Title:

USE OF METHANOL AS CRYOPROTECTANT AND ITS EFFECT ON MOLECULAR LEVEL IN CHILLED ZEBRAFISH EMBRYOS

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Effect of chilling on gene and protein expression

Abstract

Methanol is widely used cryoprotectant (CPA) in cryopreservation of fish embryos, however there is necessity to understand its effect at molecular level before being used as CPA. This study was focused on the effect of methanol on sox gene and protein expression in zebrafish embryo (50% epiboly) when they were chilled for 3 h and subsequently warmed up and cultured to the hatching stages. Initial experiments were carried out to evaluate the chilling tolerance of 50% epiboly embryos showed no significant differences in hatching rates observed for up to 6 h chilling in methanol (0.2-, 0.5- and 1 M) whilst hatching rates decreased significantly after 18 and 24 h chilling. Further to understand molecular mechanism, s*ox* genes and protein expression were studied in embryos that had been chilled for 3 h in methanol and warmed and cultured up to the hatching stages. *Sox2* and *sox3* gene expression at the hatching stage were increased significantly in embryos that had been chilled in 1 M MeOH and subsequently cultured to hatching stage when compared to controls and *sox19a* gene expression remained above control levels at all developmental stages tested. Whilst stable *sox2* protein expression were observed between non-chilled controls and 3 h chilled embryos with or without MeOH, a surge of increase in *sox19a* protein expression was observed in 3 h chilled embryos in the presence of 1 M MeOH compared to non chilled controls before being levelled up to control levels by the hatching stage. Alteration in *sox19a* gene expression could be compensatory response in order to maintain homeostasis.

Keywords

Chilling, zebrafish, 50% epiboly embryo, methanol effect, hatching, sox gene expression, protein expression

Introduction

Methanol has been widely used cryoprotectant in embryos and oocytes and other reproductive tissues cryopreservation However, success of most of cryopreservation protocol usually measured by either physical appearance of cell or survival rate. It has previously been reported that chilling alters the pattern of *sox* gene expression in zebrafish embryos (Desai et al. 2011). Simillary effect of these CPA at molecular level is still unknown. In the present study, 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. 2004). 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 cryoprotectants are toxic especially when used at high concentrations (Zhang et al. 2012). The toxicity of cryoprotectants to cells is also dependent on their type, exposure temperature and exposure time period (Tsai and Lin 2009). Cryoprotectants can cause cellular injury by osmotic trauma (Pillai et al. 2001). Cryoprotectant toxicity studies are now common practice prior to their use in cell cryopreservation. However there is very limited information on how cryoprotectants function at the molecular level and if they have a significant effect on gene or protein expression following cryopreservation. Understanding of the impact of cryoprotectants at the molecular level is important especially for reproductive materials such as embryos, oocytes, ovarian tissues. Any changes at molecular level could have lethal effect on subsequent development. Any alteration during these early stages could be replicated in long term genetic defect. Studies in mouse and rat embryos have shown that methanol (MeOH) is toxic (Lee et al. 1994) and even lethal when used at high concentrations (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. 2003). It has been found that

methanol was effective in zebrafish embryo cryopreservation because it has low toxicity compare to other most commonly used cryoprotectants (Zhang and Rawson 1995) and also be able to permit through embryo membrane rapidly (Hagedorn et al. 1997). Similar studies in medaka also demonstrated higher embryo survival rate after chilling in presence of MeOH (Zhang et al. 2012). However, it has also been shown that methanol exposure is associated with visual impairment or blindness, affecting optic nerve and retina in rats treated with MeOH (Eells 1991). Methanol has also been demonstrated to be neurotoxic where its exposure leads to severe Central Nervous System defects in mice at gastrulation periods (Degitz et al. 2004) and in drosophila embryos at 8-11 embryonic stages (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 is better understood when used as a cryoprotectant.

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

Results

Experiment 1: Impact of chilling on embryo hatching rate at 0° C for different time periods in the presence of MeOH as a cryoprotectant

Before embryos being subject to molecular studies using MeOH, embryos should be able to withstand with lower temperature for certain amount of time. To determine this present experiment were carried out assess effect of chilling on 50% epiboly stage embryos in presence of different concentration of MeOH up to 24 h. A Small amount of development was observed in 50% epiboly stage embryos when they were subjected to chilling at 0℃. It was observed (Fig. 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 were 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 were chilled with different concentrations of methanol.

Experiment 2.1: Impact 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

Sox2 gene expression (Fig 2a) in non-chilled control embryos was relatively stable throughout the tested developmental stages (from 50% epiboly stage to hatching stage). Expression of *sox2* in embryos that were 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 the 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.

Comparisons of sox2 gene expression at specific stages throughout development

Sox2 gene expression (Fig 2b) was stable at all tested stages. However, significant decreases were found in embryos that were chilled at 0°C with or without MeOH when compared to 0 h. In the embryos that were chilled in the presence of egg water and warmed at 27°±1°C, expression levels returned to the level at time 0 by the hatching stage. Similar patterns of expression were observed in embryos that were chilled with 0.2-,0.5- and 1 M MeOH and warmed at 27°±1°C until the hatching stage.

Experiment 2.2 Impact 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

In non-chilled control embryos, *sox3* (Fig 3a) gene expression was stable until the heartbeat stage before decreasing again by the hatching stage. In embryos that were 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 levels by hatching stage. For the embryos that were chilled with 0.5 and 1 M MeOH, no significant decreases of *sox3* expression were observed until the 20 somites stage. The gene expression subsequently increased and were significantly higher than in non-chilled control embryos by the hatching stage.

Comparisons of sox3 gene expression at specific stages throughout development

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

142

143 **Experiment 2.3 Impact of chilling and warming on *sox19a* gene expression in zebrafish *(Danio***
144 ***rerio)* embryos in the presence of MeOH**

145 ***Comparisons of different concentrations of MeOH on sox19a gene expression***

146 In control embryos, *sox19a* (Fig 4a) gene expression levels remained stable throughout developmental
147 stages. In treated embryos, significant gene expression decreases were observed in embryos that were
148 chilled in egg water for 3 h before the expression returned to control levels by 20 somites stage. For
149 embryos that were chilled in 0.5 M and 1 M MeOH, significant increases of *sox19a* gene expression were
150 observed after 3h chilling when compared to non-chilled controls. The *sox19a* gene expression remained
151 significantly above the non-chilled control levels throughout development stages.

152 ***Comparisons of sox19a gene expression at specific stages throughout development***

153 In non-chilled control embryos (Fig 4b), expression of *sox19a* remained stable before decreasing by the
154 20 somites stage at 27±1 ℃. In the embryos that had been chilled at 0°C in egg water and cultured at
155 27±1°C, significant decreases were observed after 3 h chilling and at the hatching stages when compared
156 to time 0. In embryos that were chilled in different concentrations of MeOH, *sox19a* gene expressions
157 increased significantly in 0.5 and 1 M chilling embryos immediately after 3 h chilling and then decreased
158 to the time 0 level after culturing at 27±1 ℃ at hatching stage.

159

160 **Experiment 3: Impact of chilling and warming on *sox* protein expression in zebrafish**
161 ***(Danio rerio)* embryos in the presence of MeOH**

162 There is a strong relationship between gene and protein expression levels as protein is usually produced
163 based on the information obtained from a specific gene or mRNA. However, sometimes this relationship
164 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). As studies on gene expression (mRNA level) do 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.

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. Expression of *sox2* protein remained stable under all treatment conditions (Fig 5b). *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 were chilled with 1 M MeOH for 3 h at 0°C.

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 MeO and then replaced with egg water and embryos were cultured at 27±1°C until the hatching stage. *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

188 subsequently cultured until the hatching stage. Protein expression remained stable in both chilled and non
189 chilled embryos at the hatching stage. Protein expression of *sox19a* remained stable from 50% epiboly
190 stage to the hatching stages. No significant differences were observed in *sox19a* protein expression in
191 embryos that had been chilled with or without MeOH and non chilled controls.

192

193 **Discussion**

194 Cryoprotectant toxicity studies are necessary before they are used in any chilling storage and
195 cryopreservation protocol in order to minimise the effect of these chemicals. Survival rate has been
196 widely used to assess cryoprotectant toxicity in embryos (Kopeika et al. 2003) and oocytes (Plachinta et
197 al. 2004) prior to their chilling storage or cryopreservation. Methanol has been demonstrated to be an
198 effective cryoprotectant in zebrafish embryo chilling storage (Zhang et al. 2003) and oocytes
199 cryopreservation (Guan et al. 2008). However, there is no information available in the literature on the
200 effect of methanol on gene and protein expression. This information is important due to the fact that
201 MeOH is neurotoxic (Degitz et al. 2004). Studies have shown that methanol exposure leads to severe
202 CNS defect in mice embryos (Degitz et al. 2004) and drosophila embryos (Mellerick and Liu 2004).
203 Therefore the aim of the present study was to investigate the effect of MeOH on gene and protein
204 expression of zebrafish embryos following short term chilling and subsequent culture after warming.

205 **Assessment of chilling tolerance of zebrafish embryos at 0°C**

206 Initial study was carried out to investigate chilling tolerance of 50% epiboly embryos for up to 24 h at
207 0°C with the presence of different concentrations (0.2-,0.5- and 1 M) of MeOH. Results from the study
208 showed that 50% epiboly stage embryos tolerated chilling for up to 6 h with/without MeOH (90 ± 5 %
209 survival rate) before it was significantly decreased after 18 and 24 h at 0°C (18 and 24h results, 10±5%
210 and 7±5% respectively). Previously, Zhang and Rawson (1995) demonstrated that over 50% of shield
211 stage (~60% epiboly) embryos were killed when they were exposed to 0 °C for 4 h without any

212 cryoprotectant and no embryo younger than bud stage survived 11 h exposure at 0°C. Due to the fact that
213 zero or low survival rates were obtained for embryos chilled at 0°C after 24 h , no longer term chilling
214 studies in the presence of MeOH were carried out with 50% epiboly stage embryos previously. Results
215 obtained in medaka embryos (Valdez Jr et al. 2005) showed that gastrula stage embryo survival rate was
216 not affected by 24 h chilling at 0°C in hank's solution. In the present study, survival rate of similar stage
217 50% epiboly was reduced to 10% following chilling in egg water for 18 and 24 h at 0°C. This is mainly
218 due to developmental pattern of medaka embryos as it is generally slower (9 days to hatch) than zebrafish
219 embryos (3 days to hatch).
220 Cell membranes are generally highly permeable to methanol as Zhang et al. (2005) reported in zebrafish
221 that methanol penetrates ovarian follicle at a rate comparable to the rate of water transport and therefore,
222 incubation of cells in MeOH does not lead to osmotic stress. In the present study, there were no
223 significant differences found in embryos that had been chilled in MeOH (at all tested concentrations) and
224 egg water at 0°C up to 24 h. This could be due to the fact that gastrula stage embryos are highly chilling
225 sensitive and the concentrations of MeOH used in the present study was not effective in protecting
226 embryos from chilling injury. Under the similar conditions e.g. 24 h chilling at 0 °C, chilling sensitivity of
227 heartbeat stage embryos was reduced significantly with the introduction of 1 M MeOH in chilling media
228 (Zhang and Rawson 1995). The mechanism by which certain cryoprotective agents protect embryos from
229 chilling injury has not been well understood. High chilling sensitivity in early stage embryos such as 50%
230 epiboly stage is believed to be associated with the large amount of intraembryonic lipids. Studies on
231 partial removal of yolk on chilling sensitivity in zebrafish embryos showed that chilling injury following
232 rapid cooling could be mitigated after partial removal of yolk at the prim-6 stage (Liu et al. 2001). A
233 study on chilling of porcine embryos also showed that the sensitivity of porcine embryos to chilling is
234 related to their high lipid contents, embryos become tolerant to chilling when their lipid contents were
235 reduced (Nagashima et al. 1994). Lipid phase transition (LPT) in cell membranes are also responsible for
236 chilling injury in mammalian sperm (Drobnis et al. 1993) and oocytes (Arav et al. 2000). At the

237 temperature around phase transition, chilled membranes lose fluidity and become leaky, which cause
238 damage to cells (Zeron et al. 1999).

239 **Impact of 3 h chilling and warming on sox gene expression**

240 Studies were carried out to investigate the effect of 3 h chilling and warming on *sox* gene expression at
241 different embryo development stages after embryos were cultured at 27±1°C until the hatching stage.
242 MeOH has been demonstrated to penetrate zebrafish embryo membrane (Zhang and Rawson 1998) and be
243 neurotoxic where its exposure leads to severe CNS defect to mice CNS at gastrulation periods (Degitz et
244 al. 2004) and in drosophila embryos (Mellerick and Liu 2004). Therefore, developmental stages were
245 selected based on their morphology during development – 20 somites (early nervous system
246 development), heartbeat (mid brain development – early touch reflexes) and hatching (first time exposure
247 to external environment). These stages are key stages to study the effect of *sox* genes due to the fact that
248 these genes play important roles in nervous system development in zebrafish embryos (Dee et al. 2008;
249 Millimaki et al. 2010; Vriz et al. 1996), any changes in these genes can have adverse effects on
250 embryonic development.
251
252 Results from the present study showed decreased gene expression when compared to RT controls for all
253 three genes (*sox2, sox3* and *sox19a)* in the embryos that had been chilled for 3 h at 0°C without
254 MeOH,.Studies have shown that chilling of embryos at fast rates could cause damage to the nuclear
255 envelope (Smith and Ane Silva E Silva 2004). In our experiments, a fast (~300 °C/min) chilling rate was
256 used to chill embryos at 0°C for 3 h. Damage to the lamina (a part of nuclear envelope) has been reported
257 to affect the gene expression as the lamina also functions as a structural nuclear protein and regulator of
258 gene expression (Smith and Ane Silva E Silva 2004). It is possible that MeOH protects the lamina of the
259 nuclear envelop during chilling and therefore reduce the chilling injury. Methanol has been reported to be
260 an effective cryoprotectant during chilling storage of zebrafish embryo at zero and subzero temperatures
261 (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 were 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 were 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. Significant increase in *sox19a* was also found at all developmental stages had remained stable in the embryos that were chilled with 1 M MeOH. The increase in *sox19a* gene expression may due to the activation of compensatory mechanism. Compensatory mechanism can be activated to prevent the loss of gene transcript in order to recover gene expression during chilling (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. 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.

Subsequent impact of chilling and warming on *sox2* and *sox19a* protein expression

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). 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 despite decreased *sox2* gene expression. 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).

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. 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. 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.

Materials and Methods

Zebrafish maintenance and embryo selection

Adult zebrafish 12-14 weeks old were maintained in 40 litre glass tanks at 27±1° C. The males and females were kept at a ratio of 1:2 and a 12 hour light/dark cycle was used. Fish were fed three times a day with TetraMin® (Tetra, Germany) and once a day with freshly hatched brine shrimp (*Artemia salini*) (ZM systems, UK). Embryos were collected in the morning and kept in a 27±1°C water bath until the desired stage was reached. Embryonic stages were determined using light microscopy (Leica MZ95, Germany) according to the morphology described by Kimmel (Kimmel et al. 1995).

Experimental Design

Experiment 1: Impact of chilling at 0° C on embryo hatching rate for different time periods in the presence of MeOH as a cryoprotectant

Embryos (50% epiboly) were chilled (Lin et al. 2009b) at 0°C in crushed ice (temperature was maintain throughout by addition of ice) 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). 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 or until they hatch. . Hatching rates were then monitored and all experiments were repeated three times in triplicate (total embryo = 2025). 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 heartbeat. 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).

Experiment 2: Impact 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, 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 below (Desai et al. 2011). 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 at 27±1°C 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). For each embryonic stage, three different samples (5 embryos/tube) were treated and stored for RNA extraction at -80°C and real time PCR. Each experiment was repeated three times. Experimental controls were kept at 27±1°C in water bath.

Experiment 3: Impact of MeOH chilling and warming on *sox2* and *sox19a* protein expression in zebrafish (*Danio rerio*) embryos

Based on gene expression results from previous experiment, 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 27 ±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)

RNA extraction and DNase treatment

RNA was extracted from embryo samples using RNAqueous Micro RNA Isolation Kit (Ambion, UK) according to the manufacturer‘s protocol. This protocol also includes a DNase I treatment step. RNA was stored at -80 ° C until further use. RNA was checked for quantity and purity using Biophotometer (Eppendorf, UK) at 260 nm and 280 nm.

Reverse transcription

1 µg of RNA was transcribed using Precision qScript Reverse Transcription Kit (Primerdesign Ltd, UK) according to the manufacturer’s protocol. For the conventional PCR undiluted cDNA was used in subsequent steps. For real time PCR experiments, cDNA was diluted 1:2 in molecular biology grade water (Sigma, UK) and stored at -80° C.

PCR Analysis

The PCR reactions were consisted of NH_4 PCR buffer (Bioline, UK), 200 µM dNTP (Bioline), 1.5 mM $MgCl_2$ (Bioline), 2 U BIOTAQ™ DNA polymerase (Bioline), 0.5 µM each primer (see Table 1), 1 µg

RNA template and PCR water. Standard conditions for PCR were initial denaturation at 94° C for 5 minutes (1 cycle), 40 cycles of amplification contains 94° C for 30 seconds , annealing temperature (see Table 1) for 30 seconds, 72° C for 30 seconds followed by 1 cycle of additional extension step 72 °C for 10 min. The PCR products were run on 2% agarose gels and stained with ethidium bromide (0.5 µg/mL, Sigma, UK).

Generation of standards for real time PCR

The standards for real time PCR of *sox2*, *sox3* and *sox19a* along with housekeeping genes EF1-α and β actin (Lin et al. 2009a) were produced using conventional PCR as described above. The primer sequences are given in the Table 1. DNA was isolated from excised bands using the EZNA Gel extraction kit (Omega Bio-Tek through VWR, UK) according to the manufacturer's instructions. The isolated DNA was quantified using a Biophotometer (Eppendorf, UK) at 260 nm and diluted to 2 ng/µl followed by 10-fold serial dilutions to generate standards for real time PCR.

Quantification of *sox2*, *sox3* and *sox19a* using real time PCR

Real time PCR was performed on RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor to quantify the expression level of *sox2*, *sox3* and *sox19a*. Reaction tubes contained 7.5 µl of sensimix 2X reaction buffer (contained heat activated DNA polymerase, Ultrapure dNTPs, $MgCl_2$, SYBR® Green I), 333 ηm of each primer (see Table 1) and 2 µl of cDNA sample, made up to 15 µl with PCR water. The reaction conditions were 1 cycle at 95° C for 10 min, followed by 50 cycles at 95° C for 10 sec, the appropriate annealing temperature (see Table 1) for 15 sec and at 72° C for 15 sec. Data were acquired on FAM/SYBR channel at the end of each extension step. Melt curves were also analysed to check for the absence of mispriming and amplification efficiency was calculated from a standard curve (efficiencies were in ranged from 0.8 to 1.0 and R^2 from 0.99 to 1). The possibility of genomic DNA amplification was eliminated by use of primers that crossed introns. Relative gene expression levels were calculated using the two standard curve quantification method in the Rotorgene software (Pfaffl 2003). Ef1 α and β

406 actin were used for this study as these genes were shown to have the highest stability during chilling of
407 zebrafish embryos (Lin et al. 2009a).

408

409 **Protein expression analysis**

410 **Extraction of protein and quantification:** Embryos (75 embryos/treatment) were washed twice with
411 embryo medium 2 (EM2) (15 mM NaCl, 0.5 mM KCl, 0.27 mM $CaCl_2$, 1 mM $MgSO_4$, 0.27 mM
412 $NaHCO_3$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4). Following washing, embryos were subjected to
413 protease treatment (2 mg/ml, Sigma-Aldrich) for 10 min where chorion was partially digested. After
414 digestion, loosened chorion was removed by gentle suction and friction, resulting from pipetting the
415 embryo up and down. Embryos were then washed three times with EM2 before being transferred to a 1.5
416 ml tube. 100 µl of protein extraction buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol) was added to
417 each tube and samples were heated to 95°C for 10 min. Following heating, samples were vortexed and
418 centrifuged at 13,000 x g for 10 min and protein containing supernatant was collected. Isolated proteins
419 were quantified using QuantiPro™ BCA Assay Kit (Sigma-Aldrich) according to the manufacturer's
420 instructions.

421 **Western blot and immunostaining:** Extracted protein was separated using sodium dodecyl sulphate
422 polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were loaded on 4% stacking gel and separated
423 on 10% resolving gel at 200 V for 40-60 min. Proteins were then transferred to PVDF membrane using
424 the semi dry method (BioRad, UK). Immunoblotting was carried out using Anti rabbit WesternDot 625
425 Western Blot Kit (Invitrogen, W10132). The membrane was blocked in 10 ml of WesternDot blocking
426 buffer for 1 hour at room temperature. Following blocking, membranes were incubated with 10 ml of
427 primary antibody solution (dilution 1:1000) overnight at 4˚C on gel rocker. Primary antibodies *sox2*
428 (Abcam, Cambridge, UK), sox19a and *β-actin* (Eurogentech, Belgium) were used at 1:1000 concentration
429 diluted in PBS. The membranes were then washed 3 times for 10 min with WesternDot Wash buffer.

Following washing, the membranes were incubated with 10 ml of Biotin-XX-Goat anti-rabbit solution for 2 hours at room temperature. After secondary antibody incubation, the membranes were washed 3 times as before. The membranes were then incubated with 10 ml of Qdot 625 Streptavidin conjugate solution for 1 hour at room temperature before washed 3 times as previously, followed by a final wash in MilliQ water for 5 min. The membranes were soaked in 100% methanol to make it transparent and then visualised under an UV trans-illuminator with images taken.

Statistical Analysis

Statistical analysis was carried out using SPSS V.16 (IBM, USA) and Microsoft Excel (Microsoft corp. USA). Densitometry analysis was carried out using ImageJ software (Maryland, USA). All protein 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. The one-sample Kolmogorov-Smirnov test was performed to determine whether the data for each gene/protein were normally distributed. Where the data were normally distributed, significant differences in gene/protein expression levels between fresh and chilled embryos at the same time point were calculated using the t-test. One way ANOVA was carried out followed by Tukey's post hoc tests to identify changes of gene/protein expression levels between treatments. Where data were not normally distributed after logarithmic transformation, the Mann-Whitney U test was used instead. All gene and protein expression data were presented as mean ± SEM and p values of less than 0.05 were considered to be significant.

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Conflict of Interest: None declared.

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Figures Legends

Fig 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).

Fig. 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).

Fig: 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).

599 Different letters show significant differences ($p < 0.05$) between different developmental stages of
600 zebrafish embryos within same chilling treatment period (n=9).

601 Fig: 3 (a)

602 Effect of chilling and warming in different concentrations of MeOH on *sox3* gene expression in zebrafish
603 embryos. The Figure shows the gene expression profiles for *sox3* in embryos chilled for 180 min at 0°C in
604 the presence of methanol. Following chilling methanol was replaced with egg water and embryos were
605 then cultured at 27±1 °C until the hatching stage. Gene expressions immediately after chilling and at 20
606 somites, heartbeat and hatching stages after culturing were assessed using reverse transcriptase qPCR. For
607 each time point, 5 embryos were collected in triplicate and each experiment was repeated three times.
608 Results represent the mean expression level relative to the control at time 0 and error bars represent
609 standard error of the mean (SEM). Asterisk (*) shows significant differences ($p < 0.05$) between different
610 concentrations of MeOH and non chilled control within the same gene (n=9).

611 Fig: 3 (b)

612 Effect of chilling in the presence of MeOH on *sox3* gene expression in zebrafish embryos at different
613 stages. Gene expression profiles, assessed by reverse transcriptase qPCR, are for *sox3* in embryos chilled
614 for 180 min at 0°C and then cultured at 27±1°C until hatching stage. For each time point, 5 embryos were
615 collected in triplicate and each experiment was repeated three times. Points represent the mean expression
616 level relative to the control at time 0 and error bars represent standard errors of the mean (SEM).
617 Different letters show significant differences ($p < 0.05$) between different developmental stages of
618 zebrafish embryos within the same chilling treatment period (n=9).

619 Fig: 4 (a)

620 Effect of chilling in different concentrations of MeOH and warming on *sox19a* gene expression in
621 zebrafish embryos. The Figure shows the gene expression profiles for *sox19a* in embryos chilled for 180

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

629 Fig: 4 (b)

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

637 Fig: 5

638 Effect of 3 h chilling with or without the presence of MeOH on *sox2* and *sox19a* protein expression in
639 50% epiboly zebrafish embryos. Protein expression profiles are for *sox2* and *sox19a* for embryos chilled
640 for 180 min at 0°C assessed by Western Blot. For each time point, 75 embryos were collected in triplicate
641 and each experiment was repeated three times. Points represent the mean expression level relative to the
642 control at time 0 and error bars represent standard errors of the mean (SEM). Different letters show
643 significant differences ($p < 0.05$) between different chilling treatments of zebrafish embryos in post 3 h
644 50% epiboly stage (n=9).

645 Fig: 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).

Table 1 Information on gene name, accession ID and primer sequences including annealing temperature and product size.

Gene Name	Accession ID	Forward/Reverse Primers	Annealing Temp. (°C)	Amplicon size (bp)
sox2	NM_213118.1	F :CTCGGGAAACAACCAGAAAA R: TCGCTCTCGGACAGAAGTTT	58	171
sox3	NM_001001811.2	F: ACCGAGATTAAAAGCCCCAT R: TTGCTGATCTCCGAGTTGTG	56	182
sox19a	NM_130908.1	F: TGTCAACAGCAACAACAGCA R: GTTGTGCATTTTGGGGTTCT	57	126
EF1 - α	NM_131263.1	F: CTGGAGGCCAGCTCAAACAT R: ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87
β actin	NM_181601.3	F: CGAGCTGTCTTCCCATCCA R: TCACCAACGTAGCTGTCTTTCTG	59	86

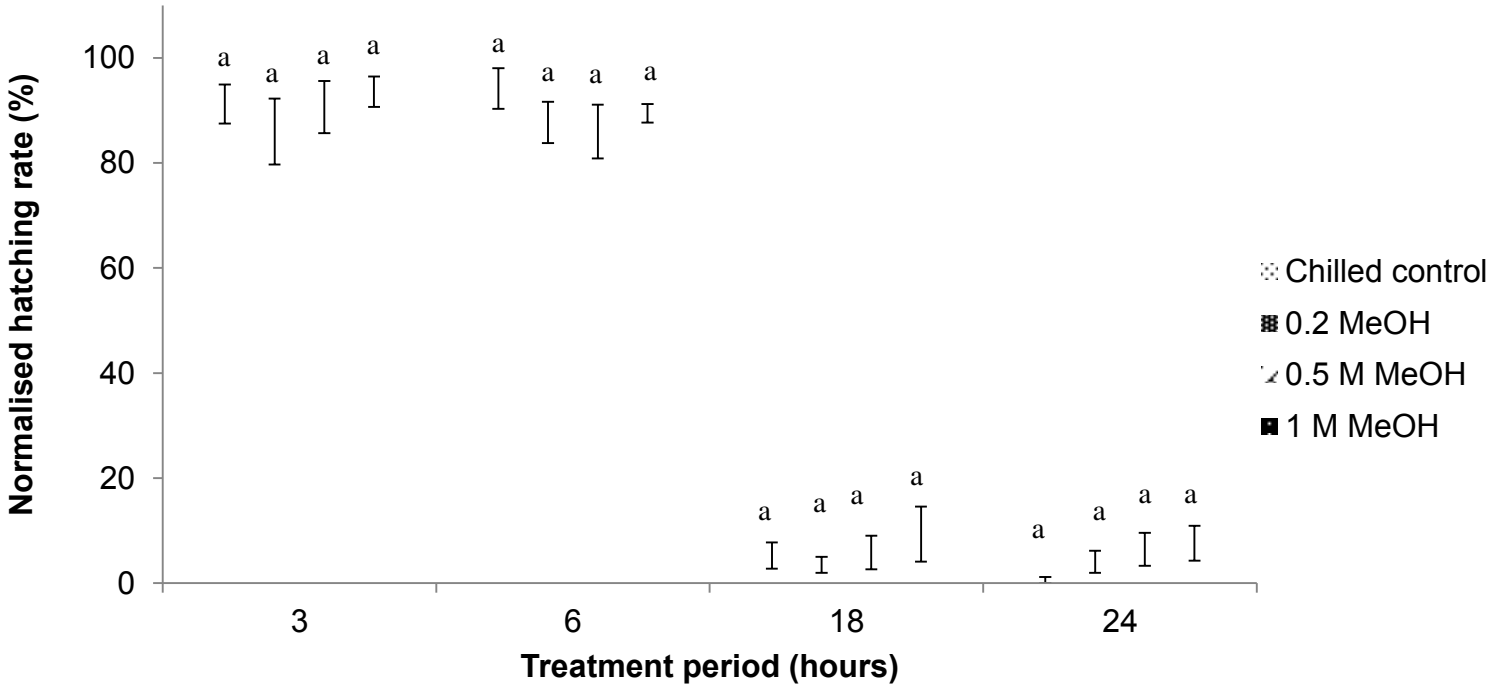

Normalised hatching rate (%)
100
80
60
40
20
0
a
3
6
18
24
Treatment period (hours)
Chilled control
0.2 MeOH
0.5 M MeOH
1 M MeOH

Figure 1


sox2 gene expression
10
1
0,1
Relative gene expression
0 h
3 h chilled
20 somites
heartbeat
hatching
Developmental stages
RT Control
Chiiled control
0.2 M MeOH
0.5 M MeoH
1 M MeOH

Figure 2 (a)

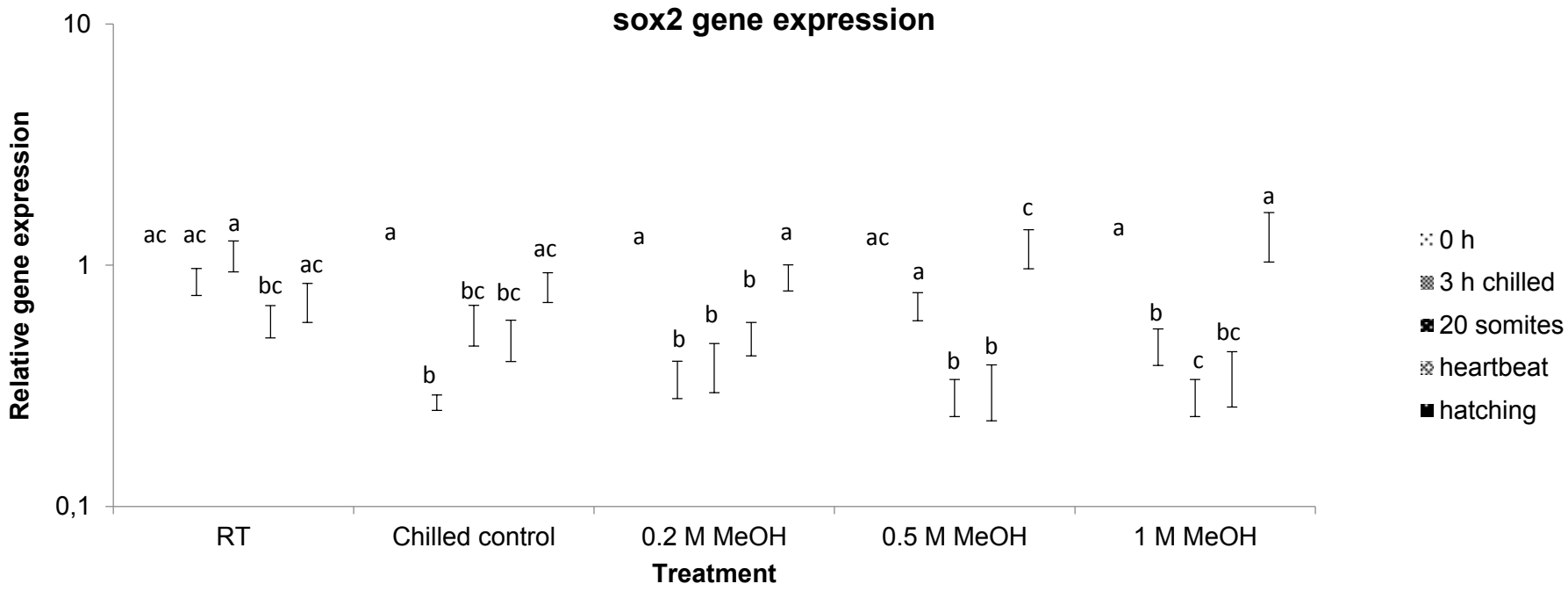

sox2 gene expression
Relative gene expression
10
1
0,1
ac ac a bc ac
a b bc bc ac
a b b b a
ac a b b c
a b c bc a
RT
Chilled control
0.2 M MeOH
0.5 M MeOH
1 M MeOH
Treatment
0 h
3 h chilled
20 somites
heartbeat
hatching

Figure 2 (b)

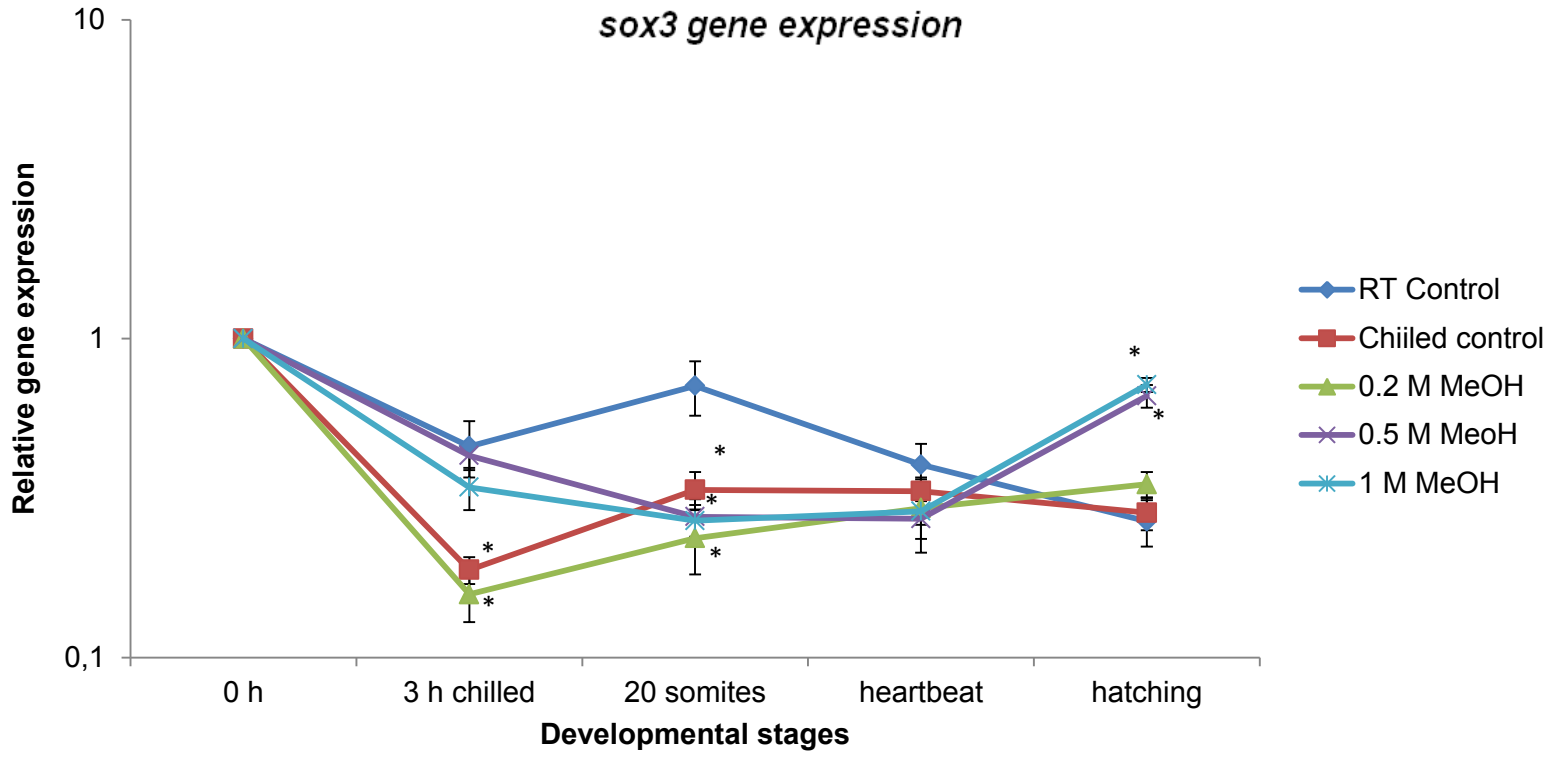

sox3 gene expression
10
1
0,1
Relative gene expression
0 h
3 h chilled
20 somites
heartbeat
hatching
Developmental stages
RT Control
Chiiled control
0.2 M MeOH
0.5 M MeoH
1 M MeOH

Figure 3 (a)

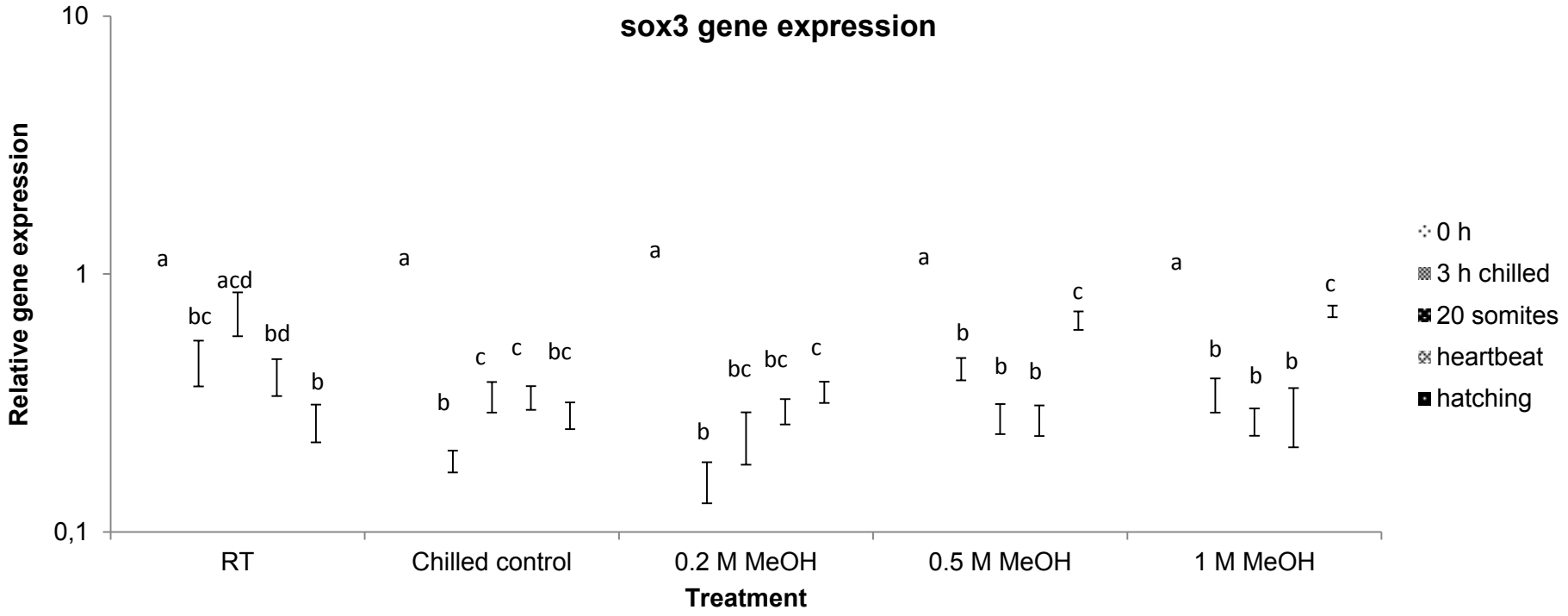

sox3 gene expression
Relative gene expression
10
1
0,1
a
bc
acd
bd
b
a
b
c
c
bc
a
b
bc
bc
c
a
b
b
b
c
a
b
b
b
c
RT
Chilled control
0.2 M MeOH
0.5 M MeOH
1 M MeOH
Treatment
0 h
3 h chilled
20 somites
heartbeat
hatching

Figure 3 (b)

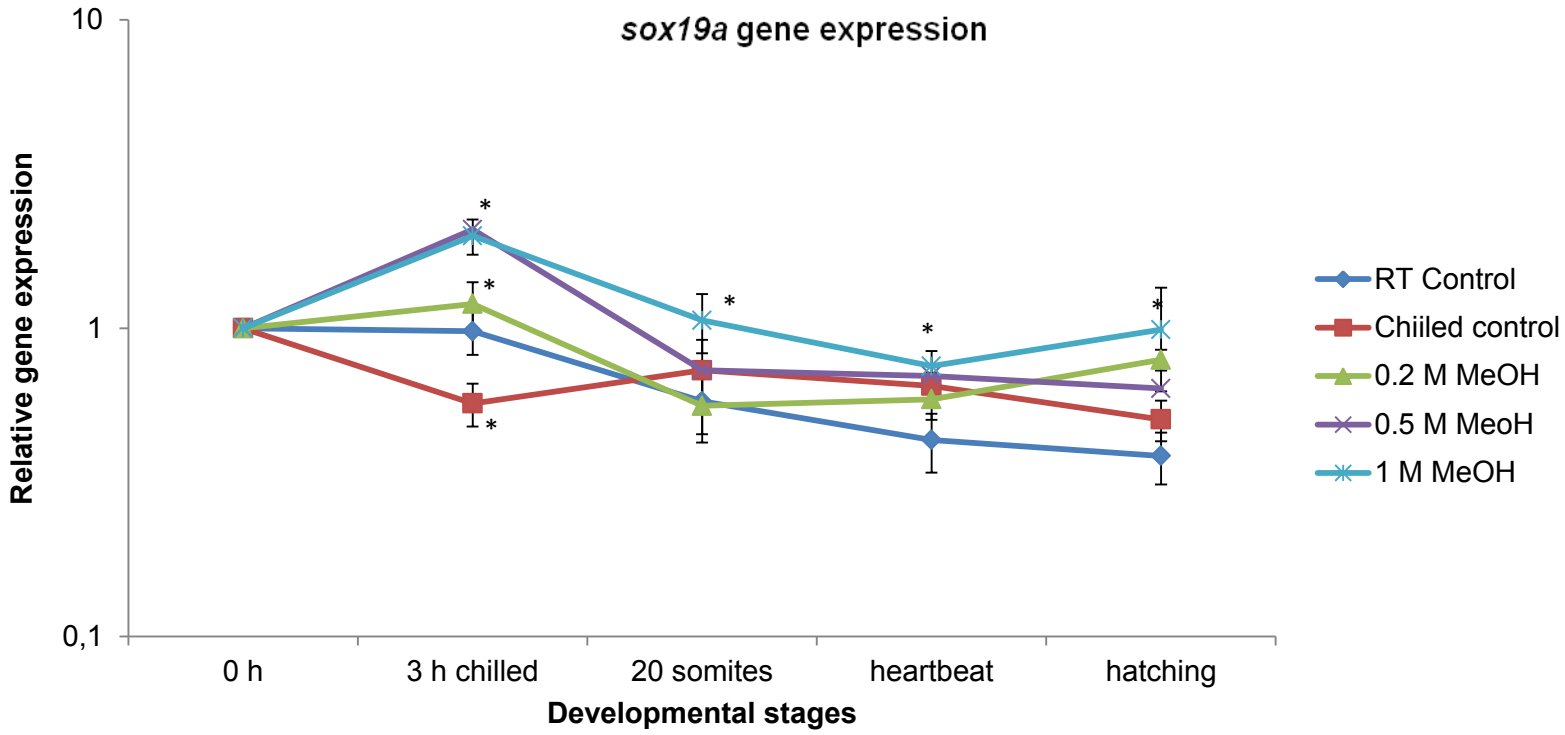

sox19a gene expression
10
1
0,1
Relative gene expression
0 h
3 h chilled
20 somites
heartbeat
hatching
Developmental stages
RT Control
Chiiled control
0.2 M MeOH
0.5 M MeoH
1 M MeOH

Figure 4 (a)

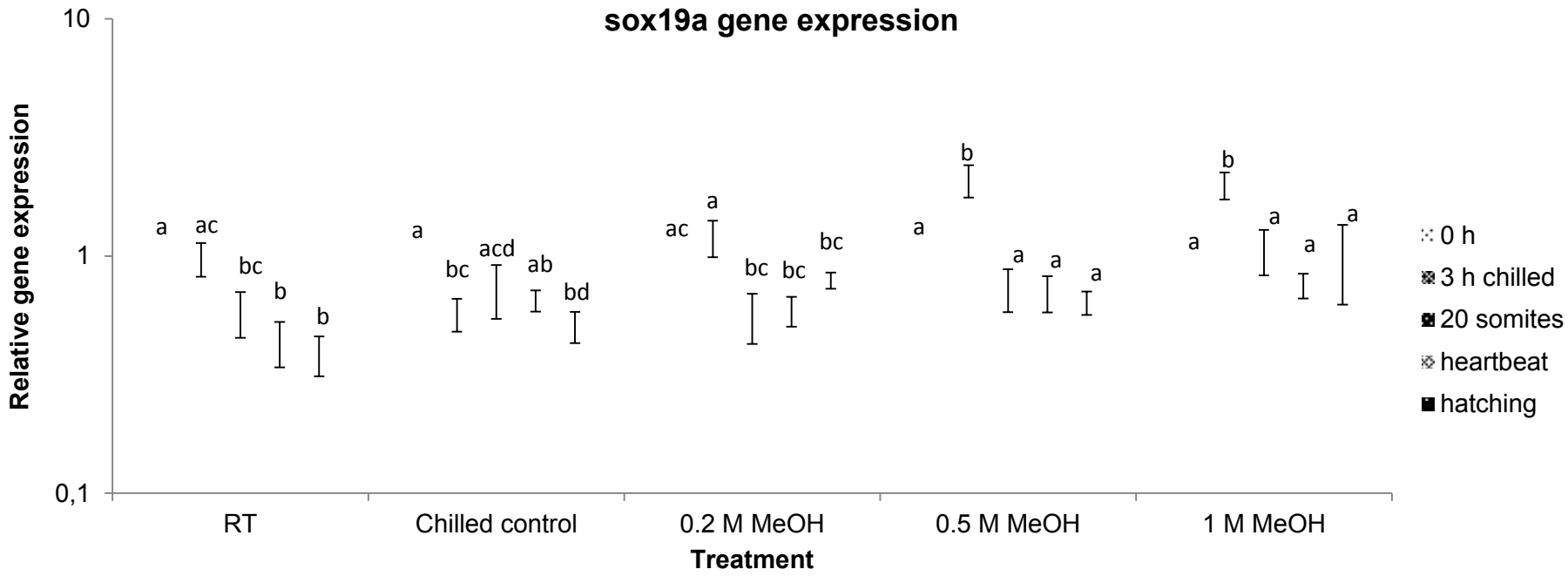

sox19a gene expression
Relative gene expression
10
1
0,1
a
ac
bc
b
b
a
bc
acd
ab
bd
ac
a
bc
bc
bc
a
b
a
a
a
a
b
a
a
a
RT
Chilled control
0.2 M MeOH
0.5 M MeOH
1 M MeOH
Treatment
0 h
3 h chilled
20 somites
heartbeat
hatching

Figure 4 (b)

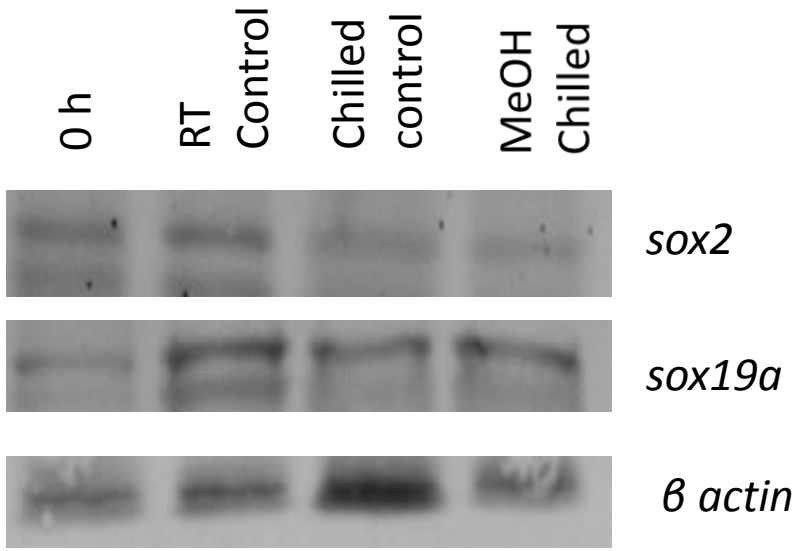
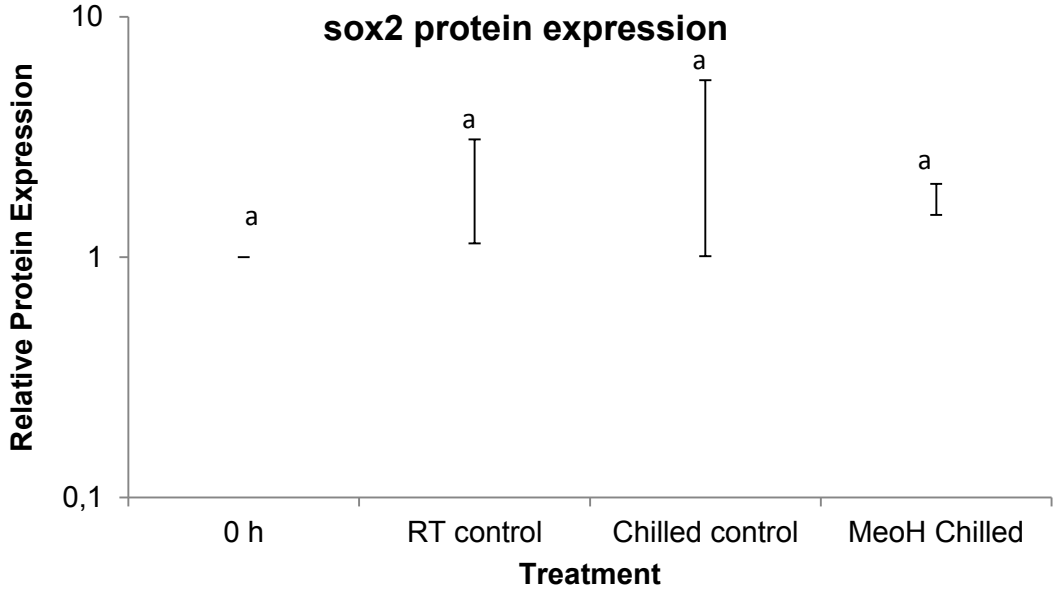


Figure 5 (a)



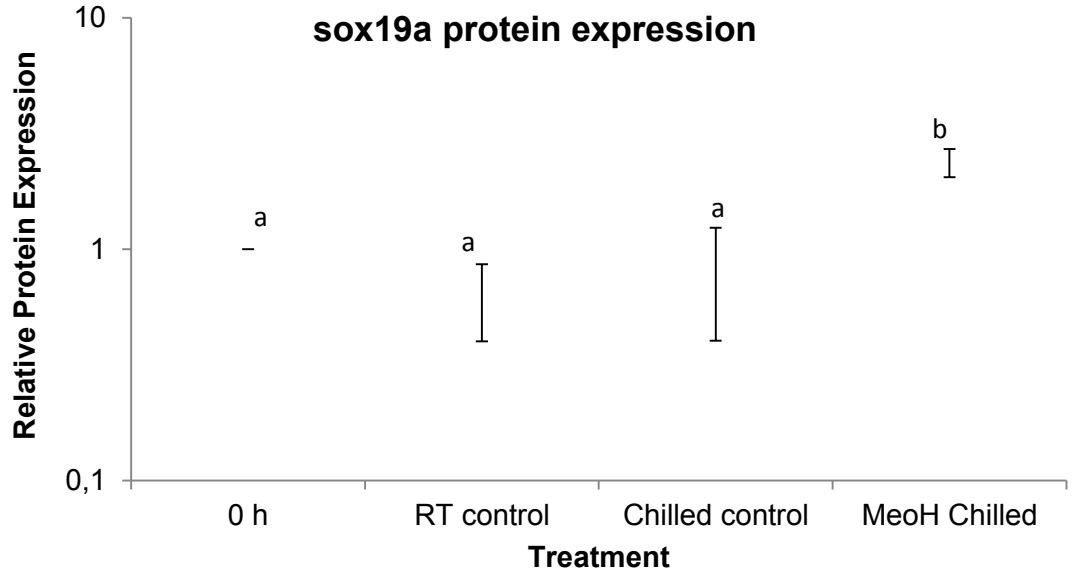


Figure 5 (b)

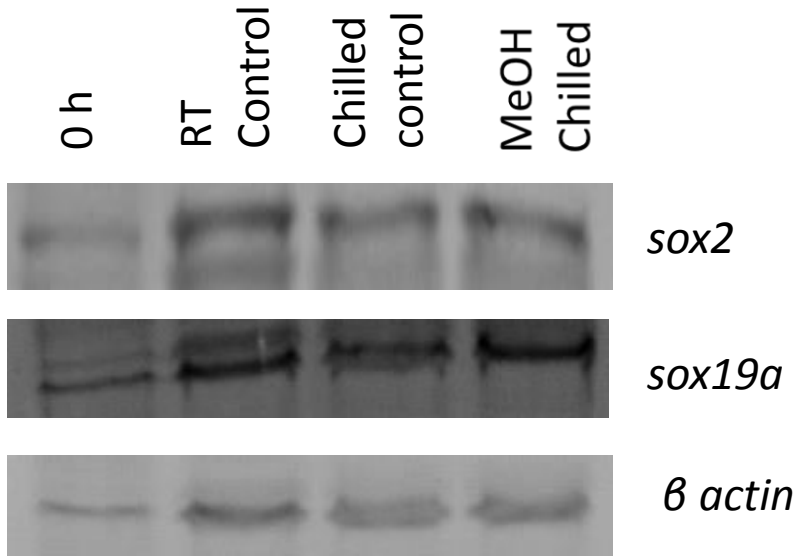
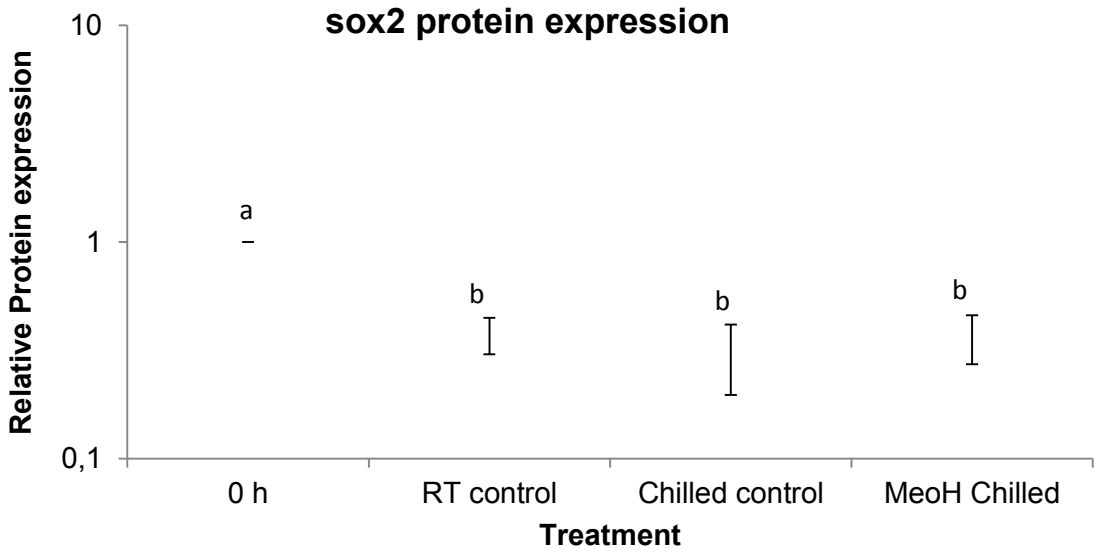


Figure 6 (a)



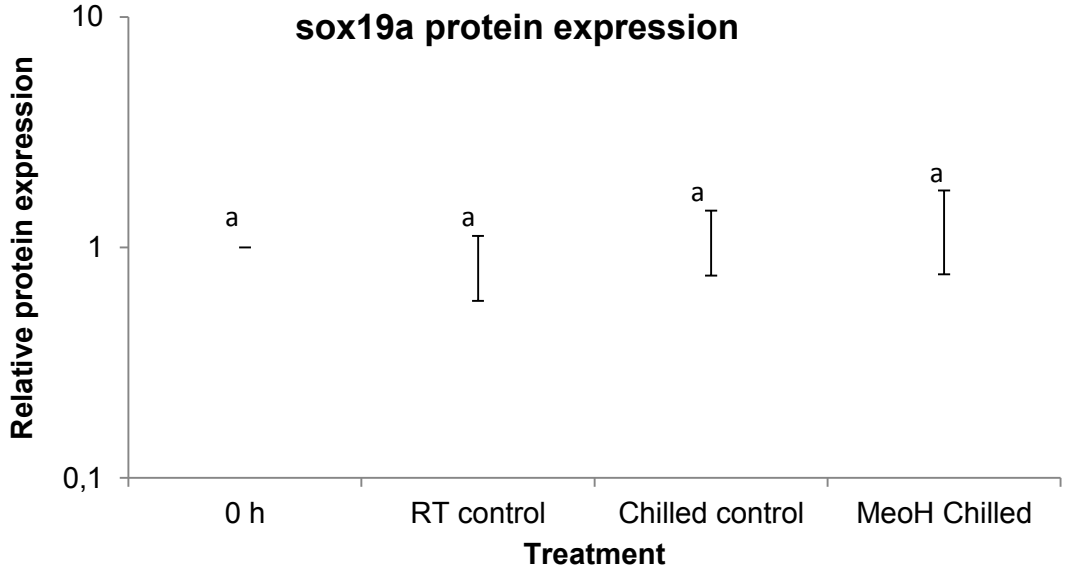


Figure 6 (b)