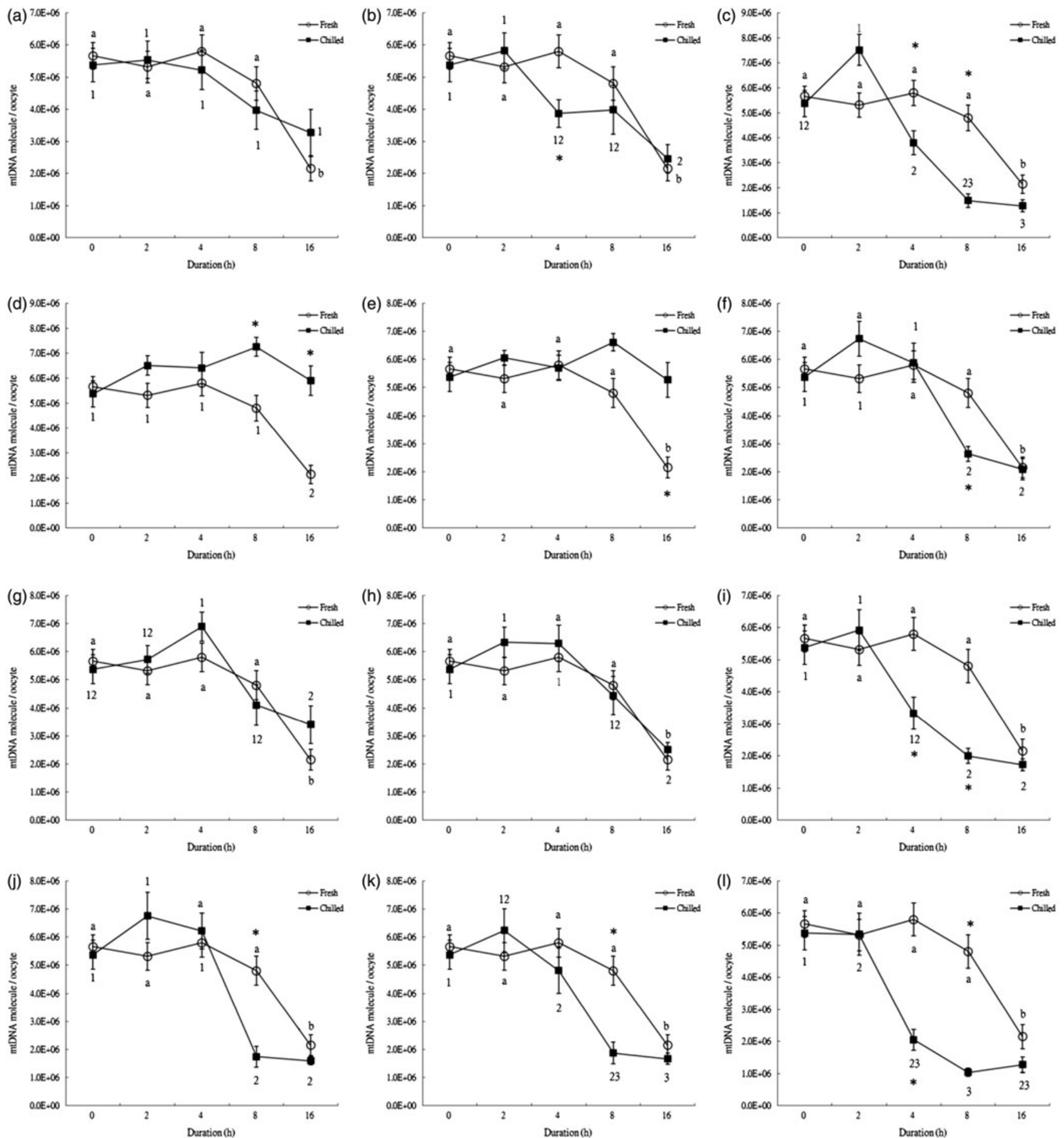

Figure 1. mtDNA copy numbers of *Echinopora* sp. oocytes were measured after exposure to cryoprotectant-free filtered seawater (a, b, and c), 0.5 M (d, e, and f), 1 M (g, h, and i) and 2 M (j, k, and l) methanol at 5, 0, and −5 °C for up to 16 h ($n = 3$ for each cryoprotectant-temperature-sampling time). Room temperature (RT) control oocytes (''fresh'') were incubated in filtered seawater at ambient temperature (25 °C). Error bars represent standard error of the mean. Asterisks indicate significant difference between control and chilled oocytes within each sampling time.

such that effective cryopreservation of corals may become a feasible achievement in the near future.

Declaration of interest

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