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Brief communication

# Preliminary studies of sperm cryopreservation in the mushroom coral, *Fungia scutaria* ☆

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**Abstract**

Coral species throughout the world are facing severe environmental pressures. Because of this, we began cryobiological studies on the sperm of the mushroom coral, *Fungia scutaria*. We determined that *F. scutaria* sperm had a mean length of 56 μm and head diameter of 2.5 μm, and a mean spontaneous ice nucleation temperature of $-37.2 \pm 1.7$ °C. When the sperm were exposed to the cryoprotectant glycerol for 5 or 20 min (at 10% v/v), no fertilized larvae were produced. However, when sperm were exposed for 20 min to propylene glycol (10% v/v), fertilizations were produced at the same rate as untreated control eggs and sperm ($P > 0.05$), but slightly less for dimethyl sulfoxide (10% v/v) ($P < 0.05$). Regardless, dimethyl sulfoxide caused less osmotic damage to the sperm membrane than did propylene glycol. Therefore, we used the dimethyl sulfoxide (10% v/v) to develop cryopreservation protocols that yielded good post-thaw morphology and motility (>95%) for coral sperm.


*Keywords:* Coral; Sperm; Cryoprotectants; Genetic banking

Coral reefs are some of the most diverse ecosystems, providing shoreline stability, major fisheries for hundreds of nations, and ecotourism [6]. Unfortunately, coral species are at risk worldwide because of anthropogenic environmental changes. This has resulted in the destruction of large portions of reefs throughout the world [2]. Given these immediate conservation needs, we began studies to examine the cryopreservation of coral sperm with the future goal of conserving cells in genome resource banks. In this type of bank, coral sperm could remain frozen, but alive for years in liquid nitrogen, and these germline cells could later be thawed and used in natural and captive breeding programs.

Mature anthozoan sperm are about 50 μm long with a blunt conical head, no acrosome, and are

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considered 'primitive' in type because of their external fertilization mode [12]. We have been using the mushroom coral, *Fungia scutaria*. It comprises a single large polyp that releases sperm or eggs into the water, and has been the subject of reproductive studies and in culture for the past 20 years at the University of Hawaii [9]. A number of reproductive characteristics are known for *F. scutaria* sperm [10,11]. Although little is known about the cryobiology of coral sperm, the sperm of other marine invertebrates have been cryopreserved [7,8,3].

We used these as guides for initial screening of potential cryoprotectants and relevant concentrations. We examined the sperm's normal morphology, intracellular ice temperature, physiological response to various cryoprotectants before freezing and after thawing, and the toxicity of various cryoprotectants with respect to fertilization. Finally, we examined cryopreservation protocols, using morphological and motility assessments. This paper describes the first preservation of coral sperm that is a combination of qualitative and quantitative results. Despite the qualitative aspects of the work, we chose presentation as a rapid communication to accelerate the worldwide preservation of coral.

The animals were collected as described in [5]. The gametes from 3 to 4 individuals were collected, pooled prior to fertilization, but not cleaned or concentrated. All solutions were made in reef seawater, and filtered to 0.45 μm. Because the sperm were already motile when collected no diluent or activation studies were performed.

To examine normal morphology, samples (20 μl; $N = 5$) of fresh coral sperm were suspended in filtered seawater and placed on a microscope slide, covered with a glass cover-slip and visualized under phase contrast dark-field with an Olympus BX41 microscope fitted with a Sony DFWV300 digital video camera (final magnification × 1000). Digital images were captured and the length of the head and tail of individual sperm were measured using morphometry software (NIH Image, 1.63). After treatments, cell morphology was assessed to determine the presence of broken or shortened flagella, headless flagella, or agglutination of cells characterized as flagella woven together in a mass. This assessment was qualitative, assigning a sample into either a high quality (few damaged cells) or poor quality (mostly damaged) category.

A qualitative quartile method was used to estimate sperm motility on the microscope. Motile cells were identified as those actively swimming and displacing liquid. After treatments, a visual estimate was made as to whether 25, 50, 75, or greater than 95% of the field had active cells, and at least three fields were assessed for each sample. If cryoprotectants produced abnormal cells without freezing, or formed precipitates, they were eliminated from further evaluation.

To determine chilling sensitivity, coral sperm were evaluated after storage in a refrigerator at 4 °C for 24 h. Samples ($N = 5$; 20 ml each) were left in filtered seawater in 50-ml loosely capped plastic centrifuge tubes. The morphology and motility was assessed after warming to 22 °C.

The fertilization methods used were slightly modified from [10,11]. Approximately, 20 fresh eggs were placed in 4.75 ml of filtered seawater and were exposed to 0.25 ml of sperm ($10^6$/ml) for 5, 10, 20, and 40 min, and 12 h (10 replicates for each time period) at 25–26 °C (repeated three times). All sperm samples were counted in triplicate with a hemocytometer. After the test period, the sperm solution was removed and replaced with 5 ml of fresh filtered seawater, except for the 12-h samples. The negative control consisted of eggs in filtered seawater (with no sperm added). Vials (15 ml) were suspended in running seawater overnight at 26–28 °C. The optimal fertilization protocol was determined by the maximum number of larva per exposure time that developed to a motile stage after 12 h.

Candidate cryoprotectants and concentrations were selected based use in other marine invertebrates. Reagent-grade glycerol, propylene glycol, and dimethyl sulfoxide solutions (10% v/v) (Sigma–Aldrich Inc.) were tested. The osmolality of filtered seawater and the test cryoprotectants were measured with a vapor pressure osmometer (Wescor VAPROR 5520 XRS. Logan, Utah). Test solutions were made by mixing 9 ml of activated sperm in filtered seawater with 1 ml of cryoprotectant solution.

A fertilization assay was used to determine whether 10% cryoprotectant solutions would have a detrimental effect on fertilization. Sperm and each of the cryoprotectants were mixed to produce 10% solutions (sperm concentration was $10^5$–$10^6$/ml) in filtered seawater, held at 25 °C for 20 min (the approximate handling time needed for loading sperm into straws), and 132 μl was added to approximately 30 fresh eggs in 3 ml of filtered seawater. The gamete–cryoprotectant mixture was swirled gently, left for 5 min, and only the eggs were transferred to 5 ml of fresh filtered seawater in a 15-ml glass vial.

Negative controls consisted of eggs in filtered seawater with no sperm added, and eggs with cryoprotectant only (no sperm added). Each treatment had 10 replicates, and was repeated twice during different field seasons (June and September, 2005).

The intracellular ice nucleation temperature was determined following the methods of [5]. Samples (5 μl; $N = 3$) of coral sperm were suspended in filtered seawater and frozen on a cryostage. In the freezing protocol for the sperm (below), a holding temperature above the intracellular ice freezing was chosen.

To optimize the freezing protocol, we used 5 and 10% dimethyl sulfoxide solutions (v/v) in filtered seawater. Samples were drawn up into 0.25-ml French straws, heat-sealed at both ends, and allowed to equilibrate in the cryoprotectant at 25–26 °C for 20 min. The straws were immersed in an alcohol bath of a controlled-rate freezer (Bio Cool III-80/SR-36, FTS Systems Missisauga, Ontario) at 0 °C, and cooled at 3 °C/min to −35 °C, held for 5 min, and plunged into liquid nitrogen. The samples were stored for 10 min in liquid nitrogen, and thawed in filtered seawater at 25–26 °C for 7 s. After thawing, a quartile method was used to estimate normal morphology and motility. We used a modified sperm scoring systems described in Ieropoli et al. [8] and Gwo et al. [4]. Because coral sperm motility was never 'vigourous,' we had to assess sperm morphology and swimming pattern. We scored coral sperm in a field in the following way: 4 = motility + no abnormality; 3 = motile with abnormality; 2 = abnormality without motility but cells still intact, and 1 = cells not intact. Samples ($N = 6$, several fields/sample) were scored as to whether cells were actively swimming and displacing liquid, and whether their cell morphology included sperm with broken or shortened flagella.

In the toxicity trials, the data were arcsine transformed and a one-way ANOVA with Tukey–Kramer Multiple Comparison post-test was performed. To examine the differences in sperm morphology, a Mann–Whitney test was performed. Both tests were performed using GraphPad InStat version 3.0a for Macintosh, (GraphPad Software, San Diego California USA, www.graphpad.com). For all tests, a 0.05 level of significance was chosen.

*Fungia scutaria* sperm have blunt triangular heads, and long tails (Fig. 1). Mature cells had a mean length of 56.1 ± 0.6 μm, and a mean head diameter of 2.5 ± 0.1 μm in the longest axis ($N = 7$ each). Fresh coral sperm swam with a few whip-like motions followed by periods (1–5 s) of inactivity. When viewing a fresh sample of sperm, only about 50% of the sample was active at any one time. Thus, the overall motility required examination for at least 1 min to yield an accurate assessment of the motility. The sperm tolerated chilling well and could be left in filtered seawater at 4 °C for 24 h or more and still retain normal morphology and near fresh levels of motility.

When eggs were exposed to sperm for varying amounts of time, the 5 min exposures produced higher rates of fertilization (mean, 50 ± 3%) than did

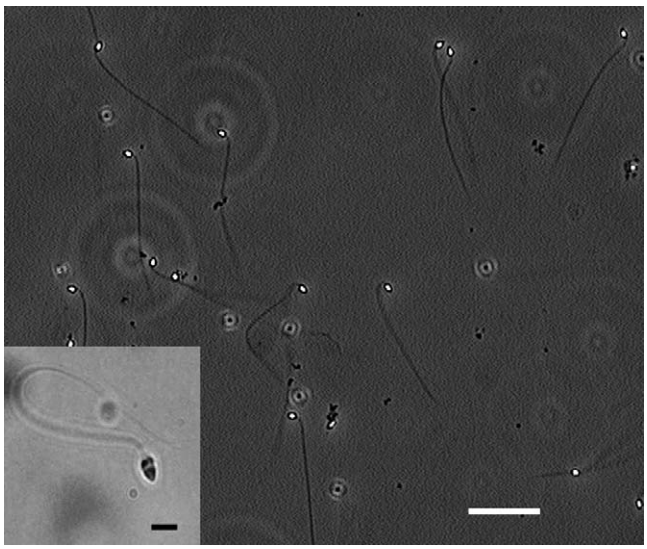

Fig. 1. Normal sperm morphology of *F. scutaria* with inset at higher magnification. Right scale bar, 20 μm; left scale bar in inset, 2 μm.

the 40-min exposure (mean, 25 ± 1%). Specifically, eggs with no sperm resulted in no successful larval development, ruling out parthenogenic development of oocytes or contamination of the egg suspension with sperm. The reduced fertility at longer time periods might be the result of polyspermy. We used these shorter exposure times (i.e., 5 min) for fertilizations in the subsequent experiments.

Filtered seawater had an osmolality of 1035 mOsm/ kg. The cryoprotectant solutions in filtered seawater were more than double this; 10% glycerol was 2841 mOsm/kg, 10% dimethyl sulfoxide was 2505 mOsm/kg, and 10% propylene glycol was 2835 mOsm/kg. When sperm were exposed to 10% propylene glycol, they fertilized at the same level as unexposed sperm ($P > 0.05$), approximately 73% for unexposed and 70% for propylene glycol-exposed sperm (Fig. 2). When the sperm were exposed to 10% dimethyl sulfoxide there was a decline in fertilization to approximately 53% from control ($P < 0.05$) (Fig. 2). Sperm exposed to 10% glycerol produced no fertilization. The two months of breeding examined (June and September, 2005) had different levels of fertilization, September being lower than June. However, the statistical relationships among the treatment groups were the same between the two months.

To determine whether the sperm or eggs were being disrupted in the 10% glycerol treatment, we reduced the sperm exposure from 20 to 5 min. This also resulted in no fertilization (Fig. 2). Exposing eggs to glycerol for 20 min, followed by washing with filtered seawater (prior to the mixture of gametes for 5 min) yielded 50% fertilizations at the same levels as the 10% dimethyl sulfoxide ($P > 0.05$). This suggested that the sperm, rather than the eggs were sensitive to the 10% concentration of glycerol. Moreover, egg exposure to 10% of propylene glycol did not induce parthenogenic development. Because of the toxicity of 10% glycerol to the sperm, only 10% propylene glycol and dimethyl sulfoxide solutions were evaluated in the subsequent experiments.

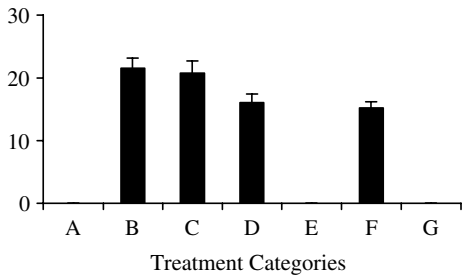


Fig. 2. Toxicity of cryoprotectants to *F. scutaria* sperm and mean fertilizations in June 2005. The average number of eggs placed in each replicate sample was 30 ($N = 10$ replicates/treatment). Eggs were mixed with treated or non-treated sperm for 5 min, rinsed in filtered seawater, and cultured for 12 h at 27 to 28 °C. *Treatment key*: (A) eggs, no sperm added; (B) eggs mixed with fresh sperm; (C) eggs mixed with sperm previously exposed to 10% propylene glycol for 20 min; (D) eggs mixed with sperm previously exposed to 10% dimethyl sulfoxide for 20 min; (E) eggs mixed with sperm previously exposed to 10% glycerol for 20 min; (F) eggs, previously exposed to 10% glycerol for 20 min rinsed, then mixed with sperm for 5 min; (G) eggs exposed to 10% propylene glycol (no sperm) for 5 min, washed, fertilized with fresh sperm.

*Fungia scutaria* sperm spontaneously formed intracellular ice at −37.2 ± 1.7 °C while the surrounding extracellular filtered seawater spontaneously formed ice at −21.7 ± 2.5 °C.

When exposed to either 10% of propylene glycol or dimethyl sulfoxide in filtered seawater, the sperm showed immediate swelling of the tail membrane. These osmotic problems did not inhibit fertilization in unfrozen tests (Fig. 2). Propylene glycol and dimethyl sulfoxide each caused osmotic damage, but it appeared that the sperm were less damaged by 10% dimethyl sulfoxide than 10% propylene glycol solutions. Only the ends of the sperm tails were curled in the dimethyl sulfoxide, whereas several areas along the tail showed membrane damage in propylene glycol.

Because glycerol caused problems with the sperm physiology and propylene glycol appeared to cause more osmotic damage to the sperm cells, 10% dimethyl sulfoxide was used in the freezing protocol. The freezing protocol caused additional morphological changes in the thawed sperm. The mean diameter of the thawed sperm head was slightly larger (mean, 2.9 ± 0 μm) and the mean length of the tail was shorter (mean, 49.2 ± 1.7 μm) than in the untreated sperm ($P < 0.05$). However, the shortening of the tail was present in unfrozen samples due to osmotic stresses, but not the enlarged sperm head (Table 1).

Table 1
Mean (+SEM) morphological traits of *F. scutaria* sperm

| Treatment | Head diameter (μm) | Tail length (μm) |
|---|---|---|
| Fresh | $2.5 \pm 0.1^{a}$ | $56.1 \pm 0.6^{c}$ |
| 10% Dimethyl sulfoxide, fresh | $2.4 \pm 0.1^{a}$ | $48.3 \pm 1.7^{d}$ |
| 10% Dimethyl sulfoxide, thawed | $2.9 \pm 0.1^{b}$ | $49.1 \pm 1.7^{d}$ |

Groups sharing superscript letter within a column were not significantly different ($P > 0.05$).

Despite these slight changes in sperm morphology, post-thaw sperm motility was >95% in the samples. Although fresh and frozen samples had >95% activity, their swimming behavior differed. Fresh sperm swam intermittently, while the thawed sperm swam slowly and continuously. Neither group produced rapid forward motion. Reducing the cryoprotectant concentration to 5% dimethyl sulfoxide reduced the osmotic damage (prior to freezing), but increased damage observed post-thaw, with motility reduced to less than 50% due to agglutinated cells.

Our finding that dimethyl sulfoxide was an effective cryoprotectant agrees with previous studies of other aquatic invertebrates. In the Pacific oyster, *Crassostrea gigas*, dimethyl sulfoxide by itself or in combination with various additives (e.g., glycine or trehalose), usually at 5–10% of solution volume, has routinely outperformed other cryoprotectants, including ethylene glycol and propylene glycol [8,3]. Dimethyl sulfoxide has also been reported as a successful cryoprotectant for small abalone *Haliotis diversicolor* [4] and sea urchin *Evechinus chlorotichus* [1]. Additionally, a few modifications, such as maintaining the sperm on ice during the procedures and using a filter to remove debris from the sperm seawater suspension may greatly benefit future protocols.

There is an immediate need to begin coral germplasm cryopreservation and form genetic banks throughout the world. One goal of this study was to draw attention to this need, and to provide tools towards this goal. The post-thaw sperm motility and morphology observed, using 10% dimethyl sulfoxide suggested promise for fertilization trials. Only fertilization trials with fresh eggs will determine this. However, this work forms the cryobiological groundwork for upcoming coral sperm cryopreservation trials in 2006, and eventual development of standardized, efficient protocols.

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## References

[1] S. Adams, P. Hessain, P. Mladenov, Cryopreservation of sea urchin (*Evechinus chloroticus*) sperm, Cryoletters 25 (2004) 287–299.
[2] D.R. Bellwood, T.P. Hughes, C. Folke, M. Nystrom, Confronting the coral reef crisis, Nature 429 (2004) 827–833.
[3] Q. Dong, B. Eudeline, C. Huang, S. Allen, T. Tiersch, Commercial-scale cryopreservation of diploid and tetrapolid oysters, *Crassostrea gigas*, Cryobiology 50 (2005) 1–16.
[4] J.C. Gwo, C.W. Chen, H.Y. Cheng, Semen cryopreservation of small abalone (*Haliotis diversicolor supertexa*), Theriogenology 8658 (2002) 1–16.
[5] M. Hagedorn, R. Pan, E. Cox, L. Hollingsworth, T.D. Lewis, J.C. Leong, D.A. Krupp, P. Mazur, W.R. Rall, D. MacFarlane, G. Fahy, F.W. Kleinhans, Coral larvae conservation: Physiology and reproduction, Cryobiology 52 (2005) 33–47.
[6] B.G. Hatcher, Coral reef ecosystems: how much is the whole greater than a sum of the parts? Coral Reefs 16 (Suppl.) (1997) S77–S91.
[7] Y. He, Q. Dong, T.R. Tiersch, R.V. Devireddy, Variation in the membrane transport properties and predicted optimal rates of freezing for spermatozoa of diploid and tetraploid pacific oyster, *Crassostrea gigas*, Biol. Reprod. 70 (2004) 1428–1437.
[8] S. Ieropoli, P. Masullo, M. Do Espirito Santo, G. Sansone, Effects of extender composition, cooling rate, and freezing on the fertilisation viability of spermatozoa of the Pacific oyster (*Crassostrea gigas*), Cryobiology 49 (2004) 250–257.
[9] D.A. Krupp, Sexual reproduction and early development of the solitary coral *Fungia scutaria* (Anthozoa: Scleractinia), Coral Reefs 2 (1983) 159–164.
[10] D.A. Krupp, L.L. Hollingsworth, J. Peterka, Elevated temperature sensitivity of fertilization and early development in the mushroom coral *Fungia scutaria* Lamarck 1801 (2005) (in press).
[11] A. Lacks, Reproductive ecology and distribution of the scleractinian coral, *Fungia scutaria* in Kaneohe Bay Oahu, Hawaii. Master's Thesis, University of Hawaii, Zoology Deptartment, 2000.
[12] D.L. West, Spermiogenesis in the antozoan *Aiptasia palida*, Tissue Cell 12 (1980) 243–253.