Cell Biochemistry and Biophysics (2026) 84:1695–1704
https://doi.org/10.1007/s12013-026-01999-0

REVIEW PAPER

Molecular Understanding of Cryopreservation of Marine Invertebrates: Achievements and New Perspectives

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Accepted: 5 January 2026 / Published online: 18 January 2026

Abstract

Cryopreservation is a promising biotechnological tool for the long-term conservation of marine invertebrates, yet its molecular impacts remain insufficiently understood. This review synthesizes current findings on how cryogenic procedures encompassing controlled slow-freezing, two-step freezing, and vitrification affect molecular structures in marine invertebrates. Cryoinjury caused by intracellular ice formation and osmotic stress remains the primary challenge, especially in lipid-rich early developmental stages with limited membrane permeability to cryoprotective agents. DNA damage, including fragmentation and methylation pattern alteration, has been reported in mollusks and corals, while variations in lipid phase transition highlight membrane destabilization under cold exposure. Cryopreservation also perturbs mitochondrial activity, leading to adenosine triphosphate depletion and reactive oxygen species accumulation. Protein alterations include degradation, shifts in energy metabolism enzymes, and stress-induced upregulation of heat shock proteins such as HSP70 and HSP90. In parallel, reductions in RNA synthesis and transcriptional responses related to antioxidant and apoptotic regulation have been documented, demonstrating species-specific redox adaptations. Despite these challenges, evidence suggests that surviving cells may recover basal metabolic activity, underscoring the resilience of some taxa. Future studies should integrate molecular biomarkers including DNA integrity, lipidomics, proteomics, and transcriptomics to evaluate cryoinjury mechanisms and optimize species- and stage-specific cryopreservation protocols. Understanding these molecular consequences will enhance the reliability of cryopreservation as an ex situ conservation strategy for marine biodiversity.

Keywords Cryopreservation · Molecular · lipids · Protein · Marine invertebrates

Introduction

Cryopreservation is an innovative technique based on freezing cells, tissues, and organs at cryogenic temperatures (−196 °C) [1, 2]. The goal is to preserve the cellular integrity of biological samples over the long term. Cryopreservation has been widely used in livestock breeding, conservation, and aquaculture [3–6]. However, studies involving marine invertebrates are still limited and often focus on species of high economic value [7, 8]. Among these, the cryopreservation of sperm and larvae has been the most developed, as oocytes present complex physical and biochemical characteristics [2, 9, 10]. There are many factors affecting successful cryopreservation (Fig. 1). One of the main limitations is the risk of cryoinjury, caused by intracellular ice crystal formation during low-temperature exposure, which can disrupt the cellular structures [11]. In early developmental stages of marine invertebrates, this cryosensitivity is often exacerbated by high lipid content, low permeability to cryoprotective agents (CPAs), and sensitivity to chemical exposure and chilling [12–17]. Other factors, such as pH fluctuations, and osmotic pressure, can also cause cryoinjuries and compromise the success of cryopreservation [1, 16, 18]. The extent of these injuries varies due to the high diversity of marine

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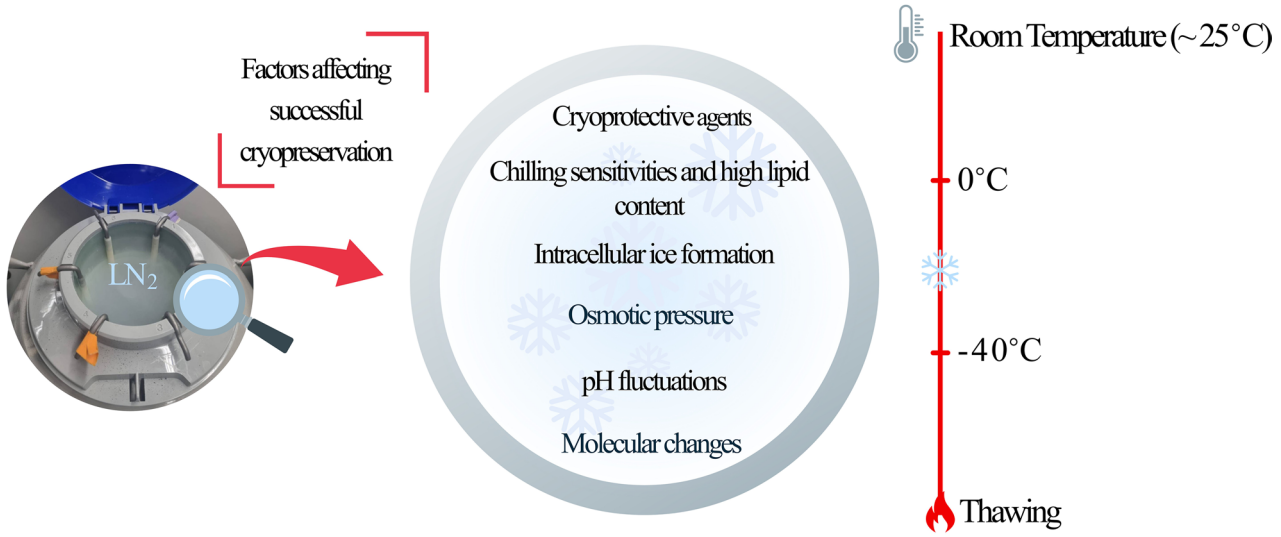

Fig. 1 Main factors affecting cryopreservation outcomes in marine invertebrate. The figure organizes the potential factors affecting cryopreservation according to a scale, ranging from ambient temperature to the stages of freezing and thawing, including CPAs at different time periods

organisms, and optimal conditions may differ between species or even between cell types of the same organism [19]. More specifically, cryopreservation can impact molecular components such as lipids, proteins, mRNA stability, and DNA integrity [20–23] (Fig. 2). Advancing this field requires elucidating how cryopreservation influences molecular and genetic processes during development, with particular attention to changes in gene expression, DNA integrity, and other biomarkers that may predict developmental success [24]. Analysing such indicators would provide valuable tools for monitoring embryo quality, guiding the optimization of species- and stage-specific protocols, and ensuring the functional viability of preserved material [1].

DNA Damage During Cryopreservation of Marine Invertebrates

Cryopreservation can induce DNA damage, compromising chromosome integrity and altering the methylation patterns, which are important indicators of cellular stress and apoptosis [23, 25, 26]. While such damage has been widely documented in mammals, it has been less studied in marine invertebrates [27, 28]. In mollusks such as *Crassostrea gigas*, increased DNA fragmentation and changes in methylation have been observed, particularly in trochophore larvae [29, 30]. These changes are associated with transcriptional modulation of epigenetic regulators, including a general downregulation of Jumonji histone demethylase orthologs, an increased expression of DNA methyltransferases as DNMT3b, and altered expression of methyl-DNA binding domain (MBD) proteins like MBD2 and MeCP2, with MeCP2 being significantly downregulated following cryopreservation [30]. This particular sensitivity of early developmental stages has been confirmed in several studies [23, 31, 32]. From a functional perspective, metabolic studies have shown that although DNA synthesis may decrease, surviving cryopreserved cells can recover a basal activity similar to their original state [33–35]. Furthermore, increased DNA fragmentation has been shown to negatively correlate with sperm motility in various species, including *Haliotis discus hannai* and *C. virginica*, thereby affecting fertilization capacity and embryonic development [21, 36–38]. Similar results have also been observed in vertebrate species (e.g., *Oncorhynchus mykiss*) [39]. Nevertheless, the mechanisms involved in vertebrates cannot be directly compared to those of marine invertebrates, and species-specific studies are essential.

Lipid Analysis After Cryopreservation

The poikilothermic nature of marine invertebrates limits their ability to adjust lipid composition in response to environmental fluctuations [20, 40]. For this reason, cryopreservation can induce specific changes in membrane lipids, leading to their reorganization [17]. This phenomenon, known as membrane lipid phase transition, occurs when low temperatures cause lipids to shift from a liquid-crystalline state to a gel phase [20, 41]. This reduces membrane fluidity and compromises its integrity, potentially causing leakage of intracellular contents or structural damage such as epithelial tissue disintegration [17, 20, 42–44]. Damage can also specifically affect polyunsaturated fatty acids (PUFAs) in the total lipid content [40]. Cryopreservation may reduce PUFA levels either through their direct loss due to stress or by increasing the relative concentration of saturated fatty acids [40, 45]. A possible adaptive response to cold stress

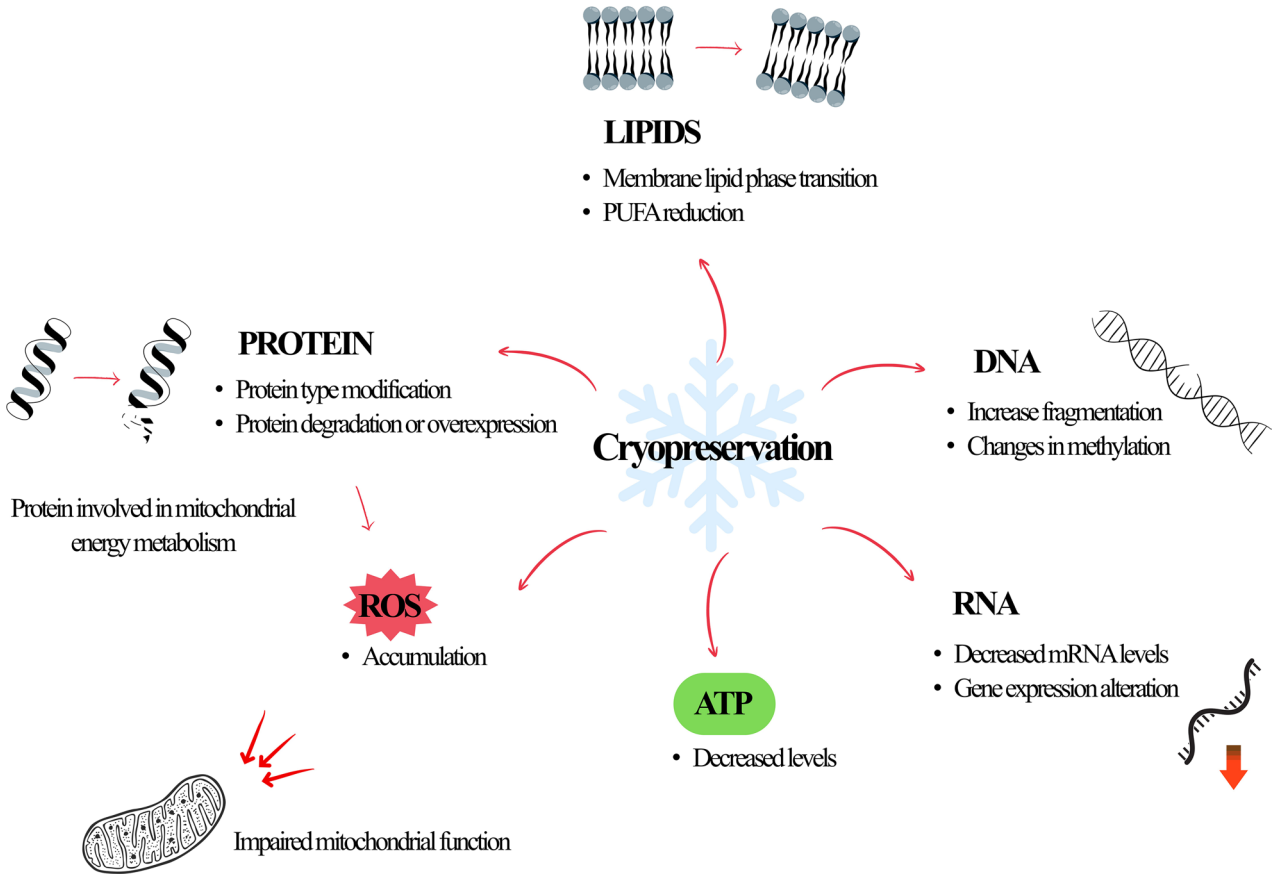

Fig. 2 Overview of the potential molecular damages associated with cryopreservation. The freezing process can lead to a variety of molecular dysregulation, from DNA fragmentation, gene expression alteration to membrane lipid phase transition. The normal mitochondrial function is also threatened, due to a possible decrease of ATP level, ROS overproduction or degradation of protein involved in mitochondrial energy metabolism

is the increase in lysophosphatidylcholine (LPC) content, which may help enhance membrane fluidity [20]. Although an overall increase in this lipid class (neutral lipids as wax ester, sterol ester, triacylglycerol, cholesterol, and polar lipids as phosphatidylethanolamine (PE), phosphatidylcholine (PC), LPC) has been observed in coral larvae of *Pocillopora verrucosa*, resulting in higher cryotolerance compared to *Seriatopora caliendrum* larvae, both species showed limited homeoviscous adaptation, a key mechanism for coping with low temperatures [20, 46]. Several studies on corals and crustaceans have further demonstrated that membrane lipid composition, particularly the abundance of phospholipid like PE, PC, and PUFAs, plays a critical role in cold tolerance, with higher lipids levels correlated with lower lipid phase transition temperatures and improved membrane fluidity [15, 32, 47–49]. The major lipid classes reported to influence membrane properties, chilling sensitivity, and cryopreservation outcomes in marine invertebrate embryos and larvae are summarized in Table 1. Additionally, later embryonic stages in some marine invertebrates, such as penaeid shrimp (*Trachypenaeus byrdi*) and coral shrimp (*Stenopus hispidus*), have shown increased resistance to chilling, possibly due to shifts in lipid composition throughout development, with early stages being more lipid-rich but more sensitive to cold stress [44, 49, 50].

Impact of Low Temperature on Adenosine Triphosphate (ATP) and Reactive Oxygen Species (ROS)

Cryopreservation can significantly impair mitochondrial function in marine invertebrates, affecting cell vitality and embryonic cell quality [51]. The main role of mitochondria is to produce ATP, a key marker of an organism's energetic state [52]. For this reason, studying mitochondrial DNA (mtDNA) can be useful for assessing sample quality, as it plays a critical role in efficient ATP production [26, 51, 53]. Previous studies have shown that exposure to cryoprotectants can reduce ATP levels, possibly as a stress response to their toxicity (*Echinopora* spp.) [21, 52]. Similarly, high concentrations of cryoprotectants (e.g., 2–3 M methanol) in the scleractinian coral *Echinopora* have been shown to

Table 1 Major lipid classes involved in chilling and cryopreservation sensitivity in marine invertebrate embryos and larvae

Lipid class	Development stage	Low temperature effect	Proposed mechanism	References
Triacylglycerols (TAG)	Embryos, larvae	High TAG content frequently associated with increased sensitivity to chilling and cryopreservation	Energy storage for early development	Lin et al. [15]; Lin et al. [44]; Cirino et al. [20]
Wax esters (WE)	Embryos, larvae	Highly abundant in early developmental stages; indirectly linked to chilling sensitivity	Long-term energy storage and buoyancy regulation	Lin et al. [15]; Lin et al. [44]; Cirino et al. [20]
Cholesterol	Larvae	Increased cholesterol content associated with enhanced chilling tolerance	Modulation of phospholipid packing and stabilization of membrane fluidity at low temperatures	Cirino et al. [20]
Sterol esters (SE)	Larvae	Higher SE content observed after chilling exposure	Intracellular sterol storage potentially supporting membrane remodeling during cold stress	Cirino et al. [20]
Phosphatidylcholine (PC)	Embryos, larvae	Higher PC content associated with lower membrane melting and lipid phase transition	Maintenance of bilayer organization, influencing membrane fluidity	Lin et al. [44]; Lin et al. [48]; Cirino et al. [20]
Phosphatidylethanolamine (PE)	Embryos	Correlated with increased resistance to chilling	Regulation of membrane surface viscosity, contributing to fluid membranes	Lin et al. [44]; Lin et al. [48]
Lysophosphatidylcholine (LPC)	Larvae	Increased following chilling exposure; associated with improved cold tolerance	Increase of membrane fluidity	Cirino et al. [20]
Polyunsaturated fatty acids (PUFAs)	Embryos, larvae	Preferentially reduced after freezing–thawing; higher levels associated with improved chilling resistance	Increase of membrane fluidity	Cook & Gabbott, [47]; Lin et al. [15]; Lin et al. [32]; Odintsova et al. [40]

drastically reduce mtDNA copy number, falling below the estimated threshold (<2,000,000 copies) necessary for normal embryonic development [51, 54]. However, in some cases, such as in *Haliotis tuberculata*, surviving cells were able to recover metabolic activity similar to fresh cells [34]. In addition, studies on Symbiodiniaceae have shown elevated ATP levels in response to certain CPAs [55–58], which may reflect a protective mechanism under stress conditions. These observations are particularly relevant given the critical symbiotic association of Symbiodiniaceae with marine invertebrates, including corals and giant clams. Cryopreservation is also known to generate reactive oxygen species (ROS), which damage mtDNA more readily than nuclear DNA [59, 60]. In the gametes and larvae of invertebrates, excessive ROS accumulation, often caused by temperature fluctuations during freezing and thawing, has been linked to lipid peroxidation, DNA damage, and reduced post-thaw viability [35, 61–64]. Although ROS at low concentrations have signaling functions, their excess leads to metabolic dysfunction [35, 65]. Not all species, however, appear to be equally affected; for example, *Mytilus galloprovincialis* and *C. gigas* showed minimal impact from ROS accumulation [35, 66].

Protein Alteration Following Cryopreservation

Cryopreservation can profoundly alter the protein profile of marine invertebrates, even in the absence of visible cellular damage or reduced fertilization rates [22]. In the oocytes of *M. galloprovincialis* and *Perna canaliculus*, the affected proteins were mainly involved in mitochondrial energy metabolism, suggesting increased metabolic activity and ROS production [22, 62, 67–69]. These changes may lead to delayed development and subsequent ultrastructural and functional damage. Moreover, exposure to certain CPAs such as dimethyl sulfoxide (DMSO) can modify specific protein types, including those associated with the cytoskeleton and meiotic resumption [22]. A study on *C. angulata* larvae reported an increase in protein degradation following cryopreservation, along with overexpression of heat shock protein 70 (HSP70), which plays a key role in cytoskeletal stabilization and apoptosis suppression [70–72]. Some marine invertebrates, indeed, express molecular chaperones constitutively, such as Heat shock protein Hsp90, Hsc70, Glucose regulated protein 78, and Hypoxia up-regulated 1, which are involved in protein folding, stabilization, and degradation control [73–75]. In *Sterechinus neumayeri*, González et al. [75] demonstrated the constitutive expression of these heat shock proteins across various tissues, suggesting a cold-adapted cellular state that ensures protein homeostasis even under continuous thermal stress. In contrast to these findings, a study on dissociated mantle cells

Table 2 Molecular evaluation following cryopreservation experiments

Molecular Component	Species	Biomaterial	Cryopreservation Method	Freezing component	Reference
DNA	*Haliotis discus hannai*	Sperm	Two-step freezing	8% Me2SO, 8% EG, 6% PG, 2% GLY, and 2% MeOH	Hossen et al. [21]
	Crassostrea gigas	Trochophore (18 h post-fertilization)	Controlled slow-freezing	10% EG + 5% Ficoll + 0.2% polyvinylpyrrolidone	Liu et al. [30]
	H. tuberculata	Mantle cells	Two-step freezing	10% Me2SO, 10% GLY	Poncet et al. [34]
	Pecten maximus	Heart cells	Two-step freezing	Me2SO 5, 10, 12, 15%, GLY 12, 15%	Le Marrec-Croq et al. [33]
	C. virginica	Gametes	Controlled slow-freezing	0, 5, 10, 15, 20, and 25% PG/ 0.5 M Me2SO, 1.75 M Me2SO, or 2 M Me2SO	Paniagua-Chávez et al. [37]
LIPIDS	*Junceela fragilis*, *J. juncea* and *Ellisella robusta*	Oocytes	Chilling	–	Lin et al. [48]
	Mytilus trossulus and *Strongylocentrotus intermedius*	Trochophore stage/ blastula stage	Two-step freezing	Me2SO 6%, trehalose 15 mg/ml, MLE 0.085–0.15% and antioxidants	Odintsova et al., [40]
	Seriatopora caliendrum and *Pocillopora verrucosa*	Larvae	Chilling	1 M PG, 1 M EG	Cirino et al. [20]
	P. maximus	Heart cells	Two-step freezing	Me2SO 5, 10, 12, 15%, GLY 12, 15%	Le Marrec-Croq et al. [33]
ATP	Symbiodiniaceae	Clade D	Two-step freezing	2 M PG	Thongpoo et al. [26]
	Junceella fragilis	Clade G *Symbiodinium*	CPA toxicity	1, 2, 3, 4, and 5 M DMSO, Gly, EG, MeOH, PG	Lin et al. [56]
	Exaiptasia diaphana	*Breviolum sp.*	Two-step freezing	2 M MeOH, 2 M PG	Li et al. [58]
	Echinopora spp.	Oocytes	CPA toxicity	0.25, 0.5, 2, 3, 4, and 5 M MEOH, DMSO, EG, PG	Tsai et al. [52]
	Echinopora spp.	Oocytes	Chilling	0.5 M MeOH, 1 M MeOH or 2 M MeOH	Tsai et al. [54]
ROS	*M. galloprovincialis*	Trochophore larvae	Controlled slow-freezing	10% EG + 7.5% Ficoll + 0.2% polyvinylpyrrolidone	Liu et al. [35]
	C. gigas	Trochophore larvae	Controlled slow-freezing	10% EG + 5% Ficoll + 0.2% polyvinylpyrrolidone	Liu et al. [66]
PROTEIN	*M. galloprovincialis*	Oocytes	Controlled slow-freezing	1.5 M DMSO or 1.5 M EG	Blanco et al. [22]
	H. tuberculata	Mantle cells	Two-step freezing	10% Me2SO, 10% GLY	Poncet et al. [34]
	Exaiptasia diaphana	*Breviolum sp.*	Two-step freezing	2 M MeOH, 2 M PG	Li et al. [58]
	C. angulata	D-larvae	Controlled slow-freezing	10% DMSO + 1% polyvinylpyrrolidone + 0.2 M Sucrose	Anjos et al. [72]

Table 2 (continued)

Molecular Component	Species	Biomaterial	Cryopreservation Method	Freezing component	Reference
RNA	*M. trossulus*, *Mizuchopecten yessoensis* and *S. nudus*	Primary cell	Two-step freezing	10% DMSO or/and trehalose (3-30 mg/ml)	Odintsova et al., [82]
	H. discus hannai	Sperm	Two-step freezing	8% Me2SO, 8% EG, 6% PG, 2% GLY, and 2% MeOH	Hossen et al. [21]
	H. tuberculata	Mantle cells	Two-step freezing	10% Me2SO, 10% GLY	Poncet et al. [34]
	H. discus hannai	Sperm	Two-step freezing	8% DMSO + 3% sucrose, 8% EG + 1% glucose, 6% PG + 2% glucose, 2% GLY + 3% glucose and 2% MeOH + 4% trehalose	Hossen et al. [64]
	C. angulata and *Chamelea gallina*	D-larvae	Controlled slow-freezing	10% EG + 1% polyvinylpyrrolidone + 0.2 M sucrose or 10% MeOH + 1% polyvinylpyrrolidone + 0.2 M sucrose	Anjos et al. [63]
	C. gigas	Trochophore larvae	Controlled slow-freezing	10% EG + 5% Ficoll + 0.2% polyvinylpyrrolidone	Liu et al. [66]
	M. galloprovincialis	Trochophore larvae	Controlled slow-freezing	10% EG + 7.5% Ficoll + 0.2% polyvinylpyrrolidone	Liu et al. [35]

Overview of the different development stages, cryopreservation techniques and relative CPA used

DMSO (Me2SO), Dimethyl sulfoxide; *EG*, Ethylene glycol; *PG*, Propylene glycol; *GLY*, glycerol; *MeOH*, methanol

of *H. tuberculata* exposed to 10% DMSO and glycerol reported no significant change in total protein content, suggesting a limited toxic effect at these concentrations [34]. Although not directly focused on protein content, a study on crustacean embryos showed that cold exposure and cryoprotectant treatments modulate the expression of specific glycans [76]. In particular, the research focused on glycans that bind to lectin proteins, indicating that changes in glycan profiles may also reflect functional modifications in associated proteins. Recent studies indicate that cryopreservation can also significantly affect the molecular profiles in symbiotic dinoflagellates. Li et al. [58] reported changes in protein expression in *Breviolum* sp., while glycan-based analyses in *B. psygmophilum* revealed comparable signs of cryoinjury, reinforcing the value of molecular biomarkers to assess damage and improve preservation strategies [77].

RNA Synthesis Reduction Following Cryopreservation

Cryopreservation induces a reduction in RNA synthesis and expression in some marine invertebrate species; however, data on this topic remain limited. In the sperm of *H. discus hannai*, a marked decrease in mRNA levels of HSP70 and HSP90, associated with sperm motility and intracellular calcium regulation, has been observed [21, 78–80]. Similarly, a reduction in mRNA levels of the ion channel regulating gene PKA-C, also involved in sperm motility, has been reported [21, 81]. In primary cell cultures of the mantle of *H. tuberculata*, mRNA synthesis, measured by [^{3}H]uridine incorporation, was significantly reduced following freeze, thaw treatment [34]. In sea urchins as well, RNA synthesis varies according to developmental stage; the gastrula stage appears to be less tolerant to cold treatment compared to the blastula stage, showing lower RNA synthesis levels after thawing [82]. Cells respond to cryopreservation-induced stress by modulating the expression of protective genes, particularly those involved in oxidative stress and apoptosis. English and Storey [83] reported a upregulation of metallothionein (MT) gene expression in *Littorina littorea* exposed to both anoxia and freezing, suggesting a key role in neutralizing ROS generated during low-temperature stress. Several studies have reported changes in antioxidant enzyme activity (superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase) following cryopreservation, though results vary, ranging from decreased activity [64, 84], to increases [66], or even no change at all [35]. In *C. angulata*, for instance, cryopreservation led to a decrease in SOD levels, counterbalanced by increased GPx activity to reduce ROS accumulation [63]. These findings suggest that redox responses are species-specific and depend on both the cell type and the freezing protocol used [35, 85]. In marine larvae, high lipid reserves, although essential for development, also make them more vulnerable to oxidative stress [62, 66]. Therefore, the development of cryopreservation protocols must always be species-specific [11].

Cryopreservation techniques

Three are the principle techniques used in cryopreservation: controlled slow-freezing, two-step freezing (ultra-rapid freezing) and vitrification [86]. Slow-freezing is a controlled process in which cells are gradually cooled below their freezing point, allowing water to exit the cell and thus

reducing intracellular ice formation [11]. This gradual dehydration, achieved with low CPA concentrations and regulated cooling rates, helps minimize cryoinjury [1]. Two-step freezing is considered a intermediate strategy between slow-freezing and vitrification, in which samples are first exposed to liquid nitrogen vapor (approximately –25 °C) to equilibrate cellular osmotic pressure and promote water efflux in the presence of CPAs [58]. Finally, they are transferred to cryogenic temperature of –196 °C for long-term storage [25]. This approach, avoid the use of a programmable machines as in the slow-freezing and employs low CPA concentrations compared to vitrification [86]. Vitrification is a recent and promising cryopreservation technique that relies on the glass-like solidification of highly concentrated CPA solutions, thereby preventing ice crystal formation [11, 87, 88]. This outcome is achieved through the combined use of an ultra-rapid cooling, and fast warming [89]. Since high CPA concentrations are toxic to cells, an equilibration step is typically required, in which samples are gradually exposed to increasing CPA concentrations prior to vitrification [87]. The molecular targets analyzed and the cryopreservation protocols applied are summarized in Table 2. Two-step freezing emerged as the most commonly used technique, followed by controlled slow-freezing, with DMSO and ethylene glycol (EG) being the most frequently utilized CPAs for marine invertebrate, although their concentrations varied depending on the species and sample type. Most studies were conducted on mussels across different developmental stages [90]. Research focusing on ATP assessment and lipids profile mainly represents the works carried out on corals; however, no cryopreservation protocol has been conducted, and the studies focused on chilling and CPA toxicity.

Summary

Cryopreservation, although a valuable technique in the field of biological conservation, involves complex procedures (such as the addition/removal of CPAs, freezing, and thawing) that expose cells to extreme conditions. As a result, the structural and functional integrity of molecular constituents such as nucleic acids, proteins, lipids, and organelles like mitochondria may be compromised; in addition, cell growth can be delayed due to the slow reactivation of metabolism, which is inhibited by low temperatures. For this reason, it is crucial to analyse cellular biomolecules and their changes to better understand and improve post-thaw survival. However, cryopreservation protocols are not easily standardized, and it is important to adopt different approaches depending on the cell type and the species under investigation.

Author Contributions Conceptualization, C.L., P.T. and S.T.; methodology, F.B, P.T. and F.W.K; validation, C.L., Z.H.W. and S.T.; formal analysis, F.B and P.T.; resources, C.L., Z.H.W. and S.T.; writing—original draft preparation, F.B. and C.L.; writing—review and editing, Z.H.W, C.L. and S.T.; visualization, C.L. and S.T.; supervision, C.L. and Z.H.W. ; funding acquisition, C.L. All authors have read and agreed to the published version of the manuscript.

Funding Coral Research and Development Accelerator Platform Foundation, CAP-2023-1391.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

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