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HPI-axis and heat shock protein (HSP) gene transcripts, and their responsiveness to stress in Atlantic salmon (*Salmo salar* L.) embryos and larvae

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Sammendrag (Norwegian abstract)

Stress kan føre til en betydelig innvirkning på fysiologien og helsen til individet senere i livet. Under en produksjonssyklus av lakse-egg, blir eggene utsatt for ulike typer behandlinger, som sjokking og transport. Slike behandlinger ville i voksen fisk kunne utløse en stressrespons. Stressresponser kan deles inn i primære, sekundære og tertiære responser. Den primære responsen består av to akser, hvor en av dem er hypotalamus-hypofyse-interrenal (HPI) aksen, som resulterer i en økning av sirkulerende kortikosteroider. På cellenivå, er heat-shock proteiner (HSP) en viktig del av den sekundære responsen. I embryo starter ikke syntesen av kortisol, som er den viktigste kortikosteroiden hos teleoster, før rundt klekking. Imidlertid har gener som er sentrale i HPI-aksen, og HSP-gener, blitt detektert i embryo i flere utviklingsstadier. Selv om HPI-aksen antagelig ikke er fullt utviklet før klekking, kan en stressor føre til endringer i genuttrykk.

Basert på dette, ble åtte sentrale gener fra HPI-aksen (*crf1, crf2, pomcA1, pomcA2, pomcB, gr1, gr2* og *mr*) og to HSP gener (*hsp70a* og *hsp90a4*) hos atlantisk lakse- embryo utsatt for sjokking og transport, analysert for å se etter en eventuell behandlingseffekt. I tillegg til dette ble nylig klekkede larver, og larver ved startfôring som var utsatt for en stress test, analysert for å kunne beskrive ontogenien, og for å analysere mulige langtidseffekter av sjokking og transport. Relativt genuttrykk av hele-dyr ble analysert med bruk av revers transkriptase real time polymerase kjedereaksjon (RT-qPCR). Resultatene viste at genene var uttrykt i alle analyserte stadier gjennom utviklingen. HPI-akse genene viste en økning i relativt utrykk gjennom utviklingen, utenom *gr1* og *gr2* som viste en forsinket økning. HSP genene derimot viste et lavere uttrykk i larver ved startfôring enn i embryo og nylig klekkede larver. Relativt uttrykk av HPI-akse genene viste ingen spesifikke kortvarig eller langvarig forskjeller etter utsettelse for sjokking og/eller transport. HSP genene derimot, viste en akutt økning etter transport, men ingen langvarig effekter.

Resultatene fra dette studiet indikerer at sjokking og transport ikke er kraftige nok stressorer til at relativt uttrykket av HPI-akse genene blir forandret i embryo. Resultatene indikerer også at HSP genene mulig spiller en viktig rolle i den cellulære stressresponsen gjennom embryogenesen.

Abstract

Exposure to stress may have a profound impact on the physiology and health of an individual later in life. During a production cycle of Atlantic salmon eggs, the eggs are subjected to different kind of handling, e.g. shocking and transport. Handling of this extent would have elicited stress responses in adult fish. Stress responses can broadly be divided into primary, secondary and tertiary response. The primary stress response consists of two pathways where one of them, the hypothalamus-pituitary-interrenal (HPI) axis, results in elevations of circulating corticosteroids. On a cellular level, heat shock proteins (HSP) play an important role as a secondary response. In embryos, cortisol, which is the main corticosteroid in teleosts, is not synthesized before around hatching. However, genes that are central in the HPI-axis and HSP genes have been detected in fish embryos at several developmental stages. Even though the HPI-axis is not fully developed a stressor may alter the gene expressions.

Based on this, eight genes central in the HPI-axis (*crf1, crf2, pomcA1, pomcA2, pomcB, gr1, gr2* and *mr*) and two HSP genes (*hsp70a* and *hsp90a4*) were examined, in Atlantic salmon embryos subjected to shocking and transport. In addition, newly hatched larvae, and larvae at start feeding subjected to a stress challenge, were analysed to map the ontogeny of the genes, and to examine any possible long-term effects of the shocking and transport. Relative gene expression of whole-animal were analysed using reverse transcriptase real time polymerase chain reaction (RT-qPCR). The results showed that all genes were present in all samples examined throughout the development. The HPI-axis genes showed an increased relative expression level during development, except for *gr1* and *gr2* that showed a delayed increase probably due to maternal transfer. The HSP genes, however, had a low expression level at start feeding compared embryos and newly hatched larvae. The relative expression of the HPI-axis genes did not show any specific short-term or long-term differences in relative gene expression after exposure to shocking and/or transport. The HSP genes, however, showed an acute upregulation after transport, but no long-term effects.

The results of this study indicate~~s~~ that shocking and transport are not high enough stressors to alter the expression of the HPI-axis genes. They also indicate that the HSP genes may play an important role in cellular stress response during development.

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1 Introduction

Atlantic salmon (*Salmo salar* L.) is intensively produced in fish farms in Norway, an industry that has had enormous growth during the last decade. This has led to an increased demand of salmon eggs, and numbers registered by the Norwegian directorate of fishery showed that 719 009 thousand eggs were transferred to hatcheries during 2014, an increase of 13 % from 2013 (Fiskeridirektoratet, 2015). When rearing salmonids, stressful events due to handling are unavoidable and include, among others, sorting, grading, transport, and shocking. Early developmental stages are sensitive and exposure to stress may have a profound impact on the physiology and health of an organism later in life (Groot, 1996; Tsalafouta et al., 2014). It has been shown that exposure to stressors during development results in permanent changes in stress coping phenotypes in mammals, birds, amphibians and fish (Tsalafouta et al., 2014).

1.1 Aquaculture and production of salmon eggs

A production cycle of salmon eggs and larvae normally starts at an egg production site, where eggs are reared until the eyed stage, after which they are transported to a hatchery (see Figure 1). The eggs hatch at the hatchery and are reared until they eventually become smolts, ready for transfer to seawater.

During salmonid development, there are periods where the embryos are more sensitive to external stimuli that need to be taken into account, to prevent increase in mortality. The first period where salmon eggs shows significant sensitivity to handling is between fertilization and the so called eyed-stage, i.e. the stage were the eyes show pigmentation (Egidius and Helland-Hansen, 1973; Groot, 1996). This is the period of early cell division, blastulation and epiboly, in which the embryo begins to take form (Gorodilov, 1996; Groot, 1996). During the eyed-stage, embryos are more robust, and this is the preferred stage where eggs can be handled without causing any harmful effects (Hayes, 1930; Groot, 1996). In production, two main handling events occur during this period of development; shocking of eggs and transport (Maren Mommens, AquaGen, pers.com). First, eggs are intentionally shocked by agitating the eggs enough to rupture the vitelline membrane surrounding the yolk in dead eggs, but not so much that normally developing eggs are damaged (Groot, 1996). This results in coagulation of the yolk proteins and causes dead eggs to turn white and opaque, and can therefore easily be

sorted out (Groot, 1996; Carls et al., 2004). Simultaneously, eggs containing embryo with unusual small eyes are also sorted out (Maren Mommens, AquaGen, pers.com). The second handling event takes place around 375 day degrees (d°C) when eggs are transported to hatcheries. Transportation occurs on ice in special designed styrofoam boxes. Depending on the location of the hatchery, the transportation can take from several hours to a couple of days (Maren Mommens, AquaGen, pers.com). At the hatchery, eggs are transferred into hatching trays and only disturbed by removal of dead eggs. During this period, head and body regions are recognizable and the embryo can be seen to move freely within the chorion. Blood vessels grows out over the surface of the yolk, and the heart is actively pumping (Gorodilov, 1996; Groot, 1996). Normally, the hatching of yolk sac larvae takes place around 500d°C. The yolk sac larvae lie on the bottom of hatching trays on a substrate that supports them with keeping a desired upright position, until they swim up at the onset of exogenous feeding. The start feeding occurs approximately at 900d°C (Groot, 1996; Maren Mommens, AquaGen, pers.com). Physical disturbances encountered in aquaculture, such as shocking and transport, usually evokes a variety of responses in fish (Barton and Iwama, 1991).

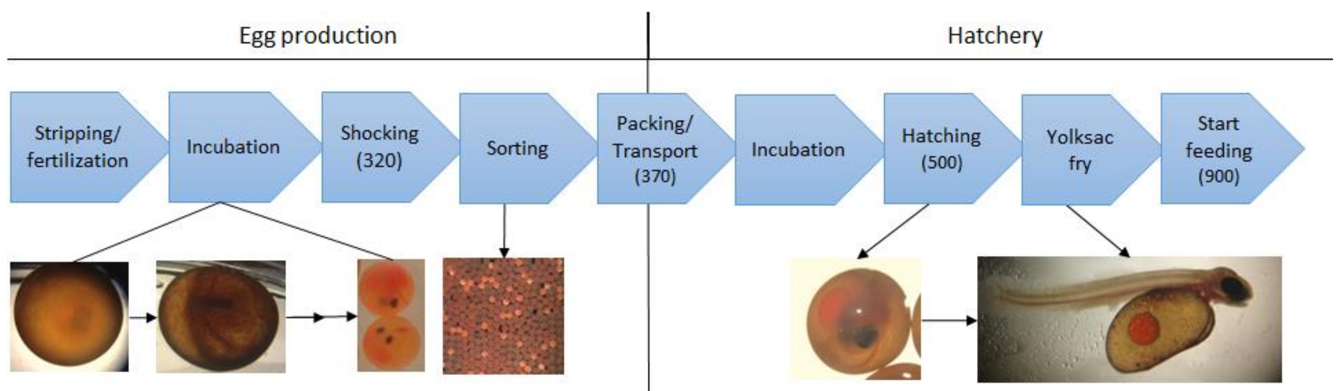

Figure 1: Normal production of salmon eggs and larvae. The pictures are showing, from left; early cell division, epiboly, eyed-eggs, embryo right before hatching, and newly hatched larvae. The number in brackets indicates the day degrees.

1.2 Stress in fish

Stress can be defined as a state where the dynamic homeostasis of an animal are threatened or disturbed by intern or extern stimuli, commonly termed stressors (Wendelaar Bonga, 1997). The stress response in vertebrates can broadly be divided into a primary, secondary and tertiary response as shown in Figure 2 (Iwama, 1998). The primary response includes neuroendocrine responses that results in measurable elevation of cortisol and adrenaline in the circulation (Sumpter, 1997). Thereafter, a secondary response is elicited, that includes cellular responses and changes in features related to metabolism, respiration, acid-base status, hydromineral balance and immune function (Mommsen et al., 1999; Gabriel, 2011). Primary and secondary stress responses are adaptive if they result in a physiological response that allows a fish to maintain homeostasis (Donaldson et al., 2008). Prolonged stress can give rise to a tertiary response, which refers to aspects of whole-animal performance, such as changes in growth, reproduction, behaviour, resistance to disease and ultimately survival (Barton, 2002). In the present study, the focus will be on the primary and secondary responses. A wide range of stressors elicits both the neuroendocrine and cellular stress responses (Ackerman et al., 2000).

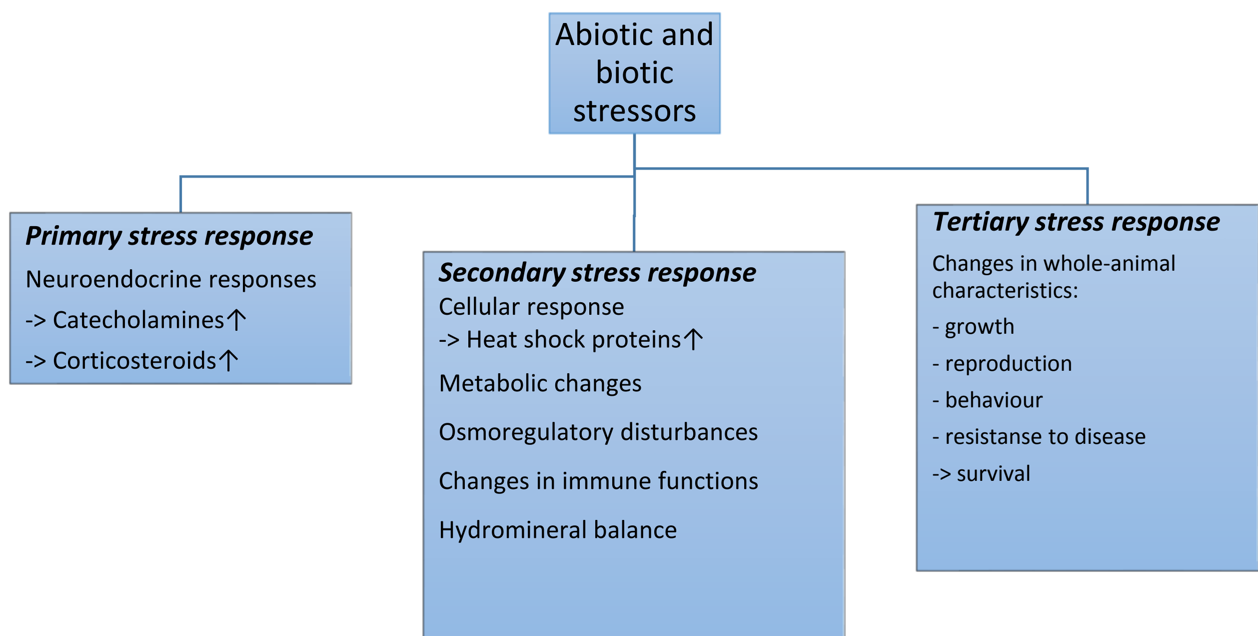

Figure 2: A figure showing the three grouped stress responses and their action (Modified from Barton et al., 2002).

1.2.1 Neuroendocrine stress response

A stressor activates a two component system in fish; the hypothalamus-sympathetic-chromaffin (HSC) axis and the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997).

The HSC-axis leads to an adrenergic response. When elicited, sympathetic nerve fibres stimulates chromaffin cells in the head kidney to release catecholamine hormones, adrenaline and noradrenaline, into the circulation (Sumpter, 1997; Wendelaar Bonga, 1997; Reid et al., 1998). Catecholamines, predominantly adrenaline in teleosts, are both synthesized and stored in the chromaffin cells and can therefore be rapidly released after stress (Reid et al., 1998; Barton, 2002). One of the primary roles of plasma catecholamines is to modulate cardiovascular and respiratory functions in order to maintain adequate levels of oxygen in the blood. In addition, they serve to mobilize energy stores to provide for the increased energy demands that often are required after exposure to stressors (Reid et al., 1998).

The HPI-axis consists of a three stage endocrine pathway as shown in Figure 3, where cortisol is the physiologically important hormone responsible for the effect of stress (Sumpter, 1997; Mommsen et al., 1999). A hormone cascade is initiated by external stimuli that stimulate the hypothalamus to release corticotropin-releasing factor (CRF). CRF will further stimulate corticotrophin cells in the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) into the circulation, which thereafter stimulates the interrenal cells, in the head kidney, to produce and secrete corticosteroids, mainly cortisol in teleost fishes (Sumpter, 1997; Wendelaar Bonga, 1997; Mommsen et al., 1999; Flik et al., 2006). The release of cortisol is delayed relative to catecholamine release (Reid et al., 1998; Barton, 2002). The main role of cortisol is to meet the energy demands in a stress response, by redirecting the metabolism, and to limit the defence reactions to stress in order to protect the body from further damage (Xiong and Zhang, 2013; Wendelaar Bonga,1997). In the present study, the focus of neuroendocrine stress response will be on the HPI-axis, and the following sections will go into more detail about some of its important components.

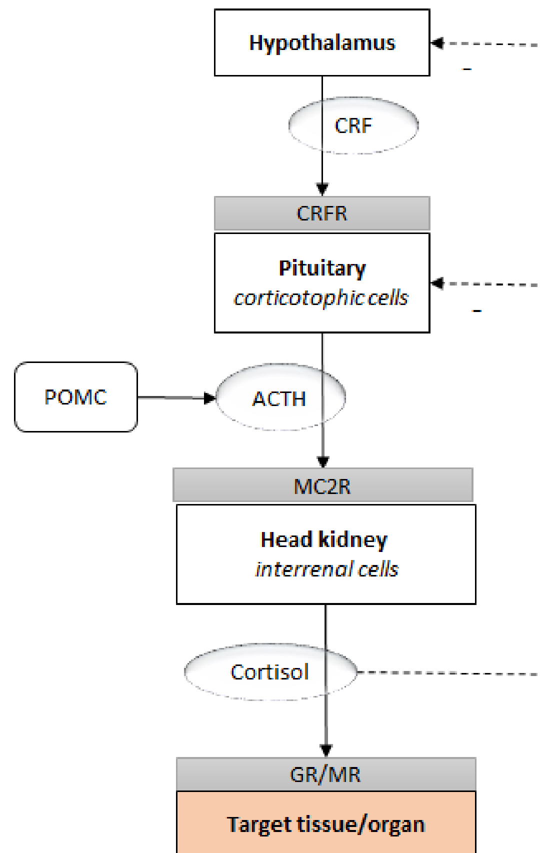

Figure 3: **The HPI-axis and the hormone cascade leading to cortisol secretion.** CRF is projected directly from the hypothalamus to the pituitary where it binds to CRF-receptors in corticotropic cells. The binding eventually elicit a secretion of ACTH into the bloodstream. POMC is the precursor for ACTH. Circulation ACTH binds to MC2-receptors in interrenal cells of the head kidney and stimulates to synthesis and eventually secretion of cortisol into the bloodstream. Cortisol enters the target tissues/organ by diffusion and binds to GR and MR, which mediates the action of cortisol by altering target gene expression. An elevated level of cortisol has a negative feedback on the hypothalamus and pituitary.

1.2.1.1 Cortisol releasing factor (CRF)

Cortisol releasing factor (CRF) is a neuropeptide that is produced in nucleus preopticus (NPO) of the hypothalamus. Various stressors are associated with an increased expression of preoptic area CRF, in adult fish (Bernier and Bristow, 2008). In teleosts, CRF controls the HPI-axis through activation of specific G-protein coupled CRF receptors (CRF-R1 and CRF-R2) and is regulated by a shared CRF binding protein (CRF-BP). In addition to the regulation of the endocrine stress response, other functions of CRF include for example food intake inhibition and behavioural modulation (Alderman and Bernier, 2009). In fishes, CRF is also produced and secreted from the caudal neurosecretory system (CNSS), a unique organ located at the caudal end of the spinal cord. At the hypothalamic level, CRF is considered to be the major regulator of adrenocorticotropic hormone (ACTH) secretion from the pituitary and thereby plays a key

role in coordinating the neuroendocrine, autonomic, and behavioural responses to stress (Alsop and Aluru, 2011).

1.2.1.2 ACTH/POMC

Binding of CRF to CRF R1/R2 in corticotropic cells of the anterior pituitary, elicits the release of ACTH into the circulation. ACTH is derived from a precursor hormone termed proopiomelanocortin (POMC), which is a large polypeptide that is progressively cleaved by prohormone convertases into several biologically active peptides (Flik et al. 2006; Nelson and Cox, 2008). In humans the POMC gene expression is stimulated by corticotrophin-releasing hormone (CRH) and vasopressin, and is suppressed by glucocorticoids (Raffin-Sanson and Bertagna, 2003). The peptides can be broadly divided into three groups: adrenocorticotropic hormone (ACTH)-like, endorphin-like and MSH-like products. POMC is primarily synthesised in two cell types of the pituitary gland: the corticotrophs of the anterior lobe and the melanotrophs of the intermediate lobe, each lobe being responsible for different peptide products (Sumpter et al.1997; Mosconi et al., 2006). In brown trout, handling and confinement has only showed to activated the corticothrophs, whereas when the handling was combined with thermal shock, both corticothrophs and melanotrophs were activated (Sumpter et al. 1985). POMC has, in addition, shown to be present in a variety of other brain regions, and peripheral tissues such as the skin (Hansen et al.2003; Karsi et al. 2004). ACTH is recognized as the principle stimulator of cortisol release (Wendelaar Bonga, 1997; Flik et al.2006). Circulating ACTH binds to melanocortin 2 receptor (MC2R) in the steroidogenic interrenal cells embedded in the head kidney in teleosts. The binding to MC2R stimulates adenylate cyclase and cAMP-dependent signalling pathways, to stimulate cortisol synthesis. This receptor has shown to be downregulated following stress due to negative feedback control (Alsop and Aluru, 2011).

1.2.1.3 Cortisol and the cortisol receptors

Free circulating cortisol enters target cells, such as hepatocytes, by passive diffusion, where its action is mediated by the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (Alsop and Aluru, 2011). These receptors belong to the nuclear receptor superfamily of ligand-bound transcription factors. The receptors require the presence of certain heat shock proteins (e.g. HSP70 and HSP90; see section 1.2.2) to form a steroid compatible heterocomplex (Norris

and Hobbs, 2006). When activated the receptors are translocated into the nucleus of the cell, where they acts as transcription factors involved in the activation or silencing of specific genes (Li and Leatherland, 2012). In fishes, GR and MR are expressed in a variety of tissues including liver, gill, muscle, kidney, blood, and brain (Norris and Hobbs, 2006). Mediated by GR, cortisol modulate aspects of metabolism, growth, reproduction and immune function during a stress elicited response (Wendelaar Bonga, 1997). The roles of MR and its ligand are less clear. The main ligand to MR in mammals is aldosterone, due to inactivation of cortisol by an enzyme, 11_HSD2, allowing aldosterone to bind. Teleosts lack the capacity to synthesize aldosterone, but another possible MR ligand that is studied in rainbow trout is 11 deoxycorticosterone (DOC) (Sturm et al.2005). Cortisol elicits a negative feedback primarily at the brain to repress the release of CRF, thereby reducing ACTH secretion and, ultimately, cortisol secretion (Norris and Hobbs, 2006; Bumaschny et al., 2007; Alsop and Aluru, 2011)

1.2.2 Cellular stress response

Cortisosteroids along with catecholamines, mediate secondary stress responses, in which a cellular response is one of them (Donaldson et al., 2008). Fish, like other organisms, produce a variety of proteins as part of the stress response, which are included in a generalized response system that exists at a cellular level. These proteins, which commonly are called stress proteins include among others, metallothioneins and heat shock proteins (Wendelaar Bonga, 1997; Basu et al., 2002). The heat shock proteins (HSP) are one of the most common and most studied groups of stress proteins in the cellular response (Deane and Woo, 2011).

1.2.2.1 Heat shock proteins

Heat shock proteins (HSPs) are a group of highly conserved intracellular proteins first detected in fruit fly when exposed to heat shock. They are classified into families based on their protein molecular size (kDa) which also gives them their names: HSP100, HSP90, HSP70, HSP60, and the small HSPs (Deane and Woo, 2011). HSPs are expressed in all tissues and cells constitutively, but some are also inducible in response to biotic or abiotic stressors. In an unstressed cell, the constitutive HSPs generally function as molecular chaperones assisting the folding of nascent polypeptides, protein folding, translocation of proteins, and degradation of misfolded proteins (Basu et al., 2002; Deane and Woo, 2011). When exposed to a stressor the inducible HSPs are upregulated, which in turn gives the cell added protection to repair and

prevent damage from cellular stress associated with protein denaturation (Iwama et al., 1998; Basu et al., 2002; Donaldson et al., 2008; Deane and Woo, 2011). Most of the inducible HSP genes do not contain introns and therefore their mRNA are rapidly translated into protein within minutes after an exposure to a stressor (Morimoto et al., 1992; Iwama et al., 1998). HSPs have been found to be upregulated when subjected to both high and low temperatures, and it is also widely accepted that their expression can alter upon exposure to a range of other abiotic, as well as biotic and chemical stressors (Deane and Woo, 2011; Donaldson et al., 2008). HSPs are also known to play key roles during embryonic development (Deane and Woo, 2011). A number of HSPs are expressed at high levels during normal cell growth and has shown to be important for reducing temperature-induced damage and deformities of fish embryos (Iwama et al., 1999; Donaldson et al., 2008).

The HSP70 family represents the most abundant and the most highly conserved HSPs. HSP70 is composed of constitutive (HSC70) and stress-inducible (HSP70) isoforms. Inducible isoforms are the best studied HSP70 in developing zebrafish and are frequently induced by thermal stress (Rupik et al., 2011). The constitutive members play important chaperoning roles in unstressed cells (Basu et al.2002). In addition, it has been shown in zebrafish that Hsp70s are required during the normal process of lens development under non-stress conditions (Evans et al. 2005).

Members of the eukaryotic hsp90 family interacts with and modulates the activity of important cellular signalling molecules, such as steroid receptors and transcription factors (Krone et al., 2003). It has been estimated that HSP90 accounts for about 1% of the total soluble protein in the cytosol of an unstressed cells, which makes it one of the most abundant proteins (Picard, 2002). Vertebrates express two hsp90 genes, hsp90α and hsp90β, and studies in zebrafish, indicate that these genes are differentially regulated (Basu, 2002; Krone et al.2003). HSP90 interacts with a large number of proteins, and its interaction with steroid receptors, including GR, results in the formation of a stable heterocomplex (Pratt and Toft, 1997). The binding of HSP90 increases the receptor stability by allowing GR to be conformational competent for ligand binding, in addition to prevent proteasomal degradation of GR. HSP90 is thereby a key molecular chaperone that is crucial for cortisol mediated cellular action, including GR signal transduction (Pratt and Toft, 1997). In addition, the isoform HSP90α

has been shown to be required for normal muscle development in zebrafish during embryogenesis (Krone et al., 2003).

1.3 The genes of the HPI-axis

In the evolution of vertebrates, several whole genome duplication (WGD) events are thought to have occurred. One of them occurred 320–350 million years ago specifically in an ancient fish, which gave rise to a number of duplicate genes that exist exclusively in teleost today (Alsop and Vijayan, 2009). In addition, salmonids have gone through another WGD, which occurred about 25-100 million years ago in a common ancestor (Meyer and Schartl, 1999). After a WGD, most duplicate genes return to single gene systems, but in some instances, duplicate genes are retained (Alsop and Vijayan, 2009).

Two CRF genes have been found in several fish species, including white sucker; *Catostomus Commersoni*, carp; *Cyprinus carpio*, sockeye salmon; *Oncorhynchus nerka*, and rainbow trout; *Oncorhynchus mykiss* (reviewed in Alsop and Vijayan, 2009), while only a single CRF system is reported in zebrafish; *Danio rerio* (Chandrasekar et al., 2007; Alsop and Vijayan, 2008). According to Alsop and Vijayan, (2009) the duplicate CRF sequences are so similar that only a few studies have been able to differentiate the two. Doyon et al. (2003) was able to differentiate between the CRF paralogs in rainbow trout brain, which showed that levels of both CRFs were highest in the preoptic area of the hypothalamus, and were expressed to the same extent.

Similar to the CRF genes, most POMC genes are identified in duplicates among studied species, including common carp; *Cyprinus carpio* (Arends et al., 1998), zebrafish (Nunez and Gonzalez-Sarmiento, 2003) and sockeye salmon (Okuta et al., 1996). In rainbow trout two POMC genes, in addition to a splice variant of one of the genes, has been identified (Salbert et al., 1992; Leder and Silverstein, 2006).

Two GR genes and one MR have been found in several teleost, except for zebrafish which only have identified a single GR, a homolog to GR2 in other teleosts (Alsop and Vijayan, 2009). Between the two GRs, GR2 has shown to be more sensitive to cortisol than GR1 in rainbow trout (Bury et al., 2003). As mentioned in section 1.2.1, deoxycorticosterone (DOC) is studied

in rainbow trout to be a possible ligand for MR (Sturm et al. 2005*).* The presence of three receptors with different affinities for cortisol raises, according to Norris and Hobbs, (2006), a possibility that the response to cortisol in tissues may change, as increasing levels of cortisol receptors with lower affinities are bound, and activated at higher cortisol levels.

1.4 Ontogeny of HPI-axis hormones in addition to HSP70 and HPS90

In fish, the most commonly used stress indicator is, to the author's knowledge, the elevation of cortisol (Gabriel, 2011). In fertilized eggs, embryos and larvae, changes in cortisol content at various developmental stages have been examined in several fish species (reviewed by Pittman et al. 2013). In fertilized eggs, the cortisol content is of maternal origin and, according to Pittman et al. (2013), seems to be necessary for the metabolic needs and for the development of various organs during early development. Most of the examined species mentioned show a general pattern of changes in cortisol content in the egg, with relatively high levels after fertilization, followed by a decrease throughout embryogenesis as the maternal deposited cortisol is depleted. The lowest levels are registered around the time of hatching and, thereafter, the larva begins to synthesize cortisol and basal levels increase (Alsop and Aluru, 2011; Pittman et al., 2013). The timing of *de novo* synthesis of cortisol varies among species. Studies on rainbow trout indicates a cortisol synthesis 6 days before hatching (Auperin and Geslin, 2008). In both chinook salmon; *Onchorynchus tshawytscha* and zebrafish *de novo* synthesis of cortisol was detected around the time of hatching (Feist and Schreck, 2001; Alsop and Vijayan, 2008). From early embryogenesis, expression of other HPI-axis genes also has been shown to undergo dynamic changes, suggesting that they are functional at this time (Alsop and Aluru, 2011). Most studies have been conducted on zebrafish, and therefore, the following description of the ontogeny of different HPI-axis genes will mainly be based on findings in zebrafish embryos and larvae.

Crf has been detected in zebrafish throughout embryogenesis and larvae showed an increase in *crf* expression levels between hatching and exogenous feeding (Alderman and Bernier, 2009). *Crf* transcripts have been detected during embryogenesis for several other teleost species including tilapia; *Oreochromis mossambicus* (Pepels and Balm, 2004), rainbow trout (Fuzzen et al., 2011), and European sea bass, *Dicentrarchus labrax* (Tsalafouta et al., 2014). A study on rainbow trout showed a pattern of *crf* expression throughout ontogeny that was

similar to cortisol, and the larvae showed a peak in CRF mRNA levels occurring at 56 days post fertilization (dpf); the onset of exogenous feeding (Fuzzen et al., 2011).

Hansen et al. (*2003) registered pomc* mRNA expression in fertilized zebrafish eggs by RT-PCR, which almost completely disappeared within the next few hours after fertilization, which demonstrates maternal expression. After 18hpf, zygotic *pomc* RNA synthesis levels significantly increased with a maximum at 28 hpf (Hansen et al., 2003).

The expression pattern of *gr* and *mr* has shown to be distinct during the embryogenesis in zebrafish. Expression levels of *mr* showed a continuous elevation during development from fertilization until start feeding, while those of *gr* followed closely the cortisol profiles seen in the embryos; i.e. showing a decrease throughout embryogenesis, followed by a rise around hatching, which continued until start feeding (Alsop and Vijayan, 2008)

HSPs studied in zebrafish, have shown to be expressed in spatial and temporal patterns, which coincided with the assumed targets of their chaperoning activity (Krone et al. 1997). It was also shown that several HSPs may be directly involved in embryonic cellular differentiation (Martin et al.2002). Both constitutive and inducible forms of *hsp70* have been detected in the developing zebrafish. During embryogenesis of zebrafish, basal levels of inducible *hsp70* showed to be low, while constitutive members of *hsp70* have shown to be more strongly expressed (Lele et al., 1997; Santacruz et al., 1997). Embryos of Atlantic salmon expressed *hsp70* mRNA transcripts at all examined stages from 62d°C until 200d°C (Takle et al., 2005). *Hsp70* expression was upregulated in early larval stage of zebrafish, which also was registered in silver sea bream (*Sparus sarba*) larvae after 14 dph (Yeh and Hsu, 2000; Deane and Woo, 2003). Deane and Woo, (2003) registered that *hsp90* increased from 1dph and onwards, where the profiles during 1-14 dph was parallel to cortisol. In addition, the two isoforms, *hsp90α* and *hsp90ß*, have shown to be expressed in zebrafish during embryogenesis (Krone et al.1997).

1.5 Stress response in early development

Several studies have registered that the necessary components for a functioning HPI axis are in place before, or at the time of hatching. However, there has not shown to be any stress-

induced elevation of cortisol during early embryogenesis (reviewed by Pittman et al.2013). In fact, stress-induced cortisol alterations have, to the author's knowledge, not been detected before hatch in most species studied. In rainbow trout, stress-induced cortisol elevation was not detected before 11 dpf (Auperin and Geslin, 2008) or 14 dpf (Barry et al., 1995a). In chinook salmon stress induced elevation of cortisol was detected one week after hatching (Feist and Schreck, 2001). In rats, a 2-week stress hyporesponsive period has been shown where stressors do not elicit an increase in circulating glucocorticoids levels, as they do in adult animals. This period is thought to be a critical time where corticosteroids may have permanent effects on the neural organization and development (Barry et al., 1995a; Barry et al., 1995b).

Early development represents a critical period during life history of fishes (Groot, 1996). At this time of the development environment may irreversibly influence the phenotype (e.g. morphology, physiology, behaviour) by allowing rapid adaptations. These adaptations may be beneficially for the animal later in life, or in contrast give adverse consequences if there is any mismatch between the anticipated and the actual environment later in life (Pittman et al., 2013). The hypothalamus-pituitary-adrenal (HPA) axis in mammals is highly susceptible to 'programming' during the development (Xiong and Zhang, 2013).

Thus, even though the HPI-axis is not fully functional before hatching in most teleosts studied, the genes of the HPI-axis may still be altered by stressors, which in turn might affect the individual later in life. Auperin and Geslin (2008) detected in rainbow trout that stress applied to eyed-egg, eggs at the time of hatching, and yolk sac larvae, resulted in a reduced cortisol response to stress in fingerlings. They suggested that stress during the development of the HPI-axis may have long lasting effects and may influence the fish's ability to cope with stress later in life.

1.6 Aim

This master project was part of a project conducted by Nofima Tromsø in collaboration with AquaGen Norway. It was important to clarify several aspects related to some of the possible effects mechanical shocking and transportation of eggs may have. The main objective of this study was to examine gene expressions of HPI-axis - and HSP genes in Atlantic salmon (*Salmo salar* L.) embryos and larvae in terms of upregulation or downregulation after exposure to shocking and/or transport by analysing total RNA from whole eggs and larvae with RT-qPCR.

Sub goal 1:

Examine the ontogeny of eight HPI-axis genes (*crf1, crf2, pomcA1, pomcA2, pomcB, gr1, gr2* and *mr*) and two HSP genes (*hsp70a* and *hsp90a4*) by using four time points in the embryo and larval development. Examine if shocking and transport conducted in rearing of salmonid eggs (mechanical shock and transportation on ice), results in alteration of the relative expression of these genes.

Sub goal 2:

Examine if the shocking and transport alters the expression of the mentioned HPI-axis genes and HSP genes over time, by examining the relative gene expression in larvae at start feeding. In addition, examine if the shocking and transport may lead to different gene expression levels when the larvae at start feeding are exposed to a stress challenge.

2 Materials and Methods

All chemicals and kits used are listed in appendix I.

2.1 Eggs, fertilization and incubation conditions

Atlantic salmon (*Salmo salar*) eggs from one female and milt from one male were sent by plane from AquaGen, Kyrkseterøra to Nofima, Tromsø. The package containing eggs and milt was immediately transported by car for 30 minutes to the Aquaculture Research Station in Kårvika. The eggs and milt were during the whole transport kept in a Styrofoam box; packed in plastic bags and laid between two layers of ice covered with newspaper. The eggs and milt held a temperature of 2-3°C at the time of unpacking. The whole transportation took less than 24 hours.

2.1.1 Fertilization

Approximately 1,825 L eggs were fertilized immediately after transportation to Kårvika, using a dry fertilization method obtained from AquaGen. The eggs were carefully poured into a tub where they were washed with a washing solution (recipe, AquaGen) until blood and ovary fluid was removed. The beam was always pointed towards one of the sides of the tub, avoiding directly contact with the eggs. After the washing procedure, the tub was filled with washing solution equivalent to 1/3 of the egg volume. Approximately 2 mL milt per litre of eggs was added. The milt and the eggs were then evenly distributed with gently stirring. After 25 seconds, activation solution (recipe, AquaGen) was added to activate the milt. The mixture was carefully stirred and left for 2,5 minutes. The milt and activation solution were washed away using the washing solution. Directly after the fertilization the eggs were treated with a disinfection solution composed of 10 parts Buffodine (Evans Vanodine) to 1000 parts water (50mL Buffodine + 5L water from the hatching column). The disinfection solution was poured into the tub covering the eggs. It was left for 10 min and then gently washed away with the washing solution. The eggs were finally divided into beakers each containing approximately 175 dL eggs, and transferred to 12 plastic incubator boxes in a hatching column (see chapter 2.2.2) for swelling.

2.1.2 Incubation

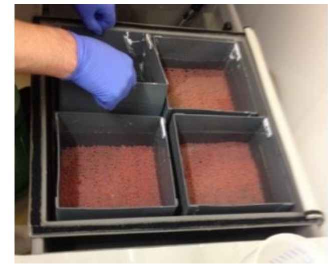

Figure 4: A picture showing one of the incubation units containing four boxes with salmon eggs.

Incubation of eggs and embryos was carried out in a specially designed hatching column with temperature regulation and continuous water supply that was filtered and free of chloride. The hatching column held three incubation units, A, B and C, each containing four special incubation boxes as shown in Figure 1. The boxes were labelled with four groups, each group having triplicates that were evenly distributed in the column to minimize ''tank effect'' (see Figure 4). Over the first nine days after fertilization, the water temperature was slowly increased from 3°C to 7°C to acclimate the eggs. During the rest of the experimental period the eggs and larvae were held under a mean (±SD) water temperature of 7,04 (± 0,16) °C and dissolved oxygen levels of 101,7 (± 1,53) %. Opaque, white eggs were counted and eliminated once a day. Both the incubation units and the whole hatching column was covered with opaque plastic sheets at all time to avoid light entering the incubation boxes.

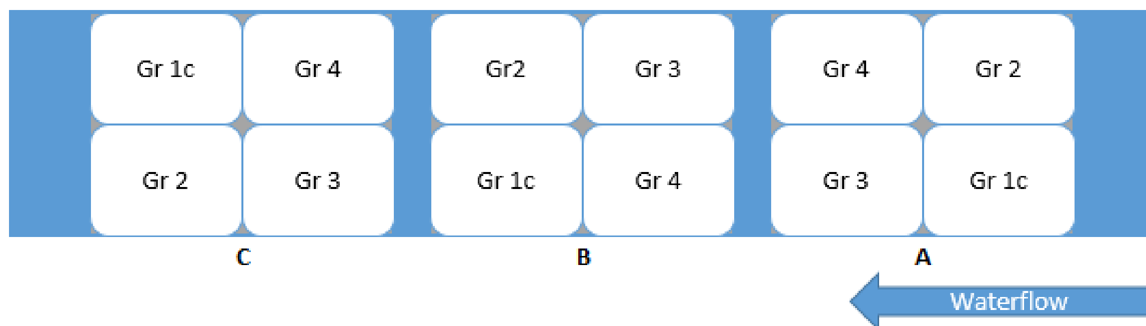

Figure 5: A flow chart showing the hatching column containing the units (A, B and C) and the incubation boxes divided into four groups of triplicates.

2.2 Experimental design

Eggs were divided into four groups of triplicates, with each group going through different treatments. Group 1 was the control group, group 2 was shocked and transported, group 3 was shocked and group 4 was transported (see figure 5). At the end of the experimental period, all groups were subjected to a stress challenge test to evaluate the effect of the previous treatments.

2.2.1 Shocking of eggs

Eggs from groups 2 and 3 were shocked at around 326 day degrees, one incubation box at a time. Eggs from an incubation box were gently poured into a bucket filled with 7,5cm water. The eggs were then poured into another bucket, containing the same amount of water, from a height of 60 cm over the water surface as shown in Figure 6. This procedure was repeated three times for all replicates within the two groups.

Figure 6: A picture showing shocking of salmon eggs as described in chapter 2.3.1.

2.2.2 Shipment on ice

Eggs from groups 2 and 4 were transported when the eggs were 377d°C. They were transferred to a Styrofoam box specially designed for transportation of fish eggs (obtained from AquaGen). The box contained three shelves, each of them divided into 12 units keeping the eggs separate. All the shelves contained small holes in the bottom to enable water to run through. During the transport, the upper shelf was filled with ice so that water could drain through to the next shelf containing eggs. It was important that the eggs were wet during the whole transportation but not soaked in water. The transportation lasted for 48 hours. The temperature in the box was measured at all times by two gauges that were placed separately in two empty units on the same shelf as the eggs. During the transportation the two gauges showed mean (±SD) temperature of 1,12 (± 0,96) and 1,41 (± 0,91)°C.

2.2.3 Stress test

Larvae from all groups were stressed when they were approximately 918d°C. Approximately 20 larvae from an incubation box were continuously transferred as described in section 2.3.4, and directly distributed into four special cylindrical incubation devices where three of the cylinders were stress challenged. The latter cylindrical incubator was immediately euthanatized and sampled as control. The stress challenge test was performed by exposing

larvae acclimated to 7°C, to ice water for 1 min and thereafter to air (14°C) for 1 min. After the challenge, the cylinders were placed in suitable containers placed in the hatching column so that the stressed larvae did not get disturbed between the challenge and the time of sampling.

2.2.4 Sampling

Samplings were conducted at four time points as shown in Figure 7. Three of the time points were sampled in accordance with the different treatments (described in section 2.3.1 – 2.3.3); before the treatment started, and 1 hour, 3 hours and approximately 24 hours after the treatment was finished. The fourth sample was taken a few days after hatching. Two types of samples were taken at all time points; one in RNA-later® (Ambion) for gene expression analyses and one immediately frozen in Liquid N_2 for other analyses. The RNA-later samples contained maximum 20 eggs or larvae and approximately 10 mL of RNA-later® (Ambion) solution in 20 mL tubes. Following samplings the tubes containing RNA-later® (Ambion), were stored as recommended by the manufacturer; overnight at 4 °C and then frozen at -20 °C the following day. The samples that were immediately frozen in liquid N_2, contained a maximum of 5 eggs or larvae in 1,8 mL Cryo Tubes (Nunc). The samples were transferred to a -80 °C freezer the same day as the sampling.

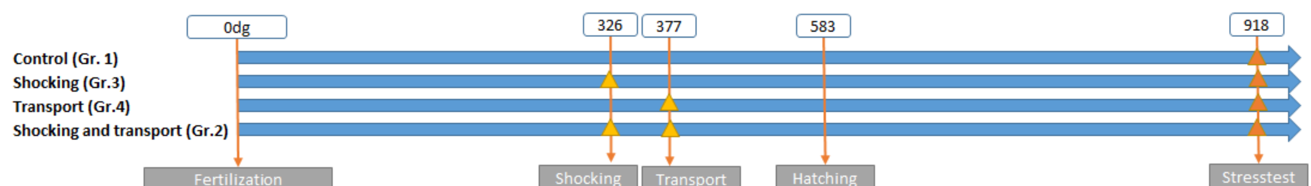

Figure 7: A flow chart containing an overview over the rearing period (blue arrows) and the time of treatments (yellow/orange triangles) performed on the four different groups. The orange arrows indicates the time of sampling.

Sampling of eggs:

A special egg tweezer or a spoon was used to collect random eggs on a petri dish kept on ice while the sampling was conducted. Normal eggs were sampled on RNA-later® (Ambion) and liquid N_2. Eggs that were developing at a slower rate (see figure 8) were sampled on separate tubes. Underdeveloped, blank eggs were discarded (see figure 8).

Figure 8: Pictures showing different developed eggs; normal, small-eye and blank (stopped developing).

Sampling of larvae:

Larvae were collected using a transparent tube and the siphon principle, which made it possible to collect an almost exact sample size without affecting the remaining larvae. All larvae that were sampled were euthanatized using an overdose of Benzocaine. They were laid on a petri dish on ice and, in the same way as the sampling off eggs; normal larvae were selected and transferred to tubes containing RNA-later® (Ambion), and tubes that were put on liquid N2. Larvae that were un-normal (see figure 9) were collected in a second tube containing RNA-later ® (Ambion).

Figure 9: Picture showing normal and un-normal larvae

2.3 Quantitative RT-PCR

Quantitative real-time polymerase chain reaction (RT-qPCR) has become the leading tool for the detection and quantification of DNA or RNA (as cDNA). Using sequence-specific primers, the number of copies of a particular DNA or RNA sequence can be determined. The RT-qPCR measures the amount of DNA or cDNA after each cycle and thereby monitors the progress of the PCR reaction as it occurs in real time. Fluorescent dyes are used as dictation agents where the yield of increasing fluorescent signal are in direct proportion to the number of PCR product molecules (amplicons) generated. One fluorescent dye that is suitable to use is SYBR Green. SYBR green is a fluorescent DNA-binding dye that binds to any double stranded (ds)DNA and provides a fluorescent signal that reflects the amount of DNA product in the sample (LifeTechnologies, 2011b, LifeTechnologies, 2014).

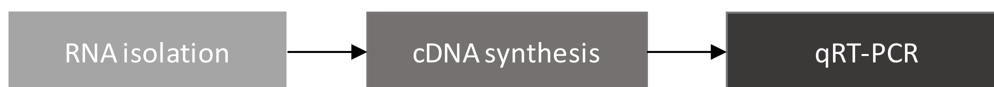

Figure 10: A flowchart that shows the main steps of quantitative RT-PCR.

In this experiment RT-qPCR was used to examine gene expression from HPI-axis genes of corticotropin releasing factor (CRF*)*, proopiomelanocorticoid A1, A2 and B (POMC A1, POMC A2 and POMC B), glucocorticoid receptor 1 and 2 (GR 1 & GR 2), mineralocorticoid receptor (MR) and heat shock protein 70a and 90a4 (HSP70a and HSP90a4). Eukaryotic Elongation factor 1 alfa (ef-1-α), Ribosomal 18S RNA (18S rRNA) and Beta-actin (ß-actin) were used as housekeeping genes. Primers were designed using the Primer Express 3 software (Life Technologies) and synthesized by Eurogentec. All primers are listed in Appendix II. Ten individuals from each group were analysed, which equals five individuals from two of the replicates (incubation unit B and C).

2.3.1 RNA isolation

RNA isolation was conducted in three steps; homogenization, purification of nuclei acids and DNase treatment. Whole eggs and larvae were used. One random egg from each group, from each sampling date, was weighed before the homogenization. This was done because the

weight of the sample tissue needed to be known for further purification. RNase-free equipment was used during the whole procedure.

2.3.1.1 Homogenization

To homogenize salmon eggs and larvae MagMAX-96 Total RNA Isolation Kit (Ambion) was used and the procedure was conducted according to the manufacturers protocol, with some minor modifications. The amounts needed to homogenize one salmon egg without making the homogenate too viscous had earlier been established in the lab. Salmon eggs on RNA-later® (Ambion) were thawed on the bench, punctured with a pipette tip, and put into tubes containing ceramic beads (Precellys) and 800 µL of Lysis/binding solution concentrate (Ambion). The samples were homogenized using the machine Precellys 24 lysis and homogenization (Bertin technologies) for 3 x (15 seconds x 6800rpm), with 30 seconds pause between each round. The tubes containing the homogenate were cooled for about 10 minutes and then added 20 µl of Proteinase K (Ambion). After incubation at 37 °C for 90 minutes the homogenate was frozen at -80 °C until further analyses.

2.3.1.2 Total nucleic acid isolation

The MagMax™- 96 Total Kit from Ambion was used to extract RNA from salmon eggs. The procedure was conducted according to the producers protocol, except from the DNase treatment which was done in a separate step (see chapter 2.4.2.1). Homogenized samples were thawed, mixed and centrifuged for 2min at 2500rpm (Kubota 1300). All solutions and plates were prepared as described in the manufacturers protocol. In brief, the reaction volumes (180µL) of the test-plate contained 5mg homogenate adjusted to 100 µL with Lysis/binding solution concentrate (Ambion), 20 µL Bead mix solution and 60 µL 100 % isopropanol Prima (Arcus). In addition to the test-plate, plates containing washing solutions, elution buffer and special tip compounds were prepared. Total nucleic acids were isolated using a magnetic purification machine; MagMAX™ Express 96 (Applied Biosystems). The machine purifies the samples by magnetically capturing the RNA binding beads in the homogenate and washing them in several steps to remove cell residues, proteins and other contaminants (AppliedBiosystems, 2008, LifeTechnologies, 2011a). A MagMax Express plate (Applied Biosystems) containing the eluate with the purified nucleic acids was put on an Ambion magnetic-ring stand (Applied Biosystems), thereby making it possible to collect the

eluate without getting remains of the magnetic beads. The now isolated total nucleic acid samples were held on ice or frozen at -80 °C until further analysed.

2.3.1.3 DNase treatment

To clean the RNA from contamination of genomic DNA the nucleic acid sample TURBO DNAfree Kit (Ambion) was used in accordance to the manufacturers protocol (LifeTechnologies, 2012). Centrifugation was conducted in a Jouan A14 centrifuge at 10000*x*g in 2min. After the procedure, the supernatant containing the isolated RNA was collected and frozen at -80 °C until analysed further.

2.3.2 NanoDrop

NanoDrop (Saveen Werner AB) is a spectrophotometer using fibre optic technology and surface tension to hold 0,5-2 µL of sample in place between two optical surfaces. (ThermoFisher, 2015). NanoDrop determines the RNA concentration by measuring its absorbance at 260nm (A_{260}). Additionally it measures the purity of the RNA sample which mainly are shown by the A260/A280 ratio (LifeTechnologies, 2011a). All the isolated RNA samples were measured on a NanoDrop 8000 (Thermo scientific) before cDNA synthesis, to assess RNA quality and quantity. Elution buffer (Ambion) was used as blank.

2.3.3 cDNA synthesis

RNA is not suitable as target for DNA polymerase and must be reversely transcribed to complementary DNA (cDNA) before it can be analysed with RT-qPCR. To reverse transcribe the isolated total RNA, High-Capacity cDNA reverse Transcription Kit (Applied Biosystems) was used in accordance with the manufacturer's protocol. The isolated RNA samples were thawed on ice and heated at 60 °C for 5 minutes to minimize secondary structures in the RNA. In brief, reaction volumes of 25 µl contained 200ng RNA, 2.5 µl 10x Reverse Transcription buffer, 1 µl 25x dNTPs, 2.5 µl 10x Random Primer, 1 µl Oligo d(T), 1.25 µl Multiscribe Reverse Transcriptase and 1.75 µl Nuclease free water (Ambion). The reaction was done in 96-well plates (Bioplastics).The plate was carefully mixed, briefly centrifuged and placed in the PCR machine 2720 Thermal cycler (Applied Biosystems) using the following cycle parameters; denaturation for 10 minutes at 25 °C, annealing for 120 minutes at 37 °C and elongation at 85 °C for 5minutes before the temperature decreased to 4 °C . The newly synthetized cDNA was diluted

1:8 in nuclease-free water (Ambion) and used as a stock solution. An aliquot of the stock-solution was additionally diluted 1:40 for use in the RT-qPCR analyses. The stock-solutions and aliquoted user-solutions were stored at -20 °C until further use.

2.3.4 RT-qPCR

Quantitative RT-PCR was used to study relative differences in gene expressions of central stress related genes. RT-qPCR assays of the HPI-axis genes and HSP genes were established by Hanne Johnsen. All primer pairs gave single distinctive melting peaks verifying the absence of primer dimers and other unwanted amplification products. The amplification efficiency of each primer pair were calculated using a 2-fold dilution series with 11 dilutions, starting with cDNA diluted 1:10 from Larvae at 900 day degrees in agreement with the following equation: Primer efficiency (E) was calculated following the equation $E = 10^{(-1/slople)}$ (Pfaffl, 2001) Quantitative RT-PCR was conducted using the 7900HT Fast Real-Time PCR system (Applied biosystems). The RT-qPCR was run in duplicates with each reaction containing 10µL Power SYBR Green Master Mix (Life Technologies), 1.2 µL (300 nM) of each primer, 0.6 µL nuclease free water (Ambion) and 7µL diluted cDNA to a final concentration of 20 µL. Two different controls were included in the RT-qPCR setup for each primer pair and plate; a no template control using nuclease free water (Ambion) as template instead of cDNA, and a positive control where a pre-made standard pool of cDNA was used as template. Additionally, random DNase treated RNA samples were used as templates to test for possible genomic contamination. When ready, the 384-well plate (Applied Biosystems) was covered with MicroAmp Optical Adhesive Film (Applied Biosystems) and briefly centrifuged in a Jouan RC 10.22. A template-file was made using the SDS 2.3 software (Applied Biosystems), and a PCR-program with the following cycling parameters were initiated: denaturation at 90 °C for 10 minutes, 40 cycles of denaturation at 95 °C for 15 seconds, annealing and elongation at 60 °C for 1 minute, and one cycle of denaturation at 95 °C for 15 seconds, annealing and elongation at 60 °C for 15 seconds, followed by denaturation at 95 °C for 15 seconds.

2.4 Data analyses and statistics

The SDS 2.3 software (Applied Biosystems) collected all results from RT-qPCR and the threshold was adjusted manually to 0.1. The dissociation and amplification curve for each amplicon was checked. Further analyses were processed in Microsoft Excel. The Pfaffl-method

was used to calculate the relative expression of the genes studied (Equation 1). This method takes into account the efficiency of the primers, in contrast to the ΔΔCt-method, which assumes that all primers are 100% effective (Pfaffl, 2001). The geometric mean of the three housekeeping genes (*ef-1-α*, *ß-actin* and *18S rRNA*) was used as reference genes in the Pfaffl-method to normalize experimental variation.

Equation 1:

$$Ratio = \frac{(E_{target})^{\Delta Ct\ target\ (control-treated)}}{(E_{ref})^{\Delta Ct\ ref\ (control-treated)}}$$

Statistics were conducted in IBM SPSS statistics 23 and all graphs were made in GraphPad Prism (version 6.07 for Windows, GraphPad Prism Software, Inc). All data, both replicates and groups, were subjected to a normality test; Shapiro-Wilk test. Replicates showed that one of the two replicates in almost all groups failed to be normally distributed. Because of this, and the small replicate sizes (n=5), a non-parametric test called, Kolmogorov Smirnov test, was used to test if the duplicates within groups could be merged. The replicates were merged into their respective groups and thereby becoming a sample size of n=10. The groups (n=10) of all the genes at all time points were tested for normality by the same test as the replicates, showing that 91 % of the groups were normally distributed. Data that failed to be normality distributed showed no trends in other distributions (e.g. Bimodal), and no trend in skewness. Since analysis of variance (ANOVA) has shown to be a robust test even with samples that have minor deviations from a normally distributed curve, and since the percentage of un-normal distributed groups were so small, one way ANOVA was used to check for significant differences between groups (Field, A. 2013). Games-Howell was used as a post-hoc test on groups that fails the Levine's test of variance, and Gabriel's procedure was used as a post-hoc test on groups that consisted Levine's test of variance. The level of statistical significance for all tests was set at $p < 0.05$. An example from the statistical method conducted on the control group of one of the genes (*crf1*) are shown in Appendix III.

3 Results

Expression levels of central HPI-axis genes (*crf1, crf2, pomcA1, pomcA2, pomcB, gr1, gr2* and *mr*) in addition to two HSPs (*hsp70a* and *hsp90a4*) were examined in embryos and larvae of Atlantic salmon, subjected to handling; shocking and transport. The study was conducted to evaluate if stress during early development of fish alters the gene expression of the mentioned genes. In order to differentiate between the treatments and the group names, abbreviations were used for the three group names; shock (S), transport (T) and shock & transport (ST).

3.1 Hatching, mortality and larval growth

A hatching profile was made by counting the un-hatched eggs of all four groups, once a day during the hatching period (Figure 11). The results showed a small difference between the groups, where 50% of the eggs in group S were hatched 1-2 days before the other three groups. At the end of the hatching period, all groups showed a similar number of total hatched larvae.

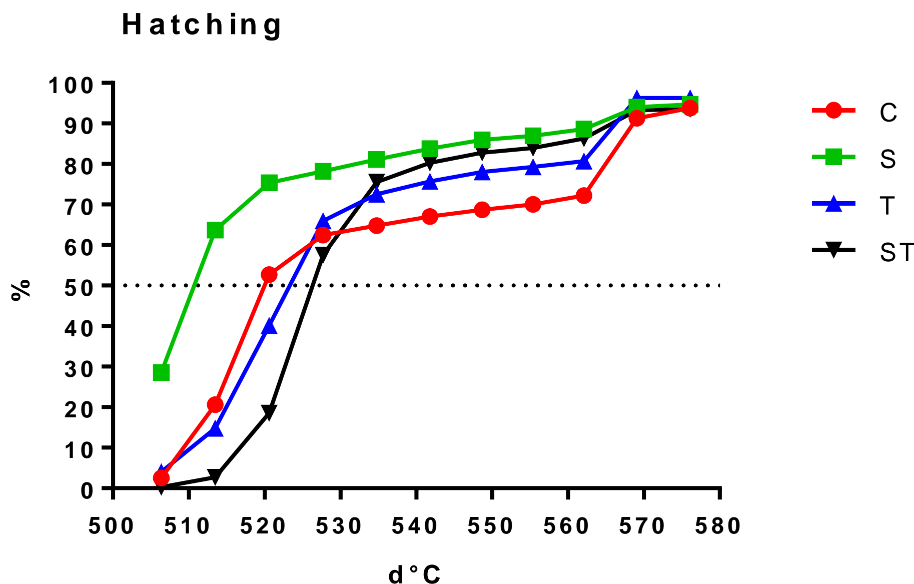

Figure 11: **Hatching (%) of Atlantic salmon eggs during normal development, and after exposure to shock and/or transport.** Abbreviations for the four groups: C = control, S = shock, T = transport and ST = shock & transport.

Dead eggs were removed from the hatching column once a day, and the amount was registered (Figure 12). Group S and ST showed a rapid increasing amount of dead eggs after shocking. Group T showed a rapid, but lower increase of dead eggs after transport. After hatching, the un-hatched eggs were removed, which led to a similar total amount of dead eggs in all four groups.

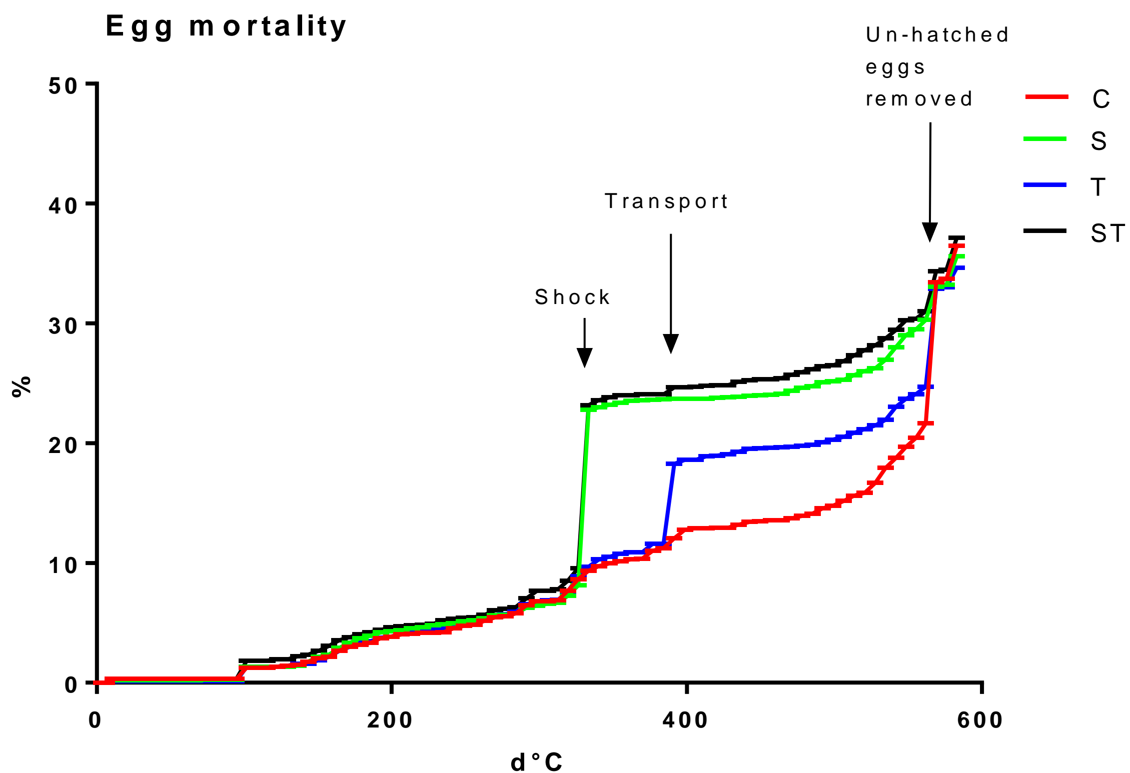

Figure 12: **Mortality (%) of Atlantic salmon eggs during normal development, and after exposure to shock and/or transport.** Abbreviations for the four groups: C = control, S = shock, T = transport and ST = shock & transport.

Yolk sac larvae was weighed at three time points; after hatching (583d°C), between hatching and start feeding (688°C) and at the time of start feeding (918d°C). The results showed a similar increase during development in all four groups (Figure 13).

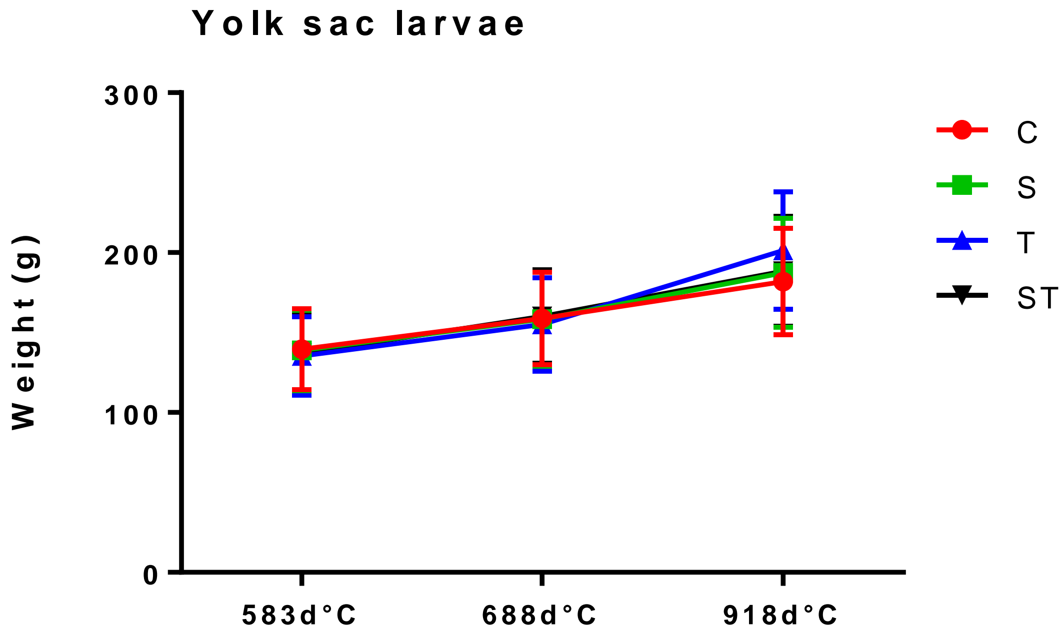

Figure 13: **Weight (g) of Atlantic salmon larvaes at 583d°, 688 d°C and 918 d°C.** Abbreviations for the four groups: C = control, S = shock, T = transport and ST = shock & transport. (n=28-30).

3.2 Ontogeny and long term treatment effects

Eggs and larvae collected previous to shocking (326d°C), transportation (377d°C) and stress challenge (918d°C; start feeding), in addition to newly hatched larvae (583d°C), were examined to study the ontogeny of different genes important in the stress response, and to examine possible long-term effect after exposure to shocking and/or transport. All genes assessed in the study (*crf1, crf2, pomcA1, pomcA2, pomcB, gr1, gr2, mr, hsp70a* and *hsp90a4*), were expressed at all studied developmental stages. The results of the examined genes will be shown in detail in the next sections.

3.2.1 Ontogeny of the HPI-axis genes and long term treatment effects

Samples from the control group (C) at the different time points were used to examine the normal ontogeny of the genes included in this study. The results showing the normal ontogeny of the genes are shown in the following figures; *crf1* and *crf2* (Figure 14), *pomcA1, pomcA2* and *pomcB* (Figure 15), and *gr1, gr2* and *mr* (Figure 16). All genes in the control group showed an increase during development, with significantly higher gene expression levels in larvae than in embryos. The two *gr*'s however, showed a significant decrease during the embryogenesis before an increase at the start of the larval period (Figure 16).

Both paralogues, *crf1* and *crf2* (Figure 14), showed a similar expression pattern in the embryos followed by a significant increase after hatching. Further, *crf1*, in contrast to an increasing *crf2,* showed a significant decrease in larvae at the time of start feeding. The groups S, T and ST showed a similar profile as the control group, with a significant increase of both *crf1* and *crf2* after hatching, and a steady or increased expression in larvae at start feeding. When it comes to any long-term effect of the different treatments, a significantly lower expression of *crf1* was detected in group T compared to the control group in newly hatched larvae. This difference, however, was also shown in the embryo prior to transport, of which T (untreated) was expected to be equal to the control group. Transcripts of *crf2* in start feeding larvae were lower in group S and ST compared to the control group. The level of expression of *Crf2* was lower compared to *crf1* throughout the development.

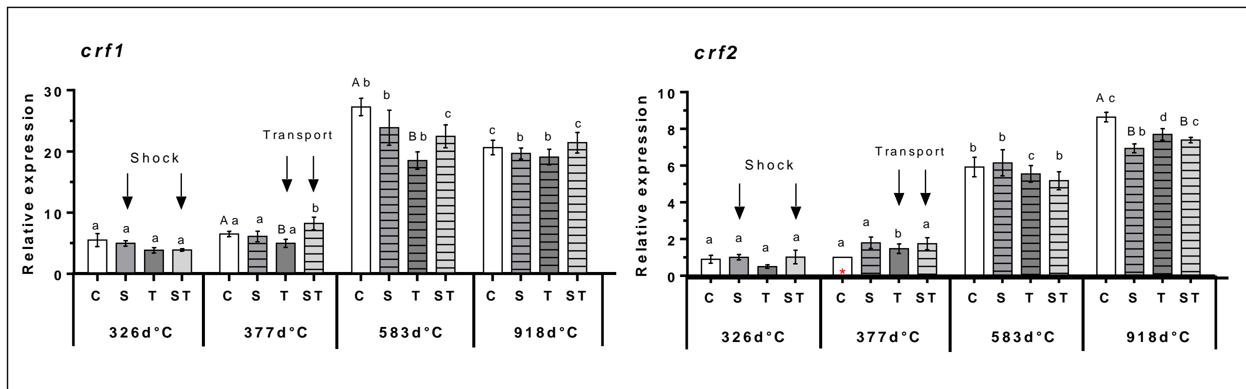

Figure 14: **Relative expression levels of genes *crf1* and *crf2* in eggs and larvae of Atlantic salmon prior to shocking (326 d°C), prior to transport (377d°C), at hatching (583d°C) and at start feeding (918d°C). Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions were measured by quantitative RT-PCR, normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression*,* and calibrated to the lowest expression of *crf2* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 6-10 individuals ± SEM. Arrows indicate in which groups and at what day degrees (d°C) shocking and transport happened, and columns with pattern are groups that have been treated. Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

In the control group, the expression levels of *pomcA1* significantly increased throughout development (Figure 15). *PomcA2* showed a similar increase in embryos prior to transport, while *pomcB* showed first a decrease in embryos prior to transport before an increase after hatching, which was steady until start feeding. A similar profile was seen in the three groups S, T and ST, except for the decrease of *pomcB.*

When it comes to any long-term effect of the different treatments, there was a significant lower expression level of *pomcA1* in group T in larvae at start feeding, compared to the control group. After hatching group T showed a lower expression level of *pomcA2* than group S. Group S also had a higher expression level/value of *pomcB* compared to control, in embryos at 377d°C. Both *pomcA2* and *pomcB* showed a much lower expression level than *pomcA1* from 377d°C and throughout the development.

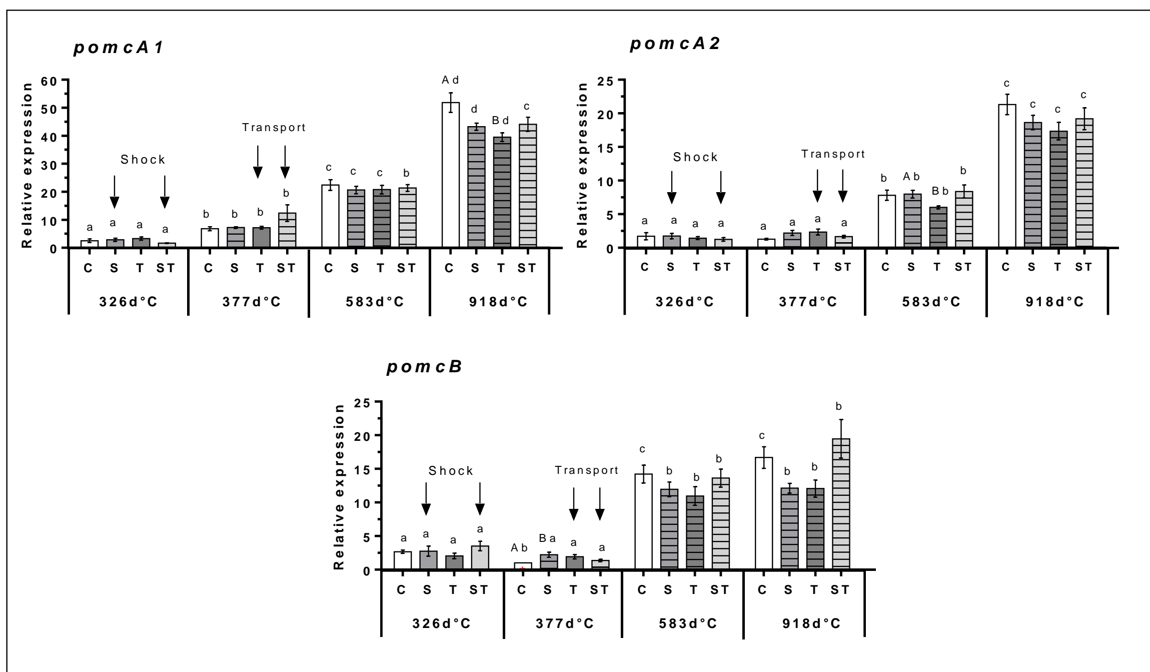

Figure 15: **Relative expression of the genes *pomcA1, pomcA2* and *pomcB* in eggs and larvae of Atlantic salmon prior to shocking (326 d°C), prior to transport (377d°C), at hatching (583d°C) and at start feeding (918d°C). Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions were measured by quantitative RT-PCR, normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *pomcB* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 6-10 individuals ± SEM. Arrows indicate in which groups and at what day degrees (d°C) shocking and transport happened, and columns with pattern are groups that have been treated. Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

As previously mentioned, transcripts of *gr1* and *gr2* (Figure 16) in the control group showed a decrease in embryos prior to transport, followed by an increase after hatching and start feeding larvae. The expression of *gr1* in larvae at start feeding was significantly higher than in embryos at prior to shocking, while the expression of *gr2* had no significant difference at these two time points. A similar pattern was shown for group S, T and ST between the embryos prior to shocking and the larvae at start feeding. In contrast to the control, which had a decrease of gene expressions in embryos prior to transport, group S and T had an increase. Expression of *gr1* in embryos at prior to shocking was significantly different between group T (untreated) and the control group, which were expected to be similar at this stage. In embryos prior to transport, the groups S and ST showed a significantly higher expression of *gr1* than the control group. However, group T (untreated), also showed a significant increase at this time point. Group S and T showed a significant lower *gr1* expression level than the control group after hatching. In embryos prior to transport, both groups S and ST had a significantly higher expression of *gr2* compared to the control group, and the *gr2* expression level of S was significantly higher than ST. However, also here a significant difference was detected between group T (untreated) and the control group at the same time point, of which they were expected to be similar.

The expression of *mr* (Figure 16) in the control group showed a significant increase after hatching and start feeding . The same trend was also shown in group ST, while group S and T showed a first significant increase in embryo prior to transport followed by a second increase in larvae at start feeding. An increase was shown in groups S and ST prior to transport, compared to the control group. However, the group T (untreated) was significantly higher than the control. After hatching the now treated group T was significantly lower than the control group.

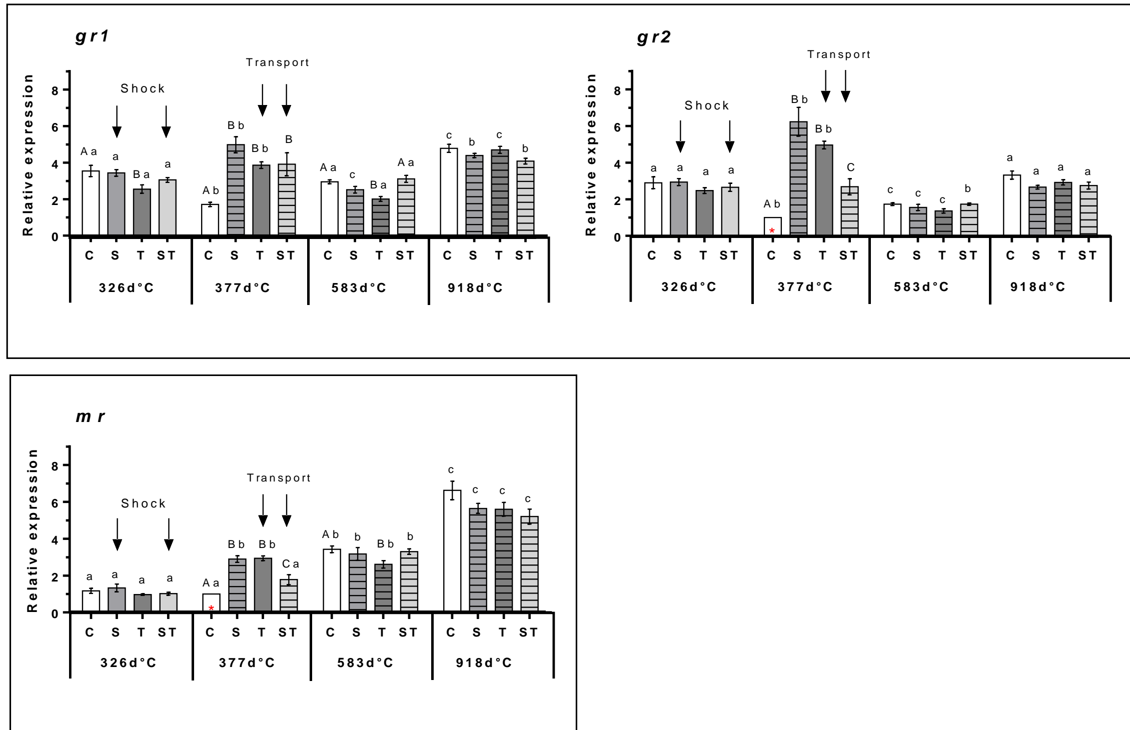

Figure 16: **Relative expression of the genes *gr1, gr2* and *mr* in eggs and larvae of Atlantic salmon prior to shocking (326 d°C), prior to transport (377d°C), at hatching (583d°C) and at start feeding (918d°C). Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *gr2 (*for *gr1* and *gr2) and mr* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 7-10 individuals ± SEM. Arrows indicate in which groups and at what day degrees (d°C) shocking and transport happened, and columns with pattern are groups that have been treated. Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

3.2.2 Ontogeny and long term treatment effects of HSP genes

Transcripts of *hsp70a* (Figure 17) in the control group showed a significant decrease in embryos prior to transport, followed by an significant increase in hatched larvae , and a significant decrease in start feeding larvae . *Hsp90a4* followed the same pattern, except that the embryos showed no significant difference in expression between embryos prior to shocking and transport. The three other groups S, T and ST also showed a significant decrease in expression levels of both *hsp70a* and *hsp90a4* in start feeding larvae, compared to the expression levels found in embryos and hatched larvae. All groups also showed a gradual increase of *hsp70a* from prior to shocking until after hatching, however, this was only significant in group ST.

The embryos in the shocked group S, showed a transient higher expression of *hsp70a* prior to transport, compared to the control group. In the untreated embryos prior to shocking there was a significant lower *hsp70a* expression in group ST compared to the control group. This was, however, temporarily, as the same group, now shocked, was similar to the control group prior to transport. No significant differences were found between the groups in expression levels of hsp90a4.

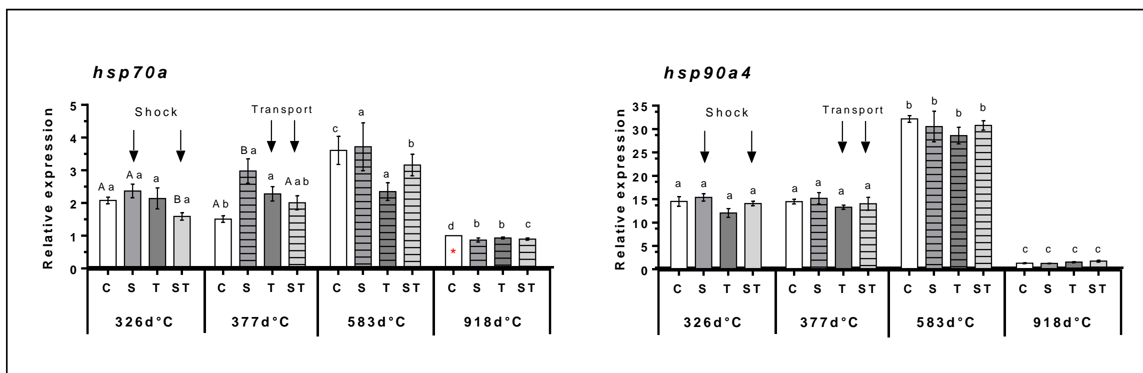

Figure 17: : **Relative expression of the gene *hsp70a* and *hsp90a4* in eggs and larvae of Atlantic salmon prior to shocking (326 d°C), prior to transport (377d°C), at hatching (583d°C) and at start feeding (918d°C). Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *hsp70a* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 8-10 individuals ± SEM. Arrows indicate in which groups and at what day degrees (d°C) shocking and transport happened, and columns with pattern are groups that have been treated. Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

3.3 Shocking

Embryos were collected before (0h), 1 hour, 3 hours and 26 hours post shocking (hps),to examine the response of important genes involved in HPI axis.

3.3.1 Influence of shocking on HPI axis genes

In general, the HPI-axis genes did not show any long-term (26hps) significant differences after treatment. Since group T was untreated at this time point, both the control group and group T should function as controls. However, they did show different levels of expression at several time points in the genes examined. This difference was temporal significant in *crf1* 1hps and 3hps, and *gr1* at 0hps and 1hps.

The *crf1* expression levels in group S (Figure 18) showed a temporal significant increase at 3hps. Group T (untreated) showed a significant increase 26hps. Between the groups there was only a significant lower level of *crf1* expression in group S 1hps, compared to the control group, if the differences between the two untreated groups were disregarded. The transcripts of *crf2* were generally lower than the *crf1* transcripts.

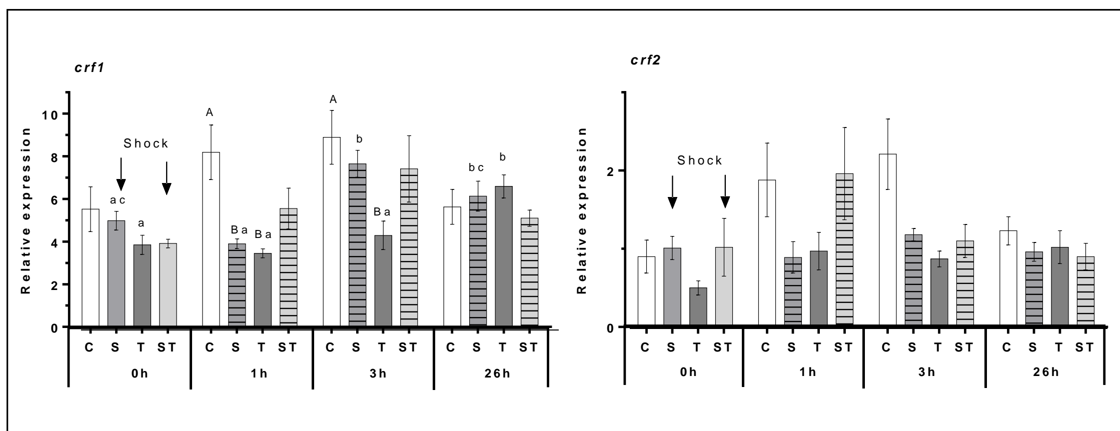

Figure 18: **Relative expression of the genes *crf1* and *crf2* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 26 hours post shock treatment. Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *crf2* in the control group, 377d°C. Each column is presented as mean of 6-10 individuals ± SEM. The arrows shows which groups that are shocked and columns with pattern are treated groups. Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

Expression levels of *pomcA1* (Figure 19) in the shocked group ST showed a long-term significant increase at 1hps that lasted until 26hps, while the shocked group S showed a significant increase 26hps. Group T (untreated) also showed a significant increase at 26hps. The expression level of *pomcA1* in group S at 1hps was significantly higher than in group T (untreated). At 1hps, the average expression level of *pomcA2* in group ST was found increased compared to the level at 0hps, however this difference was not significant. Also here there was a significant increase in group T (untreated) 24hps. Between groups, the only significance was between T (untreated) and ST at 3hps. The expression of *pomcB* showed no significant differences within all groups, and between groups.

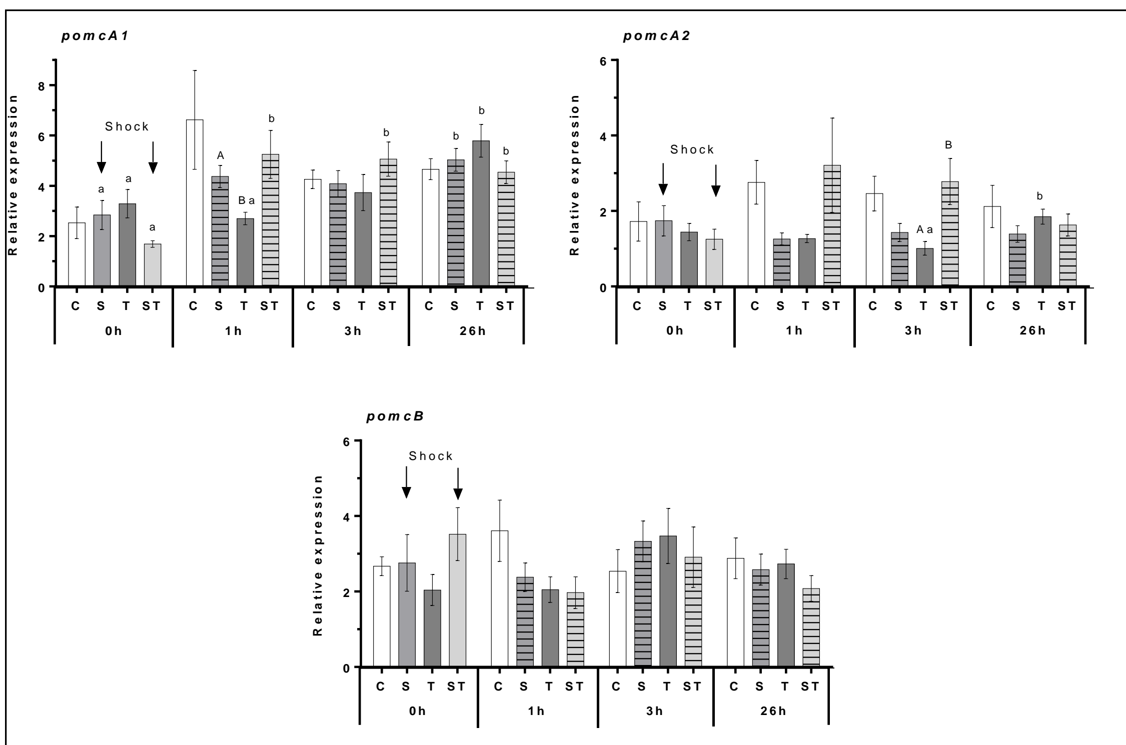

Figure 19: **Relative expression of the genes *pomcA1, pomcA2* and *pomcB* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 26 hours post shock treatment. Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *pomcB* in the control group, 377d°C. Each column is presented as mean of 6-10 individuals ± SEM. The arrows shows which groups that are shocked and columns with pattern are treated groups. Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

An increase in *gr1* (Figure 20) expression level was found in the untreated group T at 3hps that lasted until 26hps. Group S also showed an increase of *gr1* at 3hps. No significant differences were found between treated and both untreated groups. Transcripts of *gr2,* showed an increase at 3hps, which was steady until 26hps in all groups. Between treated and untreated groups, a significant difference was shown 3hps between group T (untreated) and the two groups S and ST.

All groups showed a similar *mr* expression pattern as the *gr2* transcripts, with a significant increase at 3hps that was steady until 26hps. A significant difference between ST and the control groups, was shown 1hps.

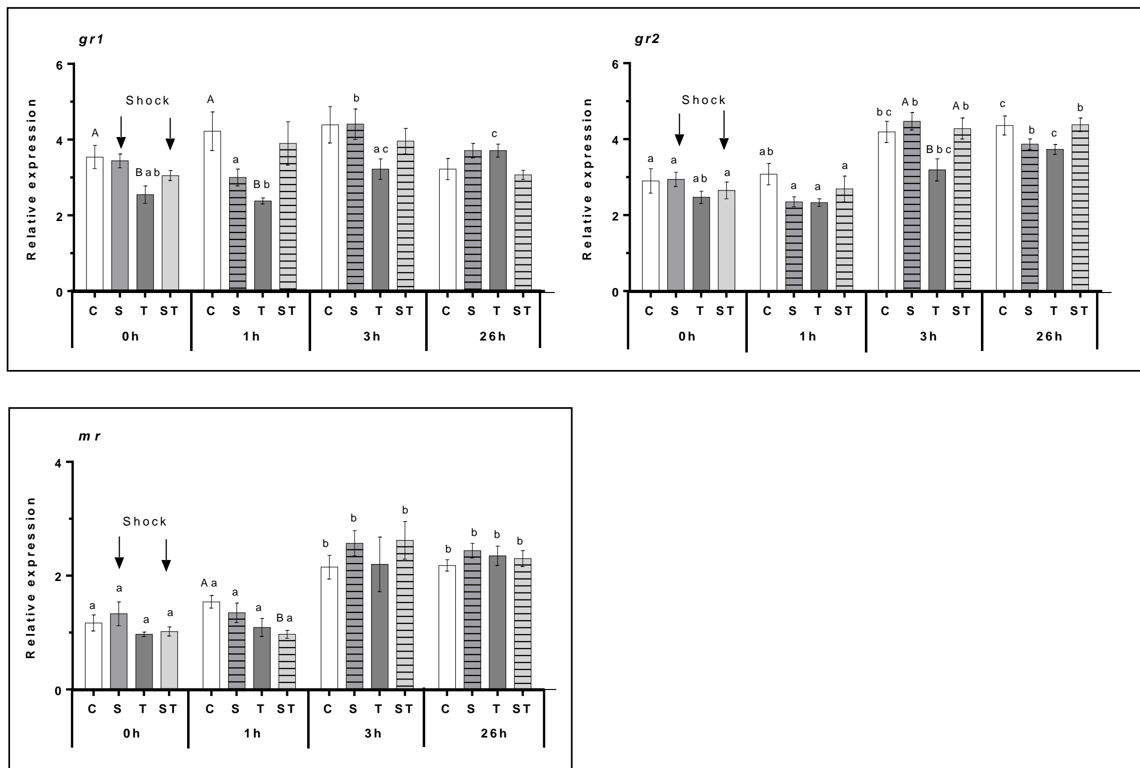

Figure 20: **Relative expression of the genes *gr1, gr2* and *mr* in eggs and larvae of Atlantic salmon before treatments (0h), 1 hour, 3 hours and 26 hours post shock treatment. Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *gr2 (*for *gr1* and *gr2), and mr* in the control group, 377d°C. Each column is presented as mean of 7-10 individuals $\pm$ SEM. The arrows shows which groups that are shocked and columns with pattern are treated groups. Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

3.3.2 Influence of shock on HSPs genes

In general, there was no evident effect on the gene expression of the two HSPs after the shock treatment (Figure 21). The two shocked groups did not have significantly different gene expressions compared to both the untreated groups simultaneously. The shocked groups S and ST, and the untreated group T, all showed a significantly increase in *hsp70a* expression at 3hps compared to 1hps (S and T) and before shocking (ST). However, this increase was not significant in the control group. An increase was also shown 26hps for *hsp90a4* expression in the group T (untreated), while the opposite was shown in the shocked group ST, compared to before shocking (0h).

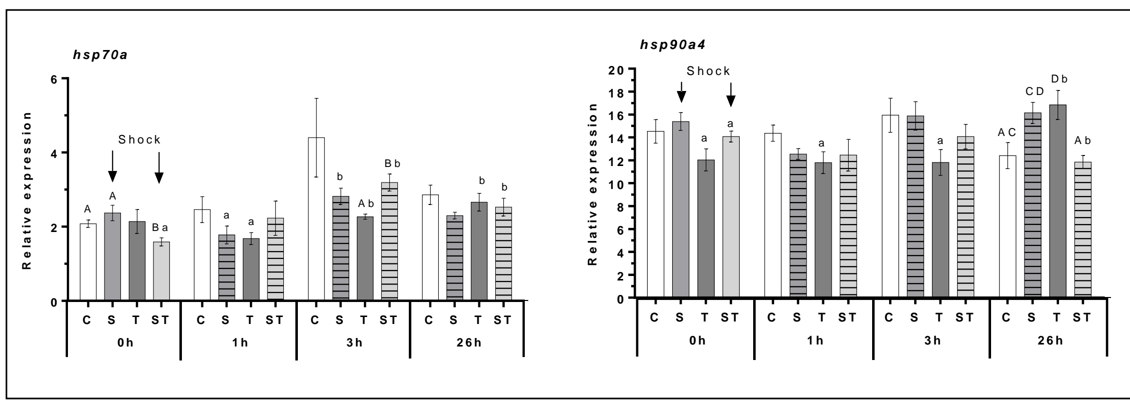

Figure 21: **Relative expression of the gene *hsp70a* and *hsp90a4* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 26 hours post shock treatment. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR compared to a calibrator and normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *hsp70a* in the control group, 918d°C. Each column is presented as mean of 6-10 individuals ± SEM. The arrows shows which groups that are shocked and columns with pattern are treated groups. Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

3.4 Transport

Eggs collected before (0h), 1 hour, 3 hours and 26 hours post transport (hpt) were examined, in order to study the response of genes associated with a stress response. It is important to stress that the transport lasted for 48 hours. The abundance of genes was measured by quantitative RT-PCR and the results of relative expression were plotted against the different time points in a bar chart. At current time point (377d°C), group S and ST had already been shocked.

3.4.1 Influence of transport on HPI axis genes

In general, most of the HPI-axis genes did not show any long-term (26hpt) significant differences after transport. The control group had a gradual increase that was significant in transcripts of all genes, except for *pomcB.* Group T was, as the control group, untreated before transport (0h). However, between the groups, *gr1, gr2* and *mr* showed significant different levels of gene expression.

Transcripts of both *crf1* and *crf2* (Figure 22) showed a significant decrease 1hpt in the shock treated group ST, followed by an increase in expression that was significant for *crf2* 29hpt. Between groups, the shock treated group S, showed a temporal significantly higher expression of *crf1* than the untreated group T before transport (0h). At 3hpt the now transported group T showed a transient significantly lower expression of *crf2* than the control group, and the same was also shown for *crf1* 29hpt. The transcripts of *crf2* were lower than *crf1*.

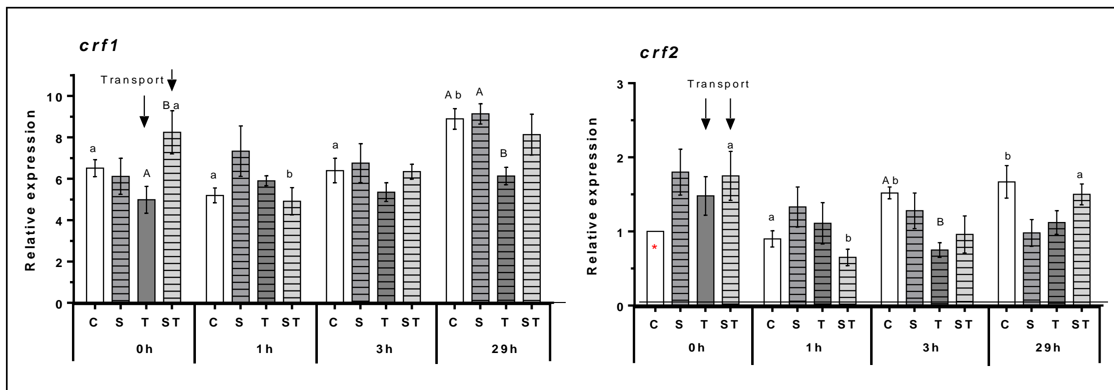

Figure 22: **Relative expression of the genes *crf1* and *crf2* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 29 hours post transport. Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression*,* and calibrated to the lowest expression of *crf2* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 7-10 individuals ± SEM. The arrows shows which groups that are transported, and columns with pattern represents groups that have been through a treatment (shock and/or transport). Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

Transcripts of both *pomcA1* and *pomcA2* (Figure 23) showed significant differences between time points within group ST. The transcripts of *pomcA1* showed a temporal, though not significant, decrease at 1hpt, followed by a significant increase 3hpt. An increase was also shown in the same group of *pomcA2* 3hpt, followed by a decrease 29hpt. The opposite was shown for *pomcB,* where the expression level in group ST increased between 3hpt and 29hpt. In addition, the not transported group S, showed a significant decrease of *pomcB* expression 1hpt, that also was significant 3hpt. Before transport (0h), a significant difference in *pomcB* expression was shown between control and the shocked group S. Of the transported groups, group T showed a temporal significantly lower expression of *pomcA1* compared to the control group at 3hpt. Both group T and ST showed a significantly lower expression of *pomcA2* 29hpt compared to the control group. *PomcA2* and *pomcB* were expressed lower compared to *pomcA1*.

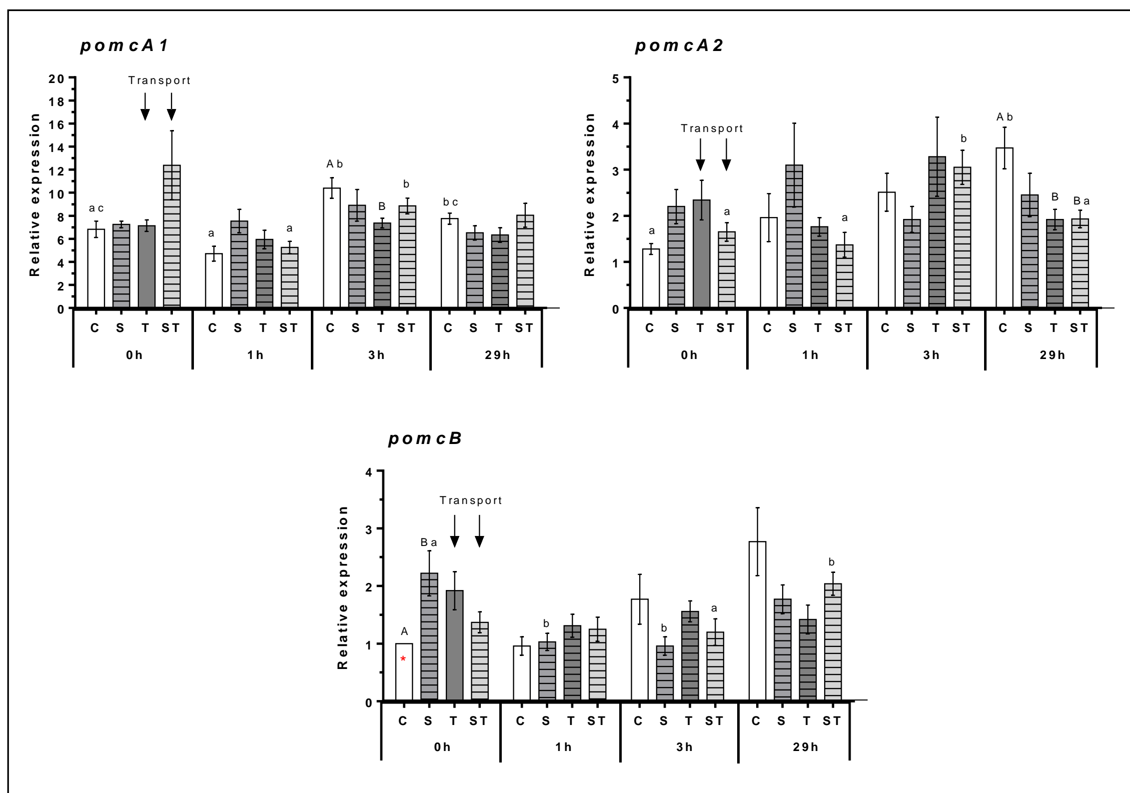

Figure 23: **Relative expression of the genes *pomcA1, pomcA2* and *pomcB* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 29 hours post transport. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression*,* and calibrated to the lowest expression of *pomcB* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 7-10 individuals $\pm$ SEM. The arrows shows which groups that are transported, and columns with pattern represents groups that have been through a treatment (shock and/or transport). Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

As a general pattern of the *gr1* (Figure 24), the three groups S, T and ST showed a significant decrease in gene expression 1hpt, which remained decreased until 29hpt. Only group ST had a minor increase of *gr1* at 3hpt and 29hpt. A similar pattern was shown for *gr2* expression, which in groups S and T decreased at 1hpt. The expression level of *gr2* in group T decreased 3hpt which remained decreased until 29hpt. Group ST showed a significant decrease 29hpt compared to before transport. Before transport (0h), both *gr's* showed a higher expression in groups S, T (untreated) and ST compared to the control group. A higher expression of *gr1* was also shown in the now transported group T 1hpt, while at 29hpt group T was significantly lower than the control group. Group T also showed a decrease of *crf2* expression at 3hpt compared to 1hpt and 0hpt.

Transcript of *mr* showed the same pattern as for the *gr1* and *gr2,* with a decrease of expression 1hpt in groups S and T, which was steady until 29hpt. Before transport, the expression pattern was similar to *gr2*, and the pattern 1hpt between control and group T was similar to that explained for the *gr1* expression.

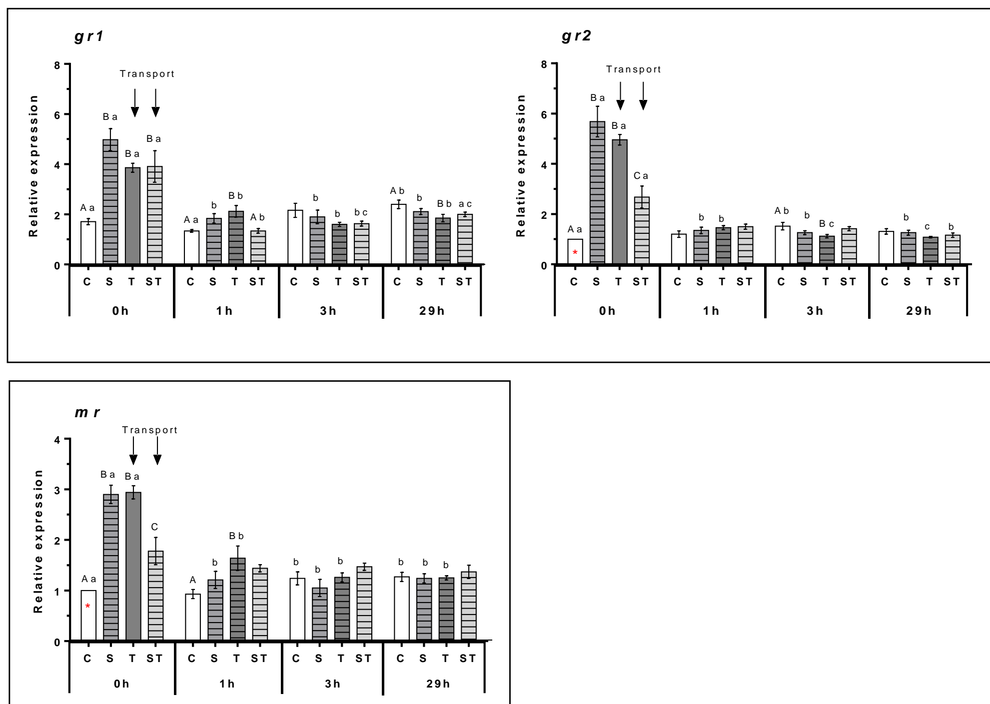

Figure 24: **Relative expression of the genes *gr1, gr2* and *mr* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 29 hours post transport. Abbreviations for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression*,* and calibrated to the lowest expression of *gr2 (*for *gr1* and *gr2) and mr* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 7-10 individuals ± SEM. The arrows shows which groups that are transported, and columns with pattern represents groups that have been through a treatment (shock and/or transport). Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

3.4.2 Influence of transport on HSP genes

Both HSP genes showed an increase after the transportation (Figure 25). *Hsp70a* expression showed a significantly increase 1hpt in the two transported groups S and T, which was transiently and at 29hpt had decreased to the same level as before transport. This increase was significantly higher than in the two groups that were not transported. The same was shown for the expression of *hsp90a4,* where the both transported groups had an increase, only group ST was a bit more delayed. In contrast to *hsp70a* expression, the *hsp90a4* expression remained elevated 29hpt. The control group showed a significant increase of both *hsp70a* and *hsp90a* expression levels at 29hpt, but *hsp90a* was significantly lower than group T at 1hpt, both group T and ST at 3hpt and group ST at 29hpt.

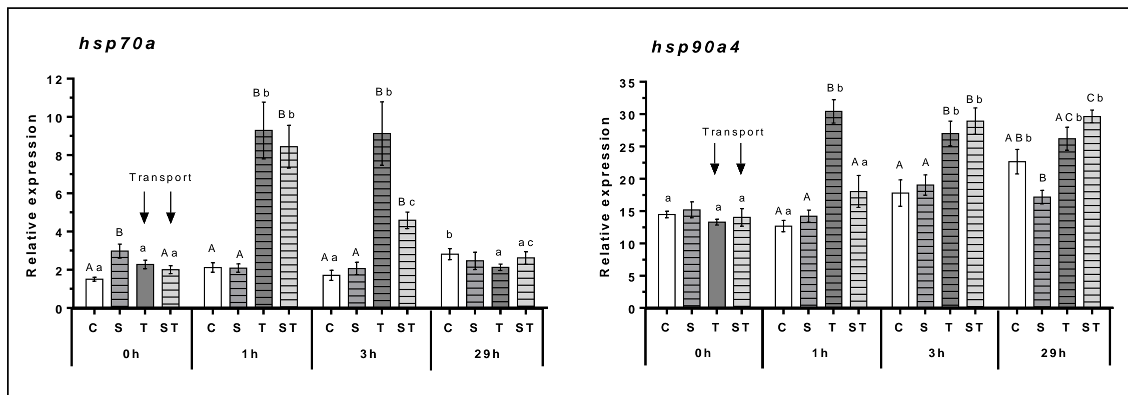

Figure 25: **Relative expression of the gene *hsp*70a and *hsp*90a4 in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 29 hours post transport. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression*,* and calibrated to the lowest expression of *hsp70a* in the control group, 918d°C. Each column is presented as mean of 7-10 individuals ± SEM. The arrows shows which groups that are transported, and columns with pattern represents groups that have been through a treatment (shock and/or transport). Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

3.5 Stress challenge

Larvae were collected before (0h), 1 hour, 3 hours and 24 hours after a stress challenge conducted on all four groups, to examine a possible effect of the treatment and possible long-term effects of previous treatments. The control group was also stress challenged.

3.5.1 Influence of stress on HPI axis genes

In general, the control group showed the highest increase in expression of *crf1, crf2, gr2* and *mr* after the stress challenge, and both the control group and group ST showed highest increase in expression of *pomcA1, pomcA2* and *pomcB.*

The treated control group was the only group that showed any significant difference between examined time points for both *crf1* and *crf2* (Figure 26). The expression level of crf1 in the control group was significantly increased after 24h, and significantly higher than groups S and T at 24h. The control group showed an increase of *crf2* after 1h, followed by a significant decrease after 3h, and then a significant increase after 24h. After 24h the control group was significantly higher than group T. Before the stress challenge (0h) *crf2* expression level was significant lower in group S and ST compared to the control.

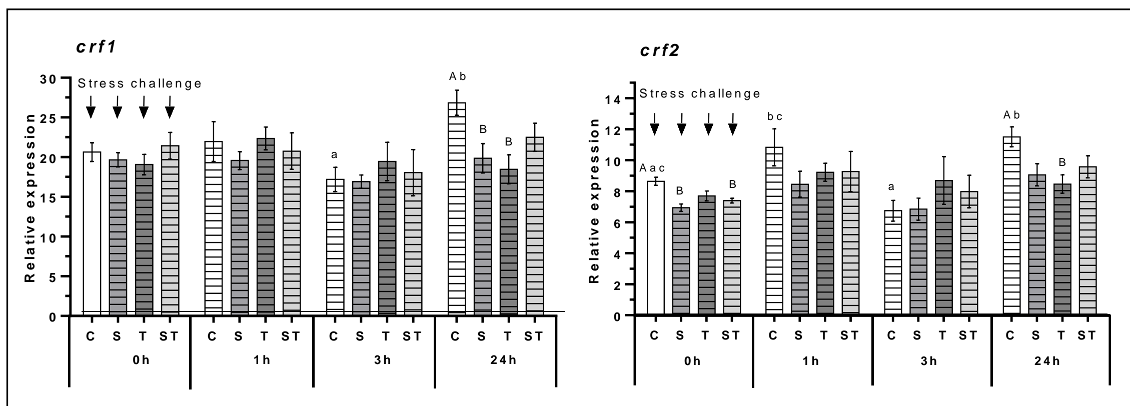

Figure 26: **Relative expression of the gene *crf1* and *crf2* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 24 hours after a stress challenge. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *crf2* in the control group, 377d°C. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 8-10 individuals ± SEM. The arrows shows all groups that are stress challenged, and columns with pattern represents groups that have been through a treatment (shock, transport and/or stress challenge). Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

Expression levels of the *pomc's* (Figure 27) showed a similar pattern from 0h to 24h, in both the control group and ST, and they both had a general higher expression levels than group S and T. Over time, *pomcA1* generally showed an increase in gene expression, but this was only significant in group ST, and between 0h and 3h in group T. *PomcA2* showed no significant expression difference within the groups. *PomcB* expression showed a gradually increase in all groups, which was statistically significant in both the treated control group (24h), and the group S (3h). General expression of *PomcA2* and *PomcB* was lower than *PomcA1*.

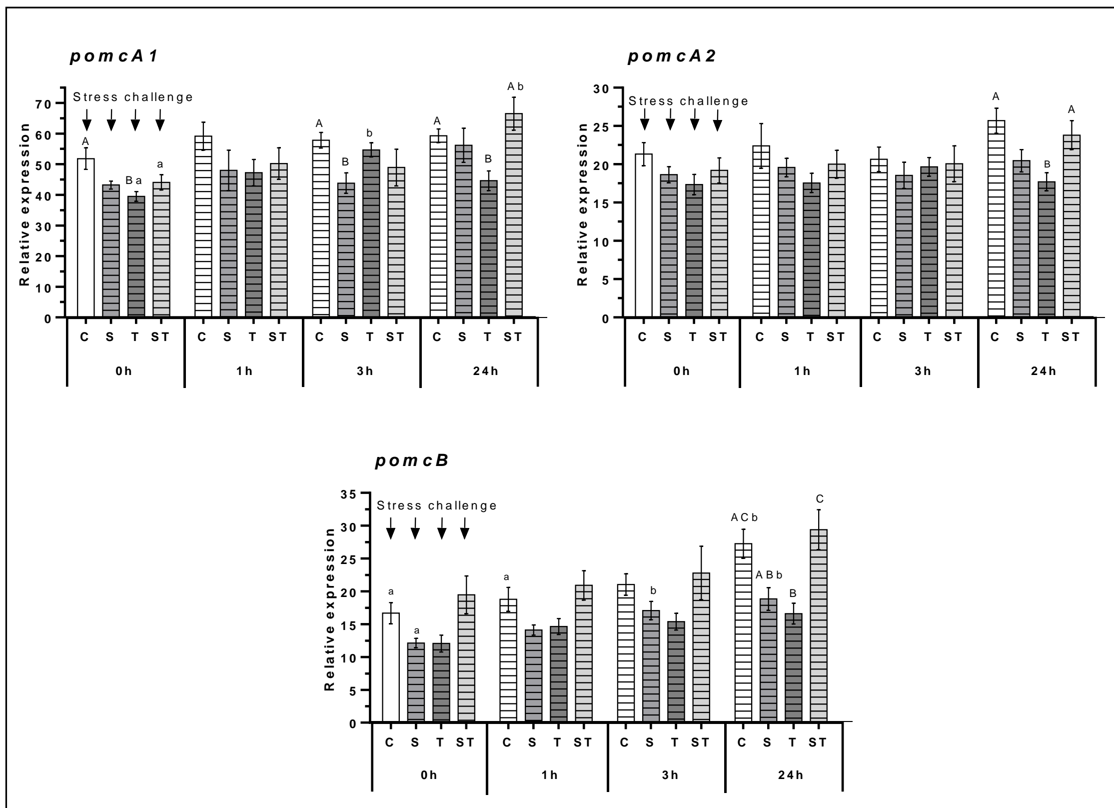

Figure 27: **Relative expression of the genes *pomcA1*, *pomcA2* and *pomcB* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 24 hours after a stress challenge. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *pomcB* in the control group, 377d°C. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 8-10 individuals ± SEM. The arrows shows all groups that are stress challenged, and columns with pattern represents groups that have been through a treatment (shock, transport and/or stress challenge). Non-capital and capital letters indicate significant differences ($p<0.05$) within a group, or between groups at the same time point, respectively.

Expression of *gr1* (Figure 28) did not show any specific alteration, except for a significant decrease in group T 24h after the stress challenge, compared to 3h after the challenge. G*r2* showed an increase in expression in both the control group and group S 24h after the challenge. Expressions of *mr* showed an increase in the treated control group and group S at 1h and a significant increase in treated control, group S and group ST at 24h. Across the four groups, *gr1* showed no significant differences. Results for both *gr2* and *mr* showed that the treated control group after 24h had significant higher gene expression than the other groups at the same time point.

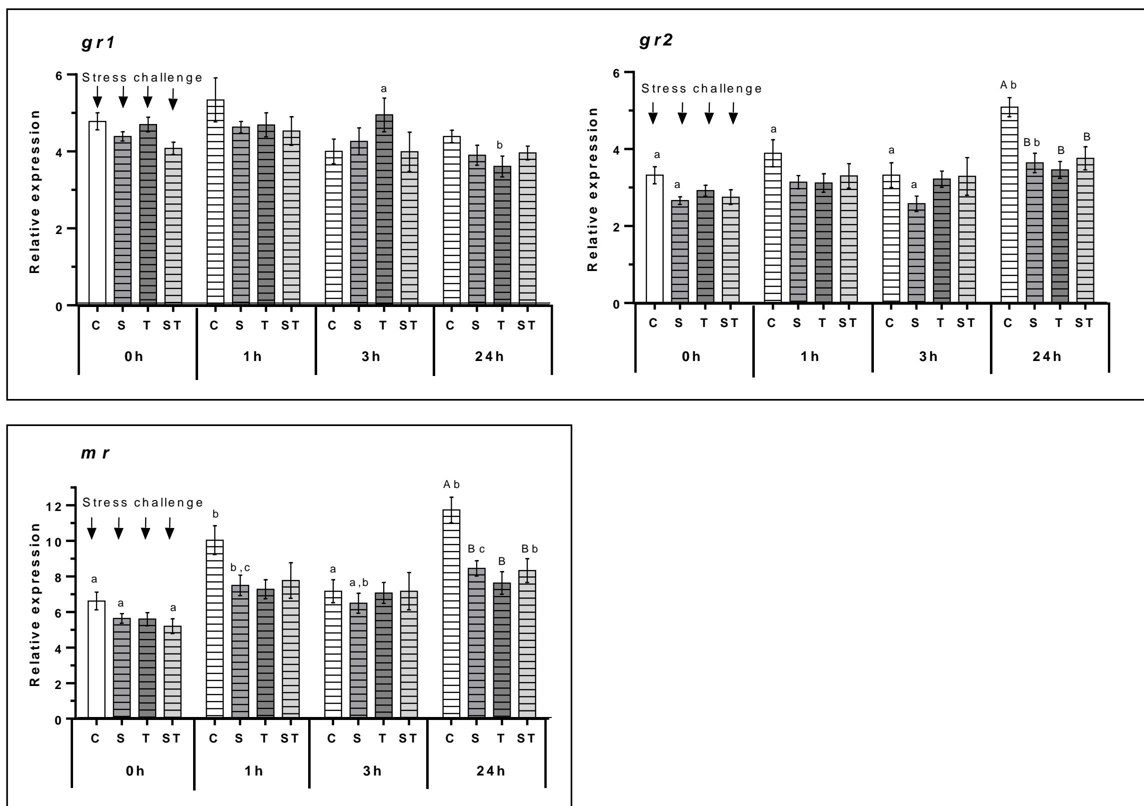

Figure 28: **Relative expression of the gene *gr1, gr2* and *mr* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 24 hours after a stress challenge. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression, and calibrated to the lowest expression of *gr2 (*for *gr1* and *gr2)* and *mr* in the control group, 377d°C. Each column is presented as mean of 8-10 individuals ± SEM. The arrows shows all groups that are stress challenged, and columns with pattern represents groups that have been through a treatment (shock, transport and/or stress challenge). Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

3.5.2 Influence of stress on HSP genes

A general trend of the HSP genes in all four groups (Figure 29) was a gradually increasing expression of *hsp70a*, with a significantly higher expression 24h after the stress challenge than before the challenge. The expression of *hsp90a4* was also generally higher in all groups 24h after the stress challenge than before (0h), but only group S showed a statistical significant difference between these two time points. Comparing the four groups, *hsp70a* showed a significant difference between the control group and group T, 1h and 24h after stress challenge. Group S was also significantly lower than the control group 24h after stress challenge.

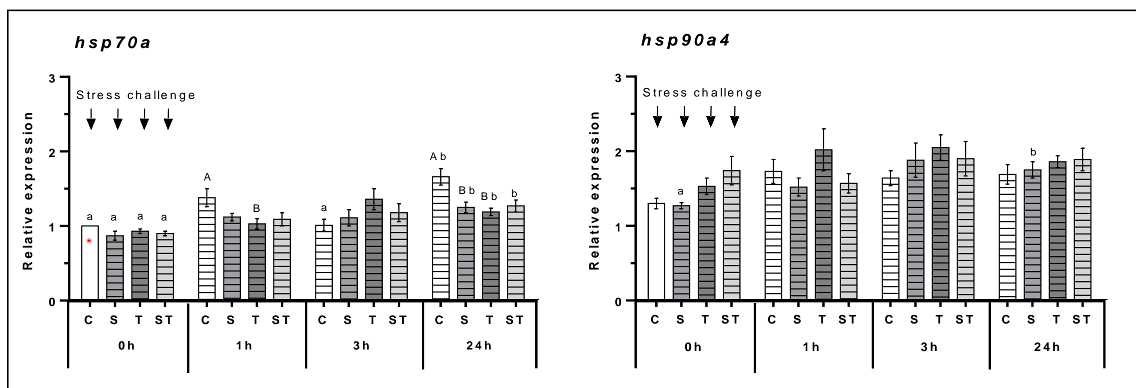

Figure 29: **Relative expression of the gene *hsp70a* and *hsp90a4* in eggs and larvae of Atlantic salmon before (0h), 1 hour, 3 hours and 24 hours after a stress challenge. Abbreviation for the four groups; C=control, S=shock, T=transport, ST=shock & transport.** The changes in relative gene expressions are measured by quantitative RT-PCR normalized to the geometric mean of *ef-1-α, ß-actin* and *18s rRNA* expression*,* and calibrated to the lowest expression of *hsp70a* in the control group. The calibrator (value 1) is marked with a red star. Each column is presented as mean of 8-10 individuals ± SEM. The arrows shows all groups that are stress challenged, and columns with pattern represents groups that have been through a treatment (shock, transport and/or stress challenge). Non-capital and capital letters indicate significant differences (p<0.05) within a group, or between groups at the same time point, respectively.

4 Discussion

The main objective of this study was to examine the influence of production related handling stress on acute and long-term expressions of HPI-axis and HSP genes in Atlantic salmon (*Salmo salar* L.) embryos and larvae. In general, the data obtained from the current study indicates that egg shocking do not result in any consistent acute changes in expression of the genes investigated. However, transport was found to initiate an acute upregulation of two HSP genes, indicating that handling may be experienced as a stressful event for the developing embryo. On the other hand, it was not found that any of the methods used for egg handling resulted in any long-term effects on basal HPI-axis or HSP gene expression.

4.1 Mortality and developmental timing

Effects of the treatment on morality, hatching and size at hatching, in addition to larval growth, were registered to account for possible bias in the sampling material. The results showed differences between groups at hatching. When it came to egg mortality the current study showed that the total amount of dead eggs eventually became similar in all four groups. However, the path was different as the three treated groups had a rapid increase in mortality following the treatments. This was probably because dead and undeveloped eggs got their vitelline membrane ruptured during the treatments. Almost all of the outgoing eggs were un-developed or small-eyed eggs and during sampling only normal eyed-eggs were collected. The weight at hatch, and larval development rate in weight was similar in all groups.

4.2 Ontogeny of the HPI- and HSP-genes

In the present study, all examined genes of the HPI-axis, except for the *gr1* and *gr2,* showed an increased expression throughout embryogenesis with a higher relative gene expression in newly hatched larvae than before hatching. This is in agreement with previous studies showing an increased expression of *crf, pomc and mr* during early life stages of zebrafish and tilapia (Hansen et al., 2003; Pepels and Balm, 2004; Alsop and Vijayan, 2008; Alderman and Bernier, 2009). To the author’s knowledge, the ontogeny of the HPI-axis is poorly investigated in salmonids, and Atlantic salmon in particular. Embryos that have been studied to date have, to the author’s knowledge, not shown a functional stress response with respect to cortisol release after exposure to a stressor. This applies to several species (reviewed by Pittman et al.

2013), including Atlantic salmon (H. Tveiten, pers. Comm) and other salmonids (Auperin and Geslin, 2008). The expression of the different HPI-axis related genes during the embryogenesis may therefore indicate that they have a different role beside the HPI-axis.

In the current study, expression of both *crf1* and *crf2* were detected during the embryogenesis, and showed a significant increase at hatching (Figure 14). This is in accordance with Tsalafouta et al. (2014) which registered that expression of *crf* in European sea bass embryos showed a peak at hatching. After hatching, however, a difference in expression pattern of the two *crf* genes was detected in the current study. Expression of *crf2* showed a second increase at start feeding, while *crf1* expression decreased. In Atlantic salmon, it has been detected that the two *crf* genes shows a decreasing expression after hatching, followed by an increase towards the time of start feeding (Johnsen and Tveiten, unpublished). The lack of increasing *crf1* expression at start feeding in the current study, may therefore be due to a delayed increase. Fuzzen et al. (2011) also detected an increase at start feeding in rainbow trout. The increase towards start feeding may correlate with *de novo* synthesis of cortisol and increase in cortisol production, and thereby indicate a developing HPI-axis. *Crf* has been shown to be involved in the regulation of appetite (Bernier and Peter, 2001), which may also explain the increase towards the time of start feeding in the current study. The detection of expressed *crf1* and *crf2* during the embryogenesis may indicate that they have a different role beside the HPI-axis, in embryos. Alderman and Bernier, (2009) detected *CRF* by *in situ* hybridization in the larval retina, and *crf* has shown to have a peak in expression at the time of eye opening in mammals (Bagnoli et al.2003). Also, in addition to being ACTH releasing factor, CRF-related peptides are also involved in secretion of pituitary thyroid hormone, thyrotropin, which have been shown to play a vital role during normal development of zebrafish (Alderman and Bernier, 2009).

POMC is the precursor for ACTH and several other biomolecules. The ontogeny of the *pomc'*s in current study showed that they all had a significant increase at hatching (Figure 15). In addition, both *pomcA1* and *pomcA2* had a second significant increase at the time of start feeding, which, due to increasing cortisol synthesis (H. Tveiten., pers. comm.) may indicate a developing HPI-axis. *PomcA1* showed a statistical significant increase in embryos at 377d°C compared to 326d°C, which may indicate that the expression increases rapidly during this time

of development. This is in agreement with a study on Atlantic salmon, which showed a rapid increase of *pomcA1, pomcA2, pomcB* expression between approximately 300d°C and 455°C (Johnsen and Tveiten, unpublished). POMC is synthesized in both corticotropic and melanotrophic cells of the pituitary, in addition to other regions of the brain and peripheral tissues (Hansen et al., 2003). Several studies have tried to assess different roles for the *pomc's*, but to the author's knowledge, there is no clear evidence of possible different functions. In the current study, there was shown a difference in the expression levels of the *pomc's* in larvae at start feeding. *PomcA2* and *pomcB* showed a relatively lower expression level compared to *pomcA1,* which may indicate that they have different roles at this development stage. However, it may also indicate a delayed increase of *pomcA2* and *pomcB* expression.

GRs and MR are receptors that mediates the action of cortisol. In the current study both *gr1* and *gr2* expressions (Figure 16) showed significant decreases in embryos at 377d°C, followed by an increase at hatching and a second increase around the time of start feeding – at a time when salmonids are able to mount a cortisol response to stress (Auperin and Geslin 2008; H. Tveiten pers. comm.). It differs only seven days between the two time points measured during embryogenesis in the current study, but the findings are supported by a study on zebrafish, which also detected a decrease in embryo *gr* expression during the embryogenesis (Alsop and Vijayan, 2008). This increase may indicate that both *gr'*s in Atlantic salmon are maternally deposited and the mRNA depleted, and that somewhere between 377d°C and hatching, the zygotic transcription is initiated. This indication is in accordance to findings in Atlantic salmon (Johnsen and Tveiten, unpublished). Alsop and Vijayan, (2008) discovered that the profile of *gr* expression was similar to that of cortisol, which is known to start the *de novo* synthesis around the time of hatching, which also seem to be the case in salmonids (Auperin and Geslin 2008; H. Tveiten pers. comm.). This indicates that the two *gr's* may play an important role in conveying the action of cortisol. The expression of *mr* did not show any decrease before hatching in the current study. A decrease, however, might have occurred before or after the two measured time points, as the level of expression not showed any differences between these seven days. Other studies have detected a continuous elevation during development of *mr*, and to the author's knowledge, there is no evidence that *mr* is maternally deposited, neither in Atlantic salmon (Alsop and Vijayan, 2008; Tsalafouta et al., 2014, Johnsen and Tveiten, unpublished). The continuous increase of *mr* expression, may indicate that also other

hormones than cortisol can be ligands for this receptor. Deoxycorticosterone (DOC) is studied in rainbow trout as a possible ligand for MR (Sturm et al. 2005).

Members of the HSP70 family are present in all intracellular compartments and are known to play a crucial role in protein folding and translocation within the cells (Krone et al., 2003). *Hsp70a* expression in current study showed a slightly decrease in embryos at 377d°C, followed by an increase at hatching, which at start feeding was significantly decreased (Figure 17). Fuzzen et al. (2011) registered in rainbow trout a basal expression of HSP70 mRNA level that increased throughout ontogeny, but it was not clarified which *hsp70* that was detected. The profile of *hsp90a4* expression in current study was similar to *hsp70a* expression pattern with an increase in newly hatched larvae, followed by a significant decrease at start feeding. The expression levels of both the examined *hsp's* in the current study, showed their lowest expression level at start feeding. This is opposite to the cortisol profile, which showed an increase towards start feeding in Atlantic salmon (Johnsen and Tveiten, unpublished). The decrease of the *hsp's* may therefore indicate that HSPs play an important role as stress proteins during embryogenesis, and are downregulated sometime after hatching because of a functional HPI-axis is developing. Deane and Woo, (2003) detected in silver seabream larvae that the expression of *hsp70* remained unchanged between 1-14dph, before increasing as larval development progressed. Also, studies conducted on fruit flies have demonstrated that expression of hsp70 can be detrimental to growth (Deane and Woo, 2003).

4.3 Does stress alter gene expression?

Even though the genes examined might have other functions than the hormone cascade that elicit the cortisol response, during the embryogenesis, they might still be altered upon stress. The genes were therefore examined to study how expected stressors like shocking and transport would be manged in embryos before the HPI-axis were functional. As mentioned earlier, the ontogeny of genes are poorly studied, and also, to the author's knowledge, very few studies have reported transcript differences in larvae after exposure to stressors, and even fewer on embryonic response.

4.3.1 Short term effects of two treatments during embryonic development

In the present study the stressors used were two main handling procedures eggs normally encounter during an egg production cycle; shocking and transportation. Both procedures occurred during the so-called eye-stage, which is associated with robust embryos (Egidius and Helland-Hansen, 1973; Groot, 1996).

Shocking exposed the eggs to mechanical stress. During shocking, eggs were agitated so that the vitelline membrane of dead eggs was ruptured, but not so much that normal eggs were damaged. By doing this, dead eggs turned white and were easily sorted out. As a general explanation of the current results, there was not detected any specific effect of the shocking on any of the genes (Figure 18-21). The findings supports that the HPI-axis is not functional at this developmental stage. Some of the studied genes showed a transient increase, but a lack of increase in the other shocked group or a simultaneously increase of an un-shocked group, made these results hard to interpret. When one disregards this, the results showed a general increase during 24 hours in several of the genes examined. This is especially evident in *gr2* and *mr* expression levels in all four groups.

The transportation was a long-lasting treatment of which the eggs were both mechanical stressed and subjected to temperature changes. The samples were taken 1, 3 and 29 hours after a 48 hours transport, which means that the samples were taken 49, 51 and 77 hours after the 0 hour sampling. All measured gene expression of the control group showed an increase during this period. Pepels and Balm, (2004) showed, that there was a correlation between body weight and the level of *crf* expression during early development in tilapia. With this in mind, and assuming that the other genes also are correlated with weight, it is tempting to suggest that the increase of the control group might be because of rapid development of the embryo. As mentioned previously, Johnsen and Tveiten, (unpublished) showed that between approximately 300d°C and 455°C the expression of *crf1, crf2, pomcA1, pomcA2, pomcB, gr1* and *gr2* had a rapid increase, which is in the period where shocking and transport happened in the current study.

During the transport, the eggs were held on a temperature that went below zero degrees, of which may indicate that they were subjected to a cold shock. The definition of cold shock according to Donaldson et al. (2008) is an ''acute decrease in ambient temperature that has

the potential to cause a rapid reduction in body temperature, resulting in a cascade of physiological and behavioural responses''. In current study a decrease of both *crf1* and *crf2* expression was shown after one hour, in the transported group that previously had been shocked (Figure 22). However, the decreased expression level was not sustained, and not significant different from the control. The same group showed an increase of *pomcA1* and *pomcA2* after 3 hours, and of *pomcB* after 29 hours (Figure 23). The other transported group showed no significant difference in expression of the HPI-axis genes during time points, except for a sustained decrease of *gr2* after 3 hours. The discussed differences in expression levels of the HPI-axis after transport were minor, and there were several factors that made them difficult to interpret. Because of this, it is tempting to conclude that there were no distinct effect on the HPI-axis genes after transport in the current study. This is in accordance to the results of a study conducted on European sea bass embryos exposed to transport for 8 hours followed by netting and air exposure for 1 minute, which did not lead to any significant effect on *crf, gr1, gr2* or *mr* expression levels (Tsalafouta et al., 2014).

Both HSP genes however, showed an effect of the transport (Figure 25). *Hsp70a* showed a significant increase after 1h in both transported groups, which were back to normal levels after 29 hours. This is in accordance with Fuzzen et al. (2011) that detected a marked stimulator effect on *hsp70* gene expression in embryos of rainbow trout 28dpf subjected to hypoxic treatment. *Hsp90a4* showed a significant increase after 1 hour in the transported group and after 3 hours in the group that had been both shocked and transported. This increase sustained in both groups until 29hour after transport. When knowing this, in addition to that the HPI-axis genes showed minor effect, one may speculate that the HSPs play a major role in stress response during embryogenesis, as the HPI-axis is not functional.

4.3.2 Does the treatments shocking and transport give any long-term effects?

Samples taken at the time of start feeding did not show any different expression between the groups of all genes except, *crf2,* which showed a significant lower expression level in both the shocked group compared to the control, and *pomcA1,* which had a significant lower expression in the transported group compared to the control (Figure 14 and 15). The lack of different expression between groups of the other genes, indicates that there was not shown any specific long-term effect after exposure to shocking and/or transport. The endocrine

system however, is a very complex system, and these findings do not exclude that stress may alter expression of other genes and pathways, e.g. immune genes and growth related genes. The increased expression of the HSP genes after transport indicates that this handling may be experienced as a stressful event for the developing embryo. One may speculate that exactly because the HPI-axis is not functional during the embryogenesis, this may be a period where other endocrine systems are more exposed and susceptible to changes after exposure to stress.

Larvae around the time of start feeding were subjected to a stress challenge, of which consisted of one minute in ice water, followed by one minute in air. The control group showed a higher level of transcripts after 24 hours of *crf1* and *hsp70a* compared to the shocked and the transported group, *crf2, pomcA1, pomcA2* and *pomcB* compared to the transported group, and *gr2* and *mr* compared to all groups (Figure 26-29). The fact that the control group showed an increase in several of the genes examined, after stress, may indicate that the HPI-axis is functional. Johnsen and Tveiten, (unpublished), showed in Atlantic salmon, a minor increase in cortisol in larvae at start feeding in response to stress, but not in the same amount as in juveniles Atlantic salmon, shown in the same study (Johnsen and Tveiten, unpublished). This indicates that the HPI-axis is functional at this time. The lack of increase in other groups may indicate that the gene expressions of previous treated groups have been depressed due to the subjected treatments. Except for the three *pomc's* none of the other genes showed any difference in expression between the previous treated groups. The group that had been submitted to both shock and transport showed a similar expression level of all three *pomc's* as the control after 24 hours. In addition, both groups showed significantly higher expression levels than the transported group. It is therefore tempting to speculate that when a gene is subjected to high enough amounts of stress, the depressed expression of the gene is reversed. Nofima has previous detected similar findings, where embryos subjected to repeated stress eventually became more similar to the control group (H. Tveiten, pers. Comm.). This may indicate that there are some epigenetic effects that possibly alters the gene expression when exposed to a stressor.

The hsp's did show a small effect of stress. However, compared to the effect of transport on the *hsp* genes, the stress challenge did not show similar response when the larvae was stress challenged at the time of start feeding. The *hsp70a* expression only showed a significant

increase over time. This is in accordance a study conducted by Fuzzen et al. (2011) which registered that *hsp70* response to stressor was lower in larvae compared to embryos. In current study, *hsp90a* showed no effect, except for an increase in the shocked group. So, also with this gene the effect of stress was more pronounced in embryos than in larvae. When knowing this, in addition to the effect of transport during embryogenesis, and the high expression levels in embryos, it is tempting to conclude that hsp's play a major role when it comes to dealing with stressors during embryogenesis.

5 Conclusions

Several aspects related to mechanical shocking and transportation of Atlantic salmon eggs were clarified through this thesis and some basic knowledge was obtained. It was clear that the treatments (shock and transport) had a direct effect on weak eggs in the sense that it speeded up the process of sorting them out. However, the overall mortality was not significantly affected through the treatments. Further, the ontogeny of the HPI-axis genes; *crf1, crf2, pomcA1, pomcA2, pomcB* and *mr,* showed an increase in relative gene expression during the development, with higher expression levels in larvae than in embryo. Relative expression of *gr1* and *gr2* showed a decrease during the embryogenesis before an increase in newly hatched larvae, indicating that they are maternally transferred. The ontogeny of the HSP genes, *hsp70a* and *hsp90a4,* showed the highest levels of relative gene expression in newly hatched larvae and the lowest levels of gene expression in larvae at start feeding. It was not shown any specific alterations of any of the genes examined after the embryo had been exposed to shocking. The HPI-axis genes examined did not show any specific alterations after exposure to transport. *Hsp70a* and *hsp90a4* however, showed an increase in relative expression after exposure to transport, which for *hsp90a4* also was significant after 29 hours. As a possible long-term effect, it was shown a significant difference in *crf2* and *pomcA1* expression between the control group and some of the treated groups at start feeding. This needs to be further analysed in future studies. For the other genes, however, significant alteration was not detected between groups. After exposure to a stress challenge, there was shown alteration of *crf1, crf2, pomcA1*, *pomcA2, pomcB, gr2, mr* and *hsp70a* expression after 24 hours. All these mentioned genes were lower expressed in the transported group than in the control group. Also in *pomcA1, pomcA2* and *pomcB* the group that had been previous subjected to both shocking and transport, showed a similar increase in expression as in the control group 24 hours after the stress challenge.

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Appendix I

Chemicals

Reagents	**Producer**
Absolute alcohol prime	
High Capacity cDNA Reverse Transcription Kit (4368814)	Applied Biosystems
Isopropanol	
Power SYBR Green PCR Master Mix (4367659)	Applied Biosystems
Proteinkinase K (AM2548)	Ambion
MagMAX Lysis/Binding Solution Concentrate (AM8500)	Ambion
MagMAX-Total RNA Isolation Kit (AM1830)	Ambion
Nuclease-free water (AM9937)	Ambion
Oligo $d(T)_{16}$ Primer, 50µM (N8080128)	Invitrogen
RNA-later® solution (AM7021)	Ambion
TURBO DNA-*free* kit (AM1907)	Ambion

Appendix II

Tabell 1: Primers used in RT-qPCR

Gene	Sequence (5’-3’)	Gene bank acc. no.	E=
Ef1α FW	CGCCAACATGGGCTGG	AF321836	2
Ef1α RW	TCACACCATTGGCGTTACCA		
ß-actin FW	CAGCCCTCCTTCCTCGGTAT	BT059604	2
ß-actin RW	CGTCACACTTCATGATGGAGTTG		
18S rRNA FW	TGTGCCGCTAGAGGTGAAATT	AJ427629	2
18S rRNA RW	CGAACCTCCGACTTTCGTTCT		
CRF 1 FW	GCGGTCAACAGCGGTCA	Unpublished	2
CRF 1 RW	TCTGGTTAGCGTAGCTGTTCAGA		
CRF 2 FW	GTCGAGAGCCCTGACGATGT	Unpublished	2
CRF 2 RW	CGTTGCCCAGTCGGATGT		
POMC A1 FW	TGGAAGGGGGAGAGGGAG	AB462418	2
POMC A1 RW	CAGCGGAAGTGGTTCATCTTG		
POMC A2 FW	TCCCTGGAGGCTGGGACT	AB462419	2
POMC A2 RW	CCAGCGGAAGTGGTTCATCTTA		
POMC B FW	ACTAAGGTAGTCCCCAGAACCCTC	DQ508935	2
POMC B RW	GCTACCCCAGCGGAAGTGA		
MR FW	TGTCCAAAACTGTGGCTGAATC	Unpublished	2
MR RW	CCGAAGCCGCCAAAGTCT		
GR1 FW	ACCGCAGCAGAACCAACAG	Unpublished	2
GR1 RW	TGGATCGATTCAAATCTGCAAT		
GR2 FW	TGTCCATGAGGACGGAGACA	Unpublished	2
GR2 RW	CCAATGTACCCTTCCTGATCCA		
HSP70a FW	CTGGGCTGAATGTGCTGAGA	Unpublished	2
HSP70a RW	CTGGACATGCCTTTGTCCATG		
HSP90a4 FW	GAAGAAGCAAGAGGAGCTGAACA	Unpublished	1.94
HSP90a4 RW	AACTGAAACCTTCTCAATCTTCTTGTC		

Appendix V

Statistics on relative gene expression of *crf1*

Table 2: Normality test of relative gene expression of the four groups; C=control, S=shock, T=transport and ST=shock & transport.

Tests of Normality						
CRF1	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
StressChallenge_0h_C	,244	10	,093	,929	10	,440
StressChallenge_0h_ST	,226	10	,159	,887	10	,157
StressChallenge_0h_S	,162	10	,200*	,956	10	,738
StressChallenge_0h_T	,180	10	,200*	,940	10	,553
StressChallenge_1h_C	,127	10	,200*	,930	10	,448
StressChallenge_1h_ST	,217	9	,200*	,956	9	,753
StressChallenge_1h_S	,203	10	,200*	,954	10	,716
StressChallenge_1h_T	,205	10	,200*	,907	10	,261
StressChallenge_3h_C	,153	10	,200*	,978	10	,953
StressChallenge_3h_ST	,209	10	,200*	,878	10	,124
StressChallenge_3h_S	,117	9	,200*	,977	9	,946
StressChallenge_3h_T	,164	10	,200*	,974	10	,927
StressChallenge_24h_C	,121	10	,200*	,982	10	,973
StressChallenge_24h_ST	,185	10	,200*	,973	10	,920
StressChallenge_24h_S	,172	10	,200*	,946	10	,624
StressChallenge_24h_T	,210	10	,200*	,927	10	,417

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

The ontogeny of CRF expression, the control group:

Tabell 3: Descriptives of the data from the analysed control group.

Descriptives										
Values conrol group (C)										
		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum	Between-Component Variance
						Lower Bound	Upper Bound			
Stress challenget_0h_C		10	20,6420	3,71933	1,17616	17,9814	23,3026	15,63	28,27	
Hatching_C		10	27,2470	4,45137	1,40765	24,0627	30,4313	19,58	31,80	
Transport_0h_C		10	6,5160	1,30486	,41263	5,5826	7,4494	4,33	8,20	
Shock_0h_C		10	5,5160	3,31534	1,04840	3,1443	7,8877	2,88	11,10	
Total		49	20,0300	16,57033	2,36719	15,2704	24,7896	2,88	77,18	
Model	Fixed Effects			9,75714	1,39388	17,2208	22,8392			
	Random Effects				6,77959	1,2069	38,8531			219,73341

Tabell 4: Levene's test for homogeneity in varians conducted on the control group.

Test of Homogeneity of Variances			
Values control group (C)			
Levene Statistic	df1	df2	Sig.
16,592	4	44	,000

Tabell 5: One way ANOVA

ANOVA					
Values control group (C)					
	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8990,769	4	2247,692	23,610	,000
Within Groups	4188,877	44	95,202		
Total	13179,646	48			

Appendix V

Tabell 6: Robust tests of equality was used because the data failed the Lavine's test.

Robust Tests of Equality of Means				
Values control group (C)				
	Statistic[a]	df1	df2	Sig.
Welch	75,895	4	19,542	,000
Brown-Forsythe	21,317	4	9,581	,000

a. Asymptotically F distributed.

Tabell 7: Games-Howell as a *post hoc* test analysing the CRF1- data from the control group.

Multiple Comparisons							
Dependent Variable:							
(I) Ontogeny, control group (C) CRF1			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Games-Howell	StressChallenge_0h_C	Hatching_C	-6,60500*	1,83434	,016	-12,1700	-1,0400
		Transport_0h_C	14,12600*	1,24644	,000	10,1064	18,1456
		Shock_0h_C	15,12600*	1,57559	,000	10,3552	19,8968
	Hatching_C	StressChallenget_0h_C	6,60500*	1,83434	,016	1,0400	12,1700
		Transport_0h_C	20,73100*	1,46688	,000	15,9504	25,5116
		Shock_0h_C	21,73100*	1,75517	,000	16,3779	27,0841
	Transport_0h_C	StressChallenge_0h_C	-14,12600*	1,24644	,000	-18,1456	-10,1064
		Hatching_C	-20,73100*	1,46688	,000	-25,5116	-15,9504
		Shock_0h_C	1,00000	1,12668	,896	-2,6048	4,6048
	Shock_0h_C	StressChallenge_0h_C	-15,12600*	1,57559	,000	-19,8968	-10,3552
		Hatching_C	-21,73100*	1,75517	,000	-27,0841	-16,3779
		Transport_0h_C	-1,00000	1,12668	,896	-4,6048	2,6048

*. The mean difference is significant at the 0.05 level.

Appendix V