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Shielding Endangered Lineages *via* Strategic Biobanking: Evaluating Extenders and Thaw Rates for Optimizing Viability of Cryopreserved Milt from Golden Mahseer (*Tor putitora*)

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ABSTRACT

Background: The Golden Mahseer (*Tor putitora*) is a coldwater fish species of paramount ecological, economic and cultural importance in India, now facing endangerment. In response to the urgency of conservation, this study focuses on the cryopreservation of Golden Mahseer milt, aiming to contribute valuable insights to conservation efforts.

Methods: To evaluate cryopreservation protocols, we tested four extender solutions (E1-E4) and applied three thawing temperatures (37, 40, 45°C). The primary indicators of sperm quality were motility rate and duration. The study employed statistical analyses to assess the significance of the results.

Result: Extender E1 (modified Ringer's solution) demonstrated remarkable performance, achieving a 42% motility rate at 37°C, surpassing E2 (31%) and significantly outperforming E3/E4 (3-5%) ($P \leq 0.05$). However, within E1, increasing thawing temperatures led to a progressive decline in motility, from 42% at 37°C to approximately 12% at 40°C and 11% at 45°C ($P \leq 0.05$), attributed to thermal shock. The success of cryopreservation was found to depend significantly on both extender formulation and controlled thawing rates ($P \leq 0.05$). These findings offer crucial insights to strategically enhance post-thaw viability, paving the way for indefinite biobanking and potential species resurrection, aligning with critical conservation goals for the endangered Golden Mahseer.

Key words: Endangered fish, Extenders, Fish cryopreservation, Sperm motility, Thawing temperatures, *Tor putitora*.

INTRODUCTION

The Indian Himalayan landscape is reported to have nearly 316 fish species, including iconic game fish from the mahseer genus *Tor* (Kunal *et al*., 2023). These large-bodied cyprinids represent an evolutionarily distinct lineage that has diversified over millennia into several ecological niches tied to specialized feeding and migratory behaviors (Michniewicz, 2019). However, in recent decades, rising threats from overfishing, habitat loss, river fragmentation and climate change have dramatically winnowed wild mahseer populations-pushing several species towards extinction (Vinod *et al*., 2007; Gupta *et al*., 2020; Sarma *et al*., 2022; Devdatta, 2023; Kitcharoen *et al*., 2023). The keystone Golden Mahseer (*Tor putitora*) has undergone catastrophic range contractions across its native distribution spanning the Ganga, Brahmaputra and Indus drainages (Pinder and Raghavan, 2013). This precipitous decline for a culturally iconic and economically prized fish warrants urgent conservation action (Sarkar *et al*., 2015).

To arrest genetic erosion and possible extinction, cryopreservation offers a powerful technological solution for indefinitely safeguarding reproductive potential (Cabrita *et al*., 2010; Wayman and Tiersch, 2011; Martínez-Páramo *et al*., 2016). Biobanking cryopreserved milt creates genetic repositories that allow selective revival of prolific or otherwise desirable lineages in the future to resurrect collapsed stocks (Liu *et al*., 2007; Martínez-Páramo *et al*., 2016). However, freezing spermatozoa imposes severe cold shock, osmotic imbalance and ice crystal damage which together decimates post-thaw fertility (Kopeika *et al*., 2003; Zhang *et al*., 2005; Martínez-Páramo *et al*., 2009). The unique biochemical traits of each fish species influence its vulnerability to cryoinjury, necessitating the creation of tailored extenders and cryoprotectants for their specific reproductive

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conditions (Kopeika *et al*., 2003; Hajirezaee *et al*., 2010). Despite its threatened status, cryopreservation research on the iconic Golden Mahseer remains limited which constrains conservation options (Basavaraja and Hedge, 2004; Basavaraja *et al*., 2006; Hajirezaee *et al*., 2010; Ponniah *et al*., 1999; Just two studies have explored freezing protocols for the congeneric Deccan Mahseer (*Tor khudree*) (Basavaraja and Hedge, 2004; Basavaraja *et al*., 2006)-but direct extrapolation to the Golden Mahseer is complicated by interspecific bio-variability (Nynca *et al*., 2012).

Therefore, this study aimed to evaluate tailored extenders and thawing temperatures for cryopreserving Golden Mahseer milt to establish optimal protocols that maximize post-thaw sperm viability. Boosting cryosurvival can enable cost-effective biobanking to selectively regenerate high-quality bloodlines when required for ecological restoration or augmentation of captive stocks (Cabrita *et al*., 2010; Martínez-Páramo *et al*., 2016). Accordingly, we assessed four extender formulations and three thawing temperatures using sperm motility rate and duration as indicators of cellular integrity and function. The findings deliver functional insights to guide strategic cryo-enhancements for boosting fertility preservation in this iconic species.

MATERIALS AND METHODS

Fish source and semen collection

Mature Golden Mahseer males (n=10) weighing 1200±30 gm (Fig 1) were sourced from captive stocks under rearing at ICAR Research Complex, Umiam, Meghalaya, India following standard husbandry protocols (DCFR, 1999). Then they were checked for presence of no disease or abnormalities. Then, by gently pressing their abdomen, milt was collected into chilled graduated collection vials (15 ml) for motility assessment (Martínez-Páramo *et al*., 2009).

Pre-freeze sperm quality analysis

Percentage motility was microscopically assessed within 30 secs of sample dilution (1:20 activation) and scored on a 5-point scale from 0 (no motility) to 4 (75-100% progressive motility) (Table 1) (Basavaraja and Hedge, 2004; Basavaraja *et al*., 2006). Motile lifespan quantified duration of forward movement post-activation. Samples exceeding 80% initial motility without abnormalities were cryopreserved.

Extender formulation and cryopreservation

Four tris-based extenders were formulated with varied buffering, cryo-protective and membrane-stabilizing agents (Basavaraja and Hedge, 2004; Basavaraja *et al*., 2006; Martínez-Páramo *et al*., 2009) (Table 2). A standard cryoprotectant (DMSO: dimethyl sulfoxide) at 10% v/v was supplemented (Basavaraja *et al*., 2006). DMSO is a widely used and effective cryoprotectant for fish sperm cryopreservation and many previous studies on various fish species have demonstrated the cryoprotective ability of DMSO at concentrations around 10%, thus adopted for this study. By using a standardized DMSO concentration, we focused on evaluating the specific effects of the different extender compositions. The diluted milt (1:10 in extender) then was aspirated into 0.5 mL French straws (Fig 2), equilibrated for 30 minutes at 4°C before 15 minutes freezing in liquid nitrogen vapors prior to plunging into liquid nitrogen for storage. The 1:10 dilution ratio is commonly used in fish sperm cryopreservation studies due to its effectiveness in maintaining the viability and functionality of the sperm cells, providing a balance between the concentration of cryoprotectants and the volume of the cryoprotectant solution, thus adopted for this study.

Thawing and post-freeze analysis

After 5 days of cryostorage, the straws were remove from liquid nitrogen and thawed at 37, 40 or 45°C for 15s. Then each thawed sample divided into four aliquots. The first aliquot was immediately activated by adding an appropriate activating solution (water) to initiate sperm motility. The activation of the remaining three aliquots by 5, 10 and 20 minutes, respectively, was delayed by keeping them at a controlled temperature. Percentage motility loss relative to initial quality indicated cryo-damage.

Statistical analysis

A two-factor ANOVA using IBM SPSS v21 was conducted to assess the effects of extenders and thawing temperature on sperm motility parameters. Duncan's multiple range of test was conducted to assess the inter-group differences at 5% level of significance. All data were presented as mean±S.D of five replications.

RESULTS AND DISCUSSION

Pre-freeze sperm quality

All four extenders maintained high initial sperm motility of 81-88% and durations around 70-75 secs before freezing ($p>0.05$) (Table 3), indicating standardized collection and handling protocols ensured uniform sample quality prior to cryopreservation.

Differential cryo-protection conferred by extenders

However post-thaw, the extenders differentially preserved sperm motility indicating distinct cryoprotective capacities intrinsic to their bioactive compositions. Across all thawing temperatures, E1 containing NaCl, KCl and $NaHCO_3$ salts buffered with NaH2PO4 markedly outperformed all other extenders in retaining motility-conferring 29-39% higher protection than E2 and enormous 39-42% greater viability over E3/E4 post-freeze ($p\leq0.05$). For example, at 37°C thawing, while E1 maintained 42% motility with 63 sec durability, E2 only achieved 31% viability with 57 sec sustainability. More dramatically, E3 and E4 rendered sperm almost entirely non-motile (~3-5%) despite optimal thawing rates-highlighting their inability to prevent cryo-injuries (Cabrita *et al*., 2005).

E1's exceptional effectiveness for cryopreserving Golden Mahseer spermatozoa aligns with previous findings by Basavaraja and Hegde (2004) that a similar formulation conferred maximal post-thaw retention of motility (~55%) and duration (~80 secs) in the related Deccan Mahseer. Collectively, the salts, buffers and osmolytes in E1 likely cooperatively stabilize cells during thermal and osmotic stresses of freeze-thaw by maintaining structural integrity. Specifically, balanced salt solutions prevent excessive dehydration or swelling by retaining optimal osmolality (Cabrita *et al*., 2005). Bicarbonate buffers stabilize pH homeostasis during temperature and liquid-solid phase transitions (Glogowski *et al*., 2002). Phosphate augments membrane architecture conservation and repairs cryo-damage. Glucose supplements provide energetic substrates to sustain motility machinery (Cabrita *et al*., 1998; Basavaraja and Hegde, 2004; Cabrita *et al*., 2005). The exact bioprotective concentrations and interactions maximizing viability in E1 warrants further investigation.

Conversely, the poor cryo-protection offered by E3 and E4 indicates suboptimal composition, concentration or absence of key osmolytes that left cells vulnerable to marked freeze-thaw injuries despite standardized cooling/warming protocols. Both formulations omitted essential salts and metabolic substrates. Furthermore, the egg yolk and milk powders in E3 seemingly failed to prevent viability loss. Their cholesterol and milk fat globule membrane supplements were clearly inadequate to offset cryo-damage compared to E1's synergistic bioactives. The organic components in E3 and E4 may have had inherent properties or interactions that compromised their ability to protect sperm cells during cryopreservation, potentially interfering with the protective mechanisms conferred by other components in the extender solution (Galeati *et al*., 2011).

While E2 containing NaCl, KCl and NaHCO3 salts performed better than E3/E4, its simpler salt-bicarbonate formulation still proved far less effective than E1's more comprehensive composition. Absent phosphates, glucoses and lower NaCl in E2 likely compromised its capacity to balance osmolality, energize motility and repair injured architecture post-thaw (Cabrita *et al*., 1998).

Overall, Extender E1, which contained a balanced salt solution along with buffer and an energy source, demonstrated remarkably better performance in retaining post-thaw sperm motility compared to other extenders. All extenders contained the same concentration (10% v/v) of the permeating cryoprotectant DMSO. However, the presence or absence of other components like salts, buffers and osmolytes influenced the overall cryoprotective ability, suggesting their critical role in complementing the permeating cryoprotectant. The findings revealed that extender salt composition, balance and permeating cryoprotectant supplementation critically govern cryopreservation success by shielding cells from osmotic, oxidative and structural damage during phase transition stresses."

Thawing temperature dramatically influences post-thaw viability

For all extenders, elevating thawing warmth from 37→40→45°C progressively halved motility at each transition - collapsing from 31-42%→8-12%→1-11% respectively ($p \leq 0.05$). Even under E1's optimal buffering, increasing thaw temperature curtailed motility 3-fold from 42% at 37°C to 12% and 11% at 40°C and 45°C respectively. Faster warming intensified thermal shock which likely disrupted sperm membrane architecture and organelle ultrastructure (Alizadeh *et al*., 2016). Sudden ambient temperature shifts can fracture phospholipid arrangements and cytoskeletal dynamics necessary for motility by exceeding membrane fluidity thresholds (Cerolini

Fig 1: Fish samples (*Tor putitora*) used for milt (semen) collection.

Fig 2: Freezing of straws filled with diluted milt.

Table 1: Five-point scale followed for motility test of fish spermatozoa.

Motility rate (%)	Motility rating (5-point scale)
0 to <l%	0
l to <25%	1
25 to <50%	2
50 to <75%	3
75 to 100%	4

Table 2: The compositions of different extenders used (*Make: HiMedia).

Chemicals*	Extender			
(g/100 ml)	E1	E2	E3	E4
NaCl	0.75	0.75	0.75	-
KCl	0.10	0.02	-	0.3
$CaCl_2$	0.016	0.02	-	-
$MgSO_4$	0.023	-	-	-
$NaHCO_3$	-	0.02	0.75	-
NaH_2PO_4	0.041	-	-	-
Glucose	0.10	-	-	-
Distilled water (ml)	100.00	100.00	90.00	95.00
Egg yolk (ml)	-	-	10.00	-
Milk powder	-	-	-	5.0
Methanol (ml)	-	-	-	5.0
pH	7.50	7.50	8.50*	7.90*
Basis	This is a modified ringer's solution. The inclusion of salts like NaCl, KCl, $CaCl_2$ and $MgSO_4$ helps mimic the ion composition of fish body fluids, preventing excessive cellular dehydration or swelling during cryopreservation. NaH_2PO_4 acts as a buffer, while glucose provides an energy source for sperm.	This extender contains fewer salts than E1, lacking calcium, magnesium and phosphate components. However, it includes $NaHCO_3$, which acts as a pH buffer.	This extender contains NaCl and $NaHCO_3$ but lacks other salts present in E1. It also includes egg yolk, which is a common additive in sperm cryopreservation media due to its membrane-stabilizing properties and potential cryoprotective effects.	This extender has a unique composition, with KCl as the only salt, along with milk powder and methanol. Milk powder has been included for its potential membrane-stabilizing effects, while methanol could have been added as a cryoprotectant.

*For E3, the inclusion of $NaHCO_3$ likely contributed to a higher pH of 8.5 and the absence of buffer components like $NaHCO_3$ might have led to pH fluctuations.

et al., 2000). Intracellular ice re-crystallization also potentially disrupts mitochrondrial and axoneme continuity. Moreover, amplified oxidative stress from warmer-induced biochemical fluctuations possibly overwhelmed endogenous antioxidant systems - permitting reactive oxygen species to accumulate and damage proteins, lipids and DNA to further affect viability (Bansal and Bilaspuri, 2010).

Effect of activation delay on post-thaw spermatozoa motility

The effect of activation delay on post-thaw spermatozoa motility was investigated by delaying the activation of spermatozoa for 5, 10 and 20 minutes. The results revealed a gradual decrease in motility with increasing storage time (the time elapsed between thawing the samples and activating them, not the duration of cryostorage), as depicted in Fig 3. Specifically, a delay in activation by 10 minutes led to a sharp decline in spermatozoa motility when extenders E1 and E2 were used. An extremely low level of spermatozoa motility, approximately 12% for E1 and 10% for E2, was observed when the activation was delayed by 20 minutes. These findings emphasize the importance of minimizing activation delay for successful cryopreservation, particularly when using extenders E1 and E2.

Therefore, independent of extender composition, strictly regulated thawing rates are imperative to peacefully rehydrate cells after cryostorage without creating thermal shock. Gentle warming around body temperature (37°C) seems optimal for smoothly transitioning vitrified samples back to liquid state without overt disruption of intracellular architecture (Cabrita *et al*., 2001). Controlling re-warming likely allows gradual permeation of external cryoprotectants to safely rehydrate cells without osmotic injury. Thus, both extending medium formulation and thawing temperature critically control post-thaw viability - highlighting target areas for strategic improvements.

Table 3: Effect of different extenders (E1, E2, E3 and E4) and thawing temperatures (T1: 37°C, T2: 40°C and T3: 45°C) on spermatozoa motility and motility duration; the data bearing same superscripts in the same column for each extender under different thawing temperatures indicates no significant difference (P≤0.05).

Different extenders	Thawing temperatures	Pre-freezing spermatozoa motility		5 days after cryopreservation	
		Motility (%)	Motility duration (seconds)	Motility (%)	Motility duration (seconds)
E1	T1	81.0±3.6^{a}	73.0±3.5 ^{a}	42.0±2.0^{a}	63.3±1.5^{a}
	T2	85.0±3.0^{a}	75.0±1.0 ^{a}	12.7±2.0^{b}	39.7±1.5^{b}
	T3	81.3±3.5 ^{a}	70.7±3.8 ^{a}	11.7±0.6^{b}	37.3±2.5^{b}
E2	T1	88.0±2.0 ^{a}	76.0±2.6 ^{a}	31.0±1.0^{a}	57.0±2.0^{a}
	T2	86.7 ±3.2 ^{a}	71.0±7.2 ^{a}	8.7±1.5^{b}	37.0±7.5^{b}
	T3	86.3±4.0 ^{a}	74.0±6.0 ^{a}	7.7±1.5^{b}	29.3±3.1^{b}
E3	T1	82.3±2.5 ^{a}	75.0±3.0 ^{a}	3.0±1.0^{a}	7.3±2.5^{a}
	T2	81.3±3.5 ^{a}	76.3±1.5 ^{a}	0.3±0.6^{b}	1.3±2.3^{b}
	T3	85.0±3.0 ^{a}	75.0±3.6 ^{a}	1.0±1.0^{b}	5.3±5.0^{a}
E4	T1	83.0±2.6 ^{a}	70.3±2.1 ^{a}	5.0±1.0^{a}	14.3±1.2^{a}
	T2	86.3±1.5 ^{a}	73.7±2.1 ^{a}	2.0±1.0^{b}	6.0±1.7^{b}
	T3	83.7±2.1^{a}	71.7±2.1^{a}	3.7±1.5^{ab}	17.7±8.5^{a}

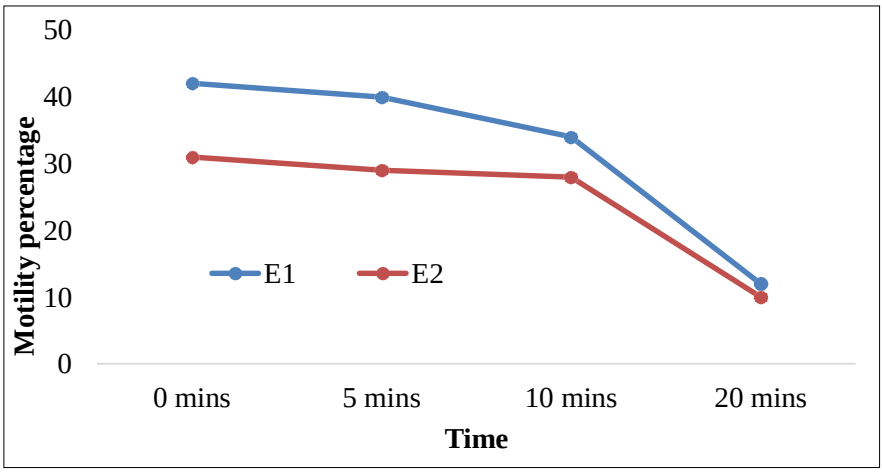

Fig 3: Declining rate of sperm motility with delay in activation time.

Despite conferring maximal protection, E1 retained only ~50% baseline motility indicating enormous potential for further bio-enhancement by supplementing additional cryoprotectants and antioxidants. Anti-apoptotics like caspase inhibitors could also potentially amplify fertilizing capacity by blocking intrinsic cell death pathways triggered by freeze-thaw stress (Martínez-Páramo *et al*., 2009). Nanocarrier delivery systems enabling timed-release of molecular shields may also minimize cryo-losses (Moraes *et al*., 2010). Selective breeding approaches that isolate hardy cryo-tolerant sperm phenotypes based on biomarkers could ultimately generate resilient lines where >90% viability becomes achievable after biobanking (Martinez-Pastor *et al*., 2004). Overall, the findings deliver a robust starting point for advancing reproducibility and amplifying the scale of biobanking for this endangered iconic species. Options now exist for efficiently regenerating selective bloodlines or resurrecting regionally extinct populations if needed to restore aquatic biodiversity. As wild stocks continue dwindling from escalating anthropogenic pressures, having an indefinite genome repository through biobanking serves as insurance against irreversible genetic erosion or extinction.

CONCLUSION

The findings provided a foundation for further optimization of cryopreservation protocols for one of the flagship Mahseer species, that is *Tor putitora*, which is presently endangered. While the optimal extender (E1) and thawing temperature (37°C) retained around 42% post-thaw motility, substantial motility loss was still observed. Additional refinements, such as supplementing with antioxidants, caspase inhibitors, or exploring timed-release nanocarrier systems, may be necessary to achieve the higher viability levels required for effective biobanking and potential species resurrection efforts.

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Conflict of interest

All authors declared that there is no conflict of interest.

REFERENCES

Alizadeh, R., Navid, S., Abbasi, N., Yari, A., Mazaheri, Z., Daneshi, E. and Agarwal, A. (2016). The effect of aminoguanidine on sperm motility and mitochondrial membrane potential in varicocelized rats. Iran Journal of Basic Medical Sciences. 19(12): 1279-1284.

Bansal, A.K. and Bilaspuri, G.S. (2010). Impacts of oxidative stress and antioxidants on semen functions. Veterinary Medicine International. doi:10.4061/2011/686137.

Basavaraja, N. and Hegde, S.N. (2004). Cryopreservation of the endangered mahseer (*Tor khudree*) spermatozoa: I. Effect of extender composition, cryoprotectants, dilution ratio and storage period on post-thaw viability. Cryobiology. 49: 149-156.

Basavaraja, N., Hegde, S.N. and Palaksha, K.J. (2006). Cryopreservation of the endangered Mahseer (*Tor khudree*) spermatozoa: Effect of dimethyl sulfoxide, freezing, activating media and cryostorage on post-thaw spermatozoa motility and fertility. Cell Preservation Technology. 4(1): 149-156.

Cabrita, E., Álvarez, R., Anel, L., Rana, K.J. and Herraez, M.P. (1998). Sublethal damage during cryopreservation of rainbow trout sperm. Cryobiology. 37(3): 245-253.

Cabrita, E., Robles, V., Alvarez, R. and Herráez, M.P. (2001). Cryopreservation of rainbow trout sperm in large volume straws: Application to large scale fertilization. Aquaculture. 201(3-4): 301-314.

Cabrita, E., Robles, V., Rebordinos, L., Sarasquete, C. and Herráez, M.P. (2005). Evaluation of DNA damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) cryopreserved sperm. Cryobiology. 50(2): 144-153.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J. and Pérez-Cerezales, S. (2010). Cryopreservation of fish sperm: Applications and perspectives. Journal of Applied Ichthyology. 26(5): 623-635. doi: 10.1111/j.1439-0426.2010.01556.x.

Cerolini, S., Maldjian, A., Surai, P. and Noble, R. (2000). Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Animal Reproduction Science. 58(1-2): 99-111.

DCFR, (1999). Himalayan Mahseer. Published by the Director, DCFR, Bhimtal, UP, pp. 1-35.

Devdatta, L. (2023). Mahseer: A bioindicator teleost. Uttar Pradesh Journal of Zoology. doi: 10.56557/upjoz/2023/v44i33415.

Galeati, G., Spinaci, M., Vallorani, C., Bucci, D., Porcu, E. and Tamanini, C. (2011). Pig oocyte vitrification by cryotop method: Effects on viability, spindle and chromosome configuration and in vitro fertilization. Animal Reproduction Science. 127(1-2): 43-49.

Glogowski, J., Kolman, R., Szcepkowski, M., Horvath, A., Urbanyi, B., Sieczynski, P., Rzemieniecki, A., Domagala, J., Demianowicz, W., Kowalski, A. and Ciereszko, A. (2002). Fertilization rate of Siberian sturgeon (*Acipenser baeri*, Brandt) milt cryopreserved with methanol. Aquaculture. 211: 367-373.

Gupta, N., Everard, M., Nautiyal, P., Kochhar, I., Sivakumar, K., Antony Johnson, J., Borgohain, A. (2020). Potential impacts of non-native fish on the threatened mahseer (*Tor*) species of the Indian Himalayan biodiversity hot spot. Aquatic Conservation: Marine and Freshwater Ecosystems. doi: 10.1002/aqc.3275.

Hajirezaee, S., Mojazi Amiri, B., Mirvaghefi, A. and Sheikh Ahmadi, A. (2010). Evaluation of semen quality of endangered caspian brown trout (*Salmo trutta caspius*) in different times of spermiation during the spawning season. Czech Journal of Animal Science. 55(10): 445-455.

Kitcharoen, P. and Youthao, S. (2023). Assessing Physical Parameters of Khadakwasla Reservoir for Mahseer Fish Conservation or "Integrated Approach to Mahseer Fish Conservation: Analyzing Environmental Variables in Khadakwasla Reservoir" In Book: Cutting Edge Research in Biology. 7: 22-30. doi: 10.9734/bpi/cerb/v7/19012d.

Kopeika, J., Kopeika, E., Zhang, T. and Rawson, D.M. (2003). Detrimental effects of cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo development are reversed by incubating fertilised eggs in caffeine. Cryobiology. 46(1): 43-52.

Kunal, K., Garima and Ganie, P.A. (2023). Fishery Resources and Ichthyofaunal Diversity in the Temperate Himalayas. In: Fisheries and Aquaculture of the Temperate Himalayas. Singapore: Springer.11-23.

Liu, Q.H., Li, J., Zhang, S.C., Xiao, Z.Z., Ding, F.H., Yu, D.D. and Xu, X.Z. (2007). Flow cytometry and ultrastructure of cryopreserved red seabream (*Pagrus major*) sperm. Theriogenology. 67(6): 1168-1174.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R. and Cabrita, E. (2016). Cryobanking of aquatic species. Aquaculture. doi: 10.1016/j.aquaculture.2016.05.042.

Martínez-Páramo, S., Pérez-Cerezales, S., Gómez-Romano, F., Blanco, G., Sánchez, J.A. and Herráez, M.P. (2009). Cryobanking as tool for conservation of biodiversity: Effect of brown trout sperm cryopreservation on the male genetic potential. Theriogenology. 71: 594-604.

Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P. and Rodriguez-Martinez, H. (2004). Use of chromatin stability assay, mitochondrial stain JC-1 and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. Animal Reproduction Science. 84(1-2): 121-133.

Michniewicz, A. (2019). Tors in central European mountains-Are they indicators of past environments? Bulletin of Geography: Physical Geography Series. doi: 10.2478/bgeo-2019-0005.

Moraes, E.A., Graham, J.K., Torres, C.A., Meyers, M. and Spizziri, B. (2010). Delivering cholesterol or cholestanol to bull sperm membranes improves cryosurvival. Animal Reproduction Science. 118: 148-154.

Nynca, J., Kuźmiński, H., Dietrich, G.J., Hliwa, P., Dobosz, S., Liszewska, E., Karol, H. and Ciereszko, A. (2012). Biochemical and physiological characteristics of semen of sex-reversed female rainbow trout (*Oncorhynchus mykiss*, Walbaum). Theriogenology. 77(1): 174-183.

Pinder, A.C. and Raghavan, R. (2013). Conserving the endangered mahseers *Tor* spp. (Cyprinidae) of India: The positive role of recreational fisheries. Current Science. 104(11): 1472-1474.

Ponniah, A.G., Sahoo, P.K., Dayal, R. and Barat, A. (1999). Cryopreservation of Tor putitora spermatozoa: Effect of extender composition, activating solution, cryoprotectant and equilibration time. Proceedings of the National Academy of Sciences, India. 69(B I): 53-59.

Sarkar, U.K., Mahapatra, B.K., Saxena, S.R. and Singh, A.K. (2015). Mahseer in India: An overview on research status and future priorities. Journal of Ecophysiology and Occupational Health. 45-52.

Sarma, D., Mohan, D., Ravindra, P., Mukul, A., Parvaiz Ahmad, G. (2022). The mighty mahseers of the genera Tor, Neolissochilus and Naziritor: A review on resource distribution, biology, ecotourism and conservation. Indian Journal of Fisheries. doi: 10.21077/ijf.2022.69.4.125074-20.

Vinod, K., Mahapatra, B.K. and Mandal, B.K. (2007). Umiam reservoir fisheries of Meghalaya (Eastern Himalayas) - Strategies for yield optimization. Fishing Chimes. 26(10): 8-15.

Wayman, W.R. and Tiersch, T.R. (2011). Research Methods for Cryopreservation of Sperm. In: Cryopreservation in Aquatic Species. [Tiersch, T.R. and Green, C.C. (Eds.)], Baton Rouge: World Aquaculture Society. (2nd ed., pp. 672-683).

Zhang, T., Isayeva, A., Adams, S.L. and Rawson, D.M. (2005). Studies on membrane permeability of zebrafish (*Danio rerio*) oocytes in the presence of different cryoprotectants. Cryobiology. 50(3): 285-293.