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Step by step optimization of a sperm cryopreservation protocol for spotted wolffish (*Anarhichas minor* Olafsen, 1772)

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ABSTRACT

Spotted wolffish *Anarhichas minor* reproduction in captivity is dependent on *in vitro* fertilization. However, low sperm volume with relatively low cell concentration and the lack of gametes synchronization (simultaneous availability of mature eggs and sperm) represent a challenge for the industry. Thus, the development of protocols for sperm storage are crucial. Four sequential experiments were conducted to optimize a sperm cryopreservation protocol for this species. First, three different cryoprotectants (DMSO; 1, 2-propanediol; and methanol) at different concentrations (5, 10, and 20%) were tested for their toxicity. No significant differences ($p > 0.05$) were detected between the control samples and cryoprotectants at concentration up to 10% DMSO, 10% propanediol, and 20% methanol in terms of motility parameters. Second, using the highest non-toxic concentrations of cryoprotectants, sperm was cryopreserved in 0.5 mL straws, at different distances from the liquid nitrogen (1.5, 2.5, 4.5, and 7.5 cm) that correspond to different freezing rates. Motility parameters after freezing/thawing decreased for all the cryoprotectants ($p < 0.001$), however, methanol had the lowest protective capacity while DMSO the highest. Afterwards, two different thawing rates (1 min at 5 °C; and 25 s at 10 °C) were tested using only 10% DMSO and 10% propanediol. Both for the DMSO and propanediol, there were no significant differences ($p > 0.05$) between the two thawing rates. The best results were obtained using 10% DMSO. Finally, the fertilization capacity of cryopreserved sperm (10% DMSO and thawed at 5 °C for 1 min) was tested against fresh sperm using two spermatozoa:egg ratios and 4 h gametes contact time. The ratio of eggs with normal cell cleavage, abnormal cleavage or undeveloped were counted at the 2–4 cell stage. Cryopreserved sperm showed lower fertilization capacity at a concentration of 5×10^4 spermatozoa:egg compared with fresh sperm ($p < 0.001$). At a concentration of 5×10^5 spermatozoa:egg, similar fertilizations rates to the fresh sperm were obtained. The presence of the cryoprotectant DMSO during the 4 h contact time did not affect the fertilization rate or the percentage of embryos with abnormal cleavage ($p > 0.05$). To cryopreserve spotted wolffish sperm it is recommended to use 10% DMSO, loaded in 0.5 mL straws, freeze at a height between 4.5 (−14.05 °C/min) and 7.5 cm (−5.9 °C/min) from liquid nitrogen for 10 min and thaw for 1 min at 5 °C (177.9 °C/min). *In vitro* fertilization with cryopreserved sperm should be performed with a concentration of at least 5×10^5 spermatozoa per egg.

1. Introduction

Spotted wolffish *Anarhichas minor* is one of the most promising species for marine cold-water aquaculture diversification in the North Atlantic [1,2]. Rearing conditions are relatively well established, and it is possible to produce this species at high densities while obtaining high growth rates. In addition to this, its valuable and tasty meat is well accepted and paid by consumers. Nevertheless, some bottlenecks regarding broodstock management and reproduction are still to be solved [3]. Due to the disruption of the reproductive behaviour in captivity, it is necessary to rely on *in vitro* fertilization. While females release a large volume of unfertilized eggs, males produce a relatively small volume of low concentration sperm [4,5]. In addition to this, usually males produce gametes in a period that does not overlap with females at the beginning and at the end of the reproductive seasons, so there is the possibility to occur spawns when males are not producing sperm or the quality is low.

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Maybe due to the presumed internal fertilization, spotted wolffish sperm characteristics are rather unusual. In opposed to other aquaculture species, spotted wolffish sperm is motile at stripping and loses viability when in contact with seawater [6]. In chilled conditions (around 4 °C), sperm remains with constant motility for at least 24 h since there is no way to deactivate it [6]. Under this scenario, sperm cryopreservation seems to be the more rational way to synchronize gametes availability.

Two previous works showed that sperm cryopreservation can be used to improve sperm management for this species, since relatively good sperm survival was observed after freezing-thawing [7,8]. Nevertheless, these studies lack a step-by-step optimization of the cryopreservation protocol in search of the best conditions. Actually, both authors admitted that the cryopreservation protocols they presented were only preliminary and needed to be refined to ensure that a detailed and standardized protocol was made available. In both occasions the results were based on the subjective analysis of sperm motility made at higher temperatures that negatively affect sperm motility, and the cryoprotectant concentration and the freezing rates were not optimized [7,8].

In this work we aimed to develop a step-by-step cryopreservation protocol for spotted wolffish sperm, to assist in reproductive management of this species in aquaculture industry, as well as in scientific research and species conservation. To ensure the best cryopreservation conditions, we optimized 1) the cryoprotectant and concentration to obtain the lowest toxicity that maximizes the best protection against cryoinjury; 2) the freezing and thawing rates that minimize the cellular damage during freezing/thawing procedures; 3) an *in vitro* fertilization protocol that makes the best use of the cryopreserved sperm.

2. Materials and methods

2.1. Broodstock rearing and gametes collection

The experiments were carried out using a farmed origin spotted wolffish broodstock from the wolffish producer AMINOR AS. Fish were kept indoors in the Mørkvedbukta research station (Nord University, Bodø, Norway), in 5 square fiberglass tanks of 2 by 2 m, and water depth of 0.4 m (1 600 L), in a semi-closed system. Fish were exposed to natural photoperiod and water parameters according to Mørkvedbukta bay patterns (67°27′N, 14°56′E).

During the reproductive season, gametes were collected by abdominal massage, following the procedures previously described [5]. Sperm was placed on a rack on ice. Only males that produced milt in the previous reproductive season were chosen to be used in the experiment. Males rested at least one month between sperm collections. Any samples containing blood or urine were discarded and, only sperm samples with motility higher than 60% were used on the trials [5]. Egg (oocyte) batches were collected from females showing close to spawn signs. Egg batches with heterogenous colour or abnormal appearance were discarded. The selected batches of sperm and eggs were kept cold (circa 4 °C) with low incidence of light due to light sensitivity [4] until use. Ongoing work at our lab has shown that both eggs and sperm quality remain stable for longer time at 4 °C than at 6 °C. Sperm samples were pooled using at least two males per pool.

2.2. Sperm quality analyses

Sperm analyses started within 3 h after sperm collection. Sperm total motility and velocity (VCL) were analysed with the CASA (Computer Assisted Sperm Analysis) system SCA 6.2 – Motility module (Microptic, Barcelona, Spain). To be able to record motility, sperm was diluted in the extender developed by Kime and Tveiten [6] (145 mM NaCl, 4.55 mM $CaCl_2$, 4.83 mM $KHCO_3$, 2.37 mM $MgSO_4$ and 1 mM glucose) with addition of 1% BSA. Images were recorded using a digital camera (Basler acA1300-200uc, Ahrensburg, Germany) attached to an optical phase-contrast microscope (Nikon Eclipse Ci, Tokyo, Japan) with ×10 negative phase contrast objective and with a stage temperature controller set to 6 °C (Linkam T95-PE, Tadworth, United Kingdom). Three CASA videos were captured right after drift movement stopped. CASA software settings were adjusted for this species' sperm analyses as followed: 50 frames/s, 1 s acquisition time, 10–50 μm^2 for head area. Cell movement bellow 9 μm/s was considered drift.

The sperm viability was measured using the LIVE/DEAD™ Sperm Viability Kit (Thermo Fisher, UK). Sperm was diluted 1:10 in an Eppendorf to the final volume of 200 μL. To this suspension, 1 μL of 40 μM from the SYBR-14 working solution (1:10 in the extender solution) was added, then incubated for 10 min. After that, 1.5 μL of PI 2.4 mM stock solution was added and let incubate for 5 min more. Two μL of this solution were loaded on a microscope slide and observed in a fluorescence microscope with × 400 magnification.

At least 100 cells were counted per slide, and three slides were evaluated per sample. The number of green and red stained cells was counted, and the percentage of viable cells was calculated as the ratio of viable cells/total number of cells.

2.3. Experimental design

2.3.1. Trial 1: Cryoprotectant solution and concentration

In order to find the highest non-toxic concentration of cryoprotectants to spotted wolffish sperm, 50 μL of the pooled semen were diluted 1:1 in the extender by Kime and Tveiten [6] in 0.5 mL Eppendorf tubes (final volume 100 μL) containing one of the different cryoprotectants: 1) DMSO, that was the selected cryoprotectant in previous studies in ocean pout from the Zoarcoidei suborder as the wolffishes [9], Atlantic wolffish *Anarhichas lupus* [7] and spotted wolffish [8]; 2) 1, 2-propanediol, which presented the second best results for ocean pout sperm cryopreservation [9]; and 3) methanol, that is recommended for other cold-water species such as salmonids [10,11], and Atlantic halibut *Hippoglossus hippoglossus* [12]. For each cryoprotectant three different concentrations (final concentration in the suspension) were tested (5%, 10% and 20%). A sub-sample with no addition of cryoprotectant was used as control. In total, 10 different treatments were tested (3 cryoprotectants × 3 concentrations + control). After 2 min incubation at 4 °C the sperm motility parameters were evaluated. The experience was replicated with five different pools and the three treatments with best results according to sperm total motility and velocity were used in trial 2.

2.3.2. Trial 2: Freezing rates

Different freezing rates were tested for sperm cryopreservation in 0.5 mL straws (MiniTube, Germany). The freezing rates were obtained using floating racks to create four different distances from liquid nitrogen (LN_2) surface (1.5, 2.5, 4.5 and 7.5 cm). Each 0.5 mL straw was filled with 400 μL sperm diluted 1:1 in the extender and chosen cryoprotectant based on trial 1, 10% DMSO, 10% propanediol and 20% methanol (highest non-toxic concentrations). After 2 min equilibration time at 4 °C, straws were placed for 10 min in the floating rack, before being submerged in liquid nitrogen. The freezing curves were recorded using a thermocouple (Hanna Instruments, Italy). The straws were thawed at 10 °C for 25 s, and sperm motility parameters immediately analysed, as described before. This initial temperature was selected to allow for a rapid thawing. A non-cryopreserved sub-sample was used as control. In total, 13 different treatments were tested (3 cryoprotectants × 4

freezing rates + control). For each treatment, three straws were used, and the experiment was repeated with five different pools. The best results according to the sperm total motility and velocity were used in trial 3.

2.3.3. Trial 3: Thawing rates

In order to evaluate the impact of thawing rates in sperm quality, the best treatments in trial 2 were chosen: 10% DMSO and 10% propanediol used as cryoprotectants to freeze sperm at 4.5 cm from LN_2 surface. Straws were thawed in a water bath at either 5 °C for 1 min or at 10 °C for 25 s. The time for each thawing temperature was the shortest needed to completely thaw the sample under the selected temperature conditions. The temperature curves during thawing were recorded using the thermocouple. After thawing the sperm motility parameters were immediately analysed. Similar to the previous trial, a non-cryopreserved sub-sample was used as control. In total, 5 different treatments were tested (2 cryoprotectants × 2 thawing rates + control). For each treatment, three straws were used, and the experiment repeated with five different pools.

2.3.4. Trial 4: Assessment of sperm viability on cryopreserved sperm

To assess if the cryopreserved sperm maintains its viability after the freezing and thawing process sperm was cryopreserved using the best treatment from trial 3 (DMSO 10%, frozen at 4.5 cm, thawed at 5 °C) and compared with fresh sperm as control. Six different pools were tested.

2.3.5. Trial 5: Cryopreserved sperm fertilization

To develop a fertilization protocol, groups of 50 eggs (oocytes) were fertilized either with fresh sperm, cryopreserved sperm with the best treatment in trial 3 or cryopreserved "washed sperm", sperm that was centrifuged to remove the cryoprotectant [13]. For this last treatment frozen-thawed sperm was centrifuged at 300 g during 10 min at 4 °C. Thereafter, supernatant was removed and replaced by the Kime and Tveiten [6] extender solution to resuspend the sperm cells. The centrifuge speed was selected according to the results reported for ocean pout [9] and preliminary trials show that did not affect wolffish sperm motility. For each treatment, two spermatozoa:egg ratios of 5×10^4 or 5×10^5 were tested. These ratios were selected based on the works from Le François and Archer [4] and Beirão and Ottesen [5], to test the minimum amount of cryopreserved sperm that is needed to fertilize the eggs. The 50 eggs were fertilized with an adjusted sperm volume to obtain the two different spermatozoa:egg ratios. Higher ratios could mask an effect of the sperm fertilizing ability in spotted wolffish [5]. Subsamples of sperm were used to analyse the sperm motility parameters immediately after thawing and then hourly during the 4 h of the gametes contact time.

Gametes were mixed every 30 min during the contact time of 4 h [4,5]. After that, seawater was added (approximately 200 mL), and the eggs left incubating at 6 °C. Three pools of eggs were incubated in duplicate (two groups of 50 eggs), for each one of the three treatments, at two different spermatozoa:egg ratios.

Fertilization rate was evaluated 22 h after the beginning of the contact time, between the 2 and 4 cell stage, and classified into fertilized with normal cell cleavage (2 or 4 cells of similar size), fertilized with abnormal cleavage, and undeveloped, following the Pavlov et al. [14] draws for Atlantic wolffish eggs.

2.4. Statistical analyses

Statistical analyses were conducted with the R 3.5.3 statistical software. A significance level (α) of 0.05 was used throughout all the statistical analysis. Data was tested for normality with Shapiro-Wilk tests and observation of quantile-quantile plots (Q-Q plots) and, for homogeneity of variances with the Bartlett's test.

Differences between the treatments on trials 1 to 3 for the dependent variables VCL and percentage of motile cells were detected using a single way ANOVA. When significant differences were obtained on the single way ANOVA, a multiple comparison *post-hoc* Tukey test was used to specify the different treatments.

A *t*-test was used to describe the differences on the percentage of viable cells (trial 4).

The results of the fertilization ratio (trial 5) were analysed with a two-way ANOVA. Spermatozoa:egg ratio and treatment (control, cryopreserved and washed sperm) were considered as independent variables. Multiple comparisons for this trial were made using a *post-hoc* Tukey test. Both sperm motility and velocity along the fertilization trial were analysed by an ANCOVA, each, to control the effect of time in the treatments. In the first model, both for motility and velocity, the interaction between time and treatment was non-significant and the model was reanalysed with the interaction removed.

3. Results

When testing the cryoprotectants toxicity in the first trial, the analysis of variance for the motility parameter revealed significant differences ($F_{9,29} = 6.24$, $p < 0.001$). According to the multiple comparisons, there were no significant differences for the percentage of motile cells between fresh sperm control (55.9 ± 14.7%) and 5 and 10% DMSO and propanediol, with sperm keeping its motility between 50.0 ± 14.0% and 60.1 ± 8.0% (Fig. 1A). Sperm incubated in DMSO and propanediol solutions at concentrations of 20% showed decreased values (29.3 ± 14.5% and 28.1 ± 9.8%, respectively) in comparison to fresh sperm. For methanol, no significant differences were found using any of the three concentrations and the sperm motility was kept between 54.4 ± 8.6% and 62.1 ± 10.2%. There were significant differences regarding VCL ($F_{9,29} = 20.60$, $p < 0.001$) in the first trial. The Tukey test revealed that there was a decrease in velocity for the cells in the treatments 20% DMSO and 20% propanediol (19.0 ± 3.4 μm/s and 18.5 ± 3.1 μm/s, respectively) in comparison to fresh sperm (24.6 ± 3.4 μm/s) (Fig. 1B). The remaining treatments did not present significant differences from the control.

In the second trial, testing different freezing rates using the highest non-toxic cryoprotectant concentrations from trial 1, the percentage of motile cells, analysed with ANOVA, presented significant differences ($F_{12,117} = 19.69$, $p < 0.001$). The *post-hoc* Tukey test detected a decrease in the percentage of motile cells in all freeze-thawed treatments when compared to the control using fresh sperm (61.4 ± 7.7%) (Fig. 2A). The best results after freezing were obtained using 10% DMSO at the four different distances from the LN_2 (between 26.0 ± 15.2% and 39.4 ± 16.9%), or 10% propanediol at 4.5 cm (26.6 ± 15.5%). The curvilinear velocity was always affected ($F_{12,117} = 19.8$, $p < 0.001$) when using 20% methanol (maximum of 9.37 ± 8.56 μm/s) in comparison with fresh sperm swimming parameters (21.43 ± 3.93 μm/s) (Fig. 2B).

Regarding freezing rates, methanol showed the highest freezing rates throughout the trial (between -20.1 and -48.3 °C/min for 7.5 and 1.5 cm from the LN_2 respectively), whilst DMSO presented a slower rate (from −5.9 to −32.8 °C/min for 7.5 and 1.5 cm from the LN_2 respectively) (Fig. 3).

The ANOVA model detected significant differences in the third trial, for the percentage of motile cells, between treatments ($F_{8,81} = 9.52$, $p < 0.001$). The *post-hoc* multiple comparisons indicate a decrease in post-thaw motility in comparison to fresh sperm (62.3 ± 14.1%). DMSO presented a maximum of 40.7 ± 15.6% when thawed at 5 °C, and propanediol only 24.5 ± 7.2% using the quicker

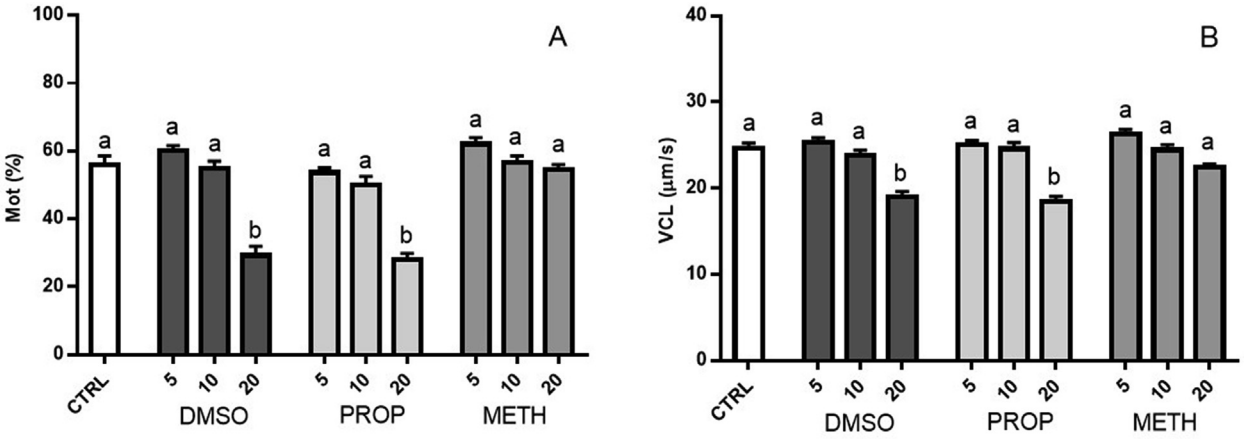


Fig. 1. Sperm motility parameters comparing the toxic effect of dimethyl sulfoxide (DMSO) (dark grey bars), propanediol (PROP) (light grey bars) or methanol (METH) (grey bars), at three different concentrations (5, 10, and 20%) on cryopreserved sperm and, compared with fresh sperm (white bars). (A) Percentage of motile cells (Mot); (B) Curvilinear velocity (VCL). Different letters refer to significant differences (n = 5; mean ± SEM; ANOVA; $p < 0.05$).

thawing rate of 10 °C (Fig. 4A). However, there were no differences on the percentage of motile cells, between the two tested thawing temperatures (5 and 10 °C) for both DMSO and propanediol. The ANOVA model for VCL also detected significant differences between treatments ($F_{8,81} = 2.61$, $p = 0.014$). However, the *post-hoc* Tukey test indicate that those differences are only between the fresh sperm control and the freeze-thawed samples, and not between the two cryoprotectants and thawing rates (Fig. 4B).

Thawing rate curves show similar behaviour between treatments (Fig. 5). Propanediol presented slightly higher rates (178.9 and 183.5 °C/min, for 5 and 10 °C, respectively) in comparison to DMSO (177.9 and 181.6 °C/min, for 5 and 10 °C, respectively).

The sperm viability was affected by the cryopreservation procedure (10% DMSO, freezing rate of −14.05 °C/min, thawing rate of 178.9 °C/min), being significantly lower (53 ± 19%) than that obtained for fresh sperm (74 ± 17%) ($t_{4,17} = 4.09$, $P < 0.001$) (Fig. 6).

Results revealed that there was a significant interaction between the spermatozoa:egg ratio and sperm treatment (fresh, freeze-thawed or thawed and washed) on the fertilization ratios ($F_{4,33} = 0.92$, $P < 0.001$). The ANOVA model detected significant differences for both variables, the spermatozoa:egg ratio ($F_{2,33} = 12.45$, $p < 0.001$) and the treatment ($F_{2,33} = 10.43$, $p < 0.001$). The *post-hoc* multiple comparisons indicate that at 5×10^4 spermatozoa:egg ratio, the percentage of fertilized eggs using freeze-thawed sperm (54.89 ± 17.78% and 45.88 ± 29.22% thawed and washed sperm, respectively) was significantly lower when compared to fresh sperm (86.29 ± 9.53%). On the other hand, when 5×10^5 spermatozoa:egg ratio was used the percentage of fertilized eggs was similar in the three treatments (fresh, freeze-thawed and washed sperm). The percentage of eggs that presented abnormal cell cleavage was not significantly affected by the treatments ($F_{2,24} = 1.28$, $p = 0.295$) (Fig. 7).

In both the ANCOVA models for the sperm motility and VCL (Fig. 8) the interaction between time and treatment was non-significant ($F_{2, 39}<0.83$, $p>0.442$). After re-run the models with the interaction removed it was observed that in both the sperm motility and the velocity models the treatment was significantly different ($F_{2, 41}>5.84$, $p < 0.001$), but not the time ($F_{1, 41}<0.08$, $p>0.783$). This means that the percentage of motile cells and their velocity remained stable along the 4 h contact time needed for the fertilization trial.

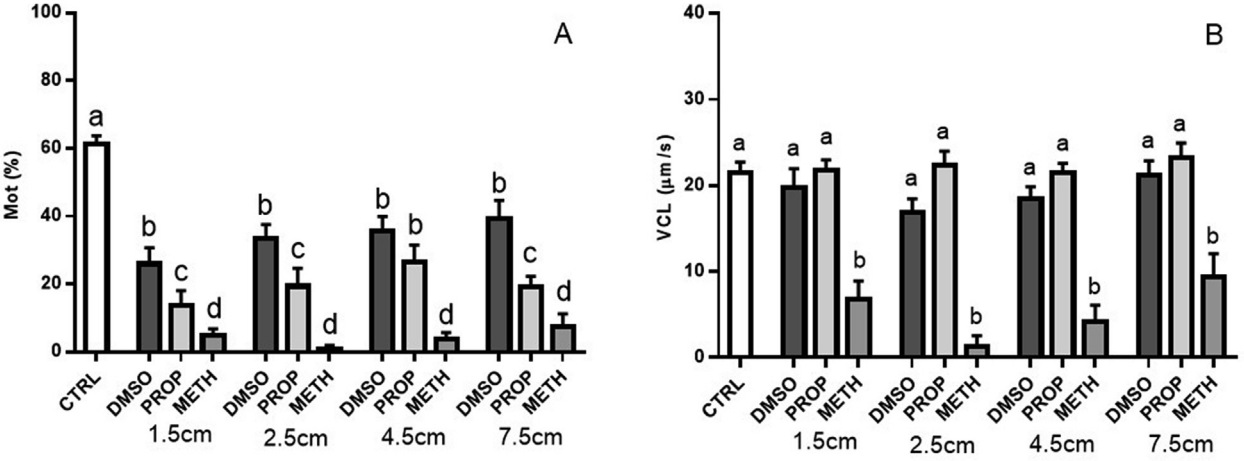
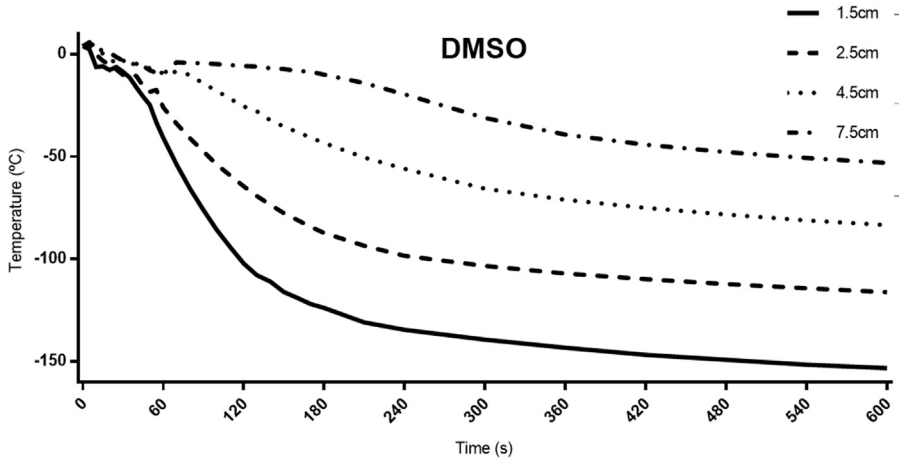
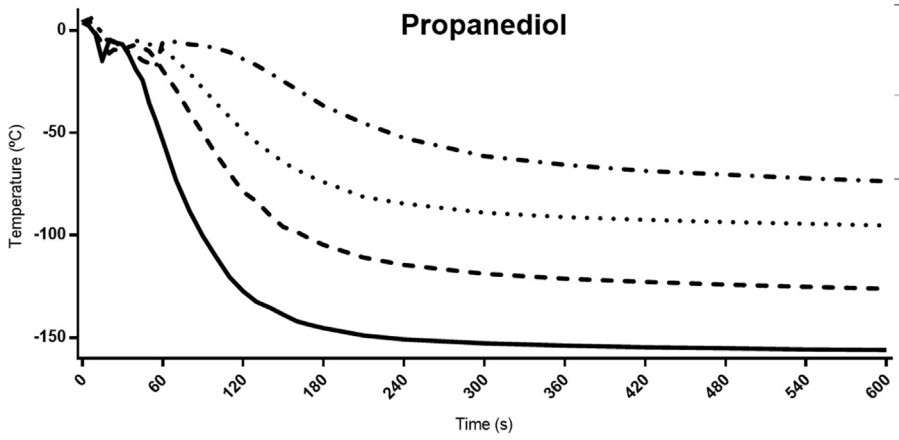


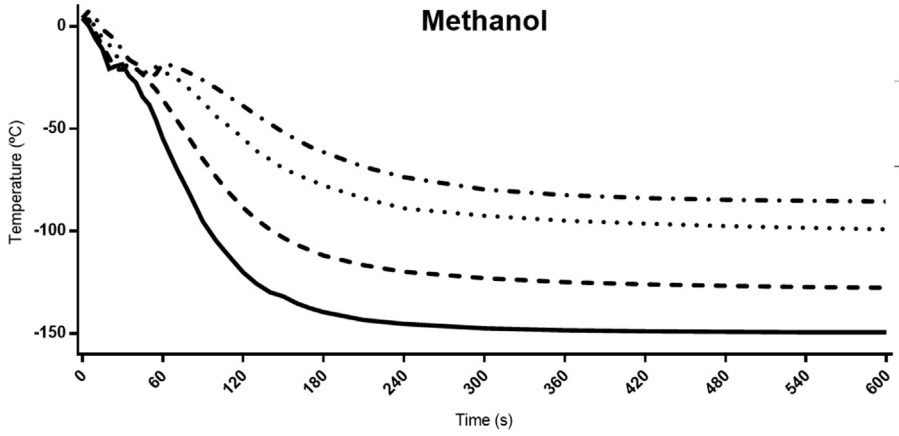
Fig. 2. Sperm motility parameters comparing four freezing rates for sperm cryopreserved with 10% DMSO (dark grey bars), 10% propanediol (PROP) (light grey bars) or 20% methanol (METH) (grey bars), with fresh sperm (white bars). (A) Percentage of motile cells (Mot); (B) Curvilinear velocity (VCL). Different letters refer to significant differences (n = 5; mean ± SEM; ANOVA; $p < 0.05$).



Distance from LN_2 (cm)	Freezing rates (°C/min^{-1}) DMSO	
	4°C to -20°C	-20°C to T_f
1.5	32.8	14.34
2.5	29.9	10.03
4.5	14.05	7.55
7.5	5.9	5.58



Distance from LN_2 (cm)	Freezing rates (°C/min^{-1}) Propanediol	
	4°C to -20°C	-20°C to T_f
1.5	37.87	14.23
2.5	23.55	11.83
4.5	18.68	8.58
7.5	10.67	6.89



Distance from LN_2 (cm)	Freezing rates (°C/min^{-1}) Methanol	
	4°C to -20°C	-20°C to T_f
1.5	48.34	13.29
2.5	36.9	11.47
4.5	26.1	8.56
7.5	20.1	7.23

Fig. 3. Freezing curves for DMSO (A); propanediol (B); and methanol (C); with different distances from liquid nitrogen surface (1.5, 2.5, 4.5 and 7.5 cm), along 10 min. Tf – final temperature after the 10 min. After 10 min the straws were submerged in LN2.

4. Discussion

One of the main bottlenecks in the implementation of new aquaculture species is related to reproduction. Spotted wolffish reproduction in captivity is dependent on *in vitro* fertilization. To assure the availability of sperm when the females are ready to spawn it is essential to keep a sperm stock in case that males are not producing gametes of enough quality. Initial attempts on sperm cryopreservation of this species proved that it is possible to use this technique [7,8], however, further work was necessary to optimize the method and deliver a step by step protocol. This study analysed the previous attempts' weaknesses and delivers a succinct sperm cryopreservation protocol for spotted wolffish that can be both used on the industry and on scientific research, making possible the assembly of a spotted wolffish sperm bank.

In a first step, we chose the cryoprotectants concentrations with lower toxicity, within the time needed for equilibration (2 min), that could provide the best protection against cryoinjury. Usually a

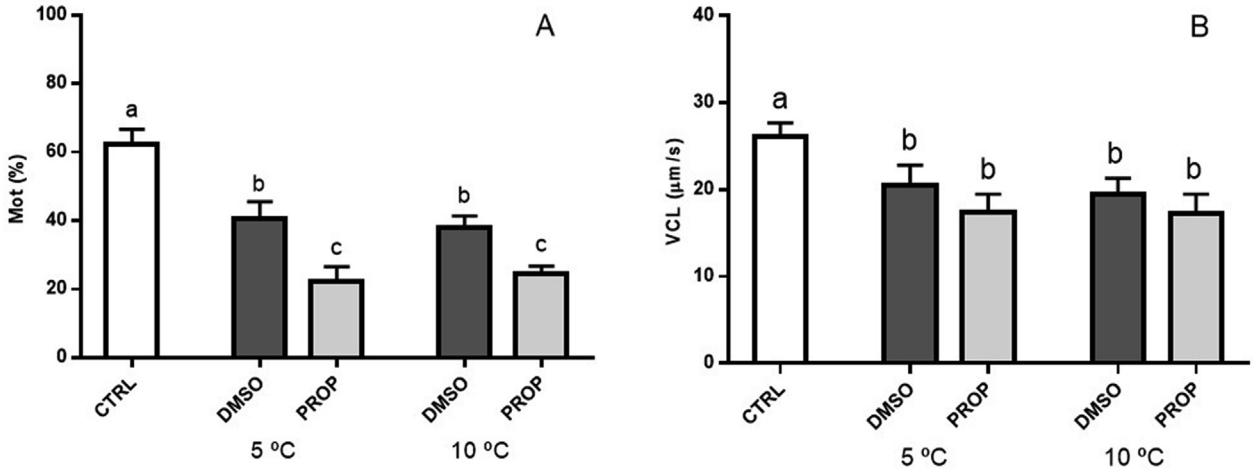


Fig. 4. Sperm motility parameters comparing two thawing rates for sperm cryopreserved with DMSO (dark grey bars) or propanediol (light grey bars), compared with fresh sperm (white bars). (A) Percentage of motile cells (Mot); (B) Curvilinear velocity (VCL). Different letters refer to significant differences (n = 5; mean ± SEM; ANOVA; $p < 0.05$).

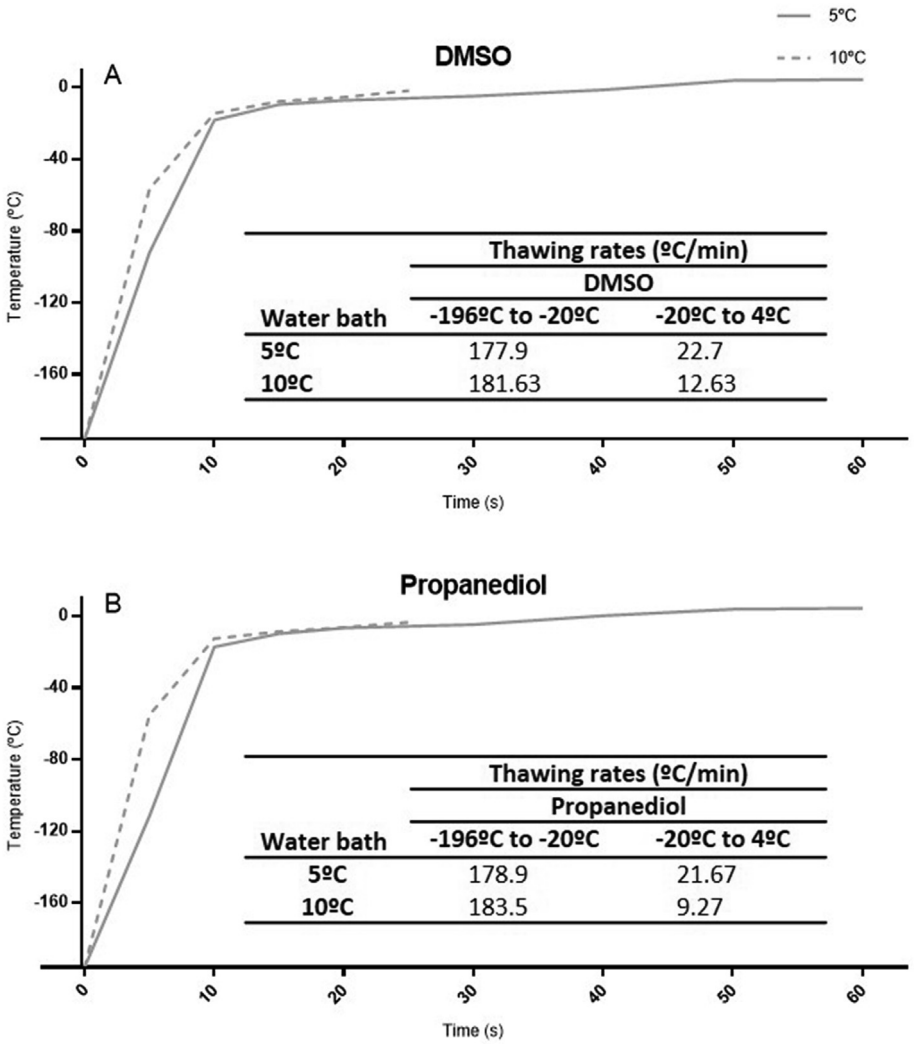


Fig. 5. Thawing curves for DMSO (A); and propanediol (B), with two different bath temperatures (5 °C for 1 min; and 10 °C for 25s).

balance exists between cryoprotectants efficiency and toxicity, that is variable between species and cryoprotectant [15]. From the different tested cryoprotectants, DMSO and propanediol, affected the motility parameters of fresh sperm, whereas methanol had no

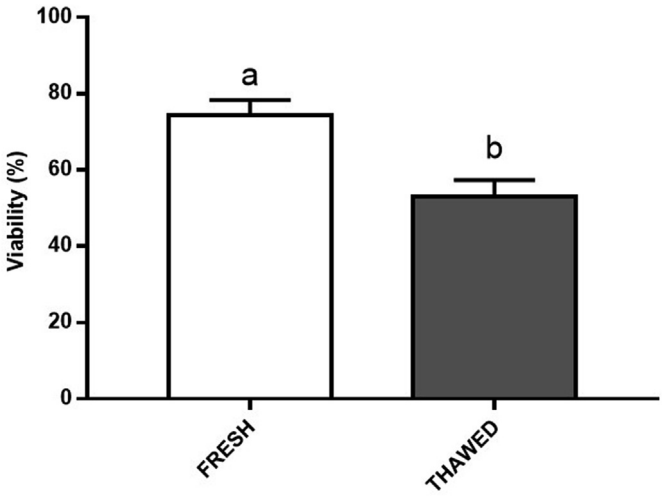


Fig. 6. Sperm viability comparison between fresh and cryopreserved sperm. Different letters refer to significant differences (n = 6; mean ± SEM; Student's t-test; $p < 0.05$).

effect at the tested concentrations. In most species, sperm motility is affected by the incubation time and concentration of the cryoprotectants [16–18]. The percentage of motile sperm and their velocity decreased in just 2 min of exposition at 4 °C to 20% DMSO and 20% propanediol. In contrast, sperm incubated in 20% methanol or DMSO and propanediol up to 10%, did not present any significant decrease. The decrease in the motility parameters after just 2 min, clearly showed that DMSO and propanediol were toxic to spotted wolffish sperm when used at these higher concentrations. This contrasts to the observations made by Gunnarsson et al. [8] that stated that for DMSO there were no significant differences for either concentration (10, 20 or 30%). This fact can be due to the different extender media used by those authors (Cryo-Fish, IMV-Technologies, France) and to the subjective sperm motility evaluation.

In a second step, we optimized the freezing rate that minimizes the cellular damage of the cryopreservation procedures. During the cryopreservation process if the freezing is too slow, then too much water leaves the cell and dehydration causes cell death; if the freezing rate is too fast, not enough water leaves the cell and large intracellular ice crystals form, causing the cell to rupture [19]. The best freezing rates are highly species-specific, and ranges from −8 to −99 °C/min are reported for different species [20]. In our study, the sperm motility parameters were affected by the freezing rate and the freezing rates were affected by the different cryoprotectants. After the toxicity assessment, spotted wolffish sperm was freeze using the three cryoprotectants at the highest concentration that did not show any toxic effects in the first trial (10% DMSO, 10% propanediol, and 20% methanol), at different distances from liquid nitrogen surface (1.5, 2.5, 4.5, 7.5 cm) to create different freezing rates. Despite the decrease in the percentage of motile cells, DMSO offered the best protection to the cells at any of the different freezing rates (−32.8, −29.9, −14.05, or −5.9 °C/min). DMSO is one of the most commonly used cryoprotectants in marine fish sperm cryopreservation, some examples are gilthead seabream *Sparus aurata* [16], summer flounder *Paralichthys dentatus* [18] or Atlantic halibut [12]. Propanediol was most effective only when the straws were placed at 4.5 cm from the liquid nitrogen corresponding to a freezing rate of −18.68 °C/min. Propanediol has also been effectively used in several marine species such as Atlantic cod *Gadus morhua* [21] or summer flounder [18]. Methanol, which is used to freeze sperm of different species such as Atlantic halibut [12] or European eel *Anguilla Anguilla* [22], seems that for spotted wolffish, on the tested conditions, was unable to keep the sperm cell characteristics after the freeze-thawing process. In most species the freezing rate in the cryopreservation protocols will affect the thawed sperm quality [16,21]. However, in our study, with the exception made for propanediol, the tested freezing rate did not affect the quality of the sperm after the freeze-thawing. By opposition, for ocean pout, a close related species, it was observed that the best post-thaw motility parameters were obtained with a freezing rate around -9 °C/min [9]. Our results are in accordance with the idea supported by Gunnarsson et al. [8] that the spotted wolffish sperm is not so sensitive to the freezing rate. Indeed, earlier studies observed the presence of antifreeze proteins in the sperm of spotted wolffish, but consider that the amounts of these proteins were too low to convey any advantage in this species [7]. However, in a small trial in our lab we managed to obtain some sperm motility (<5%) after cryopreserving fresh undiluted sperm without the addition of any extender or cryoprotectant (data not shown).

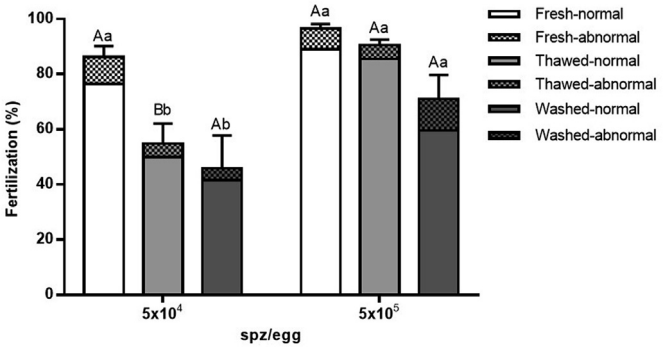


Fig. 7. Percentage of fertilized eggs with normal cleavage (open bars) and abnormal cleavage (dash bars) using fresh sperm (white bars), thawed sperm (grey bars), and washed sperm (dark grey bars). Fertilizations were performed using $5x10^4$ spermatozoa:egg and $5x10^5$ spermatozoa:egg. Different letters refer to significant differences. Uppercase letters refer to comparison within treatments and lowercase letters refer to comparison within concentrations (n = 5; mean ± SEM; 2-way ANOVA, $p < 0.05$).

As a third step, we optimized the thawing rate that minimize cellular damage of the cryopreservation procedures. The rate at which sperm is thawed is critical for the same reason as the freezing, water migration into the cell must be fast enough to avoid crystal formation but slow enough to allow the adaptation of cell membrane, reducing cell rupture [19]. In our experiment, sperm was thawed using two different water bath temperatures to obtain two thawing rates. At the highest temperature, 10 °C, it took 25 s until all the sperm solution inside the straw was completely liquefied, while at 5 °C it took 1 min. The tested thawing temperatures did not affect the quality of the sperm after freeze-thawing. No significant differences were found between both thawing rates, within cryoprotectants. However, the sperm quality was affected by the cryoprotectant. Using DMSO as cryoprotectant yielded significantly higher motility values in comparison to those of propanediol, for both thawing rates. In their work, Gunnarsson et al. [8] thawed the sperm at 5 °C because it is a favourable temperature for storage of spotted wolffish sperm. Indeed, in comparison with 10 °C is closer to the biological temperature of this species. However, sperm can be safely thawed at the highest temperature, that is usually recommended to avoid recrystallisation inside the cells [20].

Using the best cryopreservation conditions (10% DMSO, freezing at −14.05 °C/min and thawing at 177.9 °C/min), the percentage of cells with an intact sperm membrane (sperm viability) showed a decrease of approximately 20% in comparison to fresh sperm. This decrease is probably related with the cryoinjury that leads to loss of

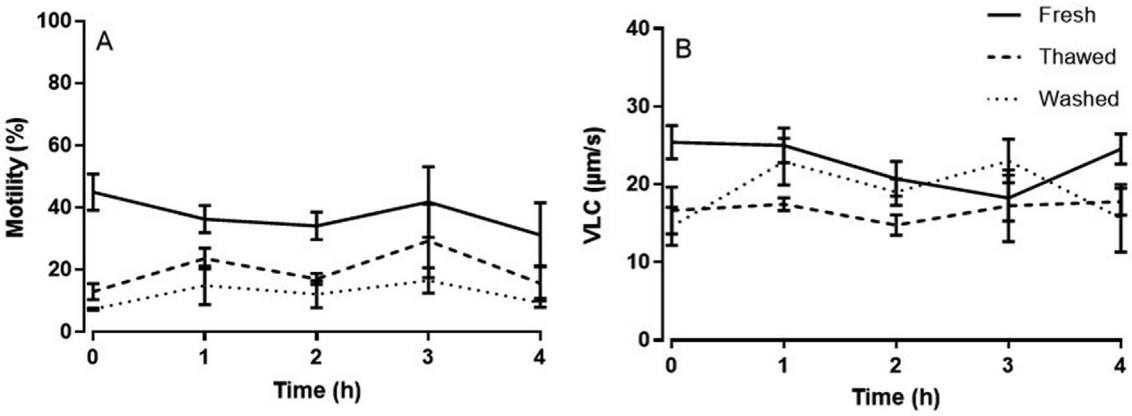


Fig. 8. Percentage of motile cells (A) and sperm velocity (B) along the fertilization trial (4 h) using fresh sperm (solid line), thawed sperm (dashed line) and washed sperm (dotted line) (n = 5; mean ± SEM; ANCOVA, $p < 0.05$).

membrane integrity in some cells [23]. Sperm viability may vary according to different factors such as species resistance to cryopreservation or the efficiency of the protocol, and in some species has been correlated with sperm motility [17,24] and fertilization success [17]. Theoretically, cells with a damage membrane will be unable to develop its functions such as fuse with the oocyte and complete the fertilization process [25].

Finally, in the fifth step we tested and developed an *in vitro* fertilization protocol for spotted wolffish cryopreserved sperm. A spotted wolffish *in vitro* fertilization protocol is presented by Le François and Archer [4] who recommended to use an excess sperm volume to make sure all the eggs were fertilized. However, sperm is usually a limiting resource in spotted wolffish fertilizations, and several males need to be stripped to fertilize a batch of eggs from just one female. Therefore, it is important to find the most efficient way to fertilize the eggs without wasting too much sperm. Beirão and Ottesen [5] adjusted the spermatozoa:egg ratio and the contact time for wolffish *in vitro* fertilization using fresh sperm. The authors recommend a ratio of at least 5×10^5 spermatozoa:egg for a contact time of 2 h, or 5×10^4 if the contact time increases (4 or 6 h). The decrease in sperm quality after freeze-thawing frequently implies the need to increase the spermatozoa:egg ratio [26]. In our study we observed that although cryopreservation affected sperm quality in comparison with fresh sperm, this loss could be overcome by using higher spermatozoa:egg ratio during *in vitro* fertilization. Using a ratio of 5×10^4 spermatozoa:egg with freeze-thawed sperm and a contact time of 4 h, fertilization ratios were significantly lower ($\approx$20%) when compared to the same ratio using fresh sperm. However, a ratio of 5×10^5 spermatozoa:egg with a contact time of 4 h, overcome the loss of sperm quality to obtain the same fertilization values as the ones obtained using fresh sperm. The required contact time (2–6 h) on spotted wolffish dry fertilization is long compared with other species such as turbot (20 min), or seabass (30 s) [26]. The cryoprotectants are not only toxic to sperm cells but also to other cells such as eggs. Considering this, it is important to have in mind that the cryoprotectant may affect fertilization due to the effect on female gametes [27]. To diminish the possible toxic effect on the eggs, "sperm washing" by centrifuging the sperm suspension allowed us to remove the extender containing DMSO and replace it with new extender, without cryoprotectant. This trial showed lower fertilization ratios when compared with the eggs fertilized with fresh and cryopreserved sperm. This may be due to changes on fertilization mechanisms, cell membrane or swimming parameters associated with the centrifuging protocol and sperm processing. Additionally, the post-thaw sperm quality remained unaltered, during the 4 h required for the fertilization protocol without the need to resort to sperm washing. Finally, we should mention that we evaluated the fertilization rate between 2 and 4 cell stage, and there is the possibility that embryos with slow rate of development might have been considered undeveloped. However, all treatments were evaluated at the same time and thus any developmental delay would have affected all treatments equally.

5. Conclusion

The present study allowed to complement the previous studies on spotted wolffish sperm cryopreservation, in order to deliver a descriptive protocol that can be used by the industry and research groups. According to the present study we recommend sperm to be diluted 1:1 (sperm:extender) in the solution developed by Kime and Tveiten [6] with 10% DMSO and an equilibration time of 2 min. The solution containing the sperm, should be loaded into 0.5 mL straws and then frozen for 10 min at −14.05 °C/min (4.5 cm from the surface of liquid nitrogen), after which, the straws are submerged into it. To thaw the cryopreserved sperm, we recommend 177.9 °C/min (5 °C for 1 min). The frozen/thawed sperm should be used at ratio of 5×10^5 spermatozoa:egg with a contact time of 4 h between gametes. This study also uses a more comprehensive analysis of post-thaw sperm quality showing evidences of the importance of objective tests to discern between cryopreservation conditions.

Author contributions

JB conceived and secured the funding for the study. JB and BE conducted the preliminary trials. JB and EC designed and supervised the experiment. JB, BE and JS collected the samples. JS analysed the samples, analysed the data and drafted the manuscript. JB and EC finalized the manuscript. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors have no conflict of interest to declare.

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