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A simple method for several-week sperm preservation applicable for various echinoid species

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Research Article

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Summary

Easy, universal, and cost-effective methods of gamete preservation for echinoids are required since echinoids are important model organisms for studying fertilization and early development, and at the same time, they are valuable fishery resources, and key components of marine ecosystems. In general, cryopreservation is the common method of animal sperm preservation, but we previously reported a simple method using a chilled condition as an alternative choice, using *Hemicentrotus pulcherrimus*. In this study, we applied this method to various echinoid species to test whether it was effective for them, and then we determined the features of the preserved sperm in detail. The fertilization capability of preserved sperm was maintained for several weeks, though higher sperm density was needed to obtain 100% fertilization in later periods. Eggs fertilized by the preserved sperm developed normally, and the morphology of the embryos was not affected. Sperm swimming in a stable, regular manner were observed even at the end of the preservation period, though the flagellar beat frequency was substantially decreased during preservation. There was almost no change of the flagellar beat form during preservation. Here, we showed that our simple method is basically applicable to various echinoid species and provides the opportunity for effective and immediate use of competent echinoid sperm by a wide variety of users, such as people in research, fisheries and education.

Introduction

Sea urchins have long served as important model organisms for studies of fertilization and early development, providing fundamental insights into sperm–egg interactions and early embryogenesis (e.g., Wolpert and Gustafson, 1961; McClay, 2011). Sea urchins are also important model organisms for fields that require reproductive experiments using gametes or embryos, such as biomedical studies (e.g., Bernardo and Carlo, 2017) and ecotoxic studies (e.g., Dorey *et al.,* 2018; Kiyomoto, 2019). Therefore, the development of reliable and practical methods for sperm preservation is essential, as it allows efficient and sustainable use of these valuable resources, particularly for experiments involving genetically modified or otherwise rare individuals.

There is a significant market for sea urchins in fisheries, and demand has been increasing for years; however, global catches are declining due to overfishing, making the protection of natural populations a critical issue (e.g., Andrew *et al*., 2002; Baião *et al*., 2021). Sea urchin aquaculture and seed production are conducted in various countries (e.g., Eddy *et al*., 2015; Unuma *et al*., 2015), and effective and easy gamete preservation methods support basic research in aquaculture and increase the chances of successful seed production. Sea urchins also occupy important ecological niches and contribute to maintaining marine ecosystems (e.g., Steneck, 2013). Thus, the development of standardized methods for gamete preservation in sea urchins is necessary from both ecological and economic perspectives (Fabbrocini *et al*., 2021).

There are three main methods for preservation of the sperm of sea urchins. The first method is cryopreservation. Detailed protocols for cryopreservation of sperm and embryos have been established in several sea urchin species (reviewed in Paredes *et al*., 2019). Cryopreservation is an effective method since it allows the stable long-term storage of sperm for years, but in many cases, it needs expensive and large-scale equipment such as a programmed freezer, and there is a limitation for shipping since the cryopreservation utilizes liquid nitrogen. It is also known that adjustment of the multiple parameters for the programmed freezing is needed due to differences among genera or species (Vacquier and Hamdoun, 2024). Thus, cryopreservation is relatively suitable for experimental usage of specific species. The second method is the chilled preservation (usually at 4°C) of dry sperm. It is traditionally known that the sea urchin sperm collected without the contamination of seawater (called 'dry sperm') can be preserved under chilled conditions and are activated when diluted in seawater (e.g., Rahman *et al*., 2009). This method is quite easy and effective for the short term, but in many cases, the fertilization capability is lost in a few days (Fernández *et al*., 2024). The third method is chilled preservation in the preservation

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medium, and it has intermediate features of these two methods, i.e., it needs only a refrigerator or ice and preservation media containing simple chemicals and enables preservation for several weeks. It is known that sea urchin eggs can be stored in seawater containing antibiotics (Epel *et al.*, 2004), and we previously utilized this method for the sperm of sea urchin *Hemicentrotus pulcherrimus* and succeeded in the storage of competent sperm at 4°C for 60 days (Kiyomoto *et al.*, 2014). Recently, it was confirmed that storage at 0°C using seawater containing HEPES and additional potassium chloride as medium enabled the preservation of competent sperm of *Strongylocentrotus purpuratus* for over 50 days and *Lytechinus pictus* for over 30 days (Vacquier and Hamdoun, 2024). Thus, these methods are thought to be potentially applicable to other echinoid species, but whether these methods are applicable to various species, and the effects of preservation on sperm features, are still unclear.

In this study, we employed the method used by Kiyomoto *et al.* (2014) for various sea urchins in Japan to examine whether the protocol is universally effective for echinoid species other than *H. pulcherrimus.* In addition, to evaluate in detail the effects of preservation on sperm competency, features such as fertilization rate, development of fertilized eggs, sperm motility, and the shape of flagella beat were investigated in detail. We investigated five species, including four regular echinoid species that have different breeding seasons and one irregular echinoid that is phylogenetically distant from regular echinoids (Figure 1). We reinvestigated *H. pulcherrimus,* which was used by Kiyomoto *et al.* (2014) and is the major echinoid used for developmental studies in Japan (Kinjo *et al.*, 2018). The other three regular echinoids we studied, *Mesocentrotus nudus, Pseudocentrotus depressus,* and *Heliocidaris crassispina,* are edible and commercially important species in Japan (reviewed in Agatsuma, 2020a, 2020b). We also studied *Clypeaster japonicus*, a common irregular echinoid in Japan that is also used in developmental studies (Hamaguchi and Hamaguchi, 2001; Hibino *et al.*, 2019).

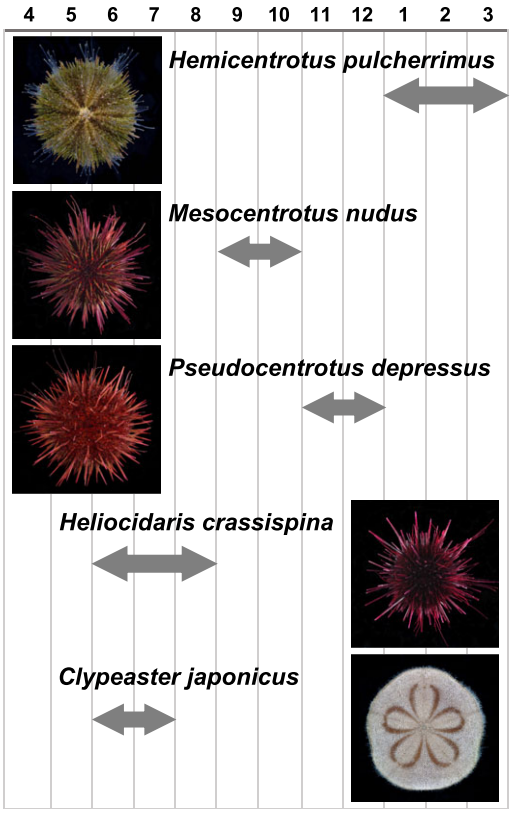


Figure 1. Echinoid species used in this study and their breeding season. Echinoids used in this study were collected from various sites and kept in outdoor flow-through tanks at Tateyama Marine Laboratory (Chiba, Japan) until sexual maturation (see also materials and methods). The columns represent months, and grey bars indicate the breeding season of each species at the laboratory.

Materials and methods

Gamete collection

Sea urchins used in this study were collected from ocean depths of 0 to 20 m during the breeding season of each species (Figure 1). The habitats and the months of sperm collection for each species were as follows:

- *H. pulcherrimus*: Choshi and Tateyama in Chiba Prefecture and Asamushi in Aomori Pref. (January)
- *M. nudus*: Otsuchi in Iwate Pref. (October)
- *P. depressus:* Tateyama in Chiba Pref. (November)
- *H. crassispina*: Tateyama in Chiba Pref. and Usa in Kochi Pref. (June)
- *C. japonicus*: Tateyama in Chiba Pref. and Usa in Kochi Pref. (June)

The detailed procedure for gamete collection is described in Kiyomoto *et al.* (2014). In brief, mature sperm and eggs were obtained by injecting 0.1–1 mL of 0.5 M potassium chloride or 2 mM acetylcholine into the body cavity of urchins. Sperm was collected as dry sperm. Before sperm collection, a male individual was wrapped in paper towels and gently shaken to remove the seawater on the body surface to prevent the contamination with seawater. Injected males were placed directly on a glass dish without seawater, with the gonopore facing downward. Semen containing little seawater (dry sperm) was then collected from the dish using a glass pipette and used for preservation. Eggs were collected and washed several times with Millipore-filtered natural seawater (MFSW) before the experiments.

Preparation for sperm preservation

Collected dry sperm was diluted with 100 times the volume of MFSW containing 200 μg/mL sulfamethoxazole and 10 μg/mL trimethoprim (ST-SW) according to Epel *et al.* (2004). Diluted sperm was dispensed in 1.5 mL Eppendorf tubes and stored at 4°C during preservation period.

Measurement of fertilization rate and observation of development

To quantify the fertilization capability of preserved sperm, the fertilization rate was measured using a sperm dilution series. Sperm stopped swimming under the storage condition and was settled on the bottom of tubes, so the tube was gently inverted to mix sperm just before the experiment. For the measurements, serial 10-fold dilutions of the preserved sperm were made using MFSW. Sperm was preserved at 10^{-2} dilution, and serial dilutions of the preserved sperm were made from 10^{-2} to 10^{-7}, so the final concentration was diluted 10^{-4} to 10^{-9} times compared to dry sperm. Dilution series were made in 24-well plates with ultra-low attachment surface (Corning 3473), and around 100 unfertilized eggs were dropped quickly into each well after the dilution. The

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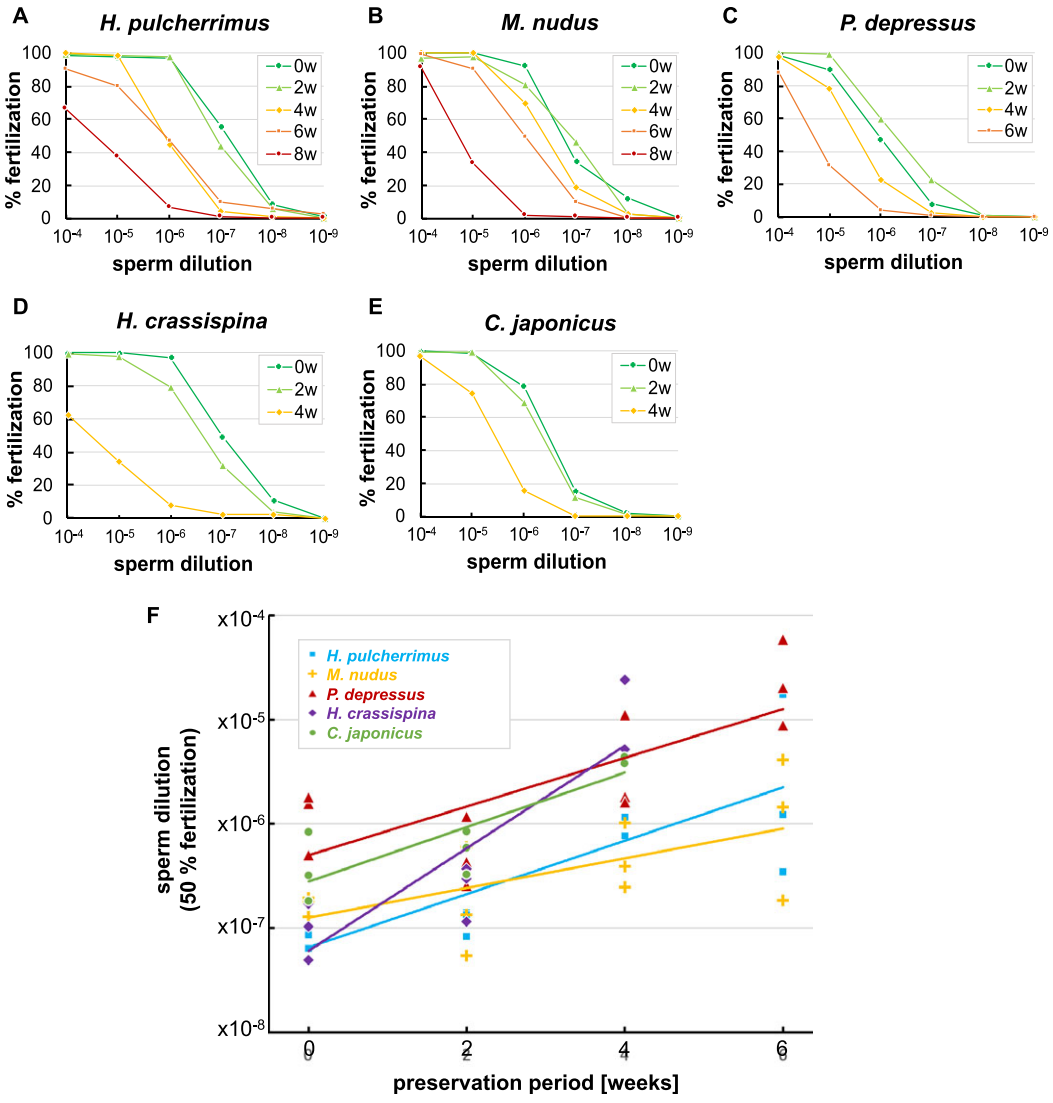


Figure 2. Fertilization assays using dilution series of preserved sperm. (A–E) Fertilization rate by preserved sperm for each species. The horizontal axis of each panel represents the final concentration calculated from dry sperm. Sperm was preserved at 10^{-2} dilution in ST-SW, and dilution series were prepared by 10^{-2}–10^{-7} dilution of preserved sperm. The vertical axis of each panel represents the fertilization rate of fresh eggs by preserved sperm. The fertilization rates were obtained by counting the eggs with fertilization envelopes. Each value is the mean of 3 biological replicates. The fertilization rates were measured on the day of the sperm sampling (0 w) and every 2 weeks during the preservation period. Individual data of each week is presented in supplementary figure 1. The standard deviations for each time point are provided in the Supplementary Figure 1. (F) Changes in fertilization competency of sperm during preservation. Dilution for 50 % fertilization was plotted for each biological triplicate for each species and preservation period. The regression curve for each species is presented as a line. Regression curves are $y = 7E\text{-}08e^{0.5893x}$ ($R^2 = 0.2979$) for *H. pulcherrimus*, $y = 1E\text{-}07e^{0.3277x}$ ($R^2 = 0.3884$) for *M. nudus*, $y = 5E\text{-}07e^{0.538x}$ ($R^2 = 0.5228$) for *P. depressus*, $y = 6E\text{-}08e^{1.1325x}$ ($R^2 = 0.6322$) for *H. crassispina* and $y = 3E\text{-}07e^{0.602x}$ ($R^2 = 0.9451$) for *C. japonicus*.

fertilization rate was measured by counting the eggs with fertilization envelope under a stereo microscope (Olympus SZX7) 5 min after the egg addition. Each measurement included 3 biological replicates. Measurement was started from the day of the sperm collection (0 weeks) and conducted every 2 weeks, up to 4–8 weeks after the collection. Eggs used in the measurement were freshly collected on every measurement day. The sperm dilution that achieved 50% fertilization was calculated using a curve fitting function for the logistic curve in ImageJ software (https://imagej.nih.gov/ij/). Regression curves for Figure 2F were calculated using Microsoft Excel. Fertilized eggs obtained after the measurement were washed with MFSW and kept in 10 mL dishes until they became pluteus larvae. The embryos in each developmental stage were placed on a hole-slide glass and observed under a microscope (Olympus BX53) equipped with a camera (Olympus DP74). Incubation temperatures for fertilization and embryos were approximately 18°C for *H. pulcherrimus*, 20°C for *M. nudus* and *P. depressus*, 24°C for *H. crassispina* and *C. japonicus*.

Measurement of flagellar beat frequency

Sperm flagellar beat frequency was measured by the previously described method (Wessel *et al.*, 2021). The preserved sperm was transferred using a toothpick to prevent co-transfer of surrounding liquid onto MFSW containing 0.01% (w/v) Bovine Serum Albumin (Wako, Japan) and placed between a glass slide and a coverslip with two strips of plastic tape (Scotch, 3 M) as spacers. The frequency of flagellar beating was measured by synchronizing the frequency of custom-made stroboscopic LED illumination equipped on a microscope (Olympus CHBS) to sperm flagellar beating. Around 30 sperm from more than three microscopic fields were measured for each preserved batch.

Analysis of flagellar beat form

After measurement of beat frequency, the observation chamber was removed from the darkfield microscope and placed upside down onto an inverted phase contrast microscope for flagellar beat form analysis. Images of motile spermatozoa were observed under a phase contrast microscope (UPlan Fl objective lens, IX2-LWUCD condenser on Olympus IX-51 inverted microscope) and recorded at 90 fps with a camera (UI-3130CP-M-GL, iDS, Germany) driven by control software (Boh Navi, Bohboh software, Japan) on a LAVIE Direct N15 (NEC, 64 bit, Windows 11). The flagellar beat waveforms were analyzed using motion analysis software (Bohboh ver. 4.94; BohbohSoft, Tokyo, Japan; Ohmuro

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et al., 2004). First, using an automatic tracking function of the motion analyzer, the XY-coordinates along the centre line of the flagella were obtained for each spermatozoa image (total 100 frames for 1.1 s). Then, using that coordinate data, we obtained the curvature of each 1 μm flagellar segment (for details, see Wada *et al.* (2015)). We used these curvature values to evaluate how long the original waveform was maintained in each preserved suspension.

Results

Fertilization capability of preserved sperm

In every species, the preserved sperm were capable of fertilization for at least 4 weeks after the gamete collection (Figure 2). Overall, there was a tendency for the fertilization rate at a given sperm concentration to decrease as the preservation period increased. In other words, a higher concentration of sperm was necessary to obtain the same fertilization rate after preservation.

For *H. pulcherrimus*, the fertilization rate was almost 100% at 10^{-6} or lower dilution on the day of sperm collection (0 weeks, Figure 2A). At 10^{-6} dilution, the fertilization rate was maintained at around 100% until 2 weeks after the sperm collection (2 weeks), but the fertilization capability was gradually reduced, and the fertilization rate was decreased to approx. 50% at 4–6 weeks. Finally, the fertilization capability was lost at this concentration, and the fertilization rate was approx. 0% at 8 w preservation. At 10^{-4} dilution, the fertilization rate was almost 100% even at 6 weeks and around 70% at 8 weeks. For *M. nudus*, the fertilization rate was approx. 90% at 10^{-6} dilution at 0 w and decreased to approx. 80% at 2 weeks, 70% at 4 weeks, 50% at 6 weeks and 0% at 8 weeks (Figure 2B). At 10^{-4} dilution, the fertilization rate was almost 100% until 8 weeks. For *P. depressus*, the fertilization rate was almost 100% at 10^{-5} dilution or higher concentration at 0 weeks (Figure 2C). At 10^{-5} dilution, the fertilization rate was approx. 100% at 2 weeks, 80% at 4 weeks and 30% at 6 weeks. At 10^{-4} dilution, the fertilization rate was almost 100% until 6 weeks. For *H. crassispina*, the fertilization rate was almost 100% at 10^{-6} dilution at 0 weeks and decreased to approx. 80% at 2 weeks and 10% at 4 weeks (Figure 2D). At 10^{-4} dilution, the fertilization rate was almost 100% at 2 weeks and around 60% at 4 weeks. For *C. japonicus*, the fertilization rate was approx. 80% at 10^{-6} dilution at 0 weeks and decreased to approx. 70% at 2 weeks and 20% at 4 weeks (Figure 2E). At 10^{-4} dilution, the fertilization rate was almost 100% until 4 weeks.

Focusing on the concentration of sperm which leads to a substantial fertilization rate (50%), the required sperm concentration increased as the preservation period was extended (Figure 2F). The rate of decrease in fertilization competency was steepest for *H. crassispina*. The competency was reduced to 1/10th in 2 weeks. For the other species, although the sperm dilution that produced 50% fertilization varied among species, the rate of decrease was approximately the same and less steep than that for *H. crassispina*. The competency was reduced to 1/10th in 4 weeks in these species.

Morphology of embryos fertilized with preserved sperm

As shown in Figure 2, the fertilization capability was gradually reduced as preservation periods increased, but fertilized eggs were obtained even after long preservation, so we observed the development of those embryos. The sperm preservation period for the observation was 8 weeks for *H. pulcherrimus* and *M. nudus,* 6 weeks for *P. depressus,* and 4 weeks for *H. crassispina* and *C. japonicus.* The morphology of the embryos fertilized by the preserved sperm was normal, and the developmental process proceeded without any defects (Figure 3). In each species, the fertilization envelope was raised after fertilization (Figure 3A–E). After the fertilization, cleavage proceeded (Figure 3F–J), the embryo became a blastula (Figure 3K–O), and then became a normal pluteus larva (Figure 3P–T). There was no recognizable difference in morphology and progression of development between embryos fertilized with preserved sperm and embryos fertilized with normal, fresh sperm.

Flagellar beat frequency

To evaluate the effect of preservation on sperm motility, we next investigated the frequency of sperm flagellar beat. Figure 4 shows the average sperm flagellar beat frequency of each sea urchin species measured every 2 weeks. In general, the beat frequency was 30–40 Hz at the date of sperm collection and then decreased as the preservation period increased. For *H. pulcherrimus,* the beat frequency was around 35 Hz and decreased by approx. 5 Hz every 2 weeks (Figure 4A). After 10 weeks of preservation, the frequency dropped to 10–20 Hz, but the sperm maintained competency for flagellar beating. In *M. nudus,* the frequency was around 35 Hz at 0 weeks, decreased by approx. 5 Hz every 2 weeks, and finally dropped to 10–20 Hz at 8 weeks (Figure 4B). For *P. depressus,* the frequency was 35–40 Hz at 0 weeks, decreased by approx. 10 Hz every 2 weeks, and was 10–25 Hz at 6 weeks, though the difference among samples was relatively large (Figure 4C). For *H. crassispina,* the frequency was around 40 Hz at 0 weeks, decreased by approx. 5 Hz every 2 weeks, and was around 30 Hz at 4 weeks (Figure 4D). For *C. japonicus,* the frequency was around 35–40 Hz at 0 weeks, decreased by approx. 10 Hz every 2 weeks, and was around 25 Hz at 4 weeks (Figure 4E). The rate of decrease of flagellar beat during the preservation period varied among species, but the frequency at the end of the period was 10–20 Hz in *H. pulcherrimus, M. nudus,* and *P. depressus* (species that mature in autumn to winter) and was higher, around 20–35 Hz, in *H. crassispina* and *C. japonicus*, species that mature in summer (Figures 1 and 4F). The average frequency at 0 w was also higher in species that mature in summer (Figure 4F).

The beat frequency of preserved sperm could be measured until 10 weeks after sperm collection for *H. pulcherrimus*, 4 weeks for *H. crassispina* and *C. japonicus*, 6 weeks for *P. depressus* and 8 weeks for *M. nudus* (Figure 4A–E). After these periods, the beat frequency could not be measured due to the unstable swimming of preserved sperm (see Discussion for details). In other words, stable swimming of sperm could not be observed beyond these periods, and the periods vary among species. At the date of sperm collection, almost all sperm swam in a circle with stable beat, but the number of sperm that swam in a circle gradually decreased, and finally, the flagellar beat frequency could not be measured at the end of the preservation period since almost all sperm swam in an unstable manner. Some examples of the types of unstable swimming were three-dimensional swimming, swimming with flagellar beat vertical to the focal plane, intermittent swimming in a typical 'quiescent' manner (Gibbons, 1980), and so on.

There was also a tendency for the duration of stable sperm swimming to decrease as the preservation period increased. Sperm in ST-SW were thought not to be moving and started swimming when diluted with MFSW. In every species, at 0 weeks almost all sperm swam in circles for more than 10 min, but the duration of stable swimming became shorter in later periods, and at the end of

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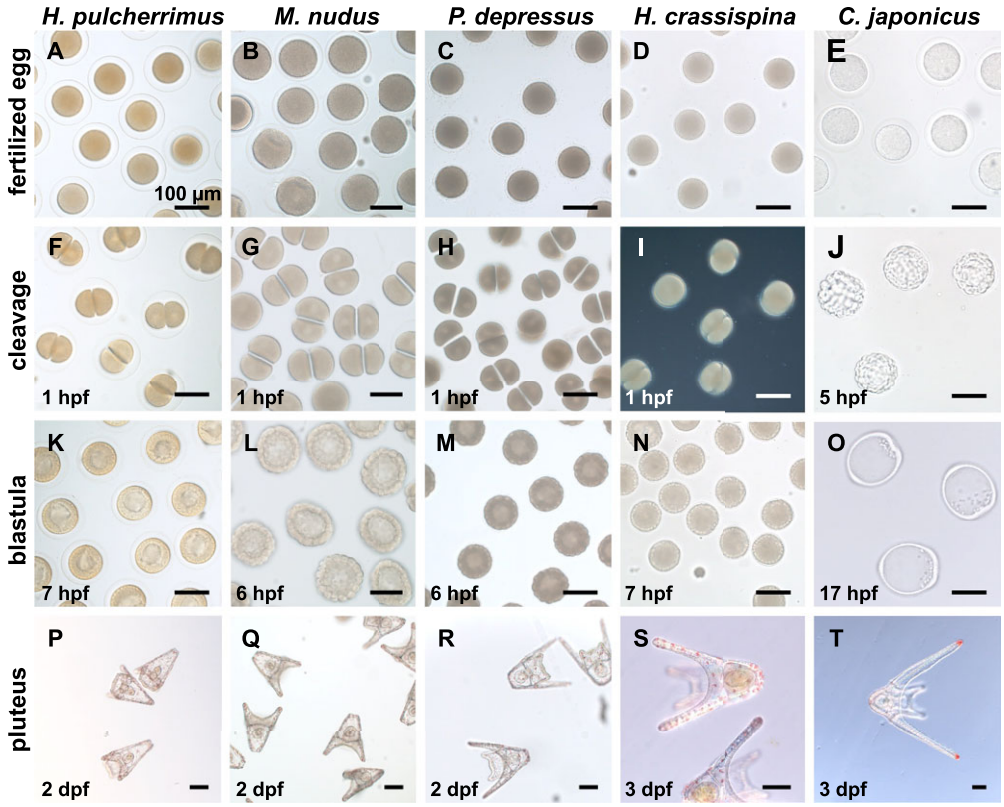


Figure 3. Development of embryos obtained by fertilization with preserved sperm. The morphology of embryos obtained by fertilization with preserved sperm was normal at each presented stage for the 5 investigated species. The columns correspond to the species, and the rows correspond to the developmental stages. Embryos of *H. pulcherrimus* (A, F, K, P) and *M. nudus* (B, G, L, Q) were obtained by fertilization with sperm preserved for 8 weeks. Embryos of *P. depressus* (C, H, M, R) were obtained by fertilization with sperm preserved for 6 weeks. Embryos of *H. crassispina* (D, I, N, S) and *C. japonicus* (E, J, O, T) were obtained by fertilization with sperm preserved for 4 weeks. Scale bars: 100 µm. The time point for each developmental stage is shown in the lower left corner of each panel. hpf, hours post fertilization; dpf, days post fertilization.

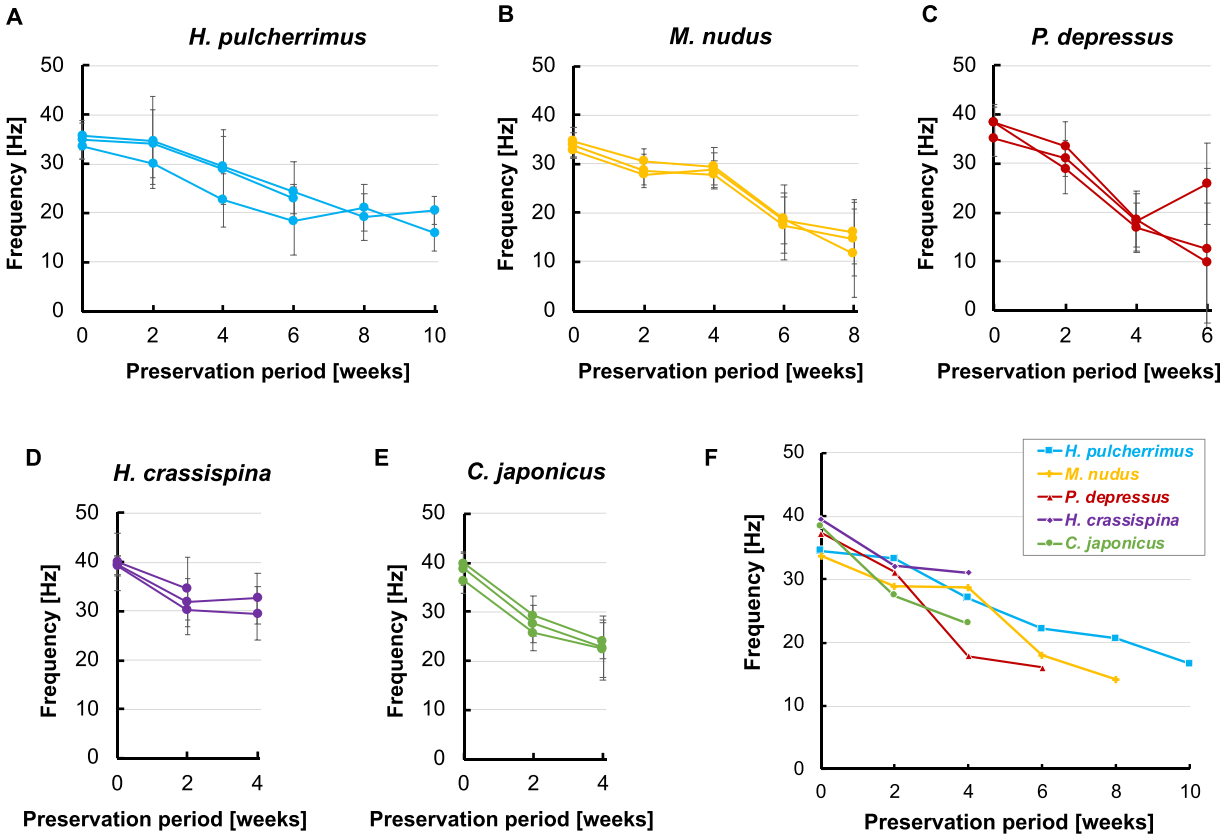


Figure 4. Changes in sperm flagellar beat frequency during preservation. (A–E) Flagellar beat frequency of preserved sea urchin sperm. Vertical axes indicate the beat frequency measured, and horizontal axes indicate the preservation periods. Each dot represents the mean value of approx. 30 sperm with circular swimming path in stable wave form, and standard deviations for each dot are shown. Sea urchin species are indicated for each graph. Three biological replicates were measured at each time point, and data from the same storage tube were connected by lines. (F) Superimposed plots of 5 species. The average frequency of three replicates is presented.

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the preservation period, flagellar beat ceased soon after the dilution. For example, in *P. depressus* after 6 weeks storage, about half of sperm swam in circles just after dilution, though most sperm stopped in 1–2 min (Movie S2).

Flagellar beat form

In addition to the beat frequency, we also observed the sperm flagellar beat form and calculated curvature of flagella along the whole length during the preservation. Figure 5 shows representative flagellar curvature and trace of a sperm in each species at the corresponding preservation period. Overall, flagellar beat form was well maintained during all preservation periods, and both the trace of flagella and the profile of curvature showed little change during storage.

For *H. pulcherrimus*, the trace of flagella and the curvature profile were almost unchanged from 0 w to 8 w. The trace of flagella indicated that flagella of preserved sperm bent uniformly from the proximal part to the distal tip at every preservation period (Figure 5A, upper row). Curvature plots showed the same tendency (Figure 5A, lower row). The width of the curvature plot was the same from the proximal part to the distal tip, indicating that the flagella curvature changed symmetrically during the measurement, and the curvature was constant regardless of the distance from the base of the flagella. This means that the bending of flagella propagated properly from the sperm head to the end of the flagella, even after the long preservation. Movie S1 shows side-by-side movies of *H. pulcherrimus* sperm swimming at 0 weeks and 8 weeks of ST-SW storage. Sperm swimming became somewhat slower due to the lower beating frequency, while the basic swimming pattern did not change much. In *M. nudus,* the overall shape of the flagella trajectory and the curvature plot did not change between 0 weeks and 8 weeks (Figure 5B). Similarly, no definitive change was observed between 0 weeks and 4 weeks in *P. depressus* (Figure 5C), 0 weeks and 2 weeks in *H. crassispina*, or 0 weeks and 4 weeks in *C. japonicus* (Figure 5E). On the other hand, although the overall shape of the curvature plot was almost the same throughout the preservation, as described above, at the end of the preservation period, the width of plot for the distal portion was slightly narrowed in some species. This tendency was observed most obviously in *H. crassispina* at 2 weeks (Figure 5D), and was also observed in other species such as *H. pulcherrimus* at 8 weeks (Figure 5A), and *P. depressus* at 4 weeks (Figure 5C). This change in shape indicates that flagella bending became attenuated at the distal part.

Discussion

Our results showed that our sperm preservation method at 4°C using ST-SW was applicable to each of the five investigated species (Figure 2A–E). In general, the fertilization rate decreased as the preservation period increased, but over 50 % fertilization rate was maintained at 10^{-4} dilution throughout all preservation periods. In addition, preservation did not affect the larval morphology (Figure 3). These results indicate that our method is widely applicable to various echinoid species, including irregular echinoids, and competent sperm is potentially preservable for 4–8 weeks.

As for the fertilization rate, decreased fertilization rate with increasing preservation period was obviously observed at 10^{-6} dilution (Figure 2A–E). The reduction in fertilization competency occurred the fastest in *H. crassispina,* which matures in summer, and occurred at almost the same rate in the other four species (Figure 2F). For these four species, fertilization competency was reduced to approximately 1/10th of the initial value after 4 weeks of preservation. This indicates that the number of active and competent sperm in preserved populations was decreased to about 10% from the beginning of preservation. On the other hand, at 10^{-4} dilution, the fertilization rate using preserved sperm was almost 100 % throughout all preservation periods. So, in general, a 10^{-4} dilution of sperm (i.e., 10^{-2} dilution of the preserved sperm) resulted in enough sperm to obtain a substantial fertilization rate even after 4–8 weeks of preservation. At this concentration, the number of competent sperm was sufficient to obtain high fertilization rates even after long preservation, since the whole population was large. In addition, these findings suggest that the addition of preserved sperm at higher concentrations to eggs would potentially be successful for sperm with longer preservation periods. Incidentally, in our experiment, the effective concentration of sperm varied even at 0 w (Figure 2F). This phenomenon is thought to be due to the difference in the number of sperm in dry sperm preparations and the inevitable contamination of seawater during the sampling procedure. In future studies, normalization by counting of the actual number of sperm will be necessary to obtain a more accurate value of the concentration of effective preserved sperm.

Observation of the embryos fertilized by preserved sperm revealed that sperm preservation did not affect the larval morphology. This result is consistent with previous research (Kiyomoto *et al.*, 2014; Vacquier and Hamdoun, 2024). The results showed that our preservation methods were also effective for obtaining normal embryos, not only for the fertilization assay. In *H. pulcherrimus,* the method using ST-SW is also applicable for unfertilized egg preservation (Vacquier and Hamdoun, 2024). Testing whether this method is applicable for eggs of other species in detail will be important for effective gamete utilization.

The flagellar beat frequency was gradually reduced during preservation (Figure 4). In addition, the ratio of sperm with a stable manner of swimming decreased, and the duration of flagellar beating became shorter, as the preservation period increased. These observations show that sperm gradually lost their activity with increasing preservation period, and the reduction of fertilization rate with the increasing preservation period (Figure 2) might reflect the gradual loss of sperm activity.

On the other hand, we observed a substantial average beat frequency of sperm flagella of all species investigated in this study throughout the preservation period. This means that stably swimming sperm were present in the preserved populations since the measurement of flagellar beat frequency requires manual synchronization of strobe frequency to the stable flagellar beat. These stably swimming sperm are thought to contribute to the substantial fertilization rate after long preservation (Figure 2). There was almost no change in sperm flagella beating form during preservation (Figure 5), so it can be said that the propagation of flagella beating was not damaged during preservation. The slight constriction of the curvature plot in the distal portion observed in *H. crassispina* (2 weeks) indicates attenuation of the curvature of flagella bending at the distal portion, and this might be due to partial damage of the flagella during preservation. It is known that flagellar beat frequency is determined by the outer arm dynein in flagella, and beat form (beat amplitude) is determined by the inner arm dynein (Brokaw and Kamiya, 1987), so one possibility is that the activity of outer arm dynein in flagella is lost faster than that of inner arm dynein under the preservation conditions of this study.

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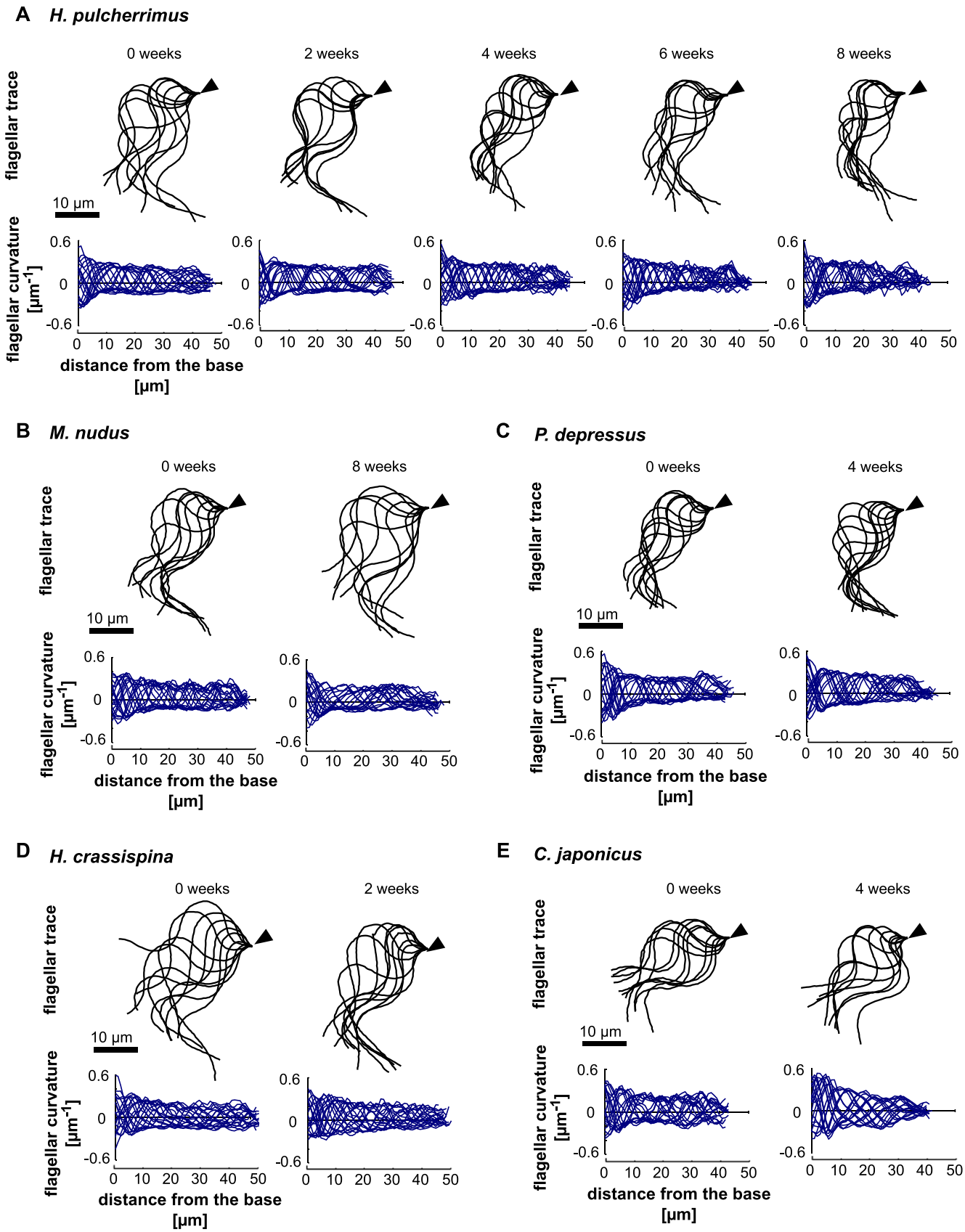


Figure 5. Changes in sperm flagella wave form during preservation. Superimposed traces of flagella (upper panel) and curvature profile(;lower panel);taken from 100 video frames are presented. For the flagellar trace, the shape of flagella in each video frame was superimposed, fixing the position of the sperm head. Sperm head position is indicated by an arrowhead. For the curvature profile, the bending curvatures obtained from the XY-coordinates of sperm tails were plotted. Vertical axes indicate the curvatures, and horizontal axes correspond to the distance from the proximal end of the flagellum. For *H. pulcherrimus,* data from every 2nd week are presented (A), and for other species, data from the beginning and latest period are shown (B–E).

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Detailed motility assays focusing on sperm with unstable beat to extract changes in beat form during preservation may offer new insights into the mechanisms of sperm deterioration during preservation.

In further studies, we will also need to accurately quantify the number of active sperm in the whole population. Our observations mainly focused on the surviving, active, and normal sperm in the preservation population, and showed that competent sperm are included in the preserved sperm population, so practically it can be said that the sperm preservation method using ST-SW is very effective. On the other hand, to know the number of inactive or damaged sperm during the preservation and to understand the mechanism of deterioration should also be important for improvement of the preservation method. Interestingly, it is known that the preservation of sperm motility is also decreased with time in cryopreserved sperm (Fabbrocini *et al*., 2023). Comparison of damage of the sperm by different preservation strategies will also help us to understand the relation between sperm activity and preservation condition.

In this study, we show that sperm obtained from various sea urchin species can be stored for up to 8 weeks at 4°C using ST-SW, and that our simple method is practically applicable across multiple species. This method allows a longer preservation period than chilled storage of 'dry sperm', thereby reducing the frequency of sperm collection from parental individuals and conserving biological resources in research, such as seed production, larval embryology, and ecotoxicological studies. It is particularly advantageous for rare species, specific strains or genetically manipulated individuals with limited opportunities for sperm collection. Although storage for several months or years cannot be achieved, as is possible with cryopreservation, our method requires only simple equipment and materials and does not require species-specific condition optimization. Therefore, it is especially suitable for repeated experiments over several weeks using multiple species and is useful for researchers as well as for applications in aquaculture and education.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0967199426100458.

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