STUDIES ON THE EFFECT OF CHILLING ON SOX GENES AND PROTEIN EXPRESSION IN ZEBRAFISH (*Danio rerio*) EMBYROS

KUNJAN DESAI

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by

KUNJAN DESAI

A thesis is submitted to the University of Bedfordshire in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Institute of Biomedical and Environmental Science and Technology
University of Bedfordshire
250 Butterfield
Great Marlings
Luton LU2 8DL

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ABSTRACT

In aquaculture, short term chilled storage has been used to transport brood stock fish embryos for genetic improvement programmes. It is therefore important to understand the effect of chilling on embryos at both developmental and molecular levels. In the present study, gene expression patterns in zebrafish embryos were studied before investigations were carried out on the effect of chilling on gene and protein expression in these embryos. The gene expression results obtained in different developmental stages using conventional PCR showed that, only *sox* genes were expressed throughout the tested developmental stages from 30% epiboly to 6 somites. Quantitative RT-PCR was then used to investigate *sox* gene expression patterns during chilling of 50% epiboly stage embryos at 0°C for up to 180 min and also after warming. Significant decreases in *sox2* and *sox3* expressions were observed when compared to those of controls following chilling whilst significant increases of expressions of the two genes were observed after warming in the embryos chilled for 30 and 60 min. Studies on the impact of cryoprotectant MeOH on *sox* genes and protein expression showed that 50% epiboly stage zebrafish embryos could tolerate chilling for up to 6 h with or without MeOH. It was observed that expression of all three *sox* genes were significantly decreased following chilling for 3 h at 0°C. However the degree of decrease was less pronounced in embryos chilled with different concentrations of MeOH. Significant increases in *sox* genes were observed in hatching stage embryos chilled with 1 M MeOH for 3h but subsequent *sox2* and *sox19a* protein expression was not affected. The effect of long term chilling (18h) on sox gene and protein expression in 50% epiboly stage embryos was also investigated. Improved hatching rates (56% ± 5) were achieved when embryos were chilled with 1 M MeOH + 0.1 M sucrose. Results from gene expression studies showed a stable *sox2* gene expression in 18 h chilled embryos in cryoprotectant mixture when compared to that of embryos chilled without cryoprotectant mixture. Similar patterns were observed when the expression of *sox2* and *sox3* protein was investigated. This is the first study carried out on the effect of chilling in early stage zebrafish embryos at the molecular level. The results obtained from the present study provided useful information on the molecular mechanisms of the effect of chilling on zebrafish embryos and will have important implications in designing chilled storage protocols for fish embryos.

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Finally, I would like to thank my parents, my brother Sarjan and my loving wife Shivali for supporting me during my PhD study. Without their support and love, this thesis would have not been completed.

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Bedfordshire.

It has not been submitted before for any degree or examination in any other University.

Name of candidate: Signature:

Date:

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Effect of chilling in the presence of MeOH on *sox3* gene expression zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox19a* in embryos chilled for 180 min at 0°C and then cultured at 27±1°C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression levels relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different

($p < 0.05$) in gene expression between control treatment within the same developmental gene.

ABBREVIATIONS

ANOVA	analysis of variance
CPAs	Cryoprotective agents
DMSO	dimethyl sulfoxide
EFTs	expressed sequence tags
hpf	hour post fertilization
IIF	intracellular ice formation
LN_2	liquid Nitrogen
MeOH	methanol
PCR	polymerase chain reaction
qRT PCR	quantitative reverse transcriptase PCR
ROS	reactive oxygen species
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard error of mean
YSL	Yolk syncytial layer

CHAPTER 1: INTRODUCTION

1.1 Significance and aims of the project

Successful cryopreservation of fish embryos has important applications in aquaculture, conservation and biomedicine. The development of cryopreservation and chilling protocol for fish gametes and embryos could provide opportunities for conserving endangered fish population (Tiersch et al. 1998, Nahiduzzaman et al. 2011). Cryopreservation also offers other benefits such as prevention of brood stock from being eliminated due to drastic epidemic disease and natural disaster. Cryopreservation significantly enhances the capability in management of brood stock in aquaculture and maintain genetic diversity (Gausen 1993).

Despite the successful cryopreservation of many mammalian embryos, cryopreservation of fish embryos is still a challenge due to structural and functional limitations of these embryos such as their large size, high yolk content, greater sensitivity to chilling and low membrane permeability (Zhang 2004). In aquaculture, short term storage of brood stock fish embryos under chilled conditions has been used to facilitate transportation of the embryos in genetic improvement programmes. It is therefore important to understand the effect of chilling on embryos not only on development but also at molecular level. Understanding of the effect of chilling on embryos also facilitates the development of cryopreservation protocols as chilling is normally the first stage of most of the cryopreservation protocols. In embryo chilling and cryopreservation studies, embryo hatching rate has mainly been used as an indicator for successful cryopreservation protocol, however information on genetic integrity of the recovered material is also important. It has been reported (Succu et al. 2008, Lin et al. 2009b, Uechi et al. 1997) that cryopreservation alters the pattern of gene expression which results in adverse effect on biological development.

In recent years, zebrafish has become an important animal model in scientific research. Zebrafish is used extensively for developmental biology studies such as determination of embryonic axis (Driever 1995), cell lineage analysis (Helde et al. 1994), formation of the central and peripheral nervous system (Strahle and Blader 1994), cardiovascular development (Stainier et al. 1993) and differential regulation of gene expression (Driever et al. 1994). Studies have also revealed a close relationship between the zebrafish and human genomes and it has been proposed that zebrafish could be a bridge between study of human disease and development due to significant homology between zebrafish genes and human disease-causing genes (Barbazuk et al. 2000, Andrew Dodd et al. 2000).

The aim of the present study was to investigate the effect of chilling and warming on molecular biological properties such as gene and protein expression using zebrafish as a model system.

1.2 Cryopreservation of biological materials

Cryopreservation is a process by which cells or whole tissues are preserved at low temperatures normally at -196°C in liquid nitrogen. At this temperature no biological activity occurs, as kinetics energy levels are too low to allow the necessary molecular motion (Grout et al. 1990). There are two main steps involve in cryopreservation, chilling and freezing step involves the reduction in temperature of cells from their physiological temperature to freezing storage temperature in liquid nitrogen and thawing which refers to warming of the cells from their freezing storage temperature to their physiological temperature. During cryopreservation, two kinds of injuries can occur: chilling injury and freezing injury. Chilling injury is associated with damages following exposure of cells at zero and subzero temperatures but without freezing and freezing injury is associated with subzero temperature exposure and ice formation. The present study is focused on the impact of various chilling treatments (with/without cryoprotectant) on gene and protein expressions in zebrafish embryos.

1.2.1 Chilling injury

Chilling injury is described as damages in cells and tissues held at critical temperature below their physiological temperature where they normally survive and function (Parkin et al. 1989). Chilling injury is normally associated with exposure of cells to low temperatures without ice formation. Studies have shown that chilling injury is caused by lipid phase transition from liquid crystalline to the gel phase in cell membrane accompanied by leakage of solutes across membranes (Hays et al. 2001, Morris and Clark 1987). Lipid phase transition can affect membrane disorganisation and membrane leakiness, which eventually affect cell viability. Another cause of chilling injury is the denaturation of protein at low temperatures (Lattman 1994). The exposure of hydrophobic region of proteins unfold as temperatures are lowered. Chilling can also induce oxidative stress leading to production of reactive oxygen species (ROS) (Scandalios 1993, Tsang et al. 1991) which results in protein denaturation, lipid peroxidation (Prasad 1996) and cell apoptosis (Wood and Youle 1995). There are two types of chilling injuries in cells: direct chilling injury (cold shock) and indirect chilling injury.

1.2.1.1 Direct chilling injury (cold shock)

Morris (1987) documented some important features of cold shock which are (1) all cell type may be considered as sensitive to cold shock provided that they are cooled rapidly enough to a sufficiently low temperature; (2) cellular viability is dependent upon rate of cooling with more injury observed following “rapid” rather than “slow” cooling; (3) cold shock injury is independent of rate of warming, (4) injury is increased as the period of isothermal incubation at the reduced temperature is extended; (5) loss of permeability occurs following rapid cooling, and in some instances, may be reversed upon rewarming; (6) the response of any cell type may be modified by the culture condition before cooling or by the addition of specific compound. Mechanism of direct chilling injury (cold shock)

is associated with lipid phase transition in cell membranes. A study in boar sperm (Drobnis et al. 1993) suggested that this form of injury is associated with thermotropic phase transition where lipid phase is undergone liquid crystals to gel phase (Fig 1.1). This phase transition is also accompanied by leakage of solutes across membranes (Morris 1987, Hays et al. 2001). Formation of "packing faults" between lipid domains of different phases may disrupt membrane permeability (Pringle 1981). Rapid rates of cooling cause membrane phase separation which results in more defects than slow cooling, rapid cooling is expected to produce more leaky membrane and cause severe damage to cells.

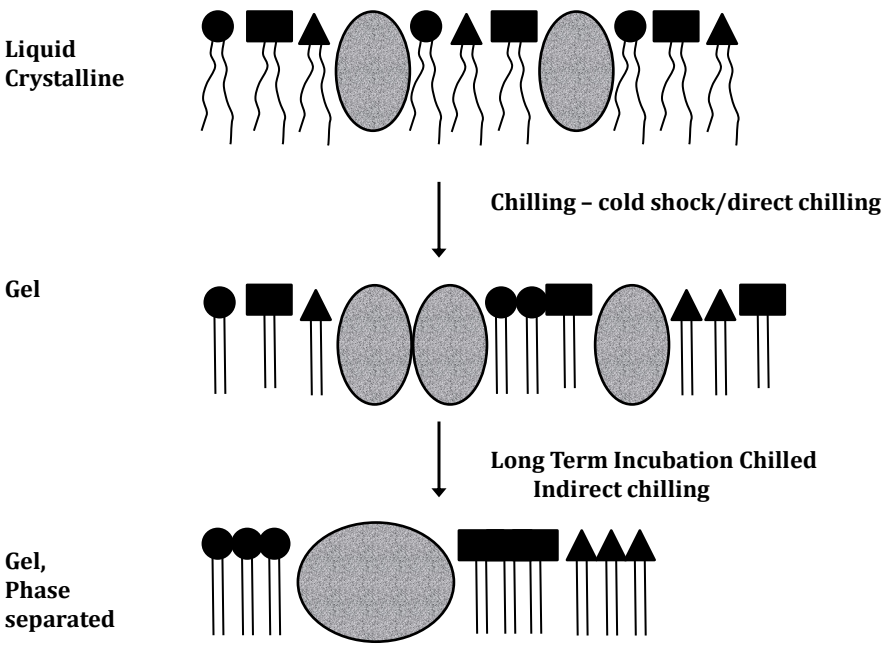


Fig 1.1 Mechanism of lipid phase transition due to chilling in cell membrane (Best 2012).

Another mechanism of cold shock was proposed by McGrath (1987) namely "thermoelastic stress theory", which described the dynamic tension within fluid membrane vesicle due to rapid cooling rate. Reduction in temperature results in membrane attempt to thermally contract laterally around an essential incompressible

aqueous interior. The contraction causes the efflux of cell water which concentrates the cytoplasmic solution and reduces chemical potential, thus create osmotic force to drive water into the cell. Sufficient force to cause damage to liposomes develops for temperature decrease of 10-20°C, larger temperature produce greater tension. The faster cooling rate produce increased tension for a given temperature reduction, therefore rapid cooling cause cold shock or injury (Liu 2000).

1.2.1.2 Indirect chilling injury

Indirect chilling injury is cooling rate independent and usually associated with long exposure at low temperature without formation of intracellular ice. Indirect chilling injury also refers to the delayed mortality and thought to be due to irreversible damage to cell (Morris 1984).

A series of events thought to occur in cellular membrane due to low temperature exposure (Fig 1.2). Initially, as temperature decreases, membrane viscosity increases. Further reduction in temperature may lead to phase separation. This phase separation depends on cell type and the extent of temperature reduction. Many cells compensate phase changes by modifying composition of their membrane, however complete compensation is rare. Cells usually be trapped in state of permanent phase change or transition after extended period of exposure to low temperature. Following phase transition, many biological properties are altered which leads to alteration of activities of membrane proteins and membrane associated enzymes (Cossins 1983). Protein and enzyme are affected by low temperature which may have downstream affect on their function and metabolic pathways. The disorder of metabolic and enzymatic processes can be detrimental in fast developing embryos like *Drosophilla* such injury increases rapidly at lower temperature due to loss of co-ordination with reducing temperature (Mazur et al. 1992). Moreover, reduced temperature may also have adverse effect on cytoskeleton system i.e.

depolymerisation of microtubules (Magistrini and Szollosi 1980, Behneke and Forer 1967, Weber et al. 1975) which could result in irreversible disruption of cellular process like cell division in oocytes (Magistrini and Szollosi 1980, Martino et al. 1995).

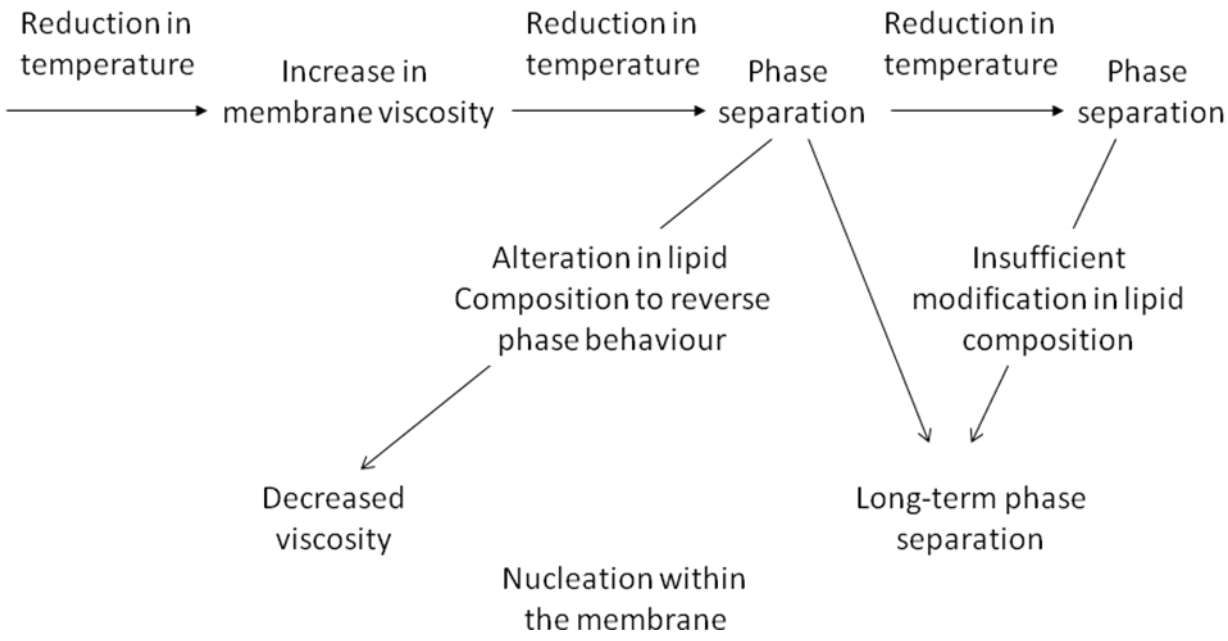


Fig 1.2 A series of events and consequence of indirect chilling injury (Morris and Clark 1987).

1.2.2 Freezing Injury

Freezing is the conversion of liquid water to crystalline ice, which results in the concentration of dissolved solutes in the remaining liquid phase and the precipitation of any solutes that exceed their solubility limit (Pegg 2007b). In other word, when a solution is cooled below its freezing point, it is initially supercooled, i.e. it remains liquid at a temperature colder than the 'true' or equilibrium freezing temperature. This is unstable condition and as the temperature is lowered further the probability of ice nucleation increases and crystals begin to form. The concentration of solution increases as pure

water convert to ice and proposition of solution remaining liquid is reduced. Formation of ice crystals is lethal to cells and membranes. The freezing injury is associated with three main factors (Grout and Morris 1987):

i. the mechanical effect of extracellular ice crystals at cell surfaces, especially in tissues with cellular interconnections

ii. alteration in physical properties of solution outside the cell, including the concentration of solutes which results from the nucleation of a proportion of extracellular water

iii. Intracellular freezing if happens

Upon freezing, the immediate cellular response is controlled by physical and chemical properties of both biological and physical systems involved to reach new equilibrium condition. As mentioned earlier, extracellular ice formation increased the solute concentration, results in chemical imbalance between extracellular unfrozen solutions and biomaterial. The supercooled water in the cells has higher chemical potential than extracellular unfrozen water which response, water flows out the cell and freeze externally (Toner et al. 1993a, Mazur et al. 1984). This physical event is cooling rate dependant.

Different types of cells have different optimal cooling rate (Konc et al. 2005, Eriksson et al. 2001). If the cooling rate is 'slow' (Fig 1.3), the cell is likely to lose intracellular water rapidly enough by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with extracellular water, results in cell dehydration and prevent intracellular ice formation (IIF). On the other hand, when cooling rate is too fast/rapid, chemical potential of water in extracellular solution decreases is much faster than the rate at which

water can diffuse out of cell, intracellular ice forms . Studies have shown that intracellular ice formation is associated with lethal injury to cells (Muldrew and McGann 1994).

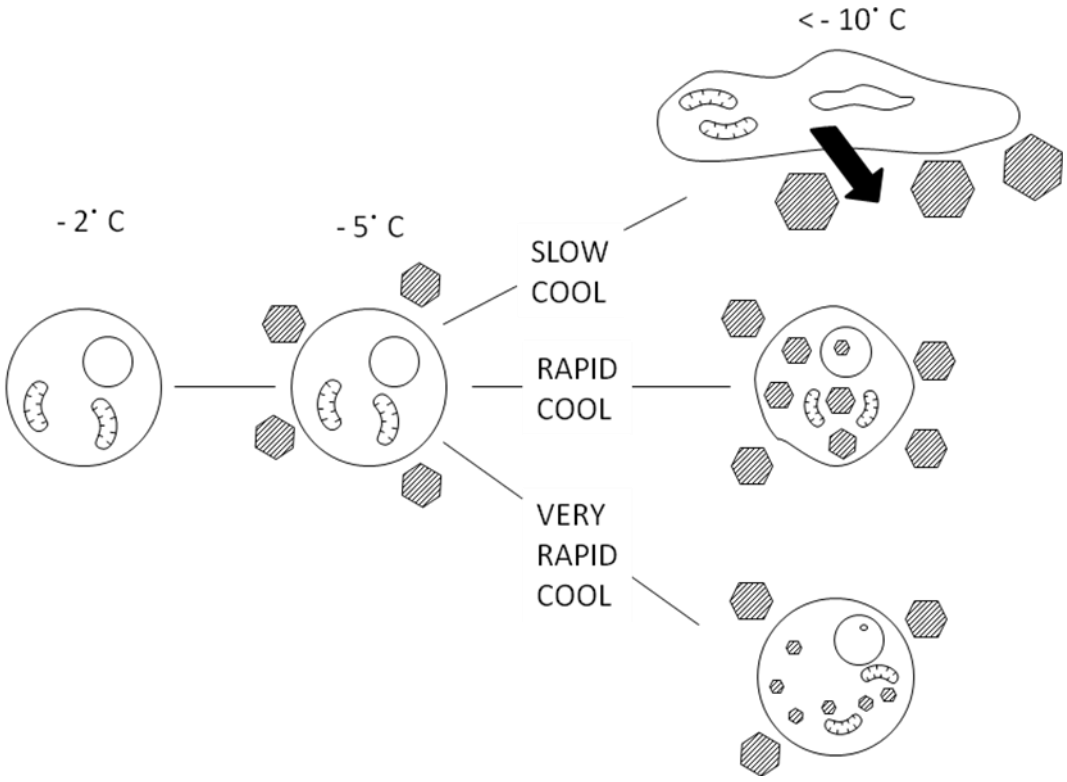


Fig 1.3 Schematic representation of the effect of different cooling rates on subsequently intracellular and extracellular ice formation. Hexagons represents ice crystals (Mazur 1977) .

Understanding the mechanisms of chilling and freezing injuries in cells is important for overcoming the adverse effect of low temperature conditions on cells and therefore assisting the development of cryopreservation protocol for biological cells. One factor also needs to be considered in developing cryopreservation protocol for cells are the selection of cryoprotectant.

1.2.3 Cryoprotectant

Cryoprotectants (CPAs) are substances characterised by their ability to reduce cryoinjury of biological materials during the course of chilling and freezing storage. CPAs are generally highly soluble to water and has relatively low toxicity to cells. However there are also disadvantages in using cryoprotectants. Most of cryoprotectants are biologically toxic at certain concentration and can be lethal to unfrozen cells (Fahy et al. 1990). Based on the mode of action, cryoprotectants are divided into two categories (i) permeating cryoprotectants and (ii) non-permeating cryoprotectants.

1.2.3.1 Permeating cryoprotectant

Permeating cryoprotectants have the ability to penetrate the cell membrane and equilibrate in the cytoplasm. They are small molecules such as methanol (MeOH), glycerol, dimethyl sulfoxide (DMSO), propylene glycol (PG) and ethylene glycol (EG) and provide protection of cells against injuries during cryopreservation process. Permeating CPAs have an effect on freezing, their presence lowers salt concentration normally found in physiological solution, which reduces the solute effect injury and lower the amount of ice (Mazur et al. 1984, Woods et al. 1999). Cryoprotective agents can provide osmotic buffering for the cells during freezing and thawing by acting as secondary solvent for salts (Pegg 2007b). Their presence results in osmotic dehydration leading to a decreasing of the amount of intracellular ice formation. The permeating cryoprotectant produces a considerate freezing point depression which results in prevention of intracellular ice formation (Shepard et al. 1976). This type of cryoprotectant diffuse into cells, they displace certain amount of water and form hydrogen bonds with protein, RNA and DNA. Hydrogen bonding can increase cytoplasm stability by keeping their structure (protein, DNA and RNA) functional and influence growth of ice. Methanol has been shown to be the most effective and least toxic cryoprotectant for zebrafish embryos (Zhang and Rawson 1995, Liu 2000) during chilling and freezing of zebrafish oocytes (Guan et al.

2008). DMSO is also a widely used cryoprotectant and has been reported to be a membrane 'modifier' and 'plastifier' (Örvar et al. 2000).

1.2.3.2 Non-permeating cryoprotectant

The non permeating cryoprotectant are long chain polymers with high molecular weight (>50,000), they are soluble in water and have large osmotic coefficient. Due to their large size, they cannot penetrate cell membrane and remain in extracellular matrix. Poly-etheleneglycol (PEG), polyvinyl pyrrolidone (PVP), polyvinyl alcohol, hydroxylethyl starch and sugars (glucose, sucrose, mannitol, fructose, sorbitol, trehalose and raffinose) are commonly used non permeating cryoprotectants. They protect cells not only during freezing but also during thawing by altering ice crystal formation to an innocuous size and shape. They enhance vitrification solution, stabilize protein and cell membrane and prevent progressive ice formation (Fahy 1986, Fahy et al. 1984).

1.2.3.3 Cryoprotectant toxicity

While cryoprotectants protect the cells from low temperature injury, it can also be toxic and lethal when use at high concentrations (Fahy 1986, Fahy et al. 1990). Cryoprotectant toxicity is cell type dependent i.e. DMSO is more toxic than methanol in zebrafish embryos (Zhang et al. 1993, Lahnsteiner 2008) and flounder embryos (Chen and Tian 2005) but the opposite was reported for oyster embryos (Chao et al. 1994) and red seabream embryos (Xiao et al. 2008). The adverse effect of glycerol (Guidet and Shah 1989) and ethylene glycol (Poldelski et al. 2001) has also been shown to cause kidney failure. The neurotoxic effect of methanol on retina and optic nerve in rat has also been reported (Eells et al. 2000). Methanol (Hoetelmans et al. 2001) and ethanol (Lindi et al. 2001) toxicity has been reported to affect cell membranes by interacting with phospholipids and destabilising the lipid bilayer of membranes. They also interact directly with the polar head group of lipids in bilayers due to their low hydrophobicity

(Patra et al. 2006). The non polar region of the alcohol creates gap between lipid chains in the membrane interior and introduce instability within bilayer (Patra et al. 2006). Use of cryoprotectant at high concentrations may cause osmotic stress to cells, disarrangement of lipid bilayer (Fahy 1986) and denaturation of proteins (Arakawa et al. 1990).

It has also been demonstrated that detrimental effect of cryoprotectant is not limited to osmotic but also biochemical injury such as inactivation or denaturation of specific enzymes, disruption of transmembranes ionic pumps or other related perturbation of cellular structure and function by direct interaction with proteins and biological membranes (Fahy et al. 1990, Fahy et al. 1984). Fahy (1986) also demonstrated that addition of cryoprotectant toxicity neutraliser into DMSO before freezing substantially reduced freezing damage following thawing. DMSO appears to interact with lysine-rich control enzyme for gluconeogenesis, fructose diphosphatase and upon removal produce effective block in glycolysis.

1.2.4 Controlled slow cooling and vitrification

There are two approaches in cryopreservation of biological materials – controlled slow cooling and vitrification. The control slow cooling procedure is characterized by the addition of molar concentration of cryoprotectant to the cell suspension and by the use of controlled freezing to the storage temperature (Zampolla 2009). There are a number of factors need to be considered while designing controlled slow cooling protocol, such as membrane permeability of the plasma membrane, cooling rate, ice seeding temperature, LN_2 plunging temperature, thawing rate, methods for removal cryoprotectant and post thawing handling. For designing optimum protocol for controlled slow cooling, knowledge of membrane permeability of cells is important for the selection of cryoprotectant. The optimum cooling rate can be obtained by balancing chemical potential of intracellular water close to the extra cellular unfrozen water (equilibrium

freezing) (Mazur 1990). In addition to cooling rate optimisation, there is also a need to optimise the temperature when extracellular ice formation is induced manually, usually termed as 'ice seeding'. Upon cooling below its equilibrium freezing point, ice forms rapidly and cell survival rate is affected by intracellular ice formation (IIF). It has also shown that IIF is drastically increased if extracellular ice formation is initiated at high degree of supercooling (Toner et al. 1993b, Diller 1975). Thawing rate and post thawing handling are also important. Cells can be damaged due to crystallisation of ice not only during cooling but also during warming. If warming is too slow, small innocuous intracellular ice crystals in rapidly cooled cells recrystallize, which can be lethal to cells (Mazur 1965). Shimada and Asahina (1975) showed improved survival rate in tumour rat cells with intracellular ice when they thawed rapidly. Rapid warming results in complete thawing of the suspension before significant embryo dehydration (Rall 1991). However there were reports that slow thawing rates resulted in higher cell survival after cryopreservation. Studies in common carp embryo cryopreservation showed that slow warming rate (8°C/min) was optimal while rapid rates killed the embryos (Zhang et al. 1989). A two step warming, a combination of slow and rapid warming, may also improve cell survival rate (Vorotilin et al. 1991). Damaging excessive osmotic swelling occurs when a thawed suspension is diluted with isotonic saline as the water moves into the cell to restore equilibrium. Upon restoration, the cells gradually shrink as cryoprotectant leave the cytoplasm. The excessive osmotic swelling is prevented using step-wise removal of cryoprotectant (Rall 1991). The step and interval is determine by permeability of the cell membrane to cryoprotectant and water (Schneider and Mazur 1984).

Vitrification is the solidification of liquid without crystallization and refers to the transformation of a liquid into a glass. It occurs when the viscosity of the solution reaches a sufficient value (arbitrary set at $10^{14.6}$ Poises) and crystallisation is inhibited (Pegg 2007a). Procedures for vitrification normally consist of five steps: (i) equilibration of

cells in a solution containing permeating cryoprotectant; (ii) dehydration of cells in concentrated solutions that will vitrify; (iii) plunging the cells in LN_2 or nitrogen slush; (iv) rapid warming of the cells; (v) removal of the cryoprotectants from the cells (Chao and Liao 2001). Vitrification has been shown to provide effective preservation for a number of cells including monocytes (Takahashi et al. 1986), early embryos (Van Wagtendonk-De Leeuw et al. 1995) and pancreatic islets (Jutte et al. 1987).

1.3 Use of Zebrafish (*Danio rerio*) as the animal model

Danio rerio , commonly known as zebrafish, are small cyprinid fish native to the stream of Himalayan region (Rainboth 1994). It has a number of advantages which make them ideal models for scientific research. They are small in size and economically easier to maintain than other vertebrates. They breed all year round and female can spawn every 2-3 days and have capacity to produce several hundred of eggs at a time. Optical clarity and large size of the embryos (0.7mm in diameter) and larvae allow the *in vivo* visualization of the cell biological events (Lieschke and Currie 2007). Due to properties such as external fertilization, high fecundity, rapid development and high stocking density, zebrafish is used extensively for developmental biology studies such as determination of embryonic axis, cell lineage analysis, formation of the central and peripheral nervous system, muscle development and differential regulation of gene expression (Lele and Krone 1996). Zebrafish has also been used as an important animal model in toxicology studies and environment studies (Ruoppa and Nakari 1988).

1.3.1 Embryonic structure and development of zebrafish embryos

The zebrafish embryo composes of two main parts: the blastoderm (developing part of the embryo) and the yolk (Fig 1.4). The yolk situated on the vegetal pole of the embryo and provides nutrients to the blastomeres (Dasgupta and Singh 1981).

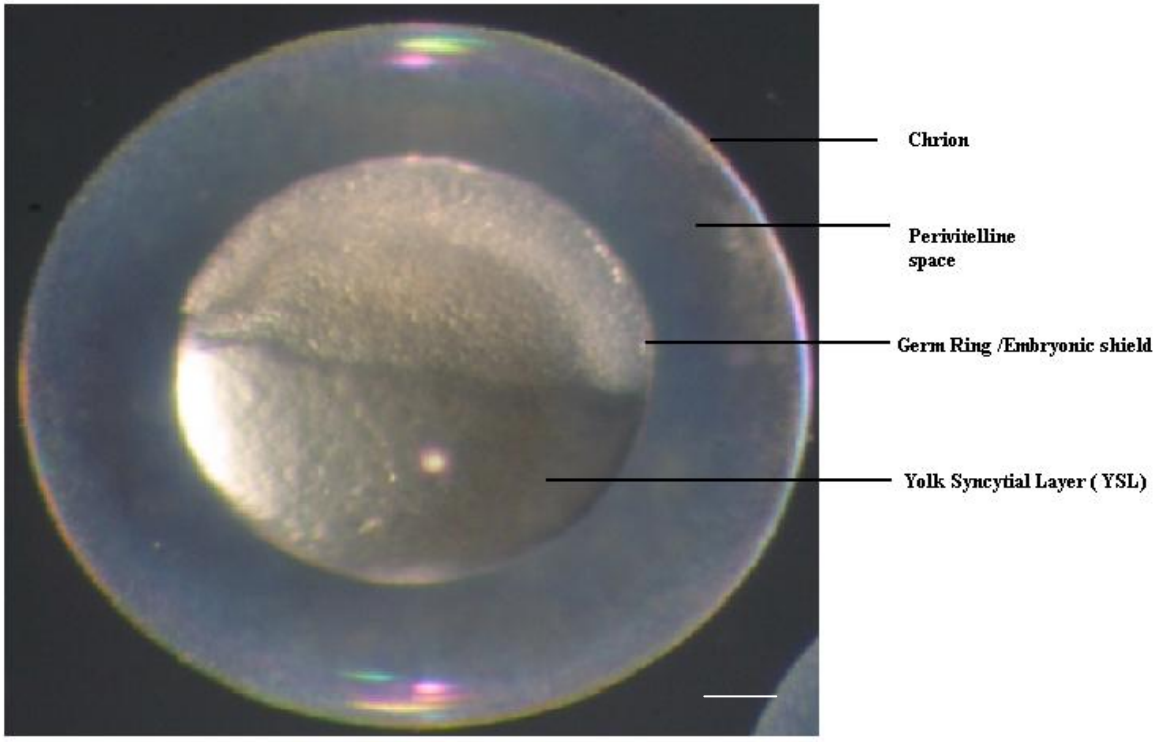


Fig. 1.4 50% Epiboly stage zebrafish embryo (Scale 1 cm= 100 µm).

In freshly laid eggs, cytoplasm and yolk are mixed and egg is surrounded by a transparent chorion which swells and lifts away from the egg on contact with water. The animal and vegetal pole axis is present during oogenesis and sperm can enter the egg only at the future animal pole through the micropyle, in a sperm impermeable chorion (Wolenski and Hart 1987). After fertilization embryonic development starts. The summary of the stages involved in the embryo development are given in Table 1.1 and Fig 1.5.

Table 1.1 Zebrafish (*Danio rerio*) embryo developmental stages (Kimmel et al. 1995)

Period / Stages	**Duration (hpf)**	**Development**
Zygote 1- Cell	0 – 0.2	Segregation of polar body/completion of second meiotic division within 5 minutes. Cytoplasm stream toward animal pole to establish animal (blastodisc) and vegetal (yolk mass) asymmetry.
Cleavage 2 cell – 64 cells	0.75 - 2	Cleavage starts rapidly every 15 minutes in synchronous fashion. Meroblastic, partial early cleavages leave connection to yolk cell. At 64 cell stage, three tiers of blastomers located on top of yolk cell.
Blastula 128-cell - 30% epiboly	2.25 - 4.7	Blastoderm sits high on yolk cell, constriction, where marginal cells meet yolk syncytial layer (YSL). Animal vegetal axis of blastoderm flattens, marginal constriction disappears and blastula has smoothly ellipsoidal shape. Continued animal-vegetal pole flattening of blastoderm resulting spherical shape of blastula. YSL bulges up toward animal pole. Marginal of blastoderm at about 30% of the distance between animal and vegetal pole.
Gastrula 50% epiboly – tail bud	5.3 – 10	Marginal of blastoderm at about 50% of distance between animal and vegetal poles. Onset of hypoblast formation and germ ring visible around margin. Convergence movements form a dorsal thickening the embryonic shield. Epiblast and hypoblast can be clearly distinguished. Embryonic axis

		extends on dorsal side; prechordal plate extends just past animal pole. Tail bud is prominent and notochord primordium distinct from neural keel.
Segmentation 1 somite – 26 somites	10.5 – 22	The somitic furrow will be the boundary between somite one and two. Somites develop at a continue speed about every 30 minutes. During this period, neurulation is completed, organogenesis is started and embryonic axis extends and straightens off the yolk.
Pharyngula Prim 5 – Prim 25	24 – 48	Prim5, early pigmentation, heartbeat. Prim 15 early touch/escape reflexes, first aortic arch. Prim 25 strong circulations through caudal artery and vein extend into tail. Pectoral fin bud's height about equal to width at its base. Xanthophores, iridophores appear mandibular and hyoid arch visible.
Hatching	48 – 72	Pectoral fin develops. Second aortic arch and branchial arches 1-4 form. Competition of morphogenesis of primary organ systems. Cartilage development.
Early larva	72- 120	First bone (cleithrum) forms, the swim bladder initiates to inflate and digestive tract fully differentiates. The embryo begins to swim. End of the day 4 and day 5 larvae is fully functional with food seeking and active avoidance behaviour.

hpf: hours post fertilization

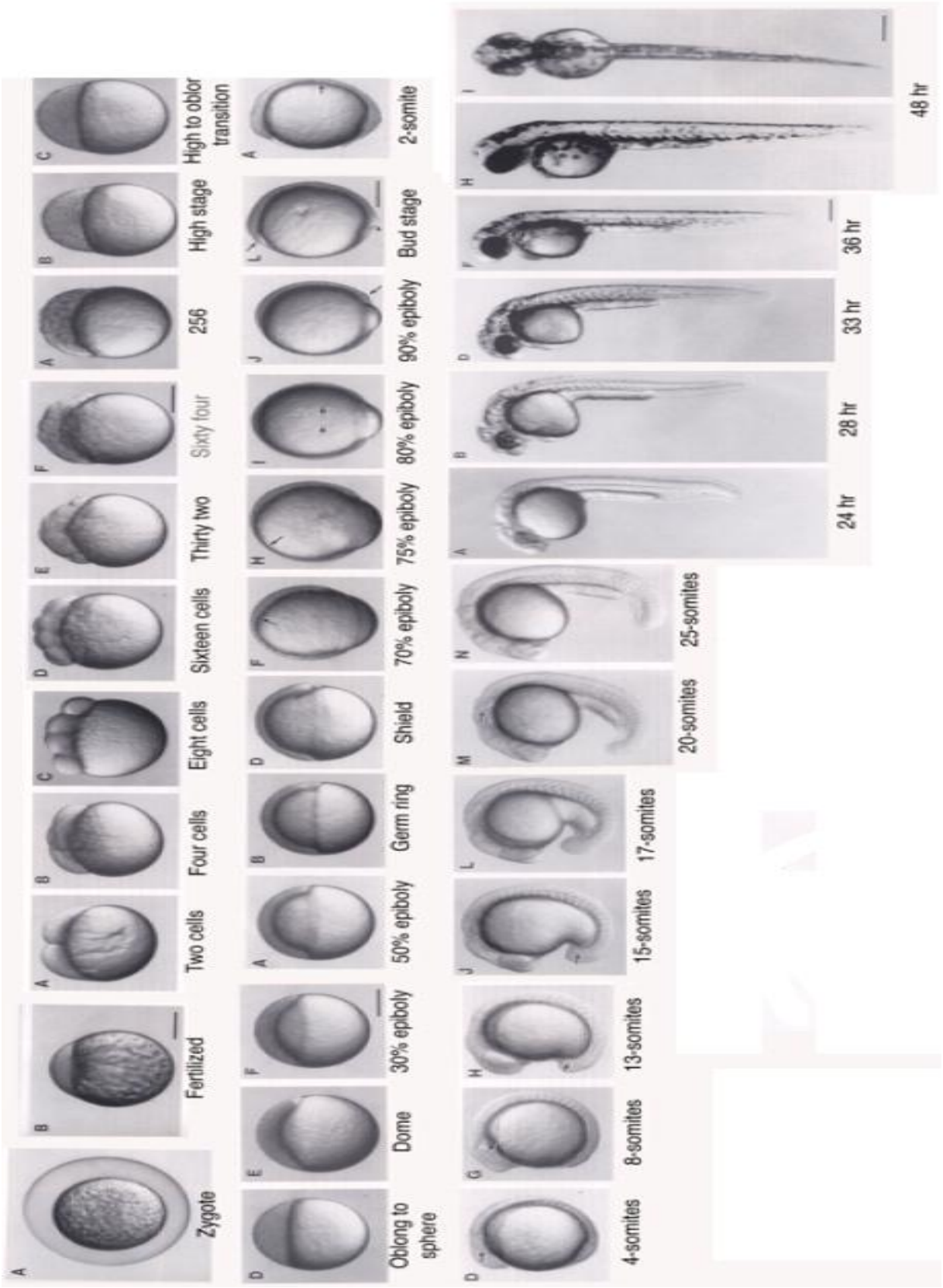


Fig. 1.5 Developmental stages of zebrafish (*Danio rerio*) embryo. The Figures show the development from zygote stage to hatching stages (Kimmel et al. 1995).

1.4 Current status in aquatic species gamete cryopreservation

Cryopreservation of germplasm of aquatic species offers many advantages in the field of aquaculture, biomedicine and conservation. Successful cryopreservation of fish gametes, eggs and embryos will offer new commercial possibilities, allowing unlimited production of fry and potentially more robust and better conditioned fish as required inn fish farming. Germplasm cryopreservation also provides a secure *ex-situ* method for preserving genome of endangered species, in diversity high enough to reconstruct stable population by facilitating the storage of gametes in gene banks. Due to their small size of genome, relative ease of analysis and sequencing, zebrafish is also an ideal model for studies on vertebrate development and human disease. Studies on zebrafish genome revealed close relationship with human genome and resemblance with disease developing human gene (Barbazuk et al. 2000, A. Dodd et al. 2000).

1.4.1 Spermatozoa cryopreservation

Knowledge of the differences between mammalian and fish sperm is important in designing cryopreservation protocol for fish species. The differences are (i) Most fish sperm do not have acrosomes like in mammals, therefore acrosome reaction preservation is not an issue for fish sperm cryopreservation; (ii) Most mammalian sperm have mitochondrial sheet active enough to ensure continuous motility between ejaculation and fertilization, while in fish, mitochondria number is less. Once activated, ATP consumes rapidly and fish sperm stay motile for short term (2-3 min for fresh water fish and 20 – 25 min for marine species); (iii) the activation signal for fish sperm motility is osmotic change of external environment. Fish sperm are activated when osmolarity of the seminal plasma lowers; (iv) Plasma membrane composition is influenced by habitat temperature of a species. Due to high body temperature of mammals, their plasma membrane lipids are less unsaturated and lipid phase transition occurs at high temperature. As a result, mammalian sperms are more sensitive to chilling injury (Zhang 2004). Cryopreservation

protocols for fish sperm have been developed for over 200 fish species and successful cryopreservation of fish sperm depends on many factors (Zhang 2004) such as sperm quality, extender composition, type and concentration of cryoprotectants, equilibrium period and temperature, freezing rate, thawing and post thaw handling and viability assessment of cryopreserved sperm. Following cryopreservation, the post thaw viability is normally assessed using the following indicators. In order to fertilize eggs, spermatozoa must conserve plasma membrane integrity and osmotic control. Moreover, spermatozoa need to maintain their moving capacity when released into the water in order to reach the oocyte. Spermatozoa motility is crucial and depends on the integrity of different aspects of the cell, such as mitochondria status, ATP production, plasma membrane channel activity and flagellar structure. DNA integrity is also important in order to obtain successful embryo development.

1.4.2 Oocytes cryopreservation

Although cryopreservation of spermatozoa of many fish species has been successfully carried out, maternal genome cryopreservation is also important as some genetic factors are only inherited maternally by the oocytes cytoplasm such as mitochondrial DNA and some mRNA that determine the early stages of embryonic development. Cryopreservation of immature and mature oocytes is still a challenge due to their structural limitations and sensitivity to chilling. Zebrafish oocytes has five stages (Selman et al. 1993) (i) stage I (primary growth), (ii) stage II (cortical alveoli), (iii) stage III (vitellogenic), (iv) stage IV (maturation) and (v) stage V (mature eggs). Each stage is different in terms of protein content, lipid distribution, membrane composition and organelle organization. Fish oocytes are larger mammals in size (stage III, IV and V) and contain large amount of yolk, which is consumed in vitellogenesis in order to provide nutrient for the development of the embryo. Yolk has been indicated to be associated with high chilling sensitivity of zebrafish oocytes (Pearl and Arav 2000). It has also been

demonstrated that early stage zebrafish oocytes (stage I and II) are less sensitive to chilling than later stage oocytes (stage III and V) and chilling sensitivity may be associated with lipid phase transition (Isayeva et al. 2004, Plachinta et al. 2004b, Tsai et al. 2009).

1.4.3 Embryo cryopreservation

Embryo cryopreservation is important for preserving both maternal and paternal germplasm as well as for breeding line regeneration or proliferation. Efforts for cryopreservation of fish embryos have been made in the last two decades; however their structural limitations and high chilling sensitivity have hindered the successful protocol development. There are several problems associated with fish embryo cryopreservation which are summarised below.

1.4.3.1 Large size

Size of cells or tissues is an important factor for successful cryopreservation. The fertilized fish eggs are larger in size (< 1 mm in diameter) (Zhang 2004) when compared to mammalian eggs (7 – 150 µm in diameter) and invertebrate eggs (< 300 µm in diameter) (Fuller et al. 2004). The large size of fish embryos results in a very low surface area-to-volume ratio and reduce the rate at which cryoprotectant can move into and out of embryos during cryopreservation (Mazur 1984).

1.4.3.2 Complex membrane systems and low permeability

Fish embryos have very complex membrane systems with two distinct membranes, the outer chorionic membrane and inner plasma (vitelline) membrane. Chorion is further divided into three distinct zones; an outer, electro-dense zone containing pore canals rich in polysaccharides, a middle fibrillar zone and inner low electron density zone rich in protein. This complex structure may play role in diffusive exchange of gases along with providing physical protection (Grierson and Neville 1981). It also plays a role as flexible

filter for transport of some chemicals and protect against microorganisms (Schoots et al. 1982). The plasma membrane is composed of actin containing cytoskeleton that functions to maintain the shape of egg and its surface specialization (Hart and Collins 1991). Studies on plasma membrane of zebrafish demonstrated low permeability to water and most cryoprotectant (Zhang and Rawson 1996a, Zhang and Rawson 1996b). Studies in the same species also showed that in addition to chorion and plasma membrane, a third layer develops following fertilization called yolk syncytial layer (Kimmel and Law 1985). Hagedorn et. al. (1996) has reported that the yolk syncytial layer is responsible for observed low cryoprotectant permeability of zebrafish embryos. The plasma membrane and yolk syncytial layer are believed to be the main permeability barriers to water and cryoprotectant movements (Hagedorn et al. 1996, Zhang and Rawson 1996b).

1.4.3.3 High chilling sensitivity

Stage dependant chilling sensitivity is demonstrated in zebrafish embryos, where developmental stage beyond 50% are less sensitive to chilling than earlier stages (Zhang and Rawson 1995). However, chilling sensitivity increases rapidly when they are subjected to subzero temperatures. The chilling sensitivity may be related to changes in cells and tissue types, number of cells, effectiveness of cell repair mechanism and enzymatic reaction (Zhang 2004). It is also hypothesized that high chilling sensitivity may be due to loss of synchrony of coupled reactions involved in embryo development (Mazur et al. 1992). Studies in mammalian embryos also demonstrated that large amount of intraembryonic lipids is responsible for high chilling sensitivity (Nagashima et al. 1994).

1.4.3.4 Two compartment nature of the fish embryos with high yolk content

Dechorinated embryos have two compartments: the blastoderm and yolk. During cryopreservation, the yolk probably acts as an independent compartment and responds

osmotically in a manner analogue to cellular cytoplasm. A single protocol for cryoprotectant permeation and osmotic dehydration of both the yolk and cells of the embryos is difficult due to the distinctive nature of the two compartments (Rall 1991).

1.4.4 Studies on the effect of chilling on fish embryos

Development of protocols for short term storage of fish embryos under chilled condition has important applications in aquaculture such as facilitating transportation of materials for genetic improvement programmes. However, fish embryos have been reported to be chilling sensitive (Zhang et al. 2003, Zhang and Rawson 1995). Stage dependant chilling sensitivity has also been reported and the later stages are less sensitive to chilling than earlier stages and chilling sensitivity increases with decreased temperature (Zhang and Rawson 1995). Cryoprotectant has been used to protect cell from chilling injury in zebrafish embryos and some success have been achieved in later stage zebrafish embryos (heartbeat stage) (Zhang and Rawson 1995), however information on the effect of chilling on early stage embryos is limited. Chilling of early stage embryos has important applications in aquaculture as it allows the synchronization of the development of the embryos collected from different spawning events (Lahnsteiner 2008). It has been demonstrated that zebrafish heartbeat embryos can tolerate 10 h chilling at 0°C. Same study also showed that sucrose and trehalose enhanced embryo chilling tolerance (Zhang and Rawson 1995). Study in pacu (*Piaractus mesopotamicus*) embryos showed successful storage of embryos at subzero temperature for 12 h in the presence of cryoprotectant (Fornari et al. 2012). Cryoprotectant has been shown to protect cells from chilling injury in many fish species (Vuthiphandchai et al. 2005, Zhang et al. 2012, Zhang and Rawson 1995, Ahammad et al. 2002). It has also been shown that early stages are more sensitive to cryoprotectant toxicity than later stages (Zhang and Rawson 1995, Chao et al. 1994, Valdez Jr et al. 2005). Cryopreservation can also cause changes in genetic material (Park et al. 2006, Takagi et al. 1996, Ahmad et al. 2010). There have been very few reports on

the effect of chilling and cryopreservation on genome in fish species. It has been shown that cryopreservation can cause mitochondrial DNA damage and increased mutation frequency in frozen blastomeres when suboptimum freezing protocol was used (Kopeika et al. 2005). Studies in zebrafish also showed that chilling of embryos at 0°C altered normal pattern of *pax* gene expression (Lin et al. 2009b). There has been no report on the effect of chilling on gene and protein expression in fish embryos. Information in this areas is important for both aquaculture and biomedicine applications.

1.5 Zebrafish and human genes

The full understanding of the role of human genes requires the use of animal models where gene functions can be examined. Understanding relationships between zebrafish and human genomes helps to identify roles for human genes. It also helps to identify genes responsible for human disease (Brownlie et al. 1998). Studies showed that 80% of zebrafish genes and EFTs (expressed sequence tags) are similar to human (Barbazuk et al. 2000). For many chromosomal loci, the synteny is obvious between zebrafish and human (Postlethwait et al. 1998). The present study is focused on hox gene family and sox gene family. Hox and sox genes are important developmental genes not only for zebrafish embryo developmental but also for human and mouse embryonic development. Hox gene functions are associated with the development of basic structure and orientation (anterior posterior development) of the embryo while sox genes have multiple functions i.e. act as transcription factor and regulate several gene regulation processes. They also play key roles in sex determination and nervous system development during zebrafish embryo development. Due to their important functions, these groups of genes serve as good markers for evaluating the effect of chilling in the present study as any changes in genes or proteins could have major impact on the development of the embryos. In order to evaluate the effect of chilling on gene and

protein expressions, it is important to understand the mechanisms of the functions of these genes.

Certain hox genes contain a DNA sequence called Homeobox which acts as a transcription factor and these kinds of hox genes as a whole are classified as Homeobox gene family.

1.5.1 Homeobox gene family

Homeobox genes are a family of regulatory genes containing a common 183 nucleotide sequence (homeobox) and the coding for specific nuclear protein (homeoproteins) that act as a transcription factor. They are usually found in higher animals including mammals and in human. Homeobox is highly conserved and is generally located in the exon nearest the 3' end of the genes in which it is found, close to the intron/exon boundary. The entire sequence share one common open reading frame encoding a protein domain called "homeodomain" (Gehring and Hiromi 1986). Homeodomain (HD) is responsible for recognition and binding of sequence specific DNA motifs (McGinnis and Krumlauf 1992). The specificity of binding allows homeoproteins to activate or repress the expression of batteries of downstream target genes (Levine and Hoey 1988). Different homeobox gene families encode different types or class of proteins (Duboule 1994). Homeobox genes account for more than 0.1% of the vertebrate genome (Stein et al. 1996).

1.5.1.1 Hox genes

The hox genes were first characterized in *Drosophila melanogaster*, where eight linked *Antennapedia* class Homeobox genes make up the homeotic complex. The zebrafish has 48 hox genes arranged over seven clusters (Fig 1.6) as compared to 39 genes organized in human (Acampora et al. 1989) or in mice (McGinnis and Krumlauf 1992, Zeltser et al. 1996) in four clusters or loci. These genes encode transcription factors that are

characterized by their role in conferral of segmental identity along the anteroposterior (A-P) axis of the body. The hox genes are arranged in genomic clusters that are strikingly collinear with their spatial and temporal expression patterns. Clusters are located at the 'anterior' end, are expressed earlier in development and more anteriorly along the main body axis, whereas at the other end of a cluster are the 'posterior' genes, which are expressed later in development and in more distal portion of the body. Present study focused on gene expression of *hoxb1b, hoxb6b, hoxc6a* and *hoxc8a* in early developmental stages of zebrafish embryo.

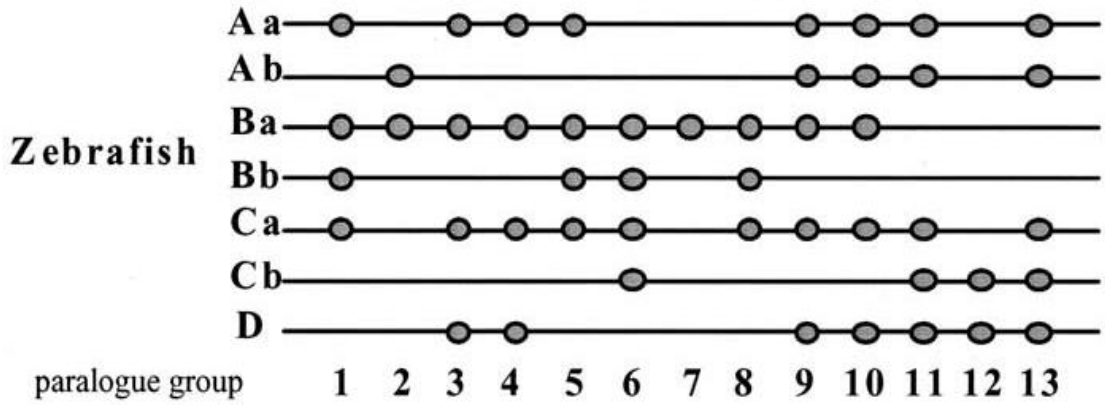


Fig 1.6 Schematic representation of hox cluster organization in zebrafish. There are total of seven hox clusters in the zebrafish i.e. Aa, Ab, Ba, Bb, Ca, Cb and D. Genes are named according to paralogue group and position (Jozefowicz et al. 2003).

1.5.1.2 Roles of hox genes in embryo development

Clustered Hox genes encode a conserved family of transcription factor implicated in conferring regional identity along anteroposterior (AP) axis of all bilateral animal embryos (McGinnis and Krumlauf 1992, de Rosa et al. 1999). *Hoxb1b* and its paralogue both play important role in the development of hindbrain. The hindbrain is subdivided along its AP length during early development into transient array of segments termed rhombomeres (r1–r7, from A to P). Rhombomeric organization allows establishment of

specific segmental identities, which provides proper neuronal organization in both hindbrain and its periphery (Lumsden and Krumlauf 1996). During early stage of gastrulation to hindbrain developmental stage in mouse, *hoxa1* and *hoxb1* are expressed together with an identical anterior expression limit at the presumptive r3/4 boundary (Wilkinson et al. 1989, Frohman et al. 1990, Murphy and Hill 1991, Barrow et al. 2000). *Hoxb1* expression is stably maintained in r4 and it is gradually lost from r5 and r6 to leave impression of *hoxb1* expression. This r4 *hoxb1* domain is maintained by an auto-regulatory positive feedback mechanism which is dependent on three defined hox/pbx binding sites upstream of *Hoxb1* (Pöpperl et al. 1995). A study revealed that *hoxa1* and *hoxb1* both play divergent roles in patterning the hindbrain. Loss of *hoxb1* function results in major alteration to the r4-derived facial (VIIth) motoneurons as *hoxb1* is important to confer r4 identity (Goddard et al. 1996, Studer et al. 1996, Gaufo et al. 2000). The zebrafish ortholog of mouse *hoxa1* and Zebrafish *hoxa1a* is not expressed in presumptive r4 and cannot play role in early patterning of this hindbrain territory (McClintock et al. 2001, Shih et al. 2001). The *hoxb1* duplicate genes, *hoxb1*a and *hoxb1*b, are both expressed in rhombomere 4 of the hindbrain (Alexandre et al. 1996, Prince et al. 1998). *Hoxb1*a and *hoxb1*b have expression profile similar to those of mouse *Hoxb1* and *Hoxa1* respectively. Zebrafish *hoxb1b* also have capacity to re-pattern r2 to an r4 phenotype (McClintock et al. 2001).

Hoxb6b is a member of the Antp Homeobox family and encodes a protein with Homeobox DNA binding domain. It is a member of cluster of Homeobox B genes located on chromosome 17 in human (Acampora et al. 1989), on chromosomes 12 in Zebrafish (Jozefowicz et al. 2003) and on chromosome 11 in mouse (McGinnis and Krumlauf 1992). They encoded protein functions as a sequence specific transcription factor that is involved in development, including lung and skin, and localized to both the nucleus and cytoplasm. Study showed that altered expression of this gene or a change in subcellular

localization of its protein is associated with some cases of acute myeloid leukaemia and colorectal cancer (Giampaolo et al. 2002). Experiments in human showed that *HoxB6* protein is expressed in the suprabasal layer of early developing epidermis and throughout the upper layer of late foetal embryos and adult human skin. *HoxB6* signal mainly express in cytoplasm throughout foetal epidermis development of embryo and substantially expressed in normal adult skin. *Hoxb6* protein is also partially nuclear in hyperproliferative skin condition, but appears to be cytoplasmic in basal and squamous cell carcinomas (Kömüves et al. 2000).

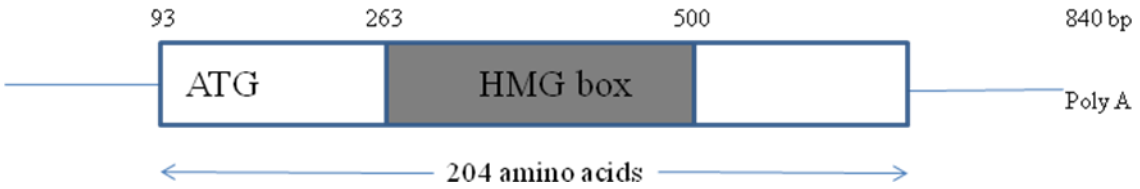
Burke *et. al* (1995) demonstrated that the anterior limit of expression of specific hox genes correlates with particular AP regional landmarks in a variety of vertebrate species, rather than with specific enumerated segments. In chick and mouse somites stages, anterior limit of *hoxc6* expression is linked with transition from cervical type vertebrae to thoracic type vertebrae and with forelimb/branchial plexus region. Expression of zebrafish *hoxc6* homologs relative to the forelimb/branchial plexus region and anterior vertebral type (Prince et al. 1998) is consistent with mouse and chick. *Hoxc8a* is identified as retinoic acid (RA) induced gene, RA is biologically active lipid mediator with important roles controlling cell fate and differentiation (Jette et al. 2004).

1.5.2 SOX genes and protein family

The SOX (**S**RY- related HMG b**ox**) genes were initially identified through their homology to the HMG box of SRY. SOX family is a group of proteins which appear to regulate cell fate decision during embryogenesis by functioning as transcriptional factor and architectural components of chromatin. The SOX genes belongs to large group of genes in which DNA binding domain (Fig 1.7a) is called high mobility group (HMG) box (Laudet et al. 1993). The SOX factor comprise a novel group of proteins characterized by

presence of *sry* box, a 79 amino acid motif that encodes an HMG-type DNA binding domain.

(a)



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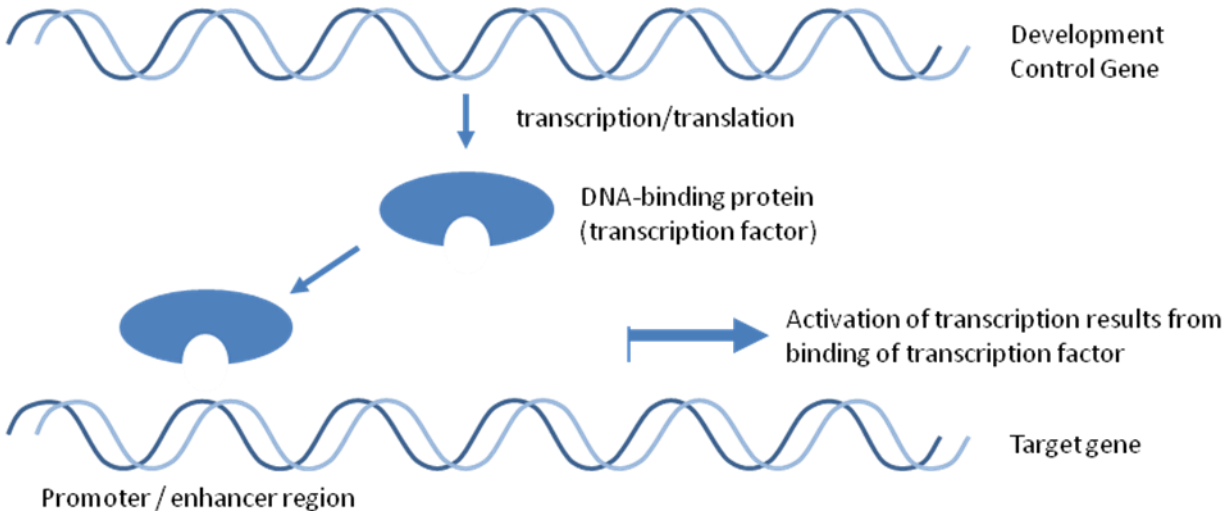


Fig 1.7 (a) Structure of the SRY Gene. The SRY gene has open reading frame (ORF) which contains a 79 amino acid HMG box (Clepet et al. 1993). (b) DNA binding of sox genes as transcription factor.

The SOX family falls into subclass of HMG box proteins, the member of which show highly restricted tissue distribution and bind specific sequence at high affinities (Ner 1992). HMG proteins have been classified into two major subgroups: according to sequence specificity of the DNA- binding (Fig 1.7b) and the number of HMG DNA binding domain within a single protein (Laudet et al. 1993). The first group comprises of

HMG box proteins which contain more than one DNA binding domain and bind preferentially to bend DNA and having a general affinity for binding DNA independent of its sequence. HMG non-histone chromatin associated proteins HMG1, HMG2, ubiquitous factor (UBF) and mitochondrial transcription factor (MT-TF1) falls under this group (Jantzen et al. 1990, Grosschedl et al. 1994). The second subgroup of proteins contains only a single HMG box, shows highly restricted expression pattern and may bind to prestructured DNA with little or no specificity. Sox1, *sox2*, sox6 and T cell factor (TCF) fall into this group of proteins. HMG box protein or genes further categorized into families on the basis of groups which share a high degree of homology within HMG box. The sox family of proteins is defined by an HMG box which has at least 50% sequence identity with the founding member of this group, mouse *sry* gene. Molecular properties of sox proteins involve DNA binding, DNA bending, protein interaction, nuclear transport, transactivation and transrepression, homodimerization and post transcriptional modification (Lefebvre et al. 2007).

1.5.2.1 Roles of sox in embryo development

Sox genes play important roles in many developmental and physiological processes *in vivo*. The major function of these genes involve sex determination, stem cell development in embryo, neurogenesis, neural crest development, skeletogenesis, hematopoiesis, endoderm and hair development, cardiogenesis and angiogenesis (Lefebvre et al. 2007). S*ry* and *sox9* were found to play important role in specifying male differentiation (Koopman 2005). In mammals, *sry* gene induced the differentiation of sertoli cells and testis development. *Sox9* is expressed shortly after *sry* in pre-sertoli cells and both genes are required for male determination. Many sox genes are expressed in the development of adult nervous system. The soxB1 genes *sox1*, *sox2* and *sox3* are expressed in nervous system and have redundant roles in maintaining the broad development potential and identity of neural stem cells. Their inhibition in the vertebrate embryo results in

premature differentiation of neural precursors and their over expression results in inhibition of neurogenesis (Kishi et al. 2000, Crémazy et al. 2000, Overton et al. 2002, Avilion et al. 2003, Graham et al. 2003). SoxB, soxC, soxD and soxE genes play crucial roles in this process (Hong and Saint-Jeannet 2005). SoxC and soxE are expressed during skeletogeneis. Overt skeletogeneis is initiated when messencymal cells commit to the chondrocyte fate and undergo early steps of differentiation (Lefebvre and Smits 2005). Sox9, sox5 and sox6 genes have shown to have roles in induction of chondrocyte differentiation of messencymal cells (Lefebvre et al. 1997). Sox genes are also important in haematopoiesis (van de Wetering et al. 1993). Sox4 is highly expressed in the thymus and promotes proB lymphocyte expansion and T lymphocyte differentiation (Schilham et al. 1997, Schilham et al. 1996).

Sox2 encodes a transcriptional factor and well known for its role in maintaining pluripotent stem cells population and differentiation during early embryonic development. This protein functions cell autonomously in both epiblast and extraembhryonic ectoderm in early embryo to maintain the pluripotency of the stem cell types (Avilion et al. 2003). *Sox2* acts in combination with POU domain protein Oct3/4 to directly activate essential genes in embryonic stem cells, such as *Fgf4* (Yuan et al. 1995). It was also shown that this combinations of c-Myc and Klf4, a quartet of transcription factor, , are sufficient to induce pluripotent stem cell properties in embryonic and adult fibroblast (Takahashi and Yamanaka 2006). Studies in human showed that heterozygous mutation of sox genes resulted in complex syndrome that reflects essential roles of *sox2* in multiple processes at later stages of embryo development (Kelberman et al. 2006, Fantes et al. 2003). *Sox2* is one of the first regulators of early specification of neuroctoderm during vertebrate gastrulation (Graham et al. 2003, Kishi et al. 2000). *Sox2* is also required for maintenance and regeneration of hair cells in the zebrafish inner ear

(Millimaki et al. 2010). Studies in mice showed that *sox2* is necessary for normal development of taste buds (Okubo et al. 2006).

Sox3 is the most common transcriptional factor in neural development of vertebrates (Rex et al. 1997, Zhang et al. 2004). *Sox3* also plays central role along with sox1, *sox2* in the maintenance of stem cells state of neural cells (Bylund et al. 2003, Graham et al. 2003). A study using gain-of-function and loss-of-function approach in zebrafish revealed that *sox3* regulates both neural fate and differentiation on both central and peripheral nervous system and for normal development of eye and ear (Dee et al. 2008). A study in chicken also showed the role of *sox3* in conjunction with Pax6, sox1 and *sox2* in the lens induction (Kamachi et al. 1998). Similar study in medaka found that ectopic *sox3* expression leads to ectopic expression of Pax6 and Eya1 in embryonic endoderm and causes ectopic lens and otic vesicle formation (Koster et al. 2000). *Sox19a* gene is unique to zebrafish and does not show any similarity with the sox sequence of any other species. *Sox19a* has also been suggested to have roles in development of central nervous system (Vriz et al. 1996).

1.6 Gene expression and analysis

Genes encode messenger RNA which translates into proteins. Expression of a particular gene can be evaluated by either RNA analysis or protein analysis.

1.6.1 Gene expression and protein expression

Gene expression is the process by which amino acid sequence or a gene transcribed into mRNA and mRNA translate into protein. This process is also called conversion of genotypic condition into phenotypes. The process includes transcription and translation (Fig 1.8) and is involved in cell differentiation, morphogenesis and varsity of any organism.

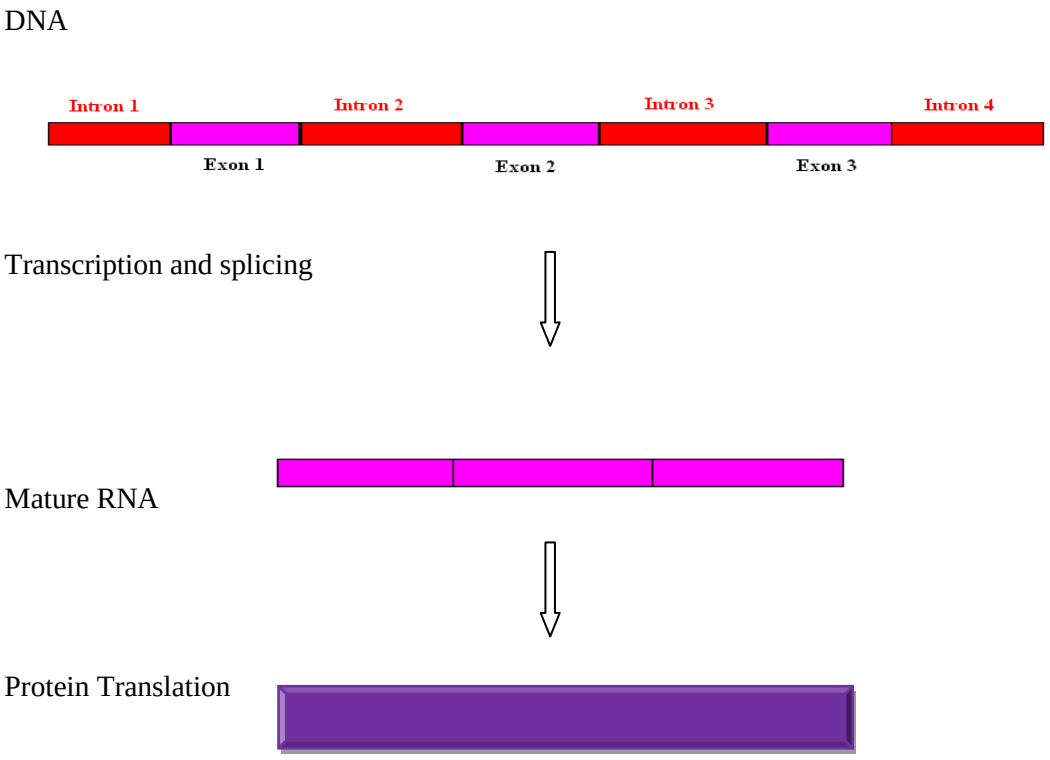


Fig 1.8 General overview of gene and protein expression

1.6.1.1 Transcription

DNA serves as the template for RNA synthesis. Major stages involved in the synthesis of m-RNA are transcription, RNA splicing (post transcription modification) and polyadenylation. The whole process occurs in nucleus of the cell. DNA dependant RNA polymerase II (Pol II, RNAP II) is the key enzyme, which binds to the complex of transcription factors and open DNA double helix. The RNA polymerase process to 3' – 5' direction and assemble ribonucleotides into the strand of RNA. Each nucleotide is inserted into growing RNA strand following base pairing rule (G-C, A–U). Synthesis of the RNA proceeds in the 5' – 3' direction. As the nucleotide triphosphate is bought in to add to the 3' end of the growing strand, two phosphates removed each time. When transcription is complete, the transcript is released from the DNA. In eukaryotes, primary transcript (pre mRNA) contains some non-coding sequence called "Introns". Therefore

pre mRNA undergoes post transcription modification which is also called as RNA splicing. The coding sequence for protein is termed as “Exons”. This process starts from “capping”. Cap is modified guanine (G) which is attached to the 5’ end of the pre mRNA. The cap protects the RNA from being degraded by enzymes and also serves as assembly point for proteins needed to recruit small subunits of the ribosome to begin translation. Step by step removal of introns and splicing of remaining exons is done by spliceosomes (complex of small nuclear RNAs – snRNAs and small ribonucleoprotein particles – snRNPs). Last step involves poly (A) tail synthesis. This is a stretch of adenine (A) nucleotides. When a special poly (A) attachment site in the pre mRNA emerges from RNAP II, the transcript is cut there and poly (A) tail is attached to the exposed 3’ which completes the RNA molecule. This process is also called as polyadenylation. The simplified process of transcription is illustrated in Fig 1.9.

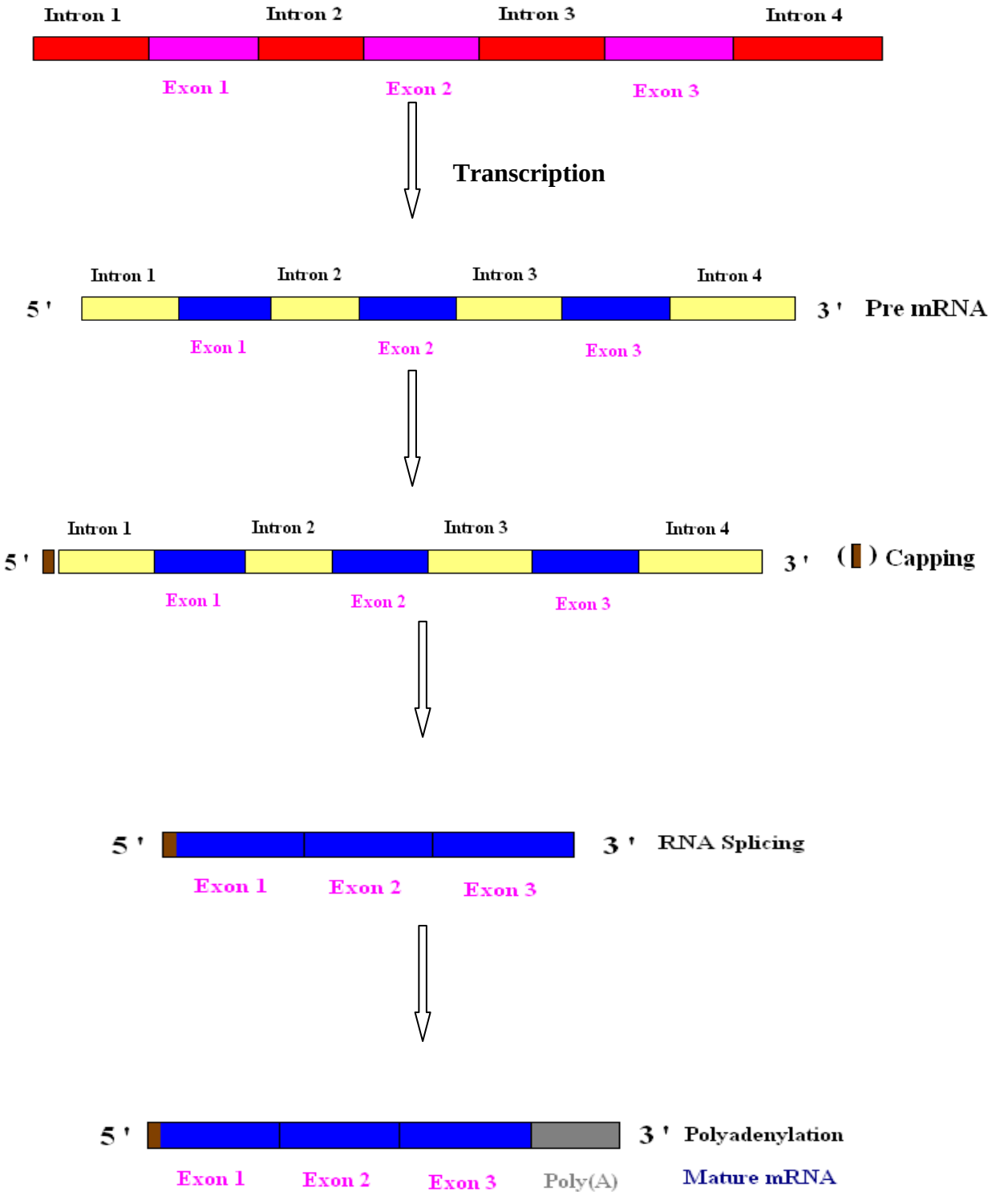


Fig.1.9 Steps involved in transcription. From DNA to mature mRNA. The process mainly involves pre mRNA synthesis, capping, splicing (post transcription modification) and polyadenylation.

1.6.1.2 Translation

A particular nucleotide sequence is a code for particular protein. A particular sequence of nucleotide in mRNA specifies a particular sequence of amino acid by the means of transfer RNA (tRNA). Each tRNA has sequence of three unpaired nucleotide - the "anticodon" which can bind with complementary triplet of nucleotide - the codon in an mRNA molecule. There are three steps involved in this process – initiation, elongation, termination. Ribosome provides the site of initiation and site for protein synthesis. Ribosome has two subunits. – The small subunit binds to the mRNA molecule. The large subunit has two sites: P site and A site. From the A site, new tRNA enters on ribosomes and from the P site tRNA leave ribosomes. The small subunit binds to the 5' site of the mRNA and proceeds towards 3' until it reads starts codon AUG. At this codon it binds with initiator tRNA. Initiator tRNA binds to the large subunit (P site) of the ribosomes. The preceding amino acid is covalently linked to the next amino acid with peptide bond. The initiator tRNA is released from the ribosomal P site and ribosomal moves one codon downstream. The tRNA which has anticodon arrives to A site. The end of translation occurs when ribosomal reaches one or more stop codon (UAG, UAG, and UGA). There is no tRNA molecule with anticodon. These are also called termination codon.

1.6.2 Gene expression analysis

There are several methods available to analyse gene expressions such as Northern blotting, SAGE (serial analysis of gene expression), DNA microarrays, polymerase chain reaction (PCR) and nuclease protection assays.

1.6.2.1 Nuclease protection assay

This assay is used to identify RNA molecule or a particular gene of interest. Oligonucleotides probe specific to gene of interest are used in this technique. The DNA probes are hybridized with RNA mixture/total RNA. DNA probe binds to mRNA / gene

of interest and form mRNA – DNA complex. Unhybridized probe and RNA are digested by nuclease specific for single stranded nucleic acids. Alkaline hydrolysis is then used to destroy the mRNA components from heteroduplexes, leaving intact probe in concentrations proportional to the amount of specific mRNA originally present. Remaining probe is analysed using chemiluminescent detection and quantification (Roberts et al. 2007). The limitation for this technique is it cannot analyse the entire genome for discovery studies.

1.6.2.2 Northern blotting

The northern blot technique is a preferred method for determination of transcript size and detection of spliced transcripts. The technique involves RNA separation using agarose gel electrophoresis, transfer to membrane and hybridization with labelled probes. There are limitations in using this technique for gene expression studies as quantitative expression is compromised due to RNA degradation and multiple probes cannot be used at same time. Northern blotting is the only technique provides information about mRNA size, alternative splicing and the integrity of RNA sample (Princivalle et al. 2012). Northern blot analysis revealed that freezing induces up regulation of fibrinogen synthesis genes in liver linked with ischemia resistance in wood frog in response to freezing survival (Storey 1999).

1.6.2.3 DNA microarray

DNA microarray allows rapid assessment of expression of many genes in parallel and helps to identify global expression pattern (Schena et al. 1995). Microarrays are usually glass or fibre chips contain thousands of different DNA clone or nucleotide probes. Each probe represents certain genes (mRNA transcripts). Microarray has been used to analyse global changes in gene expression across different treatment. Cell wall and structure

damage has been identified using this technique in yeast cells following cryopreservation (Odani et al. 2003).

1.6.2.4 Serial analysis of gene expression (SAGE)

SAGE is a technique which allows detailed analysis of thousands of transcripts in a cell. Two principles apply to this technique, a short oligonucleotide sequence defined by specific restriction endonuclease (anchoring enzyme, AE) at a fixed distance from the poly (A) tail can uniquely identify mRNA transcripts and the end-to-end connection of these short nucleotides allows multiple transcripts detection per sequencing reaction (Patino et al. 2002). There are certain limitations in using this technique. The tags generated during SAGE are extremely short (13-14 bp) sequences as it is difficult to investigate gene functions using short sequences (Yamamoto et al. 2001).

1.6.2.5 Polymerase chain reaction

Polymerase chain reaction is a unique technique for identifying gene expression. The enzymatic reactions are used to produce multiple copies of DNA or a particular gene sequence. The major components of the reactions are gene specific primers, dNTPs, *taq* polymerase and magnesium chloride (MgCl2).

The procedure involves three steps which are denaturation of the DNA template, annealing and extension. Initially the DNA strand or the starting template is denatured by heating and separated into two single stranded DNA. This step is called denaturation. The gene specific primer in the reaction is then bound to their complementary sequence according to normal base pairing rules (A-T, G-C) on the single stranded DNA strand. This step is called annealing in which primer anneal to the DNA strand. DNA polymerase then begins to add deoxynucleotides to the 3–OH group of primers, producing new double stranded DNA molecule. This step is called extension. The whole procedure is a cycle (Fig 1.10). At the next heating step, these double stranded new molecules are once

again denatured and each single stand provides a primer binding sites and acts as the template for further DNA synthesis (McPherson and Møller 2000). The successful amplification of DNA mainly depends on good primer design and selection of *Taq* enzyme, $MgCl_2$ concentration and template concentration.

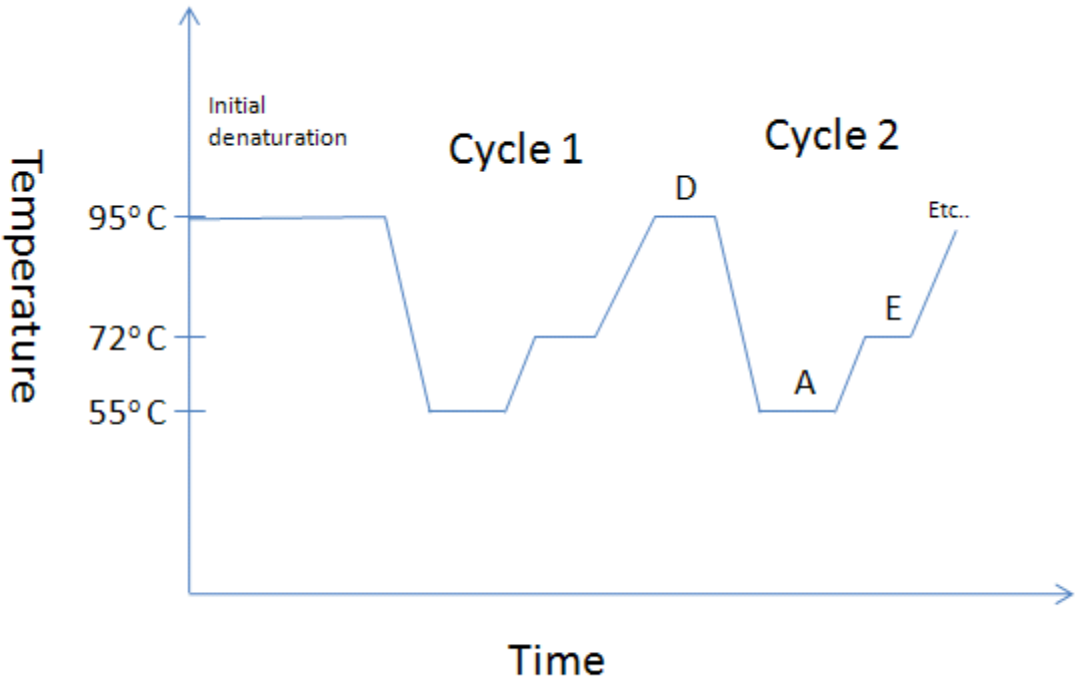


Fig 1.10 Temperature profile during PCR reaction. An initial denaturation step is followed by repetitive cycling through temperature denaturation of DNA molecule (D), annealing of primer to single strand DNA (A) and extension of primer (E) with the help of DNA polymerase and dNTPs (McPherson and Møller 2000).

1.6.2.5.1 PCR Primer design

Primer design is one of the most important factors for successful PCR and high yield of product. For the successful amplification, some factors need to be taken into account while designing primers: (1) Primer length: primer length should not be more than 18-22

bp; (2) Primer melting temperature: primer melting temperature should be in the range of 52° – 58° C; (3) Primer annealing temperature: primer annealing temperature should not be too high or too low. Primer annealing temperature can be calculated using formula Ta = (0.3 X Tm of primer) + (0.7 X Tm of product); (4) GC content: The GC content of the primer should be 40 – 60%. More than 3 G's or C's should be avoided in the last 5 bases; (5) Primary secondary structure: Avoid secondary structure (self complimentarily) particularly in 3' region to prevent primer dimer and (6) PCR product size: product size should be more than 1000bp and not cross axon-intron junction.

1.6.2.5.2 RT PCT (Reverse transcriptase PCR)

In gene expression studies, mRNA cannot be used as a template for PCR and this is why it is necessary to convert RNA into cDNA before amplification. In this process, RNA is converted into cDNA first which can be further used for PCR amplification. Reverse transcriptase enzyme is used to convert RNA into cDNA.

In the present study, real time PCR technique is used to study gene expression as this technique is more sensitive eg 10,000-to 100,000 fold more sensitive than Nuclease assay (Wang and Brown 1999) and 1000 fold more sensitive than northern blot (Malinen et al. 2003) assay.

1.6.2.6 Real Time PCR (Quantitative polymerase chain reaction- qPCR)

Real time PCR is the process of amplification of DNA in real time. In other words, copies of DNA produced during PCR cycling can be monitored in real time. For the gene expression studies, usually this process can be combined with reverse transcription step and this is called quantitative reverse transcriptase PCR (qRT-PCR). Real time PCR is a very sensitive method and can even detect a single copy of a specific transcript (Palmer et al. 2003). This assay can detect gene expression differences as small as 23% between samples (Gentle et al. 2001) and have lower coefficient of variation when compared to

band densitometry in northern blotting (44.9%) and probe hybridization (45.1%) (Schmittgen et al. 2000). Another advantage of using this technique is that it requires less RNA template as starting material than other methods for gene expression and produces relatively high throughput. Quantification of mRNA (analysis of gene expression) using real time PCR is a multi-step procedure consisting of RNA isolation, cDNA preparation, real time PCR data acquisition and data analysis (Fig 1.11).

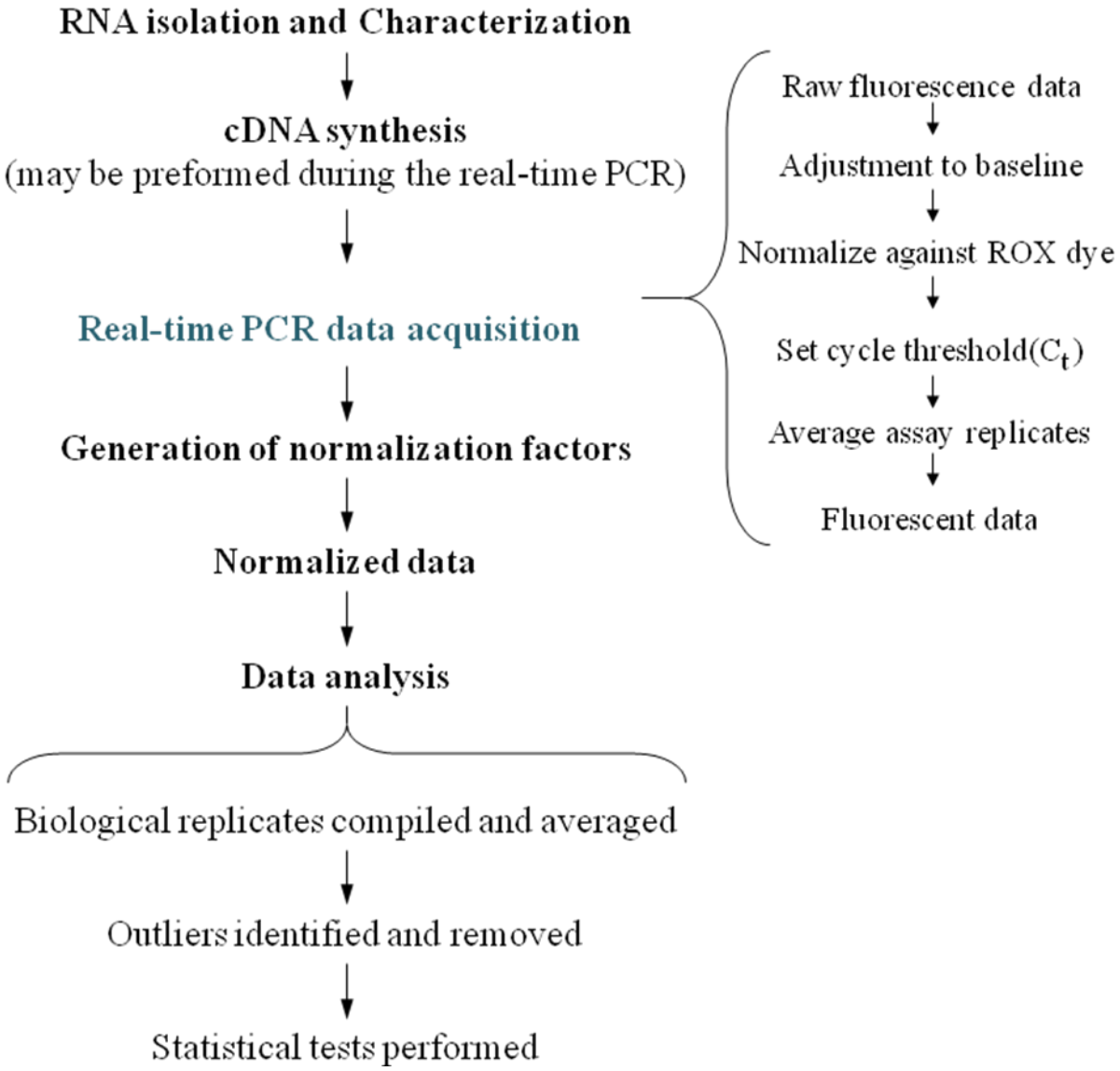


Fig 1.11 Standard procedure for quantification of mRNA using real time PCR. Main steps involved are RNA isolation and quantification, cDNA synthesis, real time data acquisition using Thermocycler machine. Sample fluoresce data is acquired by the machine and recorded onto accompanied software. Data analysis is carried out using different mathematical models in order to obtain quantitative results.

1.6.2.6.1 Detection chemistries

Different methods (assays) are available for determining the amount of PCR product present at the end of each PCR cycle, but they all follow the basic principle of measuring the amount of fluorescence associated with each newly synthesized DNA copy. The available methods are: DNA binding dyes assay (SYBR assays) , hybridization and hydrolysis probes, molecular beacons, scorpions, sunrise primers and LUX primers. Most of these techniques except SYBR assays require good knowledge of amplification transcript as the techniques involve design and optimisation of gene specialized probe which binds to complementary sequence of DNA during the annealing step. On the other hand, SYBR green I dye does not require any probes as it binds to double stranded DNA molecule directly and generate florescence which can be used to calculate quantitative data. In the present study, gene expression analysis is carried out using SYBR green I real time PCR assay.

1.6.2.6.2 DNA binding dyes (SYBR Green Assay)

SYBR green binds to all double stranded DNA via intercalation between adjacent base pair. The unbound DNA binding dye exhibits little or no florescence. DNA binding dyes emits florescence when they bound to double stranded DNA. As the PCR cycle progresses, DNA start to amplify and produce multiple copies of double stranded DNA. When monitored in real time, the increases in florescence signal can be observed during polymerisation step and decreases when DNA denatured. As a result, florescence measurements of every elongation step were performed in order to monitor the increasing amount of DNA (Fig 1.12). Increase in double stranded DNA is proportionally equal to increases emitted fluorescence (Wittwer et al. 1997).

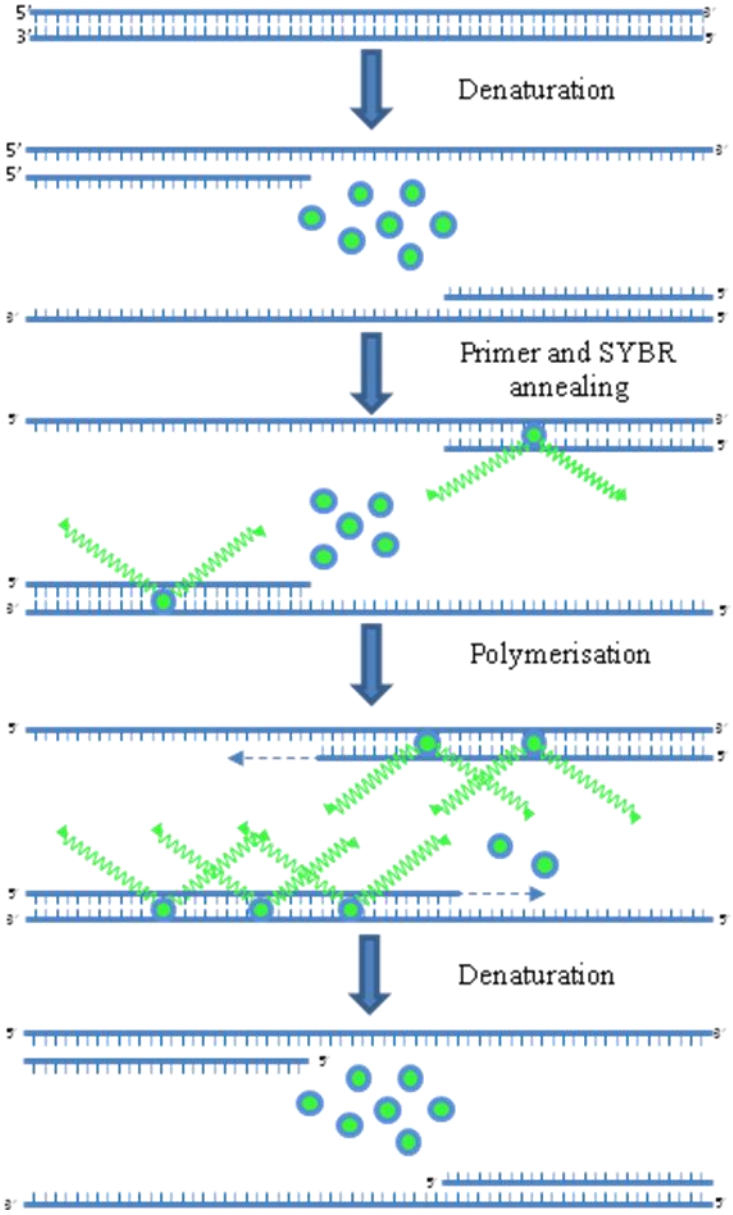


Fig 1.12 Steps involved in SYBR assay. Denaturation: Initially, upon heating, DNA strand denatured and free SYBR green dye in solution remained unattached and exhibit little or no fluorescence, this is often considered as background fluorescence. Annealing: At the annealing temperature, when primers bind to denatured DNA, a few dye molecules also bind with double-stranded DNA and excite upon light emission. Polymerisation: following annealing step, more and more SYBR green

molecule binds to newly formed double stranded DNA resulting increase in fluorescence which can be monitored in real time (Bustin 2000).

Due to the nature of this type of dye e.g. the ability in binding to all double stranded DNA, it is essential to design and use highly specific primer pair specific to the target DNA sequence to avoid generation of nonspecific product that would lead to false fluorescence signal and overestimation of target DNA. A post PCR dissociation curve (melting curve) (Fig 1.13) should be used in order to confirm that the fluorescence signal is generated from only target DNA and not from nonspecific PCR product (Ririe et al. 1997). PCR product can also be confirmed by performing agarose gel electrophoresis and by analysing target PCR product base pair (Lekanne Deprez et al. 2002).

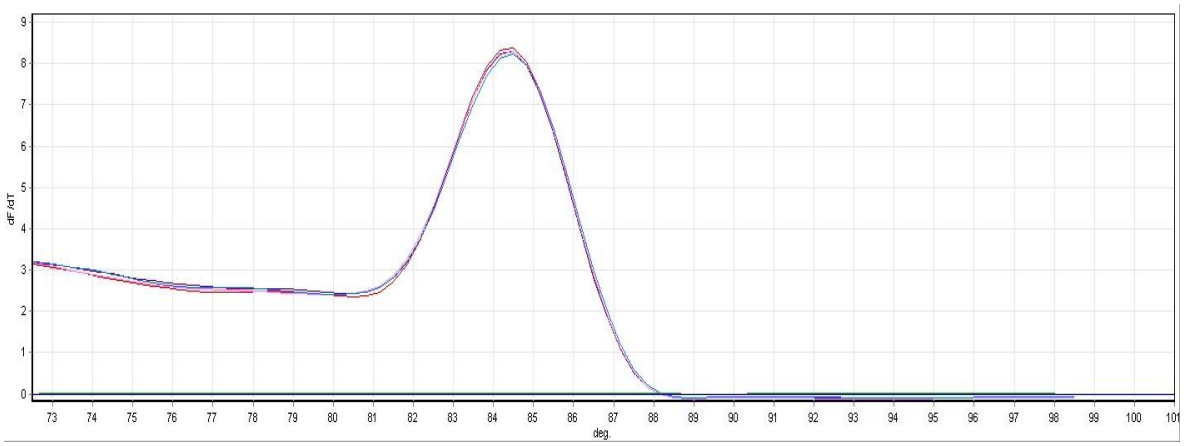


Fig 1.13 Dissociation curve (melting curve) for *sox2* gene produced using SYBR green assay. The graph was plotted for changes in florescence against increasing temperature. One single peak confirms the presence of *sox2* and the absence of any mispriming, non-specific product and primer dimer.

1.6.2.7 Quantification strategies in real time PCR

Real time PCR produces raw fluorescence data which needs to be analysed using certain strategies. There are mainly two methods (Pfaffl 2004) which can be performed based on sample size and experimental output. The level of gene expression is measured using

absolute quantification where PCR signal is converted to Ct values (copy number/cycle number) and concentration is evaluated using a calibration curve. The second method is called relative quantification where changes in level of expression are measured by comparing target gene with reference/internal control gene. In the present study, the relative quantification method is used for the quantification of gene transcript.

1.6.2.7 Absolute quantification

In this method, known concentration of oligonucleotides was run along with experimental sample and at the end of the run, calibration curve was obtained and transcript concentration was calculated. The calibration curve has to be thoroughly validated as the accuracy of absolute quantification depends upon accuracy of the standards. The calibration curve of standards can be produced using known concentrations of standard molecule e.g. RT PCR product genomic DNA (Yun et al. 2006), recombinant DNA (recDNA) (Pfaffl and Hageleit 2001) and commercially synthesized oligonucleotides (Vermeulen et al. 2009).

1.6.2.7.2 Relative quantification

Relative quantification determines the changes in gene transcript across samples and expresses these relative to the level of internal control. The reference gene is usually the housekeeping gene Housekeeping genes are responsible for basic cellular function and expressed relatively similar level across many types of cells. Quantification is based on expression levels of a target gene versus a reference gene and has been used to study physiological changes in gene expression level. There are mainly two mathematical models available to calculate mean normalised gene expression from relative quantification assay which are (1) without efficiency correction using equation below (Eqs. 1 & 2) and (2) With kinetic PCR efficiency correction (Eqs 3) (Pfaffl 2004).

$$R = 2^{-[\,\Delta CP\ sample - \Delta CP\ control\,]} \qquad \text{Eqs. 1}$$

$$R = 2^{-\Delta\Delta CP} \qquad \text{Eqs. 2}$$

$$ratio = \frac{(E_{\mathbf{target}})^{\Delta CP\,(\mathbf{target})\,(control - sample)}}{(E_{\mathbf{ref}})^{\Delta CP\,(\mathbf{ref})\,(control - sample)}} \qquad \text{Eqs. 3}$$

Fig 1.14 Equation to calculate relative quantification (Pfaffl 2004)

1.7 The present study

Cryopreservation can cause changes in genetic materials (Park et al. 2006, Takagi et al. 1996, Ahmad et al. 2010). There has been no report on the effect of chilling on gene and protein expression in fish embryos. Information in this area is important for both aquaculture and biomedicine applications. The aim of the present study was to determine whether chilling cause any changes in gene and protein expressions in zebrafish embryos. Hox and sox gene expressions in different stages of zebrafish embryos were studied first before studies on the effect of chilling and warming on gene expressions in zebrafish embryos. Investigations were also carried out on the effect of both short term and longer term chilling followed by warming on gene expressions with or without the presence of cryoprotectant. Studies were then carried out on the effect of chilling and warming on gene and protein expression. Details of these studies are provided below.

(a) Determination of *hox* and *sox* gene expression in different developmental stages of zebrafish (*Danio rerio*) embryos

The aim of this study was to investigate seven different genes for their potential use as markers for assessing the effect of chilling on gene and protein expression. Information obtained from this study formed the basis for further evaluation of the effect of chilling and warming on gene and protein expressions. Based on their importance in early embryonic development, *hox* (hoxb1b, *hoxb6b*, *hoxc6a* and *hoxc8a*) and *sox* (*sox2*, *sox3* and *sox19a*) gene expressions were investigated in early developmental stages of zebrafish embryos, the results from these studies are presented in Chapter 3.

(b) Studies on effect of short term chilling on *sox* gene expression in zebrafish embryos

This study was aimed to investigate the levels of expression of *sox* genes in zebrafish embryos following chilling at 0°C for 3 h and also following chilling and subsequent warming. mRNA was quantified to compare patterns of expression of three genes (*sox2*, *sox3* and *sox19a*) in fresh and chilled embryos using sensitive real time PCR. These results are presented in Chapter 4.

(c) Studies on the effect of chilling on gene and protein expression in zebrafish (*Danio rerio*) embryos in the presence of methanol (MeOH)

Cryoprotectant has been shown to protect cells from chilling injury in many fish species (Vuthiphandchai et al. 2005, Zhang et al. 2012, Zhang and Rawson 1995, Ahammad et al. 2002). Methanol has been widely used as a cryoprotectant in fish species to protect cells from freezing and chilling injury. This study was carried out to investigate potential impact of cryoprotectant on *sox* mRNA and protein levels in chilled zebrafish embryos. In this study, the patterns of expression of *sox* genes and proteins were compared in fresh

and chilled embryo in the presence of MeOH using real time PCR and western blotting respectively. The results are presented in Chapter 5.

(d) Studies on the effect of longer term chilling on gene and protein expressions in zebrafish (*Danio rerio*) embryo

Chilling storage of early stage embryos has important applications in aquaculture as it allows the synchronization of the development of the embryos collected from different spawning events (Lahnsteiner 2008). In this study, an 18 h chilling storage protocol was developed for the early stage zebrafish embryos. The protocol was then used to investigate the potential impact of chilling on mRNA and subsequent protein levels. mRNA levels and protein profile were investigated in fresh and chilled embryos using real time PCR and western blotting. The results are presented in Chapter 6.

CHAPTER 2 MATERIALS AND METHODS

2.1 Introduction

In the present study the effect of chilling on gene and protein expressions in zebrafish embryos was investigated with the following objectives: a) determination of *hox* and *sox* gene expressions in different developmental stages of zebrafish (*Danio rerio*) embryos, b) investigations into the effect of 3h chilling at 0°C on gene expressions in zebrafish embryos, c) investigations into the effect of 3h chilling at °0 C on gene and protein expressions in zebrafish embryos and d) identification of suitable cryoprotectant for 18 - 24 h chilling and its subsequent effect on gene and protein expressions.

All the experiments were performed at LIRANS Institute of Research In Applied Natural Science, University of Bedfordshire, UK.

2.2 General Methods

2.2.1 Zebrafish maintenance

Adult zebrafish (*Danio rerio*) of 12-14 weeks old were obtained from Aquascape Ltd., Birmingham, UK. They were maintained in 40 L glass fish tank (30 x 30 x 60 cm). The water was aerated and filtered using electric pump connected to upright funnel contained filter floss in 1 L beaker immersed in fish tank. The funnel and floss was covered by pebbles and small stones and the water temperature was maintained at 27±1° C. A 12 h light/12 h dark cycle was maintained using automatic timer switch. The water was replaced twice a week. Tap water was aged for 2 days to allow chlorine to evaporate before use.

An automatic water circulation system (Fig 2.1) was also used to maintain zebrafish. Each tank (35 x 17 x 26 cm) in this system held approximately 30 fish. The water was filtered through carbon filter located to the right of trickle tower. Water automatically

passed from refill tank from the sump tank. The Sump tank contained filter bags, the heaters and water pump and UV filters.

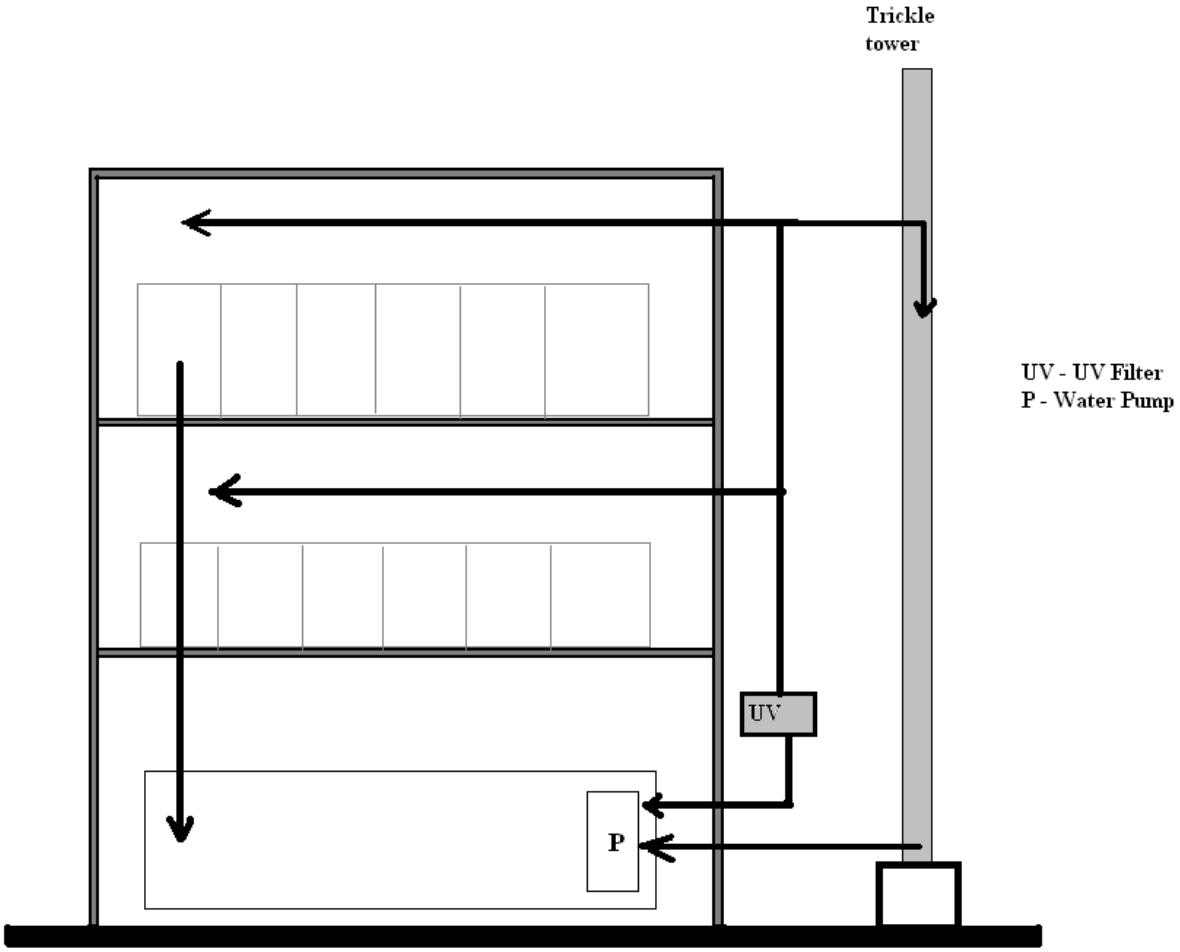


Fig 2.1 Automatic water circulating system at LIRANS.

2.2.2 Zebrafish breeding

The zebrafish were fed three times a day with TetraMin® (Tetra, Germany) flake food (ingredients: processed fish and fish derivatives, cereals, yeast, vegetable protein extracts, molluscs and crustaceans, oils and fats, derivatives of vegetable origin, algae, various sugars contains permitted colorants). The flaked food was given in such a way that it can be eaten by fish in 5 min to prevent overfeeding. Live brine shrimp was also fed in the afternoon. Live brine shrimp was produced by 24 hours culture of brine shrimp cysts in

salty aerated water made up with 52.5 gm sea salt (ZM systems, UK) in 1.5 L distilled water, temperature of the fish tanks was maintained at 27±1° C.

Female and male fish were kept together with a ratio of 1:2-3. The plastic beaker contained upright funnel covered with pebbles was used to collect embryos. Every morning, spawning was induced by first light. Males and females swam closely and eggs were fertilized as they were released. Embryos were then collected and kept in tank water at 27±1° C till desire stage. In the automatic system, an open plastic box covered by a plastic net and grass were placed in each tank for embryo collection. The plastic net protects embryos from cannibalistic parents.

2.3 Determination of *hox* and *sox* gene expression in different developmental stages of zebrafish (*Danio rerio*) embryos

In the present study, gene expression studies were carried out in zebrafish embryos at different developmental stages. Gene expression experiments were carried out for sox gene family (*sox2, sox3* and *sox19a*) and hox genes (*hoxb1b, hoxb6b, hoxc6a* and *hoxc8a*) at different developmental stages which were 30%, 50%, 75%, 100% epiboly and 6 somites stages.

2.3.1 Gene expression analysis

The process for evaluation of gene expressions included RNA extraction, DNase treatment, reverse transcription, polymerase chain reaction (PCR), and analysis of PCR product using agarose gel. The work flow of this experiment is illustrated in Fig 2.2.

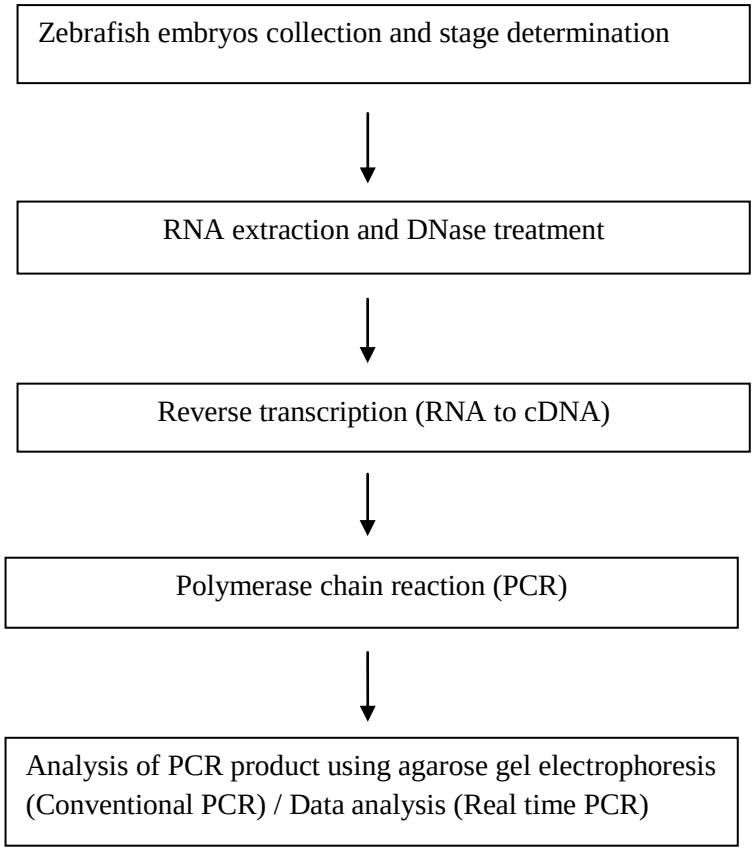


Fig 2.2 The process for evaluation of gene expression in zebrafish embryos in the present study.

2.3.2 Determination of embryo development stage

Embryo development stages were determined using light microscopy (Leica MZ95, Leica Microsystems, Germany) according to characteristic described by Kimmel et al.(1995). Embryo stages used in this study were 30%, 50%, 75%, 100% epiboly and 6 somites stages (Fig 2.3).

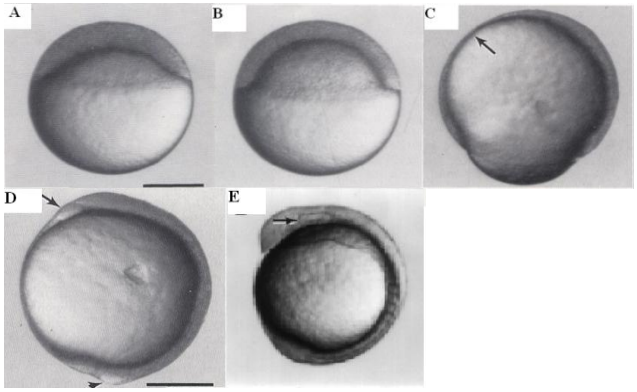


Fig 2.3 Zebrafish embryo stages used in the present study. 30%- (A), 50%- (B), 75%- (C), 100%- epiboly (D) and 6 somites (E). Pictures were taken using a light microscopy (Leica MZ 95 stereomicroscope) and stages were determined according to Kimmel et al. (1995). (A) 30 % epiboly stage – 4.7 hours post fertilization (hpf), (B) 50% epiboly – 5.3 hpf, (C) 75% epiboly – 8 hpf, (D) 100% epiboly or bud stage – 10 hpf and (E) 6 somites stage – 12 hpf.

2.3.3 RNA extraction

RNA was extracted from 5 embryos at each stage. Each extraction was done in triplicates. RNA was extracted using RNAqueous- Micro RNA Isolation Kit (Catalogue no, AM1931) (Ambion, UK), according to manufacturer's instructions. This procedure involved 3 steps - lysis, washing and elution. Steps involve in RNA extraction are given in Fig 2.4.

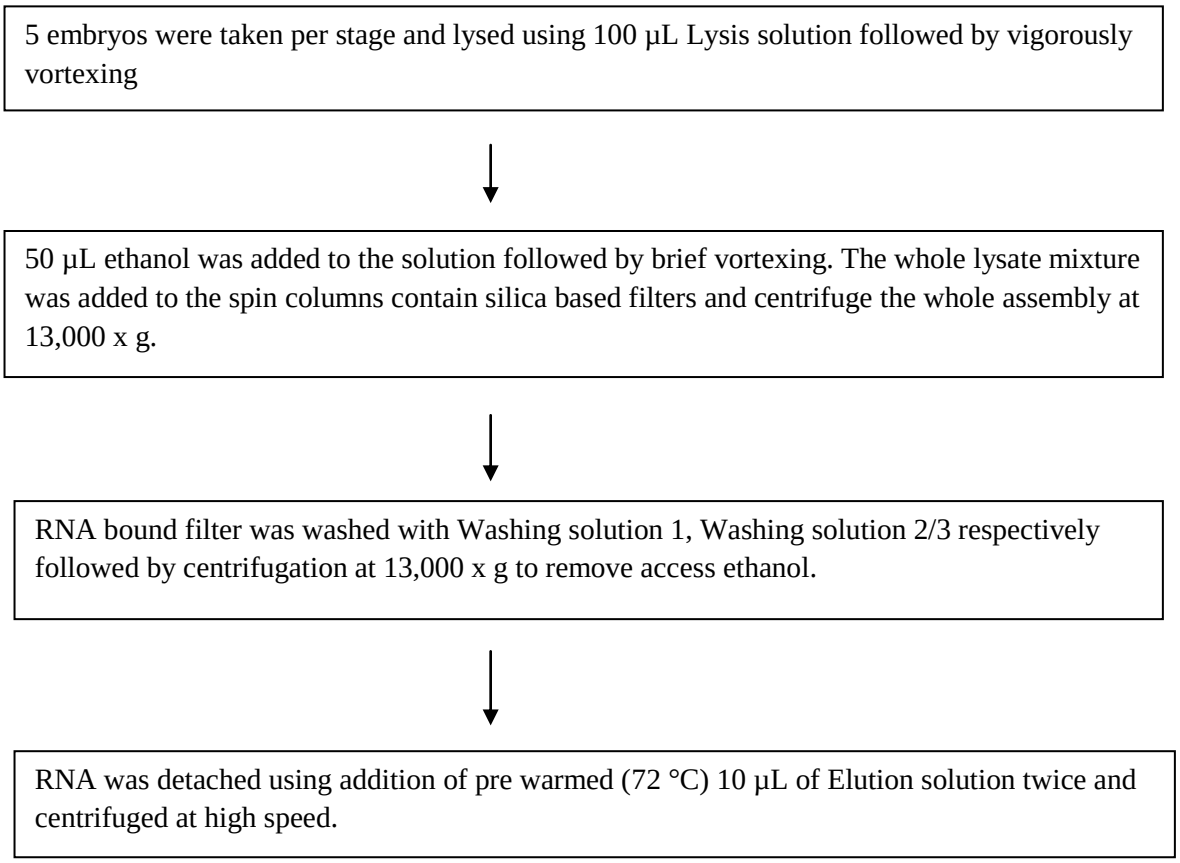


Fig 2.4 Schematic representation of the Ambion's RNAqous RNA Isolation Kit protocol

2.3.4 DNase Treatment

DNase treatment was performed in order to eliminate any genomic DNA contamination in the extracted RNA as DNA contamination in the samples may lead to negative gene expression. This procedure involved incubation of the samples with DNase enzyme and deactivation of the enzyme. RNAqous RNA Isolation Kit also included the reagent for DNase treatment. A summary of the procedure is given in Fig 2.5.

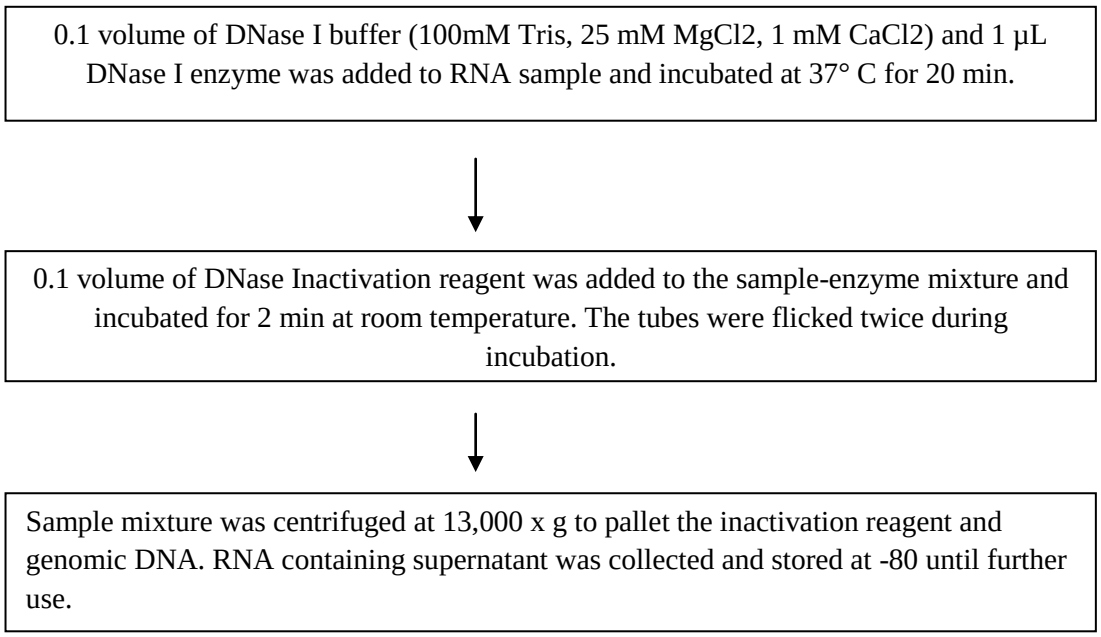


Fig 2.5 Schematic representation of the Ambion's RNAqous RNA Isolation Kit DNase treatment protocol.

2.3.5 Total RNA quantification

Quantification of RNA was carried out using Biophotometer™ (Eppendorf, Germany). RNA was diluted 1:50 with PCR water (SIGMA, UK) and placed within UVette (Eppendorf, Germany) and the absorbance was read at 260 and 280 nm. The Biophotometer™ automatically calculated the RNA concentration in the original sample using the following equation:

(Absorbance at 260nm x RNA coefficient) X Dilution factor = µg/ml of RNA

Where RNA coefficient= 40 ng/µl and Dilution factor = 50

The Biophotometer™ also showed the purity of the RNA by calculating the absorbance ratio at 260 and 280 nm. A pure total RNA sample should give a ratio between 1.7 – 2.1.

2.2.6 cDNA synthesis (Reverse transcription)

RNA was converted into cDNA using reverse transcriptase. 5 µl RNA (~1 µg) was used for each reaction. RNA was reverse transcribed using Precision nanoScript Reverse Transcription Kit (Primerdesign, Southampton, UK) according to manufacturer's instructions. The protocol involved two steps. First, RNA sample (1 µg) was mixed with oligodT primers (1 µl) and the mixture volume was made up to 10 µl using RNAse/DNAse free water. The RNA mixture was incubated at 65°C for 5 min and chilled for 2 min on ice. Second step involved the use of RNA sample from step one, plus nanoscript buffer (2 µl), dNTP mix (10 mM each -1 µl), DTT (100 mM – 2 µl), nanoscript reverse transcriptase (1 µl), the final volume of 20 µl was made up using DNAse/RNAse free water. The reaction mixture was incubated at 55 °C for 20 min, followed by termination of the reaction by inactivation enzyme at 75° C for 15 min. Thermocycler PCR machine (Techne, UK) was used for all incubation steps. No RNA template control and no reverse transcriptase reaction were also performed to check reaction purity. For conventional PCR, undiluted DNA was used subsequently. For real time PCR experiment, cDNA was diluted 1:2 in molecular biology grade water (SIGMA, UK) and stored at -80°C.

2.3.7 Conventional Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was performed for each gene and internal control housekeeping genes. For each gene specialized primer pairs were designed and used for amplification and detection. Forward and reverse primers, annealing temperatures and product sizes are given in Table 2.1.

Table 2.1 Zebrafish (*Danio rerio*) PCR primers, annealing temperature and PCR product size used in the present study.

Genes	Forward/Reverse Primers	Annealing Temp.	PCR product size
sox2	**F:** CTCGGGAAACAACCAGAAAA **R:** TCGCTCTCGGACAGAAGTTT	58	171 bp
sox3	**F:** ACCGAGATTAAAAGCCCCAT **R:** TTGCTGATCTCCGAGTTGTG	56	182 bp
sox19a	**F:** TGTCAACAGCAACAACAGCA **R:** GTTGTGCATTTTGGGGTTCT	57	126 bp
hoxb1b	**F:** TGTTGTCGTCCGGTCAATTA **R:** TCCGAATATATTCGAAATACATAA	58	107 bp
hoxb6b	**F:** TATTATCAGCAAGCTGGGGG **R:** TCTCTGTGCCCTGTTCTGTG	58	178 bp
hoxc6a	**F:** TTTATCTGGTGGGCAAGAGG **R:** TACGGTCCTCGACTTGATCC	59	171 bp
hoxc8a	**F:** TATGGTACCCAGCAAGAGGC **R:** GAGGCTCGGAGAGGAGTTTT	58	114 bp
EF1 - α	**F:** CTGGAGGCCAGCTCAAACAT **R:** ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87 bp

The PCR reaction was performed in 50 µl reaction tubes which consist of NH_4 PCR buffer (Bioline, UK), 200 µM dNTPs (Bioline, UK), 1.5 mM MgCl2 (Bioline), 2 U BIOTAQ™ DNA polymerase (Bioline), 0.5 µM each primer (forward and reverse) (see

Table 2.1), 1 µg RNA template and PCR water (Sigma, UK). PCR conditions for each gene were slightly different in temperature as well as the number of amplification cycles. Cycle number varied from gene to gene due to low/high expression levels. Standard conditions for PCR were: initial denaturation at 94 °C for 5 min (1 cycle), amplification step contains different number of cycles (see Table 2.2 for each gene) at 94° C for 30 seconds, at annealing temperature (see Table 2.1) for 30 seconds, 72° C for 30 seconds followed by 1 cycle of additional extension step at 72 °C for 10 min. PCR reaction was performed at the same time for samples from different stages (i.e. 30%, 50%, 75%, 100% epiboly and 6 somites) along with control housekeeping gene *EF1-α*. Housekeeping genes are responsible for basic cellular function and expressed relatively similar level across many types of cells. During embryonic development, embryos develop at different rates (Lonergan et al. 2000) and at also their transcription activity varies between different developmental stages (Mathavan et al. 2005). Therefore an internal reference factor is required to ensure that the data collected has acceptable level of accuracy.

Table 2.2 PCR conditions for each primer set

Gene	Initial Denaturation	Amplification condition	Additional extension
sox2	94 ° C for 5 min	45 cycle 94° C for 30 seconds 58° C for 30 seconds 72° C for 30 seconds	72 ° C for 10 min
sox3		40 cycle 94° C for 30 seconds 56 ° C for 30 seconds 72° C for 30 seconds	
sox19a		40 cycle 94° C for 30 seconds 57° C for 30 seconds 72° C for 30 seconds	
hoxb1b		45 cycle 94° C for 30 seconds 58° C for 30 seconds 72° C for 30 seconds	
hoxb6b		35 cycle 94° C for 30 seconds	

		58° C for 30 seconds 72° C for 30 seconds	
hoxc6a		45 cycle 94° C for 30 seconds 59° C for 30 seconds 72° C for 30 seconds	
hoxc8a		35 cycle 94° C for 30 seconds 58° C for 30 seconds 72° C for 30 seconds	
EF1-α		25 cycle 94° C for 30 seconds 60° C for 30 seconds 72° C for 30 seconds	

2.3.8 Analysis of PCR product

PCR product was analysed using agarose gel electrophoresis and Ethidium Bromide (EtBr) staining. 2 % agarose powder was dissolved in sterile TAE buffer (T4038, Sigma, UK) by warming the solution: 0.5 µg/mL EtBr (E1510, Sigma, UK) was added to warm agarose solution, the solution was swirled and poured onto gel cast and left to set. Comb was put to form wells where the samples were loaded. Samples were mixed with gel

loading buffer (G2526, Sigma, UK) and loaded into well along with HyperLadder™ V (Bioline, UK). Gel was run at constant voltage at 100V for 2 hours. The Gel was visualised using Genosmart UV Gel Documentation System (VWR, UK).

2.4 Studies on the effect of short term chilling on sox gene expression in zebrafish (*Danio rerio*) embryos

There are several reports on the effect of chilling on zebrafish embryos and embryo survivals were assessed by their hatching rate (Zhang et al. 2003, Zhang and Rawson 1995, Zhang and Rawson 1996a, Zhang and Rawson 1996b, Zhang et al. 1993), however information on the effect of chilling on embryos at molecular level is also needed as the analysis of gene expression following chilling may help us to understand developmental stage chilling sensitivity and make major advances in knowing the adaptations needed for low temperature survival. Therefore, quantitative analysis of mRNA abundance of certain genes would give more information on mechanism at molecular level during chilling. This experiment investigated the effect of chilling and subsequent warming on gene expressions in zebrafish embryos. It has been shown that early stage embryos are more sensitive to chilling than later stages (Zhang and Rawson 1995). In order to assess gene expression at a particular embryo stage after chilling, it is important that information on the effect of chilling on embryo survival/hatching rate is obtained first before gene expression studies. Therefore, the effect of short term chilling on embryo hatching rate at different developmental stages was studied before gene expression studies. Previously, it has been demonstrated that early embryonic stages are more sensitive to chilling than later stages (Zhang and Rawson 1995) in zebrafish and 7- to 10- h (hpf) embryos stages (50% epiboly – 100% epiboly) could tolerate 0°C up to 3 h before survival rate started decreasing significantly. In order to understand chilling sensitivity at molecular level, chilling up to 3 h (300-, 60-, 120- and 180- min) was selected to study hatching rate followed by gene expression.

2.4.1 Studies of the effect of 3 h chilling on hatching rate of zebrafish embryos

Based on the earlier results obtained from gene expression experiments in the present study, three developmental stages with consistent gene expression were selected for further studies on impact of chilling on hatching and subsequent gene expression. In these studies, 50%-, 75%- and 100%- epiboly stage embryos were collected and 15 embryos per treatment were used. Embryos in egg water (60 µg/mL sea salt in distilled water) were chilled at 0°C in a crushed ice bath for 30, 60,120 and 180 min using a previously reported protocol (Lin et al. 2009b). After chilling, embryo containing tubs were quickly plunged in 27±1°C water and then incubated at 27±1°C for up to 3 days. Control embryos were kept at 27±1°C and incubated for 3 days. Hatching rates were then monitored and all experiments were repeated three times in triplicate. Embryo hatching rates were recorded 72 hours post chilling and warming. Embryos were considered hatched when they showed natural swimming movements with functional heart beat without chorion attachment and had no obvious signs of malformation. Embryos were considered unhatched if they showed no signs of cell differentiation, yolk coagulation and no tail formation or detached tail and/or if they remained in the chorion (Lahnsteiner 2009).

2.4.2 Studies of the effect of chilling on sox genes expression in zebrafish (*Danio rerio*) embryos using quantitative PCR (qPCR)

50% epiboly stage embryos were selected for these studies as embryo hatching rates were not affected at this stage when they were chilled for up to 180 min at 0°C. To understand chilling tolerance at molecular level, the embryonic stages which survive (50% epiboly) were used to study effect of chilling on developmental genes. In this experiment, embryos at 50% epiboly stages were chilled at 0°C for 30, 60,120 and 180 min. RNA was extracted and cDNA was produced as described in Sections 2.3.3 – 2.3.6 and diluted in

1:2 in PCR molecular biology grade water (SIGMA, UK) before real time PCR. For each time point, three different biological samples (5 embryos/tube) were treated and stored. Each experiment was repeated three times. Experimental controls were kept at 27±1 ° C in a water bath for the equivalent time period.

2.4.3 Studies of the effect of chilling and subsequently warming on sox genes in zebrafish (*Danio rerio*) embryos using quantitative qPCR

In these experiments, studies were carried out on the effect of warming on gene expression after zebrafish embryos were chilled for up to 60min. Chilling periods of 30 and 60 min were selected for these experiments as the embryo gene expressions were found to be suppressed under these conditions following chilling at 0°C. Embryos were chilled for 30 and 60 min and then returned to 27±1° C for 30-, 60-, 120- and 180- min. At each time point after chilling at 0°C and warming, three different samples (5 embryos /tube) were stored at -80°C before RNA extraction and subsequent quantification using real time PCR. Each experiment was repeated three times. Experiment controls were kept at 27±1 °C in a water bath.

2.4.3.1 RNA extraction and cDNA synthesis

RNA was extracted from all the embryos (treated and untreated) using RNAqueous-Mico RNA Isolation Kit (Catalogue no, AM1931, Ambion, UK) according to manufacturer's instructions. This procedure involved lyses, washing and elution step. The protocol for RNA extraction and DNase treatment was given in Section 2.3.3- 2.3.5.

RNA was then converted into cDNA using reverse transcriptase. RNA (1 µg) was reverse transcribed using Precision NanoScript Reverse Transcription Kit (Primerdesign, Southampton, UK) according to manufacturer's instructions. The protocol involved denaturation and annealing (see Section 2.3.6). cDNA was diluted in 1:2 with molecular biology grade water before use in real time PCR.

2.4.3.2 Generation of standards for Real time PCR

The standards for real time PCR of *sox2*, *sox3* and *sox19a* along with housekeeping gene *EF1-α* and *β actin* were produced using conventional PCR. The primer sequences were given in the Table 2.1. PCR reaction was performed according to the procedure given in the Section 2.3.7. PCR product was run on 2% agarose gel and DNA was isolated from excised bands using EZNA Gel Extraction Kit (Omega Bio-Tek) according to manufacturer's instructions. The overview of the protocol is given in Fig 2.6. Isolated DNA was quantified using BioPhotometer (Eppendorf , UK) at 260 nm. The DNA was diluted to 2 ng/µl followed by 10- fold serial dilutions to generate standards for real time PCR.

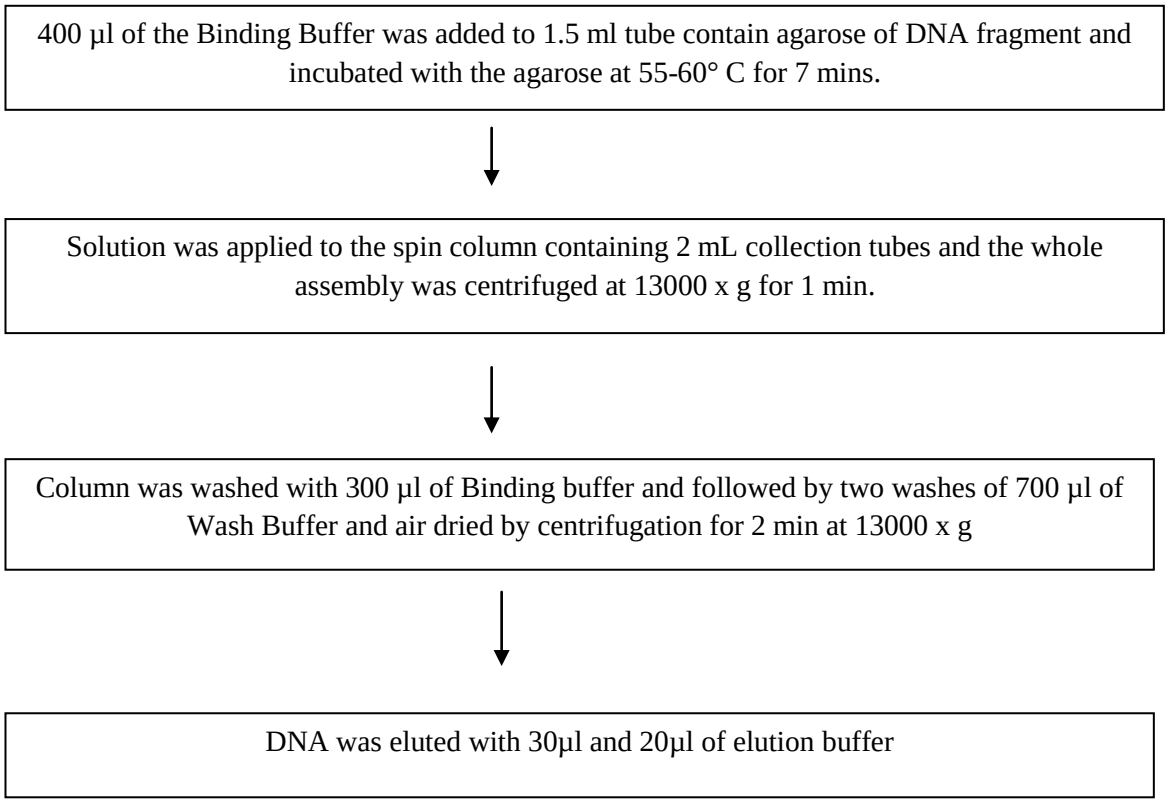


Fig 2.6 Schematic representation of the EZNA Gel Extraction Kit protocol.

2.4.3.3 Quantification of *sox2, sox3* and *sox19a* genes using Real time PCR

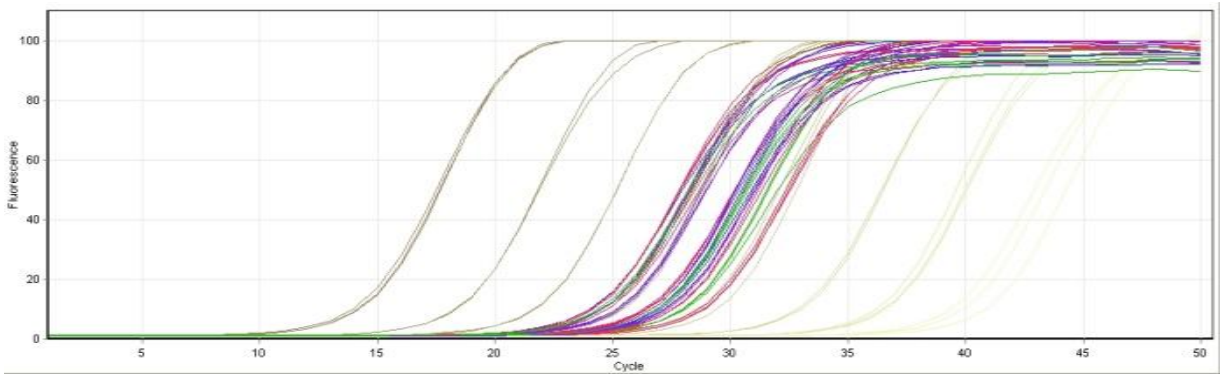
Real time PCR was performed on RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor to quantify expression levels of *sox2*, *sox3* and *sox19a*. Reaction tube contained 7.5 µl of sensimix 2X Reaction buffer (contained heat activated DNA polymerase, Ultrapure dNTPs, MgCl2, SYBR® Green I), 333 nm of each primers (see Table 2.1) and 2 µl of cDNA sample, made up with PCR water (sigma, UK). The reaction condition were 1 cycle at 95° C for 10 min, followed by 50 cycles at 95° C for 10 sec, at appropriate annealing temperature (see Table 2.1) for 15 sec and at 72° C for 15 sec. Data were acquired on FAM/SYBR channel at the end of the each extension step. Melt curve was also analysed to check for the absence of mispriming and amplification efficiency from a standard curve (R^2 should be close to 1). The possibility of genomic DNA was eliminated by use of primers that crossed introns. All the treatment time points (after chilling and warming) were analysed using real time PCR. The relative gene expression data were obtained using RototGene software (Version 1.7, Corbett research) and Microsoft Excel. Relative gene expression levels were calculated using relative quantification method. *Ef1 α* and *β actin* were used for this study as these genes were shown to have highest stability during chilling of zebrafish embryos (Lin et al. 2009a, McCurley and Callard 2008).

SYBR Green I dye fluoresces when it is bound to double strand DNA. At the end of the each extension step, florescence data was collected (Fig 2.7a). Early PCR cycle shows very low florescence which is undetectable, as the cycle progresses the florescence level also increases until plateau is reached. The standards of 10- fold dilution of DNA showed equally spaced curves on the florescence graph (Fig 2.7b). The cycle number at which threshold level is reached can be used to create a standard curve from which sample data can be quantified (Fig. 2.7c). The melting point of the PCR product is also determined using the melting curve. PCR product should give one temperature peak on melting curve

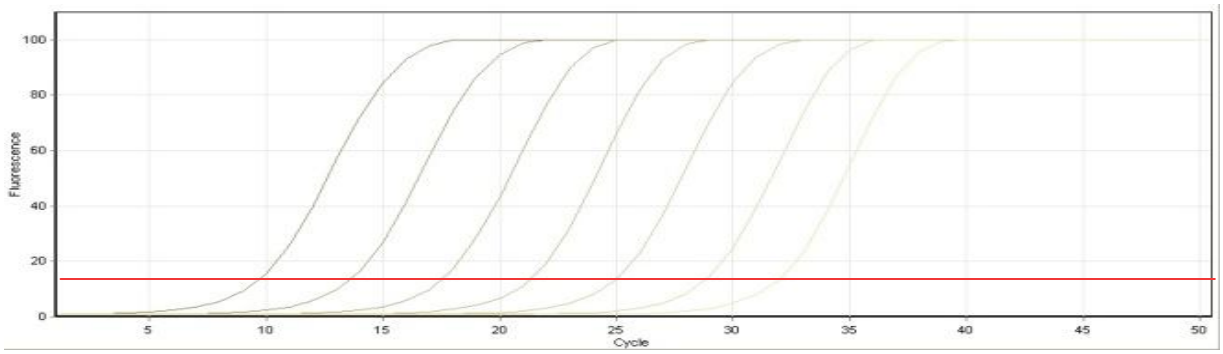
(Fig 2.7d). More than one peak was interpreted as mispriming, primer dimer or any possible contamination.

Amplification curve (Fig 2.7a) is mainly divided into four major phases (Fig 1.11) which are (1) linear ground phase (2) early exponential phase (3) log linear phase (exponential phase) and (4) plateau phase (Tichopad et al. 2003). During linear ground phase, florescence emission has not increased above background. At early exponential phase, the amount of florescence started to increase and reach threshold where it is significantly higher (usually 10 times the standard deviation of linear ground phase). The cycle number at this time called as "$C_{t\ or}$ CP2 (crossing point). This value is used to calculate gene expression or mRNA quantification (Heid et al. 1996). Ideally in log linear phase, PCR product doubled every cycle and reaches optimal amplification period before moving to Plateau stage where reaction starts lacking PCR component and florescence intensity is no longer useful for data calculation(Bustin 2000). Dissociation curve (Fig 2.7d) procedure usually carried out once amplification cycles competed. Following amplification curve, temperature is increasing slowly above the T_m over a temperature gradient and florescence levels are measured at each distinct temperature point. Due to higher temperature, DNA denatures, resulting in corresponding decrease in florescence due to SYBR dissociation from the double-stranded product (Giglio et al. 2003). The temperature at which 50% of the PCR product is denatured can be used to confirm presence of targeted product, along with presence of non specific product and primer dimer.

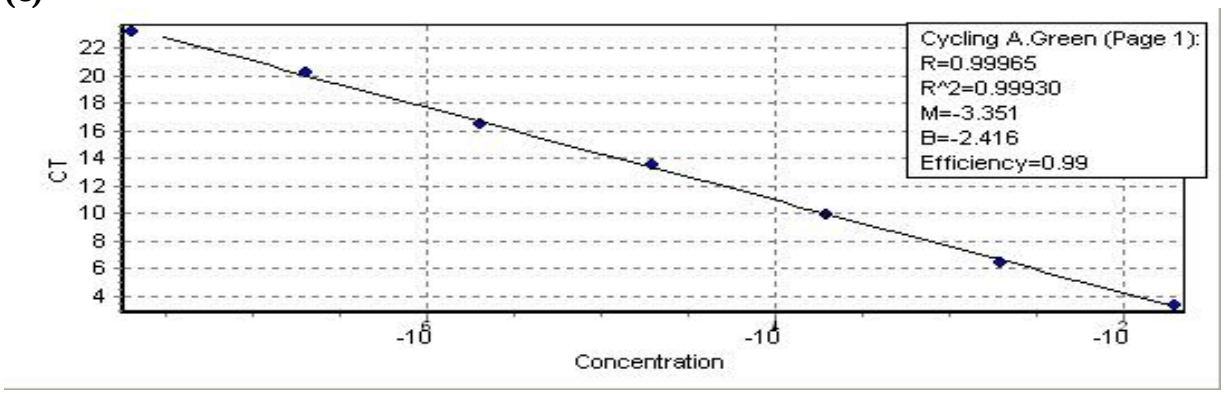
(a)


Fluorescence
Cycle

(b)


Fluorescence
Cycle

(c)


Cycling A.Green (Page 1):
R=0.99965
R^2=0.99930
M=-3.351
B=-2.416
Efficiency=0.99
CT
Concentration

(d)

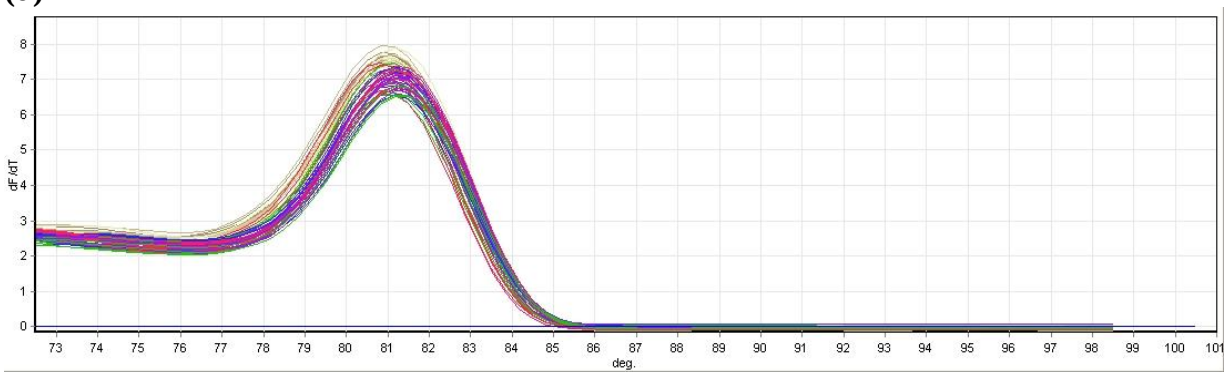


Fig 2.7 Graph produced during real time PCR experiments: (a) fluorescence measurement after each cycle (b) standards of decreasing concentration of gene of interest crossing the threshold line (red line) with increasing cycle numbers (c) standard curve produced from 10-fold dilution concentration of equally spaced standard concentration (d) melting curve with one single peak without any primerdimer formation.

2.4.3.4 Relative quantification

Relative gene expression levels were calculated using the two standard curve quantification method with kinetic PCR efficiency correction (see equation below) in the Rotorgene software (Pfaffl 2003b). Gene expression levels were normalised with respect to values at time zero and the expression levels were also normalised with housekeeping gene *Ef1 α* and *β actin*.

$$\text{Relative quantification} = \frac{(E_{\text{target}})^{\Delta CP\,(\text{target})\,(\,control - sample\,)}}{(E_{\text{ref}})^{\Delta CP\,(\text{ref})\,(\,control - sample\,)}}$$

where E is the real time PCR efficiency and ΔCP is the crossing point difference between the unknown sample and the control sample.

2.5 Studies on the effect of chilling on gene and protein expression in zebrafish (*Danio rerio*) embryos in the presence of methanol (MeOH)

Long term chilling storage or cryopreservation requires the addition of cryoprotectant in cryopreservation solution in order to protect cells from low temperature injury. Although studies on the effect of cryoprotectant on zebrafish embryo survival were carried out previously (Zhang and Rawson 1995), studies on the effect of these cryoprotectants on embryos at the molecular level have been limited (Lin et al. 2009b). In the previous experiments of the present study, it was shown that chilling suppressed *sox2* and *sox3* gene expression when embryos were chilled for 180 min at 0 °C. This experiment was focused on the effect of cryoprotectant on embryo gene expression following chilling and warming. Zhang and Rawson (1995) identified Methanol (MeOH) as the optimum cryoprotectant for chilling heartbeat stage zebrafish embryos. The present study focused on the effect of methanol on early stage (50% epiboly) embryos when embryos were chilled for up to 24 h at 0 °C. Hatching rates were monitored following chilling and warming at 27 ± 1°C. Following the results obtained from embryo survival (hatching rate), embryos were chilled for 24 h at 0°C in the presence of MeOH, gene and protein expression studies were carried out under the same treatment conditions in order to understand the effect of MeOH on embryos at the molecular level.

2.5.1 Studies on embryo hatching rate after chilling at 0° C for different time periods in the presence of MeOH as a cryoprotectant

50% epiboly embryos were chilled at 0°C for up to 24 h (3-, 6-, 18- and 24- h) in the presence of different concentrations of MeOH (0.2, 0.5 and 1M). Embryos were chilled in different concentrations of MeOH at 0°C in a crushed ice bath for 3, 6, 18 and 24 h in test tubes (Lin et al. 2009b). After chilling, cryoprotectant MeOH were replaced by egg water (60 µg/mL sea salt in distilled water) and the test tubes were quickly placed into a

27±1°C water bath and embryos were then incubated at 27±1°C for up to 3 days or until they hatched. Control embryos were kept at 27±1°C and incubated for 3 days. Hatching rates were then monitored and all experiments were repeated three times in triplicate. Embryo hatching rates were examined at 72 h post treatment. Embryos were considered to be hatched when their chorion is missing, there were no obvious signs of malformation, and there were natural movement with functional heart beat. Embryos were considered unhatched if they showed no signs of cell differentiation, yolk coagulation and no tail formation or detached tail and/or if they remained in the chorion (Lahnsteiner 2009).

2.5.2 Studies on the effect of chilling and warming on sox gene expression in zebrafish (*Danio rerio*) embryos in the presence of MeOH

Based on the results obtained from the previous experiments that no significant differences were observed in hatching rate upon 3 and 6 h chilling, further studies were carried out on the effect of 3 h chilling on gene and protein expression in 50% epiboly embryos in the presence of MeOH.

Chilling of embryos: Embryos at 50% epiboly stage were chilled at 0° C for 3 h with different concentrations of MeOH (0.2, 0.5 and 1 M) as described in earlier Section. RNA was then extracted and cDNA was produced as described previously. cDNA was diluted to 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR. For each time point, three different biological samples (5 embryos/tube) were treated and stored. Each experiment was repeated three times. Experimental controls were kept at 27±1°C in a water bath for the equivalent time period.

Warming and incubation of embryos after chilling : Embryos at 50% epiboly stages were chilled for 3 h at 0°C, they were then warmed up and incubated until three key developmental stages – 20 somites stage (hind brain development), heartbeat stage (first

heart beat starts) and hatching periods (first time when actual larvae exposed to environment). Fifteen embryos were chilled for 3 h and then returned to 27±1°C, they were then incubated until desired stage was reached. For each embryo stage, three different samples (5 embryos/tube) were treated and stored for RNA extraction at -80°C. Each experiment was repeated three times. Experimental controls were kept at 27±1°C in water bath.

2.5.3 Studies on the effect of MeOH chilling and warming on *sox2* and *sox19a* protein expression in zebrafish (*Danio rerio*) embryos

Translation is the synthesis of protein directed by an mRNA template. The information contained in the nucleotide sequence of the mRNA is read as three letter words (triplets), called codons. Each word stands for one amino acid. During translation, amino acids are linked together to form a polypeptide chain which will later be folded into a protein. There is a strong relationship between gene and protein expression levels as protein is usually produced based on the information obtained from a specific gene or mRNA. However, sometimes this relationship could be masked due to various reasons: analytical variability of the measurement technology, post transcriptional mechanism affecting mRNA stability and protein degradation and timing differences between gene and protein expressions (Tan et al. 2009). Furthermore, transcript levels detected in mRNA profiling clearly do not reflect all regulatory processes in the cell, as post-transcriptional processes altering the amount of active proteins, such as synthesis, processing and modification of proteins. Therefore, in addition to monitoring gene expression at the transcriptional level, analysis of the protein is also important for the understanding of the cellular, metabolic and regulatory networks in living organisms (Nie et al. 2007).

The result obtained from gene expression studies showed that *sox2* gene expression of 50% epiboly stage embryo was suppressed following 3h chilling in the presence of 1 M

MeOH, recovered upon warming and increased significantly following hatching. For the same treatment, different gene expression patterns were observed for *sox19a as* gene expression following chilling stayed above control level. Protein expression was studies in these time points eg. following 3 h chilling and following chilling and warming in hatching stage in presence of MeOH to see effect of cryoprotectant on protein expression of *sox2* and *sox19a.*

Embryos (~75 embryos) from 50% epiboly stages were chilled for 3 h with/without 1 M MeOH and then returned to 27±1° C and incubated until they hatched. Cryoprotectant were replaced by ±1 °C egg water (60 µg/mL sea salt in distilled water) following chilling before incubation. Experimental controls were kept at 27±1°C in a water bath for the equivalent time period. Samples were collected for protein extraction immediately after 3 h chilling and larvae (after ~ 3 day warming). Protein was extracted and separated using SDS PAGE (See Section 2.5.3.1 – 2.5.3.4). Following separation, protein was transferred to PVDF membrane and immunoblot (See Section 2.5.3.6) using a specific antibody. The experiments were repeated three times.

2.5.3.1 Protein Extraction

After treatment embryos were washed twice with embryo medium 2 (15 mM NaCl, 0.5 mM KCl, 0.27 mM $CaCl_2$, 1 mM $MgSO_4$, 0.27 mM $NaHCO_3$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4). Following washing, embryos were subjected to protease treatment (2 mg/ml, Sigma-Aldrich, 81748) for 10 min where chorion was partially digested. After digestion, chorions were removed using pipette suction method. Embryos were then washed three times with embryonic medium 2 before transferred to 1.5 ml tube. 100 µl of protein extraction buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol) was added to each tube and samples were heated to 95°C for 10 min. Following heating, samples were

vortex and homogenised and centrifuged at 13,000 x g for 10 min and protein containing supernatant was collected.

2.5.3.2 Protein Quantification

Isolated proteins were quantified using QuantiPro™ BCA Assay Kit (Sigma-Aldrich, QPBCA) according to manufacturer's instructions. This is a detergent-compatible formulation for the colorimetric detection and quantification of total proteins. This method is a combination of the reduction of cupric ions [Cu (II)] to cuprous ions [Cu (I)] by proteins in an alkaline medium (i.e. biuret reaction) and a selective and sensitive colorimetric detection of the cuprous ions. The purple-colour reaction product of BCA assay is formed by the chelating of one Cu (I) ion by two molecules of BCA. (Smith et al. 1985). The absorbance is directly proportional to protein concentration. The colour produced from this reaction is stable and increases in a proportional manner over a broad range of increasing protein concentrations. This colour complex exhibits a strong absorbance at 562 nm. The BCA method is not a true end-point method (i.e. colour development does not stop at the time of absorbance measurement). The assay was carried out by preparing bovine serum albumin (BSA) (Sigma, UK) standard concentrations (0.0 – 30 µg/mL). The BSA standard concentrations were prepared as shown in Table 2.3 from 1mg/ml stock solution of BSA. All proteins were diluted using distilled water.

Table 2.3 Concentrations of bovine serum albumin standards

Sample Buffer (µl)	Protein standard (1 mg/mL) (µl)	Final protein concentration (µg/ml)
1000	0	0
999.5	0.5	0.5
995	5	5
990	10	10
980	20	20
970	30	30

QuantiPro Buffer QA contains sodium carbonate, sodium tartate, and sodium bicarbonate in 0.2 M NaOH, pH 11.25 and QuantiPro QB contains 4% (w/v) bicinchoninic acid and QuantiPro QC contains 4% (w/v) copper(II) sulphate solution, pH 8.5, pentahydrate soultion. The desired volume of working solution was prepared by mixing QA, QB and QC according to manufacturer's instructions. BCA diluted proteins and unknown protein solutions were mixed with 1 mL of working BCA reagent and incubated on rotary incubator for 1 h at 60°C. Absorbance reading for each protein sample was taken by Biophotometer (Eppendorf, UK) at 550 nm (i.e. measured absorbance – blank absorbance) following incubation. All readings were taken three times and the mean value was used to produce standard curve. A protein standard curve was generated by plotting the absorbance for each BSA standard against its concentration in µg/µl, using Microsoft Excel software. The equation for the best fit of standard curve was used to determine the protein concentration of each unknown sample. In Microsoft Excel software, only standard curves with r^2 values (a measure of linearity of BSA standard concentrations) of 99% or better were used for quantification. A representative BCA protein standard curve is shown in Fig 2.8.

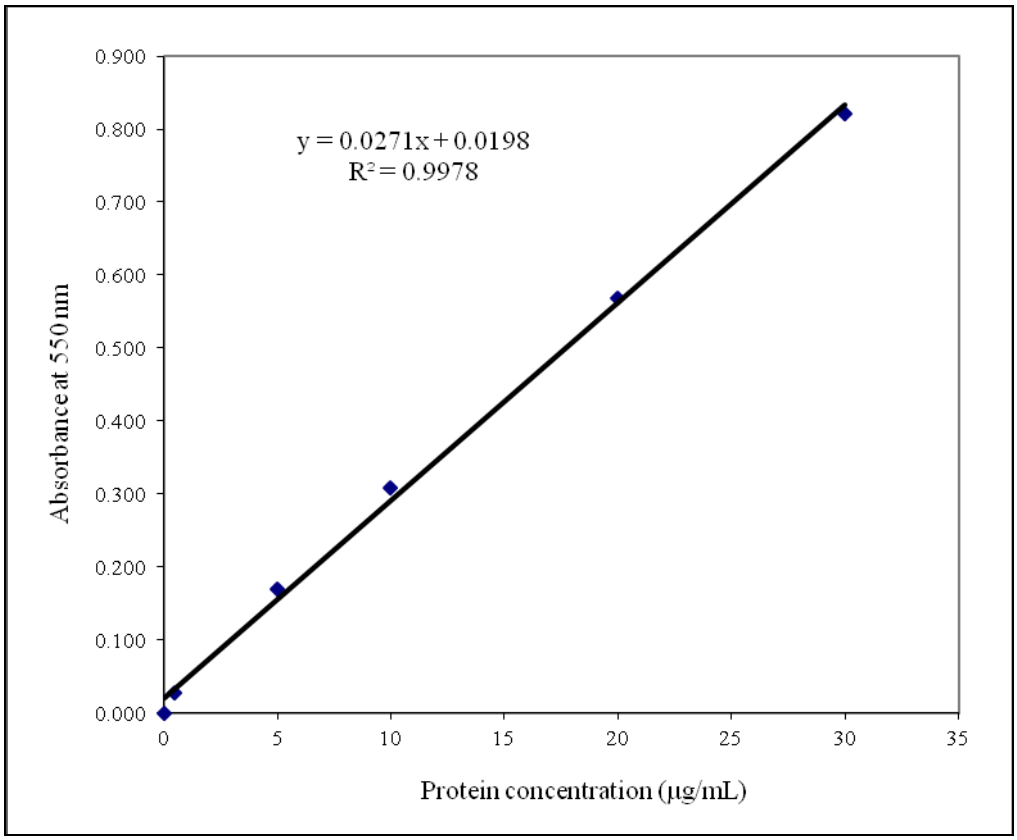


Fig 2.8 A representative BCA standard curve

The equation (y= mx + b) produced from this standard curve was used to calculate unknown amount of protein in the sample.

2.5.3.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated using sodium Dodecyl sulphate Polyacrylamide gel electrophoresis (Fig 2.9). The resolving and stacking gel was prepared on Bio-Rad Mini-PROTEAN II casting stand (Bio-Rad, UK).

For preparation of 5 mL (enough for one gel) of a 10% resolving gel, the following components were mixed in order listed : distilled water (2.03 mL), 30 % Protogel contains 37.5:1 Acrylamide: Bisacrylamide (1.67 mL, National Diagnostics, USA), 4X resolving buffer contains 1.5 M Tris HCl and 10% SDS, pH 8.8 (1.5 mL, National

diagnostics, USA), 10% ammonium persulfate (APS) (0.025ml, SIGMA, UK) and N,N,N′,N′-tetramethylethylenediamine (TEMED) (0.005ml, SIGMA, UK) .

After polymerization of gel complete, 2 mL of 4% stacking gel was prepared by adding the following components in order: distilled water (1.22 mL), 30 % Protogel contains 37.5:1 Acrylamide: Bisacrylamide (0.26 mL, National Diagnostics, USA), 4X stacking buffer contains 0.5 M Tris HCl and 0.4% SDS, pH 6.8 (0.5 mL, National diagnostics, USA), 10% ammonium persulfate (APS) (0.01ml, SIGMA, UK) and N,N,N′,N′-tetramethylethylenediamine (TEMED) (0.002ml, SIGMA, UK). The stacking gel was layered on top of resolving gel and allows polymerised.

The gel was transferred to a Bio-Rad Mini-PROTEAN II (Bio-Rad, UK) electrophoresis tank. A 1x electrophoresis tank buffer was prepared from a 10X stock consisting of: 30.28 g/L of Tris base (0.25M, pH 8.3), 144.0 g/L of glycine (1.92M), 100ml of 10% SDS (1%). Enough 1x diluted electrophoresis buffer to cover the top of the gel, was added to the gel tank. The prepared lysates along with a 2 µl MagicMark™ XP Western Protein Standard (Life technologies, UK) were heated at 95°C for 5 min prior to loading onto the gel. The gel was run at a constant voltage of 200V for 40-60 min or until the bromophenol blue front had migrated to the bottom of the gel. Negatively charged protein with low molecular weight proteins were run quickly and reach at the bottom of the gel while high molecular weight runs slower and remain at the top (Fig 2.9).

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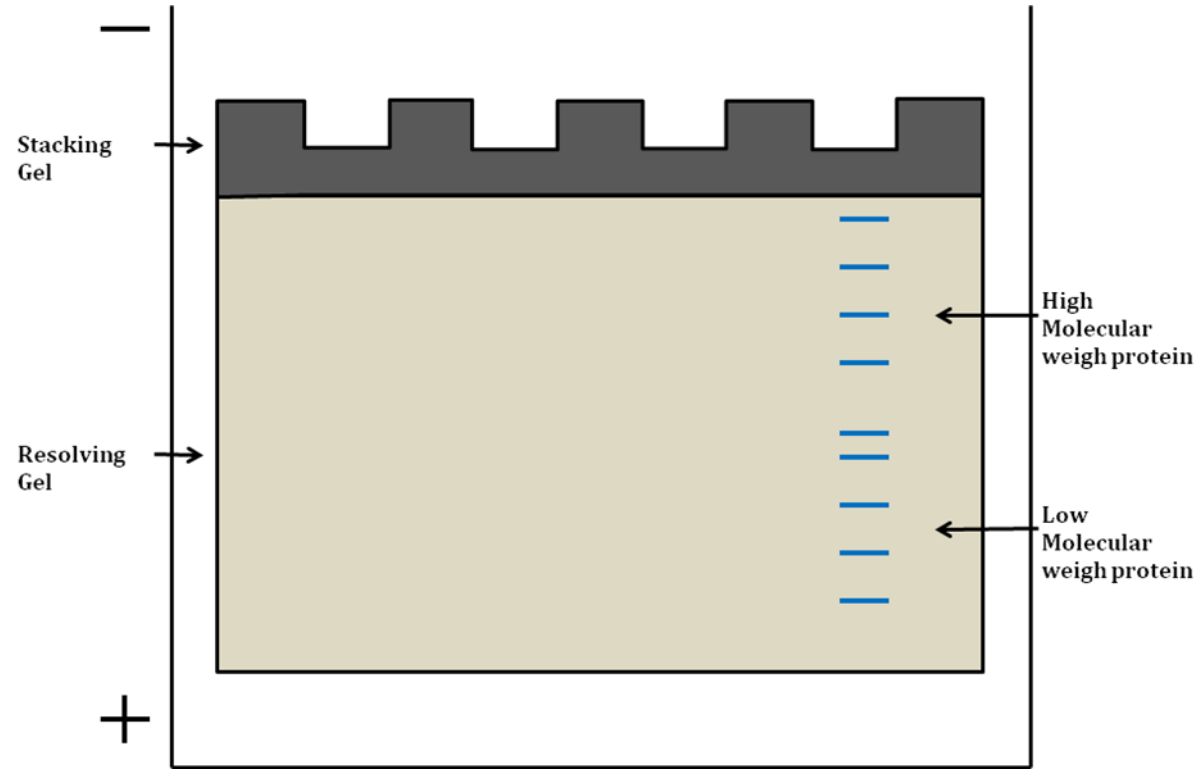


Fig 2.9 Schematic representation of SDS gel. Proteins were loaded onto each wells on stacking gel and electrophoresed. Proteins were separated according to their molecular weight, low molecular weight proteins run faster and reach at the bottom of the gel while high molecular weight proteins remain at the upper half of the gel.

2.5.3.4 Blotting

Blotting was performed in polyvenylfluoride (PVDF) membrane (Thermo Scientific, 88518) using a Semi-Dry method (BioRad, 170-3940). Following SDS-PAGE, the gel was equilibrated in blotting buffer (48 mM Tris, 39 mM glyicine, 0.04% SDS, 20% methanol) for 10 min. PVDF membrane was activated in 100% methanol for 1 min and equilibrated in blotting buffer for 10 min. A sandwich was prepared by placing the membrane onto a filter paper pre-soaked in transfer buffer, the gel was then placed onto the membrane and finally a second pre-soaked filter paper was placed on top of the gel. The sandwich was blotted for 45 min at 15 volts.

2.5.3.5 Membrane staining

Membrane staining was performed in order to check successful transfer of proteins from gel to membrane. The membrane was incubated with Ponceau S (0.1% Ponceau S and 5% acetic acid) solution (Sigma-Aldrich, P7170) for 10 min, followed by washing in distilled water until the desired contrast was achieved.

2.5.3.6 Immunoblotting and florescence detection

Immunoblotting was carried out using Anti rabbit WesternDot^{tm}625 Western Blot Kits (Invitrogen, W10132). The membrane was blocked in 10 ml of WesternDot blocking buffer for 1 hour at room temperature. Following blocking, membrane was incubated with 10 ml of primary antibody solution (dilution 1:1000) overnight at 4˚C on gel rocker. The membrane was then washed 3 times for 10 min with WesternDot Wash buffer. Following washing, the membrane was incubated with 10 ml of Biotin-XX-Goat anti-rabbit solution for 2 hours at room temperature. After secondary antibody incubation, the membrane was washed 3 times as before. The membrane was then incubated with 10 ml of Qdot 625 Streptavidin conjugate solution for 1 hour at room temperature. The membrane was then washed 3 times as previously, followed by a final wash in MilliQ water for 5 min. The membrane was soaked in 100% methanol to make it transparent and then visualised under an UV trans-illuminator with images taken.

2.5.3.7 Densitometry and data analysis

Densitometry analysis was carried out using ImageJ software (Maryland, USA) (Sheffield 2007). All bands were quantified and then normalised with respect to non-treated samples. Internal control β actin was used for normalisation of any variation in replicates.

2.6 Effect of longer term chilling on gene and protein expression in zebrafish (*Danio rerio*) embryo

Following the studies on the effect of short term chilling on gene and protein expressions in zebrafish embryos, the effect of longer term chilling (up to 24 h) on gene and protein expressions in embryos were studied as these conditions have practical applications in fish embryo handling in aquaculture eg embryo transportation between fish farms in genetic improvement programs. In order to optimise protocol for longer term storage, studies were carried out on embryo survival (hatching) rate following extended chilling periods (up to 24h). Results obtained from the earlier experiments of the present study showed that following chilling of embryos in 0.2-1 M methanol for 18 and 24h, embryo hatching rates were decreased significantly. In order to improve embryo hatching rate after chilling, cryoprotectant solution was optimised before studies were carried out on the effect of longer term chilling on embryo gene and protein expressions.

2.6.1 Studies on the effect of 18 h chilling on hatching rate in zebrafish *(Danio rerio)* embryos in the presence of sucrose and MeOH mixture

50% epiboly embryos were chilled at 0°C for 18 h with different concentrations of MeOH (0.2, 0.5 and 1- M) in combination of sucrose (0.05-, or 0.1-M). Embryos were chilled at 0°C in a crushed ice bath for 18 h in test tubes (Lin et al. 2009b). After chilling, cryoprotectant MeOH were replaced by egg water and the test tubes were quickly placed into 27±1°C water bath. Embryos were then incubated at 27±1°C for up to 3 days. Control embryos were kept at 27±1°C and incubated for 3 days. Hatching rates were then monitored and all experiments were repeated three times in triplicate. Embryo hatching rates were examined at 72 h post treatment according to morphological changes described in Section 2.4.1.

2.6.2 Studies on the effect of 18 h chilling on *sox* gene expression in zebrafish (*Danio rerio)* embryos

In earlier experiments, embryo were chilled at 0°C for 18 h with different combination of methanol (0.2-, 0.5- and 1 M) with sucrose (0.05-, or 0.1 M) mixture, they were then warmed up and hatching rates were monitored. Highest survival rates were observed in embryos that had been chilled with 1 M MeOH +0.1 M sucrose. This solution was therefore used in this study to investigate the effect of chilling on embryo gene expression. Embryos at 50% epiboly stage were chilled at 0° C for 18 h in cryoprotectant mixture (1 M MeOH+ 0.1 M Sucrose) as described in Section 2.6.2. RNA was then extracted and cDNA was produced as described in Section 2.3.6. cDNA was diluted with a ratio of 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR. For each treatment (0 h, 18 h chilling, 18 h control and 18 h chilling in cryoprotectant mixture), three different samples (5 embryos/tube) were used. Each experiment was repeated three times. Experimental controls were kept at 27±1°C in a water bath for the equivalent time period.

2.6.3 Studies on the effect of 18 h chilling and warming on *sox* genes expression in zebrafish (*Danio rerio*)

This experiment was performed in order to identify *sox* gene expression patterns following 18 h chilling and embryo incubation at 27±1 °C until hatching stage. 50% embryos were chilled in cryoprotectant mixture (1 M MeOH + 0.1 M sucrose) and warmed at 27±1°C, they were than incubated at 27 ± 1°C until hatching stages. RNA was then extracted and cDNA was produced as described previously. cDNA was diluted with a ratio of 1:2 with molecular biology grade water (Sigma, UK) for use in real time PCR. For each time point (0 h, 18 h chilling, 20 somites, heartbeat and hatching) three different biological samples (5 embryos/tube) were used. Each experiment was repeated three

times. Experimental controls were kept at 27±1°C ° in a water bath for the equivalent time period.

2.6.4 Studies on the effect of 18 h chilling and warming on *sox2* and *sox3* protein expression in zebrafish (*Danio rerio*) embryos

Based on the results obtained from gene expression studies after 18 h chilling and warming that *sox2* and *sox19a* gene expressions were increased after heartbeat stage before returned to the control level. Different gene expression patterns were observed for *sox3* as gene expressions decreased significantly at 20 somites stage and remained low before decreasing significantly in hatching stage. To further understand this different gene expression pattern of *sox3* when compared to *sox2* and *sox19a* genes, protein expression studies were carried out for *sox2* and *sox3* following 18 h chilling and after hatching to understand protein expression in chilling and warming. 50% epiboly embryos were chilled for 18 h at 0 °C in cryoprotectant mixture (1M MeOH + 0.1 M sucrose). Non chilled embryos in egg water were also incubated for 18 h at 27 ± 1°C. Following treatment, proteins were extracted immediately from embryos as described previously and stored at -80°C. These experiments were also carried out for embryos at hatching stage. 50% epiboly embryos were chilled at 0°C for 18 h and returned to 27±1°C, embryos were then incubated till they hatched. Protein was extracted and stored at -80°C for future use.

2.6.4.1 SDS PAGE, Immunoloblotting and florescence detection

Protein extracted from treated and non-treated embryos were loaded onto SDS gel and separated according to their molecular weight (see Section 2.4.3.3). Proteins were transferred using Western Blot technique onto PVDF membrane (see Section 2.4.3.4). Transferred protein were stained with a specific primary and secondary antibody (see Section 2.4.3.5 – 2.4.3.6) before they were visualized under UV transilluminator.

2.6.4.2 Densitometry and data analysis

Densitometry analysis was carried out using ImageJ software (Maryland, USA) (Sheffield 2007). All bands were quantified and then normalised with respect to non-treated samples. Internal control β actin was used for normalisation of any variation in replicates.

2.7 Statistical analysis

Statistical analysis was carried out using SPSS V.16 (IBM, USA) and Microsoft Excel (Microsoft Corp. USA). The one-sample Kolmogorov-Smirnov test was performed to determine whether the data for each gene were normally distributed. All data were normally distributed either before or after logarithmic transformation. Differences in hatching rates, gene and protein expression levels in fresh and chilled embryos at different time points and at different developmental stages were analysed using one-way ANOVA along with Tukey's post hoc tests. Where variances were not homogeneous, the Mann-Whitney U analysis was used instead. All data were expressed as mean ± SEM and P values of less than 0.05 were considered to be significant.

CHAPTER 3 DETERMINATION OF *HOX* AND *SOX* GENES EXPRESSION IN DIFFERENT DEVELOPMENTAL STAGES OF ZEBRAFISH (*DANIO RERIO*) EMBRYOS

3.1 Introduction

Gene expression is the process by which amino acid sequence or a gene transcribed into mRNA and mRNA translate into protein. This process is also known as conversion of genotypes into phenotypes. Correct gene and subsequent protein expression is important for development and physiological process as any changes in gene transcript leads to abnormalities of biological functions. The rapid development of zebrafish embryos provides opportunities for investigation of genes essential for developmental processes, the human counterparts of which might be implicated in human disease. In order to study the effect of chilling on gene expression and protein expression in zebrafish embryos, there is a need to understand the pattern of gene expressions throughout the embryo development. Understanding when genes are expressed can facilitate greater understanding of their function, and also allow the genes to be manipulated by gene knockdown in temporally and spatially specific manners (Lan et al. 2009). Selection of genes is also important as selected genes should be developmentally important eg associated with major development processes through embryonic development. In this study, *hox* and *sox* genes were studied to evaluate their patterns of expression in different developmental stages of zebrafish embryo.

The genes studied are *hoxb1b*, *hoxb6b*, *hoxc6a* and *hoxc8a* and they all belong to the homeobox gene group. Homeobox genes are a family of regulatory genes containing a common 183 nucleotide sequence (homeobox) and coding for specific nuclear protein (homeoproteins) that act as a transcription factor, characterized by their role in conferral of segmental identity along anteroposterior (A-P) axis of the body (McGinnis and Krumlauf 1992, de Rosa et al. 1999). *Hoxb1* plays a important role in the development of

hindbrain (Lumsden and Krumlauf 1996). A study also revealed that loss of *hox*b1 results in major alteration to the r4-derived facial (VIIth) motoneurons in hindbrain as *hoxb1b* is important to confer r4 identity (Goddard et al. 1996, Studer et al. 1996, Gaufo et al. 2000). *Hoxb6b* encoded protein functions as a sequence specific transcription factor that is involved in development, including lung and skin, and localized to both the nucleus and cytoplasm. Studies showed that altered expression of this gene or a change in subcellular localization of its protein is associated with some cases of acute myeloid leukaemia and colorectal cancer (Giampaolo et al. 2002). *Hoxc8a* is identified as retinoic acid (RA) induced gene, RA is biologically active lipid mediator with important roles of controlling cell fate and differentiation (Jette et al. 2004).

Studies of *sox* genes, *sox2*, *sox3* and *sox19a* are also important as they are associated with embryonic development. *Sox* family is a group of proteins which appear to regulate cell fate during embryogenesis by functioning as transcriptional factor. The major functions of these genes include sex determination (Polanco and Koopman 2007), stem cell development in embryos (Avilion et al. 2003), neurogenesis (Pevny and Placzek 2005), skeletogenesis (Smits et al. 2001), hematopoiesis (Schilham et al. 1997), cardiogenesis (Akiyama et al. 2004) and angiogenesis (Matsui et al. 2006). *Sox2* encodes a transcriptional factor and well known for its role in maintaining pluripotent stem cells population and differentiation during early embryo development. This protein functions in both epiblast and extraembryonic ectoderm of early mouse embryos to maintain the pluripotency of the stem cells (Avilion et al. 2003). *Sox3* is the most common transcriptional factor in neural development of vertebrates (Zhang et al. 2004). *Sox3* also plays a central role along with *sox1* and *sox2* in the maintenance of neural stem cells (Bylund et al. 2003). A study using gain-of-function and loss-of-function approach in zebrafish revealed that *sox3* regulates both neural fate and differentiation on both central and peripheral nervous system and for normal development of eye and ear (Dee et al.

2008). A study in chicken also showed the role of *sox3* in conjunction with *pax6*, *sox1* and *sox2* in the lens induction (Kamachi et al. 1998). Similar study (Koster et al. 2000) in medaka found that ectopic *sox3* expression leads to ectopic expression of *pax6* and *Eya1* in embryonic endoderm and causes ectopic lens and otic vesicle formation. Vriz et. al. (1996) suggested a role of *sox*19 in the development of central nervous system.

3.2 Experimental Design

The two step reverse transcriptase PCR (RT- PCR) was used to determine patterns of gene expressions in different developmental stages of early zebrafish (*Danio rerio*) embryos. Gene expression studies were performed for *sox2*, *sox3*, *sox19a* and *hoxb1b*, *hoxb6b*, *hoxc6a*, *hoxc8a*. Embryonic stages, 30%, 50%, 75%, 100% epiboly and 6 somites stages were collected and subjected to RNA extraction, cDNA synthesis and PCR. PCR products were analysed using agarose gel electrophoresis. Early stages (30% epiboly – 6 somites stage) were selected to study gene expression based on their chilling sensitivity as it was observed that early stages were more sensitive to chilling then later stages (Zhang and Rawson 1995). Housekeeping gene EF1-α was used as internal control. Housekeeping genes are responsible for basic cellular function and expressed at relatively similar levels across many types of cells. During embryonic development, embryos develop at different rates (Lonergan et al. 2000) and their transcription activity varies between different developmental stages (Mathavan et al. 2005), therefore an internal reference factor is required to ensure that the data collected has acceptable levels of accuracy.

3.3 Results

3.3.1 Determination of *hoxb1b* gene expression in zebrafish (*Danio rerio*) embryos from different developmental stages

Gene expression was evaluated in different developmental stages i.e. 30%, 50%, 75%, 100% and 6 somites. PCR product was analysed on 2 % agarose gel stained with 0.5 µg EtBr staining and visualised under Genosmart UV Gel Documentation system (VWR, UK). Each experiment was carried out with three biological samples and each experiment was done in duplicates. *EF1-α* housekeeping gene was used for each embryonic stage RNA, PCR was also performed to check any variation in RNA. No template control was used to check reagent purity.

Expression of *hoxb1b* was observed from 50% epiboly stage to 6 somites stage (Fig 3.1). Based on the band intensity, expression was increased with development from 50% epiboly to 6 somites stage. The band intensity was observed to be higher in 75% and 6 somites stages than in 50% and 100% epiboly stages. PCR product size was determined using Hyperladder™ V which was 107 bp for *hoxb1b* gene.

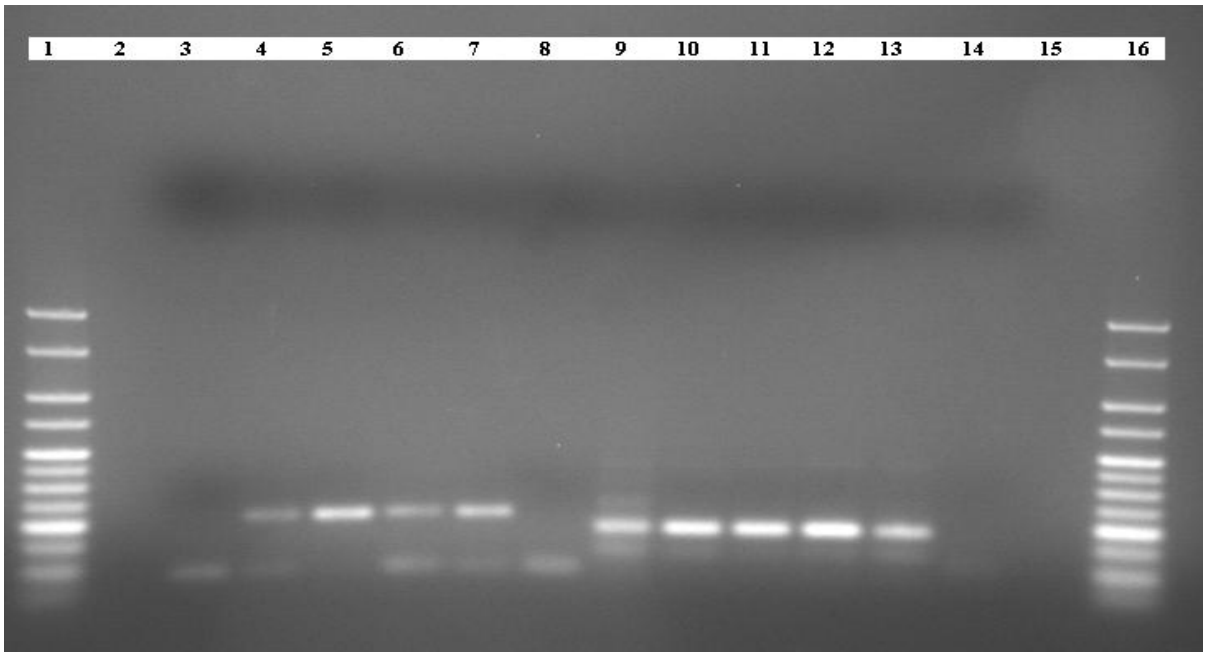


Fig 3.1 Agarose gel electrophoresis (2% agarose) of *hoxb1b* PCR product. *EF1-α* was used as an internal control. Lanes 3-7 show *hoxb1b* PCR product of 30%, 50%, 75%, 100% epiboly and 6 somites respectively. Lanes 9-13 show *EF1-α* PCR product of 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. No template controls for *hoxb1b* gene (lane 8) and *EF1-α* (lane 14) were also used in this experiment. Hyperladder™ V (25 bp ladder) (Lane 1 and 16) was used to determine PCR product size *hoxb1b* – 107 bp and *EF1-α* – 87 bp size.

3.3.2 Determination of *hoxb6b* gene expression in zebrafish (*Danio rerio*) embryo from different developmental stages

Expression of *hoxb6b* was observed in gastrula stages (75% and 100% epiboly) and 6 somites stage (Fig 3.2). Expression was increased generally with embryonic development as band intensity of *hoxb6b* increased gradually from 75% epiboly to 6 somites stage. PCR product size was determined using Hyperladder™ V which was 178 bp for *hoxb6b* gene.

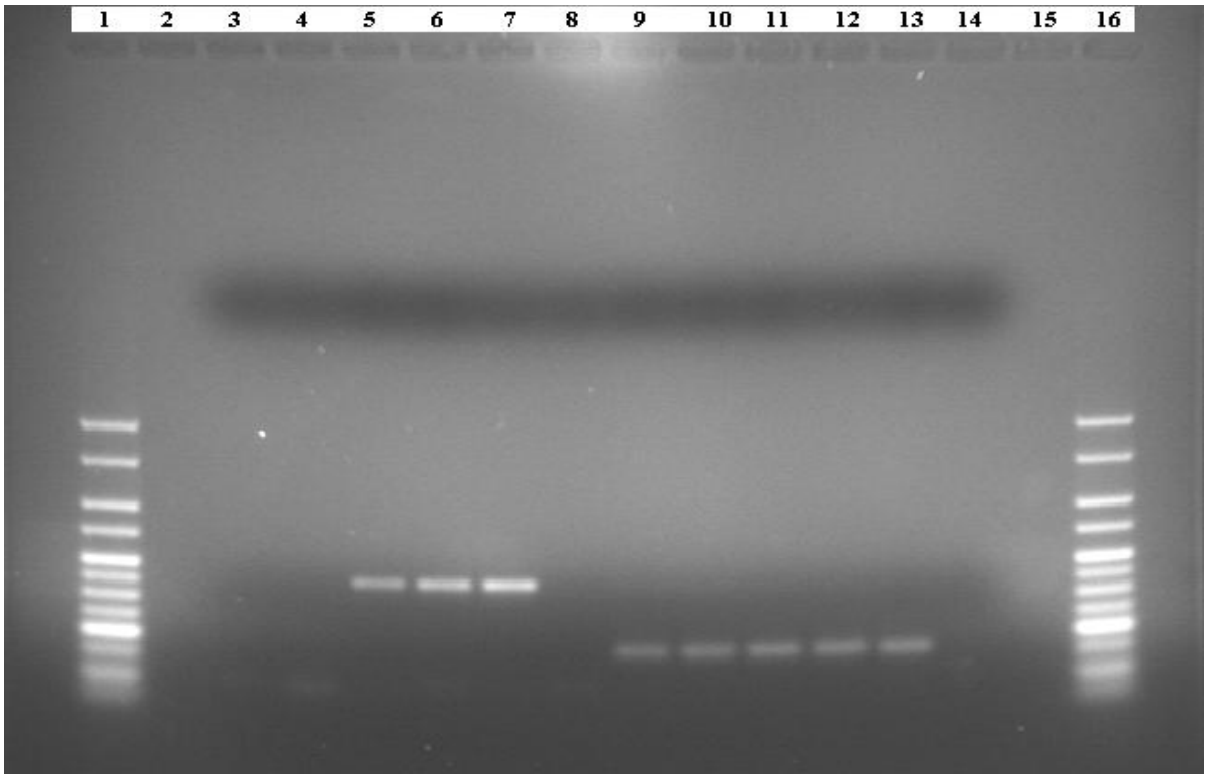


Fig 3.2 Agarose gel electrophoresis (2% agarose) of *hoxb6b* PCR product. *EF1-α* was used as the internal control. Lanes 3-7 show *hoxb6b* PCR product for 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. Lanes 9-13 show *EF1-α* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. No template controls for *hoxb6b* gene (lane 8) and *EF1-α* (lane 14) were also used in this experiment. Hyperladder™ V (25 bp ladder) (Lane 1, 16) was used to determine PCR product size *hoxb6b* – 178 bp and *EF1-α* – 87 bp size.

3.3.3 Determination of *hoxc6a* gene expression in zebrafish (*Danio rerio*) embryo from different developmental stages

There was no *hoxc6a* expression found in 30%-, 50%- and 75% epiboly stages. *Hoxc6a* started to express from bud stage although the band intensity was low. The product size for PCR *hoxc6a* gene (171bp) was determined using Hyperladder ™ V.

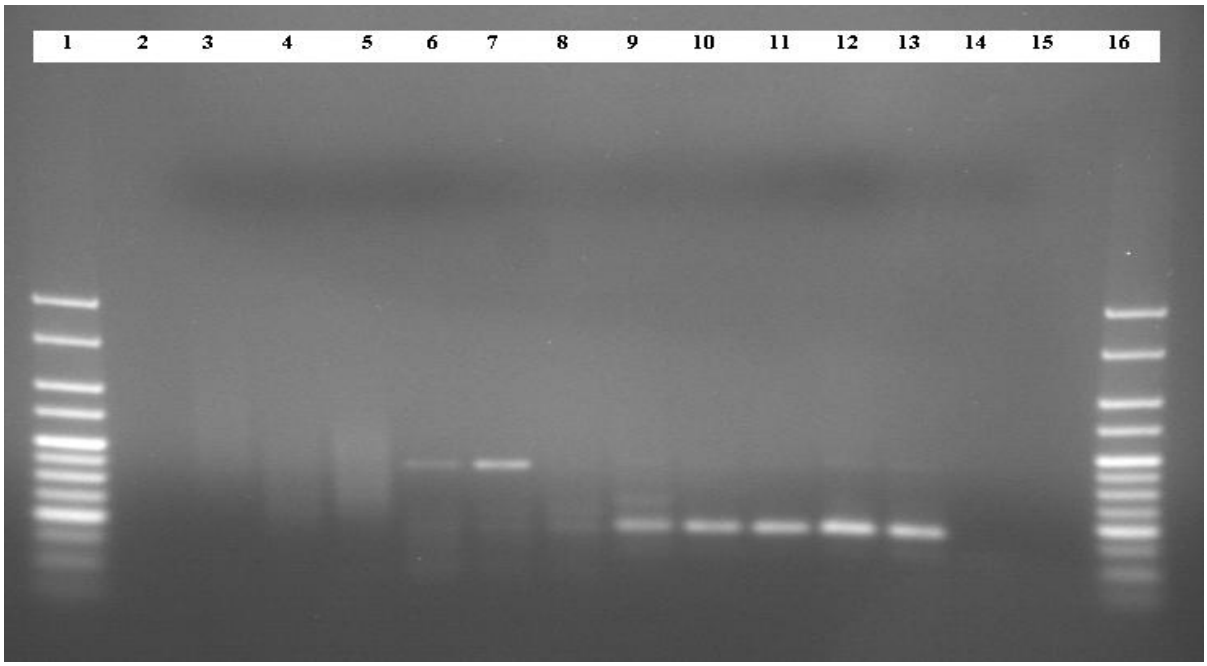


Fig 3.3 Agarose gel electrophoresis (2% agarose) of *hoxc6a* PCR product. *EF1-α* was used as the internal control. Lanes 3-7 show *hoxc6a* PCR product for 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. Lanes 9-13 show *EF1-α* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. No template controls for *hoxc6a* gene (lane 8) and *EF1-α* (lane 14) were also used in this experiment. Hyperladder™ V (25 bp ladder) (Lane 1, 16) was used to determine PCR product size *hoxc6a* – 171 bp and *EF1-α* – 87 bp size.

3.3.4 Determination of *hoxc8a* gene expression in zebrafish (*Danio rerio*) embryo from different developmental stages

There was no *hoxc8a* expression found in 30% and 50% epiboly stages (Fig 3.4). *Hoxc8a* started to express from 75% epiboly stage. Gene expression was detected from 75% epiboly stage to 6 somites stage. The product size for PCR *hoxc6a* gene (171bp) was determined using Hyperladder ™ V.

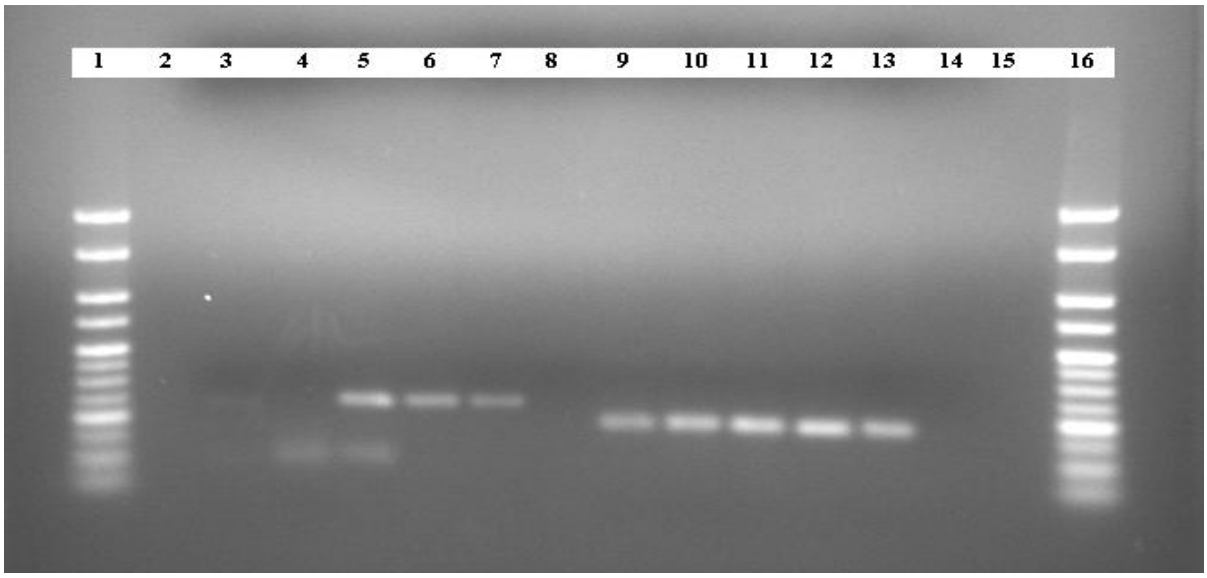


Fig 3.4 Agarose gel electrophoresis (2% agarose) of *hoxc8a* PCR product. *EF1-α* was used as the internal control. Lanes 3-7 show *hoxc6a* PCR product for 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. Lanes 9-13 show *EF1-α* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. No template control for *hoxc8a* gene (lane 8) and *EF1-α* (lane 14) were also used in this experiment. Hyperladder™ V (25 bp ladder) (Lane 1, 16) was used to determine PCR product size *hoxc8a* – 114 bp and *EF1-α* – 87 bp size.

3.3.5 Determination of *sox2* gene expression in zebrafish (*Danio rerio*) embryo from different developmental stages

Gene expression was evaluated in different developmental stages i.e. 30%, 50%, 75%, 100% and 6 somites. PCR product was analysed on 2 % agarose gel stained with 0.5 µg EtBr and visualised under Genosmart UV Gel Documentation system (VWR, UK). Each experiment was done with three biological samples and each experiment was done in duplicates. *EF1-α* housekeeping gene was used for each embryonic stage. No template control was also used to check reagent purity.

Sox2 gene expression was observed in all tested embryonic stages (Fig 3.5). Increase in band intensity was observed as the embryonic development progressed from 30% epiboly stage to 6 somites stage. PCR product size was also determined using Hyperladder™ V which was 171bp for *sox2* gene.

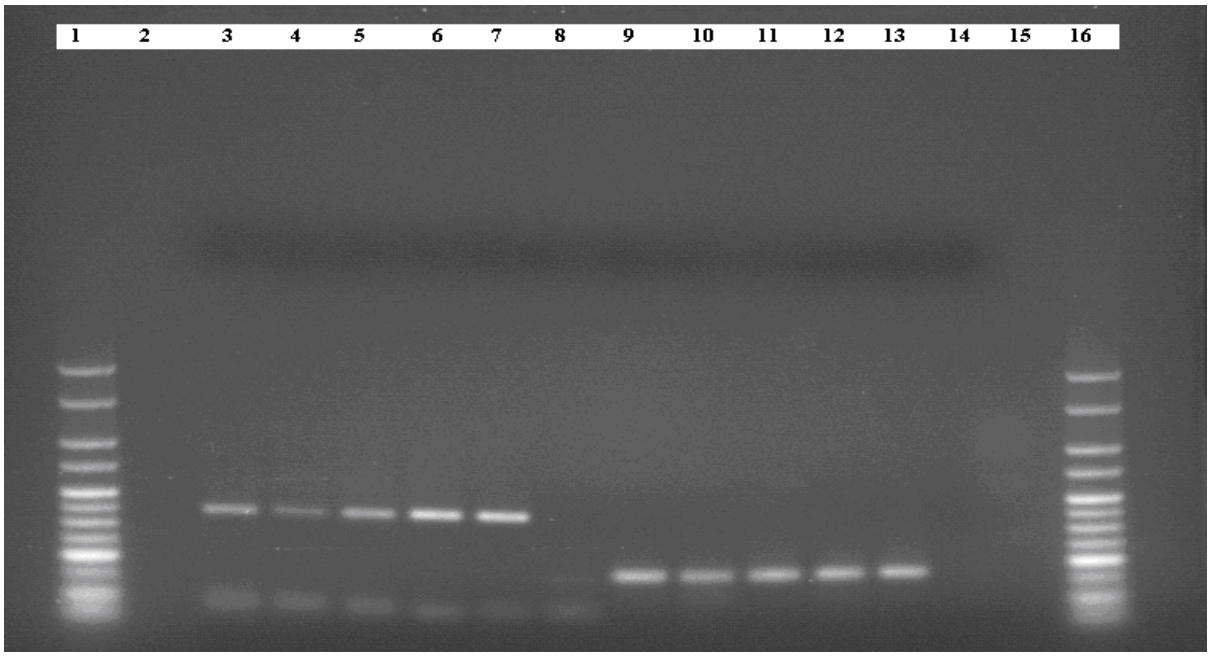


Fig 3.5 Agarose gel electrophoresis (2% agarose) of *sox2* PCR product. *EF1-α* was used as the internal control. Lanes 3-7 show *sox2* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. Lanes 9-13 show *EF1-α* PCR

product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. No template control for *sox2* gene (lane 8) and *EF1-α* (lane 14) were also used in this experiment. Hyperladder™ V (25 bp ladder) (Lane 1, 16) was used to determine PCR product size *sox2* – 171 bp and *EF1-α* – 87 bp size.

3.3.6 Determination of *sox3* gene expression in zebrafish (*Danio rerio*) embryo from different developmental stages

Consistent *sox3* gene expression was observed throughout 30% to 6 somites stages (Fig 3.6). Strong intensity band of *sox3* was observed throughout tested developmental stages. PCR product size was also determined using Hyperladder™ V which was 182bp for *sox3* gene.

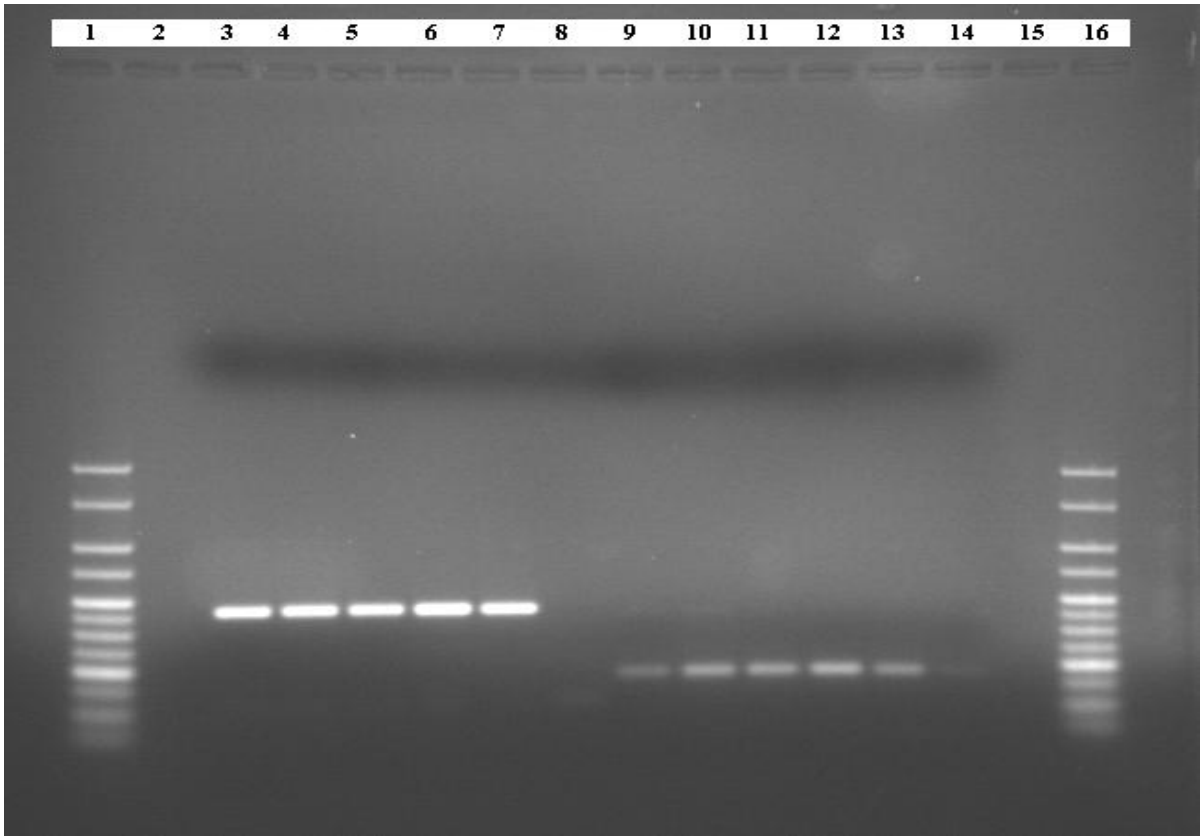


Fig 3.6 Agarose gel electrophoresis (2% agarose) of *sox3* PCR product. *EF1-α* was used as the internal control. Lanes 3-7 show *sox3* PCR product from 30%, 50%,

75%, 100% epiboly and 6 somites stages respectively. Lanes 9-13 show *EF1-α* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. No template control for *sox3* gene (lane 8) and *EF1-α* (lane 14) were also used in this experiment. Hyperladder™ V (25 bp ladder) (Lane 1, 16) was used to determine PCR product size *sox3* – 182 bp and *EF1-α* – 87 bp size.

3.3.7 Determination of *sox19a* gene expression in zebrafish (*Danio rerio*) embryo from different developmental stages

Sox19a gene expression was found in 30% to 6 somites stages (Fig 3.7). *Sox19a* band intensity was increased along with embryonic development. PCR product size was also determined using Hyperladder™ V which was 128bp for *sox19a* gene.

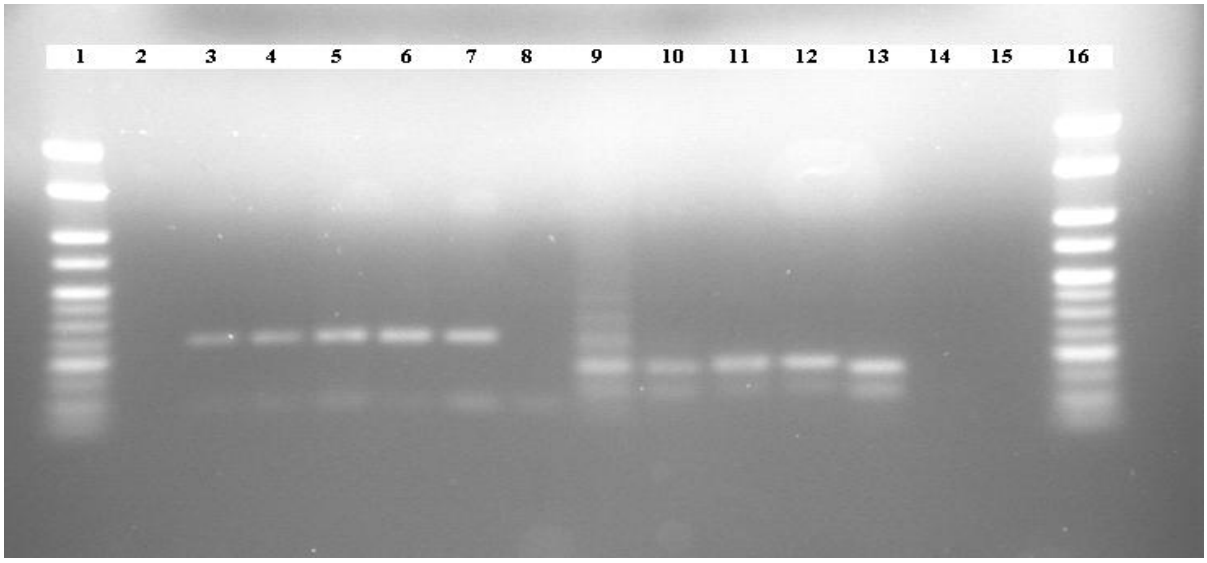


Fig 3.7 Agarose gel electrophoresis (2% agarose) of *sox19a* PCR product. *EF1-α* was used as the internal control. Lanes 3-7 show *sox19a* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites stages respectively. Lanes 9-13 show *EF1-α* PCR product from 30%, 50%, 75%, 100% epiboly and 6 somites respectively. No template control for *sox19a* gene (lane 8) and *EF1-α* (lane 14) were also used in this

experiment. Hyperladder™ V (25 bp ladder) (Lane 1, 16) was used to determine PCR product size *sox19a* – 128 bp and *EF1-α* – 87 bp size.

3.3.8 Overview of gene expression profile for *hox* and *sox* gene family

A summary of gene expressions for all *hox* and *sox* genes are presented in Fig 3.8. *Sox* genes expressed in all tested embryonic stages (30% epiboly to 6 somites stages). However some of the *hox* genes such as *hoxb6b* and *hoxc6a* genes only started to express from 75% epiboly stage whilst *hoxc8a* did not start to express until 100% stage.

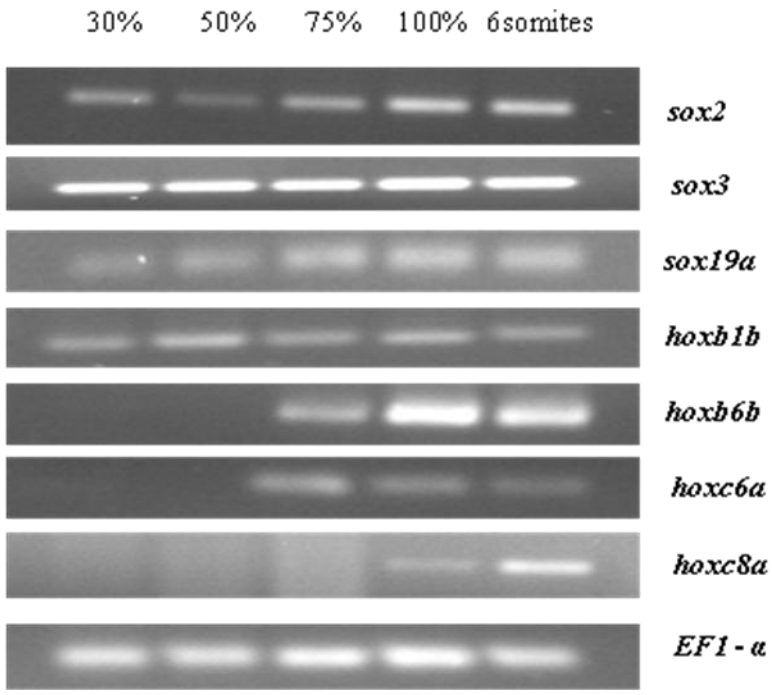


Fig 3.8 Gene expression data from different developmental stages eg 30% epiboly to 6 somites stages. Each Figure represents a different gene. EF1 – α was used as control for all experiments. No template control also used for each experiment.

3.4 Discussion

The present study investigated the expressions of *hox* and *sox* genes in different developmental stages of zebrafish embryos. The expression profiles were produced for each gene at the different developmental stages. *Hoxb1b* was found to express from 30% epiboly stage. This result is in line with a study in zebrafish which also suggested that *hoxb1b* started to express in early gastrulation from shield stage (McClintock et al. 2001). Due to specificity and sensitivity of PCR technique, *hoxb1b* was detected earlier than shield stage in presented study compare to McClintock et. al, where they used immunohistochemistry and *in situ* hybridization techniques. Another study in zebrafish showed that *hoxb1b* expressed throughout the spinal cord, caudal to the hindbrain in 24 hpf embryos (Dee et al. 2008). Studies in mouse in early somites stage showed that *hoxb1* gene was expressed in the posterior half of the embryo by the primitive steak stage. Same study also showed that *hoxb1* expression became divided into prospective rhombomere4 domain and the posterior half of 4-6 somites embryo (Conlon and Rossant 1992, Murphy and Hill 1991). In present study, *hoxb1b* gene activated as early as 30% epiboly stage, however it may not be translated until somites stages when they start contributing to nervous system development. Mutant analysis have shown that mouse *Hoxa1* is required for normal r4 (rhombomere 4) and r5 (rhombomere 5) formation (Chisaka et al. 1992) and that *Hoxa1* is required to set the appropriate anterior boundary of *Hoxb1* expression (Barrow et al. 2000). Once *Hoxb1* is activated in r4, its activation is maintained by auto-regulation (Pöpperl et al. 1995). More studies are needed to confirm the relationship of *hoxa1* and *hoxb1* genes. In the present study, gene expression of *hoxb6b* was found to start from 75% epiboly stage. This contradicted the findings by Thisse and Thisse which showed the expression of *hoxb6b* in zebrafish started from 50% epiboly stage (Thisse and Thisse 2005). The possible reason of this is that Thisse and Thisse's study was carried out using *in situ* hybridization and the experiment was only performed once (Thisse and

Thisse 2005). In the present study all experiments were performed three times with duplicates and PCR was used to study gene expression. Most other previous studies showed that *hoxb6b* expressed later and mainly from somites to prim stages in zebrafish (Krishnan et al. 2008, Horsfield et al. 2007, diIorio et al. 2007). Zebrafish *hoxc6a* gene was found to express from 75% epiboly stage in the present study. The more sensitive PCR technique used here accounts for the detection of low levels of gene expression at an earlier stage than previously reported. In mouse embryo *hoxb6* expression was detected at 11.5 days post copulation in the CNS (Central nervous system), spinal ganglia, prevertebrae and proximal region of the hind limb (Schughart et al. 1988, Graham et al. 1991, Eid et al. 1993). Another study in zebrafish showed expression of *hoxc6a* in prim-25 stage in mesenchyme pectoral fin (Gibert et al. 2006, Lan et al. 2007). These study indicated that the roles of *hox* genes are not restricted to the nervous system development. This could be a reason of early expression of *hoxc6a* and *hoxb6b* in developing embryos and their early expression could have functional implication of posterior development of zebrafish. However, more studies are needed to confirm this hypothesis. The results from the present study showed that *Hoxc8a* started to express from 100% epiboly stage. In contrary, Nadauld et al (2004) found that *hoxc8a* expressed in early stage 50% and the expression intensity peaked by bud stage (100% epiboly) using morpholino analysis in zebrafish embryos. In Nadauld et al's study, 50% epiboly zebrafish embryos were incubated with retinoic acid to produce zRDHB morphants. In the present study, fresh embryos were used to study expression of *hoxc8a.* It is clear from this the present study that PCR technique is very accurate and sensitive method for detecting gene expression in zebrafish embryos. Gene expression levels can also be quantified by using quantitative RT PCR.

In the present study *sox2* gene expressions were also investigated in 30%, 50%, 75%, 100% epiboly and 6 somites stage zebrafish embryos. The results showed that *sox2*

started to express from 30% epiboly stage although the expression level was low (low band intensity). However the expression intensity increased as embryo development progressed. *Sox3* and *sox19a* genes followed the similar pattern of expression; however *sox3* expression intensity was higher when compared to *sox2* and *sox19a genes*. These results are in line with the previous results obtained in zebrafish where *sox2* gene expression initiated at 30% epiboly stage (Okuda et al. 2006a). The same study also suggested that *sox3* transcript was usually detected from 32-cell stage and mRNA levels of *sox3* and *sox19a* began to increase from 100-cell stage. Study in Medaka showed similar pattern of expression as *sox3* initially expressed at gastrulation stage (50% epiboly) in the ectoderm (Koster et al. 2000). This study also showed that *sox3* expression was restricted to the neuroectoderm and placode of the sensory organs. Study in mouse suggested that *sox2* and *sox3* were expressed throughout epiblast (equivalent to early gastrulation) and in a band of extra embryonic boundary (Wood and Episkopou 1999). The same study also showed that *sox2* and *sox3* expression was restricted to the anterior ectoderm while extra embryonic expression limited to the chorion. *Sox19a* is expressed most widely in the central nervous system during epiboly which is supported by broad expression of *sox2* in early embryo of mouse and chicken (Uwanogho et al. 1995, Rex et al. 1997). There is very limited information in the literature available on *sox19a* gene expression in early development stages of zebrafish embryos.

3.5 Summary

The present study provided useful information on patterns of *sox* and *hox* gene expression in different stages of zebrafish embryos. It is evident from the present study that different *hox* and *sox* genes showed different expression patterns in zebrafish embryos at different development stages. It also evident from this study PCR is a very sensitive method for studying gene expression and detect gene transcript. *Sox* genes were found to express in all tested developmental stages, therefore this groups of genes (*sox2,*

sox3 and *sox19a*) will be used to study the effect of chilling on *sox* gene expression in early stage zebrafish embryos using PCR techniques.

CHAPTER 4 STUDIES ON EFFECT OF SHORT TERM CHILLING ON SOX GENES AND PROTEIN EXPRESSION IN ZEBRAFISH (*DANIO RERIO*) EMBRYO

4.1 Introduction

Fish embryos have not been cryopreserved due to their structural limitations. One limiting factor is their susceptibility to chilling injury, eg. cells are injured by cooling temperature around 0 °C in zebrafish embryos and especially at early developmental stages (Zhang and Rawson 1995, Hagedorn et al. 1997). In other fish species, including red drum (Gwo et al. 1995), fathead minnow (Cloud et al. 1988) and carp (Dinnyés et al. 1998), it has also been reported that embryos at some stages are also sensitive to chilling. However, these studies provide no information on more subtle effect of chilling at molecular level, such as gene expression. The analysis of difference in the level of mRNA transcript following chilling may help us to understand developmental stage specific chilling sensitivity and the adaptations needed for low temperature survival. Quantitative analysis of mRNA abundance of certain genes would provide useful information on mechanisms associated with chilling injury.

Limited data is currently available on the effect of cryopreservation on gene expression. For example, Tachataki et. al. (2003) demonstrated that cryopreservation does alter the normal pattern of TSC2 gene expression during preimplantation development of human embryo. Similar study on human oocytes also demonstrated down regulation of genes responsible for cell cycle and oocytes development competence following slow cooling and freezing (Monzo et al. 2012). Cryopreservation has also been shown to affect DNA stability of rainbow trout sperm (Labbe et al. 2001) and in zebrafish blastomeres when cryopreserved using suboptimal protocol (Kopeika et al. 2005). However, these studies provide no information on the effect of chilling on gene expression in fish embryos. Although Lin et al. (2009b) studied *pax* gene expression levels after zebrafish embryos

were chilled for 24 h at 1 ℃, the impact of chilling, warming and recovery has not been studied.

This study was aimed to investigate the levels of expression of *sox2, sox3* and *sox19a* genes in zebrafish embryos following chilling at 0 ℃ and also after chilling and subsequent warming. In these study 30 min chilling could demonstrate cold shock chilling injury as temperature change of embryos from 27±1 ℃ to 0℃ was very drastic. These studies were carried out to assess effect of cold shock (initially 30 min) and indirect chilling up to 180 min and warming on gene expression in 50% epiboly stage embryos.

4.2 Experimental design

Based on the gene expression profile obtained from the previous chapter, Embryo from different development stages were chilled at 0° C for up to 3 h and warmed at 27±1 ℃, embryo hatching rates were then monitored. Chilling period was selected based on previous study which showed chilling sensitivity decreased significantly after 5 h of chilling (Zhang and Rawson 1995). Following embryo hatching studies, RT PCR was used in subsequent experiments in order to compare the level of gene expression of *sox2, sox3* and *sox19a* in fresh and 3 h chilled embryos. Selection of *sox* genes were based on results obtained in previous chapter where *sox* genes were found to express in all tested developmental stages. Gene expression profiles were also investigated following cold shock (30 min chilling), indirect chilling up to 180 min and subsequent warming in zebrafish embryos.

4.3 Results

4.3.1 Studies of the effect of chilling on hatching rate of zebrafish (*Danio rerio*) embryos

Based on the gene expression data where *sox* genes found to express in all tested developmental stages, 50%, 75% and 100% epiboly stages were selected for studying the effect of chilling on embryo hatching rates. The results from these experiments showed that there were no significant differences in embryo hatching rates between the control samples kept at 28 °C and chilled samples (chilled for 30, 60, 120 and 180 min at 0 °C) for either 50% or 100% epiboly stage embryos (Fig 4.1). Significant decreases in hatching rates were found in 75% epiboly stage embryos (78% ± 5%) after they were chilled for 180 min at 0 °C when compared with those of controls (96% ± 2%) (Fig 4.1).

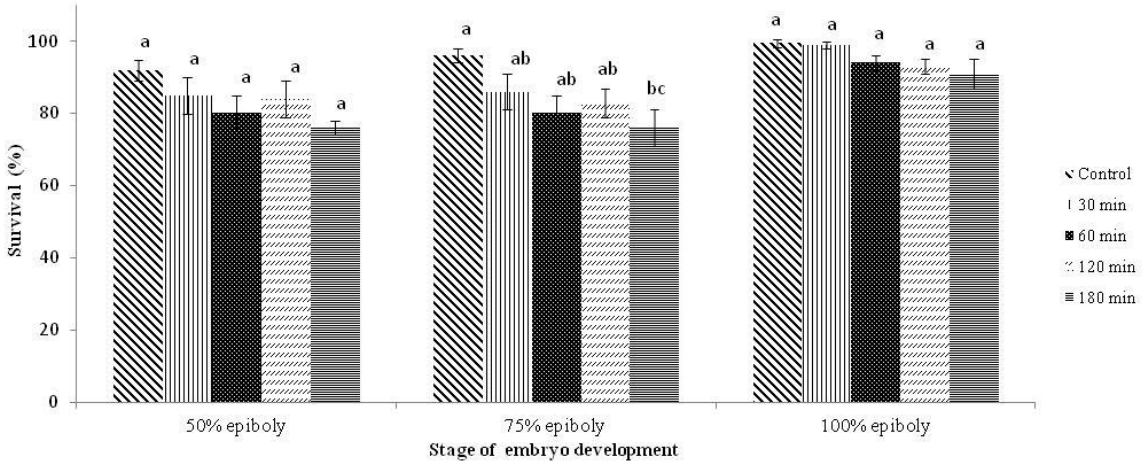
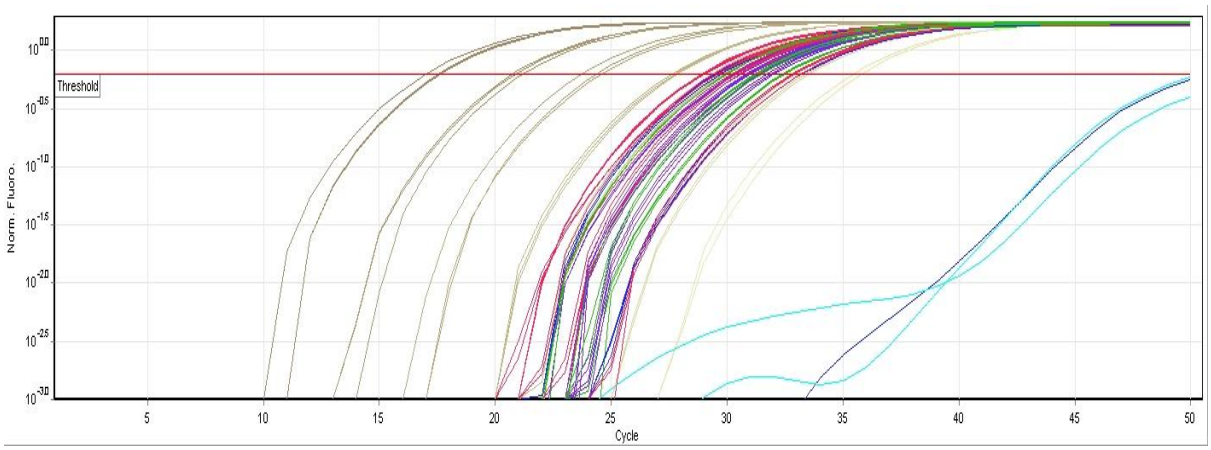


Fig 4.1 Effect of chilling on hatching rate in different developmental stages of zebrafish embryos: bars represent hatching rates of zebrafish embryos at different developmental stages after chilling at 0 °C for different time periods (0–180 min), followed by incubation at 28 °C for 3 days. Error bars represent the standard error of the mean (SEM). Different letters show significant differences ($p < 0.05$) between chilling time periods within the same developmental stage (n=9).

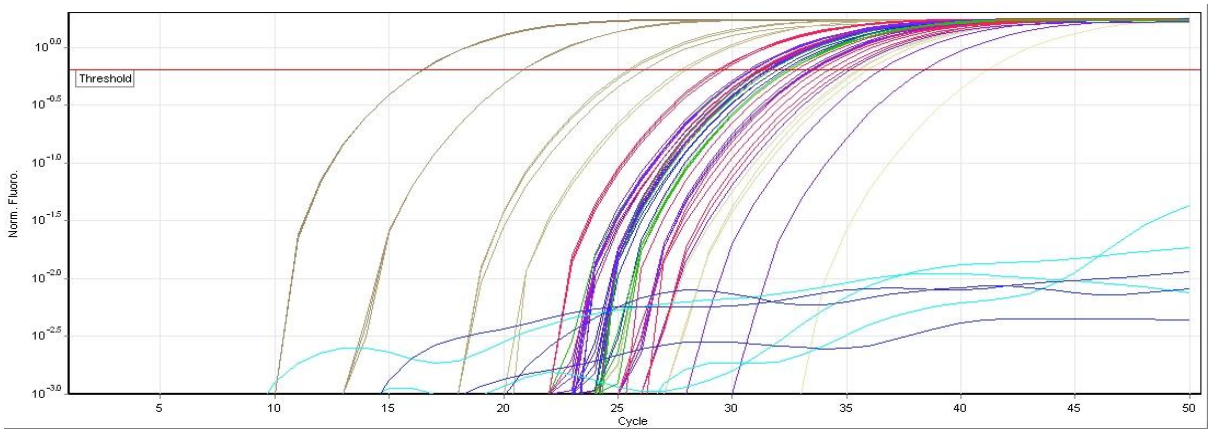
4.3.2 Fluorescence measurements melt curves and standard curve evaluation of *sox* genes

Effect of chilling on *sox* gene expression was carried out using real time PCR. Fluorescence levels within each tube were measured at the end of each cycle (Fig 4.2). Amplification efficiency and a correlation coefficient from standard curve of cDNA at different 10-fold dilution were also determined for each sox genes using accompanied real time PCR software (Fig. 4.2). All the target genes have acceptable efficiencies and correlation coefficients from 0.7 to 1.1 (Pfaffl 2003a). Melting curve was performed to ensure that the primers amplified a single product. All of the target genes had a single peak for melting curve (Fig 4.4), indicated no contamination in samples.

sox2



sox3



sox19a

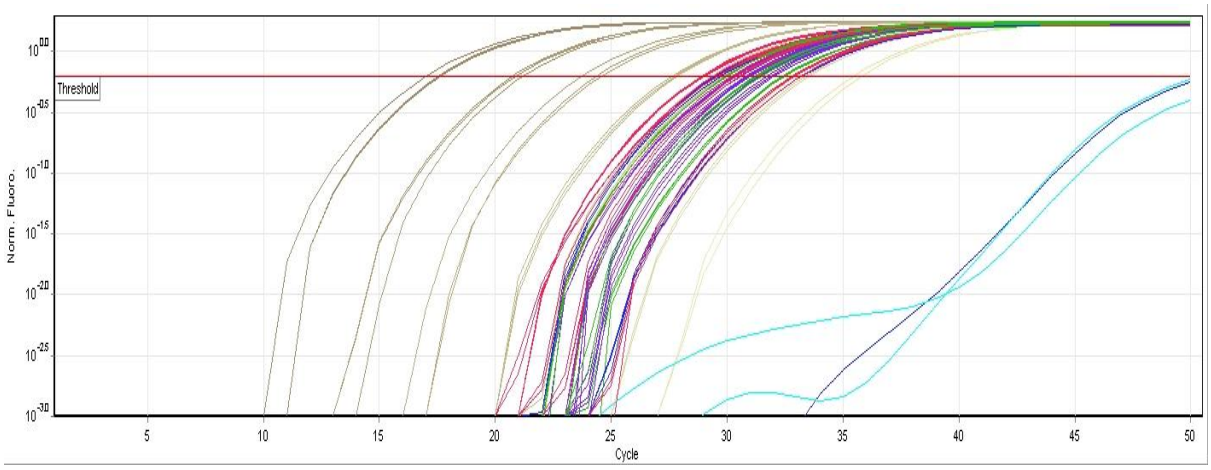
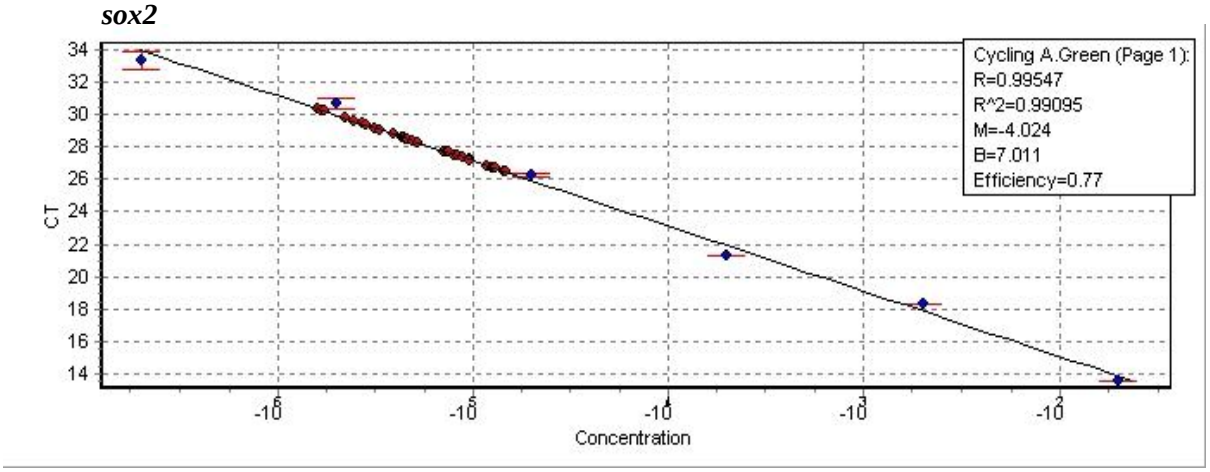
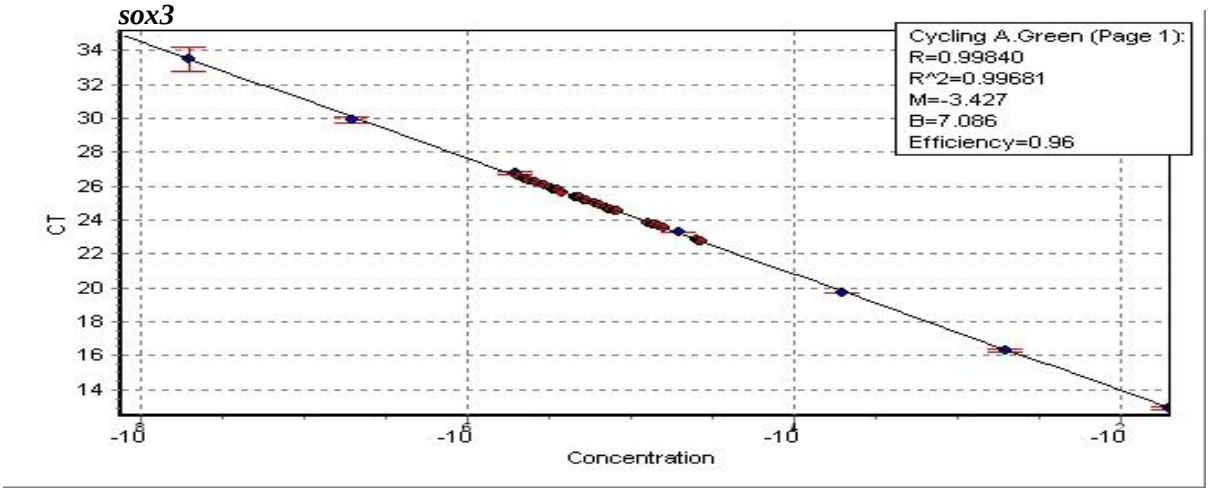


Fig 4.2 Example of fluorescence measurement of *sox2, sox3* and *sox19a* genes. Fluorescence graphs shows fluorescence measurements obtained for standard (brown), negative control (light blue) and other colours related to embryos samples.

sox2



sox3



sox19a

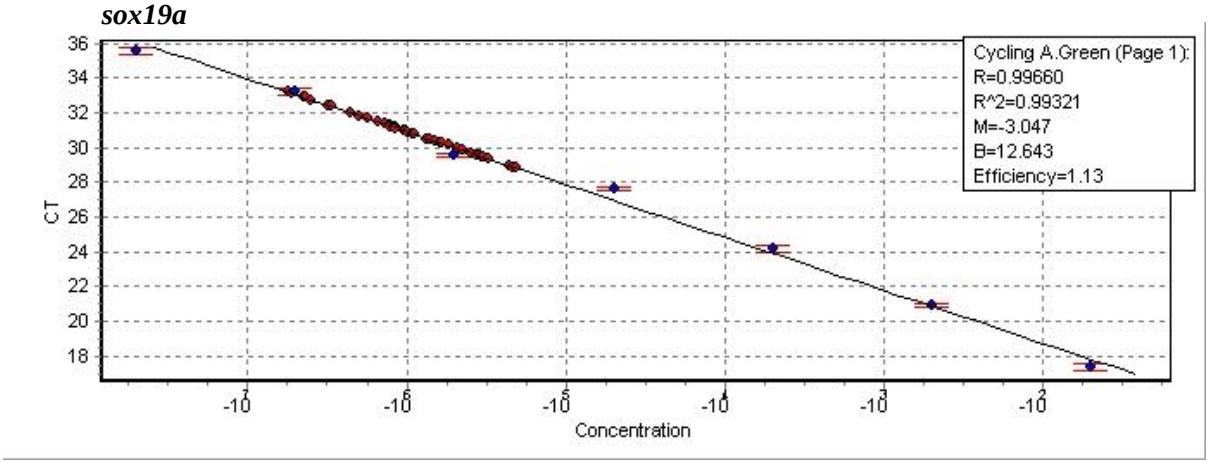
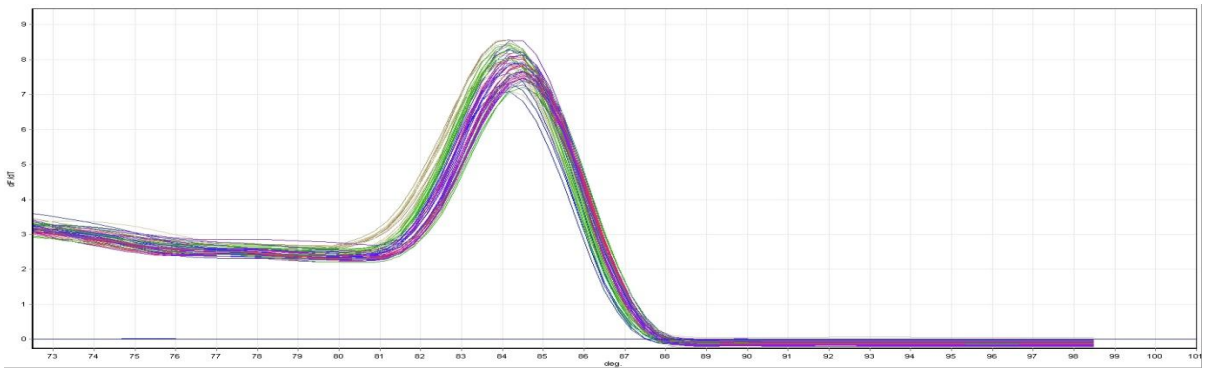


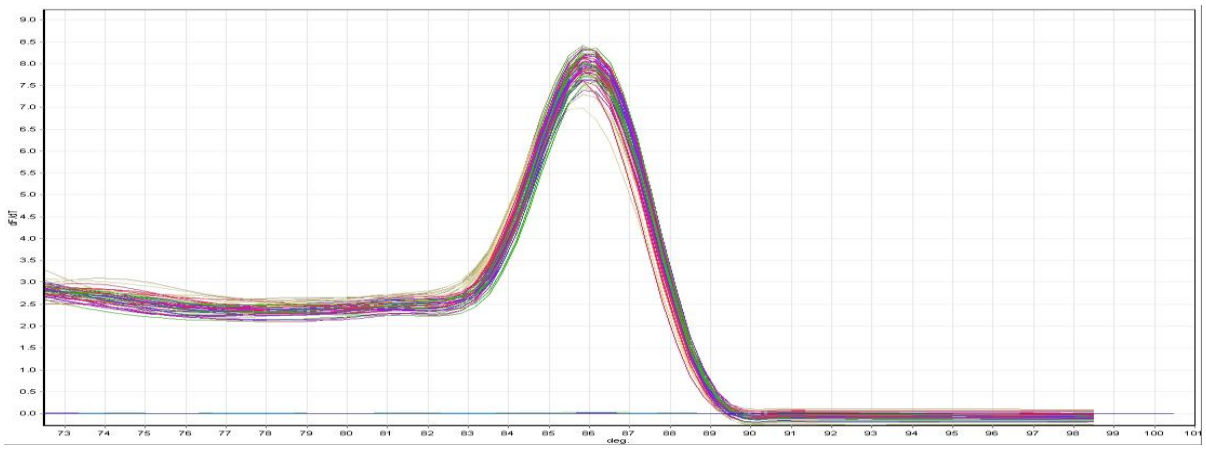
Fig 4.3 Example of standard curves for *sox2*, *sox3* and *sox19a* genes. Mean ± SEM Ct values are plotted against the log concentration of the standards for 10-fold dilutions. Each standard curve has an R^2 value > 0.99, due to an equal number of cycles separating standards of 10-flod dilution concentration difference. Each curve

has an Efficiency value within acceptable range of between 0.7 and 1.1 (Pfaffl 2003a).

sox2



sox3



sox19a

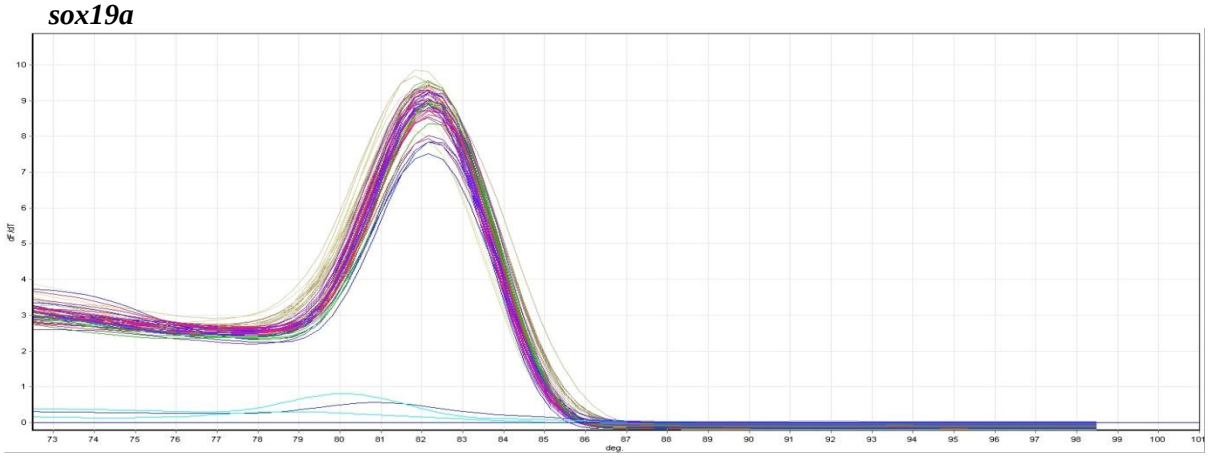


Fig 4.4 Example of melting curve profile for *sox2, sox3* and *sox19a* genes. Standard (brown), negative control (blue) and samples (other colours) melt curve from different time points. All sample produced same peak, therefore sample have no contamination, mispriming and primer-dimers.

4.3.3 Studies of the effect of chilling on gene expression in zebrafish (*Danio rerio*) embryos using quantitative PCR (qPCR)

Epiboly stage embryos (50%) were selected for these studies as there was no effect of chilling on hatching rate found at this stage (see section 4.3.1) when they were chilled for up to 180 min at 0 °C. Studies on the effect of chilling on sox gene expression showed that in the control group, *sox2* transcripts were relatively stable throughout the 180 min period (Fig 4.5a). *Sox2* transcripts in chilled embryos (Fig 4.5a) remained at similar levels as controls initially but significantly decreased after 60 min when compared with the controls. *Sox3* gene transcripts were also relatively stable in the control group at 28 °C (Fig. 4.5b). Although *sox3* gene transcript levels appeared to be relatively stable following 60 min chilling at 0 °C, they were also significantly lower than in the controls. For *sox19a*, similar patterns of gene expression were found in both control and chilled embryos and no significant differences were found between the two groups following different treatment time periods (Fig. 4.5c).

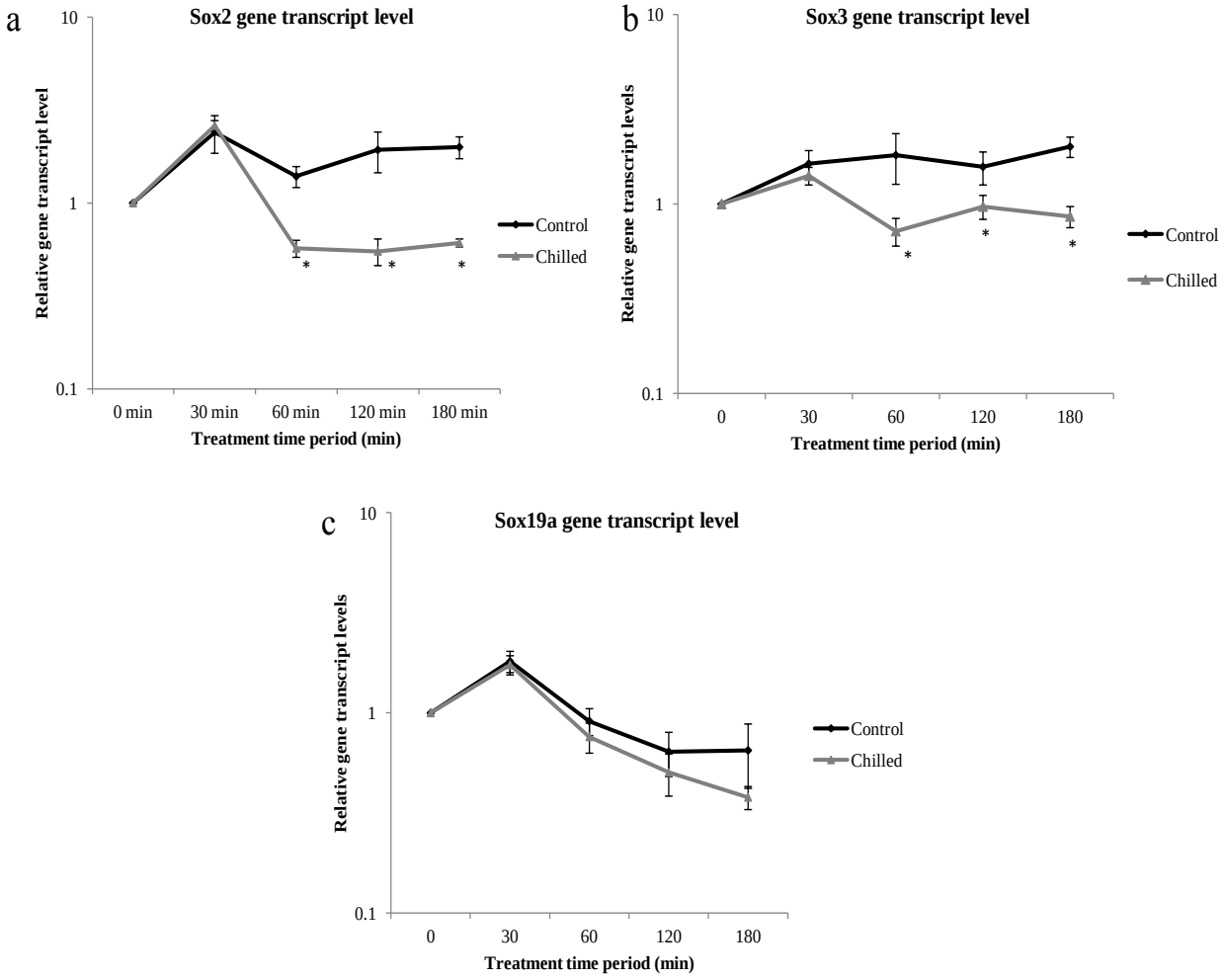


Fig 4.5 Effect of chilling on *sox* gene expression in zebrafish embryos: gene expression profiles for *sox2* (a), *sox3* (b) and *sox19a* (c) for embryos chilled for up to 180 min at 0 °C and non chilled control embryos at 28 °C, assessed by reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisks (∗) indicate significant differences between control and chilled groups at the same time point. $p < 0.05$ was considered to be significant (n=9).

4.3.4 Studies on expression of *sox* genes following chilling and warming in zebrafish (*Danio rerio*) embryos using qPCR

Based on the data obtained from the previous experiments, further studies were carried out to investigate if gene expressions were recovered after warming. In these experiments, embryos were chilled for 30 min or 60 min at 0°C before warming for up to 180 min at 27 ± 1°C.

In control embryos for the 30 min chilling experiment, the expression levels of *sox2* (Fig. 4.6a) remained stable during the incubation period. Gradual increases in expressions were observed in embryos that had been chilled, resulting in significantly higher expressions than controls by 150 min. Expressions of *sox3* (Fig. 4.6b) and *sox19a* (Fig. 4.6c) remained stable in control samples. However chilled samples showed a short burst of expression of both these genes at 60 min which subsequently decreased back to control levels.

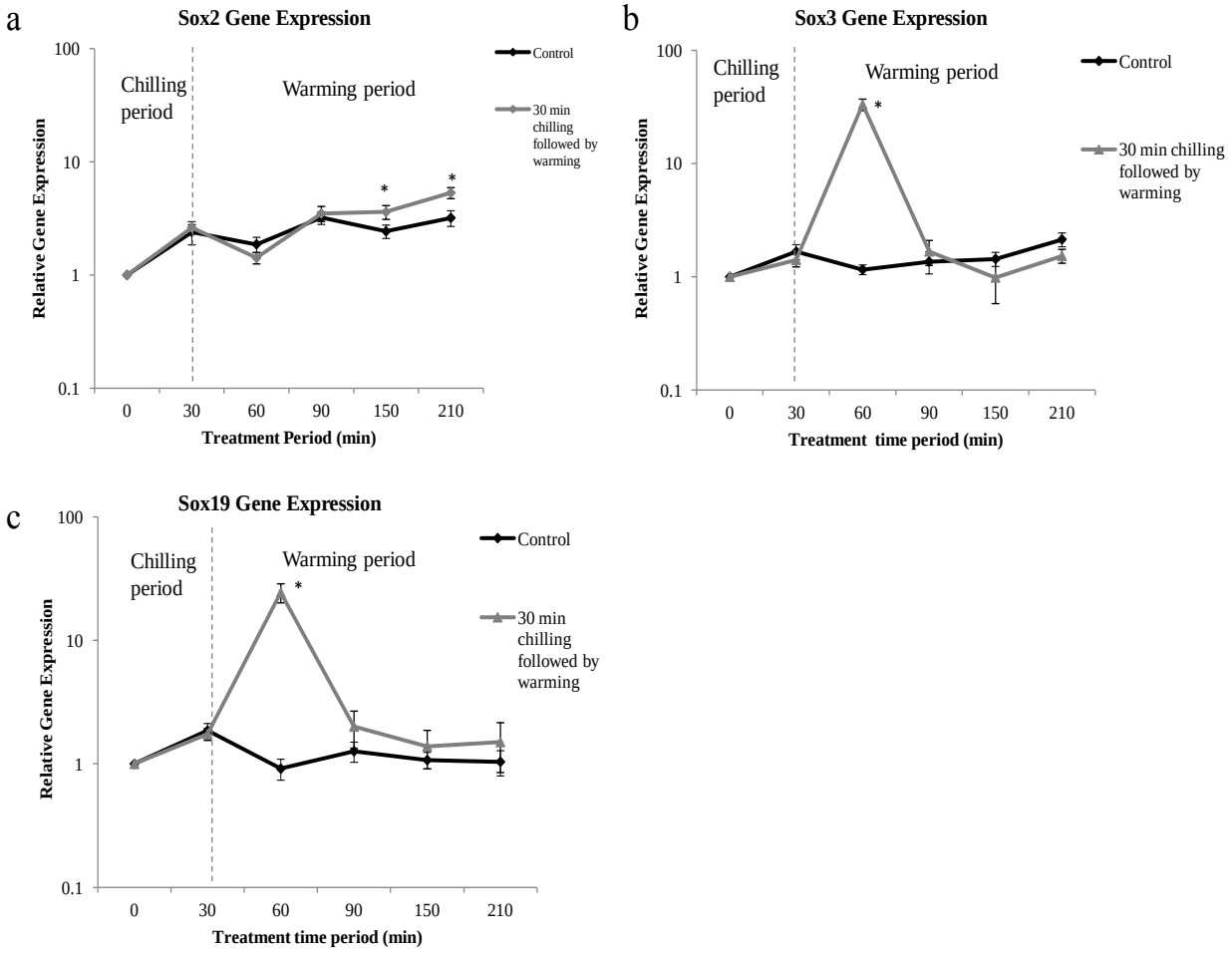


Fig 4.6 Effect of 30 min chilling on sox gene expression during warming: gene expression profiles for *sox2* (a), *sox3* (b) and *sox19a* (c) for chilled embryos and non chilled controls, assessed by reverse transcriptase qPCR. The chilled embryo samples were incubated at 0°C for 30 min and then warmed at 28± 1°C for up to 180 min. Controls were maintained at 27 ±1 °C for the entire 210 min. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisks (*) indicate significant differences between control and chilled-warmed groups at the same time point. $p < 0.05$ was considered to be significant (n=9).

Control embryos for the 60 min experiment followed a similar pattern of expression to the 30 min chilling where *sox2* (Fig. 4.7a), *sox3* (Fig. 4.7b) and *sox19a* (Fig. 4.7c) remained relatively stable throughout the treatment period. However, in embryos that had been chilled for 60 min, expressions of both *sox2* and *sox3* increased to become significantly higher than in controls until 180 min, after which levels reduced to control levels. The opposite situation was observed for *sox19a* where levels initially decreased significantly compared to those of the control before returning to control levels by 180 min.

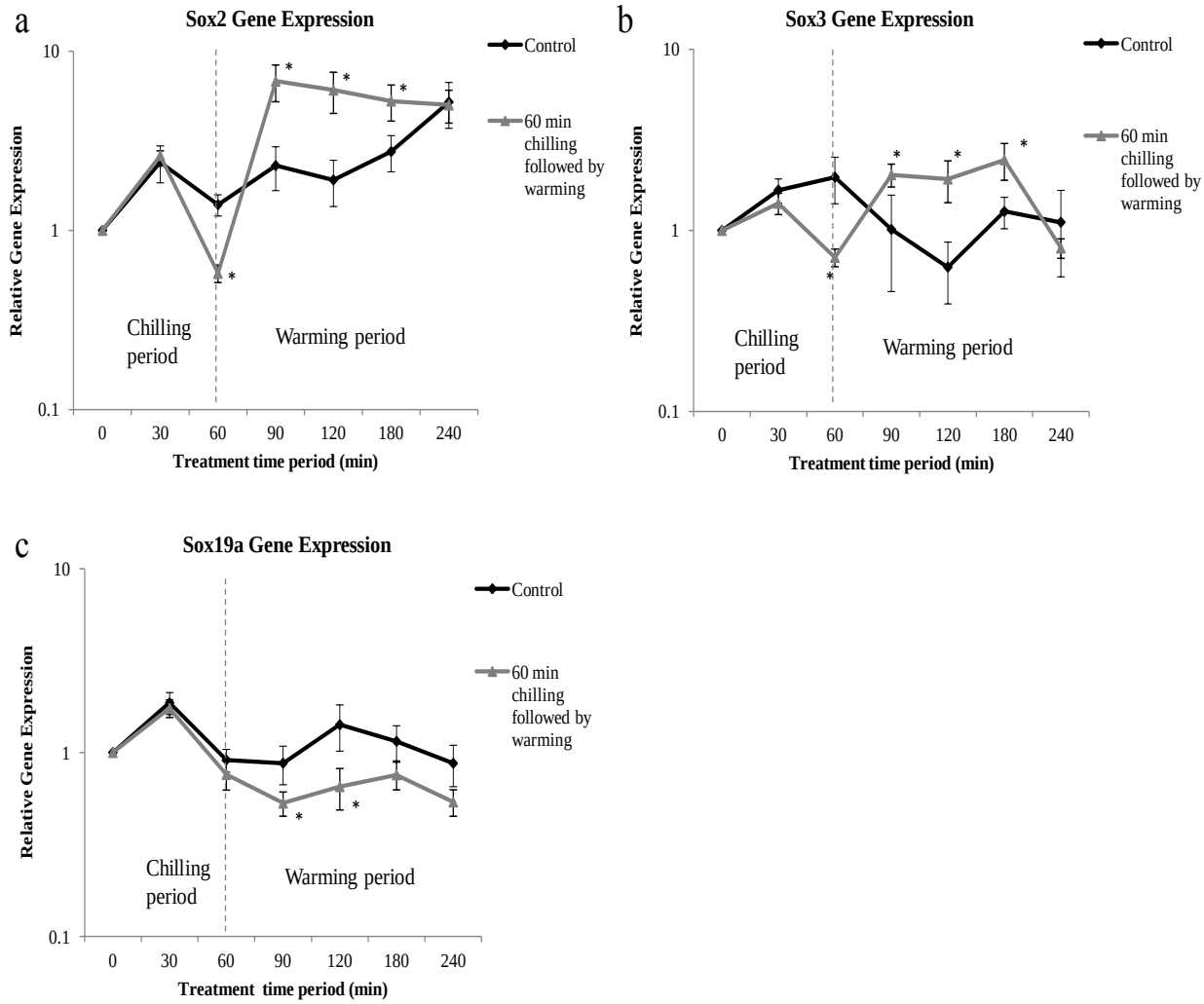


Fig 4.7 Effect of 60 min chilling on *sox* gene expression during warming: gene expression profiles for *sox2* (a), *sox3* (b) and *sox19a* (c) for chilled embryos and non chilled controls, assessed by reverse transcriptase qPCR. The embryo samples were incubated at 0 °C for 60 min and then warmed at 27±1 °C for up to 180 min. Controls were maintained at 27±1 °C for the entire 240 min. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisks (∗) indicate significant

differences between control and chilled-warmed groups at the same time point. $p < 0.05$ was considered to be significant (n=9).

4.4 Discussion

4.4.1 Studies on the effect of chilling on hatching rate of zebrafish (*Danio rerio*) embryos

Studies on the effect of chilling on hatching rate showed that there was no impact of chilling on hatching rates when 50% and 100% epiboly stage embryos were chilled at 0 °C for 30, 60, 120 and 180 min. Although early stages of zebrafish embryos were previously found to be chilling sensitive when chilled for up to 20 h (Zhang and Rawson 1995), chilling for 180 min may not have been enough for any detrimental effect to appear. These findings are similar to those obtained at equivalent stages in drosophila (Mazur et al. 1992). There were significant decreases in the hatching rate in 75% epiboly embryos after 180 min chilling which could be due to changes in blastoderm. At this stage, blastoderm develops very rapidly and become thinner and could become sensitive to chilling. Although as embryos develop to 100% epiboly, the neural plate become thickened along the entire embryonic axis and thickening is more prominent near the animal pole which could explain the reduced impact on chilling (Kimmel et al. 1995). During gastrulation at the 75% epiboly stage, *cdh* and *wnt* proteins play major roles in convergence and extension (C&E) (Okuda et al. 2010) and they are usually expressed at anterior mesendoderm, which gives rise to the hatching gland and developing epithelial tissues (Babb and Marrs 2004). A study on zebrafish embryos showed that any mutation in the *cdh1* gene resulted in severe abnormalities from flattened neural tissue to early embryonic lethality (Shimizu et al. 2005). It has been shown that B1 *sox* genes usually regulate gene expression of *cdh* and *wnt* protein expression (Okuda et al. 2010). Any changes in the sox genes following chilling may therefore affect downstream gene

regulation of *cdh* and *wnt* which may subsequently affect the gastrulation process. Subsequent studies were carried out to determine whether those treatments that did not impact embryo survival impacted gene expression.

4.4.2 Studies on the effect of chilling on gene expression in zebrafish (*Danio rerio*) embryos using quantitative PCR (qPCR)

There are very few examples available to measure the effect of cryopreservation on gene transcript (Lin et al. 2009b, Kopeika et al. 2005). In the present study, the gene expression level of *sox2*, *sox3* and *sox19a* following chilling and warming in 50% epiboly embryonic stage was investigated. In the chilling experiment when embryos were chilled for up to 180 min, significant suppression of *sox2* and *sox3* gene transcripts were found, although over 85% hatching rate was achieved during these periods. However it is interesting to note that 'relative' transcript of *sox2* and *sox3* levels decreased and not just actual transcript levels. This indicates that the sox gene transcript was less stable at low temperature than the housekeeping transcripts. Lin et al. (2009b) found no significant differences in *pax* gene expressions after chilling 50% epiboly stage embryos for 24 hours at 1° C, although these authors also reported reduced transcripts of pax2a and pax5 in cryopreserved zebrafish blastomeres. Possible reasons for suppression of transcripts could be degradation of part of the transcript such as shortening of their Poly (A) tail (Carpousis et al. 1999). This possibility is supported by data obtained using mouse oocytes where expression of TSC2 was reduced due to mRNA degradation following cryopreservation (Tachataki et al. 2003). A study in zebrafish blastomere also showed that cryopreservation can cause DNA damage when suboptimal protocol were used which could also lead to a decline in gene expression (Kopeika et al. 2005). In our experiments, a fast (~300 °C/min) chilling rate was used to chill embryos at 0°C for different time periods. Chilling of embryos at fast rates could cause damage to the nuclear envelope. Damage to the lamina (a part of nuclear envelope) has been reported to affect the gene

expression as the lamina also functions as a structural nuclear protein and regulator of gene expression (Smith and Ane Silva E Silva 2004). Reduction in *sox2* and *sox3* transcription factors could affect downstream transcription which may affect on downstream biological process i.e. protein synthesis. For example, small decrease in *sox2* gene expression leads to impaired neurogenesis in the adult mouse brain (Ferri et al. 2004). *Sox2* and *sox3* have redundant roles in maintaining the broad development potential and identity of neural stem cells (Avilion et al. 2003, Crémazy et al. 2000). Their inhibition or reduction in the chick embryo results in premature differentiation of neural precursors (Bylund et al. 2003). Studies in mouse also showed that cryopreservation slowed down biological processes such as proliferation of granulosa cells in prenatal follicles (Choi et al. 2008), cell division and blastocyst formation in embryos (Bałakier et al. 1991). Subsequently, despite significant decreases in gene transcripts of *sox2* and *sox3*, there were no obvious morphological abnormalities found following hatching. The possible reason might be that *sox19a* could compensate the B1 sox expression due to their redundancy properties (Okuda et al. 2006b). This hypothesis is supported by gene knock down studies in zebrafish embryos where Okuda et al. (2010) demonstrated significant abnormalities in *sox2, sox3* and *sox19a* knock out zebrafish embryo, subsequently no abnormalities were reported when any one of the *sox2, sox3* or *sox19a* genes were knocked down. From these studies, we conclude that chilling does alter pattern of gene expression of normal embryos development, but may have less impact on development.

4.4.3 Studies on expression of *sox* genes following chilling and warming in zebrafish (*Danio rerio*) embryos using qPCR

Sox gene expressions were also studied during the warming period after chilling for 30 and 60 min to determine whether the embryos could recover from the chilling impact. When embryos were chilled for 30 min and warmed, there was a short burst of expression

(30-fold in *sox3* and 25-fold in *sox19a*) at 30 min warming which subsequently decreased back to control levels. A shallower increase in *sox2* was also found resulting in higher expression than in the control by 120 min. This could be explained as compensatory mechanism to maintain homeostasis following the loss of transcript observed during chilling (BJ Fuller 2003). There were a similar increases observed in *sox2* and *sox3* during warming after 60 min chilling. In contrast, *sox19a* gene expressions were suppressed during the warming period following chilling for 60 min. A study in mouse embryos also demonstrated 33-fold up regulation of *Hsp70,MnSOD, CuSOD, CirpB, RBm3* and *Trp53* along with housekeeping gene *β-actin* in in-straw vitrified zygotes at 3 hr post thawing and their level dropped to normal after 7 hr (Boonkusol et al. 2006, BJ Fuller 2003). During the chilling period, the expressions of *sox2* and *sox3* genes were found to be suppressed. To compensate, expressions of these genes should be elevated in order to maintain physiological conditions and subsequent development. Significant decreases were found in *sox19a* in embryos that had been chilled for 60 min and warmed at 28°C up to 180min. The sox family genes are functionally redundant (Graham et al. 2003). This decrease may therefore have been compensated by the increased *sox2* and *sox3* expression observed. Sox genes can bind with other transcription factor proteins to activate or represses specific target genes (Kamachi et al. 2000). A study has showed that SOX proteins have the capability of pairing off with different types of transcriptional factors (Wilson and Koopman 2002). In our case, *sox2*, *sox3* and *sox19a* share a domain with heat shock proteins hsp47 and hsp90. Heat shock proteins play a vital role in protein homeostasis and cellular stress responses. These proteins are usually expressed during stress conditions like hypothermia. The increase in sox genes in the warming period following chilling could be a heat shock response. A study also showed that sox9 can interact with heat shock proteins hsp70 in chondrocytes and testicular cell lines (Marshall and Harley 2001). Liu et al, also demonstrated that cryopreservation can induce heat shock proteins *hsp78* and *hsp86* expression in mouse ovarian tissue (Liu et al. 2003). The

possibility of sox-hsp interaction could lead to increase of sox genes in our experiment. More studies are needed to understand the potential interaction of sox genes with heat shock proteins.

4.5 Summary

This is the first report to evaluate the gene expression pattern of *sox2*, *sox3* and *sox19a* following short chilling periods and subsequent warming. It is clear that chilling does have some effect on gene expression during chilling and subsequently; there may be some sort of compensatory response during warming, the extent and mechanism of which require further investigation.

CHAPTER 5 STUDIES ON THE EFFECT OF CHILLING ON *SOX* GENES AND PROTEIN EXPRESSION IN ZEBRAFISH (*DANIO RERIO*) EMBRYOS IN THE PRESENCE OF METHANOL (MEOH)

5.1 Introduction

In Chapter 4, it was shown that chilling alters the pattern of *sox* gene expression in zebrafish embryos. In this Chapter, the effect of chilling in the presence of cryoprotectant methanol on gene and subsequent protein expression was studied in order to understand the mechanisms of the effect of cryoprotectant on embryos at molecular level during chilling.

Cryoprotectants usually protect cells from chilling and freezing injury by dehydrating cells and lowering the freezing point (Plachinta et al. 2004b). The use of cryoprotectant in low temperature storage has been proven to be essential in protecting cells from chilling injury (Zhang and Rawson 1995). However, most of cryoprotectants are toxic especially when used at high concentrations (Zhang et al. 2012). Cryoprotectants can cause cellular injury by osmotic trauma (Pillai et al. 2001). Toxicity of cryoprotectant depends on the type, concentration, temperature and exposure period (Tsai and Lin 2009). Cryoprotectant toxicity studies are now common practice prior to their use in cell cryopreservation. However there is very limited information on how cryoprotectant function at the molecular level and if they have significant effect on gene or protein expression following cryopreservation. Understanding of the impact of cryoprotectant at molecular level is important and especially for reproductive materials such as embryos. Studies in mouse and rat embryos have shown that methanol (MeOH) is toxic (Lee et al. 1994) and even lethal when concentrations are high (Andrews et al. 1993). Methanol is a widely used cryoprotectant in fish embryo cryopreservation. Methanol has been found to protect cells during cryopreservation in zebrafish oocytes and embryos (Zampolla et al. 2009, Zhang and Rawson 1995) and common carp embryos (Ahammad et al. 2003a). It

has been found that methanol was an effective cryoprotectant in zebrafish embryo cryopreservation because it has low toxicity (Zhang and Rawson 1995) and permits embryo membranes rapidly (Hagedorn et al. 1997). Similar studies in medaka also demonstrated higher embryo survival rate after chilling when MeOH was used as a cryoprotectant (Zhang et al. 2012). However, it has also been shown that methanol exposure is associated with visual impairment or blindness, affecting optic nerve and ratina in rats treated with MeOH (Eells 1991). Methanol has also been demonstrated to be neurotoxic where its exposure leads to severe CNS (Central Nervous system) defect to mice at gastrulation periods (Degitz et al. 2004) and in drosophila embryos (Mellerick and Liu 2004). Rico *et. al.* (2006) showed that methanol also alters ecto-nucleotidases and acetylcholinesterase enzymes (important for neuromodulation in brain) in zebrafish brains. Therefore it is important that the effect of methanol when used as a cryoprotectant is better understood.

The present study investigated the effect of chilling on *sox* gene and proteins expression in the presence of methanol. Sox genes (*sox2, sox3* and *sox19a*) are important genes in development of nervous systems in zebrafish embryos (reviewed in Chapter 1, Section 1.5.2.1) and any changes can lead to serious abnormalities (Ferri et al. 2004). *Sox* genes expression inhibition in vertebrate embryos results in premature differentiation of neural precursors and their over expression results in inhibition of neurogenesis (Kishi et al. 2000, Crémazy et al. 2000, Overton et al. 2002, Avilion et al. 2003, Graham et al. 2003). However, a study on gene expression (mRNA level) does not provide information on protein translation as the efficacy of translation is also affected by post transcription modulation of regulatory genes. It has been demonstrated that small non-protein-coding RNAs (small nucleolar RNA, micro RNAs, short interfering RNAs, small double stranded RNA) also regulates gene expression, including translation in developmental processes (Mattick and Makunin 2006). Therefore, following gene expression studies,

subsequent protein expression studies were also carried out to understand the effect of MeOH at molecular level during chilling.

5.2 Experimental Design

In the present study, the effect of methanol on hatching rates of zebrafish embryos was investigated first following they were chilled for up to 24 h at 0°C. Embryos that survived chilling in MeOH were then used to study the gene and protein expression. Once embryo hatching rates were obtained following treatment in different concentrations of methanol, the effect of methanol on gene expression and protein expression was studied in chilled and chilled-warmed embryos throughout embryonic development.

Based on the results obtained from the previous Chapter 4, 50% epiboly embryos were chilled in different concentrations of methanol for up to 24 h at 0°C. Epiboly stage embryos (50%) were selected for these studies as no effect of chilling on hatching rate was found at this stage in earlier Chapter (see Section 4.3.1) when they were chilled for up to 180 min at 0 °C. Once chilling tolerance has been identified, zebrafish embryos were chilled and subsequently cultured until hatching stage and mRNA level was studied for key developmental stages eg 20 somites stage (initiation of nervous system development), heartbeat stage and hatching stage after chilling. Based on gene expression profiles, embryos were also chilled in cryoprotectant methanol and *sox* protein expressions were also studied.

5.3 Results

5.3.1 Studies on embryo hatching rate following embryos were chilled at 0° C for different time periods in the presence of MeOH as a cryoprotectant

Zebrafish embryos from 50% epiboly stage were chilled with different concentrations of MeOH (0.2, 0.5 and 1 M) for 3, 6. 18 and 24 h at 0°C, they were then returned to room

temperature and incubated at 27 ±1°C until they hatched. Small amount of development was observed in 50% epiboly stage embryos when they were subjected to chilling at 0°C. Embryo hatching rates (following chilling and warming) were normalised with respect to non-chilled control embryos. It was observed (Fig. 5.1) that up to 6 h chilling at 0°C, no significant differences in hatching rates (over 85%) were found between 3 and 6 h chilled embryos. Significant decreases in embryo hatching rates were observed in embryos that had been chilled for 18 and 24 h with or without MeOH. It was also observed that there were no significant differences in hatching rates between embryos chilled in MeOH and embryos chilled in egg water. Similarly, no significant differences in hatching rates were observed in embryos that had been chilled with different concentrations of methanol.

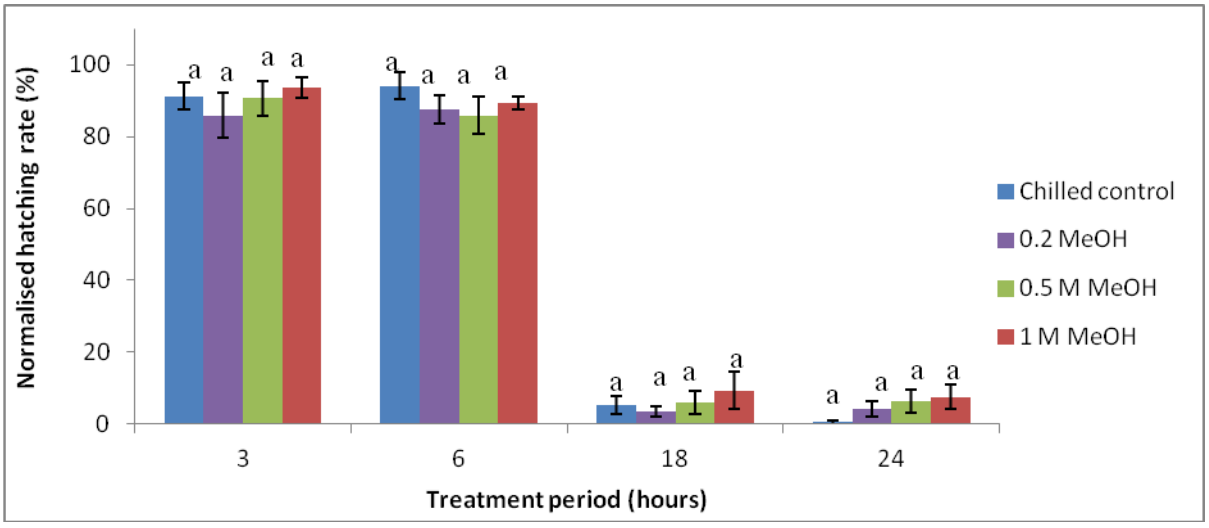


Fig 5.1: Effect of chilling on hatching rates in 50% epiboly stage of zebrafish embryos: Bars represent hatching rates of zebrafish embryos after chilling at 0° C for different time periods (3-24 h) in different concentrations of MeOH (0.2, 0.5 and 1 M), followed by incubation at 27±1°C for three days. Error bars represent the standard errors of the mean (SEM) (n=9).

5.3.2 Studies on the effect of chilling and warming on *sox* gene expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

Based on the results obtained from the previous experiments that no significant differences were observed in zebrafish embryo hatching rates upon 3 and 6 h chilling at 0°C, further studies were carried out on the effect of methanol on 3 h chilling on gene and protein expression in 50% epiboly embryos in the presence of MeOH. Different concentration of methanol has been used to chilled embryos for 3 h and warmed until hatching stage. Gene express profile relative to housekeeping gene EF1-α and β actin were studies just after 3 h chilled and warming stages 20 somites, heartbeat and hatching stages.

5.3.2.1 Studies on the effect of chilling and warming on *sox2* gene expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

Comparisons of different concentrations of MeOH on sox2 gene expression

Embryos from 50% epiboly stages were chilled in different concentrations (0.2, 0.5- and 1 M) of MeOH at 0°C for 3 h and MeOH was replaced with egg water and cultured at 27±1°C until hatching stage. Gene expression was measured immediately after 3 h chilling and also at 20 somites, heartbeat and hatching stages. *Sox2* gene expression (Fig 5.2a) in non-chilled control embryos was relatively stable throughout the tested developmental stages (from 50% epiboly stage to hatching stage). Expression of *sox2 i*n embryos that had been chilled with or without MeOH decreased significantly when compared to non-chilled control embryos and increased following warming and culturing at 27±1°C to the non-chilled control level by hatching stage. However, *sox2* gene expression in embryos chilled in 1 M MeOH was significantly increased when compared to non-chilled control embryos at hatching stage.

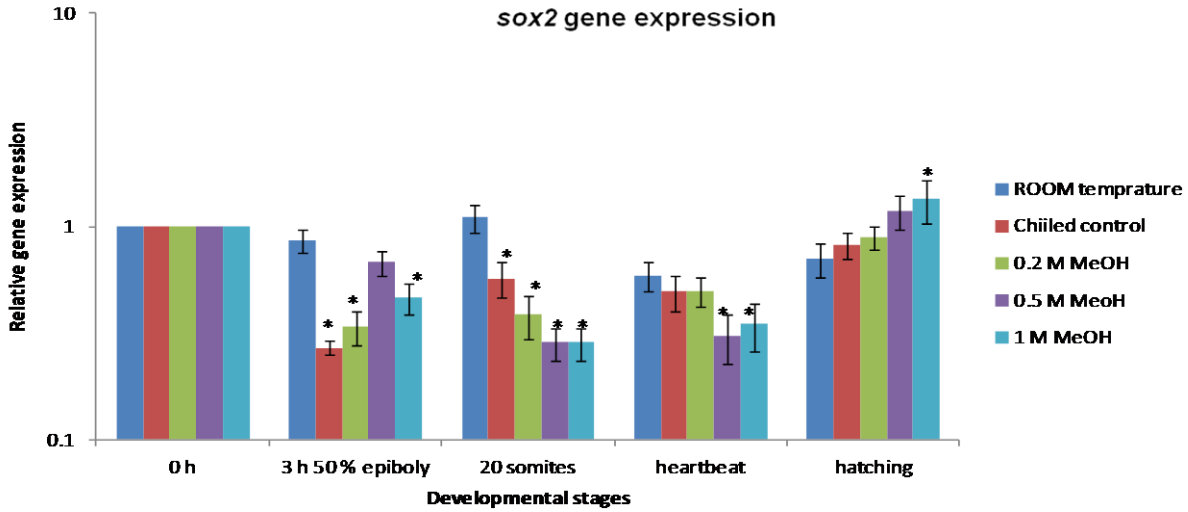


Fig. 5.2 (a) Effect of chilling in different concentrations of MeOH and warming on *sox2* gene expression in zebrafish embryos. The Figure shows the gene expression profiles for *sox2* in embryos chilled for 180 min at 0°C in the presence of methanol. Following chilling, methanol was replaced with egg water and embryos were cultured at 27±1 °C until hatching stage. Gene expressions immediately after chilling and at the 20 somites, heartbeat and hatching stages were assessed using reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Results represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different concentrations of MeOH and non-chilled control within the same gene (n=9).

Comparisons of sox2 gene expression at specific stages throughout development

Embryos from 50% epiboly stages were chilled in different concentrations (0.2, 0.5 and 1 M) of MeOH for 3 h and they were then warmed and cultured at 27±1° C until hatching stage. *Sox2* gene expression (Fig 5.2b) was stable at all tested stages. However, significant decreases were found in embryos that had been chilled at 0°C with or without MeOH compare to 0 h. In the embryos that had been chilled in the presence of egg water

and warmed at 27°±1°C, expression level returned to the level at time 0 by hatching stage. Similar patterns of expression were observed in embryos that had been chilled with 0.2-,0.5- and 1 M MeOH and warmed at 27°±1°C until hatching stage.

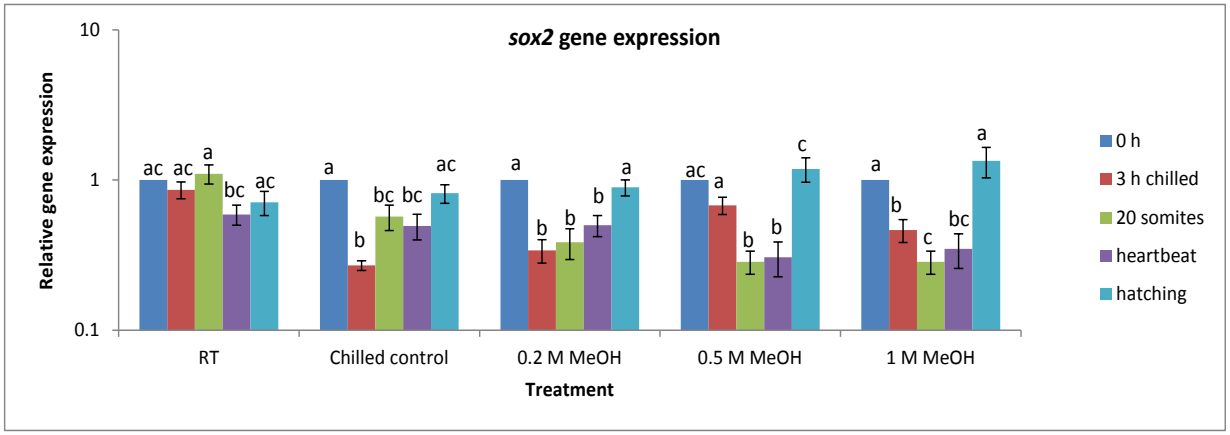


Fig: 5.2 (b) Effect of chilling in the presence of MeOH on *sox2* gene expression in zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox2* in embryos chilled for 180 min at 0°C and then cultured at 27±1°C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different developmental stages of zebrafish embryos within same chilling treatment period (n=9).

5.3.2.2 Studies on the effect of chilling and warming on *sox3* gene expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

Comparisons of different concentrations of MeOH on sox3 gene expression

Embryos from 50% epiboly stages were chilled with different concentrations (0.2, 0.5 and 1 M) of MeOH for 3 h, MeOH was then replaced with egg water and embryos were

cultured at 27±1° C until the hatching stage. In non-chilled control embryos, *sox3* (Fig 5.3a) gene expression was stable till heartbeat stage before decreased in hatching stage. In embryos that had been chilled with egg water and 0.2 M MeOH, significant decreases of *sox3* expression were observed immediately after chilling and after culturing at 27±1°C at 20 somites stage when compared with non-chilled controls. The expression level returned to non-chilled control level by hatching stage. For the embryos that had been chilled with 0.5 and 1 M MeOH, no significant decreases of *sox3* expression were observed until the 20 somites stage. The gene expressions were subsequently gradually increased and were significantly higher than that in non-chilled control embryos by hatching stage.

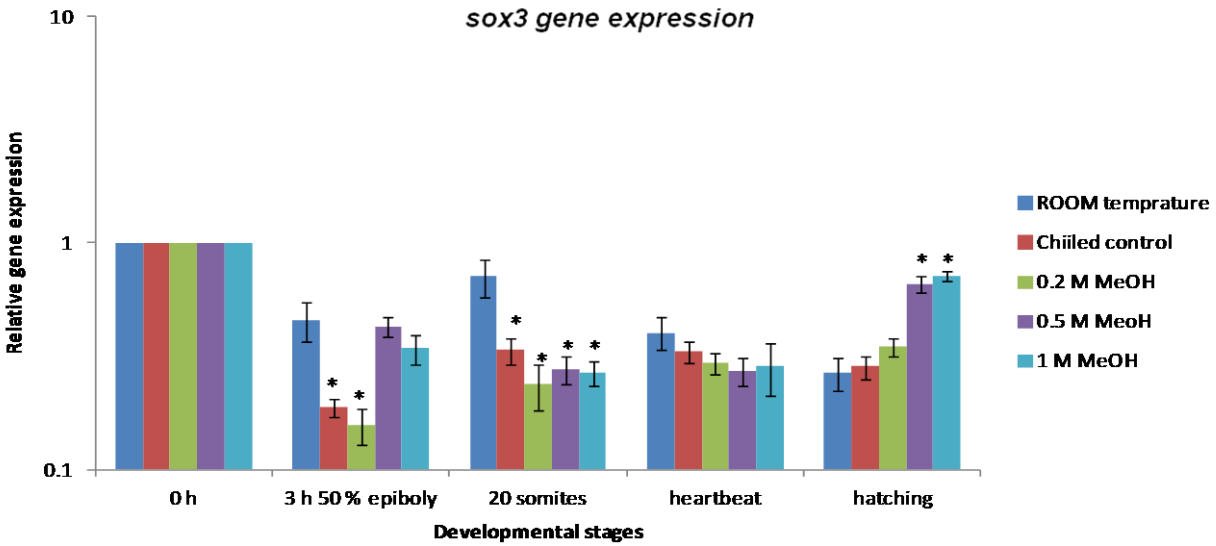


Fig: 5.3(a) Effect of chilling and warming in different concentrations of MeOH on *sox3* gene expression in zebrafish embryos. The Figure shows the gene expression profiles for *sox3* in embryos chilled for 180 min at 0°C in the presence of methanol. Following chilling methanol was replaced with egg water and embryos were then cultured at 27±1 °C until the hatching stage. Gene expressions immediately after chilling and at 20 somites, heartbeat and hatching stages after culturing were assessed using reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Results

represent the mean expression level relative to the control at time 0 and error bars represent standard error of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different concentrations of MeOH and non chilled control within the same gene (n=9).

Comparisons of sox3 gene expression at specific stages throughout development

In non-chilled control embryos, *sox3* (Fig 5.3b) gene expression was decreased after 3 h and returned to control level at the 20 somites stage before decreased at hatching stage. In the embryos that had been chilled without MeOH, the levels of expressions were decreased significantly immediately after chilling and then increased significantly following warming. Significant increases were observed at hatching stage in the embryos that been chilled with MeOH.

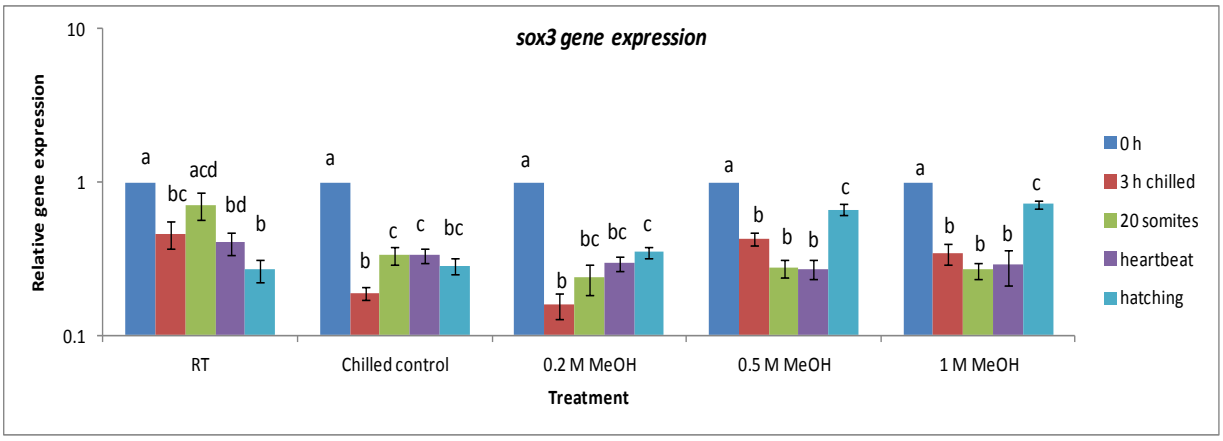


Fig: 5.3 (b) Effect of chilling in the presence of MeOH on *sox3* gene expression in zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox3* in embryos chilled for 180 min at 0°C and then cultured at 27±1°C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant

differences ($p < 0.05$) between different developmental stages of zebrafish embryos within the same chilling treatment period (n=9).

5.3.2.3 Studies on the effect of chilling and warming on *sox19a* gene expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

Comparisons of different concentrations of MeOH on sox19a gene expression

Embryos from 50% epiboly stages were chilled with different concentrations (0.2, 0.5- and 1 M) of MeOH for 3 h and MeOH was then replaced with egg water and cultured at 27±1° C until hatching stage. In control embryos, *sox19a* (Fig 5.4a) gene expression level remained stable throughout development stages. In treated embryos, significant gene expression decreases were observed in embryos that had been chilled in egg water for 3 h before the expression returned to control levels by 20 somites stage. For embryos that had been chilled in 0.5 M and 1 M MeOH, significant increases of *sox19a* gene expression were observed after 3h chilling when compared to non-chilled controls. The *sox19a* gene expression remained significantly above the non-chilled control level throughout development stages.

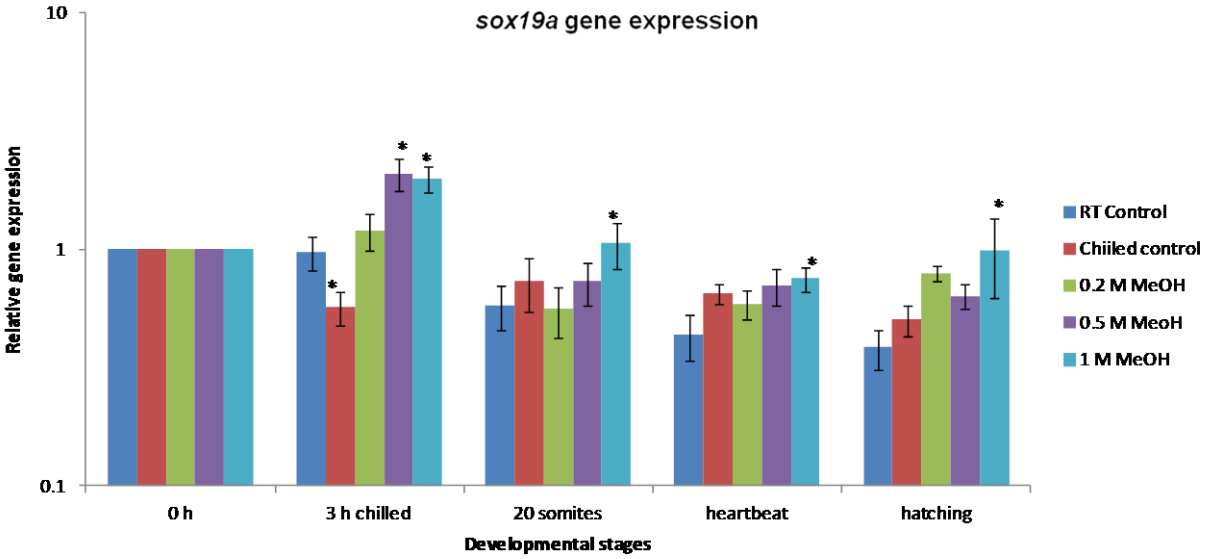


Fig: 5.4(a) Effect of chilling in different concentrations of MeOH and warming on *sox19a* gene expression in zebrafish embryos. The Figure shows the gene expression profiles for *sox19a* in embryos chilled for 180 min at 0°C in the presence of methanol. Following chilling methanol was replaced with egg water and embryos were then cultured at 27±1 °C until hatching stage. Gene expressions immediately after chilling and at 20 somites, heartbeat and hatching stages were assessed using reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Results represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different concentrations of MeOH and non chilled control within the same gene (n=9).

Comparisons of sox19a gene expression at specific stages throughout development

In non-chilled control embryos (Fig 5.4b), expression of *sox19a* remained stable before decreased after 20 somites stage at 27±1 °C. In the embryos that had been chilled at 0°C in egg water and cultured at 27±1°C, significant decreases were observed after 3 h

chilling and in hatching stage when compared to time 0. In embryos that had been chilled in different concentrations of MeOH, *sox19a* gene expressions increased significantly in 0.5 and 1 M chilling embryos immediately after 3 h chilling and then decreased to time 0 level after culturing at 27±1 °C at hatching stage.

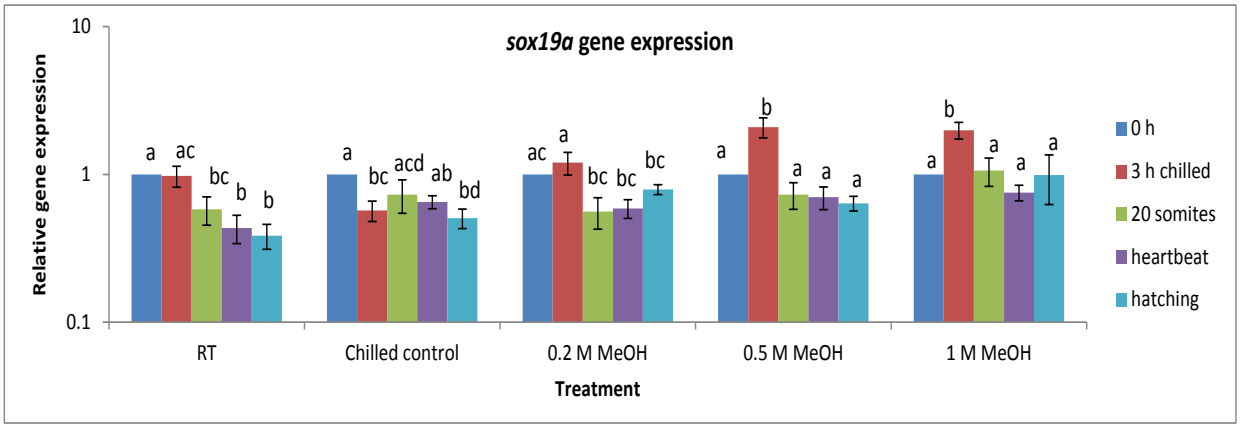


Fig: 5.4 (b) Effect of chilling in the presence of MeOH on *sox19a* gene expression zebrafish embryos at different stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox19a* in embryos chilled for 180 min at 0°C and then cultured at 27±1°C until hatching stage. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression levels relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different developmental stages of zebrafish embryos within the same chilling treatment period (n=9).

5.3.3 Studies on the effect of chilling and warming on *sox2* and *sox19a* protein expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

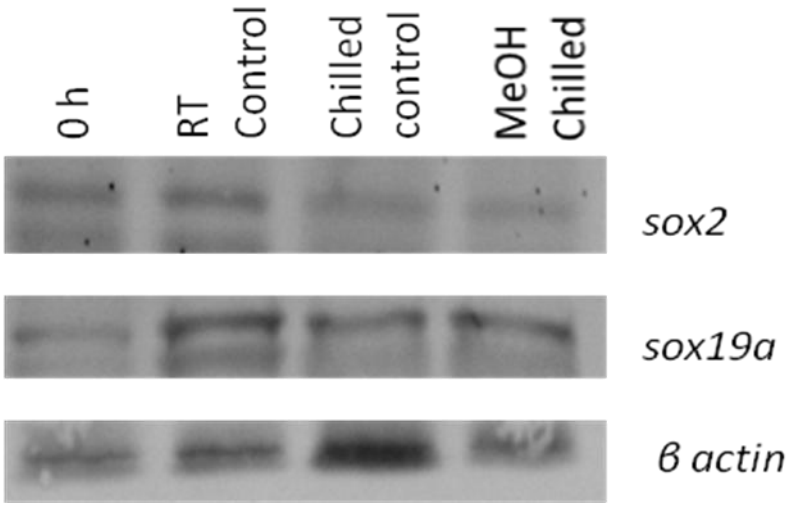
In the previous Section embryos were chilled for 3 h with or without MeOH, MeOH was then replaced with egg water following chilling and embryos were cultured at 27±1°C until they hatched. *Sox* gene expressions were measured immediately after chilling and also at 20 somites, heartbeat and hatching stages after culturing at 27±1°C. It was observed (Fig 5.2a) that *sox2* gene expressions decreased after 3 h chilling in the embryos that have been chilled with or without MeOH and gene expressions were recovered gradually upon warming and subsequent culturing until hatching stage. However, significant increases were observed in *sox2* gene expression at hatching stage in embryos that had been chilled in 1 M MeOH and cultured to hatching stage. Under the same condition, similar patterns of *sox19a* gene expression were observed in embryos that had been chilled without MeOH. Opposite *sox19a* gene expression patterns were observed in the embryos that had been chilled in 1 M MeOH and cultured until the hatching stage, *sox19a* gene expression remained significantly above control levels throughout development stages following chilling and warming. As studies on gene expression (mRNA level) does not provide information on protein translation, further studies were carried out to assess simultaneous protein expression of *sox2* and *sox19a* after 3 h chilling at 0°C and after warming and culturing at 27±1°C until hatching stage as altered patterns of gene expression were observed at different developmental stages.

5.3.3.1 Studies on the effect of chilling and warming on *sox2* protein expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

Effect of 3 h chilling on sox2 and sox19a protein expression

Embryos from 50% epiboly stages were chilled with or without 1 M MeOH and protein expression was measured. The effect of 3 h chilling on zebrafish embryos in the presence of MeoH on protein expression of *sox2* and *sox19a* is shown in Fig 5.5. Protein expression profile is shown in Fig 5.5(a) along with internal control *β actin*. Based on these results, densitometry was performed using ImageJ software and data are presented in Fig 5.5(b). Expression of *sox2* protein remained stable under all treatment conditions (Fig 5.5b). No significant differences were observed in *sox2* protein expression in embryo when they were chilled at 0°C for 3 h with or without 1 MeoH. *Sox19a* protein expression level remained stable in non chilled controls and embryos chilled in egg water for 3 h at 0°C. Significant increases in *sox19a* protein expression were observed in the embryos that had been chilled with 1 M MeOH for 3 h at 0°C.

(a)



(b)

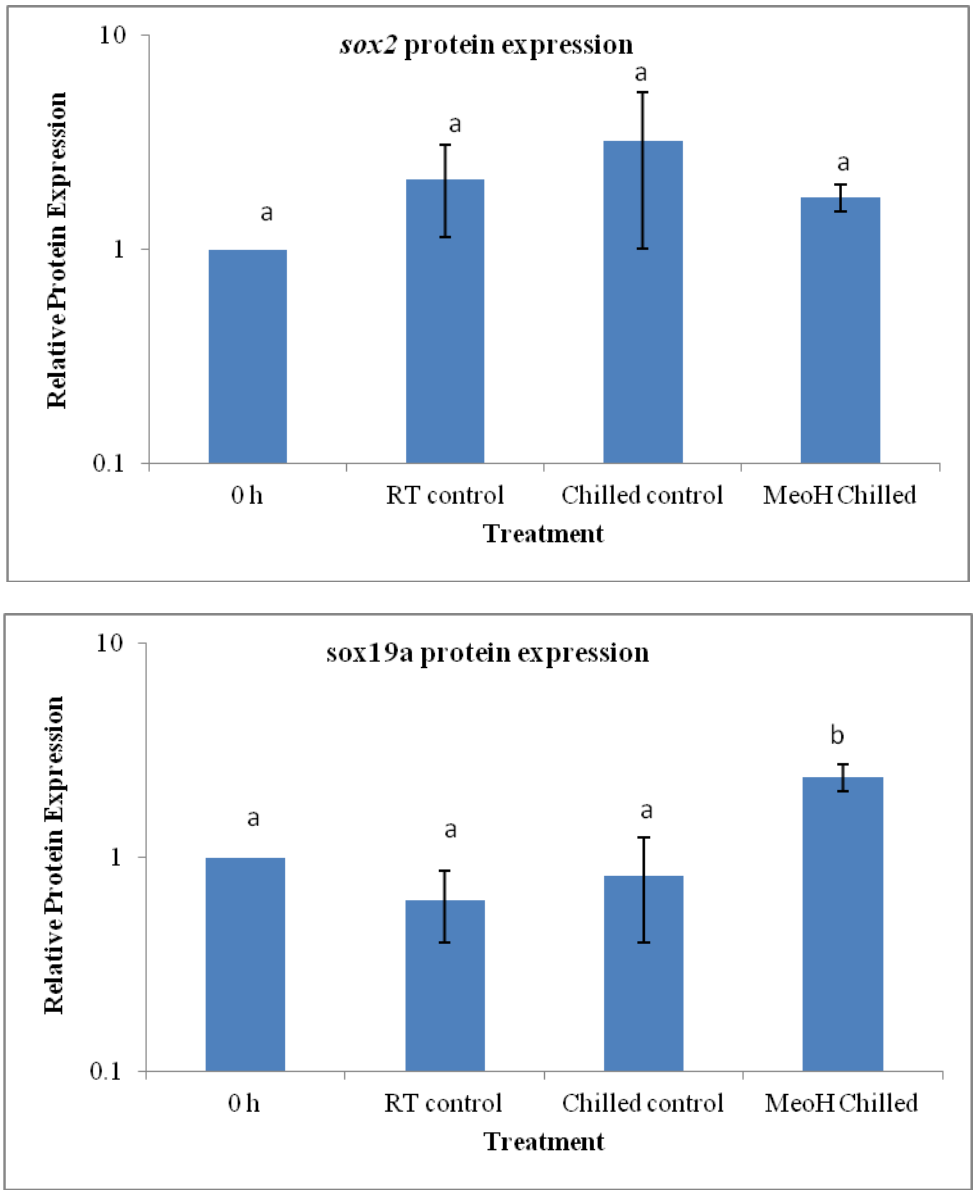


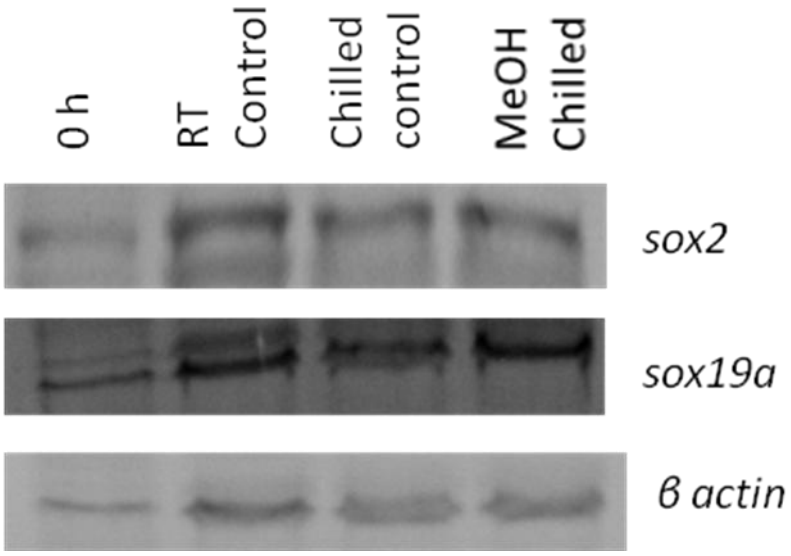
Fig: 5.5 Effect of 3 h chilling with or without the presence of MeOH on *sox2* and *sox19a* protein expression in 50% epiboly zebrafish embryos. Protein expression profiles are for *sox2* and *sox19a* for embryos chilled for 180 min at 0°C assessed by Western Blot. For each time point, 75 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between

different chilling treatments of zebrafish embryos in post 3 h 50% epiboly stage (n=9).

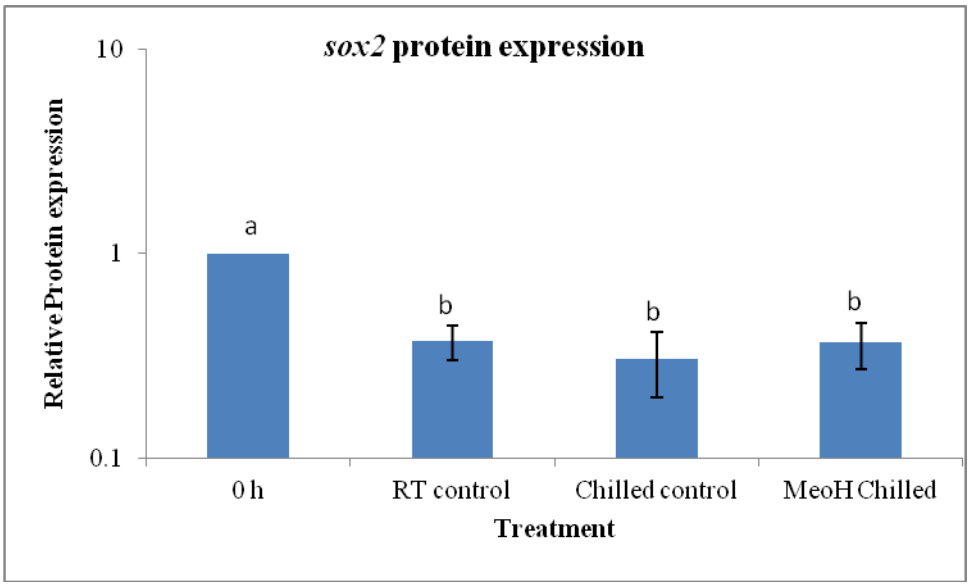
Effect of 3 h chilling and subsequent warming on sox2 and sox19a protein expression in hatching stage embryos

Embryos from 50% epiboly stage were chilled with or without 1 M MeOH, MeOH was then replaced with egg water and embryos were cultured at 27±1°C until the hatching stage. The effect of 3 h chilling in presence of MeoH and subsequent warming on protein expression in hatching stage zebrafish embryos of *sox2* and *sox19a* was shown in Fig 5.6. Protein expression profiles are shown in Fig 5.6(a) along with internal control *β actin*. Based on these results, densitometry was performed using ImageJ software and data are presented in Fig 5.6(b). *Sox2* protein expression decreased significantly in hatching stage when compared to 50% epiboly stage. No significant differences were observed in non chilled embryos at hatching stage and embryos chilled with or without 1 M MeOH and subsequently cultured until the hatching stage. Protein expression remained stable in both chilled and non chilled embryos at hatching stage. Protein expression of *sox19a* remained stable throughout from 50% epiboly stage to hatching stages. No significant differences were observed in *sox19a* protein expression in embryos that had been chilled with or without MeOH and non chilled controls.

(a)


0 h
RT Control
Chilled control
MeOH Chilled
sox2
sox19a
β actin

(b)


sox2 protein expression
Relative Protein expression
10
1
0.1
a
b
b
b
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RT control
Chilled control
MeoH Chilled
Treatment

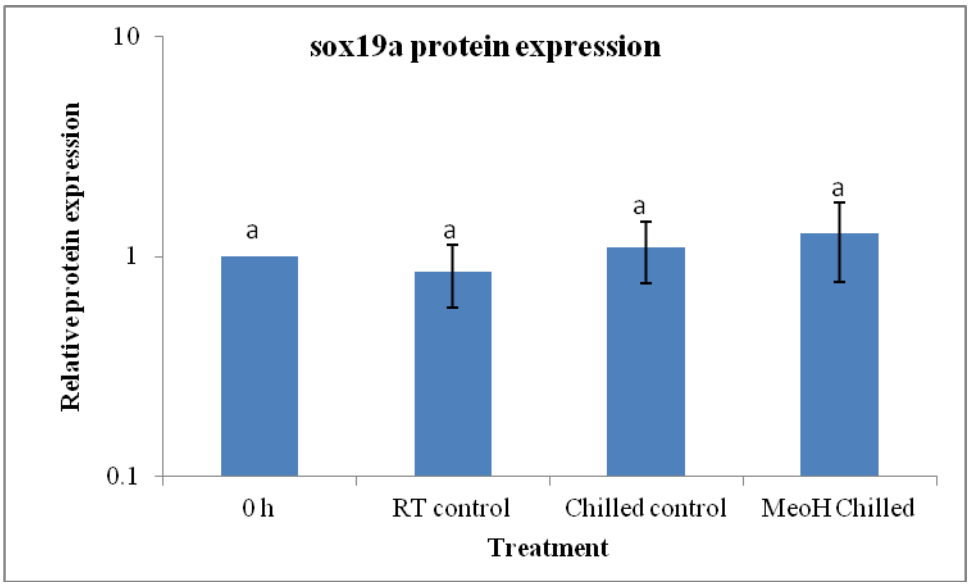


Fig: 5.6 Effect of 3 h chilling with or without the presence of MeOH and subsequent warming and culturing on *sox2* and *sox19a* protein expression in hatching stage zebrafish embryos. Protein expression profiles are for *sox2* and *sox19a* in embryos chilled for 180 min at 0°C and cultured at 27±1°C assessed by Western Blot. For each time point, 75 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos in hatching stage (n=9).

5.4 Discussion

Cryoprotectant toxicity studies are necessary before they are used in any chilling storage and cryopreservation protocol in order to minimise the effect of these chemicals. Survival rate has been widely used to assess cryoprotectant toxicity in embryos (Kopeika et al. 2003) and oocytes (Plachinta et al. 2004a) prior to their chilling storage or cryopreservation. Methanol has been demonstrated to be an effective cryoprotectant in zebrafish embryo chilling storage (Zhang et al. 2003) and oocytes cryopreservation (Guan

et al. 2008). However, there is no information available in the literature on the effect of methanol on gene and protein expression. This information is important due to the fact that MeOH is neurotoxic (Degitz et al. 2004). Studies have shown that methanol exposure leads to severe CNS defect in mice embryos (Degitz et al. 2004) and drosophila embryos (Mellerick and Liu 2004). Therefore the aim of the present study was to investigate the effect of MeOH on gene and protein expression of zebrafish embryos following short term chilling and subsequent culture after warming.

5.4.1 Studies on embryo hatching rate after chilling at 0° C for different time periods in the presence of MeOH as a cryoprotectant

Initial study was carried out to investigate chilling tolerance of 50% epiboly embryos for up to 24 h at 0°C with the presence of different concentrations (0.2-,0.5- and 1 M) of MeOH. Embryo hatching rates after chilling, warming and subsequent culture to hatching stage was investigated. Results from the study showed that 50% epiboly stage embryos tolerated chilling for up to 6 h with/without MeOH (90 ± 5 % survival rate) before it was significantly decreased after 18 and 24 h chilling at 0°C (18 and 24h results, 10±5% and 7±5% respectively) . No significant differences in embryos survival were observed in embryos that had been chilled in different concentrations of MeOH for up to 6 h. Previously, Zhang and Rawson (1995) demonstrated that over 50% of shield stage (~60% epiboly) embryos were killed when they were exposed to 0 ℃ for 4 h without any cryoprotectant and no embryo younger then bud stage survived 11 h exposure at 0°C. Due to the fact that zero or low survival rates were obtained for embryos chilled at 0°C, no longer term chilling studies in the presence of MeOH were carried out with 50% epiboly stage embryos previously. Results obtained in medaka embryos (Valdez Jr et al. 2005) showed that gastrula stage embryo survival rate was not affected by 24 h chilling at 0°C in hank's solution. In the present study, survival rate of similar stage 50% epiboly was reduced to 10% following chilling in egg water for 18 and 24 h at 0°C.

Cell membranes are generally highly permeable to methanol as Zhang et al. (2005) reported in zebrafish that methanol penetrates ovarian follicle at a rate comparable to the rate of water transport and therefore, incubation of cells in MeOH does not lead to osmotic stress. In the present study, there were no significant differences found in embryos that had been chilled in MeOH (at all tested concentrations) and egg water at 0°C up to 24 h. This could be due to the fact that gastrula stage embryos are highly chilling sensitive and the concentrations of MeOH used in the present study was not effective in protecting embryos from chilling injury. Under the similar conditions e.g. 24 h chilling at 0 °C, chilling sensitivity of heartbeat stage embryos was reduced significantly with the introduction of 1 M MeOH in chilling media (Zhang and Rawson 1995). The mechanism by which certain cryoprotective agents protect embryos from chilling injury has not been well understood. High chilling sensitivity in early stage embryos such as 50% epiboly stage is believed to be associated with the large amount of intraembryonic lipids. Studied on partial removal of yolk on chilling sensitivity in zebrafish embryos showed that chilling injury following rapid cooling could be mitigated after partial removal of yolk at the prim-6 stage (Liu et al. 2001). A study on chilling of porcine embryos also showed that the sensitivity of porcine embryos to chilling is related to their high lipid contents, embryos become tolerant to chilling when their lipid contents were reduced (Nagashima et al. 1994). Lipid phase transition (LPT) in cell membranes are also responsible for chilling injury in mammalian sperm (Drobnis et al. 1993) and oocytes (Arav et al. 2000). At the temperature around phase transition, chilled membranes lose fluidity and become leaky, which cause damage to cells (Zeron et al. 1999).

5.4.2 Studies on the effect of chilling and warming on sox gene expression in zebrafish *(Danio rerio)* embryos in the presence of MeOH

Studies were carried out to investigate the effect of 3 h chilling and warming on *sox* gene expression at different embryo development stages after embryos were cultured at 27±1°C until the hatching stage. In this experiment, embryos were chilled with different concentrations of MeOH, warmed and then cultured until hatching stage. Gene expression was investigated at different time points (after 3 h chilling, after culturing at 27 ±1°C until they reached 20 somites, heartbeat and hatching stages). MeOH has been demonstrated to penetrate zebrafish embryo membrane (Zhang and Rawson 1998). Methanol has also been demonstrated to be neurotoxic where its exposure leads to severe CNS defect to mice CNS at gastrulation periods (Degitz et al. 2004) and in drosophila embryos (Mellerick and Liu 2004). Therefore, different embryo developmental stages were selected to investigate the effect of MeOH on sox gene expression following chilling, warming and subsequent culture. Developmental stages were selected based on their morphology during development – 20 somites (early nervous system development), heartbeat (mid brain development – early touch reflexes) and hatching (First time exposure to outer environment). These stages are key stages to study the effect of *sox* genes due to the fact that these genes play important roles in nervous system development in zebrafish embryos (Dee et al. 2008, Millimaki et al. 2010, Vriz et al. 1996), any changes in these genes can have adverse effects on embryonic development.

Results from the present study showed decreased gene expression when compared to RT controls for all three genes (*sox2, sox3* and *sox19a)* in the embryos that had been chilled for 3 h at 0°C without MeOH, however, lesser degree of decrease in gene expression was observed in embryos that had been chilled for 3 h in different concentrations of MeOH and expression levels closer to RT controls in higher concentrations. Studies have shown

that chilling of embryos at fast rates could cause damage to the nuclear envelope (Smith and Ane Silva E Silva 2004). In our experiments, a fast (~300 °C/min) chilling rate was used to chill embryos at 0°C for 3 h. Damage to the lamina (a part of nuclear envelope) has been reported to affect the gene expression as the lamina also functions as a structural nuclear protein and regulator of gene expression (Smith and Ane Silva E Silva 2004). It is possible that MeOH protects the lamina of the nuclear envelop during chilling and therefore reduce the chilling injury. Methanol has been reported to be an effective cryoprotectant during chilling storage of zebrafish embryo at zero and subzero temperatures (Zhang and Rawson 1995). Methanol was also found to improve survival rate for 50% epiboly stage carp (*C.carpio*) embryos when they were cooled to 4 or 0°C (Dinnyés et al. 1998). In our study, MeoH was shown to protect gene expression following chilling at 0°C for 3 h in the embryo that had been chilled with different concentrations of MeOH and the protective effect was increased with increasing concentration as alterations in gene expression were less when compare to embryos that had been chilled without MeOH. Zhang et. al. (2003) also suggested that higher concentration of MeOH treatment generally provided better embryo survival rate when embryos were cooled at fast cooling rate of 300 °C/min. Further studies are needed on the molecular mechanisms of the effectiveness of MeOH in protecting fish embryos from chilling injury.

Following chilling, embryos were warmed up and then cultured at 27±1°C to hatching stages, investigations were then carried out on the level of gene expression in 20 somites, heartbeat and hatching stages. It was observed that *sox* gene expression was gradually increased after 20 somites stage and recovered to non chilled control level at hatching stage in the embryos that had been chilled without MeOH. However, gene expression of *sox2* and *sox3* increased significantly at hatching stage when embryos were chilled in 1 M

MeOH and cultured to hatching stage. Significant increase in *sox19a* was also found at all developmental stages and remained stable in the embryos that been chilled with 1 M MeOH. The increase in *sox19a* gene expression may be activation of compensatory mechanism. Compensatory mechanism can be activated to prevent the loss of gene transcript in order to recover gene expression during chilling (B. J. Fuller 2003). The decrease in *sox2* and *sox3* gene expression may therefore have been compensated by the stable higher levels of *sox19a expression* throughout. This could be the reason for unaffected embryo survival rates after 3 h chilling at 0°C despite the decrease of *sox2* and *sox3* gene expression. In the present study, no significant differences in hatching rate were observed between non chilled control and 3 h chilled embryo in 1 M MeOH, however significant increases were observed in *sox* gene expression at the hatching stage when compared to non chilled controls. Alteration in *sox* gene expression could have adverse implications on long term development of the embryos. A study in mice demonstrated that over expression of *sox* genes can be carcinogenic and induce large number of tumour types (Dong et al. 2004). Toxicity studies in zebrafish embryos also demonstrated that exposure of low concentrations of Perfluorooctanesulfonate (PFOS) induced upregulation of *pax8* genes (falls in the same group as sox gene) which leads to the induction of apoptosis genes in zebrafish embryos and larvae (Shi et al. 2008). More long term studies are needed in order to investigate the adverse effects of MeOH on larvae and adult fish.

5.4.3 Studies on the effect of MeOH chilling and warming on *sox2* and *sox19a* protein expression in zebrafish *(Danio rerio)* embryos

Transcript levels detected in mRNA profiling do not reflect all regulatory processes in the cell as post-transcriptional processes altering the amount of active proteins, such as synthesis, processing and modification of proteins (Mattick and Makunin 2006). Therefore, in addition to monitor gene expression at the transcriptional level, analysis of

the protein is equally important for the understanding of cellular, metabolic and regulatory networks in living organisms (Nie et al. 2007). Previous experiment on the effect of 3 h chilling at 0°C on *sox* gene expression showed that 1 M MeOH altered the pattern of *sox* expression at hatching stage by increasing transcript level significantly above that obtained in the controls.. In the present experiment, the effect of 1 M MeOH on *sox2* and *sox19a* protein expression following 3h chilling and subsequent culturing of the embryos to hatching stage was investigated. In embryos that had been chilled for 3 h at 0°C, no significant differences in *sox2* protein expression were observed in 3 h chilled embryos with or without MeOH and non chilled control. Under the same condition, gene expressions of *sox2* were decreased significantly in embryo that had been chilled with or without MeOH when compared with controls. This could be explained by the repair mechanism of *sox2* gene transcript during post transcriptional processes, such as post transcription and translation modification to repair loss of *sox2* gene transcript. Studies in *hsp90* in parasite *Giarida* chilled for 20 min on ice demonstrated post transcriptional repair mechanism by mRNA *trans*-splicing (Nageshan et al. 2011). Degradation or fragmentation of mRNA due to chilling could be repaired by similar mechanism. During the mRNA splicing of *sox2*, the splicing junction carries hallmarks of classical *cis*-spliced introns, suggesting that regular splicing machinery may be sufficient for repair of open reading frame. A complimentary sequence in the introns regions adjacent to the splice sites may assist in positioning two pre-mRNA for processing (Nageshan et al. 2011). Damage in *sox2* due to chilling, could be processed by pre-mRNA and produce protein as normal, and resulted in recovered/unaffected protein expression. *Sox19a* protein expression remained significantly above the control level following 3 h chilling at 0°C before decreasing to non-chilled control level at hatching stage. This protein expression pattern is similar to the pattern obtained in gene expression studies. High protein level of *sox19a* could be explained by compensation mechanism. To compensate, expressions of

sox19a genes and subsequent proteins should be elevated in order to maintain physiological conditions and subsequent development due to their redundant function (Graham et al. 2003).

5.5 Summary

It is evident from this study that 50% epiboly stage embryos could tolerate chilling at 0°C for 6 h with or without MeOH; however, hatching rates were significantly decreased in embryos that had been chilled with or without MeOH for 18or 24 h at 0°C. Further studies have been carried out to assess the effect of cryoprotectant MeOH on gene expression in 50% epiboly embryos chilled with different concentrations of MeOH (0.2,0.5 and 1 M) at 0°C and warmed and then cultured to hatching stage. *Sox2* and *sox3* gene expressions at the hatching stage were increased significantly when compare to non-chilled controls in embryos that had been chilled with 1 M MeOH and subsequently cultured to hatching stage. Under the same conditions, *sox19a* gene expression remained at above control levels at all tested developmental stages. Further protein expression studies were carried out in order to investigate the effect of 3 h chilling on embryos in the presence of 1 M MeOH and the subsequent culture of embryos to the hatching stage. The study showed that no significant differences in *sox2* protein expressions were observed between non-chilled controls and 3 h chilled embryos with or without MeOH. Significant increases in *sox19a* protein expressions were observed in 3 h chilled embryos in the presence of 1 M MeOH when compare to non chilled controls and the expression was decreased to control levels by hatching stage. It is clear from the present study that MeOH protected embryos at the molecular level during chilling and the protective effect was increased with increasing concentrations of MeOH. However after warming and culturing of embryos until hatching stage, higher concentration (eg. 1 M MeOH) also altered the pattern of gene expression the level of gene transcript was increased. Increased gene expression may be a compensatory response in order to recover the loss of mRNA

transcript during chilling. However, no significant differences were observed in protein expressions in the embryos that had been chilled at 0°C for 3 h and warmed then cultured to hatching stage when compared to non chilled controls. The reason for this may be associated with the embryo post transcription repair mechanism which stabilized protein translation. However the mechanisms associated with the effect of chilling and warming on gene and protein expressions require further investigation. In the present study, 3 h chilling period was studied and the results do not provide information on the effect of long term chilling on embryos in the presence of MeOH. More studies are needed to assess effect of long term chilling on gene and protein expression.

CHAPTER 6 STUDIES ON EFFECT OF LONGER TERM CHILLING ON GENE AND PROTEIN EXPRESSION IN ZEBRAFISH (*DANIO RERIO*) EMBRYO

6.1 Introduction

In chapter five, it was observed that MeOH altered the pattern of gene expression and protein expression in embryos that had been chilled for 3 h with MeOH and subsequently cultured to hatching stage. In this chapter, the effect of MeOH following longer term (18h) chilling was studied. Previous study also showed that 50% epiboly embryos could tolerate zero degree temperature for up to 6 h and but their survival rate decreased to 10% when chilled to 18- and 24 h at 0°C. Therefore the first step of the present study was aimed to improve survival rate for long term chilling storage by using different mixtures of cryoprotectants (penetrating and non-penetrating cryoprotectants). Once these studies were completed, the effect of long term chilling and subsequent warming on gene and protein expression was studied.

Long term chilling storage of zebrafish embryos is important and especially when cryopreservation of fish embryos is still not achieved. Storage of embryos at low temperature for extended period has important applications in aquaculture. Chilling storage has practical applications in fish embryo handling eg embryo transportation between fish farms in genetic improvement programs. However fish embryos of several species have been shown to be chilling sensitive including zebrafish (Zhang and Rawson 1995, Maddock 1974, Dinnyés et al. 1998, Liu et al. 1993). Zhang and Rawson (1995) showed that early stage zebrafish embryos are more chilling sensitive than later stages. They also demonstrated that methanol solution containing sucrose and trehalose enhanced survival of heartbeat embryos following chilling storage for 18 and 24 h at 0°C. In the present study, similar approach was taken to improve survival rate in early stage embryos following chilling up to 24 h at 0°C. A study in common carp (*Cyprinus carpio L.*)

embryos (12 hpf) also demonstrated that the mixture of MeOH (1.5 M) and sucrose (0.5 M) produced higher survival rate than use them individually in 12-72 h chilled embryos at zero and subzero temperatures (Ahammad et al. 2003b). However, all these studies are based on the results obtained on embryo survival rate and does not give any information on their genetic integrity. It has been shown that storage of zebrafish embryos using propylene glycol in the liquid nitrogen even for 1 min resulted in damage of mitochondria, disorganisation of ribosomes and plasma membrane of the yolk syncytial layer (Janik et al. 2000). Therefore information on the effect of chilling and cryopreservation on embryos at the molecular level is important in designing protocols for successful chilling storage and cryopreservation of the embryos.

The aims of the present study were to identify and optimise suitable cryoprotectant mixtures for improve survival rate for 50% epiboly embryos following chilling for up to 18 h. Studies were carried out to investigate the effect of chilling and warming on gene and protein expressions in the embryos that had been chilled with cryoprotectant mixtures for up to 18 h and warmed and culture until hatching stages.

6.2 Methodology

Initially, hatching rate studies were carried out to improve the survival of embryos after chilling prior to study the effect of chilling on gene and protein expression. Embryos from 50% epiboly stage were chilled for up to 18 h with different concentrations of cryoprotectant mixtures containing MeOH and sucrose. Chilled embryos were returned to 27 ± 1°C and cultured until they hatched. Embryo hatching rates were then assessed. Optimised cryoprotectant treatment conditions which improved the embryos hatching rates were further tasted on whether they had any effect on subsequent gene and protein expression. Embryos from 50% epiboly stage were chilled with optimised concentration of cryoprotectant for 18 h, gene and protein expressions were then studied using

quantitative RT PCR and western blotting at different developmental stages following chilling and culturing to the hatching stage respectively.

6.3 Results

6.3.1 Studies on the effect of 18 h chilling on hatching rate in zebrafish *(Danio rerio)* embryos in the presence of sucrose and MeOH mixture

Embryos from 50% epiboly stage were chilled with different concentrations of MeOH and sucrose for 18 h and warmed to 27±1°C before them were cultured to hatching stage. Small amount of development was observed in 50% epiboly stage embryos when they were subjected to chilling at 0°C. Significant increases in hatching rates were observed in the embryos that been chilled with 0.5 and 1 M MeOH + 0.1 M sucrose mixtures when compared to embryos that had been chilled in different MeOH concentrations with 0.05 M sucrose (Fig 6.1). No significant differences in hatching rates were observed among embryos treated in 0.05 M sucrose plus different concentrations of MeOH.

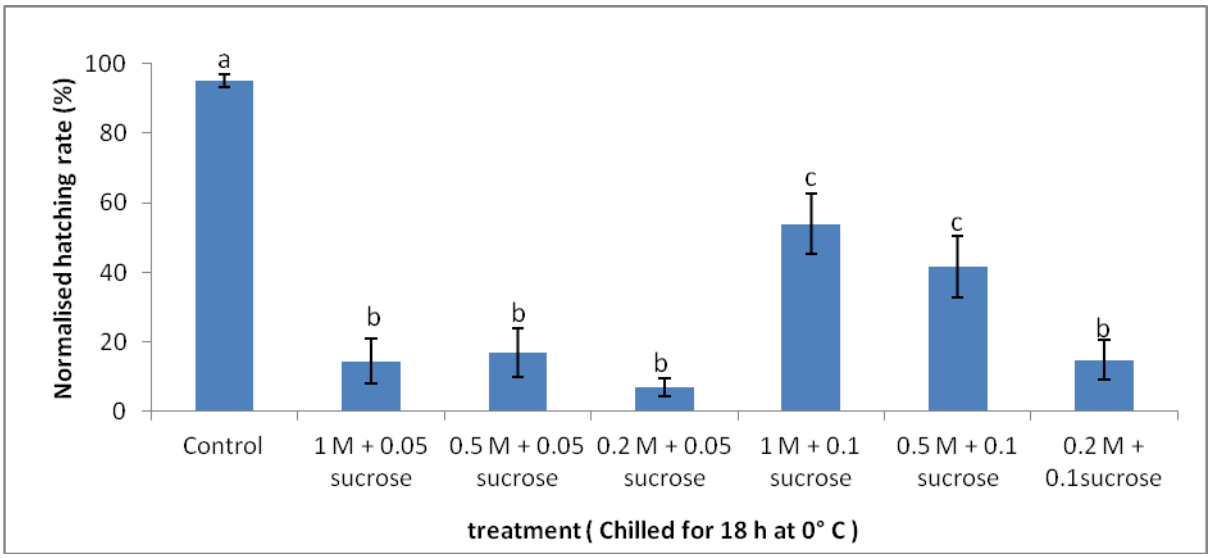


Fig 6.1: Effect of chilling on hatching rate in 50% epiboly stage of zebrafish embryos. Embryos were chilled for 18 h in different combinations of MeOH and

sucrose followed by culturing at 27±1°C for up to three days and until they hatched. Bars represent hatching rates of zebrafish embryos after chilling at 0° C for 18 h in different concentrations of MeOH (0.2, 0.5 and 1 M) plus sucrose (0.05 – 1 M), followed by incubation at 27±1°C for three days. Error bars represent the standard error of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments (n=9).

6.3.2 Studies on the effect of 18 h chilling on *sox* gene expression in zebrafish (*Danio rerio)* embryos

In the previous experiment, 1 M MeOH + 0.1 M sucrose cryoprotectant mixture improved survival rate to 56% ± 5 when compared with embryos chilled with 1-, 0.5- and 0.2 M MeOH + 0.05 M sucrose. These parts of the experiments were designed to investigate if chilling of embryos in this cryoprotectant mixture has any adverse effect on embryo gene expression.

In non chilled control embryos after 18 h at 27± 1°C, *sox2* (Fig 6.2a) gene expression increased significantly when compare to time zero 0 h. However in the embryos that have been chilled at 0°C for 18 without cryoprotectant, *sox2* gene expressions decreased significantly when compared to non chilled controls. No significant differences were observed in embryo that had been chilled for 18 h in the cryoprotectant mixture containing 1 M MeOH and 0.1 M sucrose. *Sox3* (Fig 6.2b) gene expressions were decreased significantly - after 18 h of development at RT and no significant differences were observed in chilled embryos with or without cryoprotectant when compared to non chilled 18 h controls. Similarly, for gene expression of *sox19a* (Fig 6.3c), significant decreased was observed in 18 h non chilled control embryos when compared to 0 h controls. For embryo that had been chilled for 18 h in cryoprotectant mixture containing 1

M MeOH and 0.1 M sucrose, significant increases were observed compare to 18 h non chilled controls.

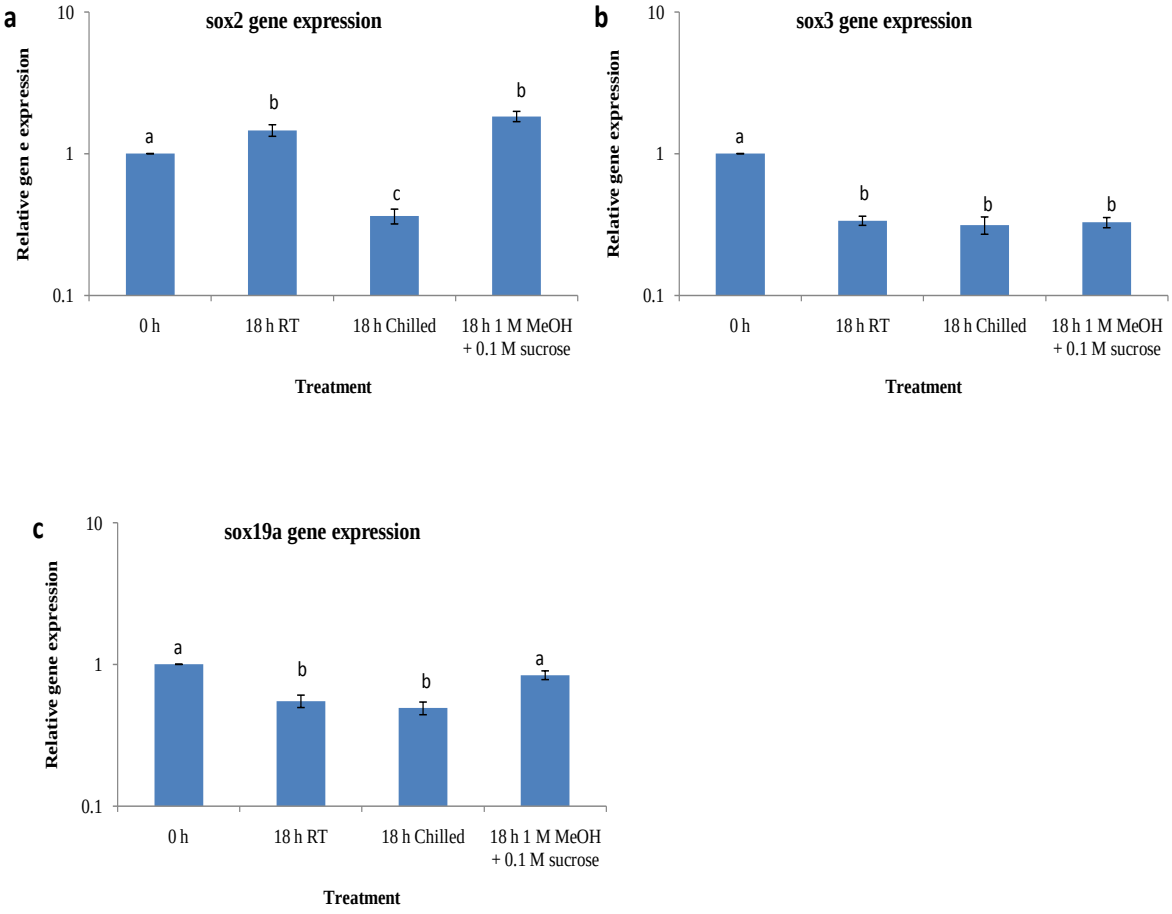


Fig: 6.2 Effect of chilling in the presence of MeOH and sucrose on *sox* gene expression in 50% epiboly stage of zebrafish embryos: gene expression profile for *sox2* (a), *sox3* (b) and *sox19a* (c) for embryos that had been chilled for up to 18 h at °C with or without MeOH + sucrose. Non chilled control embryos were kept at 27±1°C. Gene expressions were assessed by reverse transcriptase qPCR. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the non chilled

controls and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different conditions of chilling within the same developmental gene (n=9).

6.3.3 Studies on the effect of 18 h chilling and warming on *sox* genes expression in zebrafish (*Danio rerio*) embryos

Previous experiment showed that chilling in MeOH+ sucrose mixture of 50% epiboly embryos does have impact on embryo gene expression. However, information on gene expression after warming and culturing of embryos is also important. This part of the experiments is designed to study the embryo *sox* expression level following chilling and warming.

In control embryos, gene expression of *sox2* remained stable and *sox3* and *sox19a* (Fig 6.3) gradually decreased throughout development from 50% epiboly to hatching stage. In the embryos that had been chilled with 1 M MeOH + 0.1 M sucrose for 18 h and then warmed before cultured to hatching stage, *sox2* (Fig 6.3a) and *sox19a* (Fig 6.3c) *gene* expression increased just after chilling period and again increased following heartbeat stages before levelling up with non chilled control embryos at hatching stage. Opposite gene expression patterns were observed for *sox3* (Fig 6.3b) as gene expression decreased significantly when compared to controls at 20 somites stage and recovered after heartbeat stage before decreased significantly at hatching stage.

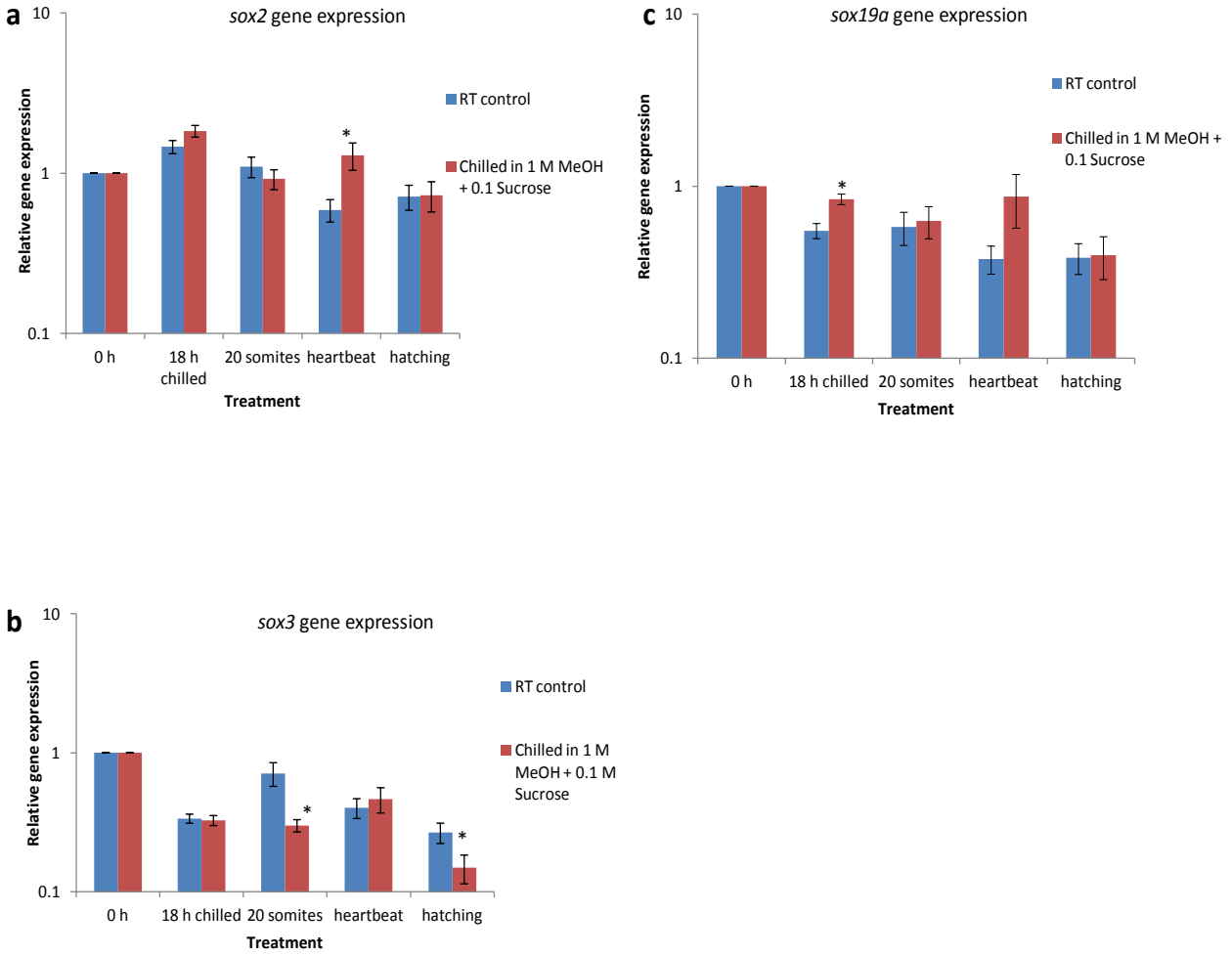


Fig: 6.3 Effect of chilling in the presence of MeOH + sucrose and warming on *sox* gene expression in 50% epiboly stages of zebrafish embryos: gene expression profile for *sox2* (a), *sox3* (b) and *sox19a* (c) for embryos chilled up to 18 h at °C with or without MeOH + sucrose followed by warming at 27±1°C up to hatching stage, assessed by reverse transcriptase qPCR. Non chilled control embryos were kept at 27±1°C. For each time point, 5 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to o h time point and error bars represent standard errors of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) in gene expression between control treatment within the same developmental gene (n=9).

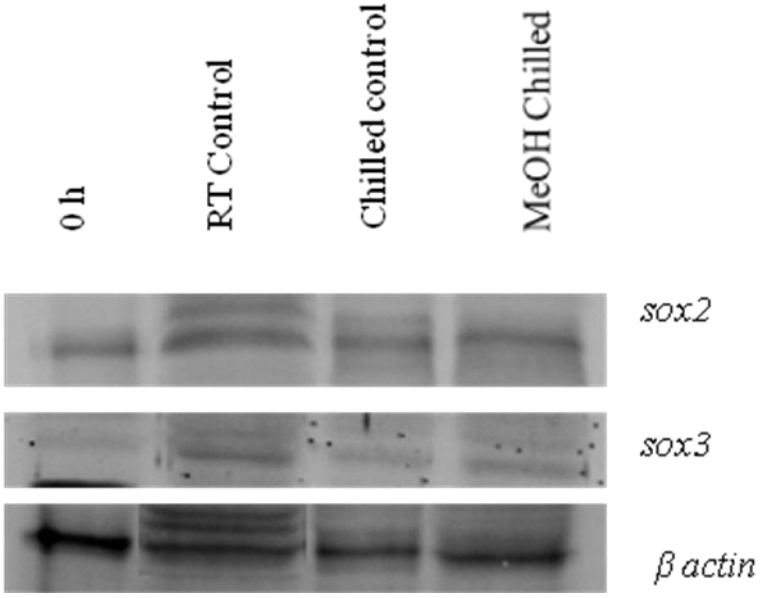
6.3.4 Studies on the effect of 18 h chilling and warming on *sox2* and *sox3* protein expression in zebrafish (*Danio rerio*) embryos

The results obtained from gene expression studies showed that after 18 h chilling and warming *sox2* and *sox19a* gene expressions were increased significantly above control levels after heartbeat stage before returned to the control level. Different gene expression patterns were observed for *sox3* as gene expressions decreased significantly at 20 somites stage and recovered at heartbeat stage before decreased significantly in embryos at hatching stage. To further understand the gene expression pattern of *sox3* when compared to *sox2* and *sox19a* genes, protein expression studies were carried out for *sox2* and *sox3* following 18 h chilling and after hatching.

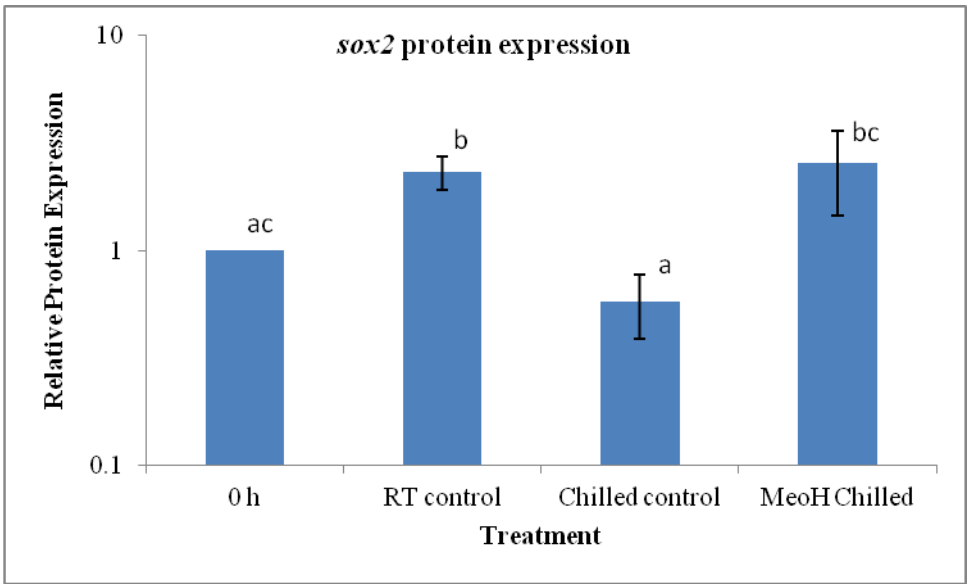
Effect of 18 h chilling on sox2 and sox3 protein expression:

The effect of 18 h chilling on *sox2* and *sox3* protein expression in 50% embryos chilled with or without 1 M MeOH + 0.1 M sucrose is shown in Fig 6.4. Protein expression profile is shown in Fig 6.4(a) along with internal control β actin. Based on these results, densitometry was performed using ImageJ software and data are presented in Fig 6.4(b). *Sox2* protein expression increased significantly from 0 h to 18 h in control non chilled embryos. *Sox2* protein expression was affected by 18 h chilling in egg water and significantly decreased when compared to non chilled 18 h controls. For embryos that had been chilled with 1 M MeOH + 0.1 M sucrose, no significant changes in *sox2* protein expressions were observed when compared to 18 h non chilled embryos. *Sox3* protein expression increased significantly in non chilled 18 h embryos compare to 0 h. No significant differences in *sox3* protein expression were observed in embryos that had been chilled with/without cryoprotectant mixture compare to non chilled 18 h control embryos.

(a)


0 h
RT Control
Chilled control
MeOH Chilled
sox2
sox3
β actin

(b)


sox2 protein expression
Relative Protein Expression
10
1
0.1
ac
b
a
bc
0 h
RT control
Chilled control
MeoH Chilled
Treatment

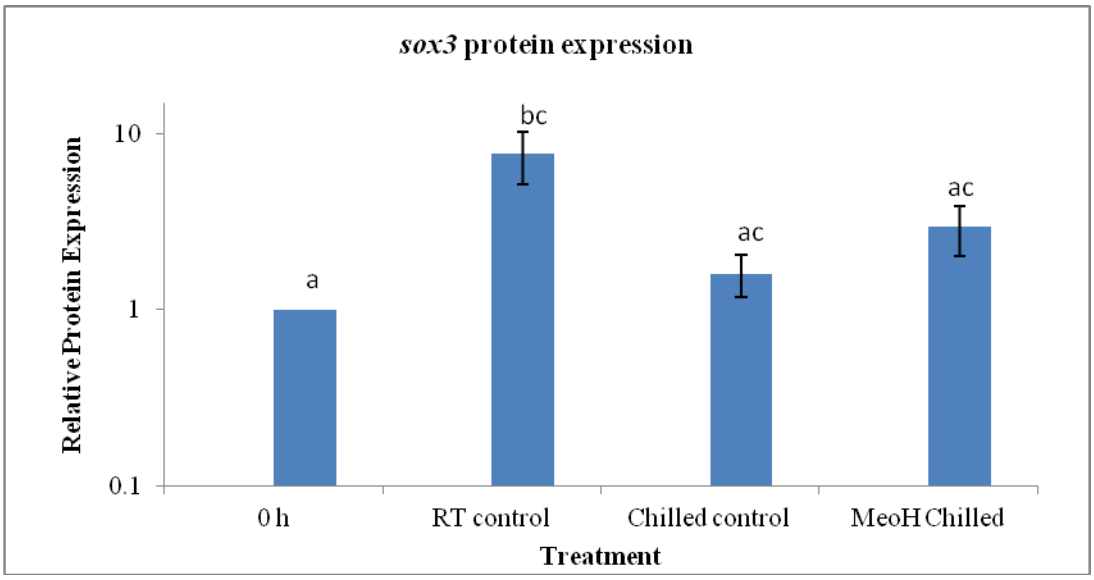


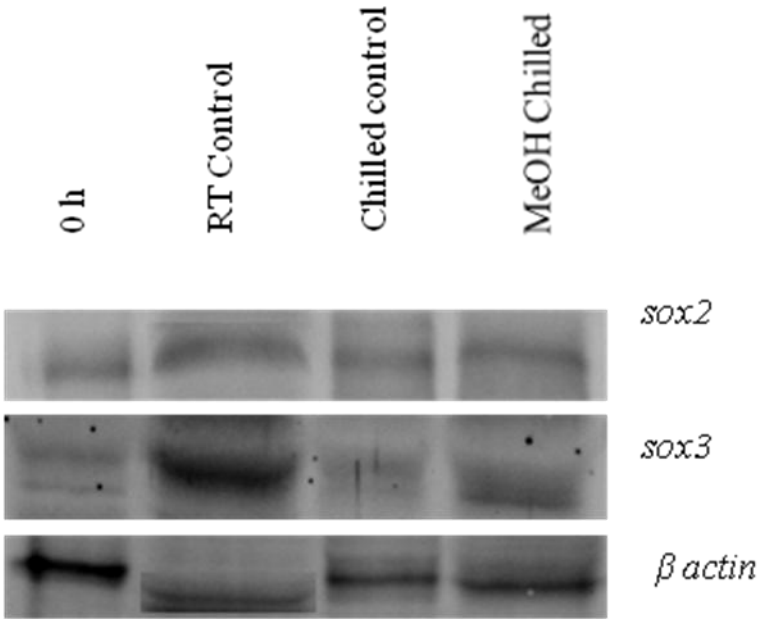
Fig: 6.4 Effect of 18 h chilling with or without 1 M MeOH and 0.1 M sucrose mixture on *sox2* and *sox3* protein expression in 50% epiboly stage zebrafish embryos: Protein expression profiles of *sox2* and *sox19a* for embryos chilled for 18 h at 0°C were assessed by western blot. For each time point, 75 embryos were collected in triplicate and each experiment was repeated three times. Points represent the mean expression level relative to the control at time zero and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos within developmental stages (n=9)

Effect of 18 h chilling, warming and culturing on sox2 and sox3 protein expression in embryos at the hatching stage:

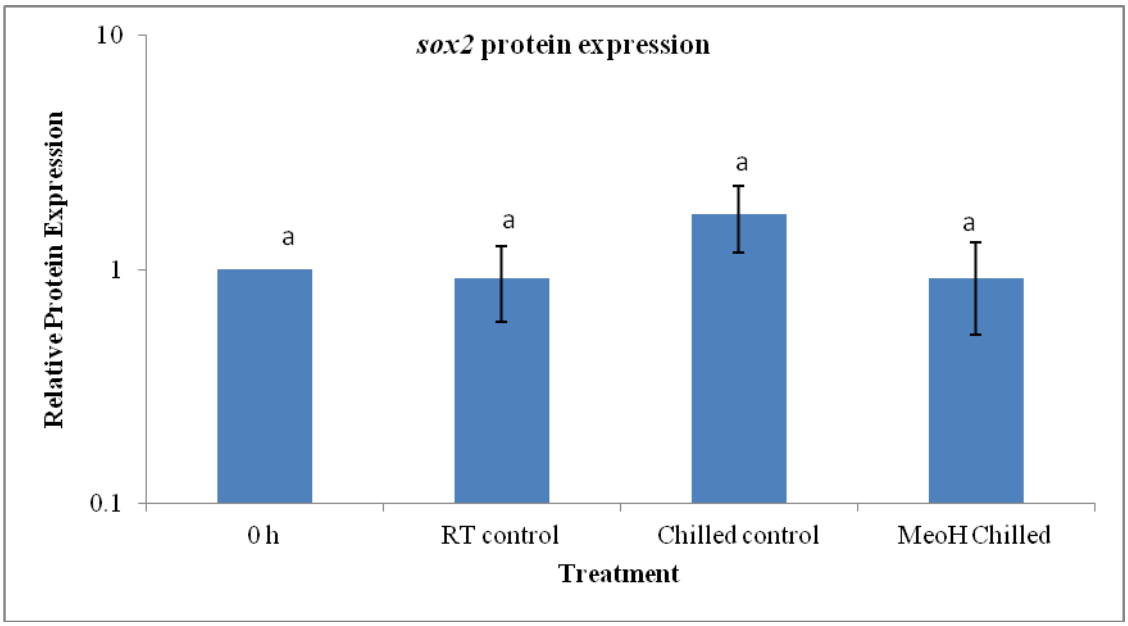
The effect of 18 h chilling on *sox2* and *sox3* protein expression in 50% epiboly stage embryos chilled with or without 1 M MeOH + 0.1 M sucrose and cultured until the hatching stage is shown in Fig 6.5. Protein expression profiles are shown in Fig 6.5(a) along with internal control β actin. Based on these results, densitometry was performed using ImageJ software and data are presented in Fig 6.4(b). *Sox2* protein expression was stable throughout treatment period and no significant differences were observed between

chilled and non chilled control embryos. *Sox3* protein expression was increased significantly with development from 0 h (50% epiboly) to hatching stages. No significance differences in *sox3* gene expressions were observed between non chilled controls and embryos that had been chilled with or without cryoprotectant mixture.

(a)



(b)



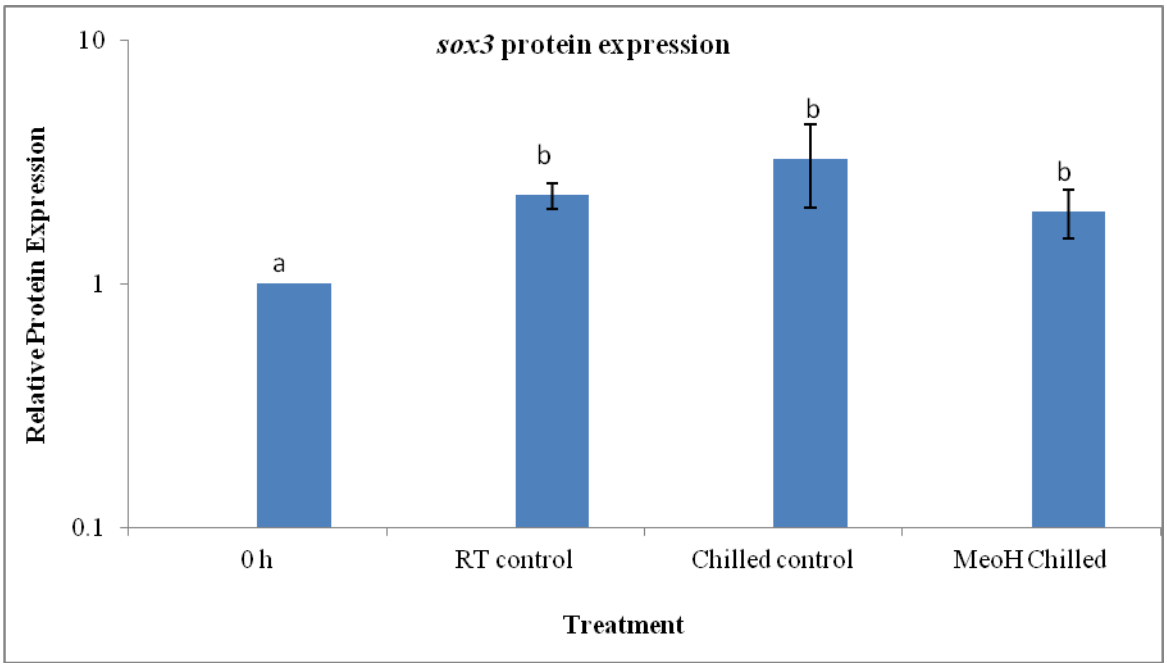


Fig: 6.5 Effect of 18 h chilling with or without 1 M MeOH and 0.1 M sucrose mixture and warming on *sox2* and *sox3* protein expression in hatching stage zebrafish embryos: Protein expression profiles of *sox2* and *sox3* for embryos chilled for 18 h at 0°C were assessed by western blot. For each time point, 75 embryos were collected in triplicate and each experiment was repeated three times. Points

represent the mean expression level relative to the control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos within developmental stages (n=9)

6.4 Discussion

Chilling storage is an important alternative to cryopreservation for fish embryos in aquaculture to synchronize the development of embryos deriving from different spawning dates or to delay the development for extended period of time. Maddock (1974) demonstrated that in the brown trout (*Salmo trutta*), embryo development can be extended for up to 4 months at 1.4°C. Studies in the vendace (*Coregonus albula*) showed that hatching of embryos can be delayed for several weeks by cooling them at 1 to 2 °C in order to synchronize mass hatching for lake stocking (Luczynski 1984). In the present study, studies were carried out in order to optimise the chilled storage protocol for zebrafish embryos for 18 h at 0°C. Different mixtures of cryoprotectants were used in the chilling medium. Once embryo survival rates were optimised after 18 h chilling at 0°C, further studies on the effect of 18h chilling in the presence of cryoprotectant mixture on the gene and protein expressions were carried out. This study was necessary and especially it has been shown in the chapter 4 that short term 3 h chilling of embryos at 0°C does alter the patterns of gene and protein expression. The chilling conditions applied in the present studies have practical applications in fish embryo handling in aquaculture eg embryo transportation between fish farms in genetic improvement programs. Storage of embryos at 0°C for prolong period of time slowing down embryo development significantly therefore providing an alternative method for accessing early stage embryos.

6.4.1 Studies on the effect of 18 h chilling on hatching rate in zebrafish *(Danio rerio)* embryos in the presence of sucrose and MeOH mixture

Embryos (50% epiboly) were chilled at 0°C for 18 h with different concentrations of MeOH (0.2, 0.5 and 1 M) in combination of sucrose (0.05 or 0.1M). It was observed that survival rates were increased significantly in embryos that had been chilled with 1 M or 0.5 M MeOH in 0.1 M sucrose when compare to those chilled without cryoprotectants, embryo hatching rates under these conditions were 56 ±5% and 45 ± 10% respectively. Methanol has been widely used as a cryoprotectant in zebrafish embryo cryopreservation and chilling sensitivity studies. In chapter 5, it was shown that when embryos were chilled for 18 h in 1 M MeOH, no significant improvement compare to non chilled control in survival rate was observed and embryo hatching rate was as low as 5% after chilling at 0°C. It is clear from the present study that 1 M MeOH along with sucrose (0.1 M) supplementation resulted in higher survival rates than that obtained when methanol was used along during longer term chilling of embryos for up to 18 h at 0°C. Zhang and Rawson (1995) obtained 88.4% survival rate for heartbeat stage zebrafish embryos that had been chilled at 0°C for 18 h in 1 M MeOH+0.1 M sucrose and survival rate was decreased to 81% as chilling period increased to 24 h. In the present study, under the similar conditions 56 ± 5% survival rate was obtained for 50% epiboly stage embryos. Decreased survival rate in 50% epiboly embryos compare to heartbeat stage could be due to the fact that early stages embryos are more sensitive to chilling than late stage embryos and chilling sensitivity decreases with development of embryonic stages (Zhang and Rawson 1995). Studies in medaka embryos (12 h hpf – early gastrulation stage which is similar to 50% epiboly in zebrafish) also showed 3-6 % increase in survival rates of embryos chilled up to 72 h at 0°C in methanol (3 M) supplemented with sucrose (0.5 M) when compared to those obtained from embryos chilled without sucrose (Ahammad et al. 2003b). Addition of sucrose in methanol also increased the survival of mrigal (*Cirrhinus*

mrigala) embryos from 0% to 25% for tail-bud stage embryos at 4°C for 12 h (Ahammad et al. 1998). This indicated the beneficial effect of sucrose when used in combination with MeOH in preventing embryos from chilling injuries. The protective effect of sucrose may be related to the moderate level of dehydration of embryos in the presence of sucrose which might helped in protecting the cell membrane from chilling injury (Dinnyés et al. 1998). The mechanisms of sugars such as trehalose (which is similar to sucrose – both are disaccharides) in protecting cells from injuries at low temperatures have been proposed by a number of studies. Trehalose acts as compatible solutes and is an excellent protector of membrane and proteins, it protects membranes during dehydration by hydrogen bonding to the phospholipid head group (Crowe et al. 1987). This interaction increases head-group spacing, hence lowering the transition temperature of the phospholipids (Rudolph and Crowe 1985). Unlike methanol, which penetrates into the cell membrane and protects cells intracellularly, sucrose does not penetrate the cell membrane and functions at the extracellular surface of the cells. At this site, the disaccharides increase the osmolarity of medium and keeping fewer water molecules from contacting the cell exterior. This process reduces the effect of low temperature on cell and stabilize cell membrane and protein (Beattie et al. 1997). However, high concentration of sucrose (1 M) alone caused 100% mortality of early gastrula stage common carp embryos after 12 h storage at 0°C (Ahammad et al. 2003b). This may be resulted from the severe dehydration of the embryos caused by high osmolarity of the sucrose solution.

6.4.2 Studies on the effect of 18 h chilling on *sox* gene and protein expression in zebrafish (*Danio rerio*) embryos

Previously, it was observed that 1 M MeOH supplemented with 0.1 M sucrose provided improved survival rates when embryos were chilled for 18 h at 0°C. In this chapter, studies were carried out to investigate whether the use of this cryoprotectant mixture had any effect on *sox* genes and protein expressions in embryo that had chilled for 18 h at

0°C. No significant differences were observed in *sox3* gene expression (Fig 6.2) and protein expression (Fig 6.4) between 18 h non chilled control embryos and chilled embryos with or without cryoprotectant. Significant decreases were observed in *sox2* gene (Fig 6.2a) and protein expression (Fig 6.4) in 18 h chilled embryo without cryoprotectant when compared to non chilled 18h control and no significant differences were observed in the 18 h chilled embryo with 1 M MeOH when compared with 18 h RT control. Previous hatching rate results showed improved survival rate in the embryos that had been chilled for 18 h at 0°C with 1 M MeOH+0.1 M sucrose cryoprotectant mixture. Chilling for prolonged period can cause indirect chilling injury (Morris 1984). Protein and enzyme are affected by indirect chilling injury at low temperature which may have downstream affect on their function and metabolic pathways. The disorder of metabolic and enzymatic processes can be detrimental in fast developing embryos like *Drosophilla* and such injury increases rapidly at lower temperature due to loss of co-ordination with reducing temperature (Mazur et al. 1992). Moreover, reduced temperature may also have adverse effect on cytoskeleton system i.e. depolymerisation of microtubules (Magistrini and Szollosi 1980, Behneke and Forer 1967, Weber et al. 1975) which could result in irreversible disruption of cellular process like cell division in oocytes (Magistrini and Szollosi 1980, Martino et al. 1995). Damage to the lamina (a part of nuclear envelope) has been reported to affect the gene expression as the lamina also functions as a structural nuclear protein and regulator of gene expression (Smith and Ane Silva E Silva 2004). Laminas have also been found to be associated with heterochromatin, at sites of DNA replication, RNA processing, replication protein and RNA polymerases. Any changes in lamina due to chilling injury may have affected such process results in alteration in mRNA transcription and subsequent translation. If such cryo-damage occurs, cells would appear morphologically normal following warming, however, future development of the cells could be compromised (Smith and Ane Silva E Silva 2004). Changes in gene

expression also affect the protein synthesis and protein expression. In this study, it was also observed that decreased *sox2* gene transcript due to 18 h chilling also suppressed subsequent *sox2* protein expression. This change in protein expression could be mainly due to changes in mRNA transcript, which is needed to initiate protein synthesis. Changes in transcription and translation level also affect phenotypical changes on embryo development as survival rate is affected and decreased significantly. Gene and protein expression of *sox2* did not show any decrease in 18 h chilled embryos in 1 M MeOH + 0.1 M sucrose and remained stable compared to 18 h non chilled control. Relatively stable gene and protein expression also resembles high survival rate in the embryos that had been chilled with cryoprotectant mixture. The precise mechanism by which sucrose act in these processes requires further investigation.

6.4.3 Studies on the effect of 18 h chilling and warming on *sox* gene and protein expression in zebrafish (*Danio rerio*) embryos

In the previous section 6.4.2, it was discussed that 1 M methanol plus 0.1 M sucrose protected gene and protein expression of the embryos following they were chilled for 18 h. When embryos were chilled for 18 h and cultured to hatching stages at 27± 1°C, *sox2* gene expression was increased significantly in heartbeat stage and *sox19a* was increased just after 18 h chilling when compared to non chilled controls and which gene expression levels decreased to non chilled control levels in hatching stage embryos. However, no significant differences were observed in *sox2* protein expression in embryos that had been chilled for 18 h with/without cryoprotectant before cultured to hatching stage when compared to 18 h non chilled embryos. Opposite was true for *sox3* gene expression in embryo that had been chilled for 18 h in 1 M MeOH + 0.1 M sucrose and cultured to hatching stage, where expression of *sox3* decreased significantly compare to non chilled control. No significant differences was observed in *sox3* protein expression in the hatched embryos chilled for 18 h in 1 M MeOH +0.1 M sucrose compare to non chilled 18 h

controls. Results obtained from this study complimented by higher survival rate in the embryo that had been chilled for 18 h in cryoprotectant mixture and cultured to hatching stage, despite the changes in *sox3* gene expression in hatched embryos. Changes in *sox3* gene expression could have been compensated by increased expression of *sox2* and *sox19a* genes. Sox genes have capability to bind with other transcription factor proteins to activate or represses specific target genes (Kamachi et al. 2000). A study has showed that Sox proteins have the capability of pairing off with different types of transcriptional factors (Wilson and Koopman 2002). A study in early blastula to gastrula stage zebrafish embryos by a quadruple knockdown technique demonstrated that B1 *sox* genes (*sox2/sox3/sox19a/sox19b)* are highly redundant and their encoding proteins are functionally interchangeable in early zebrafish embryogenesis (Okuda et al. 2010). Stable *sox3* protein expression in hatched embryo after they were chilled for 18 h in cryoprotectant mixture when compare to non chilled control could be due to the post transcriptional repair mechanism which involves the post transcription and translation modification to repair the loss of *sox3* gene transcript. Studies in *hsp90* in parasite *Giarida* after chilling for 20 min on ice demonstrated the post transcriptional repair mechanism by mRNA *trans*-splicing (Nageshan et al. 2011).

6.5 Summary

In the present chapter, long term chilling storage protocol for early stage zebrafish embryos was developed which does not compromise genetic integrity. 1 M MeOH + 0.1 M sucrose cryoprotectant mixture was found to improve embryo hatching survival when embryos were chilled for 18 h at 0°C when compared to embryos that had been chilled in 1 M MeOH + 0.05M sucrose or in MeOH alone (see previous chapter). This indicated the beneficial effect of sucrose when used in combination with MeOH in preventing embryos from chilling injuries. The protective effect of sucrose may be related to the moderate level of dehydration of embryos which prevented them from chilling injury. It was also

found that gene expression was affected by cryoprotectant mixture as significant increases in *sox19a* gene expression was observed in embryos that had been chilled for 18 h in 1 M MeOH compare to non chilled control. This increase could be the result of the compensation of the loss of gene transcript due to 18 h chilling as this group of genes are functionally redundant and interchangeable. Furthermore, no significant differences were observed in *sox* protein expression in hatching stages which also complimented by higher survival rate in embryos that had been chilled with 1 M MeOH+ 0.1 M sucrose. This may be explained by the repair mechanisms of the embryos which involved the post transcription and translation where loss of gene transcript could be repaired resulted in normal protein expression. There is no information in the literature available on the effect of long term chilling with cryoprotectant on gene and protein expression. This is the first report of successful early stage embryo (50% epiboly) chilling storage for up to 18 h without compromising genetic integrity. The method developed here for gene and protein expression studies would be very useful in providing additional information on embryo quality not only after chilling storage but also after cryopreservation. In the presence studies, we only analysed genes responsible for nervous system development following long term chilling. Further studies are needed to analyse genes and proteins responsible for metabolic activity, transcription, cell organization, signal transduction, intracellular transport etc, in order to provide a complete picture of embryo quality following chilling storage.

CHAPTER 7 CONCLUSIONS

7.1 Reiteration of aims and objectives

Cryopreservation of fish embryos is still a challenge due to structural and functional limitations of these embryos such as their large size, high yolk content, greater sensitivity to chilling and low membrane permeability (Zhang 2004). In aquaculture, short term storage of fish embryos under chilled conditions has been used to facilitate transportation of the embryos in genetic improvement programmes. It is therefore important to understand the effect of chilling on embryos not only on development but also at molecular level. In embryo chilling and cryopreservation studies, embryo hatching rate has mainly been used as an indicator for successful cryopreservation protocol, however information on genetic integrity of the recovered material is also important. It has been reported (Succu et al. 2008, Lin et al. 2009b, Uechi et al. 1997) that cryopreservation alters the pattern of gene expression which results in adverse effect on biological development. In the present study, the effect of chilling on gene and protein expression was studied using both short and long term chilling protocols including the effect of cryoprotectant.

Four areas of studies were carried out using zebrafish embryos: (1) Investigation of *hox* and *sox* gene expression in different developmental stages of zebrafish (*Danio rerio*) embryos (2) Studies on the effect of short term chilling on *sox* gene expression in zebrafish embryos (3) Studies on the effect of chilling on gene and protein expression in zebrafish (*Danio rerio*) embryos in the presence of methanol (MeOH) (4) Studies on the effect of longer term chilling on gene and protein expressions in zebrafish (*Danio rerio*) embryo

(1) Determination of *hox* and *sox* gene expression in different developmental stages of zebrafish (*Danio rerio*) embryos

In order to study the effect of chilling on gene expression and protein expression in zebrafish embryos, there is a need to understand the pattern of gene expressions throughout the embryo development. Understanding when genes are expressed can facilitate greater understanding of their function, and also allow the genes to be manipulated by gene knockdown in temporally and spatially specific manners (Lan et al. 2009). Selection of genes is also important as selected genes should be developmentally important e.g. associated with major development processes through embryonic development. In this study, *hox* and *sox* genes were studied to evaluate their patterns of expression in different developmental stages of zebrafish embryo. The genes studied were *hoxb1b, hoxb6b, hoxc6a* and *hoxc8a* and they all belong to the homeobox gene group. Homeobox genes are a family of regulatory genes containing a common 183 nucleotide sequence (homeobox) and coding for specific nuclear protein (homeoproteins) that act as a transcription factor, characterized by their role in conferral of segmental identity along anteroposterior (A-P) axis of the body (McGinnis and Krumlauf 1992, de Rosa et al. 1999). Studies of *sox* genes, *sox2*, *sox3* and *sox19a* are also important as they are associated with embryonic development. The major functions of these genes include sex determination (Polanco and Koopman 2007), stem cell development in embryos (Avilion et al. 2003), neurogenesis (Pevny and Placzek 2005), skeletogenesis (Smits et al. 2001), hematopoiesis (Schilham et al. 1997), cardiogenesis (Akiyama et al. 2004) and angiogenesis (Matsui et al. 2006).

(2) Studies on the effect of short term 3 h chilling on *sox* gene expression in zebrafish embryos

Based on the results obtained from previous section, the effect of chilling on *sox* genes was investigated. Information on the effect of short term chilling on gene expression is important before studies on the long term chilling storage protocol. Results from these studies provide important information in designing longer term chilling storage protocol for zebrafish embryos.

(3) Studies on the effect of 3 h chilling on gene and protein expression in zebrafish (*Danio rerio*) embryos in the presence of methanol (MeOH)

Long term chilling storage or cryopreservation requires the addition of cryoprotectant in cryopreservation solution in order to protect cells from low temperature injury. Although studies on the effect of cryoprotectant on zebrafish embryo survival were carried out previously (Zhang and Rawson 1995), studies on the effect of these cryoprotectants on embryos at the molecular level have been limited (Lin et al. 2009b). Cryoprotectant toxicity studies are essential prior to their use in chilling storage or cryopreservation protocols. The effect of cryoprotectant is commonly assessed by subsequent embryo development. However this type of study does not give any information on the effect of cryoprotectant at molecular level eg the effect on gene or protein expression. Methanol (MeOH) has been reported to be ocular toxic (Lee et al. 1994) and the toxicity increases with increasing concentrations of methanol in mouse and rat embryos (Andrews et al. 1993). In the present study, investigations have been carried out to assess gene and protein expression in the embryos that been chilled with or without MeOH. The effect of methanol on hatching rates in embryos chilled for up to 24 h at 0°C was investigated. Once the hatching rate had been identified, the effect of methanol on gene expression and protein expression was studied in chilled and chilled-warmed embryos throughout embryonic development.

(4) Studies on effect of longer term chilling on gene and protein expression in zebrafish (*Danio rerio*) embryo

Following studies on the effect of short term chilling on gene and protein expressions in zebrafish embryos, the effect of longer term chilling (up to 24 h) on gene and protein expressions in embryos were studied as these conditions have practical applications in fish embryo handling in aquaculture e.g. embryo transportation between fish farms in genetic improvement programs. Initial studies were carried out to identify and optimise suitable cryoprotectant mixture for improving survival rate of 50% epiboly embryos chilled for up to 24 h. Studies on gene and protein expression was then carried out in order to investigate effect of chilling and warming in the embryos that had been chilled with optimised cryoprotectant mixture for up to 18 h.

7.2 Review of main findings

7.2.1 Determination of *hox* and *sox* gene expression in different developmental stages of zebrafish (*Danio rerio*) embryos

The objective of this work was to investigate gene expression of pattern of *hox* and *sox* genes in early embryonic developmental stages. The results showed that expression of *sox* was observed throughout tested developmental stages from 30% epiboly to 6 somites stages. It was also observed that band intensities of *sox2* and *sox19a* increased with developmental stages, while band intensity of *sox3* was stable throughout tested developmental stages from 30% epiboly to 6 somites stages. Similarly expression of *hoxb1b* was also observed in all tested developmental stages. However, expression of *hoxb6b* and *hoxc6a* was only detected from 75% epiboly to 6 somites stages, whilst *hoxc8a* was detected from bud stage to somites stages. Based on the results obtained from this study, *sox* genes were selected to study the effect of chilling on gene expression due to their consistent expression throughout the tested embryonic development stages.

7.2.2 Studies on the effect of short term chilling on *sox* gene expression in zebrafish (*Danio rerio*) embryos

The aim of this study was to investigate whether short term chilling had an impact on *sox2, sox3* and *sox19a* gene expression in zebrafish embryos. Studies on the effect of chilling on hatching rate were carried out prior to gene expression studies. The results from these experiments showed that there were no significant differences in embryo hatching rates between the control samples kept at 27 ± 1 °C and chilled samples (chilled for 30, 60, 120 and 180 min at 0 °C) for either 50% or 100% epiboly stage embryos, however, significant decreases were observed in 75% epiboly embryos that had been chilled for 180 min.. It was observed that expression levels of *sox2* and *sox3* were stable throughout 180 min treatment period in control embryos. For embryos that had been chilled at 0°C for 180 min, expression levels of *sox2* and *sox3* were decreased significantly after 60 min. No significant differences were observed in *sox19a* gene expression in chilled and control embryos.

Further studies were carried out on whether the loss of transcript was recovered upon warming after embryos were chilled for 30 and 60 min at 0°C. The results showed that, gradual increases in *sox2* expressions were observed in embryos that had been chilled for 30 min, warmed and then cultured for up to 180 min, the expression levels were significantly higher than those obtained from controls by 150 min after chilling. Short term surge of expression in *sox3* and *sox19a* genes was observed at 60 min which subsequently decreased to control levels by 90 min. For embryos that had been chilled for 60 min, expressions of both *sox2* and *sox3* increased to significantly higher levels than those in controls until 180 min, the expression levels were then decreased to control levels. The opposite trend was observed for *sox19a* where expression levels initially decreased significantly when compared to those of the controls before returning to control levels by 180 min.

7.2.3 Studies on the effect of chilling on gene and protein expression in zebrafish (*Danio rerio*) embryos in the presence of methanol (MeOH)

The aim of this study was to evaluate *sox* gene expression and subsequent protein expression in the embryos that been chilled in MeOH. Initial hatching rate studies were carried out to assess chilling tolerance of 50% epiboly stage embryos in the presence of MeOH. Results from this study showed that 50% epiboly embryos could tolerate chilling up to 6 h at 0°C and no significant decreases were observed in embryos that been chilled with different concentrations of MeOH for 3 and 6 h. Significant decreases were observed in embryos that been chilled for 18 and 24 h with or without MeOH.

Further studies were carried out to assess gene and protein expression of *sox* in the embryos that survived chilling for 3 h in different concentrations of MeOH. It was observed that expression of all three *sox* genes were significantly decreased following chilling for 3 h at 0°C. However the degree of decrease was less pronounced in embryos that had been chilled in different concentrations of MeOH. The degree of significant decreases was less pronounced as the concentrations of MeOH increased. Upon warming the both gene and protein expression levels were recovered in all embryos chilled for 3h. However, short term surge of significant increases were observed in hatching stage embryos that had been chilled with 1 M MeOH. Significant increase in *sox3* was also observed in the embryos that had been chilled with 0.5 M MeOH. *Sox19a* gene expression remained higher than control and was stable throughout treatment period in the embryos that had been chilled with 1 M MeOH.

Studies were then carried out to evaluate whether significant changes (following chilling in 1M MeOH and warming) in gene expression of *sox2* and *sox19a* resulted in similar pattern of protein expression. No significant differences were observed in *sox2* protein expression in embryos that had been chilled for 3 h with 1 M MeOH and cultured to hatching stages. In contrast, *sox19a* protein expression was observed to be significantly

higher than that in controlled embryos following 3 h chilling but remained stable upon warming and cultured to hatching stages.

7.2.4 Studies on the effect of longer term chilling on gene and protein expression in zebrafish (*Danio rerio*) embryo

The aim of this study was to investigate the effect of longer term (18h) chilling on *sox* gene and subsequent protein expression in early stage (50% epiboly) embryos. The results from the previous study (Chapter 4) indicated that 50% epiboly stage embryos could tolerate chilling for up to 6 h at 0°C and hatching rate was significantly reduced after 6 h. Therefore in order to study the effect of longer term chilling, it is important to improve embryo survival rate after chilling 18 h. In these experiments, sucrose was used in methanol as the cryoprotectant mixture and different combinations of MeOH + sucrose concentrations were tested. It was shown that embryo hatching rate was improved (compared to 5% when chilled with 1 M MeOH) to 60% when embryos were chilled for up to 18 h in 1 M MeOH + 0.1 M sucrose and 0.5 M MeOH + 0.1 M sucrose. Hatching rate remained low (~20%) for the 18 h chilled embryos when they were chilled in different concentrations of MeOH + 0.05 M sucrose.

The experiments were then carried out to assess if chilling embryos in 1 M MeOH + 0.1 M sucrose has any adverse effect on gene expression and subsequent protein expression. When embryos were chilled for 18 h, it was observed that *sox2* gene expression decreased significantly without the use of cryoprotectant and in embryos that been chilled with cryoprotectant, no significant differences under these conditions were observed. Likewise, no significant differences were observed in *sox3* and *sox19a* gene expression in 18h chilled embryos without cryoprotectant and 18h RT control embryos. Significant increases were observed in level of *sox19a* expression in the embryos that had been chilled with cryoprotectant compared to 18h RT control. In the embryos that had been chilled with 1 M MeOH + 0.1 M sucrose and cultured until hatching stages, *sox2* and

sox19a gene expression increased immediately after chilling and continued to increase until heartbeat stages before reaching similar levels in those of control embryos by hatching stage. Opposite gene expression patterns were observed for *sox3* as gene expressions decreased significantly at 20 somites stage when compared with RT control and remained low before decreased significantly again by hatching stage.

To further understand the different gene expression patterns of *sox3* when compared to *sox2* and *sox19a* genes, protein expression studies were carried out for *sox2* and *sox3* following 18 h chilling, warming and embryo culture until they hatched. Significant decreases of protein expressions in *sox2* and *sox3* were observed in embryos that had been chilled without cryoprotectant and protein expression was recovered after warming and culturing of embryos until hatching stage. No significant differences in *sox2* and *sox3* protein expression were observed during chilling and warming in the embryos that had been chilled with cryoprotectant.

7.3 Conclusions

Limited studies has been carried out on the effect of low temperature on cells at molecular level as the effect of chilling or cryopreservation has largely been assessed by embryo hatching rate or vital stain viability studies in blastomeres or oocytes. In the present study, progress has been made in several key areas on the understanding of the effect of chilling on gene expression and protein expressions in zebrafish embryos after chilling and warming. The original contributions to knowledge from the present study is summarised below:

a) Zebrafish *hox* gene expression profile is reported here for the first time. So far very little is known about *hox* and *sox* gene expression in early stage of embryonic development in zebrafish. Present study on *hox* gene expression in early embryonic stages of zebrafish showed that *hoxb6b* and *hoxc6a* genes

started to expressed from 75% epiboly stage whilst *hoxc8a* did not start to express until 100% stage. Studies of *sox* gene expression in zebrafish embryonic stages showed that all three *sox (sox2, sox3* and *sox19a) genes* expressed in all tested developmental stages from 30% epiboly to 6 somites stages. *Hox* genes act as a transcription factor, characterized by their role in conferral of segmental identity along anteroposterior (A-P) axis of the body (McGinnis and Krumlauf 1992, de Rosa et al. 1999). Similarly, *sox* family is a group of proteins which appear to regulate cell fate during embryogenesis by functioning as transcriptional factor. Information obtained from the present study would help to understand embryonic development not only in zebrafish but also in human and associated genetic disease as both human and zebrafish share similar groups of genes. Information on gene expression patterns throughout embryonic developmental is essential prior to any further investigations relating to human disease.

b) The effect of short term (3h) chilling and warming on *sox* gene expression were studied for the first time. It was shown that expression of *sox2* and *sox3* genes were decreased significantly following 3 h chilling at 0°C and *sox3* and *sox19a* expression increased significantly when compared to controls upon warming at 27°C before returned to control levels after culturing at 27°C for 3.5h. *Sox2* expression remained significantly above control levels throughout warming and culturing period at 27°C. It is clear that chilling and warming of embryos does alter patterns of gene expression. However, despite of (significant changes are not subtle) changes in gene expression during chilling and warming, embryo survival rates were not affected by chilling when compared to RT controls. The present studies showed that decrease in

gene expression during chilling could have been compensated by higher levels of gene expression than those in controls after warming which may have explained the unaffected embryo hatching rate following chilling and warming process.

c) The effect of chilling in the presence of cryoprotectant methanol on gene expression in 50% epiboly zebrafish embryos is studied here for the first time. Results from embryo hatching rates studies showed that embryos could tolerate chilling for up to 6h with/without MeOH. However, for embryos that had been chilled without cryoprotectant, gene expression of *sox* decreased significantly when compared to control levels. For embryos that had been chilled with different concentrations of MeOH, the degree of decrease was less pronounced. The level of gene expression decrease was less affected as the concentrations of MeOH increased. The results showed that MeOH could protect embryos from transcript degradation. However, higher concentration of MeOH (1 M MeOH) also altered pattern of sox gene expression when compared to controls at hatching stage. The present study showed that alteration in *sox* gene expression does not necessarily mean changes in protein expression as protein expression of *sox2* and *sox19a* were stable in the embryos chilled with 1 M MeOH. This could be explained by the repair mechanisms of the embryos which involved post transcription and translation where loss of gene transcript could be repaired and resulted in normal protein expression. These results provided useful information in understanding the mechanisms associated with chilling injuries of zebrafish embryos.

d) The present study also provided new information on the effect of long term (18h) chilling on gene expression and protein expression. Early stage

zebrafish embryos (50%) were successfully chill-stored for up to 18 h in a MeOH and sucrose mixture. Embryos from 50% epiboly stage were chilled with 1 M MeOH + 0.1 M sucrose and the survival rates were improved significantly from 5% (chilled in 1 M MeOH) to 60%. The results also showed that in the embryos that had been chilled with 1 M MeOH + 0.1 M sucrose and cultured until hatching stages, *sox2* and *sox19a* gene expression increased immediately after chilling and continued to increase until heartbeat stages before reaching similar levels in those of control embryos by hatching stage. No significant differences between 18 h chilled with egg water, 18 h chilled with 1 M MeOH+0.1 M sucrose and 18 h RT were observed in *sox* protein expression.

7.4 Future work

Following the findings obtained from the present study, further investigations should be carried out in the following areas.

7.4.1 Global gene and protein expression studies using microarrays

In the present study, different gene and protein expression profiles were observed during 3 and 18h chilling and all genes function differently under different chilling conditions. It is possible that different mechanisms may be present in order to protect embryos from chilling injury. Further studies would need to be carried out in this area.

The effect of chilling on gene expression can be carried out on more than one group of genes i.e. associated with protein metabolism, transcription, cell organization, signal transduction, intracellular transport, macromolecule biosynthesis and development (Larman et al. 2011). Information from this type of study is important in further understanding the molecular mechanisms associated with chilling. Microarray can be used to study different sets of genes at same time and from same set of samples. Results

obtained from microarray are semi quantitative and does not provide accurate quantification provided by quantitative PCR, so once genes are identified, real time PCR techniques can be incorporated on specific sets of genes in order to study their functions in details. Further protein expression studies would also need to be carried to understand protein profile.

7.4.2 Studies on effect of chilling at different developmental stages of the embryos

Present study has been carried out on 50% epiboly embryos and effects of chilling on 50% epiboly embryos were studied. It has been shown that early stage zebrafish embryos are chilling sensitive than in later stage embryos (Zhang and Rawson 1995). More studies could be done to investigate chilling effect on different developmental stages. Gene and protein expression profile can be varies in different developmental stage as every genes have specific time period for activation and deactivation. Comparison of these profile and analysis would be robust approach for the genes to use them as a marker to assess embryo quality collected from different spawning and at different development stages. It would also provide information on how different embryonic development affected by chilling treatment and any adaptation which help to design more robust chilling storage protocol.

7.4.3 Heat shock proteins (HSPs) and cold shock proteins studies

Heat shock proteins (HSPs) are a family of highly conserved cellular proteins present in all organisms including fish. Heat shock proteins play important role in protein homeostasis and cellular stress responses within the cells. HSPs, also known as chaperones, play crucial roles in folding/unfolding of proteins, assembly of multiprotein complexes, transport/sorting of proteins into correct subcellular compartments, cell-cycle control and signalling, and protection of cells against stress/apoptosis (Li and Srivastava 2001). Upregulation of *hsp70* mRNA in response to low temperature was reported in

several insect species including *Drosophilla* (Goto et al. 1998, Sinclair et al. 2007). Liu et al (2003), also demonstrated that cryopreservation can induce heat shock proteins *hsp78* and *hsp86* expression in mouse ovarian tissues. It was also observed that chilling could alter pattern of expression of *sox* genes, may be due to degradation of mRNA transcript and recovered upon warming and culturing at 27°C. This recovery may be due to heat shock proteins, although the repair mechanism is still unknown. Studies of heat shock proteins could provide useful information in understanding the repair mechanisms in cells.

There are other groups of proteins namely cold induced RNA binding proteins (CIRBP) and RNA binding motif 3 (RPM3) which are induced by cold shock. Translation of mRNA appears to be key point of control in the cold-shock response. CIRBP and RPM3 protein regulate gene expression at the level of translation by binding to different transcript, thus allowing the cells to respond rapidly to environmental signal (Lleonart 2010). It has also been proposed that CIRBP binds DNA and serves as a chaperone that assists in the folding/unfolding, assembly/disassembly and transports various proteins (Fujita 1999, Gualerzi et al. 2003). Studies of these genes and subsequent proteins expressions could enhance the understanding of molecular mechanisms associated with cold shock injury.

7.4.4 Antifreeze protein studies

The antifreeze proteins (AFPI and AFPIII) as potential cryoprotectants should be investigated further following their successful use in mammalian systems (O'Neil et al. 1998). AFPs are natural cryoprotectants present in arctic species, as well as in other teleost fish (Crevel et al. 2002, Davies and Sykes 1997). AFPs non-colligatively lower the freezing point of aqueous solutions, block membrane ion channels and thereby confer a degree of protection during cooling (Baguisi et al. 1997). These proteins adsorb ice crystals and prevent their growth and also avoid crystal formation during freezing and reduce cellular damage (Fletcher et al. 2001). Studies have

showed that AFPs improved the survival rate of zebrafish embryos following chilling and vitrification (Martínez-Páramo et al. 2009). Studies also showed that the production of AFP proteins *in vivo* in drosophila melanogaster can be achieved using hsp70 promoter (Rancourt et al. 1987). Similar approach can be used with zebrafish hsp gene to produce AFP in *vivo* using molecular cloning and recombinant technology. The success of this experiment can be measured using quantitative real time PCR and western blot by using *sox* genes as a marker.

Reference:

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APPENDIX A

The chemicals used in present study

Chemicals	Source	Product No.
10x Tris/Glycine/SDS, 1 L	Biorad	161-0732
Agarose	Bioline	BIO-41025
ATX Ponceau S red staining solution	Sigma	9189
BIOTAQ™ DNA Polymerase	Bioline	BIO-21040
dNTP Mix	Bioline	BIO-39053
Ethanol	Fisher	E/0500DF/08
Ethidium bromide	Sigma	E1510
EZNA Gel extraction kit	Omega Bio-tek	A112907W
gel loading buffer	Sigma	G2526
HyperLadder™ V	Bioline	BIO-33031
MagicMark™ XP Western Protein Standard	Invitrogen	LC5602
Methanol	Fisher	M/4000/17
Phosphatase Inhibitor Cocktail 3	Sigma	P0044
Precision nanoScript Reverse Transcription kits	Primerdesign	RT-nanoScript
Protease from Streptomyces griseus	Sigma	P6911-1G
Protease Inhibitor Cocktail	Sigma	P8340
Protogel	Fisher	ELR-210-010P
QuantiPro™ BCA Assay Kit	Sigma	QPBCA
Resolving buffer (Tris/SDS),	Fisher	ELR-210-060A
RNAqueous-Micro Kit	Invitrogen	AM1931
Sample Buffer, Laemmli 2× Concentrate	Sigma	S3401
SDS solution 10%	Fisher	BPE2436-1
Sea Salt	ZM Ltd.	N/A
SensiMix SYBR No-ROX Kit	Bioline	QT650-05
Stacking buffer (Tris/SDS)	Fisher	ELR-210-070U
Sucrose	Sigma	S0389
TEMED	Fisher	T/P190/04
Tris Buffered Saline	Sigma	T5912
WesternDot™ 625 Goat Anti-Rabbit Western Blot Kit	Invitrogen	W10142

APPENDIX B

Publication

Desai K, **Spikings E**, and **Zhang T** (2011). Effect of chilling on *sox2*, *sox3* and *sox19a* gene expression in zebrafish (*Danio rerio*) embryos, Cryobiology 63(2), 96-103

Desai K, Spikings E and **Zhang T** (2011). Studies on the role of hsp47 and hsp90 in zebrafish (*Danio rerio*) embryos under chilled conditions. Cryobiology, 63(3), 334 [Abstract]

Desai K, Spikings E, **Rawson DM** and **Zhang T** (2010). Studies on impact of chilling on *sox* gene expression in zebrafish (*Danio rerio*) embryos. Cryobiology 61(3), 389 [Abstract]