

Preserving crustaceans: a structured review of cryopreservation techniques

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Abstract. Cryopreservation of crustaceans is increasingly recognized as a vital tool for maintaining genetic diversity and supporting sustainable aquaculture practices. Despite advancements in cryopreservation techniques for various aquatic species, protocols for crustaceans remain underdeveloped and challenging to implement effectively. This systematic literature review examines the current methodologies and outcomes related to cryopreserving crustacean germ cells, sperm, and larvae. The review encompasses an analysis of different cryoprotectants, freezing and thawing techniques, and their impact on cell viability and recovery. It reveals that the use of vitrification combined with specific cryoprotectants, such as dimethyl sulfoxide and glycerol, has shown promise in improving long-term preservation of shrimp germ cells and sperm. Additionally, programmable freezing techniques and optimal thawing conditions have contributed to higher post-thaw survival rates in crustacean larvae. To achieve this, we conducted an extensive search of scholarly articles from reputable databases such as Scopus and Web of Science, focusing on studies published between 2019 and 2024. The flow of study was based on PRISMA framework. A total of 23 final primary data entries identified from the database were analyzed. However, significant challenges remain, including cryopreservation-induced damage and the need for refined protocols to enhance efficacy and reproducibility. The findings highlight that while there has been notable progress, achieving reliable and commercially viable cryopreservation methods for crustaceans requires continued research and technological development. The review concludes that establishing standardized and effective cryopreservation protocols is crucial for the conservation of crustacean genetic resources and the advancement of sustainable aquaculture practices. Future research should focus on overcoming current limitations and developing practical applications to ensure the successful implementation of these techniques.

Key Words: crustacean, cryopreservation, cryoprotectants, PRISMA framework.

Introduction. Cryopreservation, the process of storing biological materials at ultra-low temperatures to halt biological activity and preserve genetic material, has emerged as a transformative technique in the field of crustacean biology. This method is of paramount importance for the conservation of genetic diversity, management of aquaculture stocks, and advancement of scientific research (Whaley et al 2021; Patel & Lukose 2023). Crustaceans, including commercially valuable species like shrimp, crabs, and lobsters, are pivotal to global aquaculture and marine ecosystems. However, they face significant threats from overfishing, habitat destruction, and climate change, which necessitates innovative approaches for their preservation and management (Mohan et al 2021; Lin et al 2023; Zhang et al 2023). Cryopreservation provides a viable solution by enabling the long-term storage of genetic resources such as sperm and embryos, which are crucial for maintaining and improving genetic diversity (Liu et al 2019; Diwan et al 2020).

Current research on crustacean cryopreservation has made significant strides, yet it remains a developing field. Early studies laid the groundwork by establishing fundamental principles and protocols for cryopreservation in crustaceans. For instance, research of Castelo-Branco et al (2015) demonstrated successful sperm cryopreservation in white shrimp (*Litopenaeus vannamei*), using dimethyl sulfoxide (DMSO) as a

cryoprotectant. Similarly, explored embryo cryopreservation techniques in the blue crab (*Callinectes sapidus*), emphasizing the importance of optimizing cooling and thawing rates to achieve high post-thaw viability. These studies have provided valuable insights into the effectiveness of various cryoprotectants and protocols, contributing to the development of standardized practices (Koopman & Siders 2013).

Despite these advancements, several gaps and unresolved issues persist in the literature. One major challenge is the variability in cryopreservation success rates among different crustacean species, which often stems from species-specific differences in physiology and cryoprotectant response (Asturiano et al 2017; Diwan et al 2020; Bøe et al 2021). Additionally, there is a lack of comprehensive protocols that address the diverse needs of various crustacean species and their developmental stages. Controversies also exist regarding the optimal concentrations and combinations of cryoprotectants, as well as the most effective cooling and thawing methods. These issues highlight the need for more detailed research to refine existing protocols and develop new methodologies that can be universally applied (Martínez-Páramo et al 2017; Raju et al 2021). This article aims to address these gaps by investigating a comprehensive cryopreservation protocol tailored for crustaceans. The primary research question guiding this study is: how can cryopreservation techniques be optimized to improve the viability and functionality of genetic material across a broad range of crustacean species? By examining current methodologies and proposing refinements, the article seeks to enhance the effectiveness and applicability of cryopreservation protocols.

The objectives of this article are twofold. First, it aims to provide a detailed analysis of existing cryopreservation techniques, highlighting their strengths and limitations. Second, it proposes a refined protocol based on recent advances and emerging technologies in cryobiology. By addressing these objectives, the article contributes to advancing knowledge in the field of crustacean cryopreservation, offering practical solutions for genetic conservation and sustainable aquaculture practices. The findings are expected to provide valuable insights for researchers and practitioners, ultimately supporting the conservation and management of crustacean populations in an increasingly challenging environmental landscape.

Material and Method. The research was conducted between September and November 2024 at Universiti Sultan Zainal Abidin (UniSZA), Terengganu and Politeknik Jeli, Kelantan, Malaysia.

Identification. Important procedures from the systematic review approach were used in this study to collect a sizable number of pertinent materials. After choosing keywords, similar terms were found by scanning dictionaries, thesauri, encyclopedias, and previous research. Search strings for Scopus and Web of Science databases were developed when all pertinent phrases were found (Table 1). Scopus and Web of Science are two of the most authoritative and comprehensive databases used for systematic literature reviews, and their inclusion in our research significantly enhances the credibility and depth of our review. Scopus is renowned for its expansive coverage of scientific journals, conference proceedings, and patents, making it a valuable resource for accessing a broad spectrum of literature across various disciplines. Its robust citation tracking and analytical tools further enable a detailed examination of research trends and the impact of individual studies (Sánchez et al 2017; Pranckuté 2021). On the other hand, Web of Science is distinguished by its meticulously curated indexing of high-quality journals and its extensive citation network, which facilitates in-depth tracking of citation relationships and the historical development of research topics. The combination of these databases provides a comprehensive and nuanced view of the literature, ensuring that our systematic review is both thorough and reflective of the most significant contributions and emerging trends in the field. A total of 571 papers from the two databases that were relevant to the study issue were found during the first phase of the systematic review.

Table 1

The search strings

<i>Databases</i>	<i>Key words</i>
Scopus	TITLE-ABS-KEY ((cryopreservation OR preserve) AND ("crustacean")) (Date of access: September, 2024)
Wos	(cryopreservation OR preserve) AND ("crustacean") (Topic) (Date of access: September, 2024)

Screening. During the screening phase, research items that appear relevant are assessed to ensure they meet the predefined research questions. This process typically involves selecting studies related to the cryopreservation protocols in crustaceans and removing any duplicates. Out of an initial 571 publications, 141 were retained for further analysis based on specific inclusion and exclusion criteria outlined in Table 2. The criteria focused on literature that provides practical recommendations, such as journal articles in recent studies. The review was confined to English-language publications from 2019 to 2024, and 13 publications were discarded due to duplication.

Table 2

The selection criteria are searching

<i>Criteria</i>	<i>Inclusion</i>	<i>Exclusion</i>
Language	English	Non-English
Time line	2019-2024	< 2019
Literature type	Journal (article)	Conference, book, review
Publication stage	Final	In press

Eligibility. In the third phase, referred to as the eligibility phase, 128 articles were readied for review. During this stage, the titles and key content of each article were meticulously assessed to confirm they met the inclusion criteria and were relevant to the current research objectives. As a result, 105 articles were excluded because they were outside the field of study, had titles that were not sufficiently relevant, contained abstracts unrelated to the research goals, or lacked full-text access with empirical evidence were retained for the next stage of the review.

Data abstraction and analysis. An integrative analysis was employed as one of the assessment techniques in this study to evaluate and synthesize various research designs, particularly quantitative methods. The aim of this thorough investigation was to identify relevant topics and subtopics. The data collection phase marked the initial step in theme development. Figure 1 illustrates how the authors carefully analyzed 23 publications for information pertinent to the study's topics. Subsequently, they assessed significant studies related to cryopreservation protocols in crustaceans, examining both the methodologies used and the research findings. The authors collaboratively analyzed the selected studies with co-authors to generate thematic categories, ensuring that each theme was directly supported by empirical evidence and aligned with the study's research objectives and conceptual framework. Throughout the data analysis process, a log was maintained to capture analyses, viewpoints, questions, or other reflections relevant to interpreting the data. Finally, the authors compared their findings to identify any inconsistencies in theme development. Any disagreements in concepts were discussed among the authors.

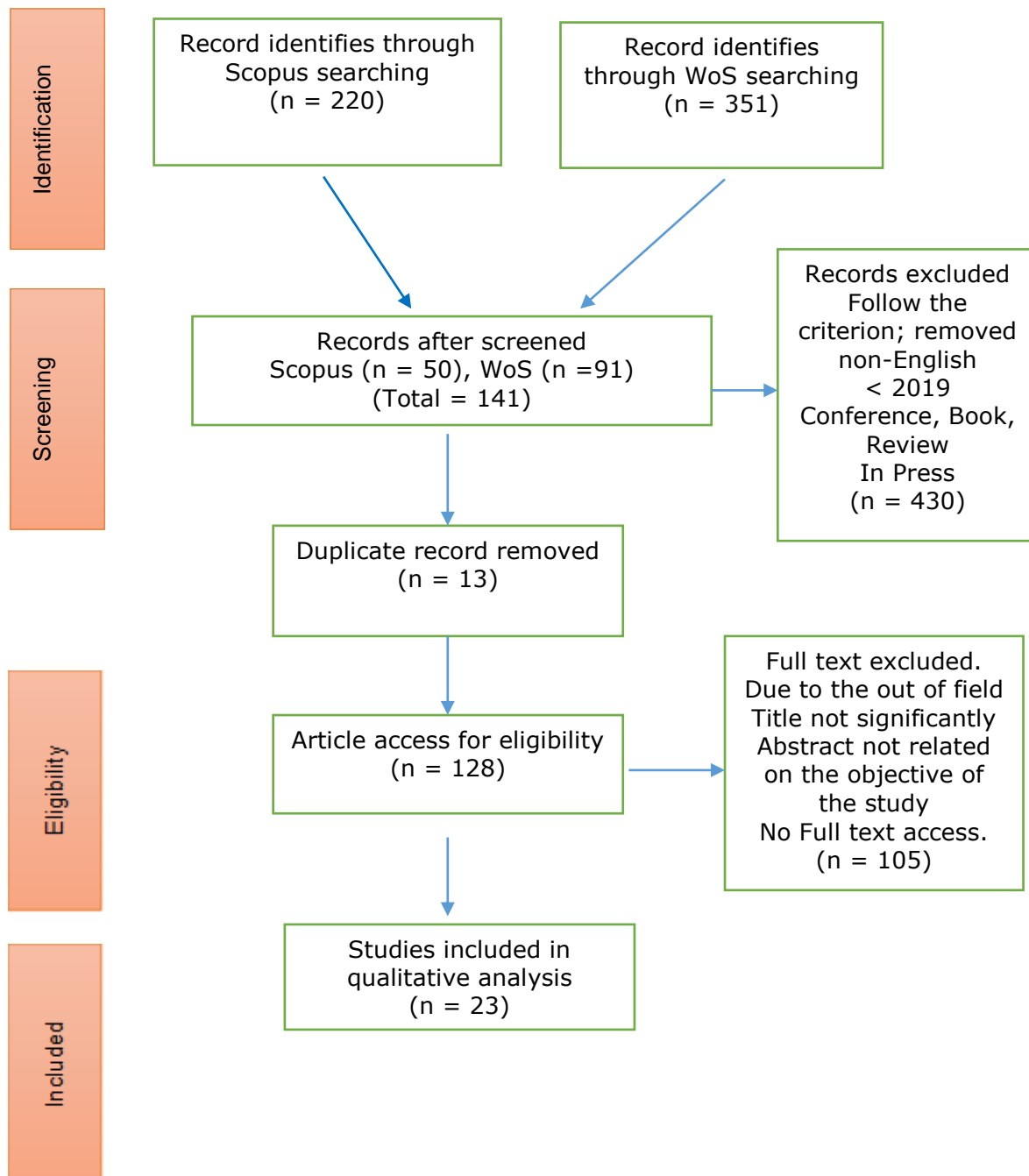


Figure 1. Flow diagram of the proposed searching study.

The authors also compared the findings to resolve any discrepancies in the theme creation process. Note that if any inconsistencies on the themes arose, the authors address them with one another. Finally, the developed themes were tweaked to ensure their consistency. To ensure the validity of the problems, the examinations were performed by two experts, one specialising in breeding technology and the other in cryobiology science. The expert review phase helped ensure each sub-theme's clarity, importance, and adequacy by establishing domain validity. Adjustments based on the discretion of the author based on feedback and comments by experts have been made.

The question is as follows: how do different cryopreservation techniques and formulations impact the viability and quality of preserved aquatic crustaceans compared to standard methods?

Results and Discussion

Cryopreservation and preservation techniques. Cryopreservation of crustaceans is an emerging field that addresses the need for effective preservation methods for various developmental stages and reproductive cells of economically important species. This literature review synthesizes recent findings and advancements in cryopreservation techniques for crustaceans, focusing on germ cells, larvae, and sperm. Recent research highlights the optimization of cryopreservation methods for different crustacean species. Nimrat et al (2020) and Rakbanjong et al (2021) investigated cryopreservation techniques for shrimp species, particularly *Fenneropenaeus merguensis* and *Penaeus monodon*. Nimrat et al (2020) compared slow freezing and vitrification methods, discovering that 10% dimethyl sulfoxide (DMSO) with vitrification was effective for *F. merguensis*, while 10% glycerol (GLY) was better for *P. monodon*. Rakbanjong et al (2021) corroborated these findings, emphasizing the suitability of vitrification and specific cryoprotectant concentrations for long-term germ cell preservation. These studies underscore the importance of selecting appropriate cryoprotectants and techniques to enhance the viability and recovery rates of preserved crustacean cells.

The technical challenges of cryopreservation are well-documented across various studies. Chang et al (2023) explored sperm cryopreservation in *Portunus trituberculatus*, noting significant reductions in sperm viability and enzymatic activity post-thaw, despite optimizing cryoprotectant concentrations and cooling programs. These findings highlight the ongoing challenges in minimizing cryopreservation-induced damage and optimizing protocols for different crustacean species. The future of cryopreservation in crustaceans includes addressing institutional and technical needs for broader application. Guo & Weng (2020) reviewed the impact of cryopreservation on various aquatic invertebrates, detailing issues such as ice crystal formation, cryoprotectant toxicity, and osmotic pressure effects. Their review stresses the need for improved techniques to mitigate freezing and thawing damage. Haagen & Blackburn (2024) expanded on these challenges by discussing the establishment of a shrimp germplasm bank in the United States. They highlighted the importance of developing validated cryopreservation methods for embryos, larvae, and spermatophores and the necessity of integrating these techniques into a national repository. Their work underscores the need for continued research and stakeholder engagement to advance cryopreservation practices and ensure the preservation of genetic diversity.

Recent studies have demonstrated significant progress in optimizing cryopreservation techniques for crustaceans. Nimrat et al (2020) developed a comprehensive cryopreservation protocol for banana shrimp (*F. merguensis*), evaluating both programmable controlled-rate and practical methods. Their research identified 5% DMSO as an effective cryoprotectant, with the best results achieved through a one-step freezing process at $-2^{\circ}\text{C min}^{-1}$ from 25 to -80°C , followed by storage in liquid nitrogen. This study also explored the efficacy of plant extracts, such as moringa and ginger, in reducing bacterial contamination in cryopreserved spermatophores, highlighting a novel approach to maintaining sperm viability during long-term storage. Similarly, Fatihah et al (2016) developed a sperm cryopreservation protocol for the mud spiny lobster (*Panulirus polyphagus*), focusing on optimizing cryoprotectant concentrations and cooling rates to enhance sperm viability. These studies collectively underscore the advancements in cryopreservation techniques but also indicate the need for species-specific adaptations to achieve consistent results. Phosphate buffer and 5% glycine are the most effective extenders and cryoprotectants for maintaining the viability of *Scylla tranquebarica* sperm cells during short-term storage, demonstrating significant initial viability and gradual decline over time (Fatihah et al 2024). In addition, the study concludes that 10% glycine is the most effective cryoprotectant for preserving the sperm of *S. tranquebarica*, achieving the highest viability at 84.75% after 60 minutes of exposure, while recommending cold preservation methods for future breeding and biochemical assessments (Fatihah et al 2018). These findings provide valuable insights for improving sperm preservation techniques in mud crabs.

The strengths of these studies include their thorough approach to optimizing cryoprotectant agents and cooling protocols. Nimrat et al (2020) and Fatihah et al (2016) provided valuable insights into the practical applications of cryopreservation techniques, offering protocols that are relevant for both research and commercial applications. However, a limitation of these studies is their focus on specific species, which may not be directly applicable to other crustaceans with different physiological and biochemical characteristics. Furthermore, while short-term efficacy is well-documented, long-term impacts on genetic integrity and reproductive success are less explored. Additional research has focused on understanding the effects of cryopreservation on crustacean physiology and development. Piazza et al (2022) investigated the effects of cold storage on the nauplii of *Amphibalanus amphitrite*, revealing that short-term storage at 4°C did not significantly affect mortality but altered immobility and swimming activity. This study highlights the importance of considering various endpoints when evaluating cryopreservation protocols and suggests that while short-term storage can be effective for certain applications, it may not be suitable for all types of bioassays or long-term studies. The research by Piazza et al (2022) emphasizes the need for further investigation into the effects of cold storage and cryopreservation on different life stages and endpoints. Haagen & Blackburn (2024) emphasized the necessity of establishing shrimp germplasm banks and developing cryopreservation methods that ensure larvae reach reproductive maturity. This highlights the need for continued research into the long-term viability and reproductive potential of cryopreserved crustaceans.

Conclusions. This comprehensive review highlights the multifaceted efforts to understand and preserve crustacean biodiversity through integrative approaches that span cryopreservation technologies. The advancements in cryopreservation techniques, particularly for economically important species such as *Penaeus monodon* and *Fenneropenaeus merguensis*, underscore the potential for safeguarding genetic resources through optimized protocols involving cryoprotectants like DMSO and glycerol. However, persistent technical challenges - such as cryoprotectant toxicity, ice crystal formation, and reduced post-thaw viability - signal the need for continued refinement and broader institutional investment, as exemplified by the development of shrimp germplasm banks.

Collectively, these interdisciplinary investigations demonstrate that preserving crustacean biodiversity requires a holistic approach. By integrating these fields, researchers and stakeholders can develop more resilient conservation strategies that ensure the long-term sustainability of crustacean species in both natural and managed ecosystems.

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Conflict of interest. The authors declare that there is no conflict of interest.

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