Development of a microinjection-based protocol for the cryopreservation of Japanese whiting Sillago japonica embryos

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Doctoral Dissertation

DEVELOPMENT OF A MICROINJECTION-BASED PROTOCOL FOR THE CRYOPRESERVATION OF JAPANESE WHITING *Sillago japonica* EMBRYOS

September 2018

Graduate School of Marine Science and Technology

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Declaration

I hereby declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research. It has neither been accepted, not submitted for any other degrees. All of the sources of information in this thesis have been duly acknowledged.

Alam Md Ariful

September 2018

Dedicated to

My beloved father

LATE MD WAZ UDDIN

CONTENTS

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September 2018   The Author

Alam Md Ariful

Abbreviations

%	:	Percentage
AFGP	:	Antifreeze glycopeptide
AFP I	:	Antifreeze protein type I
AFP III	:	Antifreeze protein type III
AFPs	:	Antifreeze proteins
ANOVA	:	Analysis of variance
ASW	:	Artificial sea water
CPAs	:	Cryoprotective agents
DSC	:	Differential Scanning Calorimetry
Me_2SO	:	Dimethyl sulfoxide
EG	:	Ethylene glycol
h	:	Hour
IAC	:	Immediate after chilling
LN_2	:	Liquid nitrogen
MeOH	:	Methanol
mg	:	Milligram
Min	:	Minute
ml	:	Milliliter
mM	:	Millimolar
ND	:	Not determined
nl	:	Nanoliter
NT	:	Not treated
^oC	:	Degree celsius
Osm	:	Osmolality
PG	:	1-2, Propylene glycol
PS	:	Perivitelline space
SD	:	Standard deviation
TIIF	:	Temperature of the intracellular ice formation
YM	:	Yolk mass

General Abstract

Cryopreservation of fish gametes and embryos is a promising tool for efficient production of seeds for aquaculture and for the conservation of endangered species. Cryopreservation of fish sperm has been successfully developed and protocols are available for numerous species but there has been little progress in the case of eggs and embryos. The insufficient impregnation of cryoprotective agents (CPAs) due to the presence of complex structural barriers in fish eggs and embryos such as the chorion and other membranes seems to be the main impediment for successful cryopreservation. In the absence of CPAs, water freezes during cooling causing the formation of ice crystals, which in turn cause damage and finally death of the cells. Among the methods for CPA incorporation in fish embryos and eggs tested so far (microinjection, electroporation, chemical treatment of eggs/embryos, sonication, dehydration, etc), microinjection stands out as one of those with the greatest possibility of development. Although more laborious and technically sophisticated than some of the alternatives, it allows direct and swift delivery of verifiable amounts of CPAs and other substances into specific locations inside the eggs or embryos. Thus, microinjection provides the means to test the relative efficiency of various CPAs to protect cells from cryoinjury in a way that is unrelated to their ability to permeate the fish egg/embryo chorion. This enables testing of whether large, organic molecules such as antifreeze proteins, which otherwise cannot penetrate the intact egg/embryo, can afford cryoprotection with lower toxicity than the chemicals tested so far. Moreover, microinjection is also a useful technique for introduction of inorganic low-permeability materials such as metallic nanorods into fish eggs or embryos for cryopreservation. In combination with the application of laser pulses, these materials have proven invaluable in avoiding recrystallization and additional cell damage during warming/thawing of the frozen materials. In this context, I intend to develop a microinjection-based protocol and evaluate the efficiency of permeable and non-permeable cryoprotective agents for the cryopreservation of fish eggs and embryos using the Japanese whiting *Sillago japonica* as the experimental model. The Japanese whiting is a small-sized, easy-to-rear, and prolific marine fish that readily spawns in captivity.

The contents of this dissertation are described in 5 chapters. The background, rationale and objectives of this research are described in Chapter 1 whereas Chapter 2

describes the optimization of the protocols for microinjection-based delivery of CPAs into Japanese whiting embryos. Chapter 3 deals with the evaluation of the efficiency of permeable and non-permeable chemicals as cryoprotective agents for cryopreservation of Japanese whiting embryos. Chapter 4 focuses on evaluation of the efficiency of antifreeze proteins (AFPs) as cryoprotective agents for cryopreservation of Japanese whiting embryos and Chapter 5 summarizes the findings of the entire study.

In Chapter 2, I examined the suitable conditions for single or combined microinjection into the perivitelline space (PS) and the yolk mass (YM) of Japanese whiting embryos at six developmental stages (2~32 cells, morula, blastula, gastrula, somites and tail elongation). The parameters examined were injection volume, CPA type and concentration, vehicle (diluent), and suitable developmental stage. Hatching rates of embryos dry-punctured by microinjection through the chorion and into the PS only were similar to those of controls (80-89%) in all groups. Embryos punctured through both the chorion and syncytial layer and into the YM had comparatively lower survival, particularly in the 2~32 cells (72%) and gastrula (66%) stages. Somites and tail elongation embryos tolerated single or combined injection with 2.1 and 15.6 nl in the PS and YM, respectively, whereas earlier embryonic stages tolerated only up to 8.2 nl in the YM. The injected solutions diffused rapidly throughout the PS and YM and remained contained within each compartment unless in the case of structural damage caused by injection of larger volumes. Yamamoto solution was marginally better as a vehicle for microinjection of CPAs than fish Ringer and phosphate buffer saline whereas ¼ artificial sea water was clearly unsuitable. A comparison of the tolerance of embryos in different developmental stages to microinjection of three common CPAs, dimethyl sulfoxide (Me_2SO), 1, 2-propylene glycol (PG), and ethylene glycol (EG) at concentrations of 30-90% into the PS and YM showed that EG was the less toxic to whiting embryos compared to Me_2SO and PG, and that tolerance decreased with increasing CPA concentration. Hatching rates of somites and tail elongation embryos injected with equivalent CPA solutions/concentrations decreased in the order PS > YM > combined injection, and tail elongation embryos showed higher tolerance than somites stage embryos. The extender distributed swiftly within minutes from injection in both the PS and YM as visualized with the aid of trypan blue dye. Microinjection was efficient in delivering CPAs swiftly to specific compartments of whiting embryos in concentrations 2-4 fold higher than previously obtained for this species by immersion-based impregnation protocols and led to significant decreases in the ice nucleation temperature (TIIF, ºC) of the embryos compared to untreated controls as

measured by Differential Scanning Calorimetry (DSC). Moreover, embryos microinjected with 30% EG showed significantly higher hatching rates in chilling tolerance tests at -10 ºC for 20 min compared to the other treatments and the controls, suggesting that EG may be a suitable CPA for cryopreservation of whiting embryos.

Chapter 3 examined the efficiency of combinations of permeable (Me_2SO, EG and PG) and non-permeable (trehalose and sucrose) CPAs for cryopreservation of Japanese whiting embryos. The CPAs were injected as single or combined solutions with concentrations between 30 and 40% and their efficiency was analyzed by DSC (TIIF, ºC) and chilling tolerance tests as performed in Chapter 1. Embryos were microinjected either at the 2 cells~morula stages with a single injection of 8.2 nl into the YM or at the tail elongation stage with a double injection of 2.1 nl into the PS and 15.6 nl into the YM. Embryos treated with 0.25 and 0.5 M trehalose had higher survival rates than sucrose-treated embryos. Among ten combinations of permeable and non-permeable CPAs, the lowest TIIF values and highest chilling tolerance for both embryo stages were obtained with T3 (15% EG + 15% PG) and T8 (15% EG + 7.5% PG + 0.125 M Tre). The results suggest that these solutions may be suitable for cryopreservation of tail elongation whiting embryos.

Chapter 4 examined the suitability of antifreeze proteins (AFPs: AFGP, AFP I and AFP III) as cryoprotective agents for cryopreservation of Japanese whiting embryos alone or in combination with other CPAs. First, embryos at the tail elongation stage were double-injected with various concentrations of the AFPs in Yamamoto solution in volumes of 2.1 nl into the PS and 15.6 nl into the YM. Embryos tolerated well up to 40 mg/ml of all AFPs in single solution and up to 20 mg/ml in combinations of two AFPs. A combined solution of 20 mg/ml AFGP and 20 mg/ml AFP I with T8 (1:1 v/v ratio) resulted in the lowest TIIF values, highest survival rates in chilling tests, and highest frequency of isolated live cells after attempted cryopreservation with tail elongation embryos, suggesting that combinations of AFPs with CPAs may be useful for cryopreservation of Japanese whiting embryos.

Overall, this study shows that microinjection provides a reliable method for introduction of all types of CPAs and other foreign substances into Japanese whiting embryos for cryopreservation. The findings of this study provide fundamental information on the development of microinjection-based impregnation protocols with applicability for this species and perhaps for other species as well.

CHAPTER 1

General introduction

In the world, there are approximately 33,400 fish species available in marine, brackish and fresh water systems (Fishbase, 2016). However, natural fish stocks are under constant threat of overfishing, habitat deterioration, and even climate change (Strayer and Dudgeon, 2010; Vörösmarty et al., 2010) and there have been dramatic worldwide declines (New, 1997). Rapid destruction of ecosystems is a global event (Hagedorn and Kleinhans, 2000) and Pullin et al. (1998) estimated that more than 20 fish species had become extinct whereas nearly 200 species have been added to the list of endangered species during the last century. In view of this scenario, it seems urgent to establish fish germ plasm repositories to preserve vanishing genetic materials and conserve fish genetic biodiversity.

In-situ approach may be the best option for conserving ecosystems and genetic resources of threatened and endangered fish species through protection/restoration of the native habitat. Unfortunately, this is not only a slow process due to a great deal of time necessary for habitat restoration but also expensive. On the other hand, *ex-situ* conservation is a potential alternative approach which deals with the preservation of components of biological diversity outside their natural environment such as in live or cryopreserved gene banks. Live gene banking requires purpose-built facilities and is costly to maintain, labor intensive, and difficult to manage. Moreover, previous attempts to maintain long-term live gene banking have often resulted in contamination of stocks. On the other hand, cryogenetic gene banking has little risk of contamination, requires little space and minimal facilities, and is comparatively inexpensive. Now-a-days, cryobiologists are trying to develop protocols to preserve genetic materials by chilling, freeze drying, refrigeration, and freezing, etc. The most desirable method is freezing (cryopreservation) which may permit the genetic materials to be stored at frozen state for thousands of years without any noticeable damage to their genetic code.

Cryopreservation of fish gametes and embryos allows the efficient propagation of fish for aquaculture and fish resource management. Its benefits include 1) enabling the preservation of rare and endangered fish from wild and captive populations, 2) helping in the establishment of genome resource banks (Harvey, 1996) and the maintenance of a large gene pool for genetic manipulation and conservation, 3) enabling the easy exchange of germplasm between hatcheries located in geographical position, 4) contributing to selective breeding

programs for commercially exploited traits (Leung and Jamieson, 1991), 5) enabling continuous supply of seeds throughout the year, 6) reducing the cost of seed production of aquaculture activities, and 7) sustaining productivity by minimizing the impact of live-culture failures resulting from human error, natural disasters, breeding failure and epidemics (Hagedorn and Kleinhans, 2000), among others. As regards the role of fish embryo cryopreservation in the conservation of endangered species, it enables to 1) maintain species diversity, 2) prevent species extinction by producing a reservoir of 'insurance' populations, and 3) allow time to rehabilitate the native environment to support species re-introduction (Liu et al., 1998).

Cryopreservation of fish spermatozoa has been successfully developed for more than 200 species (Asturiano et al., 2017). However, all attempts to cryopreserve fish eggs or whole embryos until now have failed (Rall, 1992; Martínez-Páramo et al., 2017). Cryopreserving spermatozoa, of course, saves only the parental genome and is not sufficient for preservation of genetic diversity. Maternal genomes like mRNA expression and mitochondrial DNA have an important contribution during embryonic development (Pelegri et al., 1996). Maternally-derived germplasm must be included in the germ banking process because these maternal genes are equal contributors for zygote formation and function. Some genetic manipulations such as androgenesis (using frozen sperm), chimera production (isolated blastomeres and their subsequent insertion into recipient embryos), nuclear transplantation from embryonic cells into eggs, and primordial germ cell transplantation are possible in teleosts and might help protect genetic diversity, but these techniques are extremely labor-intensive and result in low survival rates (Thorgaard and Cloud, 1993; Zhang et al., 2007). The ability to preserve both paternal and maternal lines through embryonic genome would greatly improve opportunities for conserving and protecting valuable fish species and argues strongly for cryopreservation of embryos (Hagedorn and Kleinhans, 2000). This problem could be theoretically solved by cryopreserving both sperm and eggs.

Generally, cryopreservation is a technology for the preservation or storage of living and non-living materials like cells, tissues, and entire organisms at low temperatures where biological activity including the biochemical reactions within them would be effectively slow or stopped. There are underlying fundamental mechanisms that determine how all biological systems respond to the lowering of temperature and the solidification of liquid water.

However, the cells being preserved without protective measures such as treatments with cryoprotective agents (CPAs) are often damaged due to freezing or thawing.

CPAs can minimize cell damage associated with ice crystal formation and even suppress ice crystal formation completely at high concentrations. For developing a successful cryopreservation protocol, selection of an effective and minimally toxic CPA is necessary. The target species, cell surface area, developmental stage, temperature, and concentration gradients between the intercellular and extracellular fluids, all affect the degree of CPA permeability for different types of cells (Leibo et al., 1970; Mazur et al., 1974; Schneider and Mazur, 1984). So, it is a pressing issue to select suitable CPA(s) for successful cryopreservation of biological materials. CPAs can be classified into two major categories: permeable and non-permeable agents, depending on their ability to pass through cell membranes. The most common CPAs include dimethyl sulfoxide (Me$_2$SO), glycerol, methanol, 1, 2-propylene glycol (PG), ethylene glycol (EG), sugars (trehalose, sucrose, glucose), polymers (dextran, hydroxyethyl starch, polyvinyl pyrrolidone, PVP), and proteins (egg yolk and antifreeze proteins that found in polar fish and in freeze-resistant and freeze-tolerant insects), among others.

Conventional methods of cryopreservation rely on comparatively slow and controlled cooling during early ice formation during which time cell dehydration is effected by freeze-concentration of the suspension medium. The cells are equilibrated in a solution containing a permeating CPA, and the suspension is cooled and seeded with ice crystals at a temperature slightly below its freezing point. Chilling injury and freezing damage are two major issues within temperature ranges of conventional cryopreservation along with other cryoinjuries like pH fluctuation, cold shock, ice crystal formation, osmometric effect and CPA toxicity (Morris and Watson, 1984). Most of the cryoinjuries take place over the temperature range between 0 and -40 ℃ due to heat removal and application of CPAs. This method has been proven suitable for sperm cryopreservation (Polge, 1980; Stoss and Holtz, 1983; DeGraaf and Berlinsky, 2004) but is not reliable for embryo cryopreservation (Hagedorn et al., 2004; Valdez et al., 2005).

An alternative method is vitrification, which is a process of cryopreservation that relies on rapid freezing of biological materials using liquid nitrogen (LN$_2$) along with CPA treatments to transform intracellular liquid into a glassy state or without ice crystal formation. The extracellular CPA solution also becomes super cooled and solidified into an amorphous

or glassy state. In this procedure, cell dehydration is induced by direct exposure to concentrated vitrification solutions prior to cooling in LN_2. The aim of vitrification is to preclude ice formation in both intracellular and extracellular solutions and still maintain a high level of viability. The most successful vitrification protocol for biological cells or tissues usually consists of five steps (Steponkus et al., 1992): a) loading, which results in equilibration of the cells or tissues in CPAs; b) dehydration of cells and tissues in a concentrated solution (extender + CPA) that will vitrify (vitrification solution); c) plunging the cells or tissues in LN_2; d) warming the cells or tissues; e) dilution of the vitrification solution and removal of CPAs from the cytosol. For successful vitrification, it is essential to formulate a species specific-, low toxicity-, and effective CPA solution.

Vitrification was first used as a cryopreservation technique in mouse embryos (Rall and Fahy, 1985); eventually several highly effective cryopreservation protocols have been developed for mammalian oocytes and embryos (Karlsson et al., 1996; Hurst et al., 1997; O'Neil et al., 1997). Similar protocols have recently become a viable alternative to traditional freezing for pre-hatching embryos of other vertebrates. Some of the advantages of vitrification as conservation tools include 1) it is extremely rapid, so that a large number of germplasm samples can be processed quickly; 2) it does not require sophisticated, computer-controlled equipment, but only LN_2 which is transportable and available in most areas, 3) it offers greater potential for developing cryopreservation procedures for complex tissues and organ, and 4) most importantly, it may improve post-thaw viability of cryopreserved germplasm (Rall and Fahy, 1985; Rall, 1992, 1993). However, some biological samples, such as teleost embryos, may have low plasma membrane permeability to CPAs and water, and this will result in insufficient cell dehydration and permeation by CPAs into the cells or tissues. Vitrification will not be achieved if the low permeability of the membrane cannot be overcome in advance. It is necessary to develop a technique which permit sufficient amount of CPA infiltration in cells and tissues.

The insufficient impregnation of CPAs is considered as the main problem for successful cryopreservation of fish eggs or embryos and this is supposedly related to the formation of intra- and extra-cellular ice crystals during cooling, which causes damage and finally death of the cells (Hagedorn et al. 1996, 1997, 1998; Robles et al., 2003; Cabrita et al., 2006; Edashige et al., 2006; Ding et al., 2007). It is very difficult to achieve swift permeation and homogeneous distribution of CPAs into the various compartments of fish embryos. Hence, CPAs result in toxicity to some cells while in others their concentration is not

sufficient to prevent cryoinjuries (Zhang and Rawson, 1995; Hagedorn et al., 1997). The major constraints for insufficient impregnation of cryoprotectants in fish eggs and embryos and their subsequent removal include (i) the large size of the embryos (generally, marine fish eggs are about 10 times larger than human oocytes), which hinders water and CPA efflux/influx; (ii) the large amount of yolk, which increases the likelihood of membrane disruption by intra cellular ice formation upon cooling and ice crystal growth upon warming; (iii) the presence of a complex and compartmentalized structure with a thick chorion, yolk, and the yolk syncytial layer (YSL) which surrounds the yolk (Hagedorn et al., 1996, 1997, 1998 & 2004; Rahman et al., 2008; Khosla et al., 2017). All of these membranes pose a formidable barrier to the penetration and diffusion of cryoprotectants inside the embryos and hinder the swift and uniform permeation of the CPAs into the various compartments of fish embryos. All approaches tested so far to overcome these barriers and enhance CPA permeation into the fish eggs/embryos such as electroporation (Rahman et al., 2013), treatment with aquaporins (Valdez et al., 2006), positive or negative pressurization (Routray et al., 2002), dechorionation (Hagedorn et al., 1997; Zhang and Rawson, 1998), dehydration (Rahman et al., 2011), sonication (Rahman et al., 2017), microinjection (Janik et al., 2000; Beirão et al., 2006; Robles et al., 2006), etc had only moderate efficiency and did not prevent lethal cryoinjuries. Most of these approaches have led to enhanced CPA uptake but not enough to achieve successful egg or embryo cryopreservation. Thus, more efficient, alternative methods that could provide sufficient CPA uptake, either self-sufficiently or in combination with other strategies are needed. Among the methods for CPA incorporation in fish embryos and eggs tested so far, microinjection stands out as one of those with the greatest possibility of development.

Microinjection may be a good candidate and alternative experimental strategy to reliably deliver CPAs into specific compartments (like perivitelline space or yolk mass) of the embryos than conventional methods. Actually, microinjection is a technology to introduce DNA, proteins, or liquid solutions into the cell using a micromanipulator. It may be time consuming and arguably difficult to apply in the treatment of large numbers of embryos, but in the first stage of research, it provides us with tools to test the importance of various parameters such as CPA type, concentration and distribution inside the embryos, as well as freezing and thawing rates. Equally the important fact is that microinjection allows the introduction of substances that due to their large molecular size would normally have very low or negligible permeability including proteins or even the gold nanorods recently used by

Khosla et al. (2017) in the cryopreservation of zebrafish embryos. In this context, direct delivery of CPAs into embryos through microinjection could help attaining a sufficient internal CPA concentration for preventing ice-crystallization during freezing and/or recrystallization during thawing.

By considering the above-mentioned problems and possibilities related to cryopreservation, we had set our main goal in this study to develop a microinjection-based protocol and evaluate the efficiency of permeable and non-permeable CPAs for the cryopreservation of fish embryos, using the Japanese whiting *Sillago japonica* as the experimental model. The Japanese whiting *Sillago japonica* is a Perciform species with high commercial value and is commonly found in the shallow coastal waters (Northwest Pacific) of Japan, Korea, China, Taiwan, and possibly the Philippines. The major reproductive characteristics of this species include its small-size as adult (15-30 cm body length), the easiness to spawn in captivity, its prolificity, producing daily tens of thousands of eggs daily, the small size of its pelagic eggs (diameter 0.61-0.71 mm) with a soft chorion, and its short embryonic development (hatching within 24 ± 1 h at 24°C). Previous studies on the dehydration (Rahman et al., 2011), electroporation (Rahman et al., 2013), and sonication (Rahman et al., 2017) of whiting eggs showed its potential usefulness as experimental model for cryopreservation studies on fish eggs. Thus, the Japanese whiting could be an useful model species to develop a microinjection-based protocol in the cryopreservation of fish eggs and embryos. To achieve this principal goal, the following specific objectives were taken into consideration during this study:

- Optimization of protocols for microinjection-based delivery of cryoprotective agents into the embryos.

- Evaluation of the efficiency of combinations of permeable and non-permeable chemicals as cryoprotective agents for cryopreservation of embryos.

- Evaluation of the efficiency of antifreeze proteins (AFPs) as cryoprotective agents for cryopreservation of embryos.

CHAPTER 2

Optimization of protocols for microinjection-based delivery of cryoprotective agents into the embryos

2.1. Introduction

Natural fish resources are under the constant threat of overfishing, habitat deterioration, and even climate change (Strayer and Dudgeon, 2010; Vörösmarty et al., 2010). Pullin et al. (1998) estimated that more than 20 fish species had become extinct and nearly 200 species have been added to the list of endangered species during the last century. In view of this scenario, it seems urgent to establish fish germ plasm repositories to preserve vanishing genetic materials and conserve fish genetic biodiversity. Cryopreservation of fish gametes and embryos has the added benefit of allowing the efficient propagation of fish for aquaculture. Sperm cryopreservation has been successful in numerous fish species (Asturiano et al., 2017) but there has been little progress in the case of eggs and embryos (Martínez-Páramo et al., 2017). Experimental storage of fish eggs and embryos at cryogenic temperatures has inevitably met with the formation of intra- and extra-cellular ice crystals during cooling and/or warming, causing damage and finally death of the cells (Hagedorn et al., 1996, 1997, 1998; Robles et al., 2003; Cabrita et al., 2006; Edashige et al., 2006; Ding et al., 2007; Rahman et al., 2013; Martínez-Páramo et al., 2017; Rahman et al., 2017). Theoretically, this could be solved by impregnation with cryoprotective agents (CPAs) or antifreeze proteins (Robles et al., 2004; Martínez-Páramo et al., 2008, 2009) prior to freezing but all approaches tested so far to enhance their permeation into the materials (electroporation (Rahman et al., 2013), treatment with aquaporins (Valdez et al., 2006), positive or negative pressurization (Routray et al., 2002), dechorionation (Hagedorn et al., 1997; Zhang and Rawson, 1998), dehydration (Rahman et al., 2011), sonication (Rahman et al., 2017), microinjection (Janik et al., 2000; Beirão et al., 2006; Robles et al., 2006), etc) had only moderate efficiency and did not prevent lethal cryoinjuries.

Microinjection is one of the alternative methods available for CPA incorporation in fish embryos and eggs. Although more laborious and technically sophisticated than some of the alternatives, it allows direct and swift delivery of substances into specific locations inside the embryos (Janik et al., 2000; Robles et al., 2004; Beirão et al., 2006; Robles et al., 2006). More importantly, it enables testing of whether large, organic or inorganic molecules, which otherwise cannot penetrate the egg/embryo, can afford cryoprotection with lower toxicity

than membrane-permeable chemicals. Interestingly, a recent study conducted by Khosla et al. (2017) reported promising results with embryos microinjected with CPAs and gold nanorods that allowed rapid internal warming when excited by a laser pulse and thus mitigated devitrification during thawing.

In this context, we have set to develop a microinjection-based protocol for an alternative model species in the cryopreservation of fish eggs and embryos, the Japanese whiting *Sillago japonica* (Rahman et al., 2008, 2011, 2013, 2017). Briefly, the whiting is a small-sized, prolific marine fish that spawns easily in captivity, producing daily tens of thousands of small, pelagic eggs with soft chorion and short embryonic development (hatching within 24 h). In this study, the main objective was to establish the basic protocols for microinjection of CPAs into Japanese whiting embryos and secondarily to compare the toxicity of three of the most commonly used CPAs (dimethyl sulfoxide, 1, 2-propylene glycol, and ethylene glycol; Tiersch, 2000) to whiting embryos using the established protocols. The microinjection conditions investigated in this study were microinjection volume and location of delivery inside the embryo, the sensitivity of different developmental stages to the procedure, and the CPA type, concentration, and vehicle (extender). We also examined the dynamics of distribution of the injected substances inside the embryo following microinjection and tested the efficiency of CPA microinjection to lower the temperature of intracellular ice formation and to increase the chilling tolerance of embryos.

2.2. Materials and methods

2.2.1. Chemicals

Dimethyl sulfoxide (Me_2SO), 1, 2-propylene glycol (PG), ethylene glycol (EG), potassium chloride (KCl), sodium chloride (NaCl), calcium chloride ($CaCl_2$), sodium bicarbonate ($NaHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4) and agarose were purchased from Wako Pure Chemicals (Osaka, Japan). Light white mineral oil was purchased from MP Biomedicals, Inc. (Germany) while trypan blue (0.4%) (Gibco[TM]) was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

2.2.2. Broodstock rearing and embryo collection

Broodstock fish were maintained as described according to Rahman et al. (2013) with slight modifications. In brief, sexually mature, adult females and males of Japanese whiting *S. japonica* (Temminck and Schlegel, 1843) were collected at Tateyama Bay, Japan, and reared

at a 1:1 sex ratio in 1200 L recirculated sea water tanks at the fish rearing facilities of Tokyo University of Marine Science and Technology, Tokyo, Japan. Fish were fed frozen krill once a day until satiation. Artificial sea water (ASW) was prepared using sea water salts (TetraMarin® Salt Pro., USA) and dechlorinated tap water. Water temperature and photoperiod were adjusted to $24 \pm 1°C$ and 14L: 10D (14 h light and 10 h dark), respectively, for inducing natural spawning. The fertilized, buoyant eggs were collected using a net trap set near the surface water outlet at the following developmental stages: 2~32 cells (about 40 min after fertilization), morula (2 h after fertilization), blastula (about 3 h 30 min after fertilization), gastrula (about 7 h after fertilization), somites (about 11 h 30 min after fertilization) and tail elongation (about 16 h 30 min after fertilization). All developmental stages were determined according to Oozeki and Hirano (1985).

2.2.3. General microinjection procedures and tolerance of different developmental stages to puncturing

Needles for microinjection were prepared from glass capillary tubing (GD-1, Narishige, Tokyo, Japan) using a glass micropipette puller (PC-10, Narishige, Tokyo, Japan) and sharpened in a micropipette grinder (EG-44, Narishige, Tokyo, Japan) at an angle of 35°. Microinjection was performed using a mineral oil pressure-driven microinjector (IM-9B, Narishige, Tokyo, Japan) coupled to a micromanipulator (MP-1, Narishige, Tokyo, Japan) under a stereoscopic microscope (SZX10, Olympus, Tokyo, Japan). First, the tolerance of whiting embryos at six developmental stages (2~32 cells, morula, blastula, gastrula, somites, and tail elongation) to the structural damage caused by manipulation and puncturing through the chorion and the yolk syncytial layer (YSL) was assessed in anticipation to microinjection into the perivitelline space (PS) and the yolk mass (YM), respectively (Fig. 2.1). For this test, embryos were carefully placed into parallel grooves in agar disks (3% agarose) and subjected to dry-puncturing (without injection of any substance or solution) to the two depths. Each treatment contained at least 30 embryos and was run in five replicates with batches from different spawns. Untreated embryos served as controls of the damage caused by puncturing. Punctured and control embryos were kept in plastic petri dishes (CORNING, Suspension Culture Dish, 35 mm × 10 mm, Polystyrene, USA) containing 5 ml of ASW in an incubator (CN-25C, MEE, Japan) at 25°C until hatching. Hatching rates (%) were determined as the total number of hatched embryos / total number of manipulated embryos × 100.

2.2.4. Determination of suitable microinjection location and volume

Three types of injection were tested in this study, namely a single injection into either the YM or the PS and a combined injection into these two compartments. The maximum tolerable microinjection volume for each approach was determined in tail elongation embryos by injecting an extender (Yamamoto solution; see the chemical composition in 2.2.5) containing 0.04% trypan blue as a visualization agent in aliquots of 2.1, 15.6, 24.4, 33.5 and 44.4 nl for single injection and 2.1-24.4 nl for combined injection, and observing the post-procedure survival of embryos. The injection volume was calibrated by delivering a small aliquot of Yamamoto solution into a large drop of mineral oil on an agar plate, measuring the diameter of the aqueous drop (D, millimeter), and calculating the volume of the aliquot (V, nanolitre) as $V = (4/3) \times \pi(D/2)^3 \times 1000$. The embryos were observed and photographed under a stereoscopic microscope (BX53F, Olympus, Tokyo, Japan; digital camera DP73, Olympus, Tokyo, Japan; CellSens Imaging Software v1.9, Olympus, Tokyo, Japan) at 4 minutes after injection to record their appearance and monitor possible structural changes. Each treatment contained at least 30 embryos and was run in four replicates with batches from different spawns. Uninjected embryos and embryos dry-punctured served as controls and post-procedure survival in all groups was monitored as described above.

2.2.5. Selection of a suitable extender

The suitability of four extenders: i) fish Ringer (KCl 2.99 g/l, NaCl 6.49 g/l, $CaCl_2$ 0.29 g/l, $NaHCO_3$ 0.202 g/l; pH 7.8; osmolality 285 mmol/kg), ii) Yamamoto solution (NaCl 7.5 g/l, KCl 0.2 g/l, $CaCl_2$ 0.2 g/l; pH 7.2; osmolality 243 mmol/kg), iii) phosphate buffered solution, PBS (NaCl 8 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 1.15 g/l; pH 7.3; osmolality 297 mmol/kg), and iv) diluted artificial sea water, ¼ ASW (TetraMarin® Salt Pro., USA; 8.5 g/l; pH 7.1; osmolality 217 mmol/kg) as vehicles for injection of CPAs in the PS and YM of whiting embryos was tested in six developmental stages (2~32 cells, morula, blastula, gastrula, somites and tail elongation). The osmolality and pH of the solutions was measured in an osmometer (Vapro Pressure Osmometer, WESCOR, USA) and pH meter (HM-25R, TOA DKK), respectively. Injection volumes were 15.6 nl and 2.1 nl for delivery into the YM and PS, respectively, following the findings of the previous section. The controls, number of embryos per treatment and replicates, and conditions for the determination of hatching rates were as described in the previous section.

2.2.6. Tolerance of embryos to microinjected CPAs

The tolerance of embryos to single and combined injections with 30-90% (v/v) solutions of three commonly used CPAs (Me$_2$SO, EG and PG) in Yamamoto solution was tested in 2~32 cells, morula, blastula, gastrula, somites and tail elongation embryos for single injection and in somites and tail elongation embryos in the case of combined injection. In both cases, the microinjection volumes used were 2.1 and 15.6 nl for the PS and the YM, respectively. The number of embryos per treatment and replicates and the conditions for the determination of hatching rates were as described in the previous section. Embryos without any kind of manipulation were used as controls. Embryos between the 2 cell and gastrula stages did not tolerate these volumes (see *3.4*) and for this reason an additional trial was run with injection of a lower volume (8.2 nl) and the lowest concentrations (30-50%) of the three CPAs only in the YM of 2-32 cell embryos.

2.2.7 Estimation of the internal concentration of CPAs in the YM and PS following microinjection

The internal concentration of CPA in the YM and PS of embryos after microinjection was volumetrically estimated after confirming that the introduced liquids distributed swiftly and evenly in the compartments after injection. To this aim, we first injected 2.1 nl and 15.6 nl of Yamamoto solution containing 0.04% trypan blue as a visualization agent in the PS and YM, respectively, of tail elongation stage embryos and tracked the distribution of the extender inside the embryos. Images of the embryos were recorded at 1, 2, 4, 6, 8, 10, 20 and 30 min after injection under a stereoscopic microscope (BX53F) attached with a digital camera (DP73) with the CellSens Imaging Software. Next, we measured the length of the major and minor axis of intact and dechorionated (treated with pronase 10 mg/ml for 10 min at room temperature) embryos to calculate the total embryo volume and the YM volume, respectively, and by their difference the volume of the PS. Measurements were performed under a microscope (SZX10) fitted with an eyepiece micrometer to the nearest 0.01 millimeter. Finally, the approximate concentration of CPA in each compartment after microinjection was calculated as Internal CPA concentration (mM) = Microinjected CPA Concentration (mM) × Microinjection Volume (µL) / Compartment Volume (YM or PS; µL).

2.2.8. Ice nucleation temperature of CPA-microinjected embryos

The ice nucleation temperature of treated and control embryos was measured by differential scanning calorimetry (DSC). The DSC is a thermoanalytical technique in which

the difference in the amount of heat energy required to increase the temperature of a sample and a reference material is measured as a function of temperature whilst the sample and reference material are subjected to a controlled temperature program. Prior to DSC analysis, temperature calibration was carried out using pure water. DSC analysis was then used to measure the temperature of intracellular ice formation (TIIF, °C) of untreated control and CPA-treated embryos. In this regard, 2 cells~morula stage embryos were single-microinjected with 8.2 nl in the YM whereas tail elongation embryos were double-injected with 15.6 nl in the YM and 2.1 nl in the PS. Ten to fifteen embryos (total weight around 1.0-2.8 mg) were then carefully blotted dry onto a filter paper, weighted and sealed in aluminum DSC pans, and loaded into the DSC equipment (Shimadzu DSC-50, Kyoto, Japan). Samples were loaded into the DSC equipment "without" any surrounding medium or immersion solution. To know the effects of cooling rate on TIIF, embryos at tail elongation were double-microinjected with Yamamoto solution (15.6 nl in the YM and 2.1 nl in the PS) and then the sample was cooled at rates of -2°C, -5°C, -10°C, and -20°C/min to -40°C, and then warmed to 40°C at a rate of 5°C/min. Both cooling and warming processes were recorded using a personal computer attached to the DSC. Next, embryos were microinjected with 30% concentration of single CPA (Me$_2$SO, EG, and PG) solutions, and then measurement was performed with a cooling rate of -5°C/min from room temperature to -40°C. These CPAs and concentrations were chosen based on the results of CPA toxicity (see *2.3.5*). The data was then analyzed using the equipment software (TA-60, Shimadzu, Kyoto, Japan) to calculate the TIIF values for four replicates per treatment and intact controls.

2.2.9. Chilling tolerance of CPA-microinjected embryos

The effects of the same treatments (previous section) on the chilling tolerance of embryos was determined by subjecting embryos to a subzero, non-freezing temperature. This survival test consisted of cooling from room temperature (ca. 25°C) to -10°C at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Thermal exposure was performed using a program freezer (CRYO PORTER, CS-80CP, SCINICS, Japan). The cooling/warming rates and the endpoint of -10°C for 20 min were validated empirically in a pre-trial (chilling at 0, -5, -10, and -20°C for 5, 10, 20 and 30 minutes) in which this particular combination of temperature and exposure time consistently yielded low (but non-zero) hatching rates for untreated tail elongation embryos. After warming, embryos were transferred to clean sea water at 25°C and monitored for survival as

described above. Each treatment and control were run in four replicates with at least 25 embryos each.

2.2.10. Statistical analyses

All data are expressed as mean ± SD (standard deviation). The statistical significance of the differences between means was analyzed by ANOVA followed by the Tukey's multiple comparison tests using the SPSS (Statistical Package for Social Science, version 16.0) (SPSS Inc., Chicago, Illinois, USA). Values with a $p < 0.05$ were considered as significantly different.

2.3. Results

2.3.1. Tolerance of different developmental stages to puncturing

Hatching rates of embryos dry-punctured with a microinjection through the chorion and into the PS only were similar to those of controls (80-89%) in all groups (Fig. 2.2). Embryos punctured through both the chorion and YSL and into the YM had comparatively lower survival, particularly in the 2~32 cells (72%) and gastrula (66%) stages. When hatching rates were adjusted by the respective controls, however, all groups had a minimum hatching rate of 73%.

2.3.2. Determination of suitable microinjection location and volume

Hatching rates of tail elongation stage embryos subjected to a single microinjection into the PS or the YM in the two compartments decreased with increasing injection volume (Fig. 2.3). Embryo survival dropped markedly between 15.6 and 24.4 nl in the case of a single injection into the YM and between 2.1 and 15.6 in the case of the PS. Actually, the majority of the embryos did not tolerate injection volumes higher than 2.1 nl for the PS and 15.6 nl for the YM. Hence, testing of the combined injection was limited to volumes of 2.1 and 15.6 nl for the PS and 15.6 and 24.4 nl for the YM. Hatching rates of embryos subjected to combined injection also decreased significantly with injection volume and remained above 50% only in embryos injected with a combination of 15.6 and 2.1 nl in the YM and PS, respectively (Fig. 2.3). Embryos injected with 2.1 nl (PS) or 15.6 nl (YM) or their combination presented normal appearance and structural integrity whereas those injected with larger volumes had from moderate to massive internal structural changes that were evidenced by the trans-compartmental leakage of the injected trypan blue dye (Fig. 2.4).

2.3.3. Selection of a suitable extender

Hatching rates of embryos injected with the four extenders in the PS or the YM were lower in the early stages (2~32 cells to gastrula stages; absolute hatching rates of 2-24%) than in somites and tail elongation stages (25-67%) (Fig. 2.5). There were no marked differences between extenders and the results were similar for microinjection in the YM and PS. However, Yamamoto solution and ¼ ASW gave marginally higher and lower hatching rates, respectively, than the other extenders. Hence, Yamamoto solution was selected for the preparation of the CPAs solutions for microinjection.

2.3.4. Tolerance of embryos to microinjected CPAs

The hatching rates of embryos injected in the YM (15.6 nl), PS (2.1 nl), or simultaneously in both locations (15.6 nl + 2.1 nl) with solutions containing 30-90% Me_2SO, EG or PG in Yamamoto solution are summarized in Figures 2.6-2.8. Early developmental stages (2~32 cells to gastrula) showed low tolerance to injection in the YM and PS (Figs. 2.6, 2.7) and the combined injection was not tested (Fig. 2.8). Hatching rates of somites and tail elongation embryos injected with equivalent CPA solutions/concentrations decreased in the order of PS (Fig. 2.7) > YM (Fig. 2.6) > combined injection (Fig. 2.8). In general, hatching rates with the three types of injection were highest and lowest with EG and PG, respectively, and decreased with increasing CPA concentration. Embryos in the early developmental stages (2~32 cells to gastrula) did not tolerate injection volumes of 15.6 nl (YM) and 2.1 nl (PS), either single or in combination, and hatching rates were consistently below 5% regardless of the CPA type and concentration (Figs. 2.6, 2.7). Hatching rates of 2~32 cell embryos injected only in the YM with a smaller volume (8.2 nl) and lower CPA concentrations (30-50%) improved markedly and, contrary to somites and tail elongation embryos, had highest survival rates with 30% EG and PG and the lowest with Me_2SO (Fig. 2.9).

2.3.5 Estimation of the internal concentration of CPAs in the YM and PS following microinjection

Yamamoto solution distributed homogeneously in the PS and YM of the embryos within 1 and 4 min, respectively (Fig. 2.10) and remained contained within the compartments unless as noted above (Fig. 2.4). The volumes of the entire embryo, YM, and PS increased from the 2 cell stage (0.119, 0.113, and 0.006 mm^3, respectively) to the tail elongation stage (0.166, 0.139, and 0.027 mm^3, respectively) are shown in Table 2.1, and percentage of PS and YM volume also shown in Fig. 2.11. The volumetrically estimated internal CPA

concentrations in the YM and PS of selected developmental stages and suitable injection volumes are shown in Table 2.2. A combination of this information with the survival rates shown in Figures 2.8 and 2.9 allows the estimation of the maximum load of CPAs tolerated by the embryos in different developmental stages. For example, for a cutoff survival rate of about 10% for tail elongation embryos, the maximum achievable load of EG by single injection would be 975 mM and 1406 mM in the PS and YM, respectively, attained by injection of 2.1 (PS) and 15.6 nl (YM) of 70% EG (Table 1). In the case of double injection, the maximum achievable load would be 696 mM and 1004 mM in the PS and YM, respectively, with injection of 50% EG. In the case of 2~32 cell embryos, the maximum achievable load of Me_2SO, EG, and PG in the YM for the same cutoff survival rate of 10% would be 313 mM, 520 mM, and 380 mM, respectively. These concentrations can be obtained with a single 8.2 nl injection in the YM of 30% Me_2SO, 40% EG, and 40% PG, respectively (Table 2.2).

2.3.6. Ice nucleation temperature of CPA-microinjected embryos

A typical DSC cooling and warming curve of Japanese whiting tail elongation embryos showing the temperature of intracellular ice formation (TIIF) and temperature of equilibrium melting point (Tm, ^{o}C) is represented in Fig. 2.12. In this case, cooling and warming rates were -5°C/min and 5°C/min, respectively. No significant difference ($p > 0.05$) was found among different cooling rates, but -5°C/min gave marginally lower TIIF (-24.1^{o}C) than the other cooling rates (Fig. 2.13). Thus, the remaining experiments of DSC analysis in this study were carried out at a rate of -5°C/min. For 2 cells~morula, and tail elongation stage embryos, the CPA concentration was inversely related with TIIF for EG (Fig. 2.14A). A similar pattern was found also in case of EG and Tm values (Fig. 2.14B).

Embryo survival and TIIF values decreased with time after microinjection with 30% EG in 2 cells~morula and tail elongation stages (Fig. 2.15). These results suggested that DSC analysis of CPA microinjected embryos for measuring TIIF values should be conducted between IAM and 1 h. Embryos in the 2 cells~morula stage that were single-injected in the YM (8.2 nl) and tail elongation stage embryos that were double-injected in the YM (15.6 nl) and PS (2.1 nl) with 30% Me_2SO, EG and PG had significantly lower TIIF values than untreated controls (Fig. 2.16). There was no significant difference among all treatments in both stages but EG had marginally lower TIIF than other CPA treatments and tail elongation embryos had lower TIIF than 2 cells~morula stage (Fig. 2.16). EG had clearly the best

combination (lowest values) of TIIF and Tm than other CPAs for tail elongation stage embryos whereas for 2 cells~morula stages the difference between EG, PG, and Me₂SO was not so apparent (Fig. 2.17).

2.3.7. Chilling tolerance of CPA-microinjected embryos

In a preliminary trial to determine the conditions for testing the chilling tolerance of embryos, none of the tail elongation stage embryos double-injected in the YM (15.6 nl) and PS (2.1 nl) with Yamamoto solution survived exposure to -20°C even for a period as short as 5 min (Fig. 2.18). Hatching rates also dropped markedly at all experimental temperatures during exposure for 30 min. Thus, exposure to -10°C for 20 min was chosen for the subsequent tests. Tail elongation stage embryos double-injected in the YM (15.6 nl) and PS (2.1 nl) with 30% EG showed significantly higher hatching rates in the chilling tolerance test at -10°C for 20 min compared to 30% Me₂SO or PG and untreated control embryos (Fig. 2.19A). Moreover, a comparison of different concentrations of EG (0, 30, 40, and 50%) showed higher hatching rates at 30 and 40% than in other concentrations of EG (Fig. 2.19B). It is noteworthy that 2 cells~morula stage embryos had very low chilling tolerance compared to tail elongation embryos; still, the treatment with 30% EG seemed to provide some degree of cryoprotection (Fig. 2.19).

2.4. Discussion

Successful application of microinjection for delivery of CPAs into fish eggs or embryos requires optimization of species-specific factors such as microinjection location and volume, CPA type, concentration and vehicle (diluent), and choice of a suitable embryonic developmental stage, to mention a few. In this work, we examined some of these factors for microinjection-based delivery of CPAs into specific embryonic compartments of the Japanese whiting, an alternative marine model for fish embryo cryopreservation studies (Rahman et al., 2008).

A first consideration was the location of injection and the tolerance of the embryos to the procedure. The YM and PS are the major compartments of fish embryos. The volumetric ratio between PS and YM in whiting embryos is about 16 and 84% and both compartments contain structures that need to be adequately protected from cryoinjury. The PS envelops the embryo proper and contains a fairly large amount of free water that could potentially give rise to harmful ice crystals during freezing. Khosla et al. (2017) reported the use of a combination of microinjections into the YM and PS for introduction of CPAs in zebrafish embryos

whereas in the past the emphasis has been on injection into the YM (Janik et al., 2000; Beirão et al., 2006; Kopeika et al., 2006). This study showed that whiting embryos can tolerate injections in both locations, single or in combination, under certain conditions discussed below.

After confirming that whiting embryos at all developmental stages can tolerate dry-puncturing through the chorion and the YSL without substantial loss of viability, we estimated the maximum volume of CPA vehicle that can be injected in the PS and YM of embryos during single and combined microinjection. This was examined in tail elongation embryos using Yamamoto solution and the analysis showed that survival rates remained above 50% for volumes up to 2.1 and 15.6 nl injected in the PS and YM, respectively, and that there were no significant differences in survival between location and type of injection (single or combined). We also confirmed that the solution diffused almost immediately throughout the PS and YM after injection and that it remained contained within each compartment unless in the case of structural damage caused by injection of larger volumes. The maximum volume tolerated by whiting embryos injected in the YM is in the same range tolerated by tail bud turbot embryos injected with embryo medium (e.g. 20 nl; Robles et al., 2006). Interestingly, injection of the same volume of Yamamoto solution and three other diluents caused a sharp drop in viability of embryos between 2~32 cells and gastrula stage but not in somites and tail elongation stages, suggesting that early embryonic stages of whiting, although tolerant to dry-puncturing, are sensitive to the introduction of foreign liquids including non-toxic physiological saline solutions. In fact, a reduction in the injection volume in the YM from 15.6 to 8.2 nl greatly enhanced the survival of early stage whiting embryos and may be important also in mitigating the low tolerance of these embryos to the CPAs (see further discussion below).

Next, we compared the suitability of different diluents. Diluents, or extenders, are generally saline solutions which help maintain the viability of cells during refrigeration, prior to freezing, and which are also necessary as vehicles for dilution of CPAs before impregnation/injection. We have previously used full strength ASW as diluent for immersion-based CPA impregnation aided by sonication, electroporation, osmotic, and chemical treatments (Rahman et al., 2008, 2011, 2013, 2017) but this solution is clearly osmotically incompatible with the embryo internal milieu. Studies on CPA microinjection in embryos of other fish species have used a variety of diluents, generally isotonic solutions

such as fish Ringer (e.g. in gilthead seabream *Sparus aurata*; Beirão et al., 2006), Hank' solution (zebrafish *Danio rerio*; Kopeika et al., 2006), and embryo medium (turbot *Scophthalmus maximus*; Robles et al., 2004, 2006). In this study we did not find significant differences in viability of embryos microinjected with three extenders, fish Ringer, Yamamoto solution, and PBS, but overall, Yamamoto solution was marginally better. In contrast, ¼ ASW gave clearly inferior results and therefore is considered as unsuitable for injection in whiting embryos.

As with the diluent, the choice of an adequate CPA type and concentration is critical for successful cryopreservation. In this study, we examined the toxicity of three CPAs (Me$_2$SO, EG and PG) injected in different concentrations into whiting embryos. Our results suggest that EG is the least toxic to somites and tail elongation embryos on an equal concentration basis, followed by Me$_2$SO and PG, whereas for early stage embryos EG and PG were significantly better tolerated than Me$_2$SO. The order of toxicity for late stage whiting embryos is the opposite of that obtained by Rahman et al. (2008) for immersion-based permeation through the chorion. This discrepancy is likely the result of differential permeability/uptake of the CPAs by intact embryos, which according to Rahman et al. (2008) follows the order PG < Me$_2$SO < EG, and the fact that any differences in CPA permeability through the fish embryo chorion are obviated by microinjection. Interestingly, EG was also found to be less toxic than Me$_2$SO and MeOH for injection in the YM of tail bud stage embryos of gilthead sea bream (Beirão et al., 2006). On the other hand, Khosla et al. (2017) and Janik et al. (2000) reported that PG was better tolerated by zebrafish embryos compared to other microinjected CPAs. Another difference between the current and previous results for whiting embryos is that CPAs appeared to be more toxic to earlier stages in this study whereas Rahman et al. (2008) reported the opposite. A clear explanation for this discrepancy is not yet available but it may be related to the smaller compartment sizes in early embryos, which results in comparably higher internal CPA concentrations after microinjection, and the longer exposure to the CPA until hatching that they experience compared to later stages.

After optimization, we applied the established protocols for 2 cells~morula and tail elongation stages embryos and examined the TIIF values in different treatments. A slow cooling rate of -5°C/min substantially reduced the TIIF values in embryos and was used to examine the relation between CPA concentration and TIIF values. Thus, TIIF values decreased with increasing CPA (EG) concentration in an almost linear fashion. Routray et al. (2002) also reported a linear relation between CPA (Me$_2$SO) concentration and TIIF in

pejerrey embryos. This study also found out that TIIF values of CPA-microinjected embryos decreased gradually starting from 1 hour after microinjection. A possible reason for this decrease may be diffusion of CPAs through the chorion and into the external medium during prolonged incubation. In this study, we observed that 30% CPA solutions, particularly EG, significantly depressed TIIF values compared to controls, which is a desirable feature for cryopreservation of fish embryos. We also observed that later developmental stage embryos had lowest TIIF (about -27°C) than early stage embryos (about -24°C). Thus, our results agree with those of other studies which reported that the ice nucleation temperature of fish embryos shifts to lower temperatures with development (Liu et al., 2000; Routray et al., 2001).

Next, we compared the chilling tolerance of CPA-treated embryos to exposure to -10°C for 20 min at 2 cells~morula and tail elongation embryos. These conditions were chosen based on the results of a preliminary study which showed that they consistently yield survival rates of untreated controls between 0 and 5%. The results suggested that 30% EG produced the highest survival after chilling, followed by Me$_2$SO and PG and that tail elongation embryos were more tolerant than early stage embryos. Also, there was no significant difference between 30 and 40% of EG in terms of chilling tolerance so both solutions appear suitable for future studies. Interestingly, embryos microinjected CPA solutions, particularly EG, had significantly lower TIIF values and improved chilling tolerance than untreated controls, but it is not reasonable to attribute the observed increased chilling tolerance to the lower TIIF values as both phenomena occur at different thermal ranges (e.g. -10°C for tolerance and below -20°C for intracellular ice formation. Other authors have also observed improved chilling tolerance compared to untreated embryos, although the reasons for this protection are not clear (Zhang and Rawson, 1995).

2.5. Conclusions

Perhaps the most important finding of this study is that microinjection was efficient in delivering high concentrations of CPAs swiftly to specific compartments of whiting embryos. Volumetric estimation of the internal CPA concentrations in the YM and PS achieved by microinjection in this study, assuming the injected solutions dispersed homogeneously within the respective compartments, yielded values 2-4 fold higher than those previously obtained for this species with immersion-based impregnation (Rahman et al., 2008, 2011, 2013, 2017). More importantly, embryos microinjected with single CPA solutions had significantly lower

TIIF values and improved chilling tolerance than untreated embryos. We have not yet tested the amount of cryoprotection afforded by injection of combinations of CPAs or conducted vitrification tests, but it is noteworthy that some embryos could tolerate very high internal CPA concentrations in the toxicity tests, particularly in the case of late stage embryos. This will provide valuable materials to optimize the remaining parameters that are critical for successful cryopreservation such as cooling and warming strategies (see for example Khosla et al., 2017).
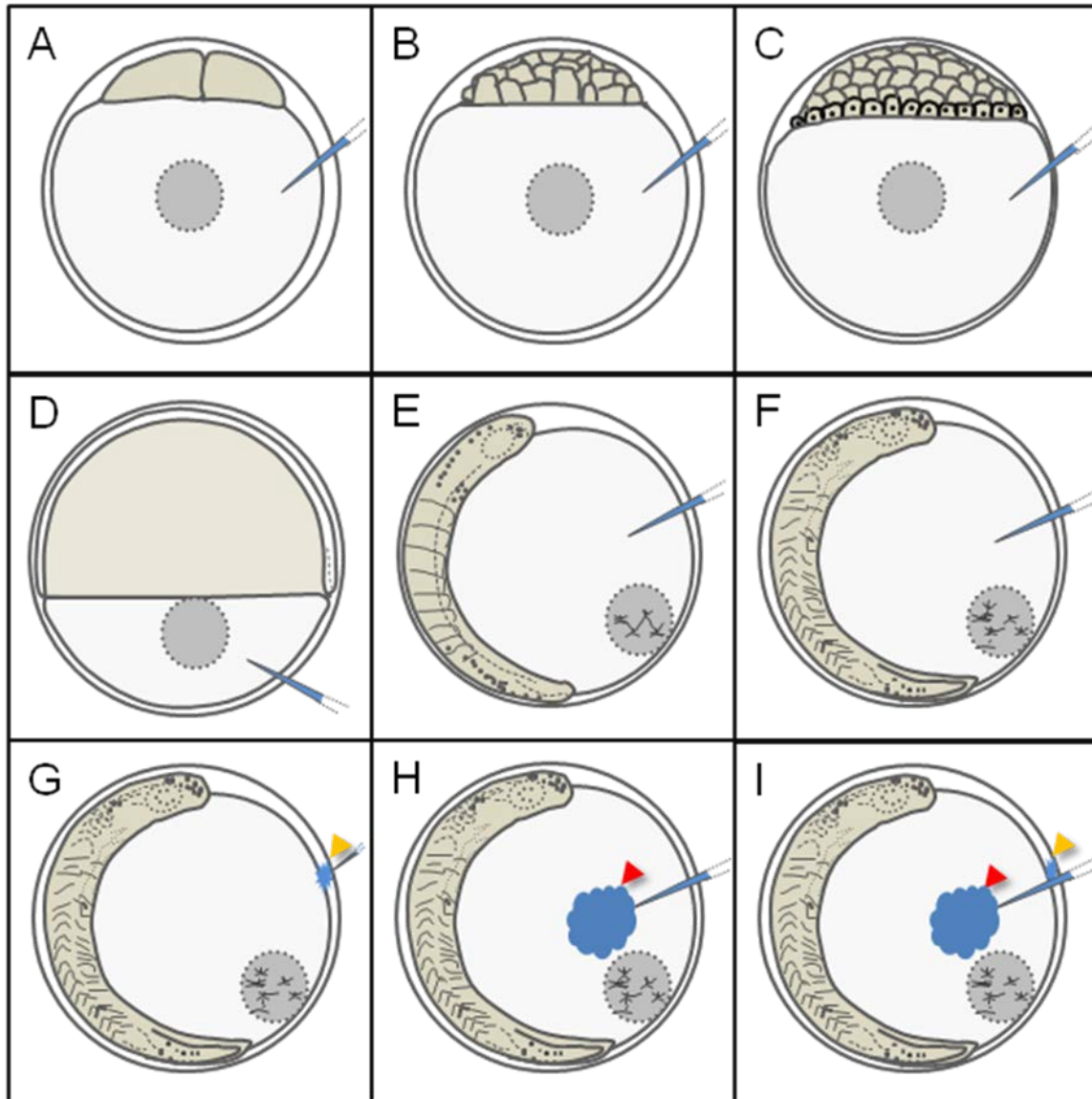
Fig. 2.1. Position of microinjection into Japanese whiting embryos at the A) 2~32 cells, B) morula stage, C) blastula stage, D) gastrula stage, E) somites, and F) tail elongation stages. Location of delivery during G) single injection into the perivitelline space (PS) or H) the yolk mass (YM) and I) double injection into the PS and YM.
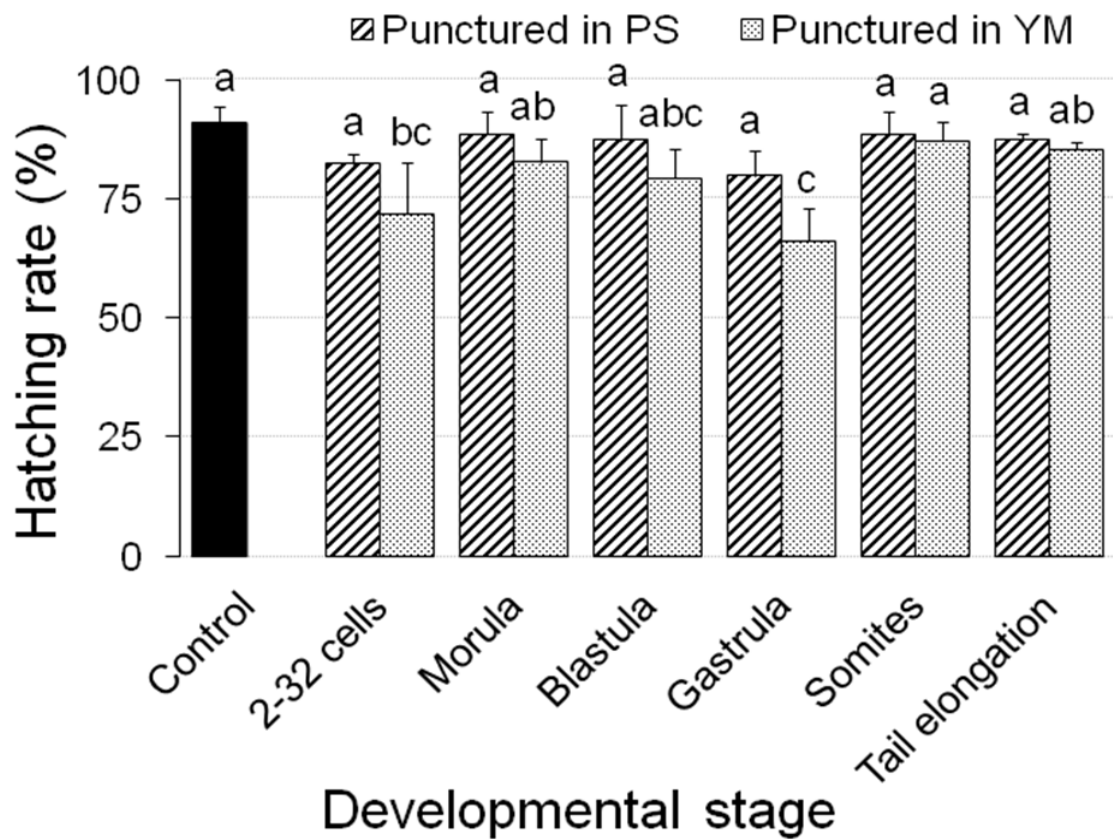
Fig. 2.2. Hatching rates of Japanese whiting embryos at six developmental stages punctured into the perivitelline space (PS) and the yolk mass (YM). Untreated, not punctured embryos were used as controls. Thick and thin bars represent the mean and SD, respectively, of five replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
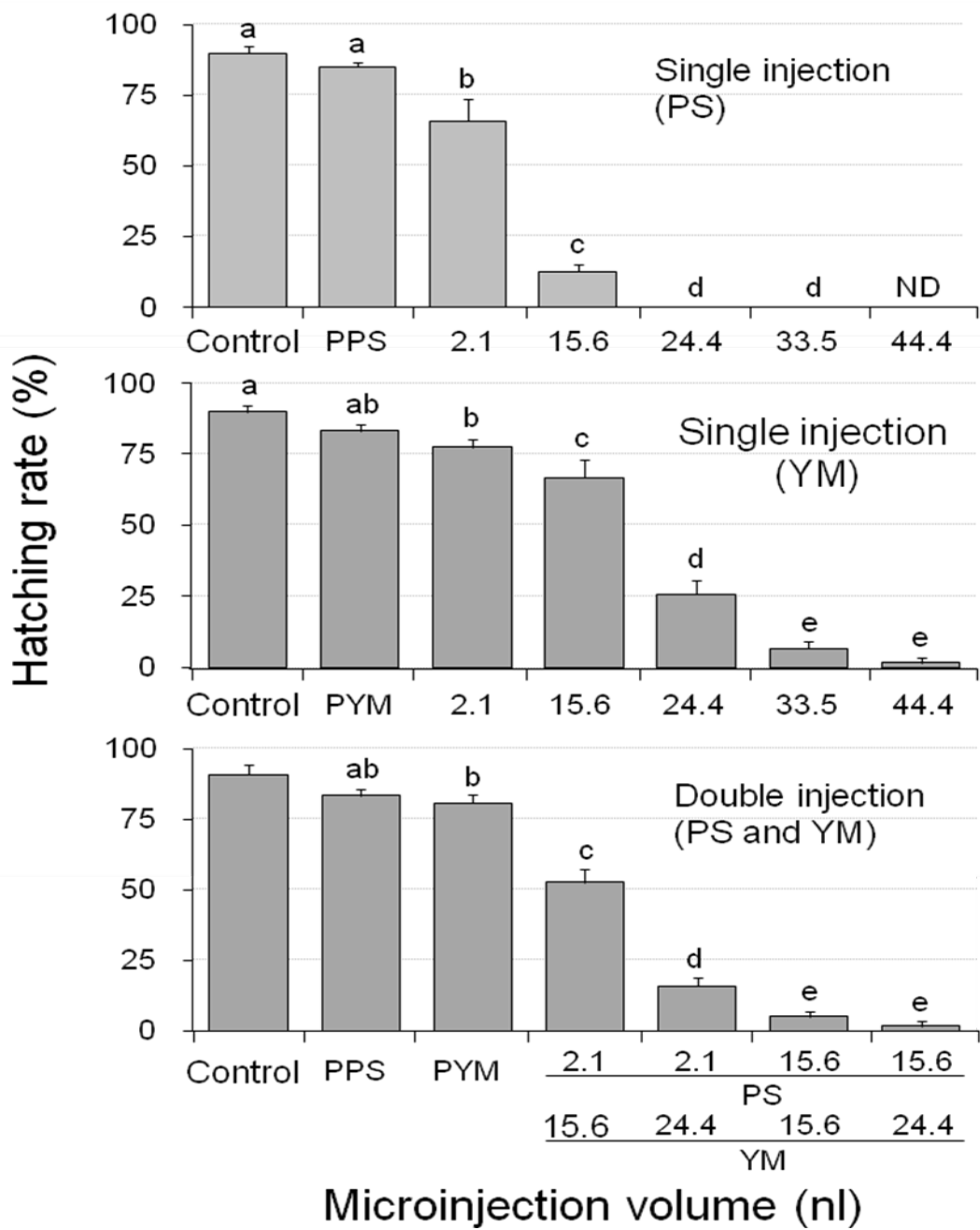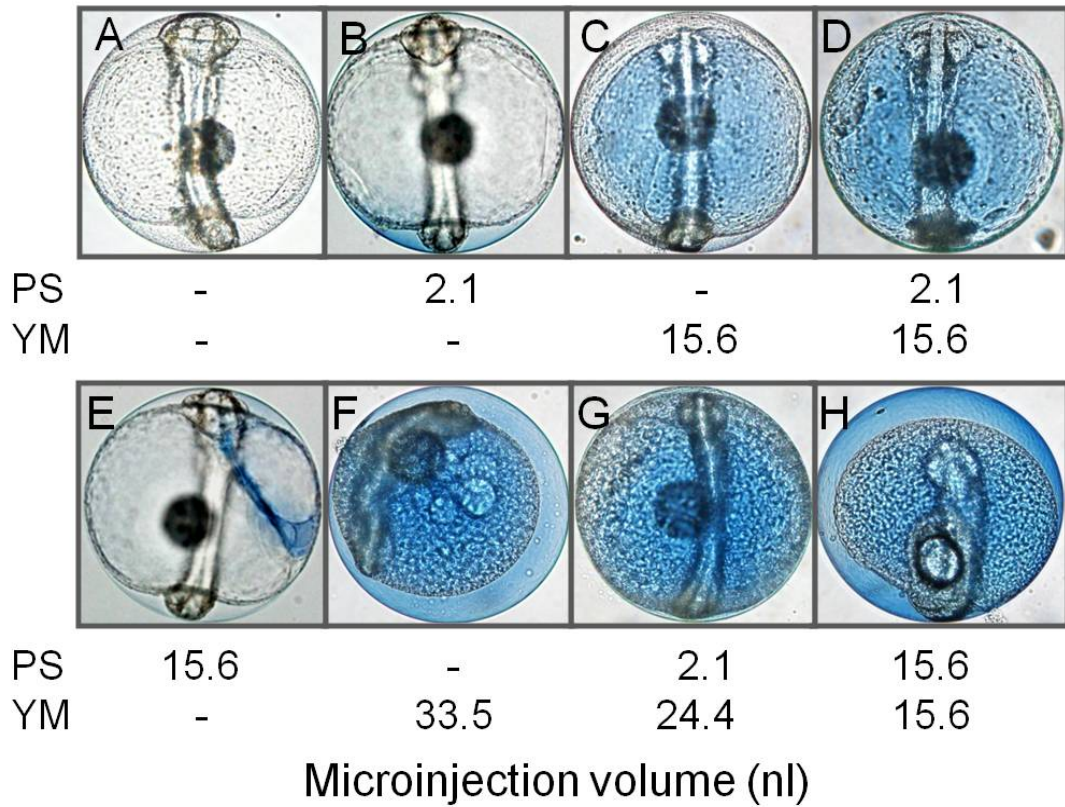
Fig. 2.3. Hatching rates of Japanese whiting embryos at the tail elongation stage single- or double-injected with different volumes of Yamamoto solution in the perivitelline space (PS) and yolk mass (YM). Controls include untreated embryos (Control) and embryos only punctured into the perivitelline space (PPS) and yolk mass (PYM) but not microinjected. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey). ND: Not determined.

	A	B	C	D
PS	-	2.1	-	2.1
YM	-	-	15.6	15.6

	E	F	G	H
PS	15.6	-	2.1	15.6
YM	-	33.5	24.4	15.6

Microinjection volume (nl)

Fig. 2.4. Appearance of Japanese whiting tail elongation embryos after microinjection into the perivitelline space (PS) and the yolk mass (YM) with different volumes of Yamamoto solution containing 0.04% trypan blue as visualization agent. Untreated embryos (A) and embryos injected with the lowest volumes (B-D) had normal appearance whereas those injected with larger volumes (E-H) had clearly altered morphology.
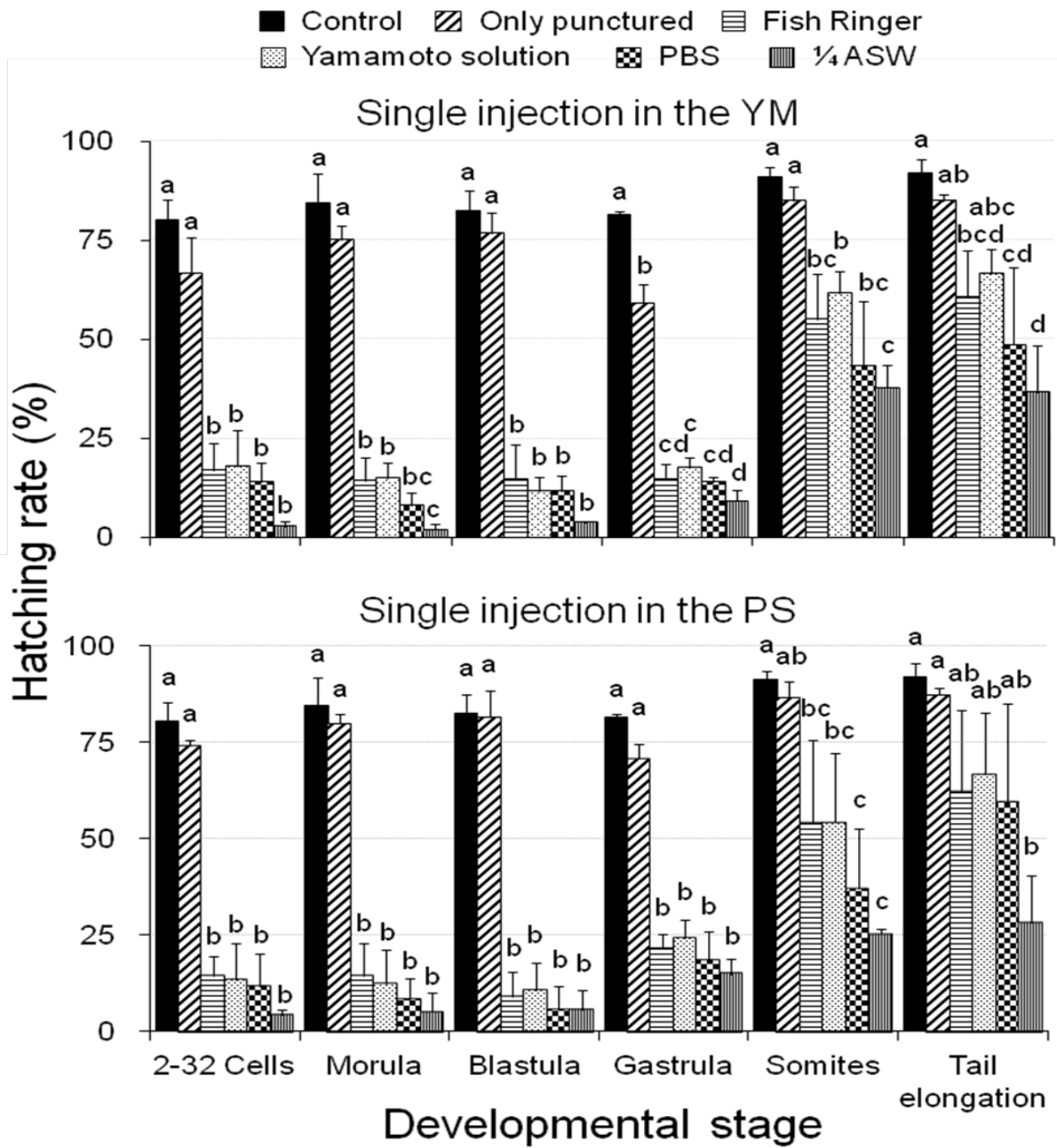
Fig. 2.5. Hatching rates of Japanese whiting embryos at different developmental stages that were microinjected in the yolk mass (15.6 nl) or the perivitelline space (2.1 nl) with four different extenders (Fish Ringer, Yamamoto solution, phosphate buffered solution, and ¼ diluted artificial sea water). Untreated embryos and embryos punctured not injected were used as controls. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey) within each developmental stage.
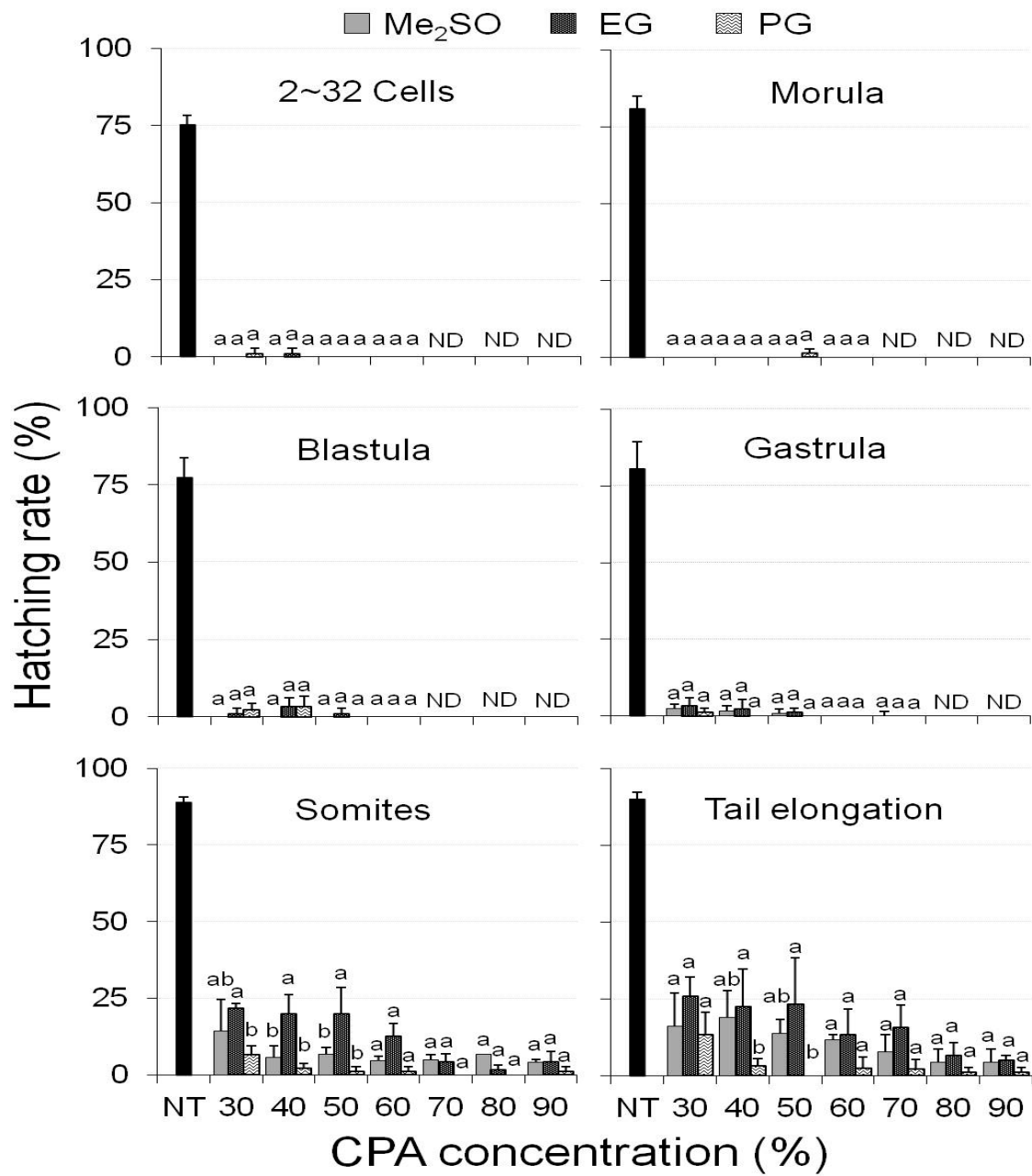
Fig. 2.6. Hatching rates of Japanese whiting embryos that were single-microinjected into the yolk mass at different developmental stages with 15.6 nl of 30-90% solutions of Me_2SO, EG and PG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA) within each CPA concentration. ND: not determined; NT: untreated controls.
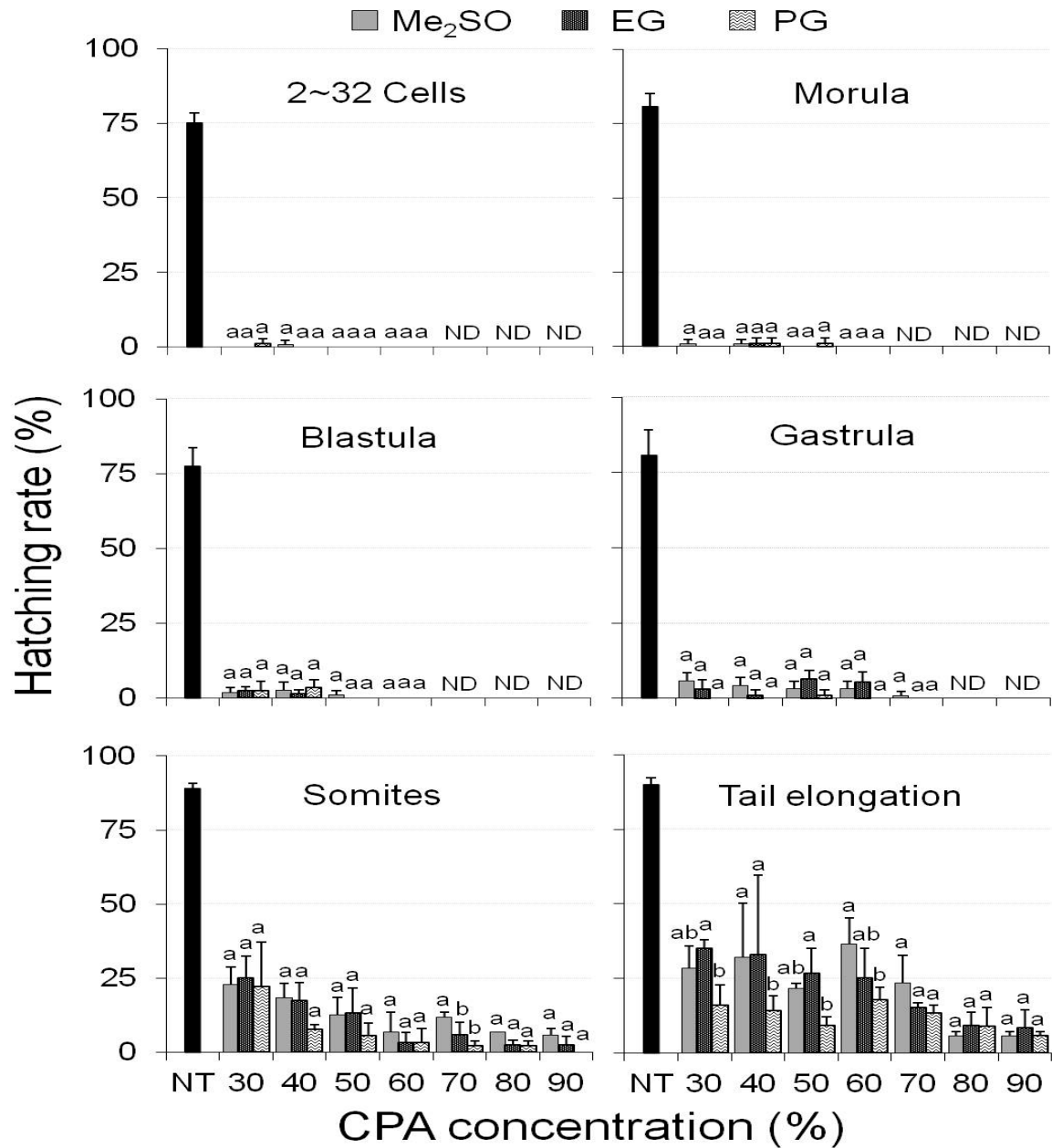
Fig. 2.7. Hatching rates of Japanese whiting embryos that were single-microinjected into the perivitelline space at different developmental stages with 2.1 nl of 30-90% solutions of Me_2SO, EG and PG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA) within each CPA concentration. ND: not determined; NT: untreated controls.
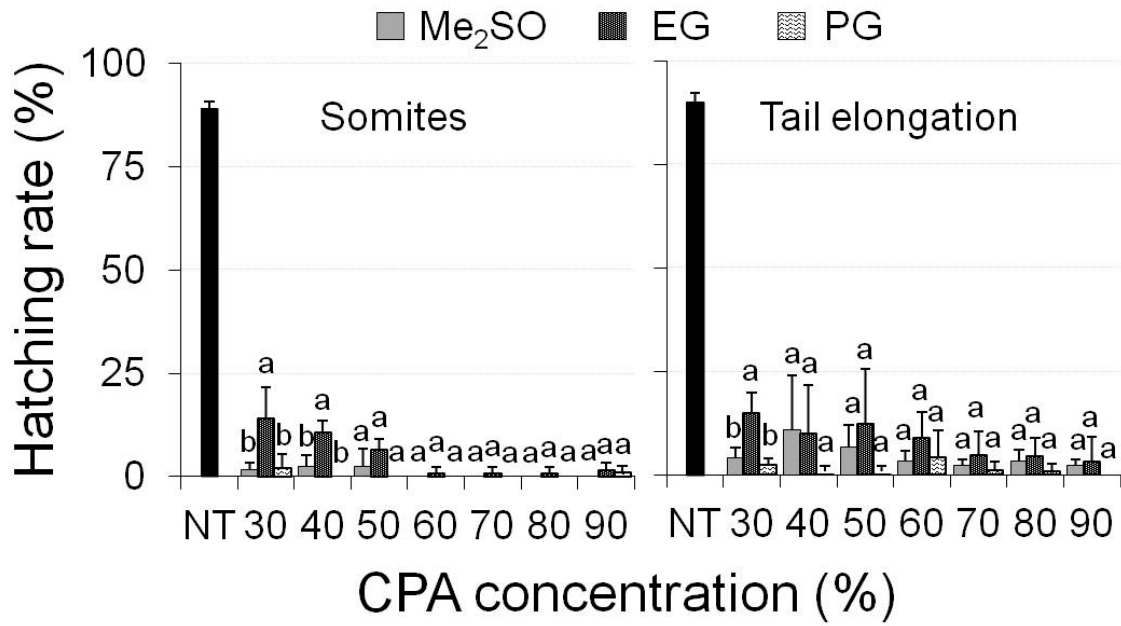
Fig. 2.8. Hatching rates of Japanese whiting embryos that were double-microinjected into the perivitelline space (2.1 nl) and yolk mass (15.6 nl) at different developmental stages with 30-90% solutions of Me_2SO, EG and PG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA) within each CPA concentration. ND: not determined; NT: untreated controls.
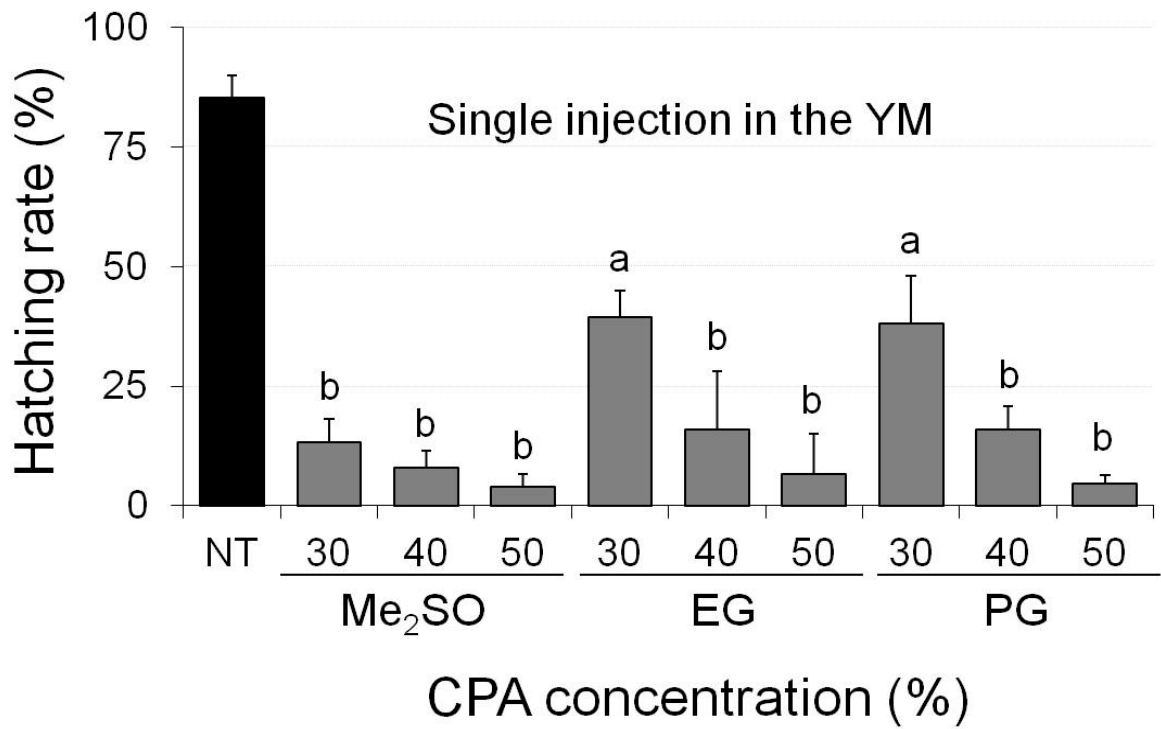
Fig. 2.9. Hatching rates of Japanese whiting 2~32 cell embryos that were injected into the yolk mass (YM) with 8.2 nl of 30-50% solutions of Me_2SO, EG and PG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey). NT: untreated controls.
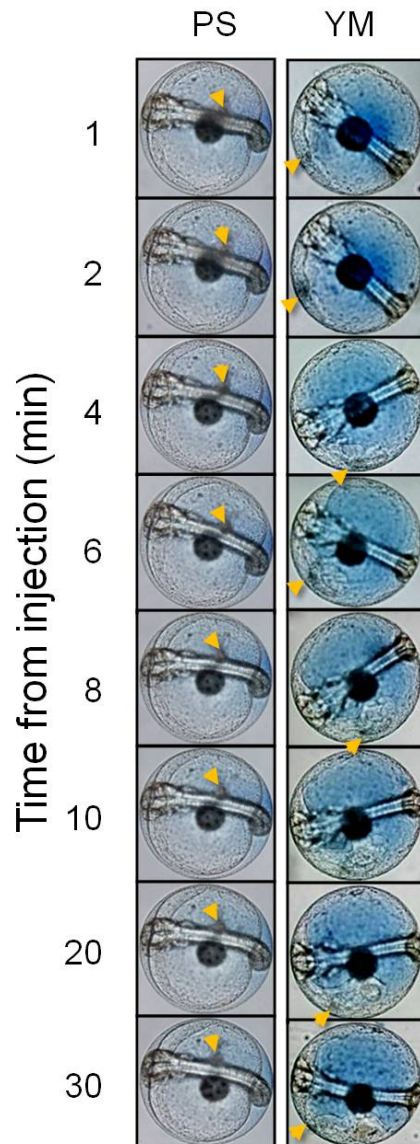
Fig. 2.10. Distribution pattern and dynamics of Yamamoto solution microinjected in the perivitelline space (PS, 2.1 nl) and yolk mass (YM, 15.6 nl) of Japanese whiting tail elongation stage embryos. Trypan blue (0.04%) was used as a visualization agent. Yellow arrows indicate the microinjection needle entry point.
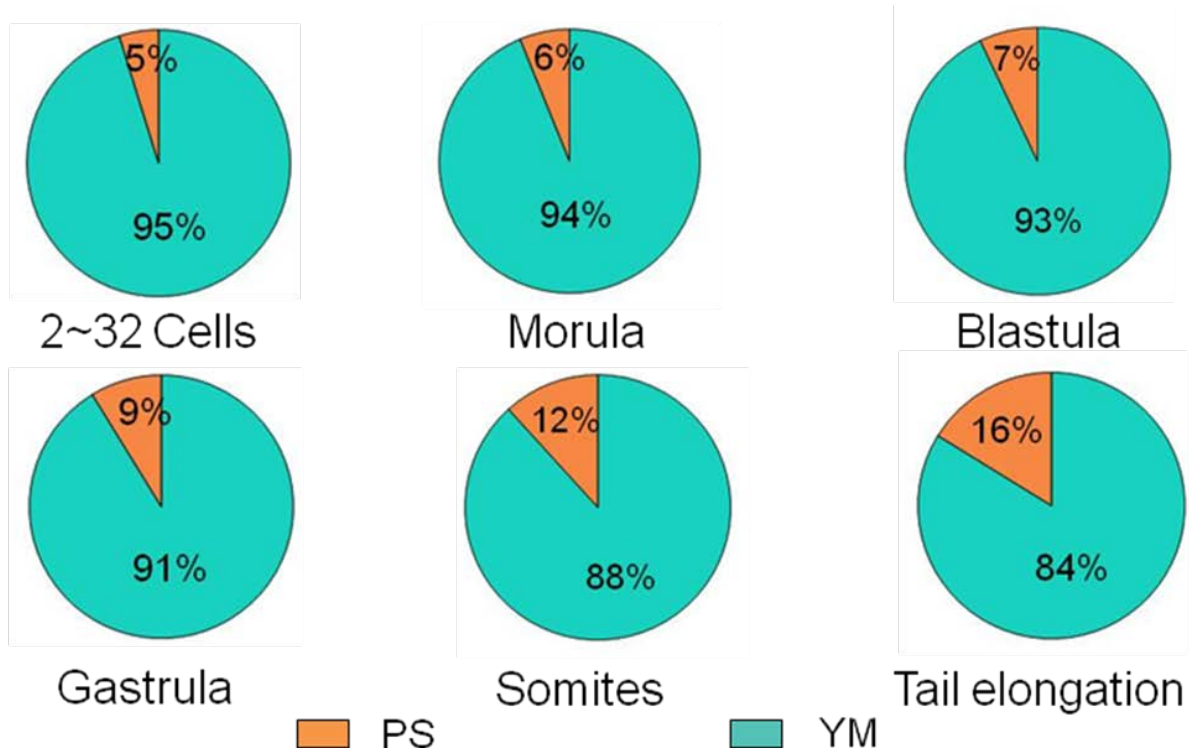
Fig. 2.11. Percentage of perivitelline space (PS) and yolk mass (YM) volume of Japanese whiting embryos at six developmental stages (2~32 cells, morula, blastula, gastrula, somites, and tail elongation).
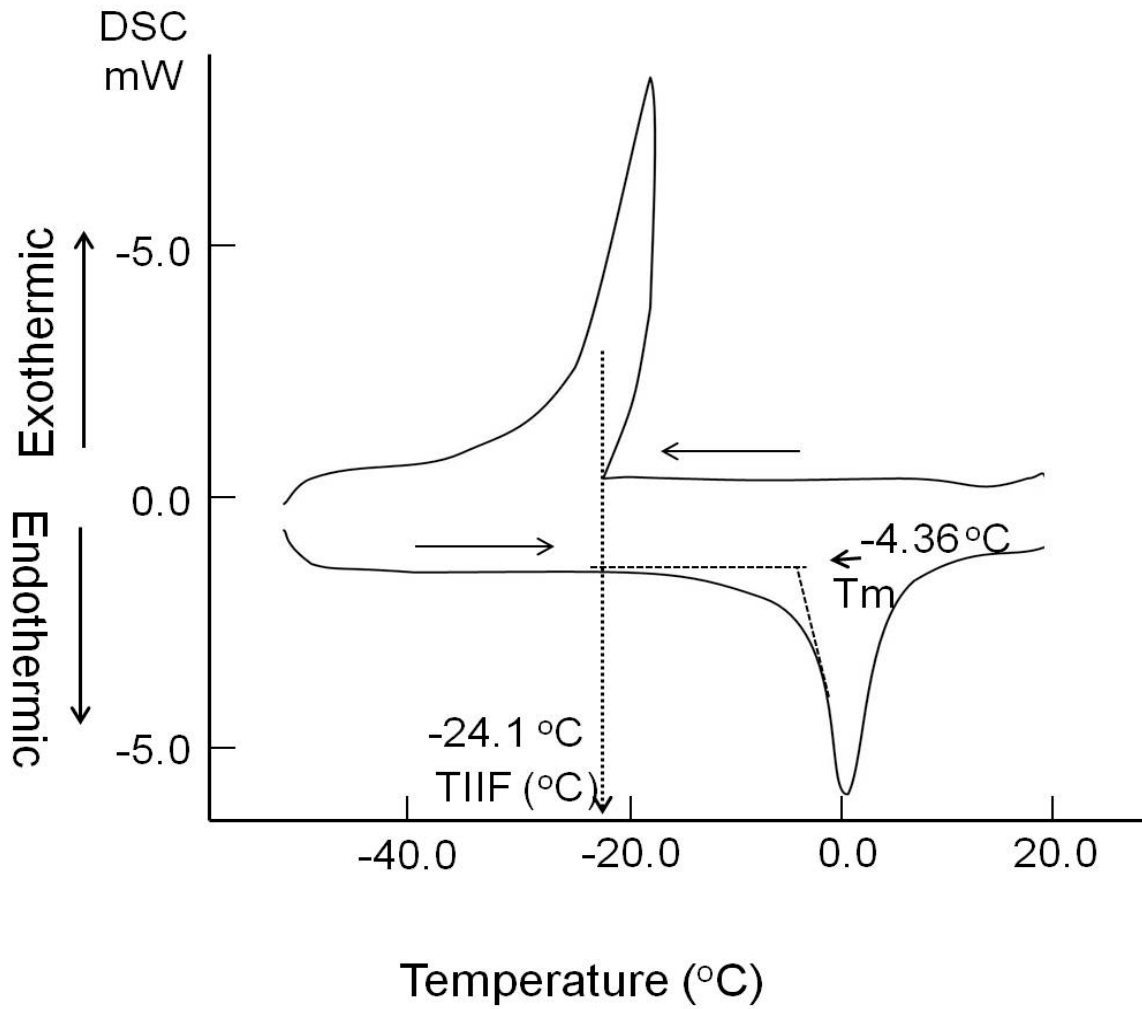
Fig. 2.12. A typical temperature and heat flow pattern of tail elongation embryos of Japanese whiting (*Sillago japonica*) obtained by DSC analysis when cooling and warming rate were maintained at -5ºC/min and 5ºC/min, respectively. Arrows indicated the temperature of intracellular ice formation (TIIF, ºC) and temperature of equilibrium melting point (Tm, ºC).
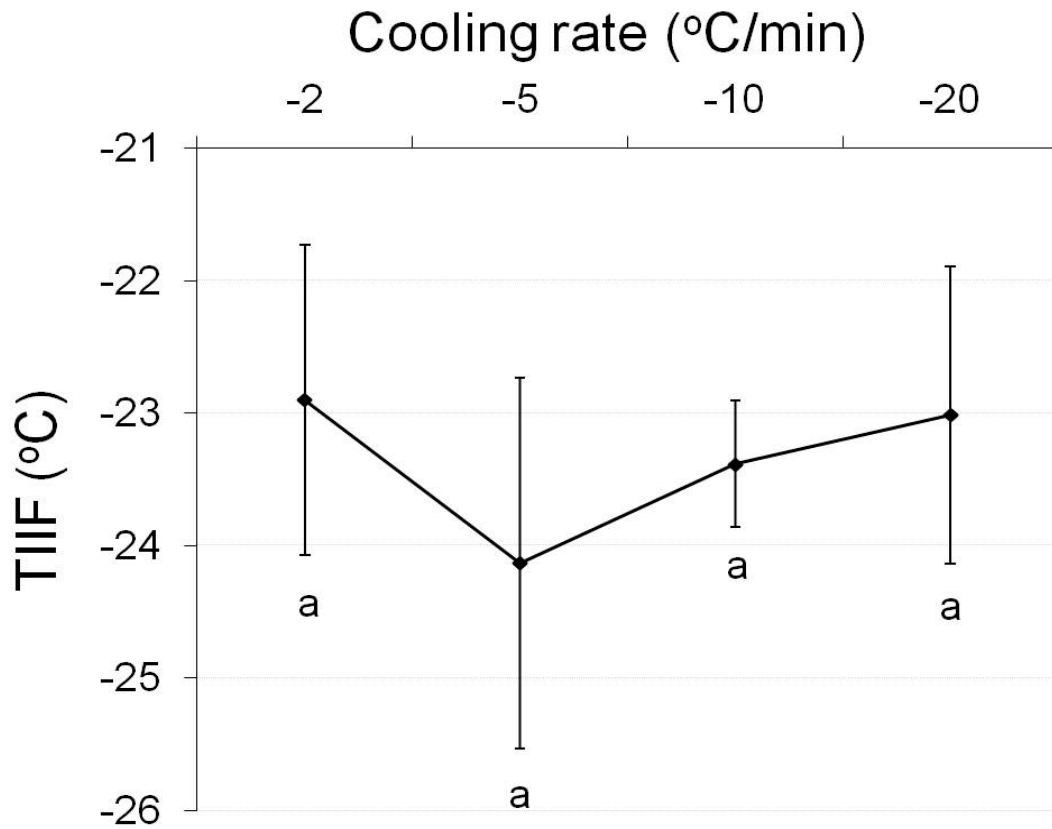
Fig. 2.13. Relationship between cooling rates and temperature of intracellular ice formation (TIIF) in Japanese whiting (*Sillago japonica*) tail elongation embryos that were double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with Yamamoto solution. Data shown as mean ± SD of four replicates. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey) within each cooling rate. Cooling rate was maintained at -5°C/min.
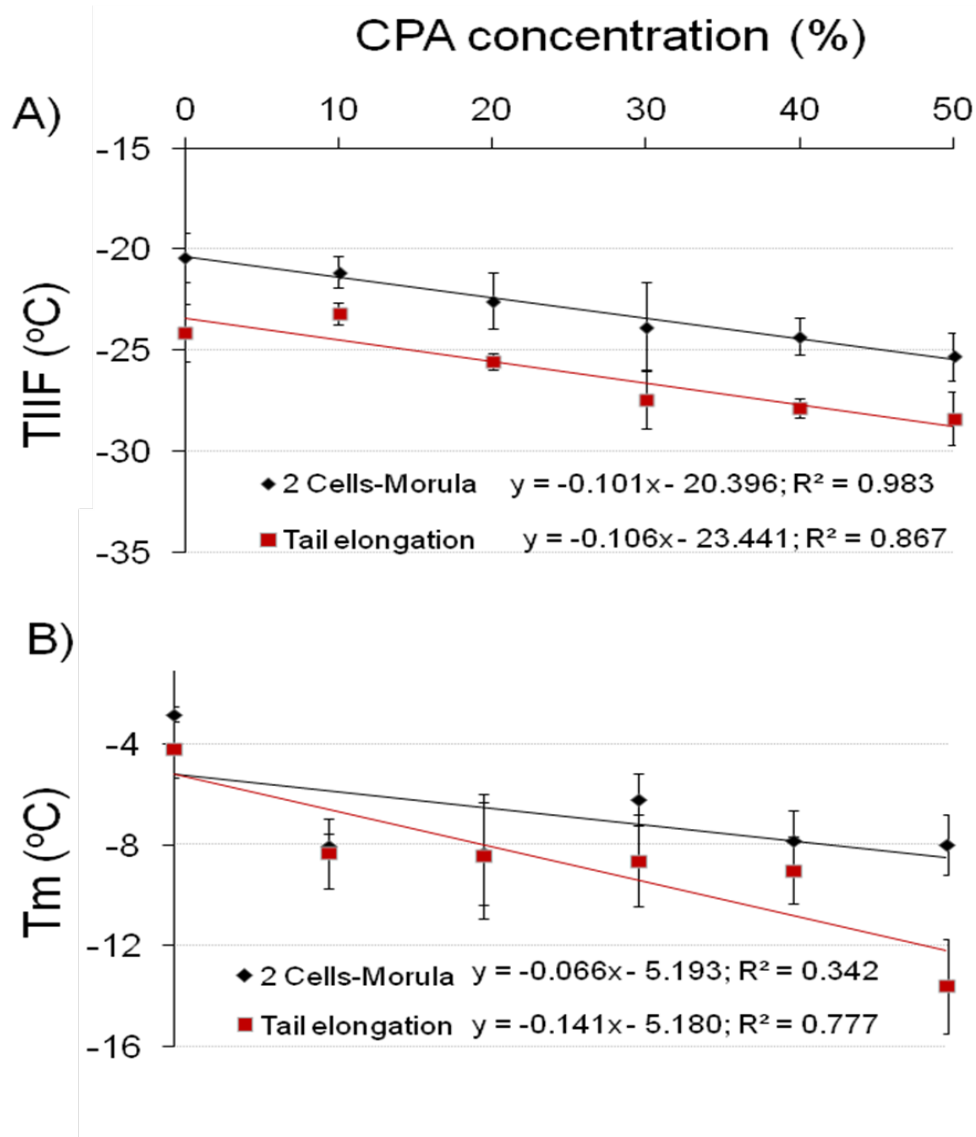
Fig. 2.14. Relationship between A) EG concentration and temperature of intracellular ice formation (TIIF), and B) EG concentration and equilibrium melting point (Tm) in 2 cells~morula embryos and tail elongation embryos of Japanese whiting. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 0, 10, 20, 30, 40, and 50% concentration of EG in Yamamoto solution. Red and black line bars indicated 2 cells~morula and tail elongation embryos, respectively. Data shown as mean ± SD of four replicates. Cooling and warming rate were maintained at -5°C/min and 5°C/min, respectively.
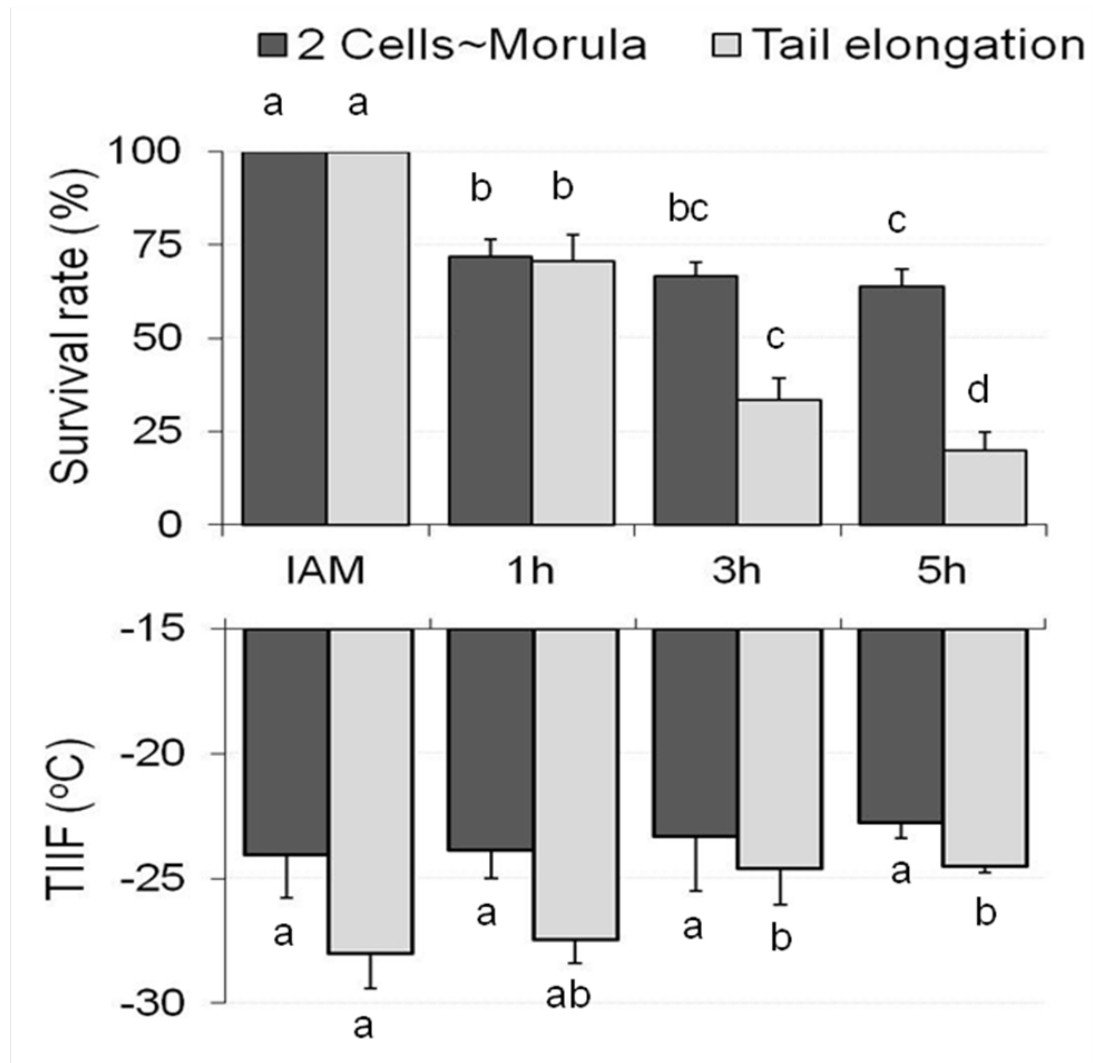
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Fig. 2.15. Chilling tolerance of embryos at different times after microinjection and temperature of intracellular ice formation (TIIF) in 2 cells~morula and tail elongation stage embryos of Japanese whiting. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 30% concentration of EG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey) within each treatment. Cooling rate was maintained at -5°C/min on DSC analysis. IAM: immediately after microinjection.
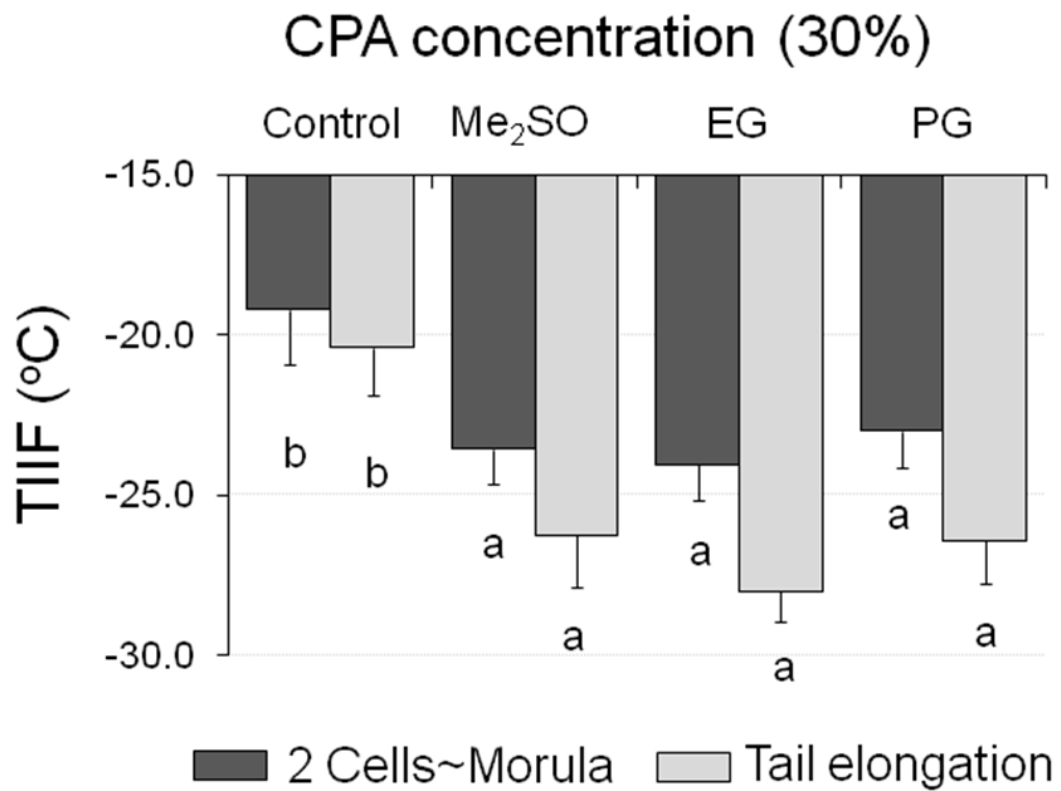
Fig. 2.16. Temperature of intracellular ice formation (TIIF, °C) in 2 cells~morula and tail elongation stage embryos of Japanese whiting. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 30% solutions of Me_2SO, EG and PG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates for each determination. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
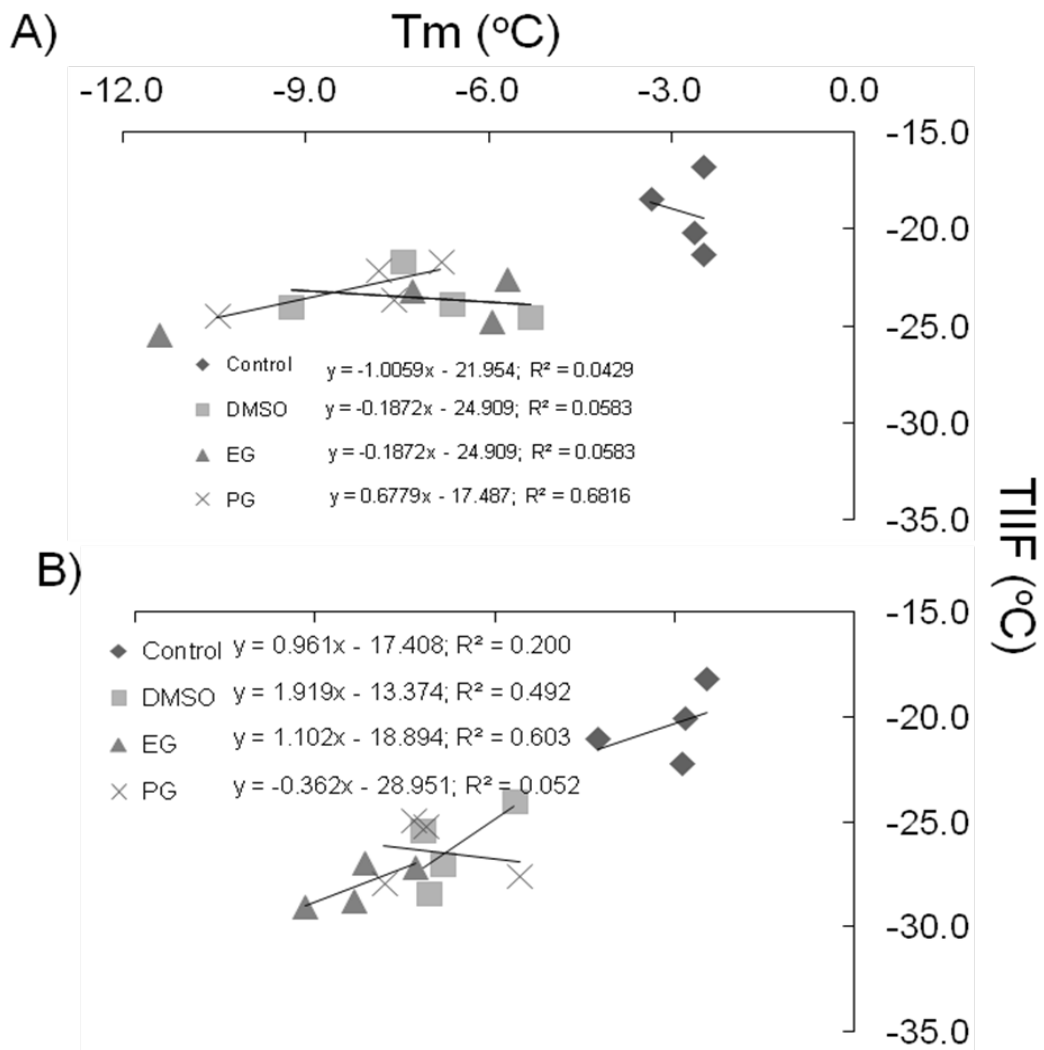
Fig. 2.17. Relationship between the temperature of intracellular ice formation (TIIF) and temperature of equilibrium melting point (Tm) in A) 2 cells~morula stage embryos and B) tail elongation stage embryos of Japanese whiting. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 30% concentration of EG in Yamamoto solution. Cooling and warming rate were maintained at -5°C/min and 5°C/min, respectively (each dot represents the result of one measurement; n = 4 for each treatment).

Fig. 2.18. Chilling tolerance of Japanese whiting embryos cooled to 0, -5, -10, and -20°C for 5, 10, 20 and 30 minutes. Embryos were double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl), respectively, with Yamamoto solution. Thick bars represent the mean of four replicates with at least 25 embryos each. The cooling and warming rates were -5°C/min and 8°C/min, respectively.
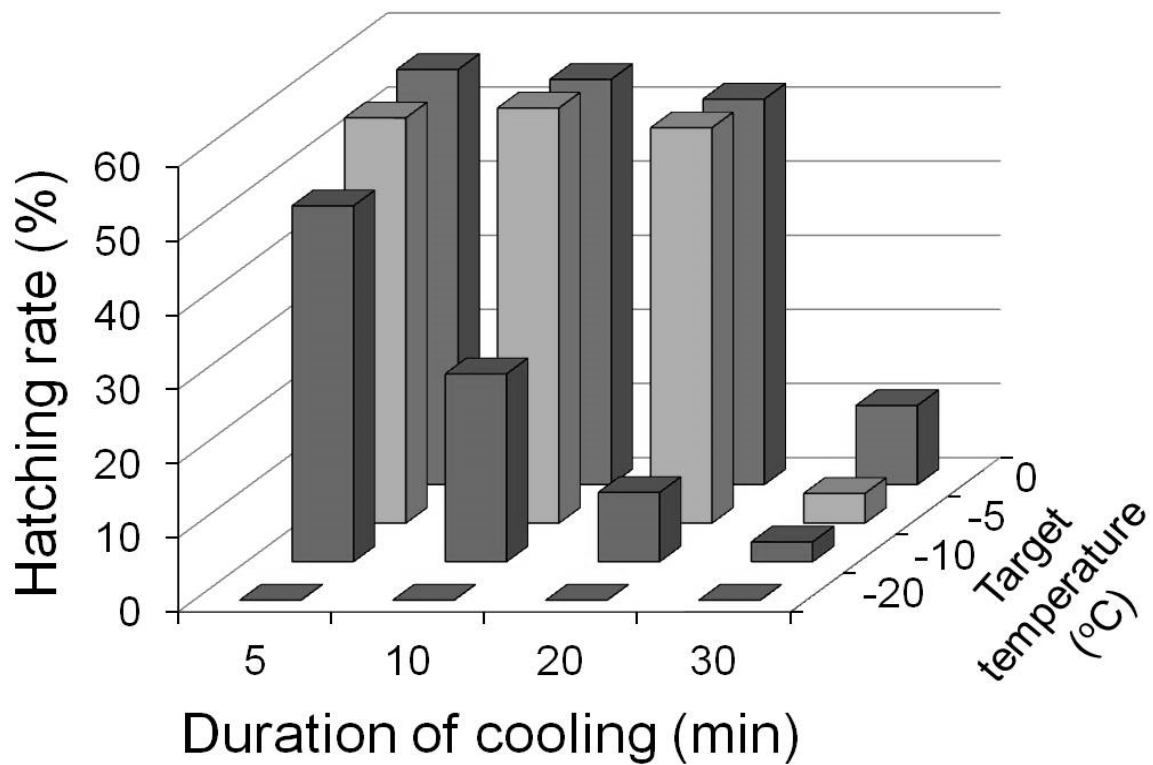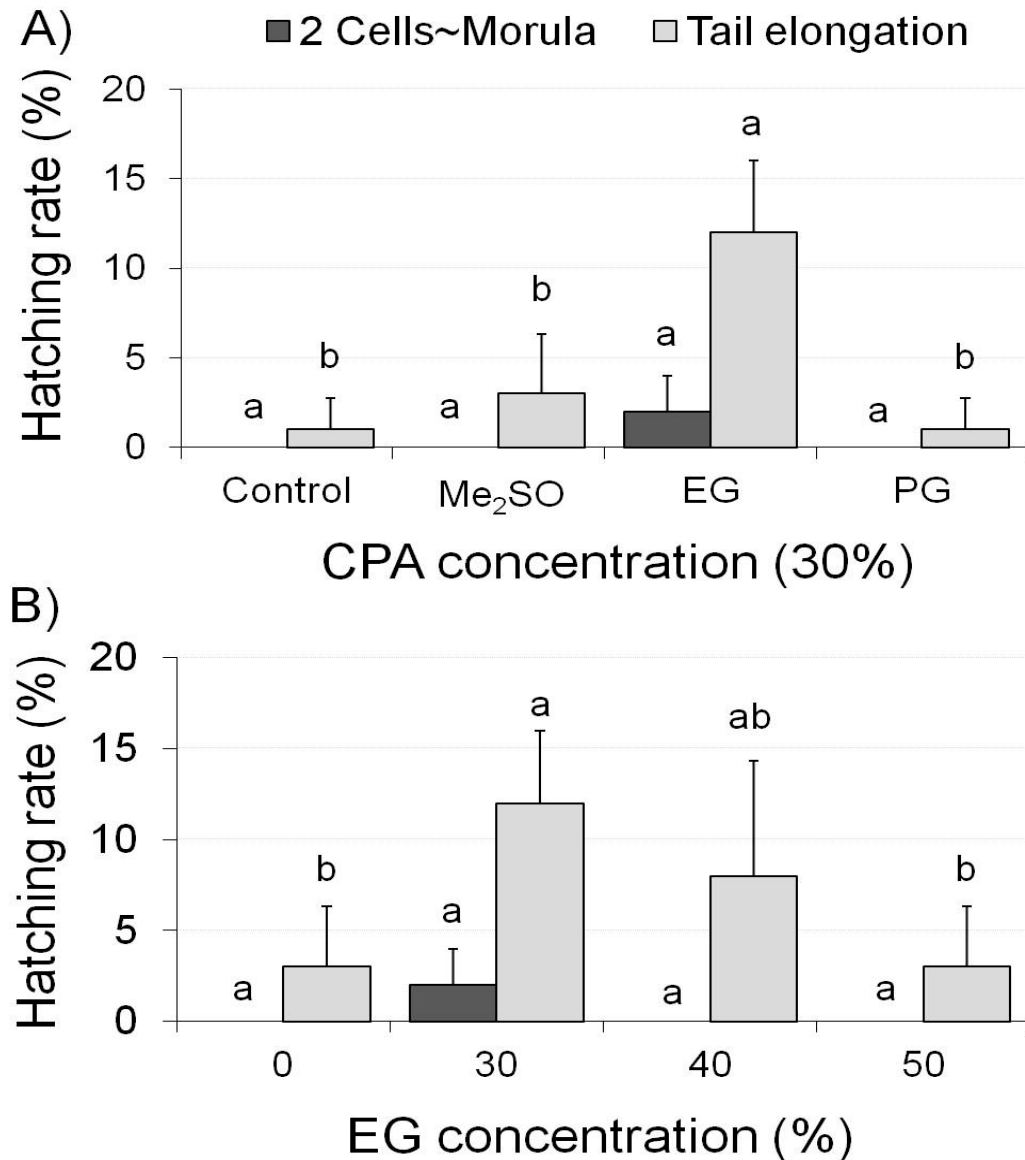
Fig. 2.19. Hatching rates in the chilling tolerance test where Japanese whiting embryos were subjected to a subzero, non-freezing temperature (-10°C). The test consisted of cooling from room temperature to -10°C at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Prior to the test, embryos at 2 cells~morula stage embryos were single-injected into the yolk mass (8.2 nl) and tail elongation stage embryos were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with A) 30% solutions of Me_2SO, EG, and PG, and B) 0, 30, 40 and 50% concentration of EG in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 25 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
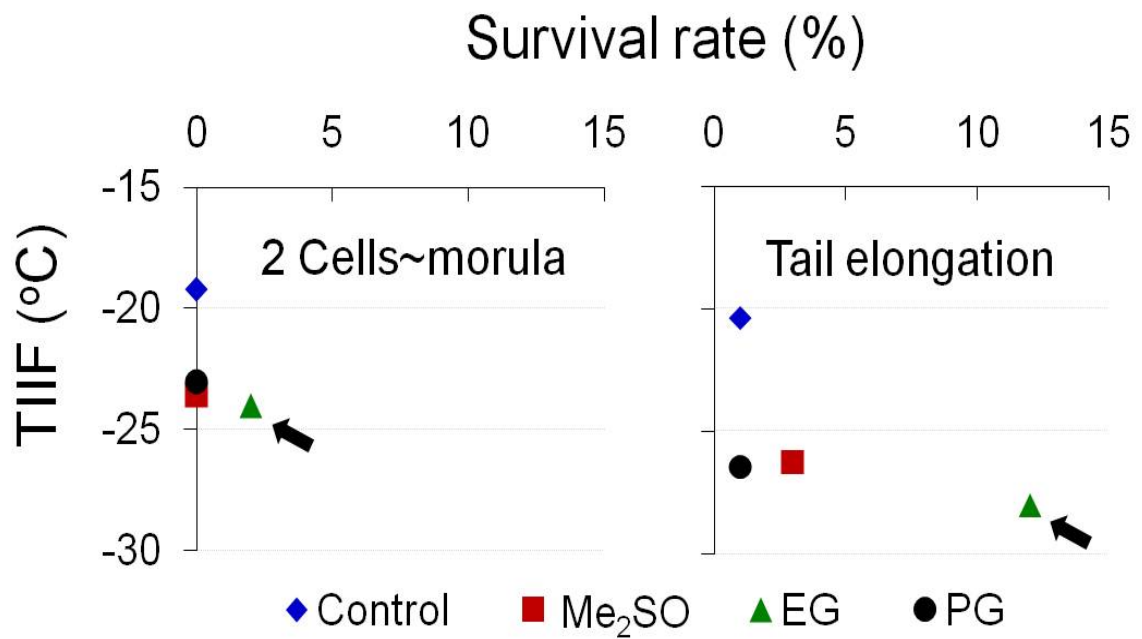
Fig. 2.20. Relationship between the survival rates (%) after the chilling tolerance test and the temperature of intracellular ice formation (TIIF) in 2 cells~morula and tail elongation stage embryos of Japanese whiting treated with 30% of Me_2SO, EG, and PG, and intact controls. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combined CPA solutions in Yamamoto solution. Black arrows indicated the highest survival and lowest TIIF.

Table 2.1. Perivitelline space (PS), yolk mass (YM) and total embryo volume (in mm^3) of Japanese whiting *Sillago japonica* at six embryonic developmental stages (n=10).

Volume	2-32 Cells mm^3	Morula mm^3	Blastula mm^3	Gastrula mm^3	Somites mm^3	Tail-elongation mm^3
PS	0.006 ± 0.000	0.007 ± 0.002	0.009 ± 0.003	0.011 ± 0.003	0.016 ± 0.003	0.027 ± 0.004
(%)	(4.8)	(6.2)	(7.1)	(8.8)	(11.7)	(16.2)
YM	0.113 ± 0.005	0.114 ± 0.007	0.115 ± 0.004	0.118 ± 0.006	0.119 ± 0.003	0.139 ± 0.010
(%)	(95.2)	(93.8)	(92.9)	(91.2)	(88.3)	(83.8)
Total	0.119 ± 0.005	0.121 ± 0.005	0.124 ± 0.004	0.129 ± 0.006	0.135 ± 0.005	0.166 ± 0.011
(%)	(100)	(100)	(100)	(100)	(100)	(100)

Table 2.2. Compartment volume and estimated internal concentration of Me_2SO, EG and PG in the perivitelline space and yolk mass of Japanese whiting embryos microinjected with CPAs at different embryonic developmental stages.

Embryo developmental stage	2~32 Cells			Somites						Tail elongation					
Compartment	Yolk mass			Perivitelline space			Yolk mass			Perivitelline space			Yolk Mass		
Compartment volume (mm^3)	0.113 ± 0.005			0.016 ± 0.003			0.119 ± 0.003			0.027 ± 0.004			0.139 ± 0.010		
Injection volume (nl)	8.2			2.1			15.6			2.1			15.6		
Internal CPA concentration (mM) per compartment															
CPA concentration (%)	Me_2SO	EG	PG	Me_2SO	EG	PG	Me_2SO	EG	PG	Me_2SO	EG	PG	Me_2SO	EG	PG
30	313	390	285	567	705	516	566	704	515	336	418	306	485	603	441
40	418	520	380	756	940	688	755	939	687	448	557	408	646	804	588
50	522	649	475	945	1175	860	944	1173	859	560	696	509	808	1004	735
60	627	779	570	1134	1410	1032	1133	1408	1030	672	835	611	970	1205	882
70	731	909	665	1323	1645	1204	1321	1643	1202	784	975	713	1131	1406	1029
80	836	1039	760	1512	1880	1376	1510	1877	1374	896	1114	815	1293	1607	1176
90	940	1169	856	1701	2114	1547	1699	2112	1546	1008	1253	917	1455	1808	1323

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CHAPTER 3

Evaluation of the efficiency of combinations of permeable and non-permeable chemicals as cryoprotective agents for cryopreservation

3.1. Introduction

Cryopreservation of fish eggs/embryos has potential application in the conservation of endangered species, including genome banking and the exchange of germplasm, as well as for aquaculture production, in which it allows improvements such as selective breeding and the continuous, low-cost production of seeds. Though protocols for fish spermatozoa cryopreservation have been developed successfully for numerous freshwater and marine water species (Asturiano et al., 2017) and practiced regularly in field and laboratory settings, all attempts undertaken in last three decades to cryopreserve fish embryos had not succeeded. Unequal or insufficient distribution of CPAs inside the eggs/embryos associated to a complex multi-compartmental structure create favorable conditions for the growth of ice crystals, resulting in physical damage and finally death of the cells (Hagedorn et al., 1996, 1997, 1998; Robles et al., 2003; Cabrita et al., 2006; Edashige et al., 2006; Ding et al., 2007; Rahman et al., 2013; Martínez-Páramo et al., 2017; Rahman et al., 2017). This is particularly evident during slow cooling and/or thawing. Consequently, it is necessary to develop swift techniques that allow sufficient impregnation of CPAs into fish embryos to prevent cryo-injuries during cooling and thawing. Microinjection is one of the alternative tools for sufficient CPA introduction into the fish embryos and an effective way of overcoming the barriers imposed by the embryo membranes (Janik et al., 2000; Beirão et al., 2006; Robles et al., 2006). It might be time consuming and arguably difficult to apply in the treatment of large numbers of embryos, but in the first stage of research, it provides us with tools to test the importance of various parameters such as CPA type, concentration and distribution inside the embryos, as well as freezing and thawing rates. Equally important is the fact that microinjection allows the introduction of non-permeable, large molecules including proteins or DNA or even gold nanorods which are useful for rapid thawing (Khosla et al., 2017; Martínez-Páramo et al., 2017). Therefore, this technique has been already tested for the introduction of chemical CPAs in embryos of zebrafish (Hagedorn et al., 1997; Janik et al., 2000) and gilthead seabream (Beirão et al., 2006), as well as in the partial removal of yolk from zebrafish embryos (Liu et al., 1999, 2001) and for the introduction of antifreeze proteins in embryos of turbot (Robles et al., 2006) and zebrafish (Martínez-Páramo et al., 2009).

The temperature of intracellular ice formation (TIIF) provides fundamental information for designing effective protocols for embryo cryopreservation since supercooling and freezing depression point (ice nucleation) are crucial factors affecting the success of cryopreservation (Liu, 2000). On the other hand, Differential Scanning Calorimetry (DSC) has been widely used to study the TIIF in a diversity of cells (Bryant, 1995; Rasmussen et al., 1997; El-Shakhs et al., 1998), in the intraembryonic water in insect embryos (Schreuders et al., 1996), and in the intraembryonic milieu of zebrafish and medaka embryos (Liu, 2000; Routray et al., 2001, respectively). It can also be used to estimate CPA permeation into fish embryos by measuring the depression of ice nucleation temperature since there is a linear relationship between the depression of the homogenous nucleation temperature by low molecular weight solutes and the solute-induced freezing point depression (Rasmussen and MacKenzie, 1972).

A number of problems must be overcome for successful development of a protocol for fish embryo cryopreservation. One of the important limiting factors is believed to be the high sensitivity to low temperatures and chilling injury of fish embryos (Zhang and Rawson, 1995). In teleost species, several studies have reported on the sensitivity of embryos to chilling, including in zebrafish (Zhang and Rawson, 1995; Hagedorn et al., 1997; Kopeika et al., 2006; Martínez-Páramo et al., 2009), gilthead sea bream (Beirão et al., 2006), sea bream (Robles et al., 2007), medaka (Routray et al., 2001; Valdez et al., 2005), rainbow trout (Haga, 1982), goldfish (Liu et al., 1993), red drum (Gwo et al., 1995), and carp (Dinnyes et al., 1998). However, the tolerance of Japanese whiting embryos to chilling has never been studied. In the present study, the main goal and objectives were to examine the toxicity, freezing point depression ability, and chilling protection afforded by permeable and non-permeable chemical CPAs administered by microinjection into Japanese whiting *Sillago japonica* embryos as single or combined solutions.

3.2. Materials and methods

3.2.1. Maintenance of broodstock and embryo collection

Five mature females and five males of Japanese whiting were collected from Tateyama Bay, Japan, and kept in 1200 L recirculated seawater tanks at the fish rearing facilities of Tokyo University of Marine Science and Technology, Shinagawa, Tokyo, Japan. Fish were reared in artificial sea water (ASW) and were fed frozen krill once a day until satiation. ASW was prepared using sea water salts (TetraMarin® Salt Pro., USA) and

dechlorinated tap water and adjusted to a salinity of 33 psu. Water temperature and photoperiod were adjusted to 24 ± 1°C and 14L: 10D (14 h light and 10 h dark), respectively, for inducing natural spawning. The fertilized, buoyant eggs were obtained using a net trap set to collect eggs flowing through the water outlet near the surface. Eggs were incubated until reaching the following developmental stages: 2 cells~morula (about 40 min to 2 h after fertilization), and tail elongation (about 16 h 30 min after fertilization). Both developmental stages were determined according to Oozeki and Hirano (1985).

3.2.2. Microinjection procedures

Microinjection procedures followed the methods developed in the previous chapter. Briefly, needles were prepared from glass capillary tubing (GD-1, Narishige, Tokyo, Japan) for microinjection using a glass micropipette puller (PC-10, Narishige, Tokyo, Japan) and sharpened in a micropipette grinder (EG-44, Narishige, Tokyo, Japan) at an angle of 45°. Embryos were carefully placed into parallel grooves in agar disks for microinjection with substances or CPA solutions. Agar plates to support the embryos during microinjection were prepared in 90 mm × 15 mm size petri dishes using 20 ml of 3% agarose (Wako, Osaka, Japan) and the grooves were created by placing glass capillaries at a depth of 1 mm during cooling. Microinjection was performed using a mineral oil pressure-driven microinjector (IM-9B, Narishige, Tokyo, Japan) coupled to a micromanipulator (MP-1, Narishige, Tokyo, Japan) under a stereoscopic microscope (SZX10, Olympus, Tokyo, Japan). Two types of injection were performed in this study. In single injection, embryos in the 2 cells~morula stage were microinjected with 8.2 nl into the yolk mass (YM). In double injection, embryos in the tail elongation stage were microinjected with 2.1 nl into the perivitelline space (PS) and 15.6 nl into the YM. The injection volume was determined by measuring the diameter (D, millimeter) of the injected drop and calculating the volume of injection (V, nanolitre) as $V = (4/3) \times \pi(D/2)^3 \times 1000$.

3.2.3. Preparation of permeable and non-permeable CPA solutions

Yamamoto solution was used in this study for preparation of CPA solutions based on the results of the previous chapter. In total, ten solutions were prepared with by combination of three permeable CPAs (Me_2SO, EG, PG; all from Wako, Osaka, Japan), and two non-permeable CPAs (sucrose and trehalose; Wako, Osaka, Japan) at different concentrations. Five solutions (T1, T2, T3, T4, and T5) were prepared by using permeable CPAs while the remaining five solutions (T6, T7, T8, T9, and T10) were prepared by using both permeable

and non-permeable CPAs. The compositions of the CPA solutions used in this study are presented in Table 3.1.

3.2.4. Tolerance of embryos to microinjected permeable and non-permeable CPAs

The tolerance of embryos to the various CPA solutions was tested in 2 cells~morula stage embryos for single injection (8.2 nl in the YM) and tail elongation embryos for double injection (2.1 and 15.6 nl in the PS and the YM, respectively). Three different trials were conducted in this study. Trial 1 was run to determine the effects of non-permeable CPAs. In this trial, embryos in both developmental stages were microinjected with 0.25 M, 0.5 M, and 1.0 M sucrose and trehalose. In trial 2, embryos were microinjected with combinations of permeable CPA solutions (T1, T2, T3, T4, and T5; see Table 3.1) while in trial 3, embryos were microinjected with combinations of permeable and non-permeable CPAs (T6, T7, T8, T9, and T10; see Table 3.1). CPA-treated embryos were rinsed in plastic petri dishes (CORNING, Suspension Culture Dish, 35 mm × 10 mm, Polystyrene, USA) containing 5 ml of ASW and 1 h later transferred to new petri dishes with clean ASW for determination of hatching rates. Treated and untreated control embryos were then kept in an incubator (CN-25C, MEE, Japan) at 25°C until hatching. Each treatment contained at least 30 embryos and was run in five replicates with batches from different spawns. Embryos without any kind of manipulation were used as controls. Hatching rates (%) were determined as $100 \times$ total number of hatched embryos / total number of manipulated embryos.

3.2.5. Ice nucleation temperature of CPA-microinjected embryos

The temperature of intracellular ice formation (TIIF) of embryos was determined by DSC. The protocols for DSC analysis were as described in chapter 2. For TIIF analysis, embryos were injected with best combinations of permeable and non-permeable CPA solutions (T3, T6, T7, T8 and T10) based on the results of the toxicity tests (see results in section *3.3.1*). After microinjection, 10-15 embryos (total weight around 1.0-2.8 mg) were carefully blotted dry onto a filter paper, weighed and sealed in aluminum DSC pans, and loaded into the DSC equipment (Shimadzu DSC-50, Kyoto, Japan) at room temperature. To know the effects of cooling rate on the TIIF, the samples were cooled to -40°C at a rate of -5°C/min and then warmed to 40°C at a rate of 5°C/min. Both cooling and warming processes were recorded using a personal computer attached to the DSC. The data was then analyzed using the equipment software (TA-60, Shimadzu, Kyoto, Japan) to calculate the TIIF values.

Embryos without any kind of manipulation were used as controls. Each treatment and untreated controls were run in four replicates.

3.2.6. Chilling tolerance of CPA-microinjected embryos

The effect of the same treatments (section *3.2.5*) on the chilling tolerance of embryos was determined by subjecting embryos to a survival test that consisted of cooling from room temperature (ca. 25°C) to a subzero, non-freezing temperature (-10°C) at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Cooling and warming were performed using a program freezer (CRYO PORTER, CS-80CP, SCINICS, Japan). After warming, embryos were transferred to petri dish containing ASW and then incubated at 25°C until hatching to monitor survival as described above. Each treatment and control were run in four replicates with at least 25 embryos each. Hatching rates were determined according to previous section *3.2.4*.

3.2.7. Statistical analyses

All numerical data were expressed as mean ± SD (standard deviation). The statistical significance of the differences between means was analyzed by ANOVA followed by the Tukey's multiple comparison tests using the SPSS (Statistical Package for Social Science, version 16.0) (SPSS Inc., Chicago, Illinois, USA) at 95% confidence level.

3.3. Results

3.3.1. Tolerance of embryos to microinjected permeable and non-permeable CPAs

The results of tests on the tolerance of Japanese whiting embryos to the microinjected permeable and non-permeable CPAs are presented in Figs. 3.1-3.3. In trial 1, hatching rates of embryos decreased with increasing CPA concentration in both developmental stages and were statistically higher at 0.25 and 0.5 M compared to 1 M (Fig. 3.1). Tail elongation embryos were more tolerant to the CPA solutions than 2 cells~morula stage embryos. Trehalose-treated embryos had slightly higher survival rates than sucrose but there were no significant differences ($p > 0.05$). This result suggested that suitability of 0.25~0.5 M trehalose for further testing. In trial 2, a significant higher hatching rate ($p < 0.05$) was obtained with T3 (15% EG + 15% PG) compared to the other treatments in both developmental stages (Fig. 3.2). In trial 3, the highest hatching rates were obtained with treatments T7, T8, and T10 for 2 cells~morula stage embryos and with treatments T6, T7 and T8 for tail elongation stage embryos (Fig. 3.3). The hatching rates in this trial suggested that the maximum tolerable concentration of trehalose in combined solutions is about 0.25 M.

3.3.2. Ice nucleation temperature of CPA-microinjected embryos

The TIIF values of 2 cells~morula and tail elongation stage embryos were lowest in the order of T8 ≤ T3 ≤ T7 ≤ T10 ≤ T6 < Control (Fig. 3.4). This result suggests that treatments T3, T7, and T8 were superior to other treatments to suppress the formation of intracellular ice. Between the two developmental stages, tail elongation embryos were shown to have lowest TIIF values. The TIIF of intact control embryos was -19.2 and -20.4°C for 2 cells~morula and tail elongation stage embryos, respectively. In the case of treated embryos, the highest and lowest TIIF values for 2 cells~morula embryos were -21.8 and -25.5°C, respectively, and for tail elongation embryos -24.6 and -28.7°C, respectively. During the tolerance tests, T8 produced the lowest TIIF value and the highest survival rate followed by T3 and T7 for both developmental stages (Fig. 3.5).

3.3.3. Chilling tolerance of CPA-microinjected embryos

The effects of CPA microinjection on the chilling tolerance of 2 cells~morula and tail elongation embryos are shown in Fig. 3.6. Embryos at the tail elongation stage microinjected with CPA solutions were much more tolerant to chilling (hatching rates from 2 to 18%) than 2 cells~morula embryos (hatching rates from 0 to 2%). None of the embryos in the 2 cells~morula stage survived injection with CPA solutions T6, T7, and T10. Treatment of tail elongation embryos with CPA solutions T3 and T8 gave significantly ($p < 0.05$) higher survival rates (about 18%) than other solutions and untreated controls (Fig. 3.6). The lowest TIIF and highest chilling tolerance were obtained with solutions T3 and T8 in embryos of both developmental stages (Fig. 3.7).

3.4. Discussion

Conventional impregnation methods do not provide sufficient CPA concentrations inside the embryos to prevent chilling injury. So, microinjection is currently considered as a promising technique to deliver CPAs directly and at high concentrations into fish eggs (Janik et al., 2000; Robles et al., 2004; Beirão et al., 2006; Khosla et al., 2017). This study examined the best combination of CPAs for introduction into specific embryonic compartments of Japanese whiting through microinjection.

First, this study examined the suitability of non-permeable CPAs (sucrose and trehalose) in 2 cells~morula and tail elongation stages embryos and found that trehalose was tolerated better than sucrose. The results of this study also indicated that the maximum tolerable concentration of trehalose as a single CPA solution for whiting embryos is 0.25 M.

On the other hand, Routray et al. (2001) reported that short time exposure of 0.5 M trehalose did not affect the viability of medaka embryos prior to impregnation with other CPAs. Some studies reported that inclusion of trehalose at low concentration in the CPA mixture solution increase the cell integrity during cooling, depress the nucleation temperature, and possibly increase the cooling tolerance of embryos (Rall et al., 1987; Chao and Liao, 2001; Rahman et al., 2008). Therefore, in the present study, we attempted to combine commonly used permeable CPAs with such non-permeable CPAs. In this regard, we first determine the toxicity tolerance of embryos to solutions containing a combination of chemical CPAs and trehalose. The results suggested that solutions containing EG (15%) and PG (7.5~15%) in the presence of small amounts of trehalose (0.125 M) were well tolerated by the embryos. This result is in accordance with the findings of Chen and Tian (2005) and Rahman et al. (2008) for flounder and whiting embryos, respectively.

The DSC analysis indicated that the TIIF values of 2 cells~morula and tail elongation embryos were significantly depressed after microinjection with solutions containing a combination of permeable and non-permeable CPAs in comparison to untreated controls. The best results were obtained with solutions T3 (15% EG + 15% PG) and T8 (15% EG + 7.5% PG + 0.125M Tre). This study also indicated that up to 0.125 M trehalose in combination with other CPAs may be suitable for depressing the ice nucleation temperature of embryos.

Moreover, this study also indicated that tail elongation embryos had lower TIIF values than early stage embryos. Liu (2000) and Routray et al. (2001) reported the same trend of decreasing intracellular ice nucleation temperature with the development in zebrafish and medaka embryos, respectively. A possible explanation for these findings is that progressive development and tissue differentiation causes water to interact with an increasing interface like the membrane, and/or being trapped in small size compartments. It is known that the homogenous ice nucleation temperature of pure water is depressed to -40°C when the volume is 10 μm in diameter (Wood and Walton, 1970). Although the nucleation in embryos may not be homogenous in nature, the depression in the ice nucleation temperature of the developed embryo, particularly at the eyed stage, might be also due to a decrease in the compartment size (Routray et al., 2001).

Another major barrier for the successful cryopreservation of fish embryos is the presence of intraembryonic lipids (Zhang and Rawson, 1995; Hagedorn et al., 1997). Beirão et al. (2006) studied on the chilling tolerance of gilthead seabream tail bud embryos through

single microinjection with three CPAs (EG, Me$_2$SO and MeOH) into yolk mass. They reported that EG produced the highest survival rates after exposure to -10°C for 30 min, followed by Me$_2$SO and MeOH. They did not perform double injection or examined the chilling tolerance after treatment with combinations of permeable and non-permeable CPAs. Therefore, this study examined the chilling tolerance of double microinjected-embryos of different developmental stages with combined CPAs. The results suggested that T3 (15% EG + 15% PG) and T8 (15% EG + 7.5% PG + 0.125M Tre) produce significantly higher survival rates (about 18%) than other treatments in tail elongation stages. To the best of our knowledge, this is the first study performing a chilling tolerance test in embryos that were microinjected with combinations of permeable and non-permeable CPA solution. Combined CPA solutions are expected to reduce toxicity to embryos compared to the use of high concentrations of single CPAs. Furthermore, a number of studies have proven that the use of small amounts of sugars in the cryopreservation medium is not detrimental for embryos (Sakkas et al., 1993; Kuleshova et al., 1999; Beirão et al., 2006). On the basis of this study, solutions containing 15% EG, 7.5~15% PG, and also small amounts of trehalose (0.125 M) are considered to be suitable for cryopreservation studies of Japanese whiting embryos.

In addition, the results of the toxicity tests supported the notion that embryos in later developmental stages tolerate CPAs better than early embryonic stages. A number of studies have reported that CPA tolerance in fish embryos during immersion-based impregnation is stage-dependent, with early stage embryos being comparatively less tolerant than late developmental stage embryos (Bart, 2000; Magyary et al., 2000; Cabrita et al., 2003; Chen and Tian, 2005; Routray et al., 2001; Rahman et al., 2008). Cabrita et al. (2003) also reported that tail bud stage embryos of turbot are more tolerant to CPAs than other developmental stages. Moreover, in this study, the lowest TIIF values and higher survival rates were observed in tail elongation stage embryos compared to earlier stage embryos. It has been reported that fish embryos in early developmental stages are damaged at low temperatures even without ice nucleation, such as in zebrafish (Zhang and Rawson, 1995; Hagedorn et al., 1997) and common carp (Magyary et al., 2000). Valdez et al. (2005) also studied the chilling sensitivity of medaka embryos and found that the 2~4 cells stage were more sensitive to chilling at 0°C compared to other stages and that sensitivity decreased with embryonic development. The results obtained in the toxicity tests, TIIF analysis, and chilling tolerance of whiting embryos suggest that tail elongation stage embryos are the most suitable for cryopreservation.

Furthermore, the findings of this study suggest that the chilling tolerance and TIIF values of tail elongation embryos can be improved by microinjection with combinations of permeable and non-permeable CPAs. It is also noteworthy that some embryos could tolerate very high internal CPA concentrations. This study provides new findings to support the development of successful cryopreservation methods for fish embryos and also for the non-frozen storage of fish embryos. However, further studies are required to clarify the usefulness of microinjection of embryos with other substances (like antifreeze proteins) for vitrification test of whiting embryos.
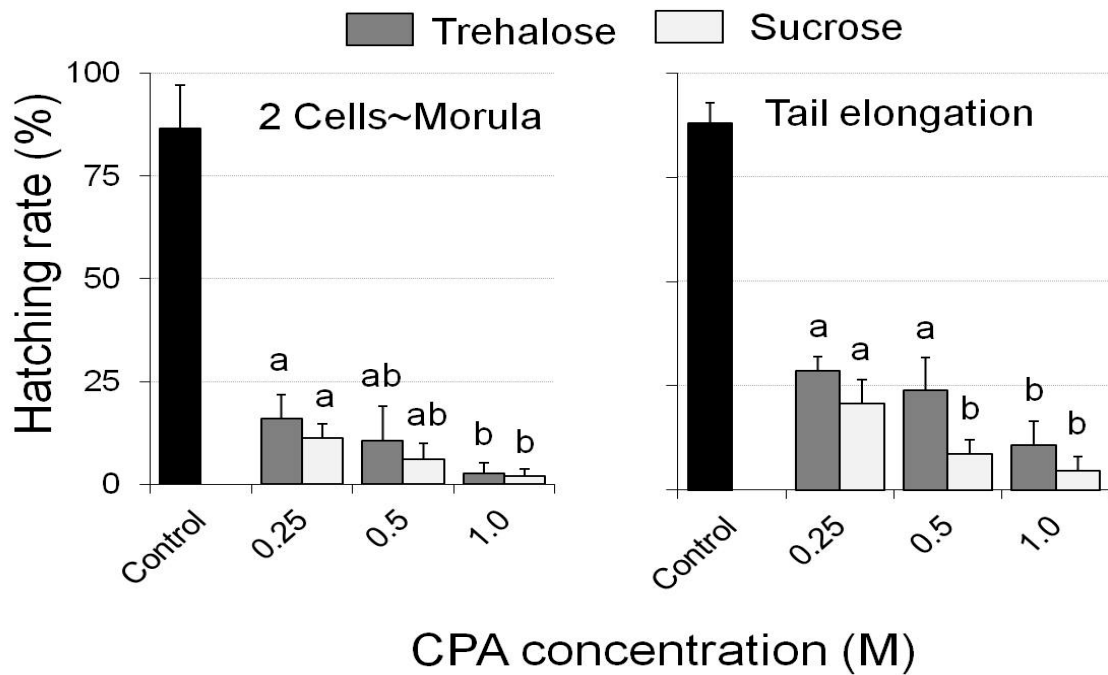
Fig. 3.1. Hatching rates of 2 cells~morula and tail elongation embryos of Japanese whiting that were single-injected into the yolk mass (8.2 nl) or double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 0.25 M, 0.5 M, and 1.0 M solutions of sucrose and trehalose in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of five replicates with at least 30 embryos each. Columns of the same solution with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
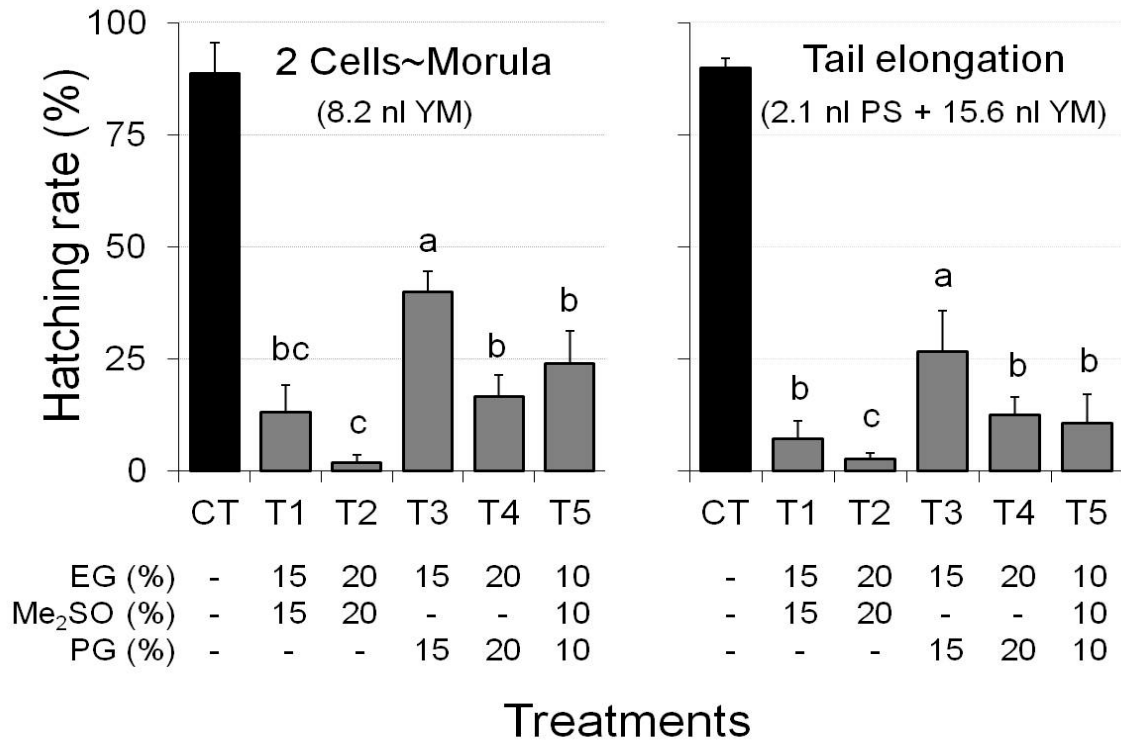
Fig. 3.2. Hatching rates of 2 cells~morula and tail elongation embryos of Japanese whiting that were single-injected into the yolk mass (8.2 nl) or double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of permeable CPAs (solutions T1, T2, T3, T4, and T5). Thick and thin bars represent the mean and SD, respectively, of five replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey). CT = Untreated controls.
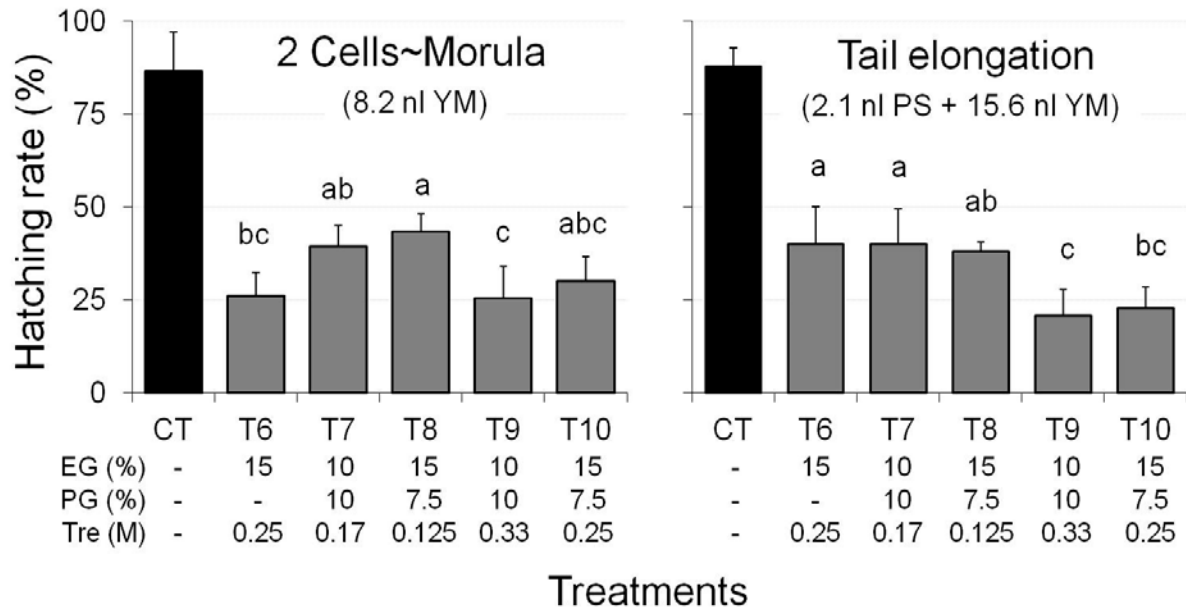
Fig. 3.3. Hatching rates of 2 cells~morula and tail elongation embryos of Japanese whiting that were single-injected into the yolk mass (8.2 nl) or double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of permeable and non-permeable CPAs (solutions T6, T7, T8, T9, and T10). Thick and thin bars represent the mean and SD, respectively, of five replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey). CT = Untreated controls.
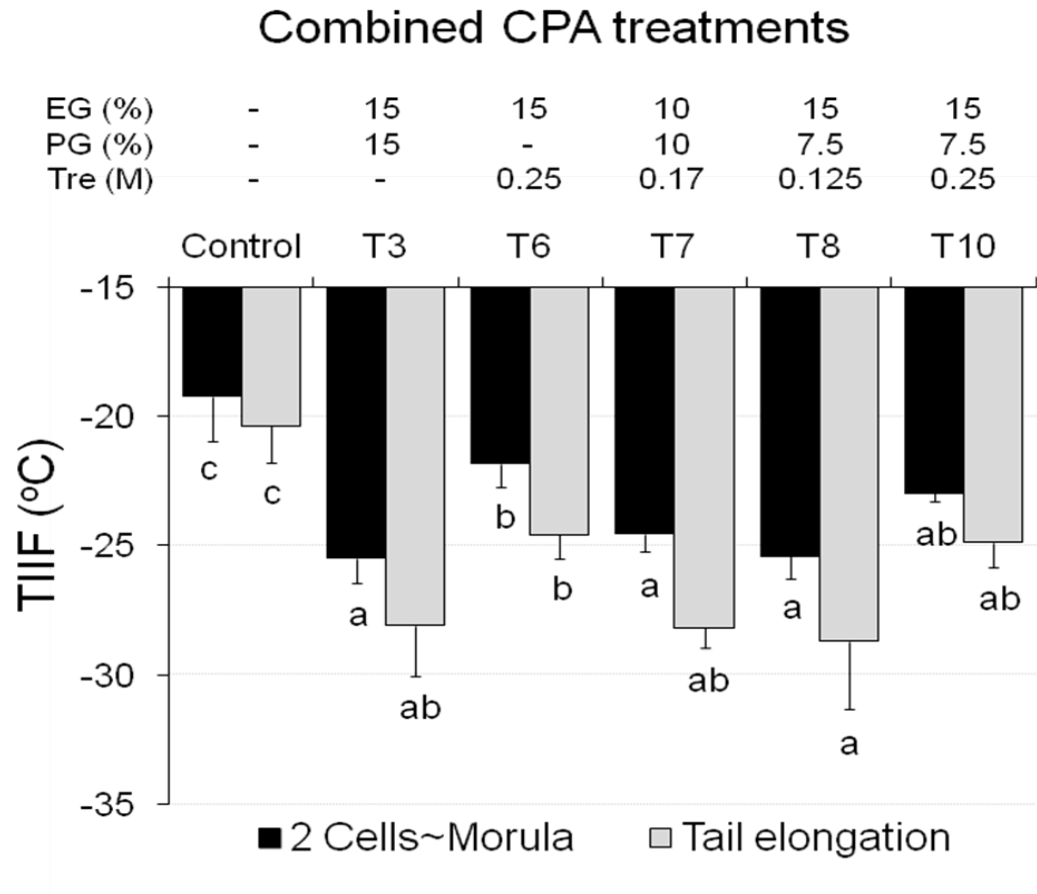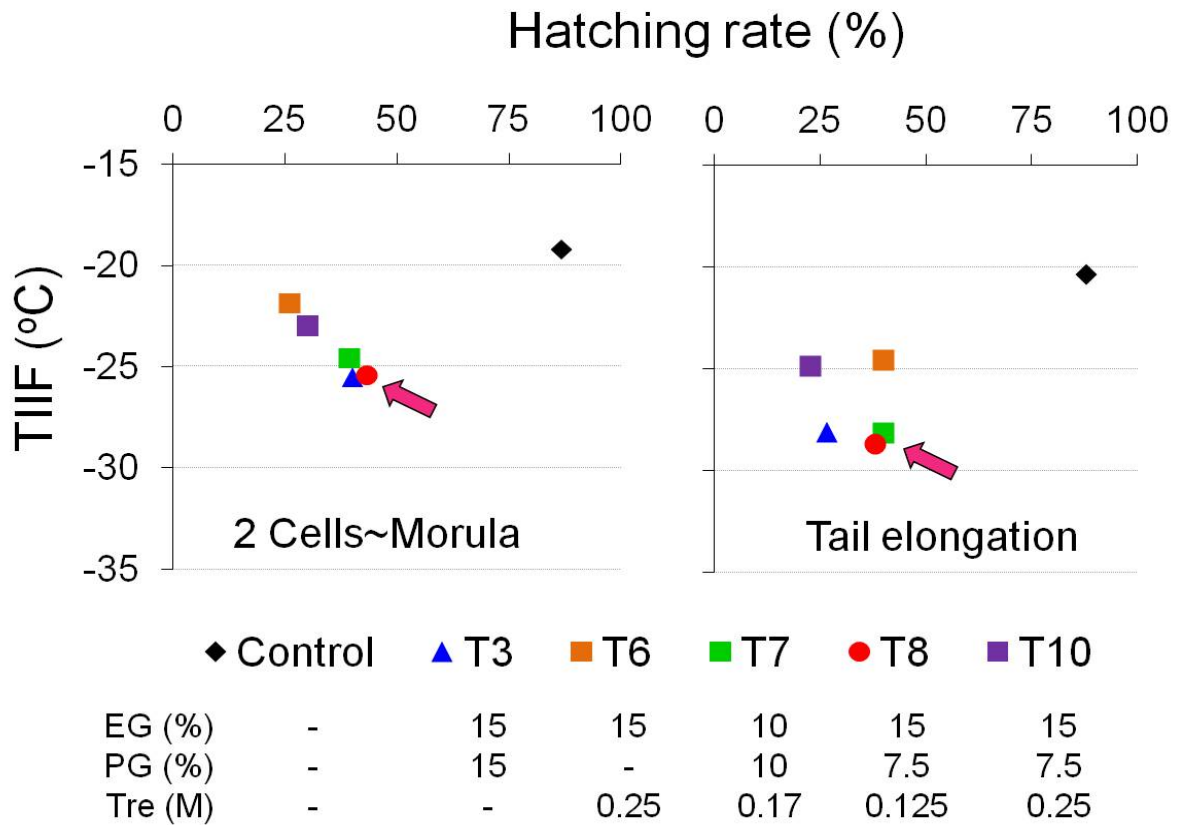
Fig. 3.4. Temperature of intracellular ice formation (TIIF, °C) of 2 cells~morula and tail elongation embryos of Japanese whiting in different treatments. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and those in the tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of permeable and non-permeable CPAs (solutions T3, T6, T7, T8, and T10). Thick and thin bars represent the mean and SD, respectively, of four replicates for each treatment. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey) in each developmental stage.

Fig. 3.5. Relationship between the survival rates (%) in the toxicity test and the temperature of intracellular ice formation (TIIF) of 2 cells~morula and tail elongation stage embryos of Japanese whiting in different treatments (solutions T3, T6, T7, T8, and T10) and intact controls. Embryos at 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and those in the tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of CPAs in Yamamoto solution.
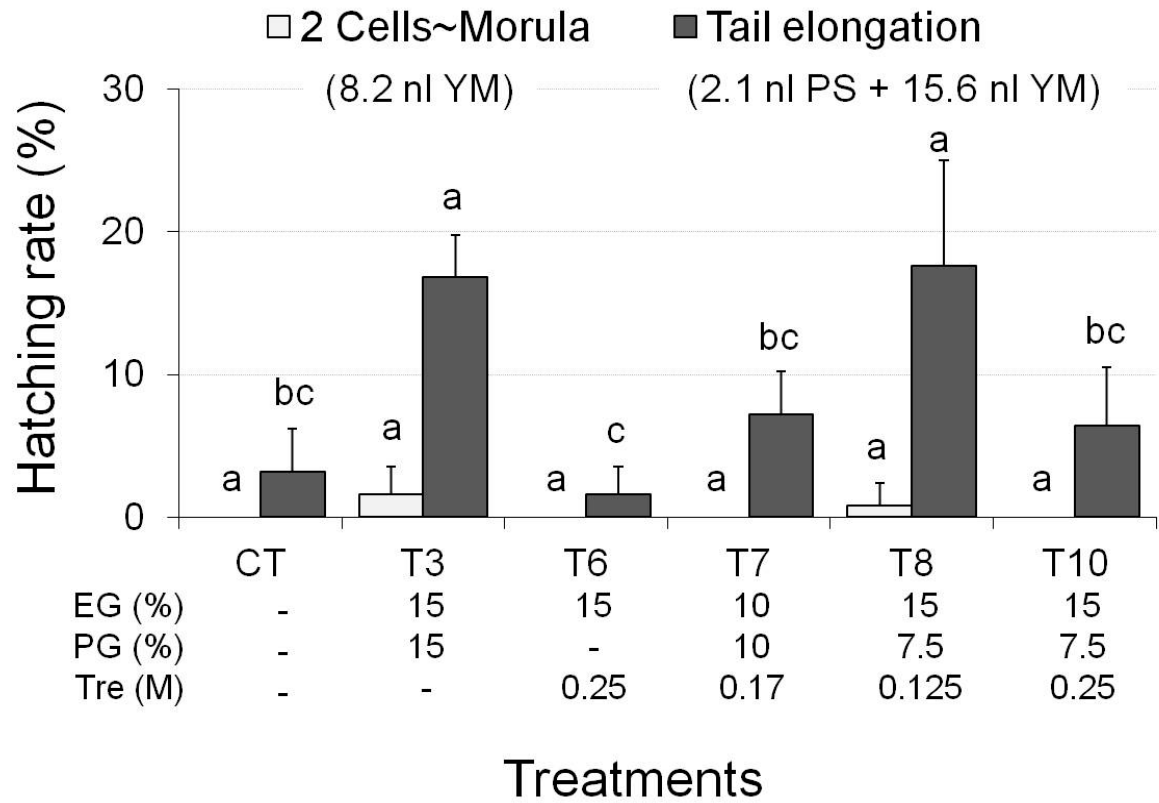
Fig. 3.6. Chilling tolerance of Japanese whiting embryos subjected to a subzero, non-freezing temperature survival test consisting of cooling from room temperature to -10°C at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Embryos at the 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation embryos were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of permeable and non-permeable CPAs (solutions T3, T6, T7, T8, and T10). Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 25 embryos each. Columns in each developmental stage with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
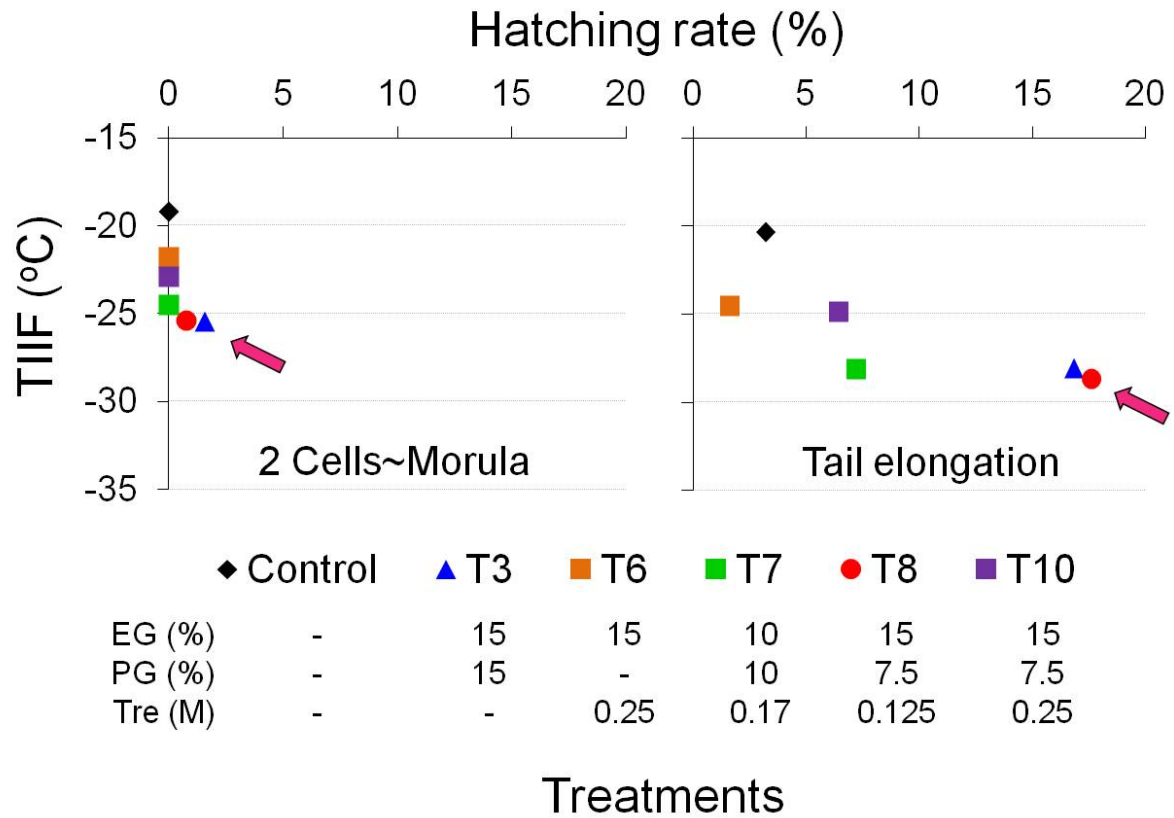
Fig. 3.7. Relationship between the survival rates (%) after the chilling tolerance test and the temperature of intracellular ice formation (TIIF) in 2 cells~morula and tail elongation stage embryos of Japanese whiting in different treatments (solutions T3, T6, T7, T8, and T10) and intact controls. Embryos in the 2 cells~morula stage were single-injected in the yolk mass (8.2 nl) and tail elongation embryos were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of CPAs in Yamamoto solution.

Table 3.1. Type and concentration of various permeable and non-permeable cryoprotective agents (CPA) used to prepare the experimental solutions. All solutions were prepared using Yamamoto solution as diluent.

Solution	CPA Concentration (v/v)			
	EG (%)	Me$_2$SO (%)	PG (%)	Trehalose (M)
Combined permeable CPA solutions				
T1	15	15	-	-
T2	20	20	-	-
T3	15	-	15	-
T4	20	-	20	-
T5	10	10	10	-
Combined permeable and non-permeable CPA solutions				
T6	15	-	-	0.25
T7	10	-	10	0.17
T8	15	-	7.5	0.125
T9	10	-	10	0.33
T10	15	-	7.5	0.25

CHAPTER 4

Evaluation of the efficiency of antifreeze proteins (AFPs) as cryoprotective agents for cryopreservation

4.1. Introduction

Fish embryo cryopreservation is a tool with biotechnological or ecological applications in fields such as aquaculture production, including selective breeding programs, and the conservation of biodiversity, including genome banking and the easy exchange of germplasm (Billard and Zhang, 2001). However, the development of successful technology for cryopreservation of fish embryos is very challenging because of their large size and large lipid content in yolk, a complex and multi-compartmental structure with several permeability barriers, and their high chilling sensitivity. Hence, although tremendous efforts have been made in the last three decades, no reliable methods have yet been developed for fish embryos cryopreservation. Recently, there has been some breakthroughs, like the use of gold nanorods and excitation with a laser beam to warm frozen materials back to room temperature while preventing recrystallization (Khosla et al., 2017), but still there is room for much improvements. Insufficient impregnation of CPAs into fish embryos appears to be the main problem, leading to physical damage to the embryos due to the formation of intra-and extra-cellular ice crystals, particularly during slow cooling and thawing. This could be solved by incorporation or introduction with sufficient CPAs or antifreeze proteins (Robles et al., 2006; Martínez-Páramo et al., 2008, 2009) prior to freezing, but all approaches tested so far to enhance their permeation into embryo by immersion-based impregnation such as electroporation (Rahman et al., 2013), positive or negative pressurization (Routray et al., 2002), dechorionation (Hagedorn et al., 1997; Zhang and Rawson, 1998), ultrasound (Rahman et al., 2017), among others, have met only with limited success. Thus, these approaches improved the CPA permeation into embryos, but not sufficient enough to prevent lethal cryoinjuries. Microinjection, a direct delivery approach, could help in attaining a sufficient internal CPA concentration for preventing ice-crystallization during cooling.

Another consideration is the type of CPAs to be used. Most traditionally used CPAs (EG, Me_2SO, MeOH, PG, etc.) have been shown to have various degrees of toxicity, particularly at the concentrations necessary for cryoprotection. Hence, there has been a constant search for novel, alternative types of CPAs. Antifreeze proteins (AFPs) are naturally occurring proteins from microorganisms, plants, insects and fish (Barrett, 2001;

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Mahatabuddin et al., 2017) that are able to bind to small ice crystals and to inhibit further growth of ice that would otherwise be fatal during freezing conditions as well as recrystallization during thawing, and therefore protect cell membranes from cold-induced damage (Robles et al., 2007; Olijve et al., 2016; Mahatabuddin et al., 2017). The introduction of AFPs into fish embryos may also reduce their freezing point and improve chilling tolerance as well as response to vitrification (Rubinsky et al., 1992; Kristiansen and Zachariassen, 2005; Martínez-Páramo et al., 2009). Artic fish species contain several AFPs for the protection from freezing during cold temperature. The most popular AFPs are antifreeze glycopeptide (AFGP), antifreeze protein type I (AFP I), antifreeze protein type II (AFP II), antifreeze protein type III (AFP III), and antifreeze protein type IV (AFP IV). Those AFPs have been used by several authors for cryopreservation studies (Robles et al., 2006, 2007; Martínez-Páramo et al., 2008, 2009). A number of studies have been conducted also in mammalian gametes and embryos to find out whether AFPs (in combination with other CPAs) could improve the viability of cryopreserved materials. The outcomes of these studies are controversial; most authors have noticed a beneficial effect on oocytes or embryo cryopreservation (Knight et al., 1988; Rubinsky et al., 1991 and 1992; Arav et al., 1993; Baguisi et al., 1997; O'Neil et al., 1998; Fletcher et al., 1999), whereas few authors have found no or even adverse effects (Ducker et al., 1995; Mugano et al., 1995). The introduction of AFPs into fish embryos helps to increase cryoprotection of the cellular structures by stabilizing membranes, as demonstrated in zebrafish (Martínez-Páramo et al., 2008 & 2009) and seabream (Robles et al., 2007). The main aim of this study was to examine the suitability of AFPs for the cryopreservation of Japanese whiting embryos, specifically to evaluate the tolerance of embryos to different AFPs single or in mixture, as well in combination with other chemical CPAs, and to elucidate the effectiveness of these combinations for cryopreservation.

4.2. Materials and methods

4.2.1. Maintenance of broodstock and embryo collection

All fishes were reared and maintained as described in details in the second chapter. In brief, Japanese whiting *Sillago japonica* brood fishes were collected from Tateyama Bay, Japan, and kept in 1200 L recirculated sea water tanks at 24°C. The tanks were maintained at a 14 h light and 10 h dark photoperiod for inducing natural spawning. The fertilized, buoyant embryos were collected using a net trap set in the surface near the water outlet. Embryos at

tail elongation stage (about 16 h 30 min after fertilization) were used in this study (Oozeki and Hirano, 1985).

4.2.2. Microinjection procedures

Microinjection procedures also followed the methods described in the previous chapter with slight modifications. Briefly, for microinjection, embryos at tail elongation stage were collected and then carefully pressed into parallel grooves in an agar plate. Microinjection was performed with a glass needle using a microinjector under a stereoscopic microscope and the injection volume was calibrated periodically to ensure accuracy during delivery. In all experiments, embryos were double-microinjected with 15.6 nl into the yolk mass (YM) and 2.1 nl into the perivitelline space (PS). After microinjection, embryos were kept in abundant, freshly prepared artificial sea water in an incubator (CN-25C, MEE, Japan) at 25°C for determination of hatching rates.

4.2.3. Preparation of experimental solutions

Antifreeze glycopeptide (AFGP), antifreeze protein type I (AFP I) and antifreeze protein type III (AFP III) were used in this study. AFGP, AFP I and AFP III are derived from sub-arctic fish family, Gadidae, Pleuronectidae, and Zoarcidae, respectively, and were purchased from Nichirei Corporation (Tokyo, Japan). Yamamoto solution was chosen as the diluent for preparation of the AFP solutions based on the results of chapter 2. First, the three types of AFPs were used for the preparation of single AFP solutions at concentrations of 10, 20, 30, 40 and 50 mg/ml. Second, three CPA solutions (T11, T12 and T13) were prepared by combination of two AFPs at the concentration of 20 mg/ml each. This choice of this concentration was based on the results of the first trial, which showed that embryos could tolerate up to 40 mg/ml of each single AFP. Finally, combined solutions (T14, T15, and T16) were prepared with the best AFP solution (T11) and with previously determined optimum CPAs or CPA mixtures (e.g. 30% EG and solutions T3 and T8) (1:1 v/v). The chemical composition of the experimental solutions used in this study is shown in Table 4.1.

4.2.4. Tolerance of embryos to microinjected AFP solutions

Embryos injected with the solutions described in the previous section and untreated control embryos were kept in plastic petri dishes containing ASW in an incubator (CN-25C, MEE, Japan) at 25°C for determination of hatching rates. Each treatment contained at least 30 embryos and was run in five replicates with batches from different spawns. Hatching rates

were determined by using the equation, Hatching rate (%) = 100 × Normally hatched embryos / Total number of injected embryos.

4.2.5. Temperature of intracellular ice formation (TIIF) of AFP-microinjected embryos

Differential Scanning Calorimetry (DSC) was used for the analysis of intracellular ice formation (TIIF) of embryos injected with 40 mg/ml of AFGP, AFP I and AFP III and with solutions T11 to T16. Immediately after microinjection, 5-8 embryos (total weight around 0.6-1.4 mg) were blotted-dried, sealed in aluminum DSC pans, and introduced into the DSC equipment. Embryos were then cooled to -40°C at a rate of -5°C/min and subsequently warmed to 40°C at a rate of 5°C/min. The data was then analyzed using the equipment software TA-60 (Shimadzu, Kyoto, Japan) to calculate the TIIF values. Each treatment consisted of four replicates.

4.2.6. Chilling tolerance of AFP-microinjected embryos

The chilling tolerance of embryos after microinjection with AFP solutions was determined for the same treatments described in the previous section. Microinjected embryos were stored in a programmable freezer (CRYO PORTER, CS-80CP, SCINICS, Japan) for the chilling tolerance test consisting of cooling from room temperature (ca. 25°C) to -10°C at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Embryos were then incubated at 25°C until hatching. Each treatment contained 4-5 batches of different spawns and each replication contained at least 25 embryos. Hatching rates were determined as described before.

4.2.7. Attempted vitrification of AFP-microinjected embryos

The suitability of the developed solutions for cryopreservation was tested by attempted vitrification. For this test, embryos were injected with solutions T14, T15 and T16. After microinjection, embryos were loaded into the 0.25 ml French straws (FHK Fujihira Industry Co., Japan), sealed, and then plunged directly into liquid nitrogen (LN$_2$) in a 2 L transparent Dewar flask for 20 min. After storage in LN$_2$, embryos were than thawed to 40°C for 5 s. After freeze-thawing, embryos were washed with ASW 2 times and then incubated in ASW for observation of morphological integrity, structural damage, and survival rates according to Rahman et al. (2017). For morphological structure observation, embryos were observed under a stereoscopic microscope (BX53F, Olympus, Tokyo, Japan) immediately after warming and digital images were taken (digital camera DP73, Olympus, Tokyo, Japan; CellSens Imaging Software v1.9, Olympus, Tokyo, Japan). Embryos were categorized as

'clearly damaged' or 'apparently intact'. Each treatment consisted of three replications with 20 embryos.

4.2.8. Live/dead cell viability test of AFPs-microinjected embryos

The PromoKine Live/Dead Cell Staining Kit II (PromoCell GmbH, (Heidelberg, Germany) was used to discriminate between live and dead cells after attempted vitrification. The Staining Kit II provides two-color, differential fluorescent stains, green (Calcein-AM stain) for live and red (EthD-III stain) for dead cells. After freeze-thawing, microinjected embryos were immersed in the Staining Kit II solution and kept at room temperature for 40 min under dark conditions. Embryos were then washed with ASW and mounted on glass slides and covered with cover slips. Finally, live/dead cells were analyzed under a fluorescence microscope (BX53F, Olympus, Tokyo, Japan) and images were taken (digital camera DP73, Olympus, Tokyo, Japan; CellSens Imaging Software v1.9, Olympus, Tokyo, Japan).

4.2.9. Statistical analyses

Values from all treatments were expressed as mean ± SD (standard deviation). The statistical significance of the differences between treatments was analyzed by one-way ANOVA followed by the Tukey's multiple comparison tests using the SPSS (Statistical Package for Social Science, version 16.0) (SPSS Inc., Chicago, Illinois, USA). A probability value of $p < 0.05$ was taken as statistically significant.

4.3 Results

4.3.1. Tolerance of embryos to microinjected AFPs and their mixtures

Hatching rates of whiting embryos microinjected with AFP solutions are shown in Figs. 4.1~4.3. Embryos tolerated well (hatching rates above 70%) AFGP and AFP-I up to 40 mg/ml and AFP-III up to 20 mg/ml but hatching rates dropped sharply for all AFPs (11-17%) at 50 mg/ml (Fig. 4.1). Hatching rates were comparable (64 to 77%) for embryos treated with all AFP-AFP combinations (Fig. 4.2). Combinations of AFPs and chemical CPAs were relatively well tolerated but T16 (survival rate of 75%) was found to be the less toxic to the embryos compared to solutions T14 and T15 (62 and 58%, respectively).

4.3.2. Ice nucleation temperature of AFP-microinjected embryos

The results of DSC analysis showed that embryos injected with 40 mg/ml of all AFPs had lower TIIF values than the untreated controls but the differences were not statistically

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significant ($p > 0.05$) (Fig. 4.4). Embryos microinjected with combinations of two AFPs at 20 mg/ml (solutions T11-T13) had lower nucleation temperature than control embryos (Fig. 4.5). The same pattern was observed for the three solutions combining AFP and chemical CPAs (solutions T14-T16) (Fig. 4.6).

4.3.3. Chilling tolerance of AFP-microinjected embryos

In the chilling tolerance test, all single AFPs solutions seemed to enhance slightly the chilling tolerance of embryos but there were no significant differences ($p > 0.05$) between the three AFPs and the respective control (Fig. 4.7). In the case of combinations of two AFPs, all solutions (T11-T13) significantly enhanced the chilling tolerance of embryos compared to the untreated control (Fig. 4.8). The solution T11 (20 mg/ml AFGP + 20 mg/ml AFP I) induced the lowest TIIF and highest survival rate of embryos after chilling (Fig. 4.9) and for this reason was used for subsequent tests in combination with permeable and non-permeable chemical CPAs. All combinations of AFPs and CPAs enhanced the chilling tolerance of embryos compared to the controls but significant differences were observed only for solutions T15 and T16 (Fig. 4.10). Solution T16, a combination of solution T11 (20 mg/ml AFGP + 20 mg/ml AFP I) and T8 (15% EG + 7.5% PG + 0.125 M Tre) on a 1:1 v/v ratio, produced the lowest TIIF and highest survival rate of embryos among the three treatments (Fig. 4.11).

4.3.4. Attempted vitrification of AFP-microinjected embryos

The CPA solutions remained transparent during freezing in LN_2 but incipient ice crystal formation, evidenced by the opaqueness of the straw, was observed during warming. The majority of the embryos became opaque during cooling, indicating the presence of intracellular ice crystals, but a few embryos remained in a glassy state (Fig. 4.12). Likewise, while the majority of the embryos after freeze-thawing showed clearly damaged morphology such as a ruptured chorion, increased volume of the perivitelline space, protrusions in the yolk mass, and mottling, a small number of embryos injected with solution T16 remained relatively intact (Fig. 4.13). These embryos were subjected to the live/dead cell viability analysis using the live/dead cell kit. The results are shown in Figs. 4.14 and 4.15. Embryos from all treatments had varied frequencies of live cells but the highest frequency was observed in solution T16. Nevertheless, none of the embryos injected with any of the three combinations of AFPs and CPAs (solutions T14-T16), survived the freeze-thawing procedure of attempted vitrification.

4.4. Discussion

Fish embryo survival after cryopreservation has never been achieved and this failure is attributed to the insufficient CPA permeation prior to cooling (Zhang and Rawson, 1996; Hagedorn et al., 1997; Zhang and Rawson, 1998; Cabrita et al., 2003; Zhang et al., 2006). Moreover, conventional impregnation with or without the aid of chemical and physical methods (Hagedorn et al., 1997; Zhang and Rawson, 1998; Routray et al., 2002; Valdez et al., 2006; Rahman et al., 2011, 2013, 2017) did not increase the internal CPA concentration in embryos enough to avoid freezing. Hence, other methods to ensure the direct delivery of CPAs into the embryos such as microinjection and alternative cryoprotectant agents have achieved much attention. Likewise, intracellular ice formation is one of the major reasons for cell death during freezing and warming (Mazur et al., 1984). Low membrane permeability to water and CPAs may be the major obstacle for successful cryopreservation in fish embryos (Zhang and Rawson, 1995; Plachinta, 2007). To overcome this problem, it is necessary to incorporate sufficient CPAs or substances that limit the formation or intracellular ice like AFPs into the embryos. In this context, this study used microinjection for introducing AFPs alone or in combination with CPAs into tail elongation stage embryos of Japanese whiting. AFPs were used in this study because they are extremely capable to prevent growth of ice crystals inside the embryos during slow cooling and at inhibiting ice recrystallization.

This study showed that tail elongation embryos of whiting tolerated well all of the three AFPs up to 40 mg/ml. Rubinsky and colleagues (1990) also used AFPs at different concentrations for short-term storage of pig oolema and found that a combination of AFPs in concentrations ranging from 1 to 40 mg/ml improved the structural integrity under low temperature. Other researchers have added AFPs to the external medium for cryopreservation (Rubinsky et al. 1991, 1992). Robles et al. (2006) reported that the highest (41%) survival in a chilling tolerance test was found in turbot embryos microinjected with 10 mg/ml AFP III into the yolk sac. Whiting embryos microinjected with mixtures of two AFP (at 20 mg/ml each), with or without combination with solutions of chemical CPAs (15% EG + 7.5% PG + 0.125 M Tre) also showed high survival (>60%). These results support the findings of Robles et al. (2006) and suggest that all three AFPs, single or in combination, as well as mixtures or AFPs and CPAs are well tolerated by Japanese whiting embryos and therefore could be useful in embryo cryopreservation studies.

We then used DSC analysis to examine the level of depression of the temperature of intracellular ice formation (TIIF) obtained with different solutions containing AFPs. We found that the TIIF values were in the order of AFPs-CPAs combinations < AFP-AFP combinations < single AFPs < untreated controls. A solution containing a combination of 20 mg/ml AFGP and 20 mg/ml AFP I and 15% EG + 7.5% PG + 0.125 M Tre (solution T16) resulted in the lowest TIIF values. We then examined the suitability of the developed solutions to prevent chilling injury during cooling of tail elongation embryos impregnated with the combinations of AFPs and CPAs. The combined AFPs and CPAs, in particular solution T16, enhanced chilling tolerance of embryos compared to single AFPs or their mixtures. Finally, we conducted a preliminary cryopreservation test with tail elongation embryos that were double-injected with different combinations of AFPs and chemical CPAs. In this trial, embryos were subjected to attempted vitrification and we observed the degree of formation of ice crystals during both cooling and warming. Most embryos were opaque during freezing and showed clear signs of structural damage after thawing. Opaqueness in an indication of the presence of intraembryonic ice crystals and hence that CPA incorporation was not sufficient.

Other studies have also reported similar findings during attempted vitrification (Magnus et al., 1995; Liu et al., 1998; Lin and Chao, 2000; Cabrita et al., 2006; Edashige et al., 2006; Ding et al., 2007; El-Battawy et al., 2009; Rahman et al., 2017). Similar results were also reported by Isayeva et al. (2004) and Lubzens et al. (2005) for zebrafish and marine fish species, respectively. However, it must be noted that another possible explanation for the opaqueness of embryos during cooling is the fusion of yolk granules into one translucent mass (Isayeva et al., 2004). Robles et al. (2003) working with turbot embryos demonstrated that the yolk mass frequently undergoes recrystallization during thawing after vitrification. We also observed the formation of ice crystals in embryos inside the straws during recrystallization. These results suggested that thawing may be another important obstacle for successful cryopreservation of whiting embryos as has been observed for other species. Recently, Khosla et al. (2017) reported that laser-based thawing is helpful to prevent or minimize recrystallization of ice during warming of vitrified embryos. Several studies also reported that rapid cooling and thawing reduced the probability of ice crystals formation and resultant cryoinjuries (Bart, 2000; Denniston et al., 2000; Wusterman et al., 2002; Yavin and Arav, 2007). Interestingly, a number of embryos apparently showing intact morphology were observed after freeze-thawing, although they also failed to resume development. We

therefore conducted a study to determine the proportions of live and dead cells in these embryos. The highest frequency of live cells in embryos after attempted vitrification and thawing was observed again with solution T16. Overall, all results obtained in this study suggest that this solution may be a useful cryoprotectant medium for microinjection in embryos intended for cryopreservation. The results also suggest that this approach could be useful for future studies on fish embryo cryopreservation.

Overall, the findings of this study show that microinjection may be a reliable method for introduction of all types of CPAs and antifreeze proteins into fish embryos for cryopreservation. It also suggests that combinations of AFPs and CPAs may be suitable for cryopreservation (vitrification) of *Sillago japonica* embryos. As such, this study provides the basis for the development of microinjection-based impregnation protocols with applicability for embryos of this species and perhaps for other species as well. In future, further studies may be required to evaluate the efficiency of gold nanorod incorporation into Japanese whiting embryos and to optimize the laser-based thawing methodology to prevent recrystallization during warming of vitrified embryos.
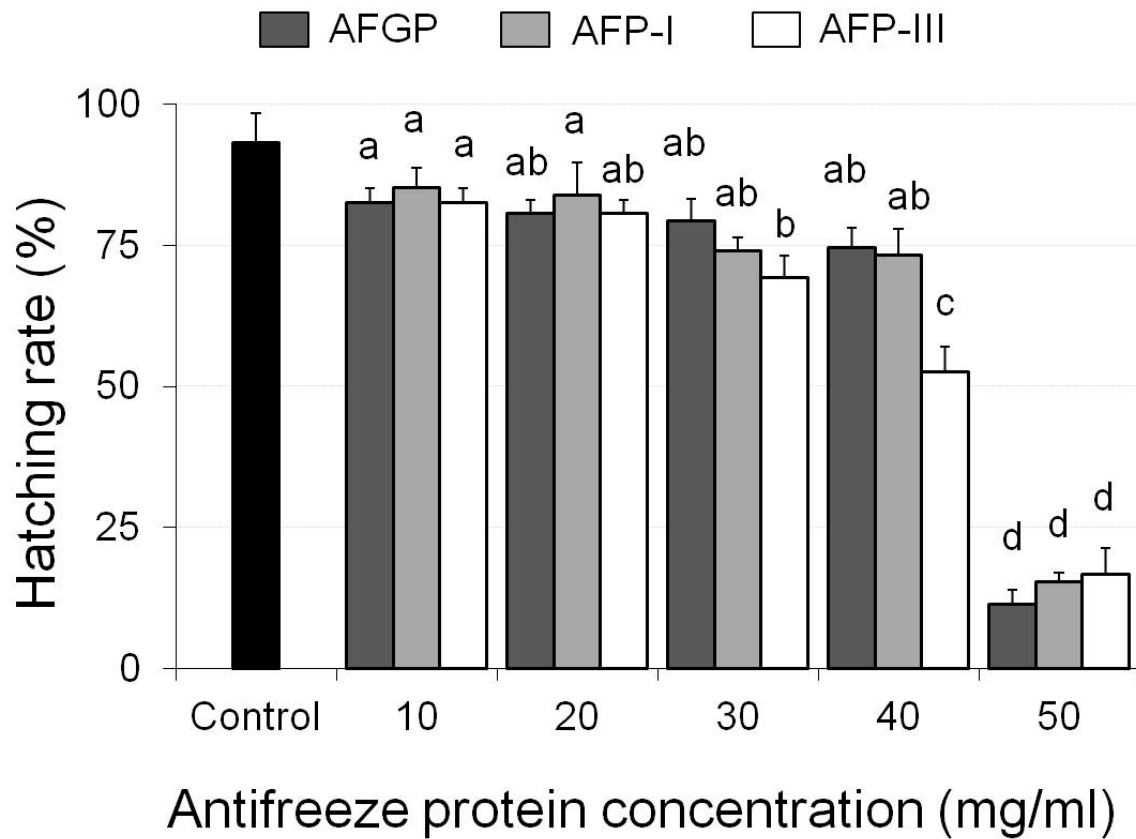
Fig. 4.1. Hatching rates of Japanese whiting tail elongation embryos that were double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 10, 20, 30, 40, and 50 mg/ml solutions of antifreeze proteins (AFGP, AFP-I, and AFP-III). Thick and thin bars represent the mean and SD, respectively, of five replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
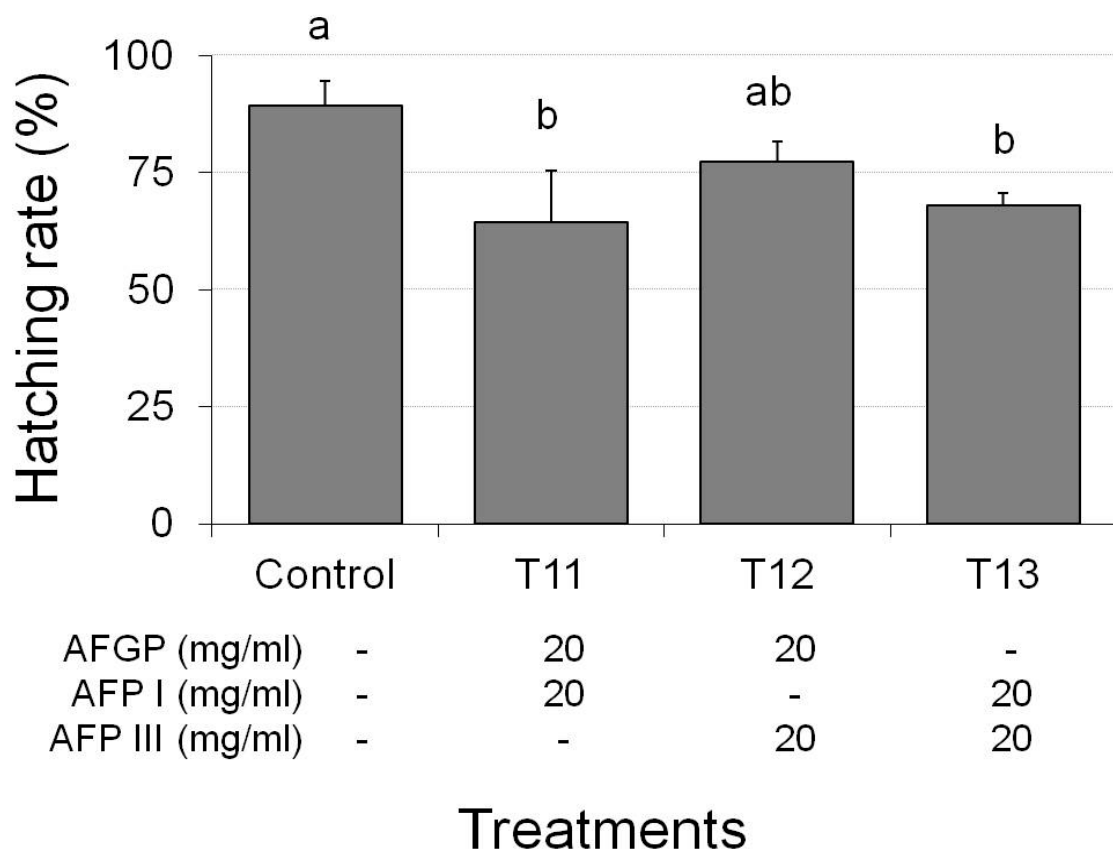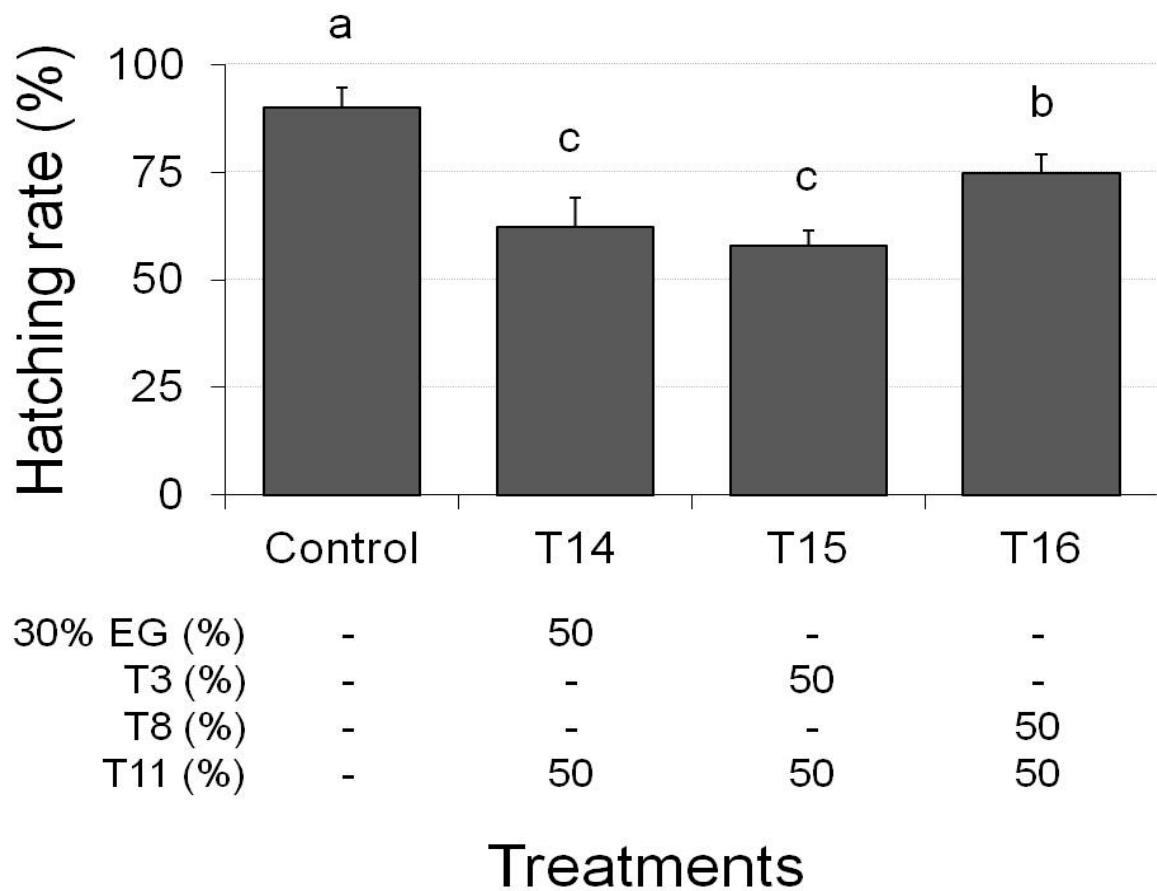
Fig. 4.2. Tolerance of Japanese whiting tail elongation embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs (solutions T11, T12, and T13). Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).

Fig. 4.3. Tolerances of Japanese whiting tail elongation embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with a combinations of two AFPs (T11) with permeable and non-permeable CPAs (solutions T14, T15, and T16). Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 30 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
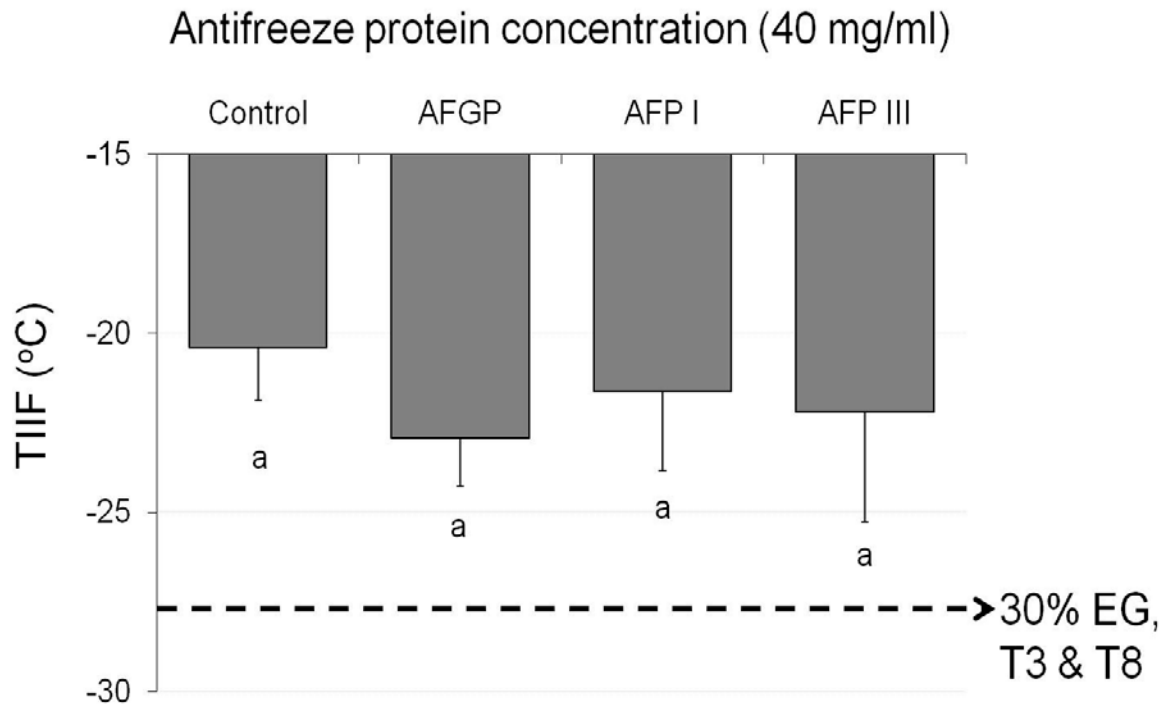
Fig. 4.4. Temperature of intracellular ice formation (TIIF, °C) of tail elongation stage embryos of Japanese whiting that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 40 mg/ml of antifreeze protein (AFGP, AFP-I, and AFP-III) solutions. Thick and thin bars represent the mean and SD, respectively, of four replicates for each treatment. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
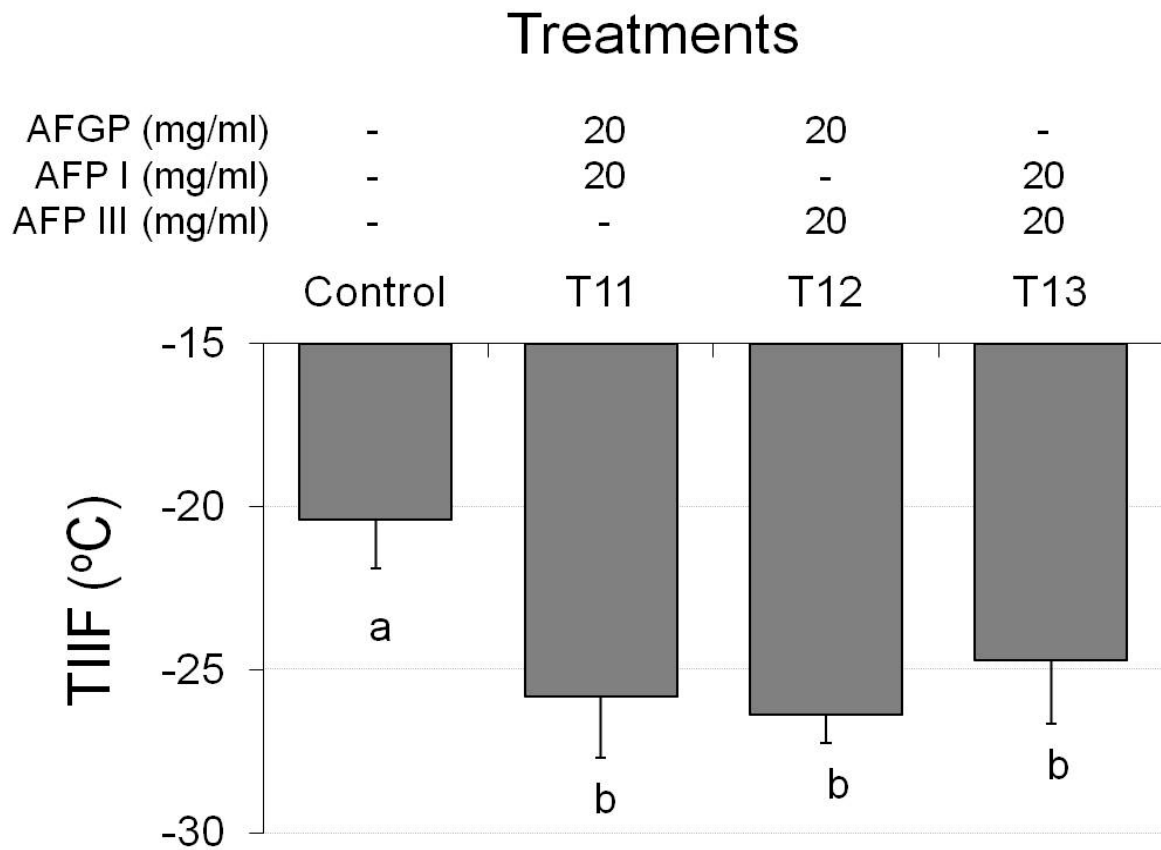
Fig. 4.5. Temperature of intracellular ice formation (TIIF, °C) of tail elongation stage embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs (solutions T11, T12, and T13) and untreated control embryos. Thick and thin bars represent the mean and SD, respectively, of four replicates for each treatment. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
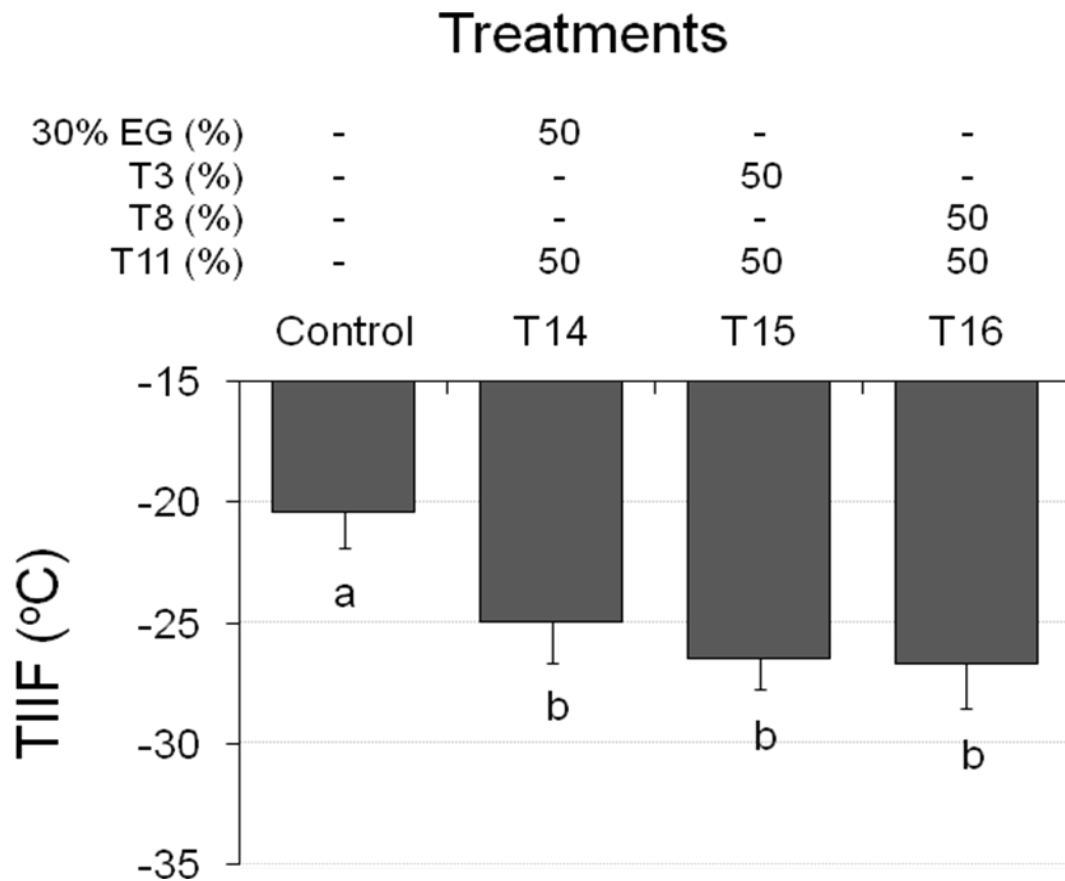
Fig. 4.6. Temperature of intracellular ice formation (TIIF, °C) of tail elongation stage embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs and CPAs (solutions T14, T15, and T16) and untreated control embryos. Thick and thin bars represent the mean and SD, respectively, of four replicates for each treatment. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
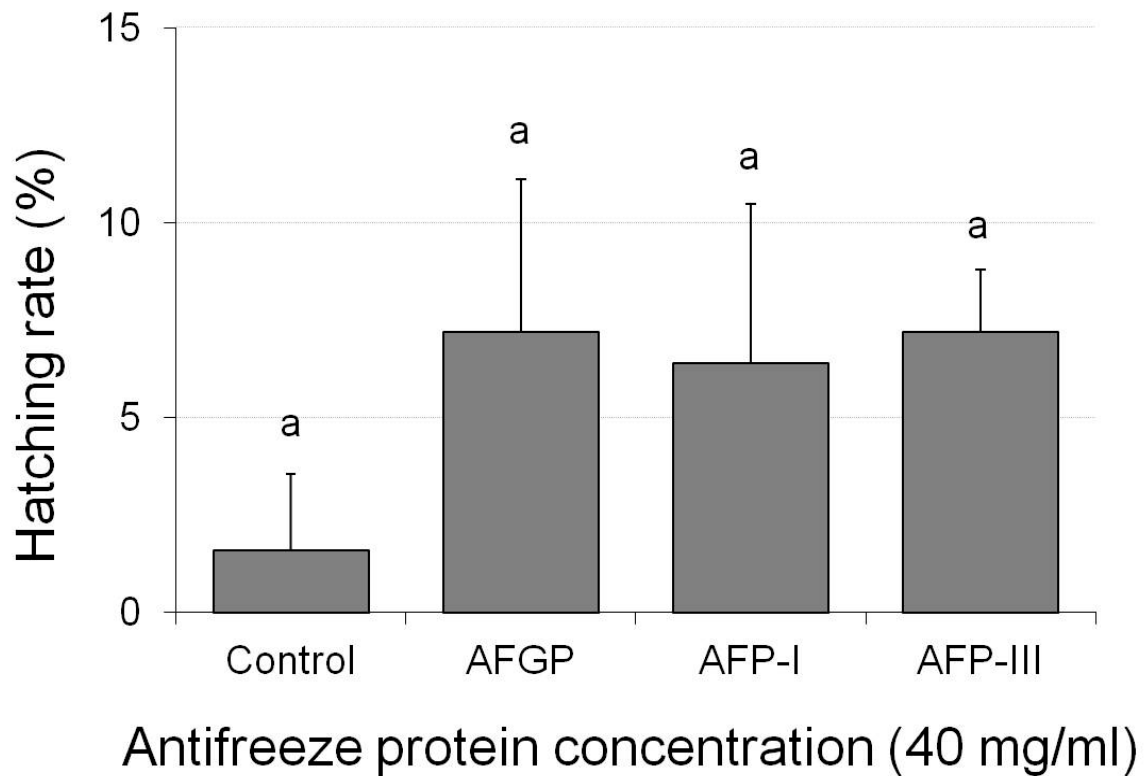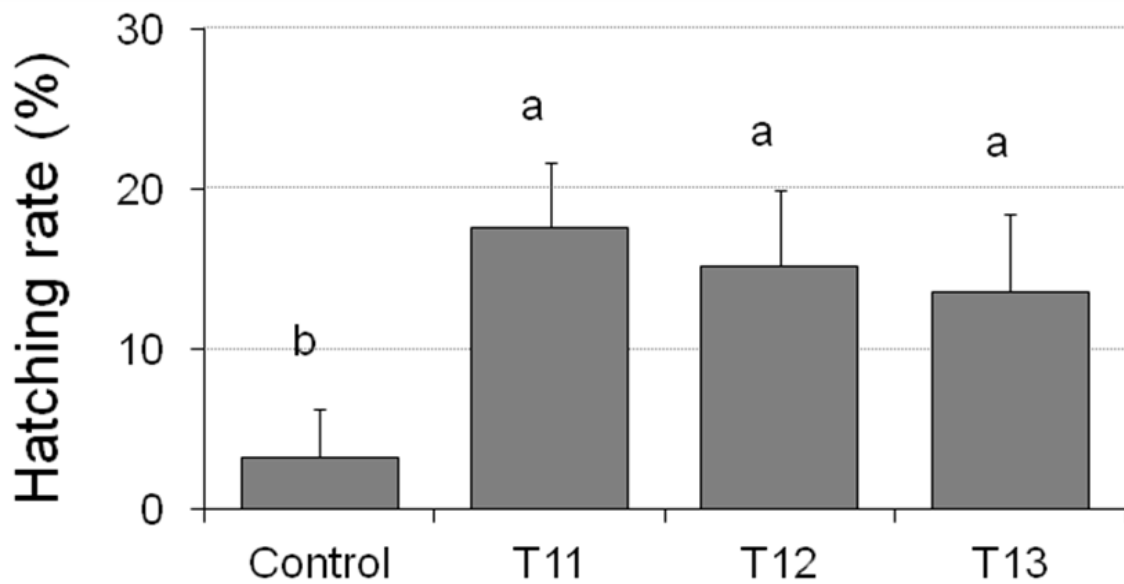
Fig. 4.7. Chilling tolerance of Japanese whiting embryos subjected to a subzero, non-freezing temperature survival test consisting of cooling from room temperature to -10 °C at a rate of - 5 °C/min, exposure to -10 °C for 20 min, and warming to room temperature at a rate of 8 °C/min. Embryos at tail elongation stage were double-injected into the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with 40 mg/ml of antifreeze proteins (AFGP, AFP-I, and AFP-III) solutions. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 25 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).

	Control	T11	T12	T13
AFGP (mg/ml)	-	20	20	-
AFP I (mg/ml)	-	20	-	20
AFP III (mg/ml)	-	-	20	20

Treatments

Fig. 4.8. Chilling tolerance of Japanese whiting embryos subjected to a subzero, non-freezing temperature survival test consisting of cooling from room temperature to -10°C at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Embryos at tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs (solutions T11, T12, and T13) in Yamamoto solution. Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 25 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
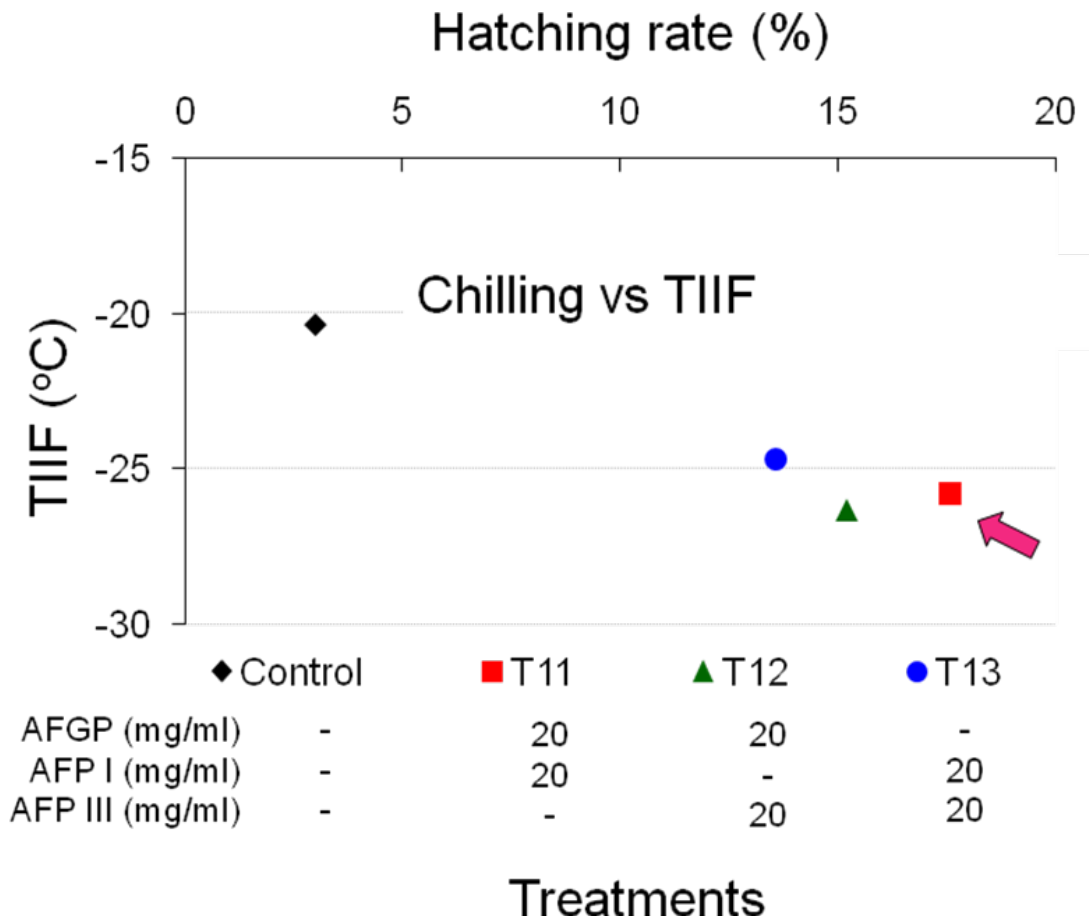
Fig. 4.9. Relationship between the survival rates (%) after the chilling tolerance test and the temperature of intracellular ice formation (TIIF) in tail elongation embryos of Japanese whiting in different treatments (solutions T11, T12, and T13) and intact controls. Embryos in the tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFP solutions.
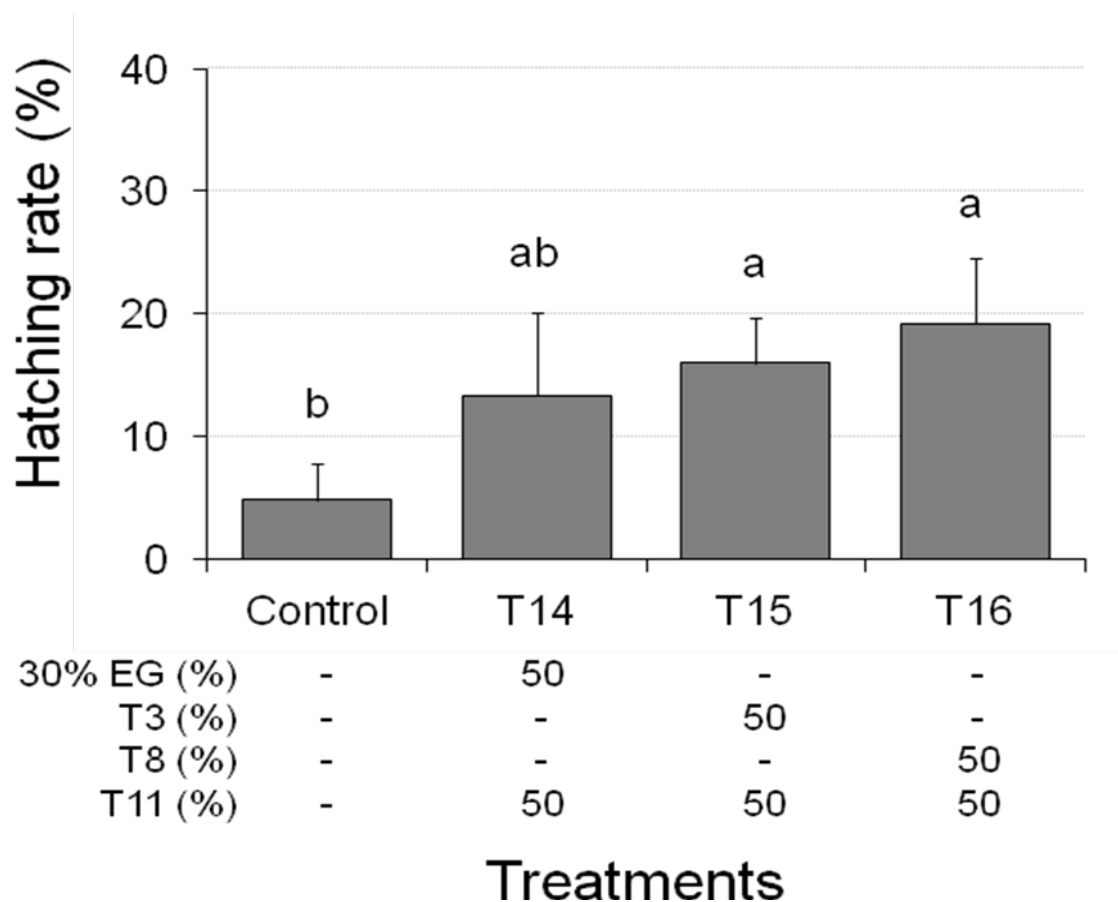
	Control	T14	T15	T16
30% EG (%)	-	50	-	-
T3 (%)	-	-	50	-
T8 (%)	-	-	-	50
T11 (%)	-	50	50	50

Treatments

Fig. 4.10. Chilling tolerance of Japanese whiting embryos subjected to a subzero, non-freezing temperature survival test consisting of cooling from room temperature to -10°C at a rate of -5°C/min, exposure to -10°C for 20 min, and warming to room temperature at a rate of 8°C/min. Embryos at the tail elongation stage were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs and CPAs (solutions T14, T15, and T16). Thick and thin bars represent the mean and SD, respectively, of four replicates with at least 25 embryos each. Columns with different letters are significantly different ($p < 0.05$, ANOVA/Tukey).
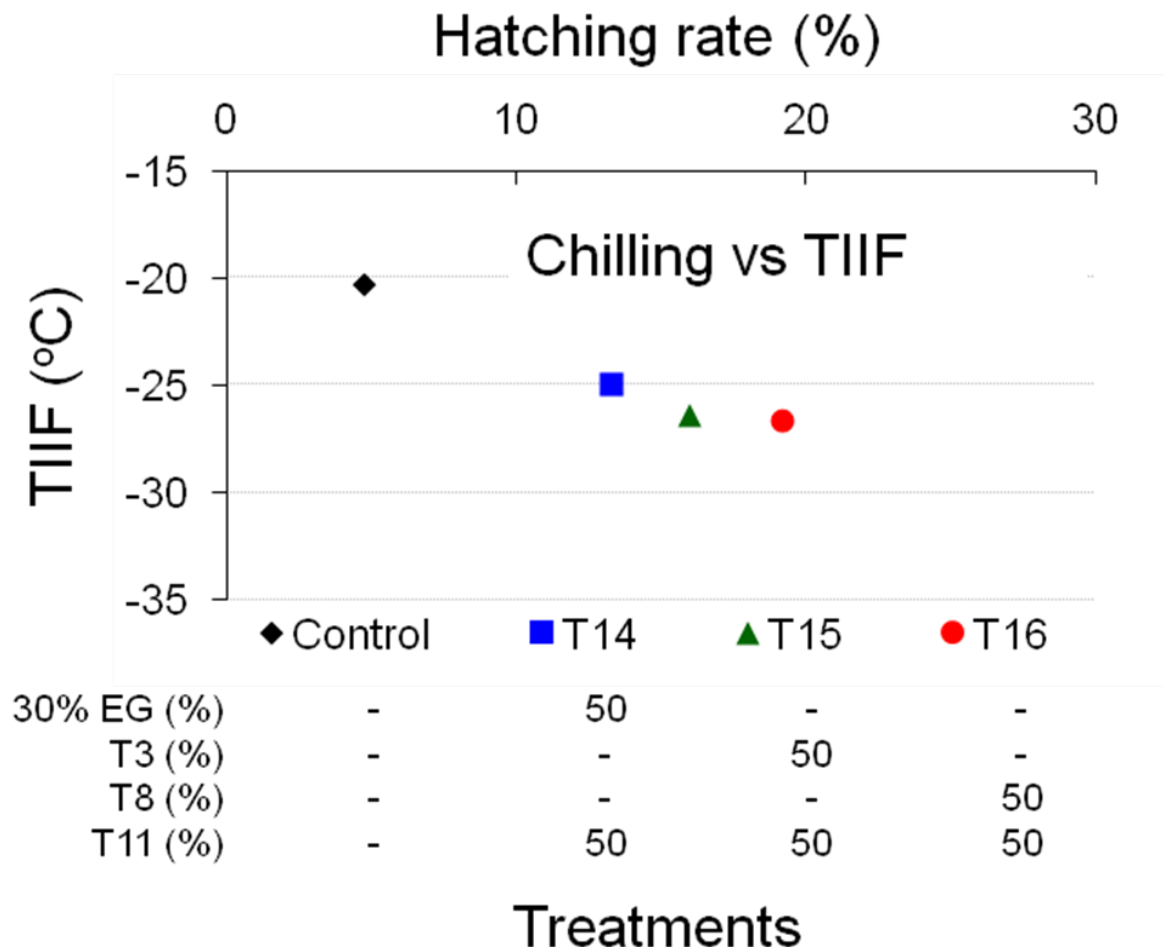
Fig. 4.11. Relationship between the survival rates (%) after the chilling tolerance test and the temperature of intracellular ice formation (TIIF) in tail elongation embryos of Japanese whiting in different treatments (solutions T14, T15, and T16) and intact controls. Embryos at tail elongation stage were double-injected in the yolk mass (YM; 15.6 nl) and the perivitelline space (PS; 2.1 nl) with combinations of AFPs and CPA solutions.

Fig. 4.12. Appearance of Japanese whiting *S. japonica* embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs and CPAs (solution T16) during attempted vitrification in liquid nitrogen.
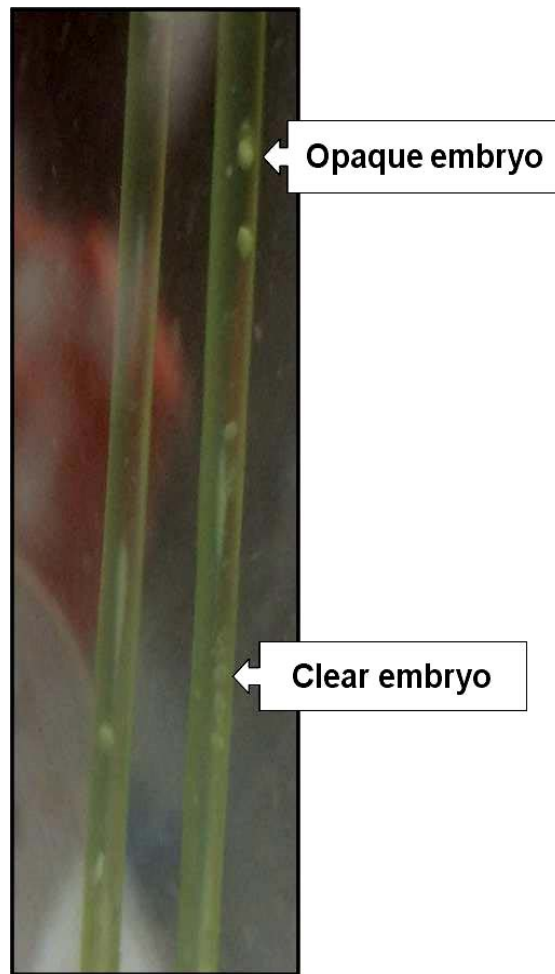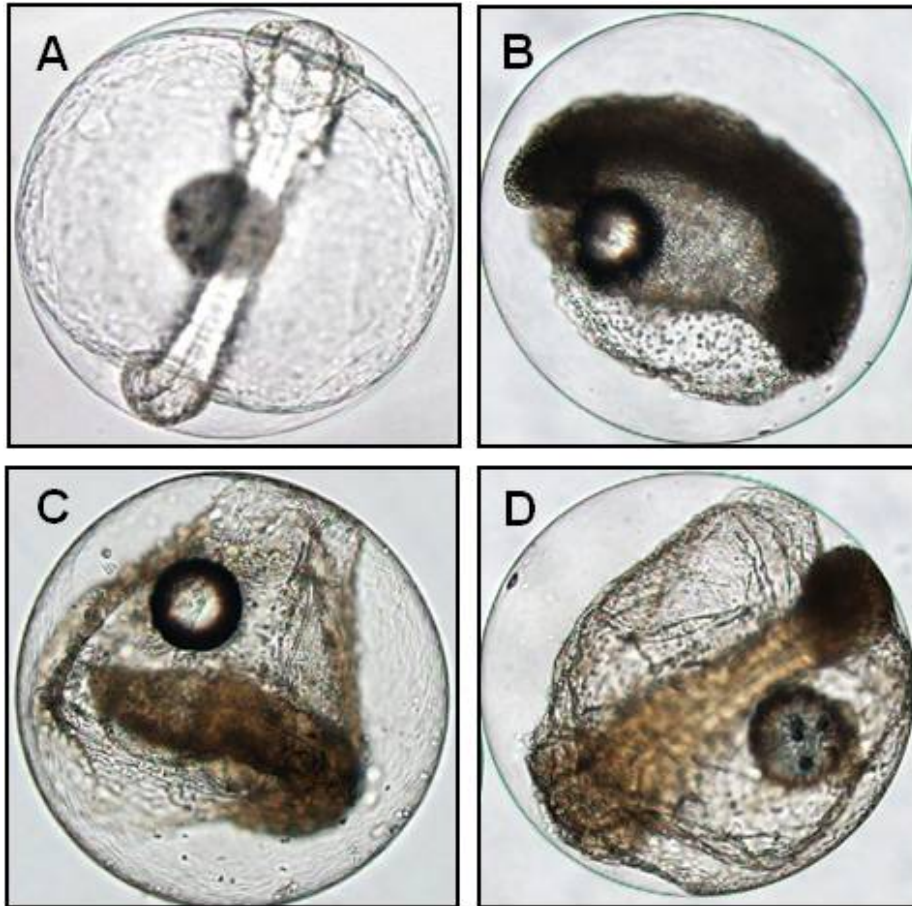
Fig. 4.13. Appearance of Japanese whiting *S. japonica* embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs and CPAs (solution T16) after freeze-thawing. A) Pre-freezing (control) embryos; B-D) clearly damaged embryos after freeze-thawing.
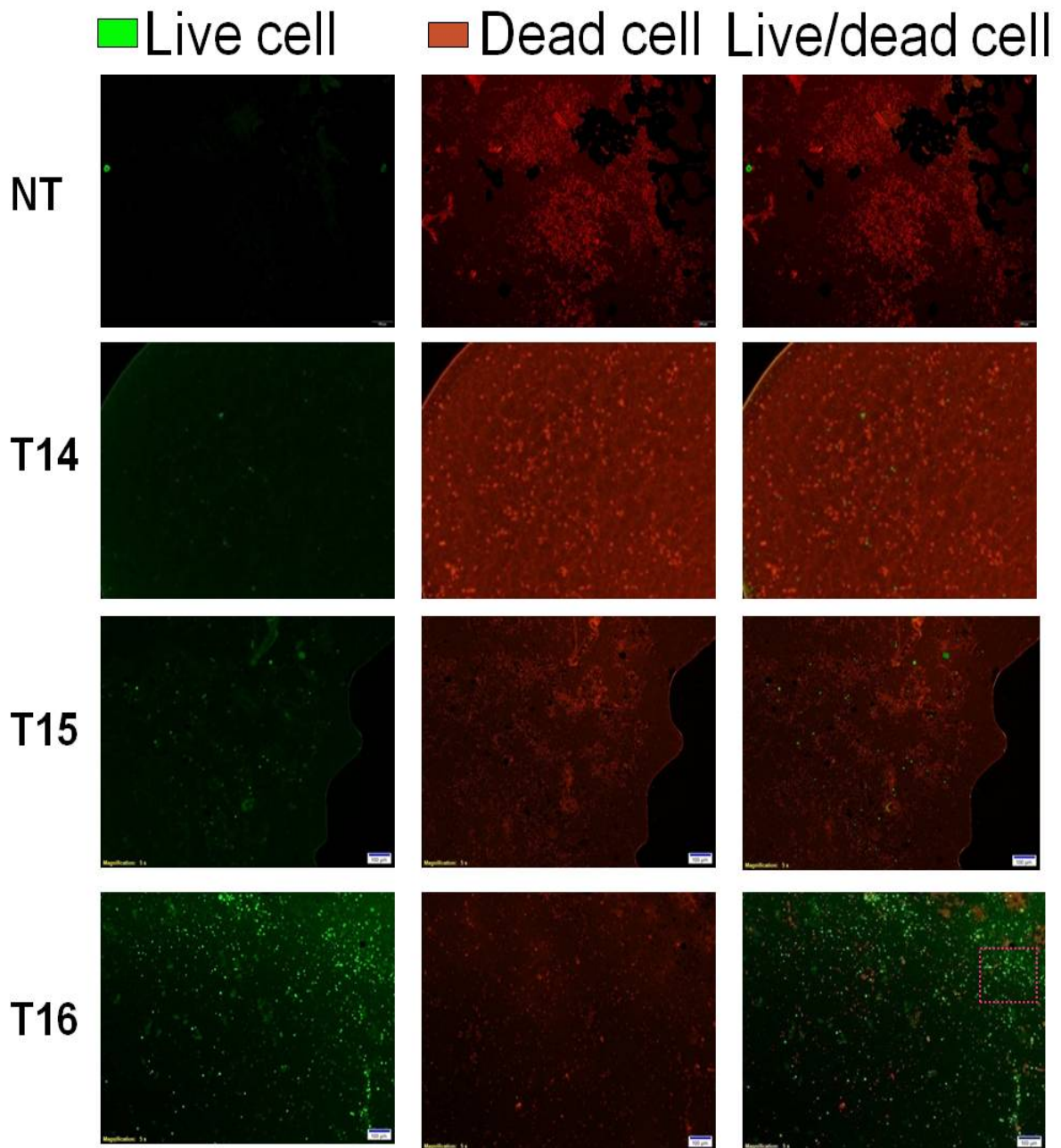
Fig. 4.14. Appearance of tissue spreads during the live/dead cell analysis of tail elongation embryos that were double-injected in the yolk mass (15.6 nl) and the perivitelline space (2.1 nl) with combinations of AFPs and CPA solutions (T14, T15, and T16) and subjected to attempted vitrification. Photographs include untreated control embryos. Scale bars represent 100 μm. The dotted area of live/dead cell in T16 is shown in higher magnification in Fig. 4.15.
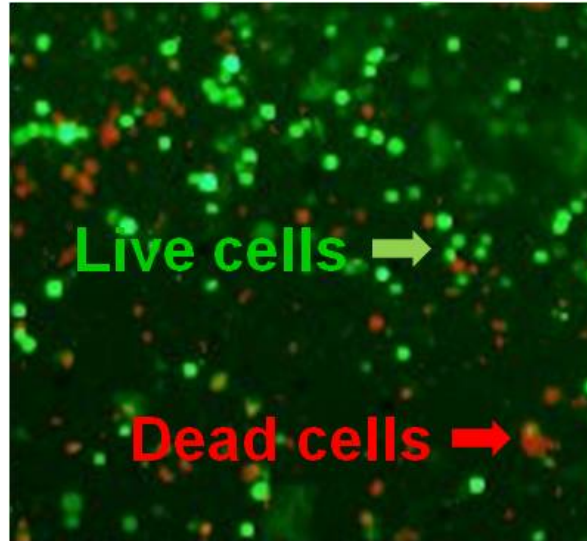
Fig. 4.15. Higher magnification of a typical observation from the live/dead cell analysis of tail elongation embryos after vitrification in solution T16 (refer to dotted box in Fig. 4.14).

Table 4.1. Composition of the solutions of antifreeze proteins (AFPs) and chemical cryoprotective agents (CPAs) used this study.

Solution	Combination of AFPs (mg/ml)		
	AFGP	AFP I	AFP III
T11	20	20	-
T12	20	-	20
T13	-	20	20
	Combination of AFPs and chemical CPAs		
	AFPs mixture	CPA solutions	Volume ratio
T14	T11	30% EG	1:1
T15	T11	T3 (15% EG + 15% PG)	1:1
T16	T11	T8 (15% EG + 7.5% PG + 0.125 M Tre)	1:1

CHAPTER 5

General conclusions

This study provides novel information about the cryopreservation of fish embryos that can be summarized as follows:

1) Embryos of Japanese whiting *Sillago japonica* were found to constitute a good model for cryopreservation studies on embryos of marine fish spawning small, pelagic, buoyant eggs;

2) The basic protocols (microinjection volume and location, suitable developmental stage, type of extender, and the type and concentration of CPAs) for injection-based CPA introduction in whiting embryos;

3) The internal CPA concentrations achieved by microinjection were 2-4 fold higher than previously obtained for this species with immersion-based impregnation methods;

4) Solutions combining EG, PG, Trehalose, and AFPs seemed to be comparatively less toxic than single-component solutions and to provide embryos with some degree of chilling tolerance and/or cryoprotection during vitrification. Hence, such solutions may be useful in the ongoing development of cryopreservation protocols.

The findings of this study provide the basis for the development of microinjection-based impregnation protocols with applicability for embryos of this species and perhaps for other species as well. However, it was found that both cooling and thawing rates are also very important constraints for successful cryopreservation of fish embryos. In this regard, it is suggested that the following points must be addressed in future studies:

1) Further optimization of solutions containing both permeable and non-permeable CPAs and AFPs;

2) Evaluation of the efficiency of gold nanorod incorporation into Japanese whiting embryos and laser-based thawing methodology to prevent recrystallization during warming of vitrified embryos;

3) Evaluation of the suitability of combining a microinjection-based protocol for CPA impregnation with the encapsulation-vitrification method.

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List of publications and presentations

Publications

1. **Md Ariful Alam**, Sheikh Mustafizur Rahman, Yoji Yamamoto, Ricardo Shohei Hattori, Toru Suzuki, Manabu Watanabe, and Carlos Augusto Strüssmann. Optimization of protocols for microinjection-based of cryoprotective agents into fish embryos Japanese whiting (*Sillago japonica*) embryos. Cryobiology 85 (2018) 25-32.

2. **Md Ariful Alam**, Sheikh Mustafizur Rahman, Yoji Yamamoto, Ricardo Shohei Hattori, Toru Suzuki, Manabu Watanabe, and Carlos Augusto Strüssmann. Optimization of protocols for microinjection-based of cryoprotective agents into fish embryos. 55th Annual meeting of the Society for Cryobiology "CRYO2018: Scientific Challenges of Cryobiology". July 10-13, 2018, Madrid, Spain. P-38. (Abstract) (Student Travel Award and also selected as a finalist for the Peter L. Steponkus Crystal Award)

Oral presentations

1. **Md Ariful Alam**, Sheikh Mustafizur Rahman, Yoji Yamamoto, Ricardo Shohei Hattori, Toru Suzuki, Manabu Watanabe, and Carlos Augusto Strüssmann. Optimization of protocols for microinjection-based of cryoprotective agents into fish embryos. 55th Annual meeting of the Society for Cryobiology "CRYO2018: Scientific Challenges of Cryobiology". July 10-13, 2018, Madrid, Spain. P-38.

2. **Md Ariful Alam**, Carlos Augusto Strüssmann, Sheikh Mustafizur Rahman, Yoji Yamamoto, and Shohei Sakai. Development of a microinjection-based protocol for impregnation of Japanese whiting (*Sillago japonica*) embryos with cryoprotective agents. 85th International Symposium "Fisheries Science for Future Generations". The Japanese Society of Fisheries Science. September 22-24, 2017, Tokyo University of Marine Science and Technology, Shinagawa, Tokyo, Japan.

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