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Optimizing the post-thaw quality of cryopreserved masu salmon (*Oncorhynchus masou*) sperm: Evaluating the effects of antioxidant-supplemented extender[1]

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Abstract

[1] Abbreviations
reactive oxygen species (ROS); glucose-methanol (GM); percentage of motile sperm (MOT); straight line velocity (VSL); curvilinear velocity (VCL); average path velocity (VAP); linearity (LIN); beat cross frequency (BCF); and amplitude of lateral head displacement (ALH); artificial seminal plasma (ASP)

The oxidative damage induced by cryopreservation significantly contributes to the overall decline in the quality of cryopreserved sperm. This underscores the necessity for exploring mitigation measures, such as supplementing the freezing media with antioxidants, aimed at enhancing sperm quality post-thaw. In this study, we determined the effects of adding five antioxidants [Vitamin C, taurine, trolox, Butylhydroxytoluene (BHT), and uric acid] to a glucose–methanol extender at three concentrations—low, intermediate, and high—on the motility, viability, concentration, and fertilization rates of the sperm of masu salmon (Oncorhynchus masou), which is an economically important fish resource in Northeast Asia. Our research reveals various sperm quality parameters of the same species react differently to different antioxidants or even different concentrations of the same antioxidants, which adds another layer of complexity to the selection criteria for an appropriate antioxidant for supplementation beyond the specific specificity of antioxidants. Moreover, the effects of the antioxidants were often nonlinear and paradoxical, highlighting the importance of applying accurate dosage. Compared with the control group (no antioxidant supplementation), only uric acid and taurine supplementation at intermediate concentrations (5 mM) significantly improved the post-thaw sperm viability and percentage of motile sperm. Meanwhile, BHT supplementation generally improved the following kinematic traits of sperm: straight-line velocity (VSL), linearity, average path velocity, and curvilinear velocity (VCL). Similarly, 5-mM taurine supplementation also significantly improved sperm VSL, VCL, and beat cross frequency. In contrast, 10-mM Vitamin C supplementation (high concentration) adversely affected multiple parameters, leading to a decline in fertilization capacity. Based on these findings, the selection criteria for antioxidants should go beyond species specificity and include sperm quality parameters the breeder considers crucial for predicting post-thaw sperm fertility, as well as the ways in which particular antioxidants influence these

parameters. We recommend 5-mM taurine supplementation for masu salmon sperm cryopreservation because of its positive effects on sperm viability, concentration, and some kinematic parameters.

Keywords: Sperm cryopreservation; Sperm quality; Reactive Oxygen Species; Antioxidants; Glucose-Methanol Extender

1. Introduction

Cryopreservation causes sperm quality to decline owing to the combined effects of various types of lethal and sub-lethal cryoinjuries on spermatozoa. The causes, nature, and severity of such cryoinjuries in fish have been extensively documented in a previous study (Ciereszko et al., 2020). However, within the context of this study, cryopreservation has also been associated with the excessive production of reactive oxygen species (ROS), such as hydrogen peroxide (H2O2) and nitric oxide radicals (•N=O), during freezing and thawing (Ball, 2008), which causes cryoinjuries such as protein carbonylation and DNA fragmentation even when a cryoprotectant is used (Sandoval-Vargas et al., 2021). Furthermore, the inclusion of diluents in all sperm cryopreservation protocols inadvertently causes a significant reduction in the levels of endogenous antioxidants in seminal plasma (Martínez-Páramo et al., 2009), which play a crucial role in protecting spermatozoa against oxidative stress damage (Félix et al., 2020), making the spermatozoa susceptible to oxidative damage. Oxidative damage to spermatozoa DNA and structure raises concerns about the efficacy of cryopreserved sperm due to the close correlation between sperm DNA integrity and the subsequent survival and quality of offspring (De Mello et al., 2017; Sandoval-Vargas et al., 2021; Bøe et al., 2021). This highlights both the severity of the problem and the need for mitigating measures.

In response to these concerns, substantial research has focused on supplementing cryopreservation extenders with antioxidant reagents to neutralize or reduce the effects of ROS compounds and ultimately enhance freeze-thaw sperm quality (Lahnsteiner et al., 2011; Cabrita et al., 2011). However, the outcomes of these studies have shown great variability. For instance, several studies have reported that supplementation with antioxidants, including butylhydroxytoluene (BHT), albumin, taurine, trolox, and ascorbic

acid, leads to increased sperm motility and viability (Sandoval-Vargas et al., 2021). However, several studies on the utilization of similar lipid- and water-soluble antioxidants have not demonstrated any significant advantages of antioxidant supplementation, and, in certain instances, the addition of these antioxidants appeared to be detrimental, resulting in the decline of post-thaw sperm quality (Ball, 2008; Cabrita et al., 2011). Therefore, the effectiveness of incorporating antioxidants into extenders to mitigate ROS-induced damage in cryopreserved sperm appears to be species-specific, possibly attributable to anatomical variations among the gametes of different fish species (Cosson, 2019; Bøe et al., 2021). This is supported Cabrita et al.'s (2011) study, where supplementation of extenders with a "commercial antioxidant supplement" had no significant impact on post-thaw cell viability in *Sparus aurata* but significantly compromised the same parameter in *Dicentrarchus labrax*. In the same study, the addition of 10 mM taurine had significant adverse effects on cell viability in *Sparus aurata* but not in *Dicentrarchus labrax*. Furthermore, Sarosiek et al. (2013) reported contrasting responses in the motility of Artic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*) sperm to Vitamin C, glutathione, and cysteine supplementation. Consequently, identifying a suitable antioxidant and establishing its optimal inclusion level requires experimentation (Len et al., 2019).

In masu salmon (*Oncorhynchus masou*), cryopreservation has emerged viable tool for both the conservation and advancement of sustainable aquaculture practices, given the species' considerable commercial and cultural significance in Northeast Asia—its native habitat (Lee et al., 2021). While cryopreservation techniques such as the pellet and straw method have been documented (Yamano et al., 1990; Fujimoto et al., 2022), the potential for enhancing post-thaw sperm quality through the supplementation of freezing media with antioxidant compounds remains unexplored. Therefore, this study aimed to determine the optimal

inclusion levels of potential antioxidants within extenders used for cryopreserving masu salmon semen, with the objective of bolstering its tolerance to cryopreservation and enhancing post-thaw sperm quality.

2. Materials and methods

2.1. Sperm collection

Sperm was collected from six sexually mature O. masou male individuals from the stock kept at the Nanae Freshwater Laboratory, a division of the Field Science Center for Northern Biosphere, Hokkaido University (Nanae town, Kameda-gun, Hokkaido, Japan), between September and October 2022. Milt was collected into 25-mL centrifuge tubes by gently massaging the abdomen of the male fish. The collected samples were promptly placed on ice and remained chilled for approximately one hour during transportation from the field station to the laboratory, where subsequent experimentation was conducted. The procedures involved in the handling of animal subjects and collecting gametes for the experiment adhered to the ethical standards outlined in the Education and Training for Animal Experimentation for Researchers at Hokkaido University protocol for the humane treatment of the experimental subjects.

2.2. Preparation of extenders

In this study, the glucose–methanol (GM) extender (0.18 M glucose and 9% methanol) that Fujimoto et al. (2022) used to successfully freeze the semen of *O. masou* was adopted. Five antioxidants—L(+)-Ascorbic acid (Vitamin C), uric acid, and taurine (all three obtained from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), Trolox (a hydrophilic analog of Vitamin E) (Sigma-Aldrich, St. Louis, Missouri, US), and BHT (Tokyo Chemical Industry, Tokyo, Japan)—were incorporated into the GM extender at three different concentrations (Table 1) representing low, intermediate, and high inclusion levels modelling after previous

works by Cabrita et al., (2011), Figueroa et al., (2018), and Merino et al., (2020). The control treatment consisted of the GM extender without any antioxidant supplementation.

2.3. Cryopreservation and thawing

Cryopreservation was conducted following the methodology outlined by Ciereszko et al., (2014) and Fujimoto et al., (2022). In summary, samples from each male were individually diluted with the various extender-antioxidant combinations (as detailed in Table 1) at a ratio of 1:5, achieved by diluting 200 µl of fresh sperm with 1 ml of the extender. Subsequently, the diluted samples were loaded into 0.25 ml clear mini plastic straws (IMV Technologies, L'Aigle, France) and sealed with sealing powder (Fujihira Industry Co., Ltd, Tokyo, Japan). These straws were then positioned on a floating rack set at a height of 3 cm and equilibrated on ice for 15 minutes. Following equilibration, the straws with the samples were cooled by floating the rack on liquid nitrogen. The straws were then packed into cryo-safe containers and stored in liquid nitrogen tanks. After a 7-day storage duration, the samples were retrieved and thawed in a water bath at 40°C for 5 seconds to facilitate post-thaw analysis.

2.4. Data collection

2.4.1. Sperm motility

Sperm motility analysis was performed on freshly collected samples (referred to as fresh sperm) to evaluate the initial sperm quality, directly following the dilution of these samples with an extender (referred to as freshly diluted sperm), and after freezing and thawing (referred to as freeze-thawed sperm). Only males with a minimum initial sperm motility of 80% were considered eligible for cryopreservation. A computer-assisted sperm analysis (CASA) system, specifically the Sperm Motility Analysis System for animal sperm (DITECT, Tokyo, Japan), was used to determine the motility of the sperm samples. To assess sperm

motility, the sperm was diluted 1,000 times (1 microliter (μL) sperm + 999 μL activation buffer) with salmon sperm activating D532 buffer, which was formulated from 125 mM sodium chloride (NaCl), 20 mM tris(hydroxymethyl)aminomethane, 30 mM glycine, and 1 mM calcium chloride dehydrate ($CaCl_2 \cdot 2H_2O$), and enriched with 1% bovine serum albumin (BSA) to facilitate sperm adhesion to glass slides for microscopic observation. Six μL of the diluted sperm were pipetted onto a 76 × 26 mm glass microslide, which was then covered by an 18 × 18 mm micro cover glass (Matsunami Glass Industries Ltd, Osaka, Japan). Motility was measured 15 seconds after activation using the CASA system. Sperm motility was assessed using the following parameters: percentage of motile sperm (MOT), straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN), beat cross frequency (BCF), and amplitude of lateral head displacement (ALH).

2.4.2. Sperm viability and concentration

Assessments of both sperm viability and concentration were made on fresh sperm, freshly diluted sperm, and freeze-thaw sperm. The sperm samples were subjected to a three-step sequential dilution process by a factor of 100,000 using artificial seminal plasma (ASP) for salmonids and a sperm count and viability kit. The ASP consisted of 110 mM NaCl, 30 mM KCl, 1.6 mM $CaCl_2 \cdot 2H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 10 mM $NaHCO_3$, and 20 mM TAPS (Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid) at pH 8.0 (Ohta et al., 2000). The dilution steps were performed as follows: (1) 10 μL of fresh milt was mixed with 990 μL of ASP (for freeze-thawed sperm, this was 60 μL of freeze-thawed milt with 940 μL of ASP); (2) 10 μL of the diluent prepared in Step 1 was mixed with 490 μL of ASP; lastly, (3) 10 μL of the diluent prepared in Step 2 was combined with 190 μL of the Muse® Count & Viability Kit. A benchtop mixer (Se-04; Taitec, Saitama, Japan) was used to ensure thorough dilution at each step. After allowing the samples to stand for 15 min, the viability of the cells (%) and

189 sperm concentration (number of viable cells/mL) in the diluted milt samples were assessed
190 using a MUSE® Cell Analyzer version 1.5.1 and a Muse® Count & Viability Kit (Millipore,
191 California, USA).

192 **2.4.3. Fertilization rates of eggs**

193 Fertilization of fresh eggs using fresh, freshly diluted, and freeze-thawed sperm was
194 conducted under standard fertilization conditions of 10 eggs per 1 mL of the D532 sperm
195 activation buffer described in 2.4.2., with an insemination dose ratio of 400,000:1
196 (sperm:egg). The mean sperm concentration determined during the assessment of sperm
197 count and viability was used to calculate the milt volume required to obtain the desired
198 insemination dose. For each treatment, fertilization was performed by placing 100 eggs in a
199 Styrofoam cup containing 10 mL of sperm activation buffer. The required milt volume was
200 pipetted into the Styrofoam cup, which was immediately placed on a reciprocating shaker
201 (NR-10; Taitec, Saitama, Japan) set at 120 rpm for 30 seconds to thoroughly distribute the
202 sperm among the eggs to fertilization. Following fertilization, the fertilized eggs were
203 incubated in labeled metal mesh trays within a recirculating system at 10°C. Thirty eggs
204 were sampled from the incubation trays 16 hours post fertilization, fixed in Bouin's fixative
205 for 24 hours, and then stored in 70% ethanol for long-term preservation. Successful
206 fertilization (or lack thereof) was determined by examining of eggs under a microscope to
207 identify the presence of cleavage furrows (Fujimoto et al., 2022).

208 The fertilization rate was calculated as:

209 $$Fertilization\ rate\ (\%) = \frac{Number\ of\ eggs\ with\ cleavage\ furrows}{Total\ number\ of\ eggs\ sampled} \times 100$$

210 **2.5. Data analysis**

The data collected as percentages were transformed using the ArcSine-square root function to facilitate parametric testing (Bhujel, 2009). Wilk–Shapiro and Fligner–Killeen tests were used to determine the normality and homoscedasticity of the data, respectively. Two-way analysis of variance (ANOVA) was performed to determine any statistically significant effects of the different antioxidants and antioxidant concentrations on sperm motility, count, viability, and fertility. Where ANOVA showed a significant interaction effect, a subsequent post-hoc analysis focused on the interaction term and analysis was performed using the R packages Post-hoc Interaction Analysis (De Rosario-Martinez et al., 2015) and Companion for Applied Regression (Fox and Weisenberg, 2019) to generate interaction means. The interaction means were then separated and ranked using Tukey's Honestly Significant Different (HSD) test. Where no significant interaction could be established, a simple main effect analysis was performed using Tukey's HSD test. All analyses were performed using the R statistical software (version 4.2.1 (R Core Team, 2022) at a 95% confidence interval.

3. Results

Across all parameters examined, the means of freshly diluted sperm showed no significant variation among the various concentrations of the same antioxidant. To enhance clarity in the graphical presentation of the results, only the mean of freshly diluted sperm across all three concentrations is presented for each parameter. All results are presented as the mean ± standard deviation (SD).

3.1. Sperm motility

233 The mean MOT of the masu salmon sperm samples at different combinations and
234 concentrations of extenders is shown in Figure 1. The mean MOT of the freshly diluted
235 sperm (84.62 ± 26.93%) was significantly lower ($p < 0.05$) than that of the fresh sperm (92.57
236 ± 1.14%). Supplementing the extender with antioxidants at different concentrations had no
237 effect on the MOT of the freshly diluted sperm. The mean MOT of the cryopreserved sperm
238 was significantly lower than those of the fresh and freshly diluted sperm ($p < 0.05$).
239 Antioxidant supplementation of the extender and its inclusion levels had a significant ($p <$
240 0.05) two-way interaction with the MOT of the cryopreserved sperm. The post-hoc analysis
241 of the interaction means revealed that supplementation with a high concentration (10 mM)
242 (22.11 ± 10.46%) and the intermediate concentration (5 mM) (23.14 ± 7.1%) of Vitamin C
243 led to the lowest mean MOT. Conversely, supplementation with the intermediate
244 concentration (5 mM) of uric acid yielded the highest mean MOT (45.48 ± 3.32%), which
245 was statistically comparable ($p > 0.05$) to the mean MOT resulting from supplementation
246 with the intermediate concentration (5 mM) of taurine (40.94 ± 6.33%). These were the only
247 treatments wherein antioxidant supplementation yielded a statistically significant
248 improvement in post-thaw MOT compared to the unsupplemented sperm (control group).
249 Alternative combinations of antioxidants and concentrations did not yield statistically
250 significant improvement ($p > 0.05$), or yielded MOTs significantly lower than that of the
251 control group ($p < 0.05$).

252 The VSL of the freshly diluted sperm was significantly affected by antioxidant
253 supplementation; the mean VSLs of the freshly diluted sperm supplemented with Vitamin C
254 (43.06 ± 3.01 μm s^{-1}), Trolox (37.95 ± 3.3 μm s^{-1}), BHT (36.6 ± 3.9 μm s^{-1}), and uric acid
255 (39.78±4.9 μm s^{-1}) were significantly higher than those of the unsupplemented (34.57 ± 5.8
256 μm s^{-1}), taurine-treated (34.0 ± 3.6 μm s^{-1}) extenders (Figure 2). Antioxidant supplementation

257 had a significant interactive effect with the inclusion level of the antioxidants on the VSL of
258 cryopreserved sperm. The highest mean VSL of the freeze-thawed sperm was produced by
259 supplementing the freezing media with 1 mM BHT (58.58 ± 2.49 μm s^{-1}) and 10 mM uric
260 acid (53.60 ± 9.87 μm s^{-1}), while the lowest mean VSL was produced by 10-mM Vitamin C
261 supplementation (18.25 ± 2.43 μm s^{-1}). The trend in the VSL response of the cryopreserved
262 sperm samples to antioxidant supplementation was nonlinear, particularly in the case of
263 Trolox- and uric acid–supplemented extenders, where the intermediate (5 mM)
264 concentration resulted in a significantly lower VSL than those elicited by the low (1 mM) and
265 high concentrations (10 mM).

266 Fresh sperm had a mean LIN of 0.45 ± 0.14. The supplementation of the extender with
267 antioxidants at varying concentrations had no statistically significant effect on the LIN of the
268 freshly diluted sperm. Post-thaw, only the sperm supplemented with 10 mM taurine and 1
269 mM BHT exhibited mean LINs significantly higher than those of the unsupplemented sperm
270 and the sperm supplemented with other combinations of antioxidants and concentrations
271 tested (Figure 3).

272 The supplementation of the extender media with antioxidants significantly affected the VAP
273 and VCL of the freeze-thawed sperm. However, for both parameters, there was no
274 statistically significant effect of antioxidant concentration and significant interaction between
275 the two independent variables ($p > 0.05$). Post-hoc analysis revealed that the VAPs of the
276 freshly diluted sperm supplemented with BHT and taurine were significantly lower than that
277 of the fresh sperm; nonetheless, there were significantly higher than those with no
278 antioxidant supplementation and the sperm supplemented with other antioxidants (Figure 4).
279 The mean VAP of the unsupplemented cryopreserved sperm was statistically similar to that

of the BHT-supplemented cryopreserved sperm. The freeze-thawed sperm supplemented with Vitamin C, Trolox, and uric had statistically similar mean VAPs at all concentrations, which were significantly lower than those of the unsupplemented sperm and BHT-supplemented sperm.

Figure 5 shows the VCL of the fresh, freshly diluted, and cryopreserved sperm. The freshly diluted sperm supplemented with uric acid had the highest mean VCL. Although the VCLs of the freshly diluted sperm supplemented with taurine, Trolox, BHT, uric acid, and Vitamin C did not significantly differ, those were significantly higher than the VCL of the control treatment ($p < 0.05$). After thawing, the concentration of all the antioxidants did not have a significant effect on the VCL of the freeze-thawed sperm. Moreover, the mean VCLs of the freeze-thawed sperm supplemented with all concentrations of taurine and BHT and a low concentration (1 mM) of uric acid were higher than that of the unsupplemented group.

The BCF of the freshly diluted sperm was significantly lower than that of the fresh sperm in both supplemented and unsupplemented sperm. Although the BCF of the freshly diluted sperm supplemented with uric acid was statistically similar to that of the unsupplemented samples, it was significantly lower than the BCFs of the freshly diluted sperm treated with Vitamin C, taurine, Trolox, and BHT, among which there were no significant differences. After thawing, the highest mean BCF was recorded in sperm supplemented with 5 mM uric acid and 1 mM Trolox, while the lowest mean BCF was recorded in the sperm supplemented with 10 mM Vitamin C (Figure 6). Lastly, the supplementation with all concentrations of the five antioxidants did not have any significant effect on the ALH of the freeze-thawed sperm (Figure 7).

3.2. Sperm concentration and viability

There were significant two-way interactions between antioxidant supplementation and concentration on sperm concentration and viability after thawing. Only the cryopreserved sperm supplemented with taurine (all concentrations) and 5 mM BHT yielded significantly higher concentrations than the control treatment with no antioxidant supplementation (Figure 8). In contrast, only cryopreserved sperm supplemented with 5 mM taurine and 5 mM uric acid had significantly higher viability ($p < 0.05$) than the control (Figure 9).

3.3. Fertilization rates

Figure 10 shows the fertilization rates of the fresh, freshly diluted, and cryopreserved sperm. The two independent factors of the fertilization rates of the freeze-thawed sperm exhibited a significant two-way interaction. The fertilization rates were comparable for all combinations and concentrations BHT, taurine, Trolox, and uric acid. However, the addition of 10 mM Vitamin C to the extender medium significantly reduced the fertilization rate of the cryopreserved sperm.

4. Discussion

In this study, possible methods of increasing the resilience of sperm to oxidative stress and improving post-thaw sperm quality were explored because oxidative damage during cryopreservation is the main factor that reduces the fertilization capacity of freeze-thawed sperm (Cabrita et al., 2011; Fang et al., 2014). Previous research has shown that supplementing freezing media with antioxidant compounds can help protect sperm cells from harmful reactive oxygen species (ROS) and improve their quality post-thaw (Cabrita et al., 2011; de Almeida-Monteiro et al., 2017). It has been reported that the initial sperm quality is the primary factor influencing the effectiveness of antioxidant supplementation for improving the post-thaw quality of sperm. Aside from this overarching factor, there have

been inconsistent findings on the suitability of various antioxidants for the sperm cryopreservation of different fish species; the antioxidants that have been demonstrated to reduce oxidative damage in some species have been found ineffective in other species (Len et al., 2019).

The findings of this study demonstrate that different antioxidants have diverse effects on sperm, and an antioxidant that optimizes one parameter can perform poorly in another due to the mode of action of the antioxidant as well as its inclusion level. For instance, owing to its amine group, taurine has been shown to exhibit a strong affinity for nucleic acids, particularly those forming DNA bases, thereby reducing DNA damage during cryopreservation (Cabrita et al., 2011). Jong et al. (2012) established that taurine regulates protein synthesis and protects mitochondria from excessive superoxide production. This is of particular significance for retaining sperm motility, as defects in sperm mitochondrial function can hinder energy production, which is necessary to initiate and maintain sperm motility (Durairajanayagam et al., 2021). Similarly, uric acid has been shown to extend the lifespan of cells and enhance their responses to oxidative stress (Tasaki et al., 2017) because it effectively protects cells against hydroxyl radicals (•OH), which cause cellular damage, including DNA damage and membrane peroxidation (Ames et al., 1981). Taken together, these observations explain the positive effects of taurine and uric acid supplementation on sperm motility, viability, and concentration after thawing (Figures 1, 8, and 9).

BHT supplementation positively affected various motility parameters of the cryopreserved sperm; 1-mM BHT supplementation elicited the highest VSL, VAP, and VCL and the third-highest LIN. These observations can be attributed to the mode of action of BHT, which is

crucial for explaining its protective effects on sperm cells during cryopreservation. Two mechanisms have been proposed to explain the beneficial effects of BHT on sperm cells: Hammerstedt et al. (1976) suggested that BHT is embedded in the sperm membrane, enhancing membrane flexibility and fluidity, and shields the sperm from cold shock during cryopreservation and improves post-thaw sperm motility; meanwhile, Aitken and Clarkson (1988) suggested that BHT actively scavenges lipid peroxyl radicals and converts them into less harmful hydroperoxides to reduce the damaging effects of lipid peroxides on the sperm cells. The demonstrated increase in the velocity of the sperm that retained motility after thawing can be attributed to the combination of increased sperm flexibility due to the membrane-embedding action of BHT and the reduction of the harmful effects of lipid peroxides, as noted by Horokhovatskyi et al. (2016). These observations indicate that BHT supplementation positively influences sperm motility by preserving the structural integrity of sperm and reducing the oxidative damage caused by lipid peroxides. When comparing the effects of taurine, uric acid, and BHT on sperm viability and motility, understanding the specific mechanisms of action of antioxidants in preserving sperm quality during cryopreservation is vital for optimizing their use.

The preceding discussion does not account for the nonlinear and paradoxical patterns observed in sperm responses to different antioxidant doses for the same parameter. For instance, the optimal MOT of the taurine- and uric acid– supplemented sperm was observed at intermediate concentrations for both antioxidants. This implies a paradoxical response to higher antioxidant dosages. BHT also exhibited paradoxical effects on the VSL, LIN, and BCF of the freeze-thawed sperm because such specimens exhibited higher mean values for these parameters when supplemented with BHT at a low concentration than at higher concentrations. This result is consistent with that in common carp (*Cyprinus carpio*)

373 (Ögretmen and Inanan, 2014), where a negative correlation was established between BHT
374 concentration and sperm motility; the motility parameters significantly decreased beyond 2
375 mM concentration. The observed nonlinear and paradoxical responses can be attributed to
376 the complex nature of antioxidants and their interactions with ROS at different
377 concentrations. Sautin and Johnson (2008) reported that at high concentrations, uric acid
378 can react with radicals to generate oxidants, which may lead to unexpected outcomes.
379 Additional evidence from Bagnati et al. (1999) suggests that oxidized lipids can convert uric
380 acid into an oxidant, further contributing to the paradoxical effects. In the case of the MOT
381 of the freeze-thawed sperm, low concentrations of taurine and uric acid (1 mM) were
382 insufficient to effectively defend sperm cells against ROS-induced damage; however, high
383 concentrations (10 mM) of these antioxidants triggered pro-oxidant reactions. This
384 observation explains why taurine and uric acid performed better at an intermediate
385 concentration (5 mM) than at low or high concentrations. Further research is required to
386 understand the mechanisms underlying the paradoxical role that BHT plays at high
387 concentrations. Overall, the nonlinear and paradoxical patterns in response to different
388 antioxidant doses presented here and in previous studies underline the importance of using
389 accurate dosage of antioxidants to ensure their protective effects against oxidative damage
390 while avoiding the potential harmful effects of excessive antioxidant levels.

391 Supplementing the extender media with 5 mM and 10 mM Vitamin C resulted in the lowest
392 mean MOT; similarly, a high dosage of Vitamin C also resulted in the lowest mean VSL, LIN,
393 and BCF. It has been reported that insufficient levels of Vitamin C in sperm plasma can lead
394 to increased free-radical damage to cellular DNA (Halliwell, 2000). However, higher-than-
395 optimal doses of Vitamin C have also been found to cause a sharp increase in 8-oxoadenine
396 levels, which indicate ROS-mediated DNA damage (Podmore et al., 1998). Therefore, the

results of our study suggest that 5 mM and 10 mM Vitamin C are too high and harmful to *O. masou* sperm, which explains the poor motility recorded under these treatments. However, it is important to emphasize that the effects of Vitamin C supplementation can be species-specific, as studies involving similar dosages in different species have reported contradictory results. Observations of the effects of Vitamin C– supplemented extender on the post-storage sperm quality of Arctic char (*Salvelinus alpinus*) and rainbow trout (*O. mykiss*) have failed to establish any significant positive or harmful effects on sperm motility (Sarosiek et al., 2013).

Given their positive correlation with post-thaw motility and fertilization outcomes, post-thaw sperm concentration and viability are key parameters for evaluating the success of cryopreservation (Ciereszko et al., 2020). In the context of the reported experiments, wherein variables such as dilution ratio, straw size, and thawing protocols remained constant (Vivieros and Leal, 2016; Nynca et al., 2017), the resilience of sperm to the cumulative effects of ROS accumulation on mitochondrial membrane depolarization and reduced ATP production determines the viability and concentration of freeze-thawed sperm. As previously established, the antioxidant added to the extender influences sperm response. In this study, the protective effects of taurine, uric acid, and BHT on mitochondria, which were synergistic with their roles in ROS scavenging (Ames et al., 1981; Jong et al., 2012; Tasaki et al., 2017), possibly explain the observed enhancement in the sperm supplemented with these antioxidants; notably, the concentration of these antioxidants also played a crucial role in eliciting such improvements.

Figure 10 shows that, except for the cryopreserved sperm supplemented with 10 mM Vitamin C, the fertilization rates of the eggs fertilized with cryopreserved sperm were

comparable to those of the eggs fertilized by fresh and freshly diluted sperm. Although the high insemination dose (400,000:1) used in this study was intended to mitigate differences among the sperm specimens in different treatments, it might have limited the ability to elucidate differences in the fertilization capacity of the freeze-thawed sperm in different treatments. We observed that in their studies, Ciereszko et al., (2014) could not establish significant differences in the fertilization rates in cryopreserved sperm at an insemination doses beyond 300,000:1 with only significant differences documented at 100,000:1. It is possible that a similar ratio lower could have provided more insight into the differences in fertility among the treatments.

However, it is noteworthy that even at this high insemination dose, the damage to the sperm supplemented with 10 mM Vitamin C, as demonstrated by the decreased MOT, VSL, LIN, sperm viability, and sperm concentration, adversely affected sperm fertility. This ultimately explains why Vitamin C supplementation elicited a significantly lower fertilization rate than that elicited by the other treatments.

There were some limitations on the study's scope and design. Masu salmon sperm shows significant variation in motility, fertility, and the physio-chemical properties of seminal plasma across the breeding season (Fujimoto et al., 2022). It was not taken into consideration how these changes throughout the breeding season impact how sperm react to the different antioxidants added to the extender. Furthermore, this study focused on the motility and fertility response of sperm rather than analyzing the effects of the different antioxidants on specific cryoinjuries.

5. Conclusion

The findings of this study reveal a complex and nuanced interaction between antioxidant supplementation and the post-thaw quality of masu salmon sperm. It is evident that there is no one-size-fits-all solution, as no specific antioxidant or inclusion level emerged as universally optimal for enhancing all post-thaw quality indicators. Rather, the selection of antioxidants should be guided by parameters considered crucial for preserving and predicting post-thaw sperm fertility, along with an understanding of how specific antioxidants influence these parameters. Our recommendation is to incorporate 5 mM taurine into sperm cryopreservation protocols for masu salmon as has been shown to significantly enhance sperm viability while also improving certain kinematic motility traits. Furthermore, exploring the effects of incorporating a combination of antioxidants could be an area for further investigations. For instance, the results from this study suggest that a combination of 5 mM taurine and 1 mM BHT could be promising. This combination might not only optimize viability but also enhance kinematic traits that are not significantly improved by the taurine supplementation alone. Experimental validation is necessary as the effects of mixing antioxidants cannot assumed to be merely additive with both antioxidant antagonism and synergism possible outcomes of combining antioxidants.

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Table heading

611 Table 1. Antioxidant levels in the glucose–methanol extender

Antioxidant reagent	**Inclusion level (concentration)**
Vitamin C (Ascorbic acid)	1 mM, 5 mM, 10 mM
Taurine	1 mM, 5 mM, 10 mM
Trolox	0.1 mM, 0.5 mM, 1 mM
Butyl-hydroxytoleune (BHT)	1mM, 5 mM, 10 mM
Uric acid	1 mM, 5 mM, 10 mM

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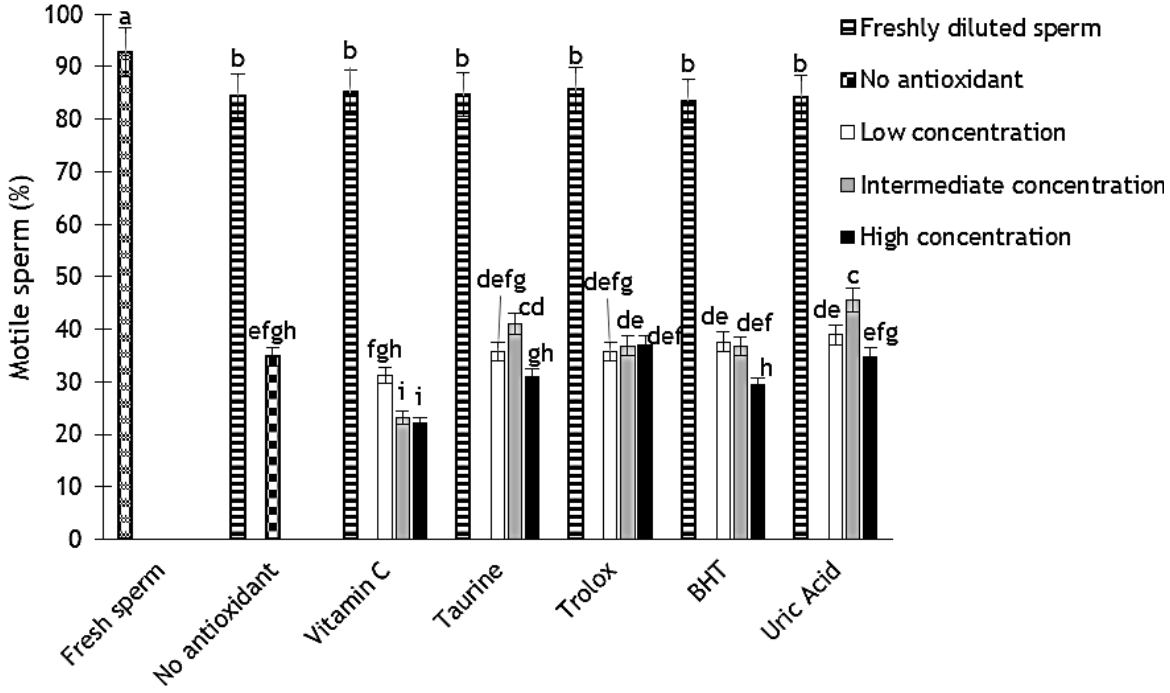


Figure 1: Mean percentage of motile sperm (MOT) of sperm in in fresh, fresh-diluted, and freeze-thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents the control with semen frozen in an extender without antioxidant supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).

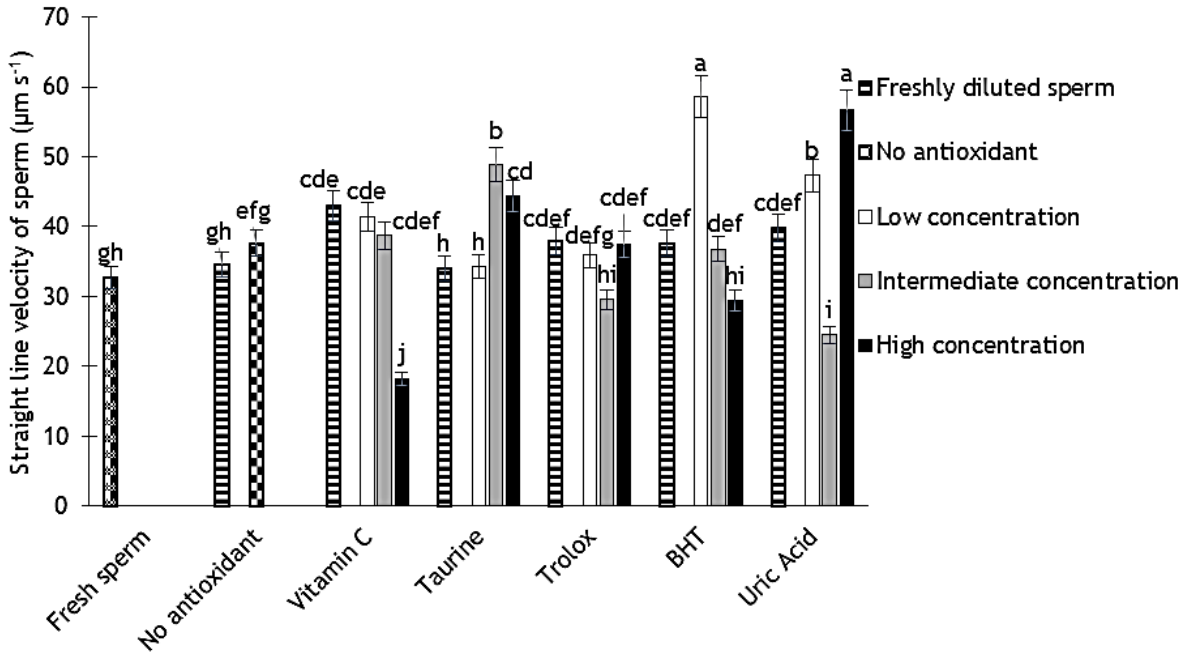
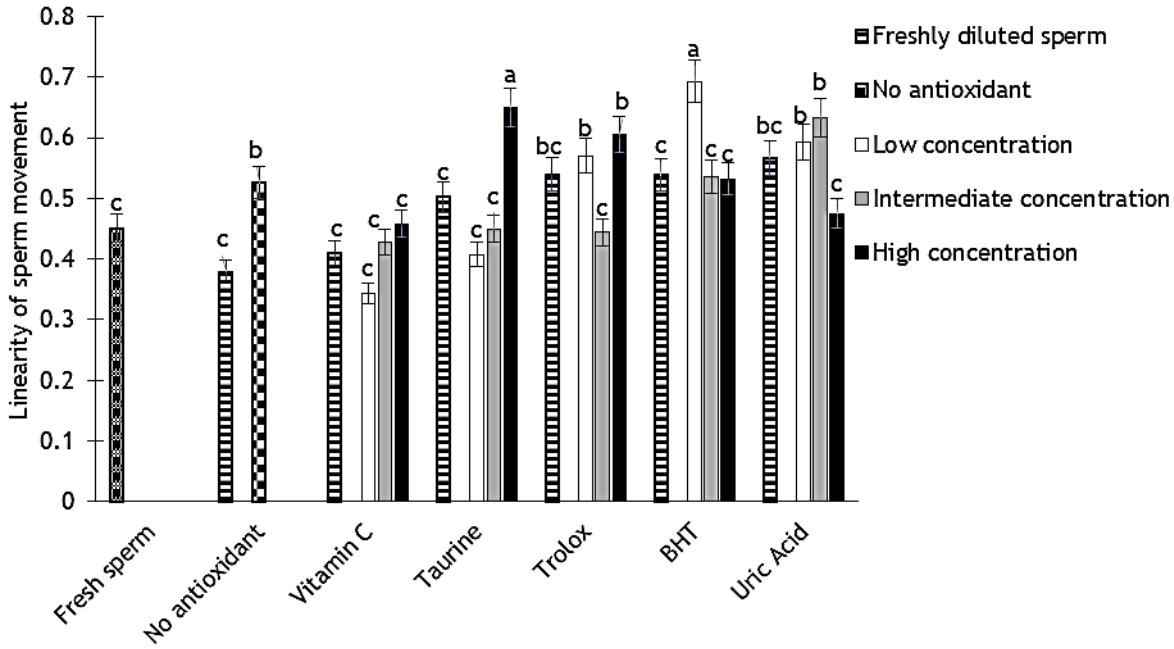


Figure 2: Mean Straight Line Velocity (VSL) of sperm in fresh, fresh-diluted, and freeze-thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents the control with semen frozen in an extender without antioxidant supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).



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661 Figure 3: Mean linearity (LIN) of the movement of sperm in fresh, fresh-diluted, and freeze-
662 thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with
663 various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine,
664 BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high
665 concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM,
666 intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant"
667 group represents the control with semen frozen in an extender without antioxidant
668 supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).

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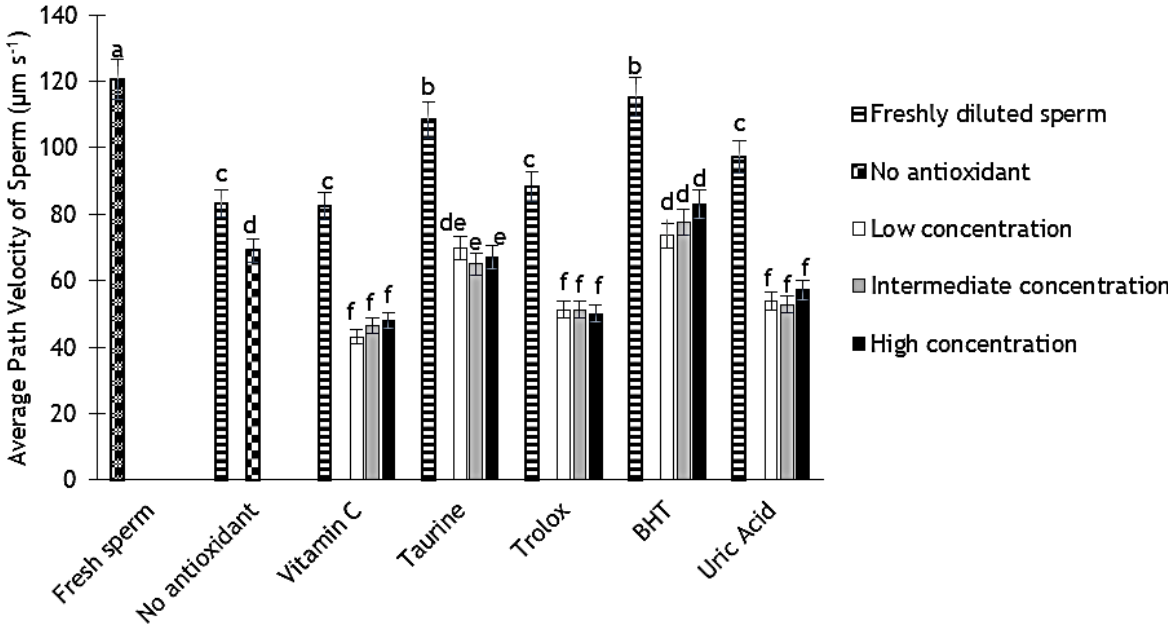


Figure 4: Mean Average Path Velocity (VAP) of sperm in fresh, fresh-diluted, and freeze-thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents the control with semen frozen in an extender without antioxidant supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).

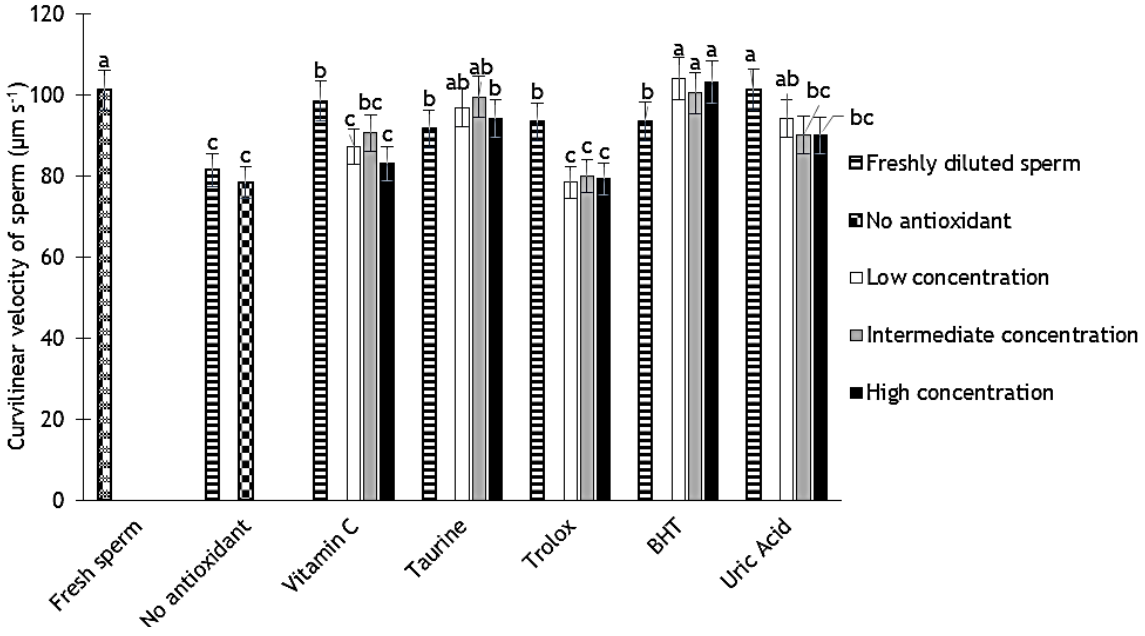


Figure 5: Mean Curvilinear Velocity (VCL) of sperm in fresh, fresh-diluted, and freeze-thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents the control with semen frozen in an extender without antioxidant supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).

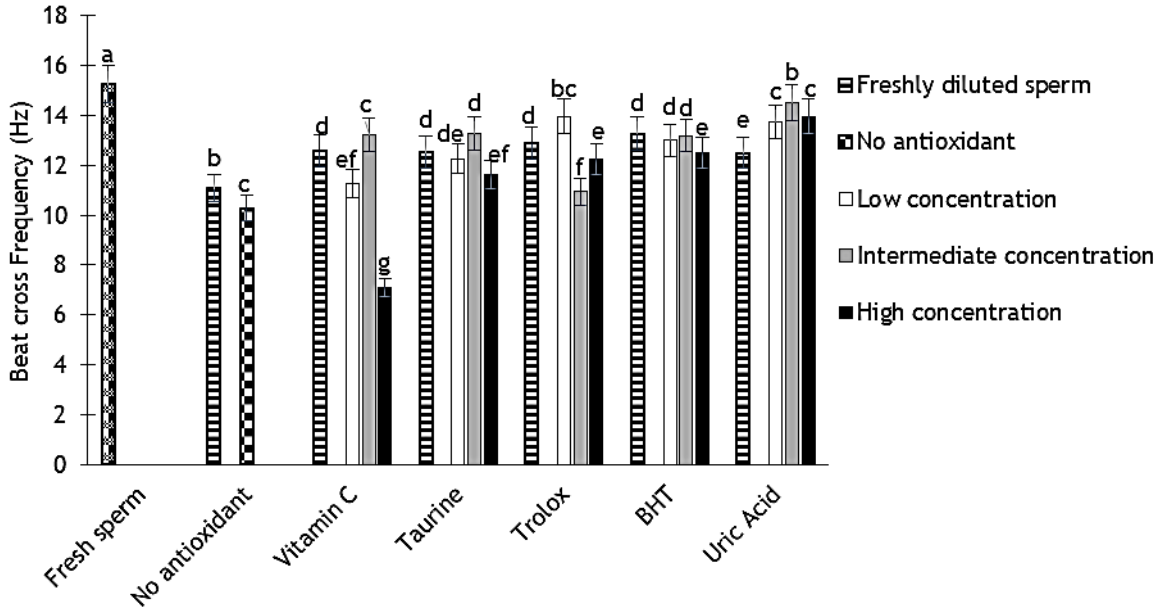


Figure 6: Mean Beat Cross Frequency (BCF) of sperm in fresh, fresh-diluted, and freeze-thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents the control with semen frozen in an extender without antioxidant supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).

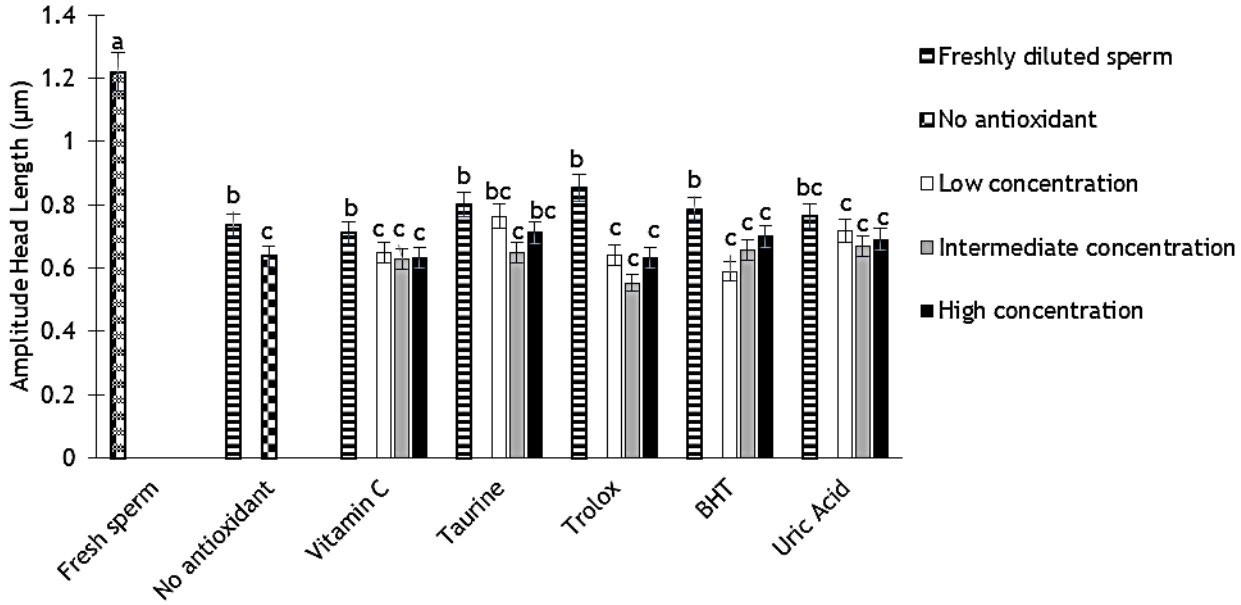
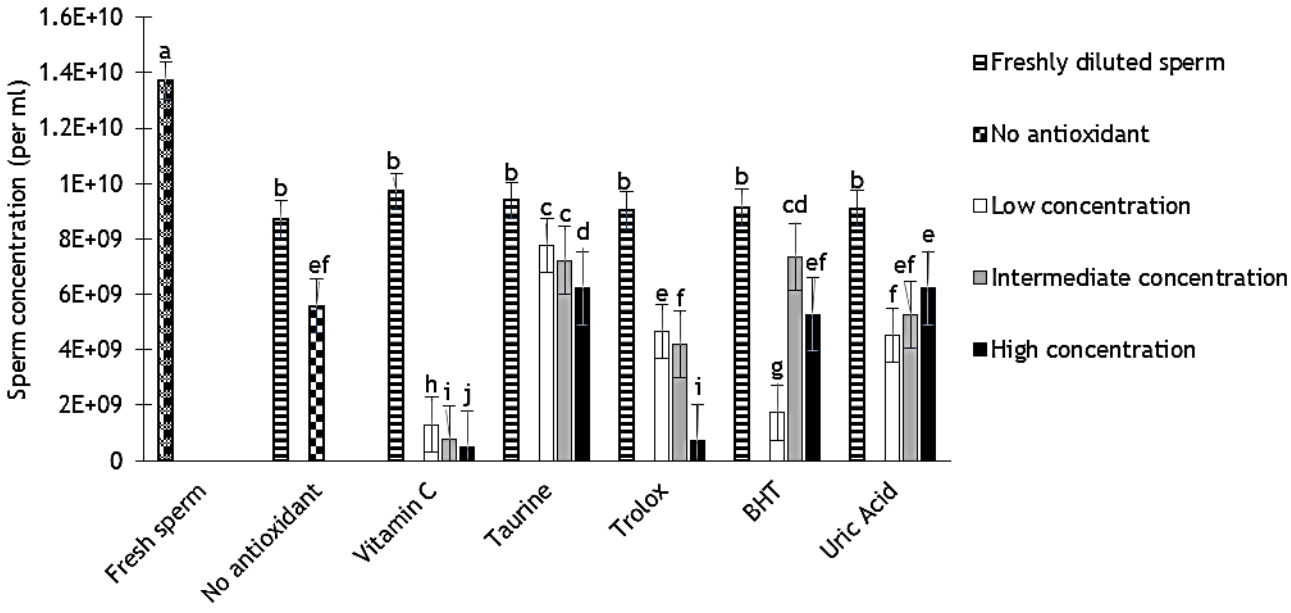


Figure 7: Mean Amplitude Head Length (ALH) of sperm in fresh, fresh-diluted, and freeze-thawed masu salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents the control with semen frozen in an extender without antioxidant supplementation. Means with the same superscript do not significantly differ ($p > 0.05$).



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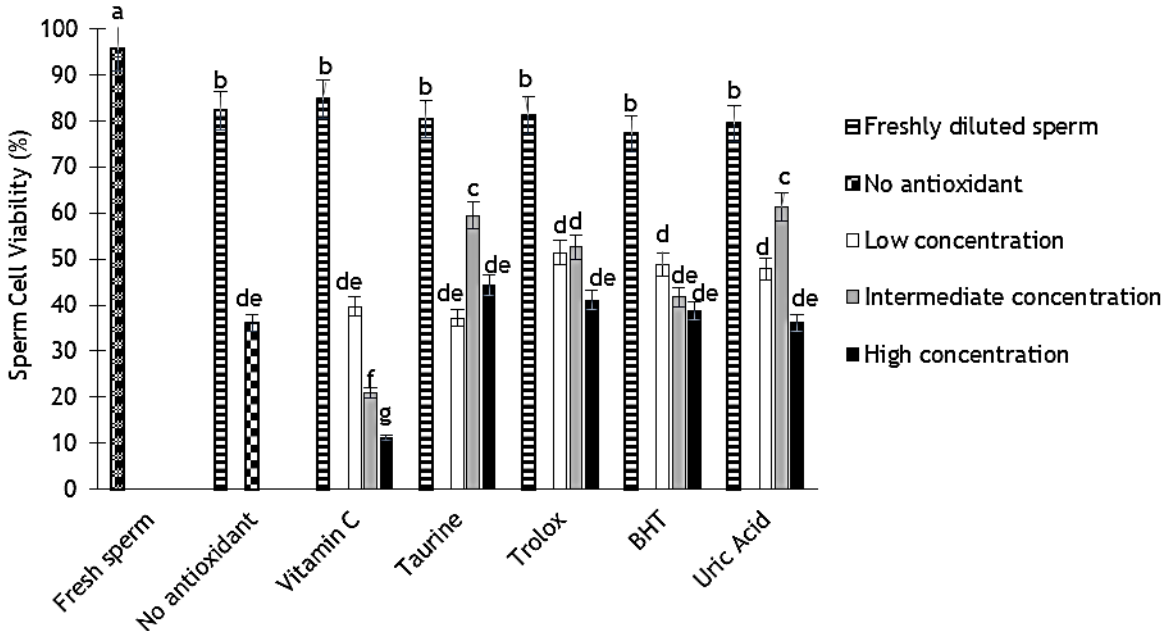
723 Figure 8: Mean concentration of sperm in fresh, fresh-diluted, and freeze-thawed masu
724 salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various
725 concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and
726 uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration
727 = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate
728 concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents
729 the control with semen frozen in an extender without antioxidant supplementation. Means
730 with the same superscript do not significantly differ ($p > 0.05$).

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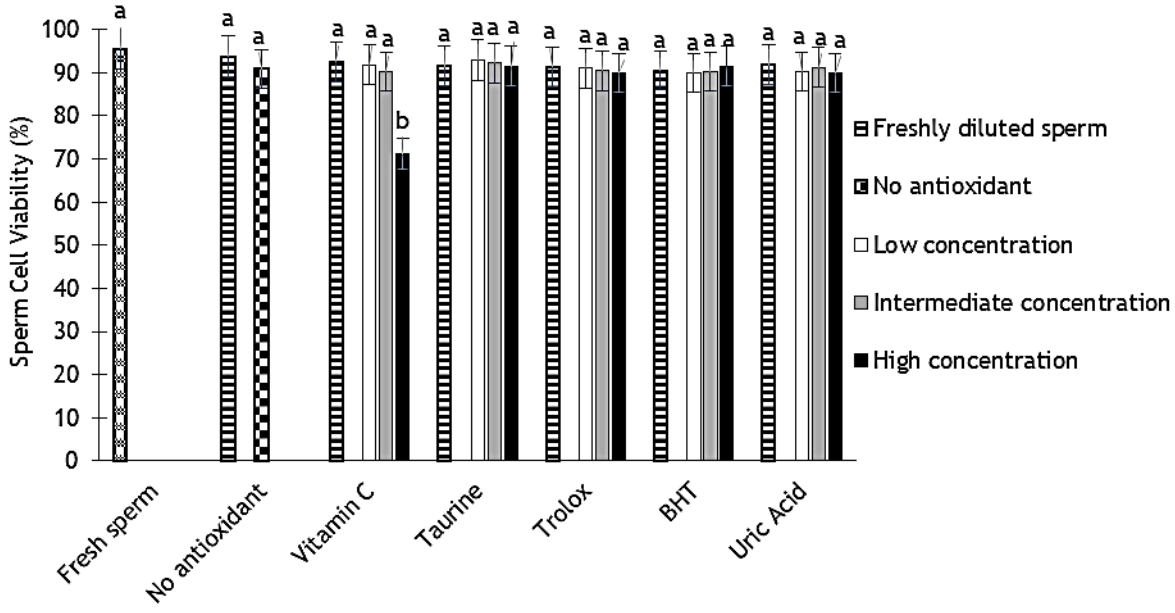
736 Figure 9: The percentage of viable sperm in fresh, fresh-diluted, and freeze-thawed masu
737 salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various
738 concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and
739 uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration
740 = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate
741 concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents
742 the control with semen frozen in an extender without antioxidant supplementation. Means
743 with the same superscript do not significantly differ ($p > 0.05$).

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749 Figure 10: The fertilization rates of sperm in fresh, fresh-diluted, and freeze-thawed masu
750 salmon (*Oncorhynchus masou*) semen with the extenders supplemented with various
751 concentrations of antioxidants. For semen supplemented with Vitamin C, taurine, BHT, and
752 uric acid, low concentration = 1 mM, intermediate concentration = 5 mM, high concentration
753 = 10 mM. For semen supplemented with trolox, low concentration = 0.1 mM, intermediate
754 concentration = 0.5 mM, high concentration = 1 mM. The "No antioxidant" group represents
755 the control with semen frozen in an extender without antioxidant supplementation. Means
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