Int Aquat Res (2015) 7:75–84
DOI 10.1007/s40071-015-0094-x

ORIGINAL RESEARCH

Cortisol levels and expression of selected stress- and apoptosis-related genes in the embryos of Atlantic cod, *Gadus morhua* following short-term exposure to air

Christopher Marlowe A. Caipang · Fernand F. Fagutao · Effrosyni Fatira · Carlo C. Lazado · Michail Pavlidis

Received: 3 November 2014 / Accepted: 9 January 2015 / Published online: 27 January 2015

Abstract Embryos (morula stage) of Atlantic cod, *Gadus morhua* L., were collected and subjected to air exposure for 2 min. followed by recovery at ambient conditions in the rearing container. Total immunoreactive cortisol and transcription of selected stress- and apoptosis-related genes of the embryos were determined before the application of the stressor and at 0.5, 1 and 24 h post-exposure. There was no significant difference in the total cortisol levels of the fertilized eggs before and after handling stress. There was high expression level of *hsp70* and *sod* before application of the stressor and significantly increased at 0.5 h post-exposure. The expression levels of *cat* and *gpx* were weak to moderate and were not affected by the stressor. The apoptotic genes, *mcl1* and NR-13 were highly expressed and significantly increased after exposure to air. Bcl-X1 and Bcl-X2 were moderately expressed in the control samples, but only the expression level of Bcl-X1 significantly increased following exposure to air. Cluster analysis of the different gene expression levels indicated three categories: those genes that did not show any change in the expression levels post-air exposure; those that had low expression level in the control followed by a significant increase after air exposure; and those that had high expression levels in the control followed by a further increase in expression after air exposure. These results clearly demonstrate that there are potential molecular biomarkers of the response in cod embryos as a consequence of air exposure at a time when cortisol is not fully active.

Keywords Atlantic cod · Cortisol · Early development · Gene expression · Stressor

C. M. A. Caipang (✉)
School of Applied Science, Temasek Polytechnic, Singapore 529757, Singapore
e-mail: cmacaipang@yahoo.com

F. F. Fagutao
Laboratory of Aquatic Animal Diseases, College of Veterinary Medicine, Gyeongsang National University, Jinju, Gyeongnam 660-701, Republic of Korea

E. Fatira · M. Pavlidis
Department of Biology, University of Crete, 71409 Heraklion, Crete, Greece

C. C. Lazado
Aquaculture Genomics Research Unit, Faculty of Biosciences and Aquaculture, University of Nordland, 8049 Bodo, Norway

Present Address:
C. C. Lazado
National Institute of Aquatic Resources, Technical University of Denmark, 9850 Hirtshals, Denmark

Introduction

The culture of Atlantic cod, *Gadus morhua,* has gained much attention in recent years. The interest in commercial aquaculture of this species is brought about by various factors including reduced capture of cod from the wild, good market value, high growth rates, anti-freeze protein production and the integration of this species for grow-out in existing culture operations for Atlantic salmon, *Salmo salar* (Goddard et al.1999; Otterlei et al. 1999; Brown et al. 2003). Artificial fertilization has been developed and routinely used to produce embryos and larvae of Atlantic cod to supply the growing demand of the seedstock for cod aquaculture (Trippel and Neilson 1992; Rideout et al. 2004). At present, there are commercial cod hatcheries that are located in the USA, UK, Canada, Iceland and Norway (Rosenlund and Halldórsson 2007).

Modern hatchery methods for Atlantic cod include supplemental feeding with cultivated plankton such as rotifers and *Artemia* (Howell 1984). The provision of supplemental feeds ensures year-round mass production of larvae. However, bulk production of cod eggs and larvae is also confronted with high mortality rates during the early stages of embryonic and larval development (Seppola et al. 2009). These high mortality rates in cod hatcheries are due to poor egg quality and infection with either bacterial or viral pathogens. The identification of the different defense mechanisms, particularly maternal-derived immune factors in cod during early embryogenesis has been explored in previous studies (Seppola et al. 2009; Lanes et al. 2012; Rise et al. 2012). In those studies, the various immune response genes that are related to innate immune defense have been identified and believed to have roles in protecting the early embryos and the larvae against those pathogens. Aside from identifying the key immune factors that will improve survival of the embryos and larvae of cod, egg quality is also one aspect that is considered when predicting survival rates in cod juveniles.

The variability in the quality of eggs and embryos is one of the most important constraints on the yield of production in any aquaculture facility (Bromage et al. 1991). As such, it is important that good quality eggs are produced to ensure good quality larvae and juveniles that will be used for grow-out culture operations. Good quality eggs exhibit low mortality at fertilization, eyeing, hatching and at first feeding (Bromage and Shepherd 1992). There are several factors that contribute to egg quality in fish, and these include: nutrition, stress, genetics, egg buoyancy, egg overripening, fertilization rates,age of the broodstock, hormonal induction of spawning, environmental factors and the like (Brooks et al. 1997; Bobe and Labbé 2010). The effects of these different factors on egg and embryo quality are species-specific (Rideout et al. 2004). In Atlantic cod, the preceding factors contribute to good quality eggs as well as other requirements for a successful production of juveniles (Rosenlund and Halldórsson 2007).

One of the factors that are likely to affect egg quality in fish is the exposure of the eggs to a variety of stressors. There have been previous studies on the effects of stress on the broodstock and spawners and the resulting progenies that are produced (Stratholt et al. 1997; Mileva et al. 2011; Li and Leatherland 2012). On the other hand, the primary response of the eggs and embryos during exposure to various stressors is somewhat difficult to assess because cortisol is not synthesized during early embryogenesis due to a lack of functional interrenal cells (Barry et al. 1995; Szisch et al. 2005; Auperin and Geslin 2008). Hence, there is no direct measure of what is happening to eggs and early embryos immediately after application of the stressor. The effects of stress are only seen at later development of the fish larvae, including decreased hatching rate, higher incidence of deformities and higher larval mortality, which were observed in milkfish, *Chanos chanos,* eggs following exposure to mechanical stress and simulated transport (Hilomen-Garcia 1998).

Exposure of Atlantic cod eggs and embryos to elevated temperature, ultraviolet and environmental contaminants resulted in high mortality of the embryos, differential expression of heat shock proteins and stress-related genes, changes in the activities of antioxidant enzymes and DNA damage (Lesser et al. 2001; Olsvik et al. 2012; Skjærven et al. 2011, 2013). These are few of the stressors that cod eggs and embryos are exposed to and there are other hatchery-related activities that could contribute to embryonic stress. Routine collection and handling of cod eggs including the pouring of fertilized eggs from one container to the other, siphoning as well as dispensing eggs in the incubation tanks make the fertilized eggs as well as the developing embryos vulnerable to being exposed to air even for a short period of time. There have been no studies conducted on the effects of these handling procedures on the fertilized eggs of cod; hence, this study aimed to find out the effects of short-term air exposure on the fertilized eggs of cod by measuring the cortisol content of the early embryos and profiling the transcription of selected oxidative stress- and apoptosis-related genes that could

serve as biomarkers of the response to a stressor and immune status during the early stages of embryonic development in cod.

Materials and methods

Sampling of eggs

Fertilized Atlantic cod eggs (morula stage), approximately 36–48 h post-fertilization, were kindly provided by a commercial cod hatchery in Bodø, Norway. These eggs were obtained from the same batch of fertilized eggs and hence had more or less similar stage of development. The fertilized eggs (approximately 150–200 eggs) were placed in a 500-ml beaker (filled up to 80 % capacity) containing seawater (salinity 32–35 ppt) at 6 °C and provided with ample aeration. The eggs were immediately transported to the laboratory for the experiment and used immediately for the assays. The beakers were placed on top of a wooden plank during transport to prevent severe mechanical disturbance to the eggs. Upon reaching the laboratory, the eggs were subjected to handling stress by scooping them out with a fine-meshed net and exposing them to air for 2 min. The duration of 2 min is most likely the time that the eggs are exposed to air during normal hatchery operations when they are transferred from one tank to the other. Except for a study done by Ramsay et al. (2009) on the effects of air exposure for 3 min on the resting egg cortisol of adult zebrafish, no such data on the length of exposure to air as a potential stress factor in fertilized fish eggs have been collected. After exposure to air, the eggs were placed in individual beakers to allow recovery. Each beaker contained 50–60 eggs and four beakers with eggs were sampled at each time point. Samplings were done at 0.5, 1 and 24 h post-exposure to air. Analyses of gene expression were done over a 24-h period to ensure that the fertilized eggs are still within the morula stage. Egg samples that were not exposed to air were used as control for the experiment.

Determination of egg cortisol

Total immunoreactive cortisol from the fertilized eggs (20–30 eggs per cortisol extraction) was extracted following the procedures described by de Jesus et al. (1991). Egg cortisol was quantified by Enzyme-linked Immunosorbent Assay (ELISA) with a commercial kit (Cortisol EIA Kit, Oxford Biomedical Research, MI, USA) following the protocol supplied by the supplier. A series of dilutions of the egg cortisol samples were tested to determine the effective range for cortisol determination.

Isolation of total RNA and analyses for gene expression

Total RNA was extracted from fertilized eggs using TriZol® (Invitrogen) following standard procedures. To obtain significant amounts of RNAs, 20–30 eggs were pooled for each extraction. The samples for each time point as well as the control had four batches of fertilized eggs for the extraction. The RNA purity of all the samples was >1.85 and hence were suitable for the synthesis of the complementary DNA. First-strand complementary DNA (cDNA) was synthesized from the total RNA (1 μg) using qScript™ cDNA SuperMix (Quanta Biosciences, USA). The cDNAs were quantified and normalized at a concentration of 10 μg mL^{-1} with 1× TE buffer. The samples were stored at −20 °C until use for subsequent PCR reactions. The expression of selected genes that are related to oxidative stress response (catalase, *cat*; glutathione peroxidase, *gpx*; Hsp70, *hsp70*; and Cu,Zn-SOD, *sod*) and apoptotic activity (Bcl-X1; Bcl-X2; Mcl-1, *mcl1*; and NR-13) was determined using the published primers in previous studies (Caipang et al. 2008a, b; Feng and Rise 2010).

PCR amplification was carried out at the following conditions: an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s and elongation at 72 °C. A final elongation step at 72 °C for 5 min was done to complete the reaction. Five microliters (5 μl) of the PCR product was used for electrophoresis on a 0.8 % agarose gel and stained with SYBR Safe™ (Molecular Probes, USA). The intensity of the band on the image was visualized using a gel documentation system (Kodak Electrophoresis Documentation and Analysis System 120, Eastman Kodak Co., USA) and

compared with the intensity of the band from the reference gene, β-actin using Image J software (http://rsb.info.nih.gov/nih-image/). The ratio of expression for each gene was measured and used for the Cluster analysis.

Cluster and TreeView analysis

Relative expression levels of the genes were grouped into categories based on the patterns of their expression using Cluster Program 3.0 (Clustering library version 1.27) (De Hoon et al. 2004) and visualized with TreeView program (Version 1.60) (Saldanha 2004). The genes that belong to specific clusters were grouped based on their expression patterns in the fertilized eggs before and after air exposure.

Data analysis

The data are represented as mean + SD. One-way ANOVA was used to determine significant differences in the total cortisol in the eggs. For the gene expression analyses, the assumptions for ANOVA were checked prior to carrying out the computations. If the data did not follow the Gaussian distribution, transformation was conducted prior to parametric one-way ANOVA; thereafter, the differences between the groups were checked by Tukey's Multiple Comparison Test. Kruskal–Wallis test followed by Dunn's Multiple Comparison Test was used for non-parametric data. All significance levels were set at $P < 0.05$.

Results

Cortisol content of the fertilized eggs

Fig. 1 shows the cortisol content of the fertilized eggs before exposure to air and several time points during recovery. The cortisol content of the fertilized eggs was less than 1 ng per gram of fertilized eggs and the levels were not significantly affected upon exposure of the eggs to air as a stressor.

Gene expression

The expression levels of the different stress-related genes in the fertilized eggs before and after handling stress are shown in Fig. 2. The levels of *hsp70* and *sod* prior to application of the stressor were high, whereas there was weak expression of *cat* and *gpx*. The expression level of *hsp70* significantly increased at 0.5 h post-air exposure and maintained until 24 h post-exposure. On the other hand, significant increase in the expression of *sod* was observed at 0.5 h post-air exposure and then the expression returned to its pre-exposure level. The expression level of *cat* was still weak but significantly increased at 0.5 h post-air exposure. However, the levels of expression returned to their pre-exposure levels after 1 h post-exposure to air. No significant change in the levels of expression in *gpx* was observed before and after exposing the fertilized eggs to air.

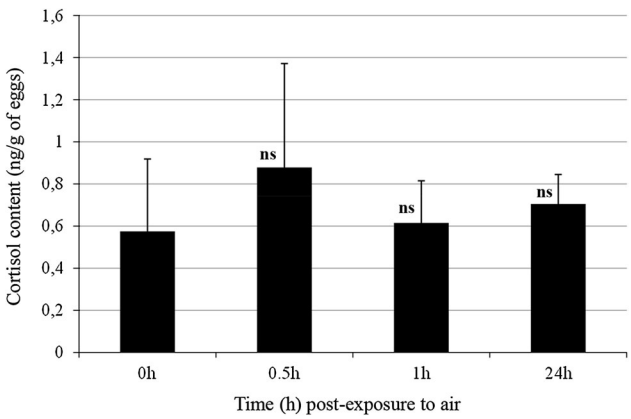

Fig. 1 Immunoreactive cortisol content of the fertilized eggs of Atlantic cod before and after exposure to air for 2 min. Each bar represents the mean ± SD of $n = 4$ egg batches. *Ns* denotes no significant difference from the initial value (time 0) at $p > 0.05$

Fig. 3 shows the expression profile of the different apoptosis-related genes in the fertilized eggs of cod as a consequence of handling stress due to air exposure. The expression levels of Bcl-X1 and Bcl-X2 in the eggs were weak before air exposure; however, the expression of Bcl-X1 significantly increased at 0.5 h post-air exposure and gradually returned to the pre-exposure levels. No significant change in expression of Bcl-X2 in the fertilized eggs was observed before and after exposure to air. There were high expression levels of both *mcl1* and NR-13 in the fertilized eggs before exposure to air. The expression levels of both genes significantly increased at 0.5 h after exposing the fertilized eggs to air and continued until 24 h post-exposure.

Cluster analysis

The expression levels of the different transcripts that were analyzed in this study are categorized into three categories (Fig. 4). Category 1 included the genes that did not show significant change in their expression levels post-exposure to air. These were the *cat*, *gpx* and Bcl-X2. Category 2 included the genes that had low expression level in the control followed by a significant increase after air exposure; and this included Bcl-X1. Category 3 is classified as the group of genes that had high expression levels in the control followed by a further increase in the expression after air exposure. The genes in this category included: *hsp70*, *sod*, *mcl1* and NR-13.

Discussion

The present study demonstrated that immunoreactive cortisol can be detected in fertilized eggs of Atlantic cod and corroborated the earlier findings of Kleppe et al. (2013). However, the egg cortisol was not responsive when the eggs are exposed to air for a short period of time as shown by no significant changes in the cortisol levels following exposure to air. In several species of fish including rainbow trout, *Oncorhynchus mykiss* (Barry et al. 1995; Auperin and Geslin 2008) and gilthead sea bream, *Sparus aurata* (Szisch et al. 2005), cortisol was detected in the fertilized eggs. It is believed that the hormones, which are present in the oocytes and during the early embryonic stages in teleosts are of maternal origin (Greenblatt et al. 1989; Hwang et al. 1992). The data that were obtained in this study together with the findings obtained in other species of fish are concrete evidence that the cortisol in the fertilized eggs of Atlantic cod is maternal in origin and is utilized during early embryonic development similar with other species of fish (de Jesus et al. 1991; Yamano et al. 1991; Hwang et al. 1992). The cortisol level of the fertilized eggs in Atlantic cod was comparable to the cortisol levels in the fertilized eggs of gilthead sea bream (Szisch et al. 2005). The fertilized eggs of rainbow trout had higher cortisol content than Atlantic cod (Barry et al. 1995; Auperin and Geslin 2008) and this could be due to differences in egg size and the amount of hormones and other bioactive substances that are transferred from the mother to the developing oocytes. The amount of cortisol in the oocytes as well as in the fertilized eggs is largely determined by the amount of the circulating cortisol in the female spawner as shown in coho salmon, *O. kisutch* (Stratholt et al. 1997); thus, if the female spawner is subjected to stressors, the level of circulating cortisol could increase and subsequently be transferred to the eggs. Hence, the initial cortisol level in the fertilized eggs is reflective of the condition of the female spawner during oocyte production. It is difficult to ascertain whether the low level of egg cortisol in cod obtained in the present study is an intrinsic factor or due to the physiological status of the female spawners because the condition of the spawners was not assessed in the study. It is important to consider this aspect of stress physiology in female spawners when assessing cortisol content during embryogenesis.

The effects of a hatchery-related stressor on the fertilized eggs of cod were determined by measuring the amount of circulating cortisol before and after application of a potential stressor. Exposure of the fertilized eggs to air for 2 min followed by placing the embryos back in the rearing container for recovery was the method of choice as a stressor for the embryos because this practice is common in hatcheries, especially when fertilized eggs are scooped out from the spawning tanks and transferred to the hatching tanks. Exposing the eggs to air during handling is inevitable and is a likely stress factor. No significant changes in the cortisol content of the embryos were observed following air exposure, indicating that there was no mechanism in the developing cod embryo that can synthesize cortisol. This is expected because at this stage in cod development, the embryos have the ability to metabolize cortisol but do not have the capacity to produce this corticosteroid

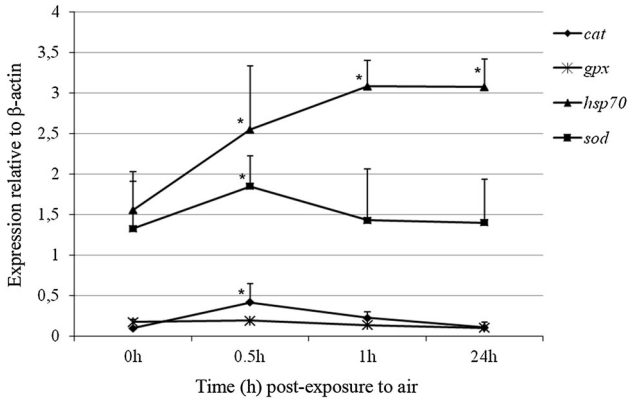

Fig. 2 Changes in the expression levels of oxidative stress-related genes in fertilized eggs of Atlantic cod before and after exposure to air for 2 min. Each value represents Mean + SD of $n = 4$ egg batches. An *asterisk* denotes significant differences from the initial value (time 0) at $p < 0.05$

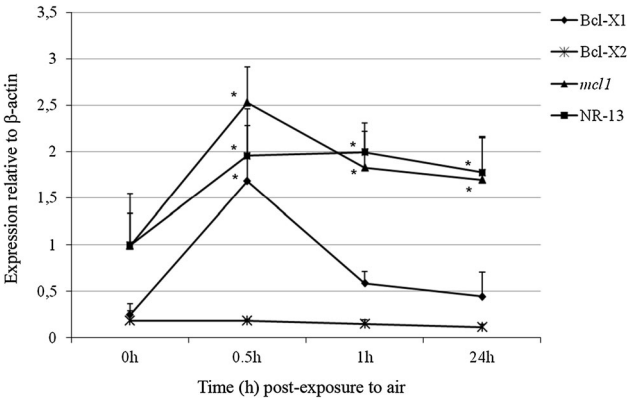

Fig. 3 Changes in the expression levels of apoptosis-related genes in fertilized eggs of Atlantic cod before and after exposure to air for 2 min. Each value represents Mean + SD of $n = 4$ egg batches. An asterisk denotes significant differences from the initial value (time 0) at $p < 0.05$

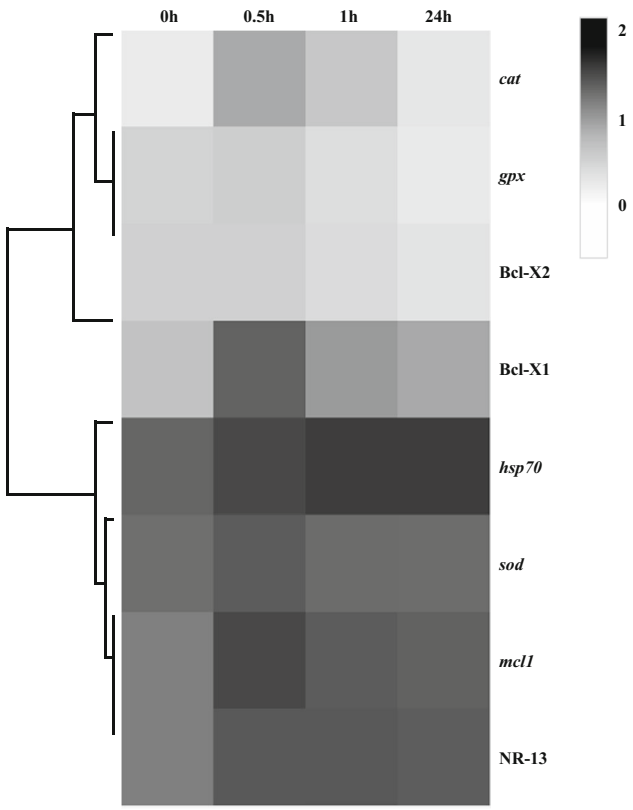

Fig. 4 Clustering of genes involved in oxidative stress and apoptosis in the fertilized eggs of Atlantic cod following exposure to air for 2 min. *Black shade* indicates highest expression levels and *light gray shade* indicates lowest expression levels. Analyses were done for the expression of the individual gene from $n = 4$ egg batches

(Stratholt et al. 1997). Studies in other fish species including the temperate flatfishes, tilapias, salmonids and sea breams also demonstrated the inability of embryos to synthesize cortisol (de Jesus et al. 1991; Hwang and Wu 1993; Barry et al. 1995; Tanaka et al. 1995; Sampath-Kumar et al. 1997; Szisch et al. 2005; Auperin and Geslin 2008). In rainbow trout, changes in the cortisol levels due to acute stress have been observed 9 days after hatching, and this species of fish is capable of cortisol synthesis at least 6 days before hatching (Auperin and Geslin 2008). This was also observed in chum salmon at 1 week before hatching (de Jesus and Hirano 1992). In this study, the cod embryos were at the morula stage; hence, the cells that are responsible for cortisol production are not likely to be fully functional. It is unlikely that the hypothalamus-pituitary-interrenal axis (HPI) that produces and regulates cortisol in fish is fully functional during the embryonic stage (McCormick and Nechaev 2002). Because cortisol is regulated by the hypothalamus and pituitary in fish (Mommsen et al. 1999), the changes in the levels of this hormone in the fertilized eggs as a consequence of exposure to various exogenous factors may not be possible.

At several time points after exposing the embryos to air, there were changes in the expression levels of genes related to oxidative stress and apoptosis. This indicates that even if there was no increase in cortisol production, the embryonic cells were already capable of synthesizing transcripts that respond to an exogenous stimulus. The changes in the expression levels of these different genes are likely a result of the response of the embryos to an external stimulus rather than a response due to a shift in the developmental stage. This is because embryonic development in cod takes a longer time as compared to warm-water fish, and the duration of sampling that was done over a 24-h period ensures that the stage of the embryonic development was more or less similar over that time period. All the genes that were used in the analyses were upregulated following exposure of the eggs to air, and it would be interesting to determine what other genes that could be down-regulated as a consequence of air exposure. Large-scale analysis using microarray or bulk transcript sequencing could be done in future studies.

The oocytes and early embryos largely depend on the barrier defense that is provided by the vitelline envelope as well as by the innate immune factors in the ooplasm (Li and Leatherland 2012). These defense systems are maternal in origin and protect the eggs from opportunistic pathogens in the aquatic environment. Moreover, the transcriptional profile of the eggs includes various mRNA transcripts of the immune response that contribute to the proteomic profile of early embryos (Aegerter and Jalabert 2004; Keyvanshokooh and Vaziri 2008; Li and Leatherland 2012). In cod, maternal-derived transcripts that are involved in immune defenses have been detected in developing embryos (Seppola et al. 2009; Lanes et al. 2012; Rise et al. 2012). Lysozyme activity was also observed even in unfertilized eggs, suggesting the presence of a functional protein (Seppola et al. 2009). In the present study, the oxidative stress-related genes, *sod* and *hsp70,* responded to the handling stress, where significant upregulation of the expression has been observed for both genes. The increased expression of *sod* is a likely consequence of the response of the embryos against oxidative stress. The by-products of oxidative stress are known as reactive oxygen species (ROS) and these include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO.) (Halliwell and Gutteridge 2007). These ROS by-products are degraded or converted to non-toxic metabolites by both enzymatic and non-enzymatic antioxidant defense systems (Romero et al. 2011). Superoxide dismutase (SOD) is an anti-oxidant enzyme that prevents oxidative stress by transforming O_2^- to H_2O_2 (Paital et al. 2013). Exposure of embryos to a stressor leads to increased production of ROS as demonstrated in zebrafish embryos when exposed to an environmental contaminant (Xu et al. 2013). Although in both studies the early embryos were subjected to different stressors, the responses that were observed for *sod* expression were similar. Unlike in aquatic invertebrates where there have been some studies done on the effects of air exposure to the production of antioxidant enzymes (Almeida and Bainy 2006; Weihe et al. 2010; Romero et al. 2011), in fish very few studies have been done in such effects. Hence, the results generated on this study will provide a platform for future studies on assessing gene expression and activity of these oxidative stress-related genes when early embryos are exposed to various handling stressors.

There was also increased expression of *hsp70* when the early embryos were exposed to air for 2 min. Heat shock proteins function as molecular chaperones during embryonic development and they are involved in cell movement, proliferation, morphogenesis and apoptosis (Rupik et al. 2011). In fertilized eggs of cod, basal expression of *hsp70* was detected (Lanes et al. 2012), indicating the role of this gene during early development. Exposing fertilized eggs of cod to heat resulted in the enhanced expression of *hsp70* and *hsp90a* transcripts (Skjærven et al. 2011). While *hsp70* is constitutively expressed in fertilized eggs of cod, this gene

had low expression level in any developmental stage of *Xenopus laevis* but was induced upon heat exposure in the embryos at the post-blastula stage (Lang et al. 2000). Although the specific function of *hsp70* during embryogenesis is not clearly understood, it was clearly evident that various kinds of stressors upregulated the expression of *hsp70* in fish (Deane and Woo 2003). Whether the upregulation of *hsp70* in early embryogenesis would protect the embryonic cells or ensure proper folding of proteins that are required for normal metabolism and development needs further studies.

In addition to the upregulation of key oxidative stress-related genes in the fertilized eggs of cod during exposure to air, there was also increased transcription of apoptosis-related genes, *mcl1*, Bcl-X1 and NR-13. These genes belong to the anti-apoptotic gene sub-family, Bcl-2. These anti-apoptotic genes bind and sequester the pro-apoptotic Bcl-2 proteins, thus preventing mitochondrial membrane permeabilization (MMP)- induced apoptosis (Brunelle and Letai 2009). These genes have been identified in cod and were differentially expressed when the fish were exposed to both bacteria and virus (Feng and Rise 2010). In the fertilized eggs of cod, *mcl1* and Bcl-X2 were detected and believed to be maternal-derived transcripts (Lanes et al. 2012). The roles of these anti-apoptotic genes during short-term exposure of fertilized eggs of cod to air are not clearly defined due to a limited number of studies. However, it is known that the damaged, unwanted and misplaced cells are continuously replenished through apoptosis during early development in multicellular organisms (Krumschnabel and Podrabsky 2009), but such process may only occur during the gastrula stage as demonstrated in zebrafish (Hensey and Gautier 1997; Ikegami et al. 1999). The early embryos that were used in this study were at the morula stage, which is a few stages earlier than the gastrula stage; hence it is possible that the upregulation of the anti-apoptotic genes following air exposure prevented the unwarranted programmed cell death during the morula stage. This is also to ensure that the early embryos undergo normal growth and development. Additional studies are needed to firmly establish the roles of these anti-apoptotic genes during early embryonic development of cod and in other fish species as well.

Conclusion

In conclusion, the study demonstrated that the embryos of Atlantic cod during early development contained immunoreactive cortisol, which has been derived from the female spawner. The embryonic cortisol does not show changes in the amount when the fertilized eggs are exposed to air for a short period of time, indicating this hormone is not synthesized by the cod embryos during the early stages of development. On the other hand, there are potential molecular biomarkers in the cod embryos that could offer clues on how these embryos respond to a potential stressor. Transcription analyses of two oxidative stress-related genes, *sod* and *hsp70,* demonstrated increased expression levels following exposure of the embryos to air. In addition, the apoptotic genes: *mcl1*, Bcl-X1 and NR-13 showed enhanced expression levels after exposure to air. These are anti-apoptotic genes and their upregulation in the embryos following air exposure could indicate their role in arresting premature cell death during early embryogenesis. These molecular biomarkers that are involved in stress response and apoptosis could be potentially used to assess the impacts of any drastic conditions that the developing embryos are subjected to as well as the immune status of these eggs during early development. Transcription profiles and the subsequent products of translation can be determined in the embryos in lieu of measuring cortisol at a time when this hormone does not exhibit a dynamic response during the early stages of development. Future studies should focus on establishing the relationship between gene expression and stress-induced abnormal development in the fish following exposure of the fertilized eggs to various stressors at a longer duration as well as the nutritional status and stress levels of the female spawners.

Acknowledgments A postdoctoral research fellowship at the Faculty of Biosciences and Aquaculture, University of Nordland, Norway and a travel grant awarded by the Short-term Scientific Mission of the COST scientific programme (FA0801) on "Criticial success factors for fish larval production in European Aquaculture: a multidisciplinary network" (LARVANET) awarded to the first author are greatly acknowledged.

Conflict of interest On behalf of the co-authors, I declare that there is no conflict of interest regarding the manuscript.

References

Aegerter S, Jalabert B (2004) Effects of post-ovulatory oocyte ageing and temperature on egg quality and on the occurrence of triploid fry in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 231:59–71

Almeida EA, Bainy ACD (2006) Effects of aerial exposure on antioxidant defenses in the brown mussel *Perna perna*. Braz Arch Biol Technol 49:225–229

Auperin B, Geslin M (2008) Plasma cortisol response to stress in juvenile rainbow trout is influenced by their life history during early development and by egg cortisol content. Gen Comp Endocrinol 158:234–239

Barry TP, Malison JA, Held JA, Parrish JJ (1995) Ontogeny of the cortisol stress response in larval rainbow trout. Gen Comp Endocrinol 97:57–65

Bobe J, Labbé C (2010) Egg and sperm quality in fish. Gen Comp Endocrinol 165:535–548

Bromage NR, Shepherd CJ (1992) Intensive fish farming. BSP Professional Books, Oxford 404 p

Bromage NR, Jones J, Randall C, Thrush M, Springate J, Duston J, Barker G (1991) Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout *Oncorhynchus mykiss*. Aquaculture 100:141–166

Brooks S, Tyler CR, Sumpter JP (1997) Egg quality in fish: what makes a good egg? Rev Fish Biol Fish 7:387–416

Brown JA, Minkoff G, Puvanendran V (2003) Larviculture of Atlantic cod (*Gadus morhua*): progress, protocols, and problems. Aquaculture 227:357–372

Brunelle JK, Letai A (2009) Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci 122:437–441

Caipang CMA, Brinchmann MF, Berg I, Iversen M, Eliassen R, Kiron V (2008a) Changes in selected stress and immune-related genes in Atlantic cod, *Gadus morhua* following overcrowding. Aquac Res 39:1533–1540

Caipang CMA, Brinchmann MF, Kiron V (2008b) Short-term overcrowding of Atlantic cod, *Gadus morhua*: effects on serum-mediated antibacterial activity and transcription of glucose transport and antioxidant defense related genes. Comp Biochem Physiol A 151:560–565

Deane EE, Woo NYS (2003) Ontogeny of thyroid hormones, cortisol, hsp70 and hsp90 during silver sea bream larval development. Life Sci 72:805–818

De Hoon M, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. Bioinformatics 20:1453–1454

De Jesus EG, Hirano T (1992) Changes in whole body concentration of cortisol, thyroid hormones, and sex steroids during early development of the chum salmon, *Oncorhynchus keta*. Gen Comp Endocrinol 85:55–61

De Jesus EG, Hirano T, Inui Y (1991) Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. Gen Comp Endocrinol 82:369–376

Feng CY, Rise ML (2010) Characterization and expression analyses of anti-apoptotic Bcl-2-like genes NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in Atlantic cod (*Gadus morhua*). Mol Immunol 47:763–784

Goddard SV, Kao MH, Fletcher GL (1999) Population differences in antifreeze production cycles of juvenile Atlantic cod (*Gadus morhua*) reflect adaptations to overwintering environment. Can J Fish Aquat Sci 56:1991–1999

Greenblatt M, Brown CL, Lee M, Dauder S, Bern HA (1989) Changes in thyroid hormone levels in eggs and larvae and in iodine uptake by eggs of coho and Chinook salmon *Oncorhynchus kisutch* and *O. tschawytscha*. Fish Physiol Biochem 6:261–278

Halliwell B, Gutteridge JMC (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, New York

Hensey C, Gautier J (1997) A developmental timer that regulates apoptosis at the onset of gastrulation. Mech Dev 69:183–195

Hilomen-Garcia GV (1998) Sensitivity of fertilized milkfish (*Chanos chanos* Forsskal) eggs to mechanical shock and simulated transport. Aquaculture 159:239–247

Howell BR (1984) The intensive rearing of juvenile cod, *Gadus morhua* L. Fløedevigen Rapp Ser 1:657–675

Hwang PP, Wu SM (1993) Role of cortisol in hypoosmoregulation in larvae of the tilapia (*Oreochromis mossambicus*). Gen Comp Endocrinol 92:318–324

Hwang P, Wu S, Lin J, Wu L (1992) Cortisol content of eggs and larvae of teleosts. Gen Comp Immunol 86:189–196

Ikegami R, Hunter P, Yager TD (1999) Developmental activation of the capability to undergo checkpoint-induced apoptosis in the early zebrafish embryo. Dev Biol 209:409–433

Keyvanshokooh S, Vaziri B (2008) Proteome analysis of Persian sturgeon (*Acipenser persicus*) ova. Anim Reprod Sci 109:287–297

Kleppe L, Karlsen O, Edvardsen RB, Norberg B, Andersson E, Taranger GL, Wargelius A (2013) Cortisol treatment of prespawning female cod affects cytogenesis related factors in eggs and embryos. Gen Comp Endocrinol 189:84–95

Krumschnabel G, Podrabsky JE (2009) Fish as model systems for the study of vertebrate apoptosis. Apoptosis 14:1–21

Lanes CFC, Fernandes JMO, Kiron V, Babiak I (2012) Profiling key apoptotic, stress, and immune-related transcripts during embryonic and postembryonic development of Atlantic cod (*Gadus morhua* L.). Theriogenology 78:1583–1596

Lang L, Miskovic D, Lo M, Heikkila JJ (2000) Stress-induced, tissue-specific enrichment of hsp70 mRNA accumulation in *Xenopus laevis* embryos. Cell Stress Chaperones 5:36–44

Lesser MP, Farrell JH, Walker CW (2001) Oxidative stress, DNA damage and p53 expression in the larvae of Atlantic cod (*Gadus morhua*) exposed to ultraviolet (290–400 nm) radiation. J Exp Biol 204:157–164

Li M, Leatherland JF (2012) The interaction between maternal stress and the ontogeny of the innate immune system during teleost embryogenesis: implications for aquaculture practice. J Fish Biol 81:1793–1814

McCormick MI, Nechaev IV (2002) Influence of cortisol on developmental rhythms during embryogenesis in a tropical damselfish. J Exp Zool 293:456–466

Mileva VR, Gilmour KM, Balshine S (2011) Effects of maternal stress on egg characteristics in a cooperatively breeding fish. Comp Biochem Physiol A 158:22–29

Mommsen TB, Vijayan MM, Moon TW (1999) Cortisol in teleosts: dynamics, mechanism of action, and metabolic regulation. Rev Fish Biol Fish 9:211–268

Olsvik PA, Berntssen MHG, Hylland K, Eriksen DØ, Holen E (2012) Low impact of exposure to environmentally relevant doses of ^{226}Ra in Atlantic cod (*Gadus morhua*) embryonic cells. J Environ Radioact 109:84–93

Otterlei E, Nyhammer G, Folkvord A, Stefansson SO (1999) Temperature- and size dependent growth of larval and early juvenile Atlantic cod (*Gadus morhua*): a comparative study of Norwegian coastal cod and northeast Arctic cod. Can J Fish Aquat Sci 56:2099–2111

Paital B, Kumar S, Farmer R, Tripathy NK, Chainy GBN (2013) *In silico* prediction of 3D structure of superoxide dismutase of *Scylla serrata* and its binding properties with inhibitors. Interdiscip Sci Comput Life Sci 5:69–76

Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB (2009) Whole-body cortisol response of zebrafish to acute net handling stress. Aquaculture 297:157–162

Rideout RM, Trippel EA, Litvak MK (2004) Predicting haddock embryo viability based on early cleavage patterns. Aquaculture 230:215–228

Rise ML, Hall JR, Alcock BP, Hori TS (2012) Dynamic expression profiles of virus-responsive and putative antimicrobial peptide-encoding transcripts during Atlantic cod (*Gadus morhua*) embryonic and early larval development. Gene 509:232–246

Romero MC, Tapella F, Sotelano MP, Ansaldo M, Lovrich GA (2011) Oxidative stress in the subantarctic false king crab *Paralomis granulosa* during air exposure and subsequent re-submersion. Aquaculture 319:205–210

Rosenlund G, Halldórsson O (2007) Cod juvenile production: research and commercial developments. Aquaculture 268:188–194

Rupik W, Jasik K, Bembenek J, Widłak W (2011) The expression patterns of heat shock genes and proteins and their role during vertebrate's development. Comp Biochem Physiol A Mol Integr Physiol 159:349–366

Saldanha AJ (2004) Java TreeView—extensible visualization of microarray data. Bioinform Appl Note 20:3246–3248

Sampath-Kumar R, Lee STL, Tan CH, Munro AD, Lam TJ (1997) Biosynthesis in vivo and excretion of cortisol by fish larvae. J Exp Zool 277:337–344

Seppola M, Johnsen H, Mennen S, Myrnes B, Tveiten H (2009) Maternal transfer and transcriptional onset of immune genes during ontogenesis in Atlantic cod. Dev Comp Immunol 33:1205–1211

Skjærven KH, Olsvik PA, Finn RN, Holen E, Hamre K (2011) Ontogenetic expression of maternal and zygotic genes in Atlantic cod embryos under ambient and thermally stressed conditions. Comp Biochem Physiol A 159:196–205

Skjærven KH, Penglase S, Olsvik PA, Hamre K (2013) Redox regulation in Atlantic cod (*Gadus morhua*) embryos developing under normal and heat-stressed conditions. Free Radic Biol Med 57:29–38

Stratholt ML, Donaldson EM, Liley NR (1997) Stress induced elevation of plasma cortisol in adult female coho salmon (*Oncorhynchus kisutch*), is reflected in egg cortisol content, but does not appear to affect early development. Aquaculture 158:141–153

Szisch V, Papandroulakis N, Fanouraki E, Pavlidis M (2005) Ontogeny of the thyroid hormones and cortisol in the gilthead sea bream, *Sparus aurata*. Gen Comp Endocrinol 142:186–192

Tanaka M, Tanangonan JB, Tagawa M, de Jesus EG, Nishida H, Isaka M, Kimura R, Hirano T (1995) Development of the pituitary, thyroid and interrenal glands and application of endocrinology to the improved rearing of marine fish larvae. Aquaculture 135:111–126

Trippel EA, Neilson JD (1992) Fertility and sperm quality of virgin and repeat-spawning Atlantic cod (*Gadus morhua*) and associated hatching success. Can J Fish Aquat Sci 49:2118–2127

Weihe E, Kriews M, Abele D (2010) Differences in heavy metal concentrations and in the response of the antioxidant system to hypoxia and air exposure in the Antarctic limpet, *Nacella concinna*. Mar Environ Res 69:127–135

Xu H, Shao X, Zhang Z, Zou Y, Wu X, Yang L (2013) Oxidative stress and immune related gene expression following exposure to di-n-butyl phthalate and diethyl phthalate in zebrafish embryos. Ecotoxicol Environ Saf 93:39–44

Yamano K, Tagawa M, de Jesus EG, Hirano T, Miwa S, Inui Y (1991) Changes in whole body concentration of thyroid hormones and cortisol in metamorphosing conger eel. J Comp Physiol B 161:371–375