Review of Molluscan Larval Cryopreservation and Application to Germplasm Cryobanking and Commercial Seed Production

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Running title: Review of molluscan larval cryopreservation

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Abstract

This review is focused on the status and summary of larval cryopreservation in aquaculture mollusks. A total of 26 publications were identified addressing 14 molluscan bivalve species. Most studies were conducted on trochophores and D-larvae, a few studies were on umbo larvae, and no study was found on pediveliger. Based on the post-thaw viability, there is no general conclusion about the best larval stage for cryopreservation. The research topics of most publications focused on developing or improving cryopreservation protocols through exploring one or more vital factors of the cryopreservation procedure. The most dominant cryoprotectant agents (CPAs) used for molluscan larval cryopreservation were dimethyl sulfoxide (DMSO), propylene glycol (PG), and ethylene glycol (EG) at final concentrations of 5-15%. Non-permeable CPAs, such as glucose, fructose, sucrose, trehalose, and polyvinylpyrrolidone (PVP), were dominantly used as additives. Most cooling processes started from 0 °C, 4 °C, or larval culture temperature to -10 °C or -12 °C at -1 °C min^{-1} and holding at this temperature for 5-15 min with or without "seeding", and then continued to cool to around -35 °C at -0.3 to -2.5 °C min^{-1} (mostly at -0.5 and -1 °C min^{-1}) followed by a 5-10 min holding period. All frozen samples were then transferred into liquid nitrogen (-196 °C) for long-term storage. Evaluation of post-thaw viability was reported over a wide range of parameters with no standard criteria, for example, motile post-thaw larvae were considered alive but no definition of swimming velocity. A sharp decrease of survival was often reported after a period of culture although immediate post-thaw survivals were reported high in several studies. Overall, no general optimal protocol could be concluded. Post-thaw larvae were reported to survive beyond metamorphosis in Pacific oysters and even to the adult stage and reproduced successfully after 3 years of culture, indicating the promising future of this technology. Till now, no study has been reported on germplasm cryobanking by use of molluscan larval cryopreservation. Pathways and aspects for promoting molluscan larval cryopreservation technology for germplasm repository establishment and commercial seed production were discussed.

Key words: Mollusk, larval cryopreservation, germplasm repository, out-of-season seed production, review.

1. Introduction

Cryopreservation is a technology through which biological materials are frozen at a low temperature (usually at -196°C in liquid nitrogen). Since the first report (Polge et al., 1949), this technology has been developed into an effective medical treatment for infertility in humans (Di Santo et al., 2012) and into a multi-billion-dollar industry for artificial insemination in livestock (Ugur et al., 2019). The concepts, history, principles, and development of cryopreservation technology have been reviewed in previous publications (Mazur, 1970; Mazur, 1984; Pegg, 2002; Walters et al., 2009; Yang and Tiersch, 2020). For aquaculture species, cryopreservation technology has been studied for over 200 species of fish (majorly salmonids and cyprinids) (Tiersch et al., 2007; Martinez-Paramo et al., 2017), 27 species of molluscan shellfish (Liu et al., 2015; Yang, 2017), and 35 species of macroalgae (Yang et al., 2021). Past research has majorly focused on developing cryopreservation protocols or addressing certain factors of the cryopreservation procedure. Applications of cryopreservation technology for establishment of germplasm repositories or commercial aquaculture production have been limited (Martinez-Paramo et al., 2017). In 2019, FAO published the first report as guideline to address the use, exchange, cryopreservation, and policy of aquatic genetic resources (FAO, 2019)

Globally, molluscan aquaculture is the primary component of marine production. The annual molluscan production worldwide in 2018 was 17.5 million tonnes, accounting for 56.8% of the total marine aquaculture production (30.8 million tonnes) with a 3.5% annual growth between 2000-2017 to support global seafood markets (FAO, 2020). Molluscan aquaculture includes about 65 reported species, primarily marine bivalves, including clams (together with cockles and ark shells), oysters, mussels, scallops, and pearl oysters (Yang et al., 2016a; Naylor et al., 2021). Additionally, molluscan aquaculture plays a significant role in ecosystem service to accumulate nitrogen and phosphorus due to the filter-feeding lifestyle of molluscan bivalves and can provide habitat structure and coastal stabilization (Naylor et al., 2021).

In general, molluscan bivalves have two reproductive modes (Verdonk et al., 1983): 1) broadcast spawning (most species of oysters, mussels, clams, and scallops), a form of sexual reproduction through releasing gametes into the surrounding water environment for fertilization, and 2) spermcast spawning (e.g., *Ostrea* oysters), a form of sexual reproduction in which males broadcast sperm, females inhale sperm for fertilization of retained oocytes, and then brood

developing embryos until they are released as larvae. Aquaculture abalones (gastropod) also use broadcast spawning for their reproduction. After fertilization and embryo development, molluscan bivalves and abalones have a swimming larval stage, which usually lasts for about two weeks (this could be different depending on species and geographical distribution), and a metamorphosis stage before turning into juveniles with lifestyles like the adults. Considering these reproductive characteristics, germplasm materials for cryopreservation in cultured molluscan species would include sperm, oocytes, embryos, and free-swimming larvae.

Germplasm cryopreservation in mollusks was first reported for sperm cryopreservation of the Pacific oyster *Crassostrea gigas* in 1971 (Lannan, 1971). To date, over 80 publications have been published and the studied species were exclusively aquaculture species, including oysters, mussels, scallops, clams, and abalones (Liu et al., 2015; Yang, 2017) with the majority on oysters (Hassan et al., 2015). These studies primarily focused on developing laboratory protocols instead of establishment of germplasm repositories or commercial use.

Sperm was the most studied germplasm in mollusks for cryopreservation in 20 species (Liu et al., 2015; Yang, 2017). Different cryopreservation protocols were reported with varied post-thaw survival. Overall, dimethyl sulfoxide (DMSO), with or without sugar additives, was predominantly used as the cryoprotectant, and propylene glycol (PG) or ethylene glycol (EG) were also used as cryoprotectants in several studies. For sperm collection, seawater (natural or artificial) or calcium-free buffers were mostly used. Cooling of samples were performed by use of programmable freezers or many different homemade methods, such as use of nitrogen vapor in storage/shipping dewars or Styrofoam boxes (Hu et al., 2017; Huo et al., 2021, in review). Overall, cryopreservation sperm from mollusks reported a post-thaw motility ranging from 0 to 50% among the different species or different reports for the same species. The fertility of post-thaw sperm was reported from 0 to 97%, with different sperm-to-oocyte ratios for fertility testing (Liu et al., 2015).

Oocyte cryopreservation in mollusks were reported only in a handful species with limited success. In *Perna canaliculus*, cryopreserved oocytes yielded less than 1% of post-thaw survivals to D-stage larvae (Adams et al., 2009), and the addition of oxidants (α-tocopherol at 0.1mM plus 1 mM taurine or 0.1 mM EDTA) in cryomedium (solution used to suspend germplasm samples for cryopreservation) showed effects to ameliorate oxidative stress in oocyte during cooling process (Gale et al., 2015). In Pacific oysters, cryopreserved oocytes yielded 0-30% survival to

D-stage larvae (Tervit et al., 2005), and 0-25% post-thaw fertilization (Naidenko, 1997; Smith et al., 2001). In *Haliotis diversicolor diversicolor*, osmometric characteristics of oocytes showed different permeability of five cryoprotectants PG > DMSO > acetamide > ethylene glycol (EG) > > glycerol (Lin et al., 1992), and cryopreserved oocytes yielded a hatching rate of 24% (Yang et al., 2013).

For embryo cryopreservation in mollusks, survival of post-thaw late embryo/early larvae were reported as 64% in *Ruditapes philippinarum* (Kang, 2021), 72-78% in *Meretrix lusoria*, and 62-75% in *Crassostrea gigas* (Chao et al., 1997), but no post-thaw survival was achieved in small abalone *Haliotis diversicolor diversicolor* (Lin and Chao, 2011). Comparison of cryopreservation at different embryo stages in *Crassostrea gigas* revealed that morula and gastrula stage embryos are less tolerant to cryoprotectants than the trochophore stage, while 2- to 8-cell embryos did not survive after thawing (Gwo, 1995), and similar results were reported in *Mytilus edulis* (Toledo et al., 1989).

This review is focused on summarizing the research advances in molluscan larval cryopreservation. Literature collection was made through searching of database of “web of sciences core collection”, “PubMed”, and “google scholar” using key words: oyster, scallop, mussel, clam, abalone, mollusk, cryopreservation, larvae, trochophore and pediveliger with different strategic combinations for the search. Collected literatures were saved in an Endnote library and further sorted to identify the related literature. Additionally, more papers were identified and added to the collection by reading the bibliography list of the literatures in collection. Readers interested in molluscan sperm, oocyte, or embryo cryopreservation are referred to other review publications (Gwo, 2000; Chao and Liao, 2001; Tiersch et al., 2007; Hassan et al., 2015; Liu et al., 2015; Diwan et al., 2020).

In this review, we will summarize the status of molluscan larval cryopreservation, analyze the reported research protocols, provide insights into the cryopreservation mechanisms, discuss the aspects for promoting larval cryopreservation technology for germplasm repositories and, most importantly, investigate the potential use of larval cryopreservation for commercial seed production for the aquaculture industry.

2. Summary of Molluscan Larval Cryopreservation

Swimming larvae is a unique life stage for molluscan bivalves and abalones. In general, the swimming larvae stage is about two weeks after fertilization, and larval size changes from 60-175 µm (starting to swim) to 200-300 µm (ready for metamorphosis). The larval stages are termed as follows in this review (**Figure 1**).

1) Trochophore: After fertilization, embryos go through multiple cell divisions and develop into motile larvae with a row of cilia around the middle and a long apical flagellum which enable them to swim (Figure 1A).
2) D-stage: Larvae, after shell formation, are referred to as D-stage. D-stage larva has a complete digestive system and a velum for swimming and feeding by use of the cilia (Figure 1B).
3) Umbo larvae: As D-stage larvae continue to grow, the shells near the hinge became protuberated after 7-10 days. Larvae at this stage are called umbo stage larvae (Figure 1C).
4) Pediveliger: When a foot develops and gill rudiments become evident, the larvae are called pediveliger. Soon, a small dark circular dot develops near the center, larvae at this stage are called eye-larvae at 250-300 µm (Gosling, 2003), and ready for metamorphosis (Figure 1C).

The trochophore and early D-stage larvae of molluscan bivalves are about 60-175 µm depending on species (Helm et al., 2004). This small size provides advantages for the penetration of conventional cryoprotectants during the cooling process (Fuller et al., 2004) and makes larval cryopreservation feasible. Although there are shells formed from D-stage larvae, the swimming behavior of larvae can allow the interaction of cells/tissues with cryoprotectants because D-stage larvae continued to swim after mixing with cryoprotectant medium (personal observation).

To date, a total of 14 molluscan bivalves from 5 families have been studied on larval cryopreservation and a total of 26 publications were identified (**Table 1**). Two studies reported toxicity of cryoprotectants only without sample cryopreservation (Choi and Nam, 2014; Heres et al., 2019), and thus were not included in the summary. No research has been reported on abalone larval cryopreservation. The first report on molluscan larvae cryopreservation was on trochophores of *Mytilus edulis* using a two-step controlled rate cooling method and DMSO as cryoprotectant (Toledo et al., 1989).

Molluscan larvae at different developmental stages posses different cryoresistance (Gwo, 1995), therefore, selection of suitable developmental stages is vital for the success of

cryopreservation (Paredes et al., 2012; Paredes et al., 2013; Liu et al., 2020b; Liu et al., 2020a). To date, larval cryopreservation in most studies has focused on trochophores (21 publications) and D-larvae (14 publications), a few studies were conducted on umbo larvae (5 publications), and no study was found on pediveliger (**Table 1**). The oldest larval stage for cryopreservation was late umbo veliger at 12 days post fertilization of *Crassostrea gigas* (Choi and Nam, 2014), umbo at 12 days post fertilization of *Pinctada fucata* (Choi and Chang, 2003), and umbo at 16 days post fertilization of *Spisula sachalinensis* (Choi et al., 2008). The small size of trochophore and early D-stage larvae (60-175µm) could allow better penetration of conventional cryoprotectants better during the cooling process (Fuller et al., 2004; Best, 2015). Embryos and early-stage larvae, with high lipid content prior to natural feeding (Gosling, 2003), would be more sensitive to cryopreservation (Liu et al., 2020b).

Based on the post-thaw viability, no general conclusion was made about the best larval stage for cryopreservation, which could be trochophore larvae (Gwo, 1995; Usuki et al., 2002; Labbe et al., 2018; Liu et al., 2020b; Heres et al., 2021), D-stage larvae (Choi and Chang, 2003; Liu and Li, 2008; Kim et al., 2013; Labbe et al., 2018; Rodriguez-Riveiro et al., 2019; Heres et al., 2020; Liu et al., 2020b; Liu et al., 2020a; Heres et al., 2021), or umbo larvae (Choi et al., 2008; Choi and Nam, 2014). Post-thaw larval survival was found to increase with the age of developmental stage (Choi et al., 2008; Choi and Chang, 2014). Further investigation, with parallel comparison of all larval stages, is needed for toxicity evaluation or post-thaw viability. In view of the post-thaw larval applications, cryopreservation of larvae at later stages could be beneficial because less time would be needed to culture post-thaw larvae to juveniles.

Overall, the research topics of most publications on molluscan larval cryopreservation focused on developing or improving laboratory larval cryopreservation protocols through exploring one or more vital factors of the cryopreservation procedure (**Table 1**). These factors included (1) larval developmental stages and concentrations; (2) type, concentration, and combinations of cryoprotectant agents (CPAs); (3) cooling strategies and processes; (4) thawing processes with post-thaw amendments; and (5) evaluation of post-thaw survival, growth, and reproduction. Although post-thaw larvae of *Crassostrea virginica* were reported to survive to beyond metamorphosis at 4 months of age (Paniagua-Chavez et al., 1998), and post-thaw larvae of *Crassostrea gigas* were reported to survive to the adult stage and reproduced successfully after nearly 3 years of culture in the open sea (Suquet et al., 2014), molluscan larval

cryopreservation in general yielded low post-thaw viability to metamorphosis. Consequently, till now, no study has been conducted on the development of a molluscan larval germplasm repository or the application of cryopreserved molluscan larvae in breeding programs and commercial hatchery seed production.

Cryopreservation includes interconnected steps, including sample collection, selection of cryoprotectants and cryomedium, packaging of samples after mixing with cryoprotectants, cooling process at suitable cooling rates, thawing of frozen samples, and viability assays with post-thaw amendments of samples. Optimization of each cryopreservation step is crucial for protocol development with high post-thaw viability because any error at any step could cause final failure (Leibo and Pool, 2011). Therefore, a detailed summary of research updates on molluscan larval cryopreservation is made according to the cryopreservation procedure for readers to follow easily.

2.1. Collection of larval samples for cryopreservation

Naturally spawning by thermal induction or strip spawning, depending on working species, were used for gamete collection and fertilization. For example, *Mercenaria mercenaria* (Simon and Yang, 2018) and *Mytilus* species (Liu et al., 2020a) need to be thermal induced for natural spawning, while *Crassostrea* species (Paniagua-Chavez et al., 1998; Liu et al., 2020b) can be strip spawned. Upon fertilization, the fertilized eggs were cultured at a concentration of no more than 50-100/ml (Helm et al., 2004) for larval collection at different developmental stages. Specifically, larval collection from spermcast spawning of *Ostrea* species could be directly through dissecting of females (Horvath et al., 2012).

To ascertain good quality larvae for cryopreservation, gamete quality needs to be monitored. Sexual maturity of male and female broodstock could be estimated by visual observation of gonad development and sizes. For sperm, motility and swimming velocity could be estimated by use of microscopic observation, or if necessary, sperm viability analysis could be tested by flow cytometry after dual fluorescent staining of SYBR green and propidium iodide (PI) (Yang et al., 2016b). For oocytes, quality could be estimated by microscopic observation (round shaped oocytes with dark yolk are good quality). Based on our years of experience in working with molluscan gametes and larvae, fertilization rate is an important index to reflect the gamete and

larval quality. However, no fertilization data were reported for larval cryopreservation in the 26 published reports.

After fertilization, larvae can be concentrated by filtering through a 25-μm screen into targeted concentrations at different targeted stages for subsequent cryopreservation procedure. To prevent damages due to oxygen deficiency, highly concentrated larvae can be put on ice or in a 4 °C refrigerator to reduce larval metabolism (Wang et al., 2011; Liu et al., 2020a). For D-larvae and subsequent developmental larval stages, microalgae, such as *Isochrysis* sp. and *Pavlova* sp., need to be supplied as food for larvae to maintain healthy status for successful cryopreservation (Choi and Nam, 2014; Rodriguez-Riveiro et al., 2019).

2.2. Choice of cryoprotectant types and concentration

Cryoprotectant agents (CPAs) are essential for protection of cells during cooling process. For molluscan larval cryopreservation, CPAs included permeable and non-permeable types (**Table 2**), and the most dominant permeable CPAs were dimethyl sulfoxide (DMSO), propylene glycol (PG), and ethylene glycol (EG) at the final concentration of 5-15 %. For non-permeable CPAs, glucose, fructose, sucrose, trehalose, and polyvinylpyrrolidone (PVP) were dominantly used as additives by combination with permeable CPAs (**Table 2**).

It was reported that combination of non-permeable and permeable CPAs could improve the post-thaw larval survival rates in several species, such as sugars with permeable CPAs in *Crassostrea gigas*, *Pinctada fucata martensii* (Usuki et al., 2002; Choi and Chang, 2003; Kim et al., 2013; Heres et al., 2020), Ficoll PM 70 and PVP with EG in *Crassostrea gigas* and *Mytilus galloprovincialis* (Liu et al., 2020b; Liu et al., 2020a). However, combination of permeable and non-permeable CPAs also reported without improvement of post-thaw survivals. For example, combination of 0.2 or 0.4 M trehalose and 1% PVP with 10% EG did not yield improved post-thaw survival of *Crassostrea gigas* trochophores (Paredes et al., 2013), and combination of 0.25 M sucrose with 5%-25% PG did not improve the post-thaw survivals of *Crassostrea virginica* trochophores (Paniagua-Chavez and Tiersch, 2001). It is not completely elucidated how the permeable CPAs and additive non-permeable sugars can or cannot work together to improve post-thaw survivals. Probably, the intercellular ice formation is limited by addition of suitable non-permeable CPAs and the osmotic balance intra- and inter-cellular would be changed (Gao and Critser, 2000).

Choice of cryoprotectants could be based on a toxicity evaluation on fresh larval samples. If CPAs are toxic to fresh larvae, their protection during cooling process would be of no use. Systematic assessment of the acute toxicity of cryoprotectants on fresh samples can screen effective cryoprotectant types and concentrations and save time and effort for subsequent cooling and thawing experiments. Out of the 26 publications, six studies conducted toxicity evaluation of CPAs on fresh larvae (Gwo, 1995; Horvath et al., 2012; Paredes et al., 2012; Paredes et al., 2013; Simon and Yang, 2018; Heres et al., 2021), and, as stated above, two publications did toxicity estimation only without cooling process (Choi et al., 2008; Heres et al., 2019). Based on the toxicity evaluation, glycerol was concluded to be harmful than EG, PG, or DMSO (Gwo, 1995; Simon and Yang, 2018), similar to the results obtained for toxicity evaluation of oyster sperm (Yang et al., 2012). Among EG, PG and DMSO, toxicity showed different results in different publication depending on species, larval stage, and authors. For example, for *Mercenaria mercenaria* DMSO and PG at 5% and 10% had the least toxic effects on fresh D-stage survival within 75 min, while EG at 5% and 10% had fewer toxic effects on fresh D-stage survival within 15 min (Simon and Yang, 2018). For *Venerupis corrugata*, *Ruditapes decussatus* and *Ruditapes philippinarum*, EG and PG showed to be the least toxic cryoprotectants to older clam development stages, whereas DMSO and glycerol were more detrimental with a dose - response relationship (Heres et al., 2021).

Among the commonly used CPAs, EG has been the most often used permeable CPA for molluscan larval cryopreservation (**Table 2**). Majority of the studies used one CPA, such as EG, PG, or DMSO, with different concentrations or non-permeable additives, such as trehalose, Ficoll PM 70, and PVP (see summary in **Table 2**), and parallel comparison was only conducted in a handful reports (Choi et al., 2008; Wang et al., 2011; Choi and Chang, 2014; Simon and Yang, 2018). Overall, 5-10% EG was the widely used CPAs for molluscan larval cryopreservation (**Table 2)**, following by 5-10% DMSO or PG. EG has a lower molecular weight (62.07) than DMSO (78.13) and PG (76.09), and thus has higher permeability which maybe a reason for its use in molluscan larval cryopreservation. It is worth to mention that DMSO has been the most widely used cryoprotectant for marine fish sperm cryopreservation (Martinez-Paramo et al., 2017), molluscan sperm (Yang et al., 2012; Liu et al., 2015), and macroalgae (Yang et al., 2021).

2.3. Solvent and formula for making cryomedium

To make cryomedium, seawater or fresh water (Milli-Q water or distilled water) were used as solvent to mix with CPAs (**Table 2**). In general, the commonly used CPAs for larval cryopreservation have high osmolalities, for example osmolality of PG is 15,200 mOsm/kg, thus, after mixing with cryomedium, the larvae usually face osmotic shocks besides the toxicity of CPAs (Best, 2015). Depending on species, molluscan larvae usually live-in seawater with a salinity from 25 to 33 ppt (full-strength seawater has an osmolality of about 1000 mOsmol/kg). Mixing of larval sample with cryomedium was commonly performed at a 1:1 ratio with pre-made cryomedium with a double strengthened CPA. Use of freshwater as solvent for cryomedium would reduce the osmolality of cryomedium, and thus reduce osmotic shock to larvae after mixing. This could be extremely important for larval cryopreservation because slow cooling rates are commonly employed for larval cryopreservation (see Section 2.3 for details), use of different solvent for cryomedium would yield different "solute effects" (Mazur et al., 1972) which is damage to the cells during cooling process. Direct comparison of freshwater and seawater used for making cryomedium was conducted in only two reports (Usuki et al., 2002; Heres et al., 2020). For the *Perna canaliculus* larval cryopreservation, higher survival rates were obtained when freshwater was used for cryomedium than that when seawater was used (Heres et al., 2020). For the *Crassostrea gigas* larval cryopreservation, different ratios of seawater (1/4, 1/6, 1/8, 1/10, and 1/30) was used to make cryomedium, and larval preserved in the 1/4 seawater medium showed the highest survival at 4 days after thawing (Usuki et al., 2002). It is highly possible that the tolerant capability to salinity range of the working molluscan species would make a difference.

Besides cryomedium osmolarity, pH and other physical or chemical characteristics of cryomedium need to be considered for their compatibility with molluscan larvae after mixing and during cooling process. To date, no research was reported on the effects of cryomedium osmolarity, pH, or other physical and chemical characteristics on post-thaw molluscan larval viability. Based on our thorough review of molluscan larval cryopreservation (which resulted in the current publication), one systematic evaluation was performed on cryomedium osmolality and pH in the author's laboratory for cryopreservation of trochophore and D-stage larvae of *Crassostrea virginica*. Significant progress was made with a high post-thaw larval survival

343 (almost 100%) and the swimming speed of post-thaw larvae was comparable to that of fresh
344 larvae (Unpublished data). One manuscript is in preparation to report the detailed results.
345
346 *2.4. Sample cooling profiles*
347 Cooling rate is considered as one of the most critical factors for cell viability during
348 cryopreservation (Mazur et al., 1972). The optimal cooling rate could be empirically determined
349 depending on germplasm types, cryoprotectants (type and concentration), packaging containers
350 (volume, shape, and material), and other factors (Mazur, 1977; Pegg, 2007). All biological
351 materials and the cryomedia should be completely frozen by single step or multiple step cooling,
352 and then frozen samples can be directly immersed into liquid nitrogen at -196 °C for long-term
353 storage. This approach is often called controlled-rate slow-cooling cryopreservation. Another
354 way of cryopreservation, named 'vitrification', in which samples are cooled at ultra-fast cooling
355 rates to yield a glass-like ice transformation rather than ice crystallization (Fahy et al., 1984). For
356 molluscan larval cryopreservation, only two studies reported vitrification of molluscan larvae,
357 but all failed to produce post-thaw survival (Chao et al., 1997; Choi and Nam, 2014). In the
358 current review, only controlled-rate slow-cooling approach was discussed.
359 A two-step cooling procedure was dominantly applied for molluscan larval cryopreservation
360 (**Table 3**). The first step of cooling was usually to -30 to -40 °C to completely freeze the larvae
361 and cryomedium, and the second step of cooling was to plunge samples into liquid nitrogen. The
362 vital parameters were in the first-step cooling, including: initial temperature, cooling rates, the
363 holding temperature and holding time for seeding or no seeding, the ending temperature before
364 plunging into liquid nitrogen. Overall, most cooling processes started from 0 °C, 4 °C, or larval
365 culture temperature to -10 °C or -12 °C at -1 °C min^{-1}, and holding at this temperature for 5-15
366 min with or without "seeding", and then continued to cool to around -35 °C at -0.3 to -2.5 °C
367 min^{-1} (mostly at -0.5 and -1 °C min^{-1}) followed by a 5-10 min holding period, and then all frozen
368 samples were transferred into liquid nitrogen (-196 °C) for long-term storage. Slow cooling rates
369 can allow cryopreserved cells to have enough time for intracellular water transport during
370 freezing to avoid intracellular ice crystal formation (Mazur, 1970; Mazur et al., 1972; Mazur,
371 1977; 1984). The final temperature of -40 °C was reported to result in low post-thaw larval
372 viabilities (Toledo et al., 1989; Gwo, 1995; Usuki et al., 2002). Later, most research used -35 °C

as the final temperature of the first step cooling process. However, no reports have addressed the mechanism for the difference.

For most studies on molluscan larval cryopreservation, “ice seeding”, an action to induce ice nucleation and growth, was performed at -10 °C or -12 °C for 5-15 min (**Table 3**) by dipping the straws into liquid nitrogen (LN) or using a LN cooled cotton bud (Liu and Li, 2008; Kim et al., 2013; Paredes et al., 2013). During the cooling process, cells are slowly dehydrated because of exosmosis driven by the elevated extracellular osmolality as the extracellular water slowly transforms into ice during a slow cooling process. This allows intracellular water to move across the plasma membrane to minimize intracellular ice formation during the cooling process (Mazur, 1984; Karlsson et al., 1994; He, 2011). For the seeding temperature, it was reported that seeding at a high subzero temperature (e.g., –4 °C) during cooling can release the free energy, and prevent recrystallization-induced cell injury (Huang et al., 2017). Seeding at a temperature close to the melting point of the solution can prevent intracellular ice formation and maximize the post-thaw survival (Trad et al., 1999). The action “ice seeding” is not commonly employed for cryopreservation of molluscan sperm (Hassan et al., 2015; Liu et al., 2015), fish sperm (Tiersch et al., 2007; Yang and Tiersch, 2009), or macroalgae (Yang et al., 2021). For molluscan larval cryopreservation, no studies have addressed the necessity of “ice seeding” and how the temperature was chosen for the “ice seeding”. Further investigations are needed.

2.4. Larval concentration and equilibration with cryomeium before cooling

The germplasm concentration is an important factor for cryopreservation and could affect the effectiveness of cryoprotectants during the cooling process (Tiersch et al., 2007), especially when the concentrations are super high. For molluscan larval cryopreservation, sample concentration could be related to the application of post-thaw samples (see discussion in Section 3.5.). High concentrations would benefit the efficiency of the cooling process and the application of post-thaw samples to breeding programs and commercial seed production, while a minimum concentration enough for reconstitution of a family or a strain would benefit conservation of endangered species.

Overall, molluscan larval concentrations used for cryopreservation varied over a wide range from 1×10^1 to 2.1×10^6 larvae/ml (**Table 4**). As the larval concentration increased, the post-thaw larval survival was reported to decrease in eastern oysters, which was concluded by the

404 authors as the results of accumulated toxic substances and oxygen deficiency (Paniagua-Chavez
405 and Tiersch, 2001). In other studies, larval concentration yielded no significant influences on
406 post-thaw survival. However, it would be better to handle high-concentrated larval samples at
407 low temperatures, such as on ice or in refrigerator at 4 °C, to alleviate larval damage from
408 accumulation of toxic substances and metabolic problems due to oxygen deficiency.
409 Mixing of larval suspension and cryomedium were reported to be performed by a single step
410 for molluscan larval cryopreservation in most publications (**Table 4**). To avoid osmotic shock,
411 adding of cryomedium into larvae suspension was also performed by a gradual manner within a
412 time frame (Usuki et al., 2002; Horvath et al., 2012; Suquet et al., 2012; Suquet et al., 2014).
413 This approach has been widely employed for sperm cryopreservation in livestock (Grötter et al.,
414 2019). For molluscan larval cryopreservation, it was not reported if the gradual addition of
415 cryomedium to larval suspension made any significant differences in post-thaw survival.
416 Equilibration is the period of time from mixing cryomedium with larval suspension to
417 initiation of sample cooling. During the equilibration time, samples need to be packaged into
418 freezing containers, and the cryoprotectant needs to have time to reach equilibration to minimize
419 intracellular water (Pegg, 2002; Pegg, 2007). In general, optimum equilibration time could be
420 different depending on cryoprotectant type, concentration, incubation temperature, larval size
421 and concentration, and other factors. This is a step which was often overlooked and missed to
422 report in most research on cryopreservation (Martinez-Paramo et al., 2017). For molluscan larval
423 cryopreservation, the equilibration time reported in most studies was one single time ranging
424 from 10 min to 30 min, regardless of the species and larval stages (**Table 4**). Only in one
425 research, the effects of equilibration time (20, 40, and 60 min) on post-thaw D-stage larval
426 survival in *Perna canaliculus* was studied and 20 min was considered to be the optimal
427 equilibration time (Heres et al., 2020). Overall, the equilibration time reported in most studies
428 was based on the experimental tests, and no calculations of permeability was conducted for
429 optimal equilibration time. Based on this research status, systematic assessment of equilibration
430 time is warranted by combing with acute toxicity of cryoprotectants on fresh samples.
431
432 *2.5. Sample packaging, sealing, cooling, and storage*
433 Choice of packaging container is important for cryopreservation because the volume, shape,
434 and material type are directly related to sample cooling and warming. Currently, the

commercially available containers include straws (0.25 ml, 0.3 ml, 0.5 ml, 5 ml) made of different materials (polyvinyl chloride for French straw, and ionomeric resin for CBS™ straw), cryovials made of polypropylene, and blood freezing bags made of polyvinyl chloride or ionomeric resin. Choice of packaging containers need to consider those factors, including heat transfer, high-throughput processing, protocol development, sample safety, storage efficiency, inventory, and convenience.

For molluscan larvae cryopreservation, straws at 0.25 ml and 0.5 ml were the most commonly used containers (**Table 4**), and no significant difference in post-thaw larval survival was found between larvae cryopreserved in 0.25-ml and 0.5-ml straws (Liu et al., 2020a). Large volume macro-straws (5 ml) were used for cryopreservation of trochophore larvae of *Crassostrea virginica* (Paniagua-Chavez et al., 1998; Paniagua-Chavez and Tiersch, 2001). Direct comparison of straws (0.25-ml and 0.5-ml) and cryovials (2-ml and 4-ml) as package container was made on trochophore cryopreservation in *Perna canaliculus*, and a significant reduction of post-thaw survival to normal D-larvae was found when trochophores were cryopreserved in cryovials (Paredes et al., 2012). In section 3.5., use of larger sample packaging containers for larval cryopreservation is discussed as a necessary adaption for potential application to hatchery seed production. With different packaging containers, the cooling rate will have to be modified accordingly.

After sample packaging, straws or cryovials would be sealed, labeled (or prelabelled), and loaded into the freezer system for cooling at the selected cooling profiles, and plunged into liquid nitrogen in storage dewars. Proper labelling and sealing can guarantee sample tracking, biological safety, and inventory management. Frozen samples in liquid nitrogen would be sorted and packed into goblets and canes with proper labels for long-term storage. For molluscan larval cryopreservation, long-term sample storage was all directly in liquid nitrogen (-196 °C) in storage dewars. Theoretically, cryopreserved samples need to be stored and maintained at temperatures below -135 ºC/-140 ºC, which is the glass transition temperature. Therefore, liquid or vapor nitrogen can be employed for long-term storage of cryopreserved sample. Evidences in human sperm proved that little or no detectable decline of post-thaw viability was found after many years storage at -196 °C (Yogev et al., 2010) . Liquid nitrogen level in the storage tanks is extremely important and needs to be maintained as a weekly routine with alarm setup.

Alternatively, electric ultra-freezers at -150 ºC can be used for long-term storage of cryopreserved samples. These ultra-freezers are commercially available, and many types have built-in liquid nitrogen back-up systems, which can be self-activated if a power outage occurs. For application of larval cryopreservation to commercial seed production in farms, long-term storage of cryopreserved larvae in -80 °C freezer will be financially beneficial, but no study was reported on storage of cryopreserved larvae at no-cryogenic temperatures yet. Further investigation is needed. For macroalgae, storage of cryopreserved samples in non-cryogenic temperatures (e.g., -80 °C) is feasible for relatively long term (e.g., 12 month) without viability loss (Yang et al., 2021).

2.6. Thawing of cryopreserved samples

Warming process is equally important as the cooling process, and the factors causing cell injury during cooling process would potentially cause cell injury during warming process. Therefore, optimal thawing temperature or ultra-fast warming rate (similar to vitrification) needs to be determined.

For molluscan larval cryopreservation, thawing of cryopreserved samples were conducted by immersing the frozen samples into a warm water bath at a specific temperature until there was a complete thawing of samples. To date, only two studies have reported the effects of thawing temperature on the post-thaw larval survival (**Table 5**). For trochophore cryopreservation in *Mercenaria mercenaria*, thawing at 50 °C was considered suitable with the highest post-thaw D-larval survival rate of 27 ± 14% (Simon and Yang, 2018), and for trochophore of *Mytilus galloprovincialis,* thawing at 28 °C was suitalbe with the highest post-thaw D-larval survival rate of 80 ± 6% (Liu et al., 2020a). In other publications, thawing temperatures, ranging from 18 to 37 °C, were used to thaw 0.25-ml and 0.5-ml sample straws (**Table 5**). For the 5-ml macrotubes used for trochphore cryopreservation in *Crassostrea virginica*, thawing at 70°C for 15 s was used (Paniagua-Chavez and Tiersch, 2001).

In recent years, ultra-rapid warming has been used for thawing of vitrified samples of large-sized cells, such as mouse embryos and oocytes (Mazur and Paredes, 2016). The first one was infrared pulse laser with an ultra-rapid warming rate of 10,000,000 °C/min, and application to vitrified mouse oocytes yielded nearly 100% post-thaw survival (Jin and Mazur, 2015) and a 43% post-thaw survival of vitrified coral *Fungia scutaria* larvae (Daly et al., 2018). The second

one was the inductive heating system with nanoparticles, ultrarapid warming rate was achieved and applied on vitrified tissues and organs (Manuchehrabadi et al., 2017). Molluscan larvae are similar sized with the mouse oocytes, and ultra-rapid or rapid warming could have a significant effect on post-thaw survival, which is worthy for further investigation.

2.7. Post-thaw sample amendments

As one of the post-thaw sample amendments, removal of cryoprotectants from the post-thaw samples has been widely applied for human, livestock, poultry, and fish sperm cryopreservation (Elliott et al., 2017). Similar to mixing of cryomedium and sample suspension (Section 2.4.), removal of cryoprotectants could be gradually or one step to avoid osmotic shock. For molluscan larval cryopreservation, removal of cryoprotectant from post-thaw samples was reported in most of the publications before viability assays. The approach was to dilute post-thaw larvae with seawater or seawater with additives to avoid osmotic shock, such as, 9 % sucrose (Liu et al., 2020b; Liu et al., 2020a), 0.1% bovine serum albumin (BSA) (Paredes et al., 2012; Paredes et al., 2013; Suneja et al., 2014), and 12 μM EDTA plus 0.1% (w/v) BSA in fresh seawater (Heres et al., 2020) (**Table 5**). Furthermore, post-thaw samples after dilution were reported to be filtered through a 20-μm screen, and return to fresh seawater for viability assay (Usuki et al., 2002; Kim et al., 2013; Labbe et al., 2018). However, it was not reported whether the post-thaw viability was increased with or without removal of CPAs or dilution in different solutions, and no documentation was reported about the osmolality changes before and after dilution.

2.8. Viability assays of post-thaw samples

Viability assays are essential for evaluation of cryopreservation success. For molluscan larvae, post-thaw viability was measured by many different methods, and no uniform evaluation criteria were established. Therefore, it is hard to compare the post-thaw viability from these published research protocols. A summary of the viability results and estimation methods for molluscan post-thaw larvae are listed in **Table 5**.

The definitions for the viability assay methods used for post-thaw larval viability assays included:

1) Motility rate: The percentage of motile (swimming and rotating movements) larvae estimated by counting among total observed post-thaw larvae.

2) Survival rate: The percentage of motile larvae estimated by counting among total observed post-thaw larvae. For D-stage and umbone larvae, survival rate also assessed by heartbeat and movement of cilia.
3) D-larvae rate: The percentage of D-stage larvae developed from the post-thaw trochophore larvae. This assay was used only for post-thaw trochophore evaluation.
4) Feeding rate: The percentage of larvae with feeding ability (defined by presence of microalgae in stomach) out of the total post-thaw larvae. The assay was usually performed at 24-48 h after thawing and feeding with microalgae.
5) Mortality rate: The percentage of dead larvae out of the total observed post-thaw larvae.
6) Recovery rate: The percentage of post-thaw larvae with a normal morphology out of the total post-thaw larvae.
7) Abnormality rate: The percentage of post-thaw larvae with an abnormal morphology out of the total post-thaw larvae. The abnormal larvae were defined by their abnormal morphology (such as deformed hinge), damaged organ (such as missing velum of D larvae) or delayed development (trochophores), or presence of clear protruding mantle.
8) Shelled larval ratio: The percentage of post-thaw larvae with shells out of the total post-thaw D-stage larvae.
9) Normal larval ratio: The percentage of post-thaw normal larvae out of the total post-thaw larvae. No definition was described for “normal larvae”.
10) Larval velocity: The velocity of average path estimated by specific software.
11) Larval activity index: The motile ability of post-thaw larvae was evaluated according to their motility characteristics.
12) Morphologic parameters: Larval perimeter, area, and circularity.
13) Shell length parameters: Observations by scanning electron or light microscopy were used to assess post-thaw larval morphology and organogenesis.
14) Survival to beyond metamorphosis or even to adult stage: The long-term survival was the ultimate goal for applications of this technology.
15) Others: Mean weight, larvae size, shell size, etc.

Motility rate and survival rate were the two commonly used viability assays of post-thaw larvae immediately after thawing or after culture for a period (**Table 5**). D-larvae rate was specifically used to evaluate post-thaw trochophore viability after culturing for ~24 h post

558 thawing and was considered as an effective method to qualify the viability of post-thaw
559 trochophore larvae. Feeding rate was used to evaluate post-thaw D-larvae for their feeding
560 ability, such as in *Crassostrea gigas* (Suneja et al., 2014) and *Perna canaliculus* (Heres et al.,
561 2020; Rusk et al., 2020), and *Mytilus galloprovincialis* (Rodriguez-Riveiro et al., 2019). The
562 larval swimming velocity (Suquet et al., 2012; Kim et al., 2013) and larvae activity index (Kim
563 et al., 2013) were important factors for viability assays because slowly swimming or wriggling
564 larvae occurred often in post-thaw larvae and usually categorized as alive. Therefore, motility
565 rate combined with larvae swimming velocity would reflect more accurately the post-thaw larval
566 viability. Overall, no uniform criteria exist for post-thaw viability assays, and a combination of
567 different assays could be useful to evaluate the post-thaw survival from all aspects and to make
568 the results among different studies more comparable and reproducible.
569 Viability of post-thaw larvae in mollusks usually showed a sharp decrease after culture for
570 some period of time, most likely because the damaged larvae would die off after thawing (Suneja
571 et al., 2014; Rodriguez-Riveiro et al., 2019; Rusk et al., 2020). For example, the post-thaw
572 viabilities of *Spisula sachalinensis* umbo veliger and *Crassostrea gigas* late umbo veliger could
573 reach up to 96% and 99% just after thawing, but decreased sharply (Choi et al., 2008; Choi and
574 Chang, 2014). For post-thaw larvae of *Crassostrea gigas*, only 0.1-0.9% survival was obtained
575 after 21 days (Suquet et al., 2014), and less than 1% of thawed *Perna canaliculus* larvae was
576 obtained after 18 days post-fertilization (Rusk et al., 2020). Therefore, further investigation
577 should focus on improving the long-term viability of cryopreserved molluscan larvae for
578 application to germplasm repository or commercial use.
579 Due to the complicated post-thaw viability assay methods, it is impossible to compare
580 viability results among studies even for the same developmental larvae stage of the same species.
581 Additionally, reported survivals in some reports were normalized to the controls (Paniagua-
582 Chavez et al., 1998; Wang et al., 2011; Paredes et al., 2012; Rodriguez-Riveiro et al., 2019; Liu
583 et al., 2020b; Liu et al., 2020a). Harvest of juveniles (beyond metamorphosis) or adults reported
584 in several publications (Paniagua-Chavez et al., 1998; Suquet et al., 2014; Liu et al., 2020a)
585 indicated the promising future of this technology. However, the survival rates of post-thaw
586 larvae to beyond metamorphosis were extremely low, which is the bottleneck for application of
587 this technology to germplasm repository or commercial seed production. Finally, no genetic
588 confirmation was reported to verify the harvested juveniles were from the post-thaw samples.

3. Application of Molluscan Larval Cryopreservation for Aquaculture

As summarized in Section 2, most of the reports on molluscan larvae cryopreservation focused on research protocol development, similar to the situation for most aquatic organisms (Martinez-Paramo et al., 2017). In general, the aim of "research protocol development" is to attain reproducible post-thaw viability through evaluating the factors at each step along the cryopreservation process. An optimized cryopreservation protocol with assured post-thaw viability is the essential core component for application of cryopreservation technology. Beyond the core research protocol, further considerations are equally important for transition of research protocols to users for application.

3.1. Applications of larval cryopreservation technology

Compared to sperm cryopreservation, larval cryopreservation can secure the full, diploid genome of the studied species, providing advantages for its applications. Importantly, cryopreserved larvae could be thawed and directly used for out-of-season hatchery seed production. Considering the high fecundity (tens of millions of gametes) of most molluscan bivalves and abalones, larval cryopreservation technology has the potential to bring a revolutionary change to commercial hatchery seed production.

1) Germplasm banking of natural wild populations. Ongoing issues, including diseases, overfishing, and environmental pollution, have greatly changed natural resources. For example, the oyster harvesting in the iconic Apalachicola Bay in Florida, USA was stopped in December 2020, and will be closed for at least five years because of damage to the wild oyster beds (Florida Fish and Wildlife Conservation Commission (FWRI), 2020); the historical bay scallop harvest in the Florida panhandle area has suffered continuous decline since early 2000s (Arnold et al., 2005), and the abalones and Olympia oysters have been in decline along the U.S. Pacific coast (Ben-Horin et al., 2016; Ridlon et al., 2021). Restoration activities have focused on habitat enhancement (e.g., recycling oyster shells to restore oyster beds or seagrass restoration for scallops) and limited access and quotas. Germplasm resources have received little attention. The preservation of natural population germplasm will act as a repository of genetic diversity and allow for the continued adaptive genetic variation within natural populations. In addition, a

germplasm repository of wild populations can provide easy access to study materials for molluscan researchers for genetic diversity, ecology, and population biology.

2) Assistance of breeding programs. Genetic breeding of aquacultured mollusks have produced valuable specific strains or lines, for example, the various strains from the largest oyster breeding program at the Virginia Institute of Marine Sciences (VIMS). These valuable strains have been supporting a major part of the oyster aquaculture industry along the U. S. east coast. To avoid accidental loss, larval cryopreservation of these valuable strains is a sound approach for their long-term (or in perpetuity) preservation. In addition, germplasm cryopreservation can assist genetic breeding programs as a useful tool to preserve the base population or select/control populations each breeding generation (Yang et al., 2021 in review). This operation will save the resources that were used to maintain population of distinguish traits, i.e., "pure line", thus, reduce the burden of holding large numbers of oysters in systems and avoid accidental loss of these populations.

3) Commercial-scale out-of-season seed production. Seed production is always the top priority for the aquaculture industry. However, natural spawning seasons for almost all aquacultured mollusks are limited to about 4-5 weeks every year, which is the bottleneck for seed production in molluscan aquaculture. Through manipulating culture temperature profiles, the spawning season can be expanded (Loosanoff and Davis, 1952). Year-round spawning and indoor culture of broodstock to sexual maturity is extremely expensive (personnel, space, and microalgal culture). Larval cryopreservation can overcome this hurdle by cryopreserving larvae at the peak spawning season and using them for out-of-season seed production. This application could greatly benefit out-of-season triploid oyster seed production, because the production of triploid seed is reliant on the limited availability of sperm from tetraploids (Yang et al., 2018).

3.2. Standardization of cryopreservation protocol with quality controls and analysis

Cryopreservation process includes a series of interconnected steps, and an error at any step would cause failure of the cryopreserved products (Leibo, 2011). Therefore, reproducibility of post-thaw viability is the foundation for efficient transition of laboratory protocols to users for repository development or commercial-scale application. Thus, standardization of protocol with established quality control criteria is necessary for reliable and repeatable outcome (Torres et al., 2017; Torres and Tiersch, 2018). The scope of work for standardization should cover germplasm

quality analysis, procedure precision, and factor standardization along the cryopreservation procedure at each step (Torres and Tiersch, 2018). Implementation of standard operating procedures can guarantee reproducible outcomes (post-thaw survival) and support the users to make predictable project management and business plan.

For example, *Standardization of sample concentration.* During protocol development stage, a range of sample concentration may or may not have been identified (**Table 4**). Determination of sample concentration is necessary for application of larval cryopreservation protocol. A high germplasm concentration can reduce sample processing time and straw number, and less space is needed for long-term germplasm storage, while a low germplasm concentration may favor the efficient use of valuable germplasm materials with maximum sample straws. Therefore, standardization of sample concentration is required based on germplasm type (e.g., trochophore or D-stage larvae, or sperm), species, and repository use (e.g., preservation of wild populations, breeding populations, or conservation of endangered species). For cryobanking of natural wild populations, a concentration of 1×10^5 larvae/ml for 20 straws (0.5 ml/straw × 20 straws × 1×10^5 larvae/ml = 1 million larvae) may be suitable as a germplasm repository for each family, and the determination depends on post-thaw survival from D-stage to beyond metamorphosis, expected juvenile number representing a family, and available storage space for frozen samples.

For quality evaluation criteria, standardization could be at different stages such as fresh sample, after equilibration, or post-thaw. In Section 2, the use of different definitions for evaluation of post-thaw larval survival were discussed. With standardization at each step with quality control, an effective cryopreservation protocol will be streamlined and ready for applications.

Cryopreserved germplasm will be the foundation of global genetic resources exchange networks. Current live animal international shipping is constrained by the specific biology of each species, e.g., developmental stage and stress tolerances. However, for the cryopreserved germplasm, the standardized packaging and handling across the species enable industrial scale-up on bio-security assays (e.g., mass screen for pathogens), quality assessment, mass transportation, and supply chain (e.g., multi-source inventory management).

3.3. Data management plan with quality report for germplasm repository

To apply larval cryopreservation protocol for establishing germplasm repositories, a complete data management plan must be set up before sample collection and processing to guarantee the proper storage and use of cryopreserved germplasm in the future (Yang et al., in review). Determination of data parameters need to be related to the purpose of germplasm repository and could include in the following categories:

Category 1. Sample sources. Description of sample collection site, animal number, and basic biological measures of each animal used for germplasm collection. Collection site can include geographical location; date and time; water quality, and total collection number. Animal biological measures can include body sizes, weight, gonad index, fecundity (gamete number), photography, and tissues for genotyping. Depending on the purpose of germplasm repository, strategy for sample collection to preserve genetic diversity and representation is an important consideration for *in vitro* conservation (FAO, 2019). This consideration is essential for germplasm repositories of wild populations, breeding populations, and endangered species conservation. For larval cryopreservation technology, larvae from different families need to be included in a germplasm repository. For example, to maintain the inbreeding coefficient changes per generation less than 1%, larvae from a minimum 50 families (100 parents with 1 female crossing 1 male family construction) need to be included in the repository. Additionally, sample collection sites need to be considered because geographic populations may exist. In addition, traits at the population or family level (e.g., disease resistance, pearl yield, growth rate) should be recorded, because that information will be beneficial in the decision-making of breeding program.

Category 2. Fresh sample quantity and quality. Parameters about fresh sample quantity and quality should be collected in the database, including fresh germplasm fertility, hatching rate, sample volume, and concentrations.

Category 3. Cryopreserved germplasm sample. Parameters about cryopreserved sample quality and quantity should be collected and included in the database. Basic information of the cryopreserved sample should include animal number (out of total number), germplasm type, straw color and labelling, and cryopreservation protocol used for sample cooling. Germplasm quality should cover post-thaw survival (fertility) with evaluation method. Germplasm quantity should include number of sample straws and post-thaw sample concentration.

Category 4. Inventory of cryopreserved samples. This category includes information for repository management, such as cryopreserved sample location, canister number, goblet number, inventory of the straw numbers, and correlated database.

3.5. Promotion and adaptation for application to commercial seed production

Molluscan larval cryopreservation has a great potential for application to out-of-season commercial seed production. From laboratory to farm, there is a long way to go. Two major adaptations on current cryopreservation protocols of commercial seed production are immediately needed.

1) Use of large-volume packaging containers and balanced high larval concentration. For laboratory protocol, straws at 0.25 or 0.5 ml were used as the package container (**Table 4**). With the highest larval concentration (1×10^6 larvae/ml) in current cryopreservation protocol (**Table 4**), one straw will be able to hold 0.5 million larvae, and 40 straws (0.5 ml per straw, 20 million D-larvae) will be needed to fill one larval culture tank in many commercial hatcheries (250 gallon, about 1000 liter) at 15-20 larvae/ml. Based on our working experience in larval cryopreservation of Eastern oysters, a concentration of 1×10^6 larvae/ml shows to be viscous and hard to pipette for packaging. Therefore, use of large-volume packaging needs to be coordinated with larval concentration for commercial application. In most studies, larval concentrations in cryopreservation protocols were reported much lower at $1\times10^{4\text{-}5}$ larvae/ml, subsequently, a large number of straws (over 400) will be needed to fill one commercial larval culture tank, which may not be practical. Therefore, adaption of laboratory larval cryopreservation by use of large-sized packaging containers (e.g., 6-ml, 10-20 ml, 55-100 ml cryogenic bags for cell cryopreservation which are made of ionomeric resin or polyvinyl chloride) would be warranted.

2) Cost-effective freezing systems for industry use for seed production. Cooling process of samples was usually performed using the computer controlled programmable freezers for molluscan larval cryopreservation. The manufactures for liquid nitrogen controlled-rate freezers include CryoMed™ controlled-rate freezers from Thermo Scientific; TurboFreezer and IceCube from Minitube Inc.; DigitCool series from IMV technologies, and Kryo series from Planer. Liquid nitrogen free controlled-rate freezers include VIA freezers from Cytiva and Benchtop CRF-1 or CYTO sensei freezers. One programable freezer can cost between US$15,000 and US$60,000 based on the commercially available products (Hu et al., 2017). This

will be a hurdle for promoting cryopreservation technology to commercial farms. Portable and cost-effective freezing systems, by use of Styrofoam boxes and specific designs, would be a suitable alternative. One aeration freezing system was developed at a cost of US$700 using Styrofoam box as cooling chamber in the authors' laboratory and proved to be effective for a cooling capacity ranging from 1.5 to 32.1 °C/min (Huo et al, in review). Such a device could be used by small commercial entities for larval cryopreservation activities.

4. Future Research Topic

Overall, development of larval cryopreservation technology in aquacultured mollusks has made progress in recent years, although it is still at the stage of protocol development. Overall, no general optimal protocol could be concluded. Evaluation of post-thaw viability was reported over a wide range of parameters with no standard criteria. Often, sharp decrease of post-thaw survivals was reported after a period of culture although immediate post-thaw survivals were high or even 100% in several studies. However, post-thaw larvae were reported to survive to beyond metamorphosis in Pacific oysters and even to the adult stage and reproduced successfully after 3 years of culture, indicating the promising future of this technology. Till now, no application of larval cryopreservation has been reported to germplasm repository establishment and commercial seed production yet.

Based on the status of current research on molluscan larval cryopreservation discussed in Section 2, future investigations to advance this technology could be:

4.1. Improvement of research protocol to increase post-thaw survival

As discussed in Section 2.7., post-thaw larval survivals to beyond metamorphosis were extremely low (less than 1%) although the post-thaw survival immediately after thawing were reported high. To apply this technology to germplasm repository or commercial seed production, a protocol with reliable post-thaw survival is essential. Further improvement of protocol for high survival to beyond metamorphosis is needed. In Section 2, the status of current research updates at each cryopreservation step was summarized and analyzed, providing potential aspects for further investigation for protocol improvement.

4.2. Effects of the ultra-fast thawing on improvement of post-thaw survival

In recent years, ultra-rapid warming was emphasized to address the low post-thaw survival of large-sized germplasm materials, especially embryos or oocytes (Mazur and Paredes, 2016) (See the discussion in Section 2.5.). New technologies, such as inductive heating systems (Manuchehrabadi et al., 2017) or infrared laser pulse (Jin et al., 2014) , have been developed and employed successfully for coral larval cryopreservation (Daly et al., 2018). For molluscan larval cryopreservation, no attention has been made to thawing process. Considering the size of molluscan larvae, ultra-fast thawing would have potential to increase post-thaw viability. Specifically, when large-sized sample containers are employed for commercial application of larval cryopreservation, ultra-fast thawing will be essential to thaw the cryopreserved samples.

4.3. Vitrification – ultra-fast cooling for molluscan larval cryopreservation

The review in Section 2 revealed that no successful research has been performed on molluscan larval cryopreservation by vitrification approach (ultra-fast cooling to transform samples into a glass situation). Theoretically, vitrification can skip the ice crystal formation to transform samples into glass directly, and thus benefit the post-thaw survival of large-sized germplasm cryopreservation. For example, vitrification has been widely used for human oocyte, embryo, and blastocyst cryopreservation as a routine medical treatment (Rienzi et al., 2016). Vitrification technology, including its two derived encapsulation-vitrification and droplet-vitrification, can be achieved by direct immersion in liquid nitrogen and therefore can be performed in field locations easily (Penzias et al., 2021) However, vitrification is usually performed in a microliter level volume to achieve ultra-fast cooling rate, its application for molluscan larvae cryopreservation may only have academic significance for comparison with the controlled slow cooling.

4.4. Confirmation and well-being of survived individuals

Survival of post-thaw larvae to spat or even adult stage were reported in Pacific oysters and eastern oysters in several studies (**Tables 1 and 5**), indicating the promising future of this technology. However, no confirmation was made to verify that these survived individuals were from the cryopreserved cohorts, especially when the post-thaw larvae were cultured side by side with controls in hatcheries. Confirmation could be performed by analysis of parentage of the

individuals with the tissue from their parents, or culture of post-thaw larvae in a dedicated quarantine area. To strengthen the confidence, the well-being of survived individuals needs to be studied. Not only the health of the survivors, but also their offspring and survivors from the cryopreserved offspring will determine how cryopreservation of larvae should be implemented. For example, the heritability of cryo-resistance will have great impact on the economic scale due to the fecundity-based multiplier factor.

A recent study on the gene expression of post-thaw sperm from the Pacific abalone *Haliotis discus hannai* showed that cryopreservation reduced mRNA expression levels of protein kinase A and heat protein genes (Hossen et al., 2021). In the cryopreservation research field, no attention has been paid to post-thaw physiology and genetic changes. To apply this technology to germplasm repository or assistance of breeding programs, it would be worth investigating genetic changes of fresh and post-thaw germplasm.

ACKNOWLEDGEMENTS

We sincerely thank Dr. Chuck Cichra for reviewing this manuscript and his valuable suggestions and corrections. This review was supported by funds from the Gulf States Marine Fisheries Commission (No. ACQ-210-039-2019-USM - Gulf of Mexico Oyster Genetics and Breeding Research Consortium Project) and the National Institute of Food and Agriculture, United States Department of Agriculture (Hatch project FLA-FOR-005385). This study was partly supported by a National Sea Grant Aquaculture Initiative Award (NA18OAR4170344).

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Table 1. Summary of molluscan larval cryopreservation for research species, targeted larval stages, research purposes, and conclusions. *hpf: hour post fertilization. CPA: cryoprotectant agents.

Family/Species	Larval stage for cryopreservation (hpf*)			Research purpose	Conclusion	Reference	
	Trochophore	D Larvae	Umbo			Year	First author
Ostreidae							
Crassostrea gigas	3.5, 8, 24	45		Protocol development for different larval stages	Trochophore were more resistant to cooling	1995	Gwo
	9, 12, 15, 18, 21			Protocol development for different larval stage	Highest viability was obtained in trochophore	2002	Usuki
		yes		Assessment of post-thaw larval quality	Post-thaw larval velocity was a reliable index	2012	Suquet
	13	24, 43		Post-thaw larval survival, growth, and reproduction	Post-thaw larvae survived to reproductive stage	2014	Suquet
		yes		Protocol development - CPAs and cooling rate	Optimized CPA and cooling rate	2014	Suneja
	18	24, 28		Revisiting protocols to address limitations	A reliable protocol was summarized	2018	Labbe
	10, 15, 18, 21			Protocol development	Post-thaw larvae survived to spat	2020b	Liu
	14			Protocol comparison of two species	A protocol was improved	2013	Paredes
	yes	yes	yes	Protocol development - three larval stages	Post-thaw larvae did not survive to next stage	2013	Kim
	13, 16	20, 41	4, 8, 12 d	Protocol development - CPAs and larval stage	High survival rate on late umbo veliger	2014	Choi
Crassostrea virginica	12			Post-thaw larval growth and settlement	Spat were obtained	1998	Paniagua-Chavez
	12			Protocol development	A basic protocol was summarized	2001	Paniagua-Chavez
Ostrea edulis	yes	yes		Post-thaw survival using an adopted protocol	None survived over 24 h after thawing	2012	Horvath
Saccostrea glomerata	6, 12	24, 48, 96		Protocol development - larval concentration	A protocol was summarized	2008	Liu
Mytilidae							
Mytilus edulis	yes			Preliminary study on larval cryopreservation	Trochophore could survive -196℃	1989	Toledo
Mytilus galloprovincialis		30		Protocol development - CPAs	Post-thaw larvae survived to eye-stage	2011	Wang
	20			Protocol comparison of two species	A protocol was improved	2013	Paredes
	Yes	48, 72		Protocol improvement on CPA and larval stage	Post-thaw larvae survived to settlement	2019	Rodriguez-Riveiro
	10, 20, 25, 30			Protocol Development - evaluation of five factors	Post-thaw larvae survive to spat	2020a	Liu
Mytilus trossulus	24			Fatty acid profile change during cryopreservation	Freezing-thawing process affected fatty acids	2009	Odintsova
Perna canaliculus	16-20	--		Protocol development - toxicity	Low post-thaw viability was obtained	2012	Paredes
	--	48, 72		Protocol development - larval density	The protocol was improved	2020	Heres
	16	48		Effects of larval stage on post-thaw development	D-larvae showed better post-thaw survival	2020	Rusk
Pteriidae							
Pinctada fucata martensii	13, 17	24, 72	5, 7, 12 d	Protocol development - cooling, larval stage, CPAs	A protocol was summarized	2003	Choi
Veneridae							
Mercenaria mercenaria	15			Protocol development - toxicity, cooling, and thawing	A basic protocol was summarized	2018	Simon
Venerupis corrugata	18-20	48, 72		Protocol development – evaluation of CPAs, equilibration time, and larval stage	A basic protocol was summarized	2021	Heres
Ruditapes decussatus	18-20	48, 72					
Ruditapes philippinarum	18-20	48, 72					
Mactridae							
Spisula sachalinensis	20, 25	5 days	16 d	Protocol development - larval stage and CPAs	Umbo larvae had the best post-thaw survival	2008	Choi

Table 2. Summary of cryoprotectant type, concentration, sugar additive, solvent, and concluded optimal cryoprotectant in cryomedium for molluscan larval cryopreservation. n/a: not available. ASW: artificial seawater. CPAs: cryoprotectant agents. DMSO: dimethyl sulfoxide. DW: distilled water. EG: ethylene glycol. FIC: Ficoll. Fru: fructose. FSW: fresh seawater. Glu: glucose. Gly: glycerol. HBSS: Hanks' balanced salt solution. Met: Methanol. MQ: Milli-Q water. PG: propylene glycol. PVP: polyvinylpyrrolidone. Suc: sucrose. Tre: trehalose.

Family/Species	CPA/concentrations	Sugars or additives	Solvent	Optimal CPAs	Reference	
					Year	First Author
Ostreidae						
Crassostrea gigas	DMSO, Gly, PG, EG at 10, 20, 30%	n/a	ASW	10% PG	1995	Gwo
	1.0, 1.5M DMSO	0.25 M Tre	FSW, DW	1 M DMSO+0.25 M Tre in 1/4 FSW	2002	Usuki
	10% EG	1% PVP, 0.2 M Tre	MQ	n/a	2012	Suquet
	10% EG	1% PVP, 0.2 M Tre	DW	n/a	2014	Suquet
	10% EG	1% PVP, 0.2 or 0.4 M Tre	MQ	n/a	2014	Suneja
	10% EG	1% PVP, 0.2 M Suc	MQ	n/a	2018	Labbe
	10% EG	1, 3, 5, 7% FIC, 0.2% PVP	n/a	10 % EG+5% FIC+0.2% PVP.	2020b	Liu
	10, 15, 20, 25 % EG	0.2, 0.4 M Tre, 1% PVP	MQ	10% EG with or without TRE and PVP	2013	Paredes
	0.5, 1.0, 1.5, 2.0, 2.5, 3.0M EG	0.2 M Suc	ASW	2.0 M EG+0.2 M Suc	2013	Kim
	1.0, 2.0M DMSO, 0.5-3.0M EG	Fru, Glu, Suc at 0.2, 0.5M.	ASW	2.0 M or 2.5 M EG + 0.2 M Suc	2014	Choi
Crassostrea virginica	15% PG	n/a	ASW	/	1998	Paniagua-Chavez
	5, 10, 15, 20, 25% PG	0.25 M Suc	ASW	10 or 15% PG	2001	Paniagua-Chavez
Ostrea edulis	5, 10, 15, 20% DMSO	n/a	/	10 % DMSO	2012	Horvath
Saccostrea glomerata	10% DMSO, 10% PG	n/a	FSW	10 % DMSO	2008	Liu
Mytilidae						
Mytilus edulis	1.5 M DMSO	n/a	FSW	/	1989	Toledo
Mytilus galloprovincialis	DSMO, EG, PG at 5, 10, 15%	0.2 M Tre	MQ	5% DMSO	2011	Wang
	10, 15, 20, 25 % EG	0.2, 0.4 M Tre, 1% PVP	MQ	10% EG, 15% EG + 0.4 M Tre	2013	Paredes
	10%, 15% EG	0.2, 0.4 M Tre	FSW	10% EG + 0.2 M Tre	2019	Rodriguez-Riverio
	10% EG	7.5% FIC, 0.2% PVP	/	10% EG +7.5% FIC +0.2% PVP	2020a	Liu
Perna canaliculus	0, 10, 15, 20, 25% EG	0, 0.2, 0.4 M Tre	MQ	10% EG + 0.4M Tre; 15% EG + 0.2M Tre	2012	Paredes
	8, 10, 12, 14, 16% EG	0.2,0.4,0.6 M Tre	MQ, FSW	14 % EG + 0.6 M Tre + 1% PVP in MQ	2020	Heres
	10% EG	0.4 M Tre	MQ	n/a	2020	Rusk
Pteriidae						
Pinctada fucata martensii	1.0, 2.0, 2.5, 3.0 M DMSO	Fru, Glu, Suc 0.2 or 0.5 M	n/a	2.0M DMSO + 0.2M Glu or Suc	2003	Choi
Veneridae						
Mercenaria mercenaria	DMSO, PG, EG, Gly at, 5, 10, 15, 20%	n/a	HBSS	DMSO, PG at 5, 10%	2018	Simon
Venerupis corrugata *Ruditapes decussatus* *Ruditapes philippinarum*	DMSO, PG, EG, and Gly at 0.5, 1, 1.5, 2, 3 M	0.4 M Tre	FSW	10 % EG + 0.4 M Tre	2021	Heres
Mactridae						
Spisula sachalinensis	2.0 M EG, 2.0 M DMSO	0.2, 0.5M Suc or Fru	ASW	2.0M EG + 0.2M Suc	2008	Choi

Table 3. Summary of cooling profiles used in molluscan larval cryopreservation and the concluded optimal cooling variables. *From 21°C to 0 °C samples were cooling at -7°C/min; ** seeding was performed. n/a: not available.

Family/Species	Initial (°C)	Cooling rate (°C /min)	Holding		End of cooling			Optimized cooling variables	Reference	
			Temp. (°C)	Time (min)	Cooling (°C/min)	Temp. (°C)	Holding (min)		Year	First Author
Ostreidae										
Crassostrea gigas	15	0.5, 1.5, 2.5, or 5.0	-7	10 s	n/a	-10, -20, -30, or -40	n/a	-2.5 °C/min; -30°C	1995	Gwo
	10	1	-5 or -8	15	n/a	-35, -40	n/a	-1 °C /min; -35°C	2002	Usuki
	0	1	-10	5	0.3	-35	n/a	n/a	2012	Suquet
	0	1	-10	5	0.3	-35	n/a	n/a	2014	Suquet
	0 for 5min	1	-10	5	0.5, 1, or 2	-35	n/a	No difference	2014	Suneja
	0	2.5	-10	5	0.3 to -20°C, then 2.5	-35	n/a	n/a	2018	Labbe
	0 for 5min	1	n/a	n/a	0.3	-34	n/a	n/a	2020b	Liu
	0 for 5min	1	-10 **	5	0.5 or 1	-35	5	0.5°C/min	2013	Paredes
	0	1	-12 **	10	1	-35	30	n/a	2013	Kim
	0	1	-12 **	10	1	-35		n/a	2014	Choi
Crassostrea virginica	15	2.5	n/a	n/a	n/a	-30	5	n/a	1998	Paniagua-Chavez
	15	2.5	n/a	n/a	n/a	-30	5	n/a	2001	Paniagua-Chavez
Ostrea edulis	13 cm above the LN surface for 10 min, and 10 cm above the LN surface for either 12 or 23 min.							12 min of the second step	2012	Horvath
Saccostrea glomerata	0*	1	-12**	5	2	-36	10	2.5 °C/min	2008	Liu
		2.5	n/a	n/a	n/a	-36	10			
Mytilidae										
Mytilus edulis	n/a	5	-5 **	5	0.5	-20, -30, or -40	5	-30 °C	1989	Toledo
Mytilus galloprovincialis	0 for 15 min	1.5	-7	10	0.4	-33	n/a	n/a	2011	Wang
	0 for 5 min	1	-10 **	5	0.5 or 1	-35	5	0.5 or 1°C/min for 10% EG 0.5°C/min with 15% EG	2013	Paredes
	4 for 2 min	1	-12	5	1	-35	n/a	n/a	2019	Rodriguez-Riverio
	0 for 5 min	1	-10	/	0.3	-34	n/a	n/a	2020a	Liu
Perna canaliculus	0 for 5 min	1	-10 **	5	0.5 or 1	-35	5	0.5 °C/min	2012	Paredes
	0 for 5 min	1	-10 **	5	0.5	-35	5	v	2020	Heres
	0 for 5 min	1	-10 **	5	0.5	-35	n/a	n/a	2020	Rusk
Pteriidae										
Pinctada fucata martensii	0	0.25, 0.5, 0.75, or 1	-12 **	10	1	-35	n/a	1 °C/min	2003	Choi
Veneridae										
Mercenaria mercenaria	4	1, 3, 5, 10, 20, or 30	n/a	n/a	n/a	-80	n/a	5°C/min	2018	Simon
Venerupis corrugata *Ruditapes decussatus* *Ruditapes philippinarum*	4 for 2 min	1	-12**	2	1	-35	n/a	n/a	2021	Heres
Mactridae										
Spisula sachalinensis	0	1	-12 **	10	1	-35		n/a	2008	Choi

Table 4. Summary of larval concentration, the way of mixing larval samples with cryomedium, equilibration time, and packaging container used for larval cryopreservation in mollusks. Single: cryoprotectant medium was mixed with larval samples at a 1:1 ratio in a single step. Gradually: cryoprotectant medium was mixed with larval samples in a gradually way. n/a: not available.

Family/Species	Larval (larvae/ml)	Method to mix with cryomedium	Equilibration (min)	Straw Type (ml)	Reference	
					Year	First author
Ostreidae						
Crassostrea gigas	1.5×10^3	Single	10	0.5	1995	Gwo
	$2.7–6.5 \times 10^4$	Gradually	20	0.5	2002	Usuki
	1×10^4	Gradually	20	0.5	2012	Suquet
	1×10^4	Gradually	20	0.5	2014	Suquet
	$1.6 - 2.1 \times 10^6$	Single	20	0.25	2014	Suneja
	6×10^4	Single	n/a	0.5	2018	Labbe
	$4 \times 10^5, 1 \times 10^6$	Single	10	0.25	2020b	Liu
	n/a	Single	15	0.25	2013	Paredes
	400-600	Single	10	0.5	2013	Kim
	n/a	Single	15	0.5	2014	Choi
Crassostrea virginica	1.2×10^4	Single	20	5	1998	Paniagua-Chavez
	$10 - 1 \times 10^5$	Single	20	5	2001	Paniagua-Chavez
Ostrea edulis	800	Gradually	n/a	0.5	2012	Horvath
Saccostrea glomerata	$1 \times 10^3 - 3 \times 10^4$	Single	20	0.5	2008	Liu
Mytilidae						
Mytilus edulis	1×10^3	/	30	0.5	1989	Toledo
Mytilus galloprovincialis	1.5×10^5	Single	10	0.25	2011	Wang
	n/a	Single	15	0.25	2013	Paredes
	n/a	Single	15	0.25	2019	Rodriguez-Riveiro
	2×10^5	Single	10	0.25,0.5	2020a	Liu
Perna canaliculus	n/a	Single	15	0.25, 0.5, (2 or 4-ml cryovials)	2012	Paredes
	$2\text{-}6 \times 10^5$	Single	20,40,60	0.25	2020	Heres
	$3\text{-}10 \times 10^5$	Single	20	0.25	2020	Rusk
Pteriidae						
Pinctada fucata martensii	n/a	Single	15	0.5	2003	Choi
Veneridae						
Mercenaria mercenaria	500	Single	15	0.5	2018	Simon
Venerupis corrugata *Ruditapes decussatus* *Ruditapes philippinarum*	400-600	Single	15, 30, 60	0.25	2021	Heres
Mactridae						
Spisula sachalinensis	n/a	Single	15	0.5	2008	Choi

Table 5. Summary of thawing temperature, post-thaw dilution, viability, and evaluation methods for larval cryopreservation in molluscans. *Normalized data by comparing to controls; n/a: not available; ASW: artificial sea water; SW: sea water.

Family/Species	Thawing temp (℃)	Post-thaw Dilution	Post-thaw culture		Viability	Methods	Reference	
			Period	Stage				
Ostreidae								
Crassostrea gigas	RT	ASW	24 h	D-larvae	*0-38% D-larvae rate	Survival rate, D-stage rate	1995	Gwo
	24	SW/filtering	4 - 6 d	29 d	26-47% post motility rate	Motility rate, survival rate, shelled larval ratio, and normal larval ratio	2002	Usuki
	37	ASW	2-7 d	D-larvae	83% (day 2) and 2.2% (day 7)	Motility rate, larvae velocity, Morphologic parameters	2012	Suquet
	37	ASW	21 d	Juvenile	0.1-0.9 % at day 21	Survival rate, mean weight, motility rate, larvae velocity, D-larval rate	2014	Suquet
	28	0.1% BSA in SW	22 d	n/a	<5% survival rate at day 11	Survival rate, feeding rate, and shell length parameters	2014	Suneja
	37	SW/filtering	10 d	n/a	9 ± 5% at day 12	Motility rate, recovery rate, and survival rate	2018	Labbe
	28	SW, 9% sucrose	27 d	D-larvae /Juvenile	*5-61 % survival rate at day 1-27	D-larval rate, survival rate	2020b	Liu
	28	SW, 0.1 % BSA	24 h	D-larvae	*60 ± 7% D-larvae rate	D-larvae rate	2013	Paredes
	20	SW/filtering	75 h	D-larvae	16-84% post-thaw survival	Survival rate, motility rate, larvae activity index, abnormality rate	2013	Kim
	25	ASW	1 h	D-larvae	99% survival for umbo larvae	Survival rate	2014	Choi
Crassostrea virginica	70	SW	10 d	Juvenile	*24 % D-larvae rate	D-larval rate, survival rate	1998	Paniagua-Chavez
	70	ASW	n/a	n/a	~100% post-thaw survival rate	Motility rate, survival rate	2001	Paniagua-Chavez
Ostrea edulis	40	SW	24 h	n/a	59% post-thaw survival rate	Survival rate	2012	Horvath
Saccostrea glomerata	30	SW	1-2 h	n/a	93% post-thaw survival rate	Survival rate	2008	Liu
Mytilidae								
Mytilus edulis	18	SW	24 h	n/a	49% post-thaw survival rate	Motility rate, survival rate	1989	Toledo
Mytilus galloprovincialis	26	SW	3 h to 21 d	Eyed larvae	*12.5% survival rate at 21 d	Survival rate, mortality rate	2011	Wang
	28	SW, 0.1 % BSA	24 h	D-larvae	*48.9 ± 7.6% D-larvae rate	D-larvae rate	2013	Paredes
	35	n/a	48 h to 33 d	D-larvae	*64% survival to settlement	D-larvae rate, feeding rate	2019	Rodriguez-Riveiro
	18, 28, 38, 48,58	SW, 9% sucrose	48 h to 32 d	D-larvae	*> 80% D-larvae rate	D-larvae rate, survival rate, mortality rate	2020a	Liu
Perna canaliculus	28	0.1 % BSA in SW	24 h to 18 d	Pediveliger	*40–60% D-larval rate; 3% to pediveliger	D-larvae rate, survival rate, larval size	2012	Paredes
	28	SW, 12 µM EDTA + 0.1% BSA	4 d	n/a	> 50% survival rate at day 4	Survival, swimming activity, feeding rate, shell size	2020	Heres
	28	n/a	4 d	n/a	<1% survival rate at day 18	Survival rate, shell size, feeding rate	2020	Rusk
Pteriidae								
Pinctada fucata martensii	25	ASW/filtering	1 h	n/a	43% trochophore and 91% D-larvae	Survival rate	2003	Choi
Veneridae								
Mercenaria mercenaria	30, 40, 50	n/a	24 h	D-larvae	27% D-larvae rate	Survival rate, D-stage rate	2018	Simon
Venerupis corrugata *Ruditapes decussatus* *Ruditapes philippinarum*	35	SW	2 d	D-larvae	72±5.69% of normal D-larvae 28±0.58% of D-larvae rate 38±9.71% of D-larvae rate	Survival rate, D-stage rate	2021	Heres
Mactridae								
Spisula sachalinensis	25	ASW	1 h	n/a	96% survival rate (for umbo larvae)	Survival rate	2008	Choi

Figure 1. Swimming larval stages in molluscan bivalves (examples from *Crassostrea virginica*). Larval cryopreservation has studied on trochophores (21 publications), D-stage larvae (15 publications), and umbo larvae (5 publications), but no study has reported on pediveliger larvae.