NTNU

Kunnskap for en bedre verden

Bacheloroppgave

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Optimisation and validation of RT-qPCR assay for relative quantitation of reference genes and stress related regulatory genes in CHSE-214 cells

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Preface

This topic was chosen because of our interest in RT-qPCR and cell cultivation. All our work was conducted at the laboratories of NTNU Ålesund. We would like to extend our gratitude to all of those who put up with our disruptive presence, lack of respect for authority and penchant for asking dumb questions. Specifically, we would also like to thank our patient supervisors Ann-Kristin Tveten and Gro Audveig Hagen Bjørnøy. They have given us excellent support and guidance over the last three years, but especially now in the last few months when we needed it the most. May you never sigh in vain.

Abstract

Understanding how short and long-term stress affects salmonid cells *in vitro*, may help understanding the physiological effects in live salmon. In this thesis a RT-qPCR method for normalisation of two reference genes, EF1Aa and EF1Ab, and the gene expression profiles of HSP70 and p38 MAPK during the heat stress response of CHSE-214 cells was established

A method for RT-qPCR expression studies of CHSE-214 cells was optimised, and EF1Aa and EF1Ab were validated as suitable reference genes. Reference gene stability was demonstrated to be within acceptable bounds, between 1.187- and 0.382-fold expression change. Primer and probe concentration for the two reference genes yielded reproducible results. A RT-qPCR method was established for HSP70 and p38 MAPK to verify a heat shock response. HSP70 expression increased considerably, peaking at 2-4 hours and then returned to baseline expression levels after 16 hours. Expression of p38 MAPK was relatively stable without any peaks of interest, indicating that p38 MAPK does not differentially express under given conditions.

Sammendrag

Å forbedre forståelsen rundt effektene av korttids- og langtidsstress har på *in vitro* celler, kan gi et bedre bilde på de fysiologiske følgene i levende laks. I denne tesen ble en RT-qPCR metode for normalisering av to referansegen, EF1Aa og EF1Ab, og genekspresjonen til HSP70 og p38 MAPK i varmestressede CHSE-214 celler etablert.

En metode for RT-qPCR ekspresjonsstudier av CHSE-214 celler ble optimalisert, og EF1Aa og EF1Ab ble validert som passende referansegen. Stabiliteten til referansegenene var innenfor akseptable rammer, mellom 1.187 and 0.382 ganger uttrykksendring. Primer og probekonsentrasjonene for de to referansegenene ga reproduserbare resultater. En RT-qPCR metode ble etablert for HSP70 og p38 MAPK, for å verifisere varmestress responsen. HSP70 transkripsjonen økte betraktelig, og nådde en topp etter 2-4 timer for så å falle under opprinnelig nivå etter 16 timer. Ekspresjonen av p38 MAPK var relativt stabil uten klare trender, som indikerer at p38 MAPK ikke er aktivt regulert under de gitte forholdene.

Contents

1 Introduction

1.1 Background

Norwegian aquaculture industry has unrealised potential for growth hindered by high mortality and loss (1). In recent years higher densities of fish, more intensive rearing practices and *Lepeophtheirus salmonis* (salmon louse) resistance has increased disease prevalence. When epidemics occur, expansion is restricted by government agencies until the issues have been resolved. Losses in fish stock also reduce profit, and result in higher labour and material costs. A report from 2012 reported a 16.3% loss in *Salmo salar* (Atlantic salmon) from transference to sea to slaughter (2). The 2017 annual report from Norwegian Food Safety Authority (Mattilsynet) lists treatments of salmon louse as one of the main causes of death and disease problems in fish cages (1).

More Atlantic salmon are dying from complications of delousing than from lice infection, showing the need for gentler delousing techniques (3). NFSA received 963 reported anomalies in 2017, and of those, 625 concerned delousing. Thermic delousing has been implemented as an alternative to medications and chemical treatments, as lice have developed resistance to chemical treatments. In 2018 thermic delousing was the most used non-medical method in Norway, but thermic delousing techniques used today might cause fin, epithelial, gill and eye damage, brain haemorrhage and general pain (4, 5). Some of the injuries from thermal delousing might not be directly from hot water, but from panic that ensues, where fish try to escape and swim head on into the container and other fish (4).

This acute stress, that can be caused by the external stimuli of thermic delousing causes a sudden shift in hormonal and metabolic levels. These short-term experiences, if experienced repeatedly, can lead to chronic stress and distress where growth is slowed and health declines. On a cellular level, looking at metabolic and transcriptional changes can indicate when chronic stress is induced. Understanding these inducers and changes is important when trying to understand what happens to fish in stressful situations, long and short term.

Reverse-transcriptase real time PCR (RT-qPCR) allows researchers to observe the physiology of fish, and epidemiology of common diseases at the transcriptional level, and makes a good diagnostic tool in fish welfare. In research it is a highly reproducible and specific method of analysing transcriptional changes of cells both *in vitro* and *in vivo,* with a high throughput. Specificity derives from the use of sequence specific primers and hydrolysis probes, and accuracy of quantitating any transcriptional changes. Validating them to an actual change in transcription, depends on stable reference genes, thus the importance of validating *in vitro* studies before clinical studies for that specific purpose.

This thesis presents a pre-clinical *in vitro* study of CHSE-214 exposed to heat. The primary objective was to validate the suitability of Elongation Factor 1-Aa (EF1Aa) and Elongation Factor 1-Ab (EF1Ab) as reference genes for further in-house research on Atlantic Salmon and the salmonid cell line CHSE-214. EF1Aa and EF1Ab are established

reference genes, but there has been reported fluctuation in expression, so in-house validation is recommended (6, 7). Based on previous studies, it was hypothesised that EF1Aa and EF1Ab would express stably in both a normal and stressed state. A secondary objective was to observe HSP70 and p38 MAPK transcription over a long period of heat stress. This was done to validate heat stress in cells, to demonstrate that EF1Aa and EF1Ab are stable in heat stress conditions, both before and after the peak of HSP70, which is a common gene marker of heat stress.

1.2 Dictionary

cDNA – complementary/copied DNA
CHSE-214 – Chinook Salmon Embryo cells
Ct – Cycle threshold. Synonyms: Cq - Cycle of quantitation
ΔΔCt – delta delta, cycle threshold
Distress – stress of a magnitude that causes significant or lasting harm to the cell or organism
EDTA – Ethylenediaminetetraacetic acid
FRET – Fluorescence Resonance Energy Transfer
HSP70 – Heat Shock Proteins weighing 70 kDa
LOD – Limit of detection
MAP3K7IP1/TAB1 – Mitogen Activated Protein Kinase Kinase Kinase 7 Interacting Protein 1
MGB – Minor Groove Binding
MKK protein – MAP Kinase Kinase protein
NLS - Nuclear Localisation Signal
NTC – No template control
No-RT – No reverse transcriptase
PCR – Polymerase Chain Reaction
p38 – 38 KDa Mitogen activated Protein kinase MAPK14
Reference gene – gene used for normalising RT-qPCR data. Synonyms: normalisation genes, housekeeping genes
RT-qPCR – Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction
T_M – Oligonucleotide melting temperature

1.3 Theory

Fish welfare and stressors in aquaculture

In aquaculture there are multiple stressors, these can be categorised as chemical, physical or psychological (8). General handling, crowding, smoltification, water quality, diseases and delousing are examples of stressors commonly associated with the rearing of fish. These can affect the fish in different ways, as they vary in length and severity. Stress

response can be defined as “the physiological cascade of events that occurs when the organism is attempting to resist death or re-establish homeostasis in the face of a threat.” (9). The responses can be categorised as primary, secondary and tertiary responses (8, 10-12).

Many stressors might lead to a primary stress response, which the fish can recover from fast without causing lasting physiological harm. It is characterised by the immediate and significant release of stress hormones like adrenaline and cortisol, whilst a change in neurotransmitter activity occurs (9, 10).

A primary response might induce a secondary response, and subsequently a tertiary response (*Figure 1*). Symptoms of the secondary and tertiary responses may arise without the primary responses if the stressors are persistent. Chronic pain, unsuitable environment and dietary restrictions are examples of stressors that can lead to this (9, 10).

The secondary response is characterised by metabolic, cellular, homeostatic, hematologic and immunologic changes. These changes can shift the glucose and ion levels in tissue, blood, and produce heat shock and stress proteins. Secondary response changes take more time to revert to a normal state than that of a primary response (10).

A tertiary response is seen as severe changes in the fish’s behaviour, were energy is being used to cope with the stressors rather than growth and reproduction. This is seen as changes in the overall resistance to disease, growth, condition and survival. These tertiary changes may lead to distress, if the stressors are not controlled (8, 11).

Visual indicators of stress are vertical swimming, increased swimming speed, grouping of fish or slowed growth. Options for measuring stress look at physical changes in the fish, examples are hormonal changes, like cortisol and adrenaline, and blood glucose levels. These changes happen at a cellular level, affecting the whole fish. Knowing more about what happens at the cellular level will benefit the whole organism (8, 13, 14).

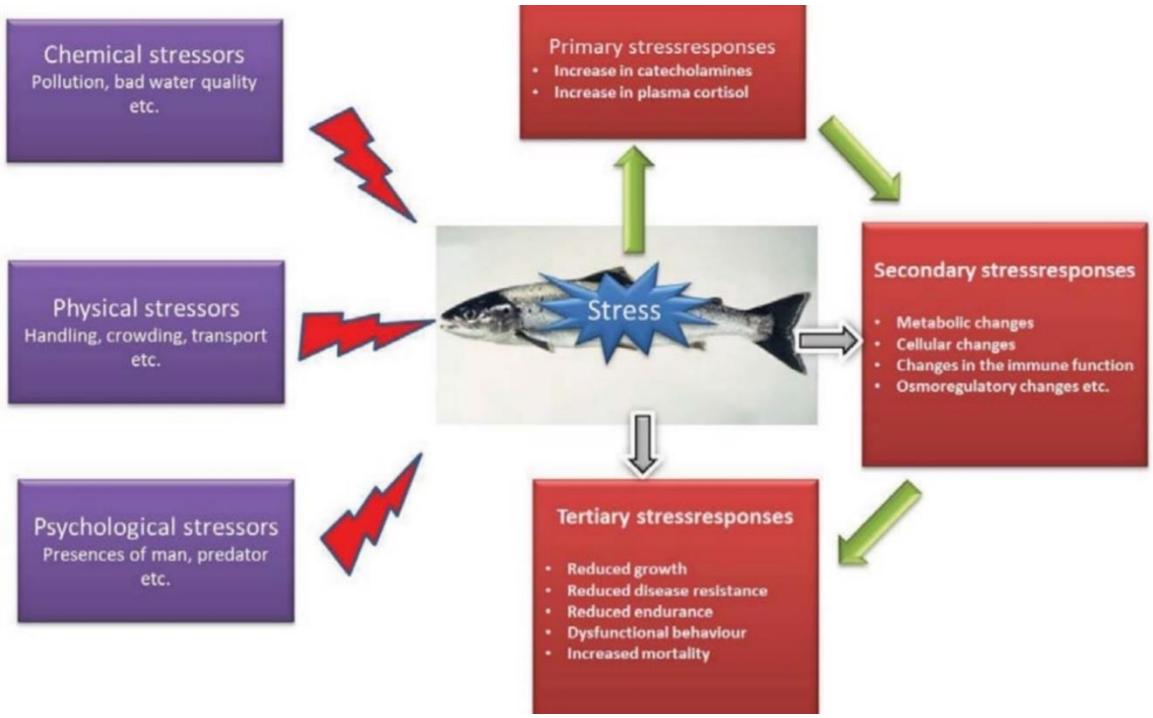


Figure 1. *Physical, chemical and other perceived stressors act on fish to evoke physiological and related effects, which commonly are grouped as primary, secondary and tertiary responses. The arrows indicate the various response routs, a primary response might induce a secondary response, and subsequently a tertiary response. Although a secondary and tertiary response might occur without a prior response.*
Reproduced with permission (9).

Cellular stress

Cells, being part of an organism or *in vitro*, react to stressors. Stress can be defined, with some modification, in the same way as for the whole organism; a cascade of events that occur when the cell is attempting to resist death or to re-establish homeostasis in the face of a threat. The cell can respond to stressors in four different ways; cell repair, adaption, autophagy or cell death. What mechanism the cell chooses to employ depends on the cell type, severity and type of stress, for example: damage to the DNA can be mended, but in severe cases it can activate pathways leading to cell death. Stressors such as light, heat and access to nutrients cause different adaptions. After a period of adaption, a cell can assume a new steady state and will no longer express a stress reaction (12, 15).

Organism level stress markers, physical damage and fish death are visible indicators, however understanding the metabolic changes due to stress might be a challenge.

On the cellular level different stress responses cause different adaptions. Measuring these adaptions is a way to determine the level of stress, and the only way to determine the difference between secondary and tertiary stress (12). A common method is analysing gene expression to see if the expression of certain genes changes under stressful conditions.

CHSE-214 cells – model cells

For *in vitro* studies, biological sample material is produced and collected from laboratory grown cells. Lab conditions allow for controlling bias and to obtain results that have a higher correlation with the intended experimental conditions. These cells should be as representative for the organism you want to investigate as possible, while being easily handled in the lab. A common model cell for aquaculture is the CHSE-214 cells, as used in this thesis.

The CHSE-214 cells were isolated from pooled *Oncorhynchus tshawytscha* (Chinook salmon) embryos by researches from Oregon state university in 1964 and exhibit characteristics similar to epithelial cells (16). The cells grow in a single adherent layer and display relatively uniform morphology. A few cells grow to considerable size (*Figure 2*). Cells were originally cultured in Eagles Minimum with 10% FBS. It was later discovered that their growth is faster in L-15 Glutamax with 10% FBS (17). Their growth maxima lie around 21°C and cell count after 7 days drops considerably if the cells are incubated at 27°C (16).

Oncorhynchus tshawytscha (GCF_002872995.1) and *Salmo salar (GCF_000233375.1)* have been fully sequenced and their genome is available through refseq. NCBI and other databases simplify oligonucleotide design which enable PCR assays. Several reference genes have been verified for use in Atlantic salmon by independent research teams, which makes CHSE-214 cells a candidate model organism for *in vitro* studies of salmon cells (7).

Maintaining and making use of cells in laboratory work is the backbone of biology. The cells are taken out of their natural environment and given optimal growth conditions. This allows shorter timeframes for experiments and reduces the need for fieldwork. These cells naturally do not act exactly like their wild counterparts and live parent organisms, but can still yield general information about their biology.

Isolation and maintenance of a eukaryotic cell line requires the cells to be perpetual, and their growth conditions must be known and met. Immortalised cells are produced either by isolating embryonal stem cells, that express telomerase and can divide endlessly. Alternatively, cancer cells or immortalised hybrid cells (cancer cells that have been genetically modified to include a desired genetical sequence) can be used. Growth of eukaryotic cells usually depend on; specific temperature, specific gas composition, specific cell density, specific media salinity and pH, amino acids, sugar, and specific growth factors. When all these are met, model cells usually grow well.

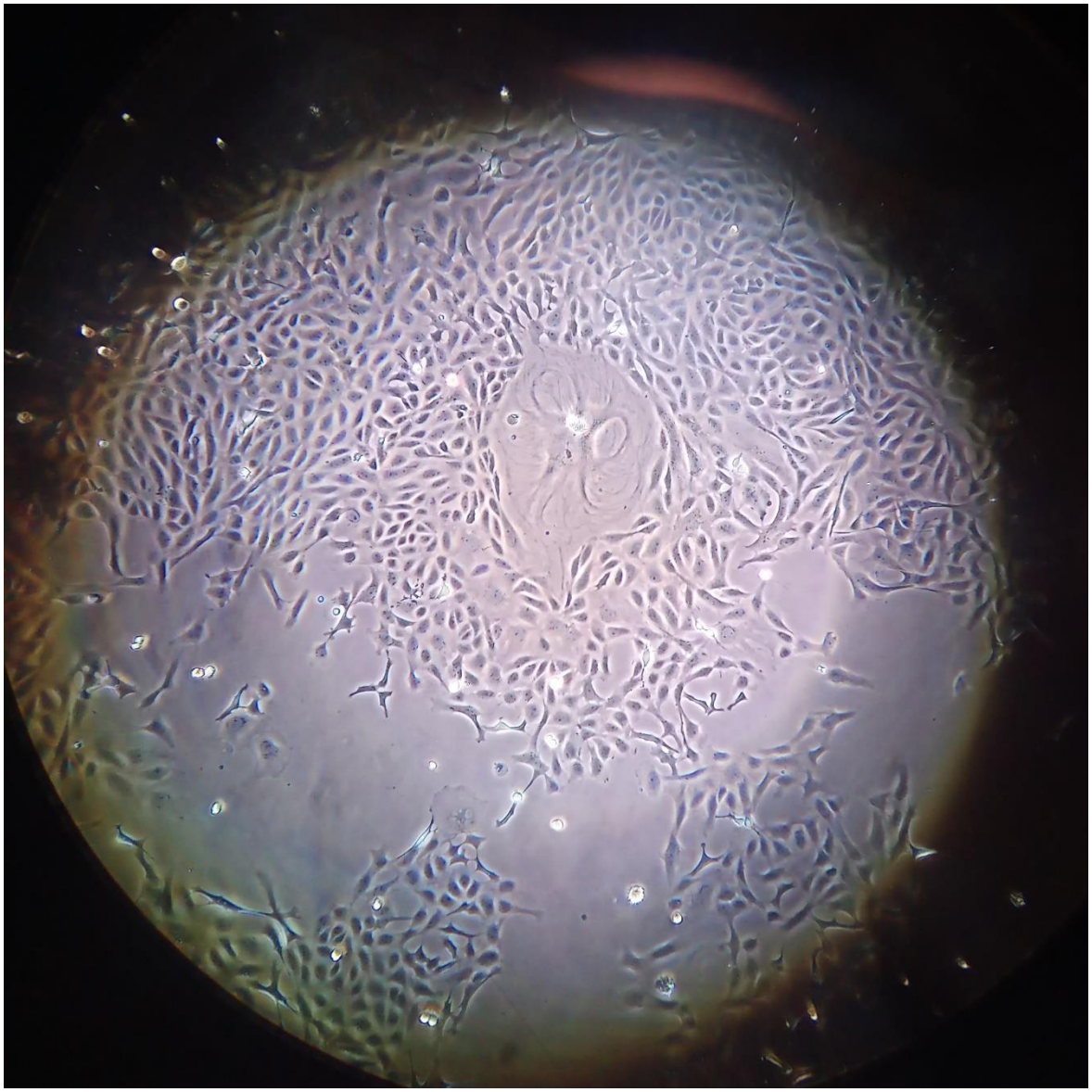


***Figure 2**. CHSE-214 cells under phase contrast microscopy. 65-70% growth density. Notice the big cell in the middle of the figure. The cells were for the most part relatively uniform, except for some cells growing to a considerable size, although these were not many.*

Reference genes

EF1Aa and EF1Ab

The eukaryotic Elongation Factor 1 (EF1) is a protein complex, consisting of two subunits: EF1A and EF1B. The EF1A subunit exists in two paralogous forms EF1Aa and EF1Ab. The complex is involved in the elongation phase of translation. This is considered the canonical function of the EF1 complex. The architecture and other functions of the complex are still under investigation, but findings list interactions with the cytoskeleton and nuclear export of tRNAs. Reports also show direct correlation between the rate of apoptosis and levels of EF1A, and EF1A directing damaged proteins to the proteasome for proteolysis (18).

EF1Aa and EF1Ab are established reference genes in Atlantic salmon and other species (19, 7). The rate of protein transcription in a cell is relatively constant, and the role of EF1A genes are closely related to translation. It has been demonstrated that the genes are subject to transcription regulation (20). mRNA abundance of these genes may be kept stable due to their relatively constant demand. Previous findings indicate that EF1Ab is more stable than its counterpart, EF1Aa. This may be because EF1Aa is recruited to perform non-canonical functions that EF1Ab is not involved in. Specifically, roles in apoptosis may cause EF1Aa to be unstable during acute stress (6, 7). In any case after EF1Ab, EF1Aa is still more stable than the known alternatives (7).

Differentially expressed genes

HSP70

70 kDa Heat Shock Proteins, shortened to HSP70, are a group of chaperone proteins. When a cell is subject to stress the abundance of HSP70 protein and mRNA increases, which is why the gene or protein group is usually referred to as "stress genes" or "stress proteins". They were first discovered in connection to heat stress but were later determined to be involved in many other mechanisms. HSP70 interacts with proteins' hydrophobic segments in an ATP-dependent way. These proteins have diverse cellular functions. They assist in protein folding by helping newly synthesised proteins fold correctly, deal with misfolded and aggregated proteins, convey membrane translocation of proteins and control the activity of some regulatory proteins and cell signals. They cooperate with co-chaperones, ex. Bag-proteins which have been related to cellular stress responses like apoptosis and proliferation. They also cooperate with other chaperones causing a broad activity spectrum (21-27).

p38 MAPK

p38 MAPK (Mitogen Activated Protein Kinase 14) is a serine/threonine specific kinase. The functions and interactions of this protein are well studied in humans. Computational models infer its function in zebrafish (28). Primarily it phosphorylates serine or threonine residues in a substrate specific and ATP driven enzymatic reaction. It is also implicated in the formation of both apoptosis and cell cycle regulating protein complexes (29). Its functions are assumed to be homologous in CHSE-214 cells. p38 MAPK is one of the effectors of the MAP pathway. This pathway is of great interest to understand the cellular stress response and apoptosis (30, 31). It also interacts with the heat shock response pathway (*Figure 2*).

It has been proposed that the relocation of p38 MAPK to the nucleus is performed by HSP70. This claim is substantiated by a lack of a nuclear localisation signal (NLS) in p38 MAPK. HSP70 does have an NLS, as well as a nuclear localisation-related signal and a nuclear export signal. Furthermore, the localisation of HSP70 depends on the co-import of proteins. A study finds a co-localisation of p38 MAPK and HSP70 to the nucleus after stress stimulus, and that HSP70 may play a role in p38 MAPK's phosphorylation of MAP

kinase 2 (MK2) (32). HSP70's role in p38 MAPK's phosphorylation of MK2 was indicated by no change in phosphorylated p38 MAPK, but an increase in phosphorylated MK2 as HSP70 increased. This implies a relationship between the two proteins in the stress response (32).

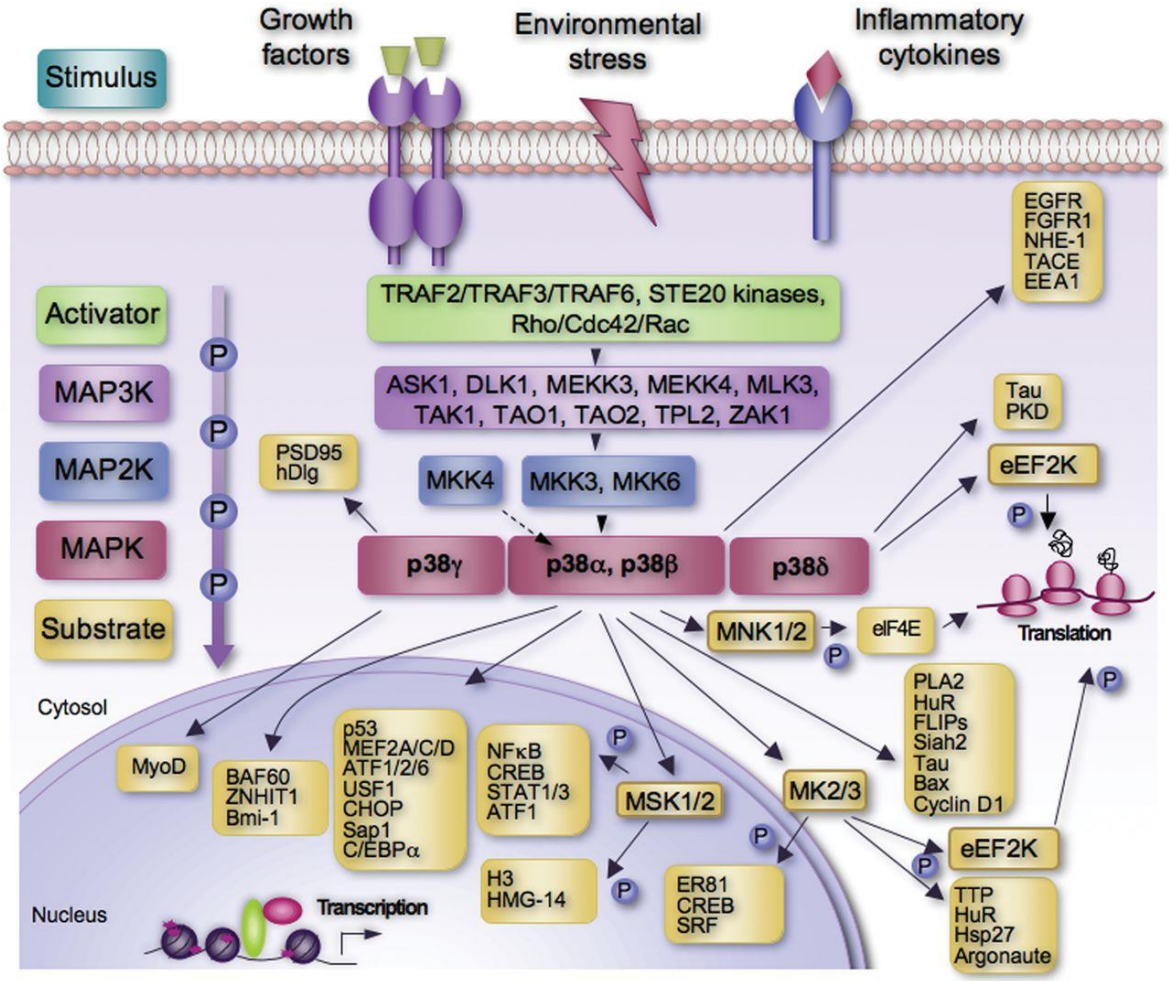


Figure 3. *The p38 MAPK MAPK pathway*
Different stimuli such as growth factors, inflammatory cytokines or a wide variety of environmental stresses can activate p38 MAPKs. A number of representative downstream targets, including protein kinases, cytosolic substrates, transcription factors and chromatin remodellers, are shown. CHOP, C/EBP-homologous protein; DLK1, dual-leucine-zipper-bearing kinase 1; EEA1, early-endosome antigen 1; eEF2K, eukaryotic elongation factor 2 kinase; eIF4E, eukaryotic initiation factor 4E; HMG-14, high-mobility group 14; NHE-1, Na+/H+ exchanger 1; PLA2, phospholipase A2; PSD95, postsynaptic density 95; Sap1, SRF accessory protein 1; STAT, signal transducer and activator of transcription; TAO, thousand-and-one amino acid; TPL2, tumour progression loci 2; TTP, tristetraprolin; ZAK1, leucine zipper and sterile-α motif kinase 1; ZNHIT1, zinc finger HIT-type 1. Reproduced with permission (29)

An observed increase in the transcription of p38 MAPK could serve as an indicator of cellular distress. This could precede G-1 cell cycle arrest. If p38 MAPK is not downregulated, it could lead to apoptosis. The transcriptional changes in apoptosis associated proteins, could serve as a yardstick to calibrate the temperatures conducive to salmon health. It is important to note that these interactions are inferred from research on humans and zebrafish. In CHSE-214 cells or live salmon these systems may function differently.

Gene expression analysis

mRNA represents the transcriptional level of expressed genes, to analyse cellular adaptions to stress on a transcriptional level, mRNA must be measured and quantitated. RNA is unstable and has a limited shelf life due to the ubiquity of RNase. There are two common workflows; two-step RT-qPCR, were cDNA is synthesised in a separate reaction, or a one-step RT-qPCR.

The synthesis converts mRNA to cDNA, facilitated by oligo-dT primers, which are selective for mRNA, and random hexamers, that are selective for any RNA molecule without a conformed structure (33). cDNA is further analysed with qPCR and target genes are defined by primers and probes.

In q-PCR amplification of template is detected by fluorescent probes after each cycle, in real time, detection may be specific or nonspecific. Nonspecific dyes such as SYBR Green, which is a commonly used dye, adhere uncritically to any double stranded nucleic acids. Hydrolysis probes are sequence specific and will only fluoresce when its specified target has undergone amplification (34).

The quantitative aspect of RT-qPCR makes it a powerful tool for gene-expression studies. It allows quantification of fold change in expression between independent samples. The quantification is dependent on a correction for the technical variance. Reference genes allow this to be done for all technical steps at once, because the mRNA of the target reference gene was present in the cells at the time of control or test.

ROX dye is used as a reference dye to normalise each well against a constant and known amount of active fluorophore. ROX dye is not bound to a nucleotide and its fluorescence is impacted only by the sum of interferences in the well. This include absorbance or scattering caused by fingerprints, dust bubbles or scratches along the light path, as well as chemical interference like quenching molecules or photodegradation of the fluorophores. It also accounts for instrumental error, except for module specific faults (fluorescence from different dyes are measured by different detector modules). The ROX signal is affected equally as the experimental dye and subtracting the intensity of the ROX signal accounts for many of these factors (Correspondence with teacher).

Hydrolysis probes

Hydrolysis probes such as TaqMan probes are sequence specific oligonucleotides with a fluorophore and a quencher at the 3’- and 5’-end respectively. These probes have a higher specificity than that of dyes, and this specificity is enhanced by the addition of a Minor Groove Binding (MGB) moiety, which increases melting temperature (Tm). The TaqMan MGB probe has additional specificity due to its short sequence length of 13-18 nucleotides, which decreases the possibility of nonspecific binding to highly similar sequences.

TaqMan MGB probes' quencher absorbs emissions at a wide range of frequencies and emits them as heat. Heat does not interfere with the instrument's fluorescence detector. Non-fluorescent quenchers eliminate the issue of quencher noise, and the necessity of longer probes as is the case of TAMRA-dye probes. Fluorescence Resonance Energy Transfer (FRET) only occurs if the fluorophore and quencher are compatible, and they are in proximity of each other. Compatibility between fluorophore and quencher requires only an overlapping spectrum of emission from the fluorophore with a spectrum of absorbance from the quencher (35, 36).

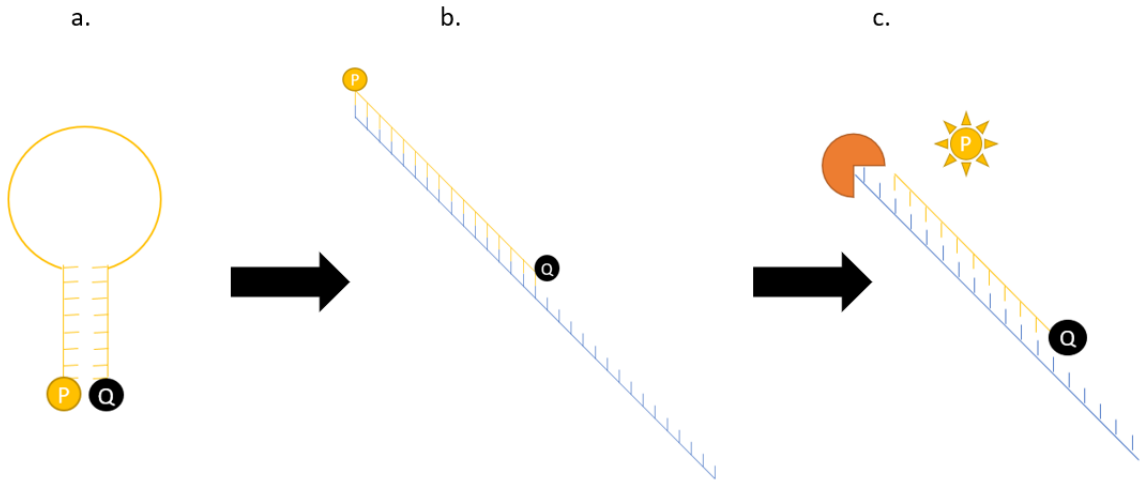


Figure 4. *General mechanism of action of a TaqMan hydrolysis probe. a. Unbound probe wraps in on itself and the proximity between quencher (Q) and fluorescence probe (P) inhibits signal. b. During annealing the probe binds to a complementary sequence. c. During extension the exonuclease activity of the DNA polymerase cleaves the fluorescence probe and quenching desists.*

Statistics and quality control in RT-qPCR

Limitations on interpretation methods

qPCR efficiency should be measured, to precisely interpret results from RT-qPCR. The efficiency is a number that describe how fast amplification progresses. The ideal amplification has [2n] copies where [n] is the cycle number, in practice reactions contain [Xn] copies. The Livak method for relative quantitation assumes nearly 100% efficiency and similar efficiency between each primer sets (37). While the Pfaffl method can be used to account for different efficiencies (38).

The measured PCR effectivity may exceed 100% if nonspecific product or primer-dimers are produced. The proportion of signal derived from nonspecific amplification can be difficult to assess. Such nonspecific product or primer-dimer products will cause lowered efficiency. The primer-dimer directly impact available primers, and nonspecific products will spend both primers and nucleotides reserved for the target product. To avoid this,

asymmetric concentrations of primers may be used in specific RT-qPCR assays, but in nonspecific assays this will lead to a false positive result.

Delta fluorescence

The absolute change in fluorescence follow a gentle downward curve, an abrupt increase and a resuming downward curve. To interpret the analysis, it is necessary to construct a reference curve from the cycles preceding the measurable signal amplification and expresses the results as fold deviation from this expected trend. This gives more reliable and precise results especially in assays that yield low signals.

Quality control of obtained sample material

Sample concentration was measured with the Qubit ® Fluorometer. It detects a light signal from a nucleic acid binding fluorophore. The fluorophores are specific to a single type of nucleic acid, and the instrument can detect most natural and laboratory nucleotides. The Qubit® Fluorometer can detect from 10pg/µl to 100 ng/µl dsDNA and 250pg/µl to 100ng/µl of RNA. It detects the emitted light from the sample/fluorophore complex and calculates the concentration of RNA or dsDNA, calibrating the calculation to the measurements from the standard solutions (*Appendix 14, 15*).

2^-ΔΔCt method for normalising RT-qPCR data

The 2^-ΔΔCt method was developed by Livak and Schmittgen as a mathematical tool for calculating fold expression change of selected assumed differentially expressed genes in RT-qPCR data (37). This method requires Ct values from one or more reference genes and assumed differentially expressed genes from samples taken under reference conditions and experimental conditions. The method makes some assumptions and is only precise if the efficiency of every assay is nearly equal and nearly 100%. The relationship between Limit of detection (LOD) of assumed differentially expressed genes and reference genes must also be constant. The Pfaffl method accounts for variation in efficiency and is applicable in assays where the 2^-ΔΔCt method is not useful (38). One of the strengths of the 2^-ΔΔCt is the use of an endogenous reference to normalise and correct results of differing amounts of input RNA (37). The mathematics behind the ΔΔCt method as described by Livak and Schmittgen and paraphrased:

Amplification in PCR is described by this equation. X_0 is the initial amount of target sequences, X_n is the amount of target sequences at cycle number n, and E is the efficiency of the amplification:

$$Xn = X0 \times (1 + EX)n$$

This equation describes the cycle threshold number: X_T and R_T is the threshold number of target molecules, $C_{T,X}$ and $C_{T,R}$ is the cycle threshold for their respective assays and K_R and K_X are constants:

$$XT = X0 \times (1 + Ex)C, TX = KX$$

$$RT = R0 \times (1 + ER)CT, R = KR$$

The relationship between target molecule thresholds must be constant, but does not have to be 1:

$$(X0 \times (1 + Ex)CT, X) \div (R0 \times (1 + ER)CT, R) = Kx \div KR = K$$

$$(X0 \times (1 + Ex)CT, X) \div (R0 \times (1 + ER)CT, R) = Kx \div KR = K$$

If efficiencies are equal for all assays, the following equation is true:

$$(X0 \div R0) \times (1 + E)CT, X - CT, R = K$$

ΔCt is defined as:

$$\Delta Ct = (CT, X - CT, R)$$

substituting $C_{T,X}$-$C_{T,R}$ for ΔCt gives:

$$(X0 \div R0) \times (1 + E)\Delta Ct = K$$

Substituting (X0/R0) for X_N representing the normalised amount of target. Or "Reference-fold" amount of experimental molecules present in the initial sample, and solving for X_N gives:

$$XN = K \times (1 + E) - \Delta Ct$$

This expression from samples 0-i is divided with the expression from the calibrator sample (that has not been subjected to experimental conditions):

$$(XN, i) \div (XN, c) = (K \times (1 + E) - \Delta Ct, i) \div (K \times (1 + E) - \Delta Ct, c)$$
$$= (1 + E) - \Delta\Delta Ct$$

Since this method presumes an efficiency of 100% or 1; (1+E) is always 2, the final equation is simplified to:

$$Fold\ expression\ change = 2 - \Delta\Delta ct$$

Reference gene stability

Reference gene stability can be evaluated by investigating variation of a reference gene Ct ($X_{i,j}$, where i=1, 2, 3…n_j and j=1, 2, 3…r) within a single point in time:

$$\left(Q1 = \sum_{i,j} \left(X_{i,j} - \bar{X}\right)^2\right)$$

This can be compared to variation across all time points:

$$\left(\mathrm{Q2} = \sum_j n, j \left(\overline{\mathrm{X}}_j - \overline{\mathrm{X}}\right)^2\right)$$

Variation between time points across a series can be evaluated with an F-test

$$\left(F = \frac{Q_{2(mA)}}{Q_{1(mB)}}\right)$$

A large value for F indicates high variation. For a data array with m_A= 40 and m_B=14 degrees of freedom the critical α=0.05 F-value is 2.266.

Significance testing of experimental data

Expression patterns of assumed differentially expressed genes was analysed with a chi square distribution test.

[X^2] values were calculated as:

$$X2 = \sum \frac{(o - v)^2}{v}$$

Where [o] is the observed value at [T(1-x)] and [v] is the expected value. To test the probability of the observed value deviating from the null hypothesis, [v] is defined as 1. The calculated values are then referenced against a chi square distribution table (*Appendix 1*).

2 Method and materials

2.1 Cell culturing

CHSE-214 cells were obtained from Sigma-Aldrich (91041114, Sigma-Aldrich, St. Louis, USA). The cell line was cultured in growth cabinets at 20°C in Gibco™ L-15 media GlutaMAX™ (31415029), with 10% Gibco™ Fetal Bovine Serum (FBS) (A3840401) and 100 µg/ml gentamicin (15705-60) (all from Thermo Fisher Scientific, Waltham, USA).

All handling of the cells happened aseptically in a laminar flow cabinet. Sub-culturing was performed by removing culturing media and rinsing twice with Hank's Balanced Salt Solution (HBSS) (SH30588.02, GE healthcare Life sciences, Chicago, USA), new pipettors used each time. Cells were detached with Gibco™ Trypsin-EDTA, phenol red (25200-072, Thermo Fisher Scientific, Waltham, USA) until >80% of the cells were detached. Trypsin was neutralised by adding culturing media, depending on the splitting ratio and vessel, from 1.0ml in wells and 4.0ml in medium flasks (10062-860, VWR, Radnor, USA). Cell suspension were not centrifuged to remove culturing media. Culture plates (10062-892, VWR, Radnor, USA) with 9.6cm^2 wells were chosen for the stress experiment. Eight 25.0cm^2 flasks were at approx. 70% confluence and each flask was seeded out to a total of 6 wells on two different plates.

2.2 Thermal stress induction

The thermal stress experiment was carried out with cells grown for ≈ 36 hours in 9.6cm^2 wells. All wells had an initial confluence of 65% - 70% and were presumed to be in a state of exponential growth. A sample size of N=3 at every time interval (*Table 1*) were stressed and harvested by the following method: medium was removed and 1,5ml RNAlater (1018087, Qiagen, Hilden, Germany) was added. The wells were scraped with a cell scraper (VWR, Radnor, USA), pipetted over to sterile collection tubes and directly stored at 4˚C. Two stress experiments were performed, first T_0 to T_{10}, and then later T_{11} to T_{14}, two 24 hours samples are overlapping.

***Table 1**. Showing when the different samples were harvested and what temperature they were held at.*

Sample	T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	T_{10}	T_{11}	T_{12}	T_{13}	T_{14}
Time (hours)	0	0,5	1	1,5	2	4	8	12	16	20	24	24	28	32	36
Temp. °C	20	26													

2.3 Lysis and RNA extraction

Lysis and homogenisation were performed in a TissueLyser II (Qiagen, Hilden, Germany). RNA extraction was performed with RNeasy mini spin kit (74104, Qiagen, Hilden, Germany). Cells suspended in RNAlater were centrifuged at 5000g for 5 minutes, supernatant was removed, cells were resuspended in Buffer RTE (74104, Qiagen, Hilden, Germany) and pipetted over to tissuelyser tubes on ice. Lysis and homogenisation by tissuelyser for 3min at 25Hz on each side. RNA extraction proceeded according to manufacturer's protocol for harvested cells (*Appendix 19*). Elution of RNA from the membrane was repeated twice with 40µl Nuclease free water (100030795, Thermo Fisher Scientific, Waltham, USA). RNA concentration was quantified with a Qubit Fluorometer (Invitrogen, Carlsbad, USA) and the RNA HS kit (Q32852, Invitrogen, Carlsbad, USA). RNA yield varied between 7 and 35ng/µl.

2.4 First strand cDNA synthesis

cDNA synthesis was performed on the RNA samples with qScript cDNA Synthesis kit (95047-100, QuantaBio, Beverly, USA) according to manufacturer's protocol (Appendix 13). 15µl of RNA sample was added as this corresponded to an amount of RNA within the specified range in all samples. Synthesis was performed on a 2720 Thermal Cycler (applied biosystems, Waltham, USA). cDNA was directly stored at -18˚C before qPCR analysis. DNA yields were between 7 and 35ng/µl. During the optimisation stages cDNA concentrations were measured with Qubit fluorometer and the DNA HS kit (dsQ32854, Invitrogen, Carlsbad, USA).

2.5 RT-qPCR primers and probes

Primers for EF1Aa, EF1Ab HSP70 and p38 MAPK MAP kinase were obtained from different suppliers (*Table 2*). TaqMan MGB hydrolysis probes were designed by and acquired from various suppliers (*Table 2*). EF1Aa and EF1Ab oligos' sequences were obtained from Olsvik *et al.* 2005 (7). HSP70 and p38 MAPK oligonucleotides were designed by Ann Kristin-Tveten using NCBI primer design tool. Primer specificity was confirmed by end-point PCR *(Appendix 12, 20*). Probe-binding and hydrolysis was confirmed in initial RT-qPCR optimisation runs.

Primers were BLASTed with the NCBI primer blast function. No cross specificity to any non-relevant genes were found in the genome of Chinook salmon (39). Pseudogenes and/or allelic copies were present for EF1Aa, EF1Ab and HSP70 genes, but minimum interference is expected from these as they are expected to be coregulated homologues and/or unexpressed.

***Table 2**. Oligonucleotides used in the qPCR assay: function, sequence, modifications and suppliers.*

Type/Target gene	Sequence 5'-3'	Modifications	Supplier
MGB Probe EF1Ab	CCAATACCGCCGATTTT	VIC, MGB	Applied Biosystems UK
MGB Probe EF1Aa	ATCGGTGGTATTGGAAC	6-FAM, MGB	Applied Biosystems UK
MGB Probe HSP70	GCCTGTGGAGAAAGCCCTCGG	CY5, MGB	Eurogentec
MGB Probe p38 MAPK	CCTTGCCTCAGATGCCCAAGAGG	JOE, MGB	Eurogentec
EF1Aa FWD	CCCCTCCAGGACGTTTACAAA	-	Invitrogen
EF1Aa RWD	CACACGGCCCACAGGTACA	-	Invitrogen
P38MAPK FWD	CGAGGCCAGGAACTACATCA	-	Eurogentec
P38MAPK RWD	GCTCCGATGAACACGTCAGA	-	Eurogentec
HSP70 FWD	TTCCGACTTCAGGGGAA	-	Eurogentec
HSP70 RWD	TTGGGCCTTGTCCATCTTGG	-	Eurogentec
EF1Ab FWD	TGCCCCTCCAGGATGTCTAC	-	Invitrogen
EF1Ab RWD	CACGGCCCACAGGTACTG	-	Invitrogen

2.6 Optimisation

Culturing, lysis, RNA extraction and RNA concentration protocols were verified in house. Well plates with 9.6cm^2 wells were chosen to limit the amount of cell material required and avoid clogging the RNeasy mini spin columns. Primer optimisation was performed with asymmetric forward (FWD) and reverse (RWD) primer concentrations (*Appendix 5, 10, 12*). Preliminary RT-qPCR results showed significant inhibition. This was resolved by lowering the template additions to below 400pg/µl. Positive results were yielded down to a template addition of 6.25pg total to the reaction. Probe optimisation was performed by choosing the optimum FWD and RWD concentrations and running a RT-qPCR with different probe concentration (*Appendix 5, 11, 12*).

2.7 qPCR

qPCR reactions were performed with qPCR PerfeCta® Multiplex qPCR SuperMix, Low ROX™ kit (95108-200, QuantaBio, Beverly, USA) on the AriaMX (Agilent Technologies, Santa Clara, USA) instrument with a 96 well-plate (VWR, Radnor, USA) and a reaction volume of 15µl according to the PerfeCta® Multiplex protocol (*Appendix 7*).

In the main experiment, a total of 4 plates were run. One of the biological replicate series was analysed in duplicate, and a second biological replicate was partially analysed in duplicate. These are referred to as parallel C and B respectively. Primer and probe concentrations used were obtained from the optimisation (*Table 3)*. Each plate had NTCs for all genes (*Appendix 3*). The first plate was run with 200pg/µl cDNA, the rest with undiluted cDNA. The program run was 3 minutes hot start at 95˚C, and 40 cycles of 15 seconds of denaturing at 95˚C and 60 seconds of annealing at 60˚C.

2.8 Data processing and statistical analysis

Data was processed in the AriaMX qPCR software, Ct values were exported and analysed according to Livak *et al.* (37). A 10x background fluorescence commonly gives good results and is the default setting of the AriaMX instrument (40).

3 Results

After the performed RT-qPCR assay, the Ct-values were exported to excel, where calculations were performed. Complete calculations and data of gene expression analysis are available (*Appendix 1*). These results have some missing parallels and were obtained over a time period of several months.

3.1 Primer and probe optimisation

Optimised concentrations of the primers and probes are shown in table 3. Optimisation tables are available (*Appendix 5, 10, 11*). 100nM of probe is enough for the HSP70 and EF1Ab assays, but 200nM of probe is utilised for EF1Aa quantitation and should be considered in the p38 MAPK assay. Amplification of p38 does not occur exponentially [2^n] and this indicates less than optimal PCR chemistry (*Figure 5*).

Table 3. *The concentrations of primers and probes used in this paper.*

Gene	FWD (nM)	RWD (nM)	Probe (nM)
EF1Aa	100	500	200
EF1Ab	500	100	100
HSP70	500	100	100
P38*	500	100	100
Optimisation of the p38 MAPK assay shows that a lower forward primer concentration and higher probe concentration may yield better results.			

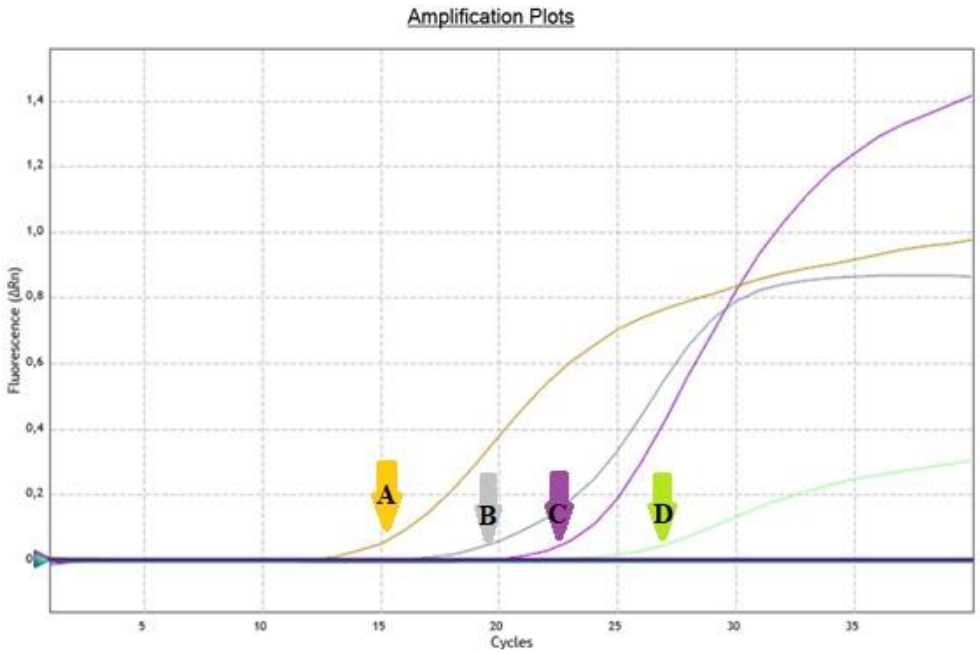


Figure 5. *Showing the amplification of the four genes, HSP70 (orange), EF1Ab (grey), EF1Aa (purple) and p38 MAPK (green) post optimisation. p38 MAPK is a candidate for re-optimisation as p38 MAPK is a candidate for re-optimisation because similarity to an exponential curve [2^n] is not present in its amplification plot, marked D. Similarity is adequate for other amplification plots, as marked by A, B, and C.*

3.2 Gene expression

Gene expression levels were calculated from Ct values, using previously stated mathematics and statistical approaches in excel (*Appendix 1*).

Reference gene stability

Table 4. *Ct-values of EF1Aa and standard deviations between technical parallels. Empty cells indicate lack of data.*

Time pts.	Gene	C-1	C-2	B-1	B-2	SD C	SD B
0	EF1Aa	27.81	27.76	21.43	21.22	0.03535534	0.14849242
1	EF1Aa	27.34	28.33	21.55	21.6	0.70003571	0.03535534
2	EF1Aa	27.49	28.44	21.22	21.47	0.67175144	0.1767767
3	EF1Aa	28.78	27.88	21.39	21.53	0.6363961	0.09899495
4	EF1Aa	27.54	28.74	21.58	21.66	0.84852814	0.05656854
5	EF1Aa	28.35	29.83	24.75	25.01	1.04651804	0.18384776
6	EF1Aa	27.64	29.56	21.96	22.07	1.35764502	0.07778175
7	EF1Aa	26.69	22.57	24.19	22.02	2.91327994	1.53442172
8	EF1Aa	22.28	22.75	21.88		0.33234019	
9	EF1Aa	23.76	23.81	22.31		0.03535534	
10	EF1Aa	22.33	23.07	22.74		0.52325902	
11	EF1Aa	21.76	22.32	22.76		0.3959798	
12	EF1Aa	21.87	22.3			0.30405592	
13	EF1Aa	21.43	21.18			0.1767767	
14	EF1Aa	21.97	22.74			0.54447222	

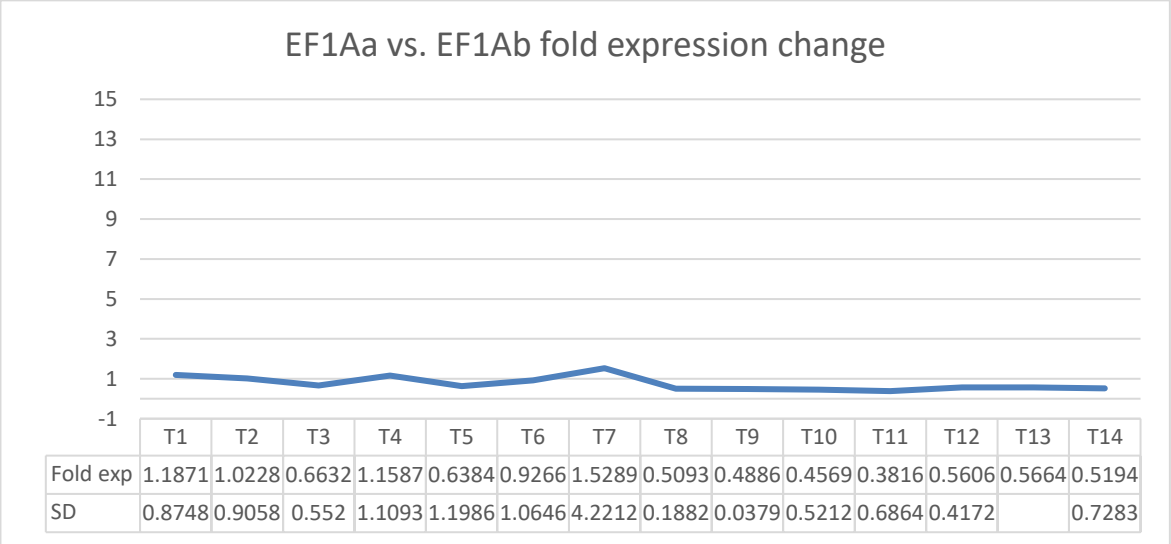
Table 5. *Ct-values of EF1Ab and standard deviations between technical parallels. Empty cells indicate lack of data.*

Time pts.	Gene	C-1	C-2	B-1	B-2	SD C	SD B
0	EF1Ab	25.08	25.48	17.01	17.41	0.28284271	0.28284271
1	EF1Ab	25.24	25.75	17.55	18.03	0.36062446	0.33941125
2	EF1Ab	25.11	25.82	17.08	17.5	0.50204581	0.29698485
3	EF1Ab	24.62	24.79	17.34	17.22	0.12020815	0.08485281
4	EF1Ab	25.66	26.29	17.39	17.79	0.44547727	0.28284271
5	EF1Ab	25.82	26.42	19.91	19.96	0.42426407	0.03535534
6	EF1Ab	25.55	26.53	17.67	17.8	0.69296465	0.09192388
7	EF1Ab	24.26	25.59	17.32	17.51	0.94045202	0.13435029
8	EF1Ab	18.12	18.56	17.38		0.31112698	
9	EF1Ab	19.46	19.45	17.94		0.00707107	
10	EF1Ab	18.49	18.37	17.96		0.08485281	
11	EF1Ab	17.77	17.57	17.4		0.14142136	
12	EF1Ab	18.02	17.86			0.11313708	
13	EF1Ab	17.3					
14	EF1Ab	18.23	17.97			0.18384776	

Table 6. *Variability of reference gene ΔCt as previously described*

$\bar{Q}$**1**	**Q2**	**F**	**Q1**$_{T7}$
0.450	0,365	1.31	13,5

The standard deviations of EF1Aa and EF1Ab are higher from T_5-T_7 (*Figure 6*). These values are affected by high standard deviations between the technical parallels of EF1Aa C-series (*Table 4, 5*). F-tests of the variance within each point in time against the variance over time indicate that the variance of each time point is equal to or smaller than the variance over time, except T_7 where variance is significantly higher. When excluding T_7 from the F-test the variation $\bar{Q}1$ and Q2 are statistically equal [α=0.05] (*Table 6*) (*Appendix 1*). Because of this, all results from this time point were excluded from further calculations. Excluded Ct values are available (*Appendix 1*). EF1Aa and EF1Ab mRNA levels were expressed stably. There is a near two-fold difference in mRNA levels from T_8 onwards, when compared to T_0.



	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
Fold exp	1.1871	1.0228	0.6632	1.1587	0.6384	0.9266	1.5289	0.5093	0.4886	0.4569	0.3816	0.5606	0.5664	0.5194
SD	0.8748	0.9058	0.552	1.1093	1.1986	1.0646	4.2212	0.1882	0.0379	0.5212	0.6864	0.4172		0.7283

***Figure 6**. Showing reference gene interstability, calculated as ΔΔCt of EF1Aa referenced against EF1Ab. Time point 7 (T_7) displays a much greater SD. The X-axis shows the time series, and the Y-axis shows fold expression change.*

Analysis range and viability

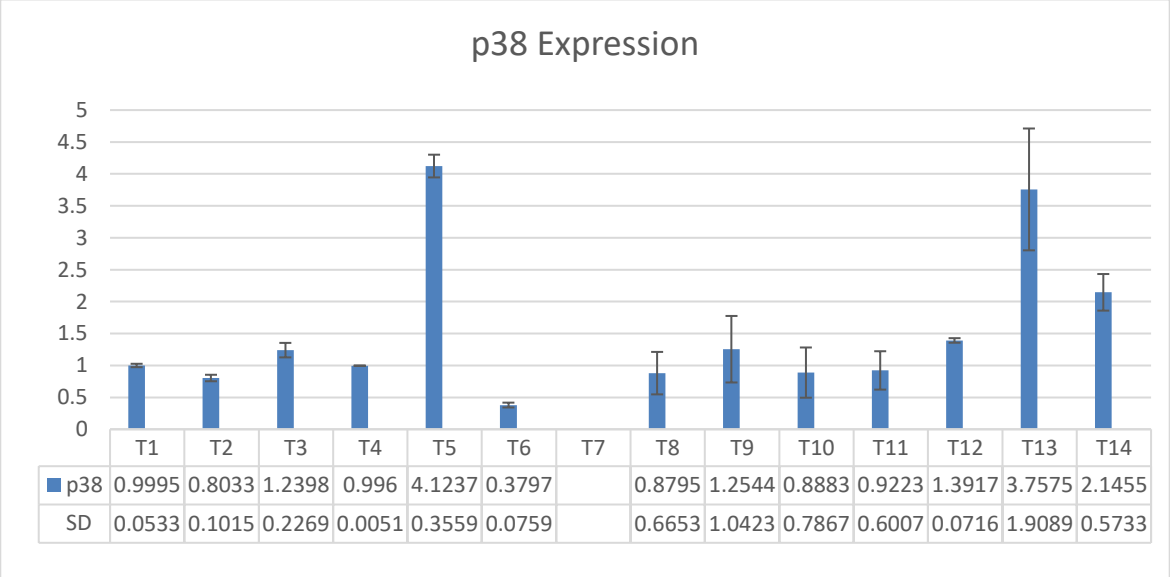
Abundance of EF1Aa is lower than the abundance than EF1Ab, but both yield Ct values between HSP70 and p38 MAPK assays at baseline cellular conditions. They are both detectable at very low additions of template (6.25pg).

HSP70 and p38 MAPK expressions

Fold expression change in p38 normalised against EF1Aa and EF1Ab on the same plate, was calculated with Livak and Schmittgen ΔΔCt (12) (*Figure 7*) (*Appendix 1*). Standard deviations were calculated by separation of each parallel, and for p38 MAPK expression standard deviations are minor compared to the [2^-ΔΔCt] calculations. This could indicate p38 MAPK expression regulation independent of the heat shock (*Figure 7*).

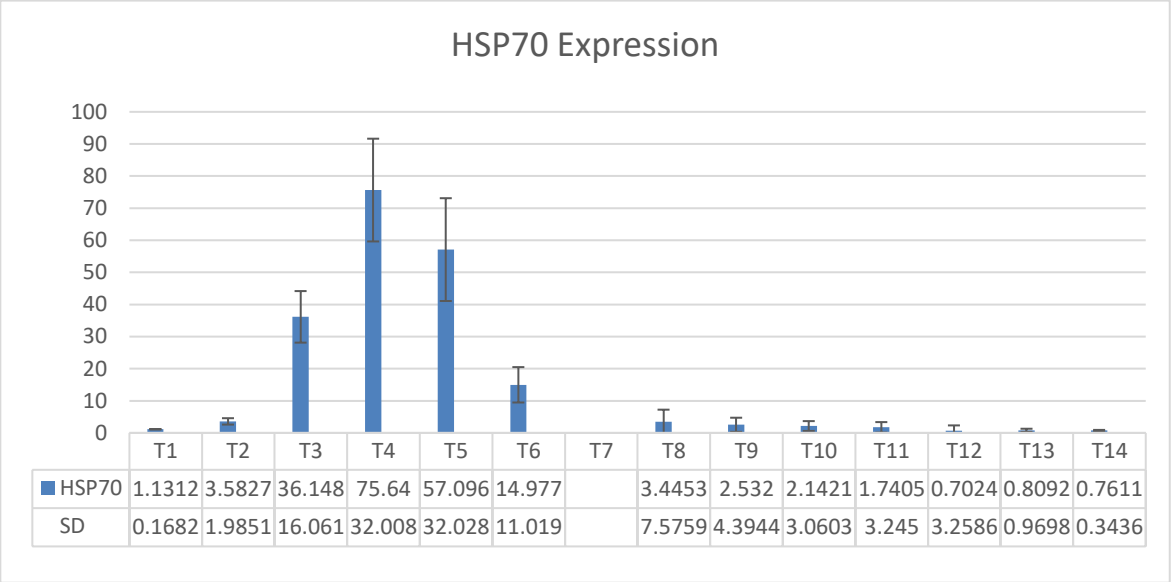
Fold expression change in HSP70 were calculated by the same method as p38. HSP70 expression increased rapidly after T_2, but returned to baseline levels around T_6-T_8. There were no significant changes after T_8 (*Figure 8*).

Fold expression change was also calculated from the average of the Ct values of parallels of p38 and HSP70 for each time point this shows expression changes over time of HSP70 and p38 (*Figure 9*) (*Appendix 1*). HSP70 has a peak at T_4 and a return to baseline expression from T_6 onwards. Increased expression of HSP70 is statistically significant [$p \leq 0.05$] (*Figure 9*) (*Appendix 1*). p38 expression changes are smaller and not statistically significant [$p \geq 0.05$] (*Appendix 1*).



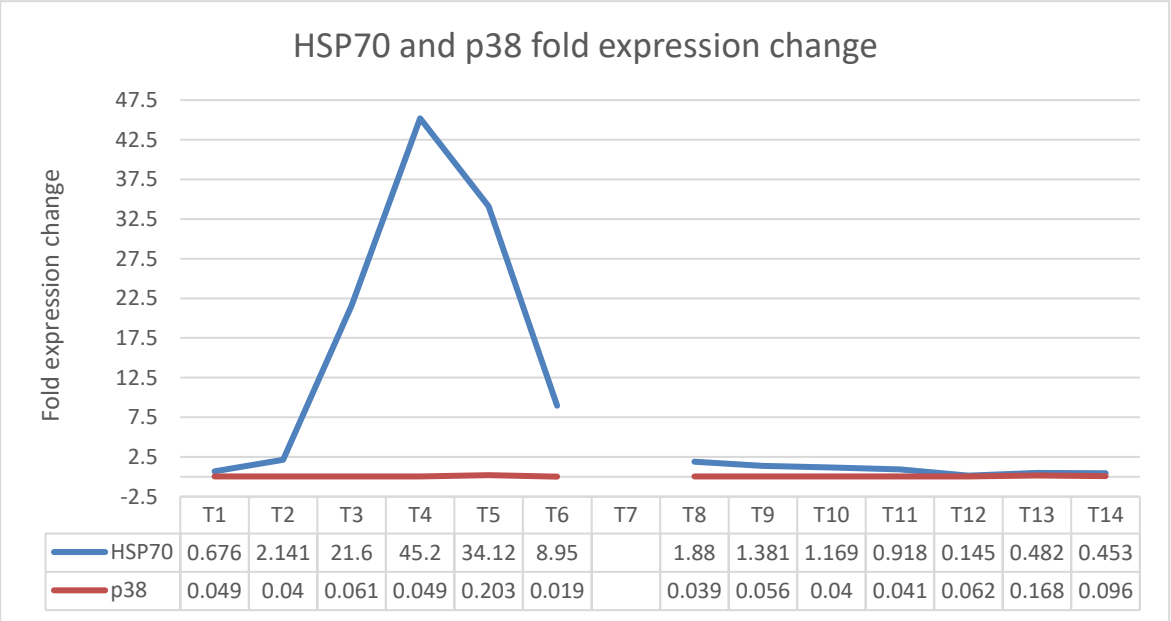
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
p38	0.9995	0.8033	1.2398	0.996	4.1237	0.3797		0.8795	1.2544	0.8883	0.9223	1.3917	3.7575	2.1455
SD	0.0533	0.1015	0.2269	0.0051	0.3559	0.0759		0.6653	1.0423	0.7867	0.6007	0.0716	1.9089	0.5733

Figure 7. *Fold expression change of p38 MAPK calculated with Livak and Schmittgen ΔΔCt method against the geometric mean of corresponding reference gene analyses (12). Each parallel time series has been separated to allow calculation of a standard deviation. Blue bars represent average of each parallel's fold expression change and the error bars represent the standard deviation for each time point. The X-axis shows the time series, and the Y-axis shows fold expression change.*



	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
HSP70	1.1312	3.5827	36.148	75.64	57.096	14.977		3.4453	2.532	2.1421	1.7405	0.7024	0.8092	0.7611
SD	0.1682	1.9851	16.061	32.008	32.028	11.019		7.5759	4.3944	3.0603	3.245	3.2586	0.9698	0.3436

***Figure 8**: Fold expression change of HSP70, calculated by the same method as p38 MAPK. Blue bars represent average expression and the error bars represent the standard deviation for each time point. Blue bars represent average of each parallel's fold expression change and the error bars represent the standard deviation, for each time point. The X-axis shows the time series, and the Y-axis shows fold expression change.*



	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	T13	T14
HSP70	0.676	2.141	21.6	45.2	34.12	8.95		1.88	1.381	1.169	0.918	0.145	0.482	0.453
p38	0.049	0.04	0.061	0.049	0.203	0.019		0.039	0.056	0.04	0.041	0.062	0.168	0.096

***Figure 9**: Fold expression change over time of the average of HSP70 and p38 MAPK's Ct relative to the geometric mean of EF1Aa and EF1Ab. Each data point was normalised against the corresponding reference gene on the same plate. The X-axis shows the time series, and the Y-axis shows fold expression change.*

4 Discussion

Primer and probes were optimised for *in vitro* expression studies in CHSE-214 cells, and EF1Aa and EF1Ab were validated as suitable reference genes in heat stress. The reference gene stability was statistically significant by F-test at [α=0.05] (*Appendix 1*), although there are indications that EF1Ab might be more stable than EF1Aa (*Appendix 1*). Primer and probe concentration for the two reference genes yield consistent and interpretable results. HSP70 transcription showed considerable increase from 2 hours through 8 hours, and the increase is significant [p=0.005] (*Appendix 1*). No statistically significant change in p38 MAPK expression was observed [p=0.10] (*Appendix 1*).

4.1 Applicability and impact

This study was a pre-clinical *in vitro* study of heat stress in salmonid cells and its applicability is to serve as a foundation for a clinical study of heat stress in salmonids. This study provides validated Real Time PCR protocols that can be utilised to measure transcriptional response to thermal stress in CHSE-214 cells, in such clinical studies. The protocols can be adapted for use in salmon tissue samples, and the adapted assays may assist in identifying thermal stress. Knowledge of the thermal stress levels following thermic delousing could assist in optimising the delousing protocols to maintain good fish health. Expansion of the assay pool should be possible to improve normalisation and provide a broader sense of the transcriptional changes.

The main principle of thermic delousing is temporary paralysis of detached salmon lice by immersion in 28-34˚C water for about 30 seconds (49). The lower temperatures are in use during the cold season, though it seems unlikely that 26˚C will be sufficient to delouse salmon in 30 seconds. A weakness of the thermic delousing strategy is that it does not remove attached lice, if 26˚C is not permissible to lice survival, or otherwise impact their ability to remain attached a longer-term immersion in 26˚C water could be a viable method for delousing (49).

4.2 CHSE-214 cell behaviour

Cell proliferation rate in CHSE-214 cells is influenced by cell density, most likely due to paracrine signalling. If cell density is too low initially, the exponential growth phase is delayed. Because of this a small change in split ratio influences the time it takes for cell dishes to reach 70% density greatly. In this experiment, to engineer 70% cell density at a given time cells were split 1:2 about 36 hours before the experiment, or 1:3 48 hours before the experiment.

After incubation at 26˚C for more than 24 hours a few cell dishes appeared to resume growth. Lannan *et al.* observed low cell numbers after incubation for 7 days at 26˚C, however those observations were made with culturing in glutamine free media, which is shown to slow growth significantly (16, 17). Resumed growth along with the return of HSP70 mRNA to baseline levels seems to indicate successful adaption to the elevated temperature.

For the duration that the cell culture was maintained abnormal morphologies, like elongated, flattened, translucent or sometimes abnormally large cells, were observed (*Figure 2*). The cause of morphological deviancy is not clear and is not thought to affect any experimental results. It may be that some select cells temporarily differentiate away from the baseline expression pattern of CHSE-214 cells.

4.3 Optimising and establishing RT-qPCR-assay

Primer optimisation

Uneven amounts of primers were utilised in every assay, 1:5 ratios. Optimal combinations were unambiguous for EF1Aa, EF1Ab and HSP70. In the p38 MAPK assay the most suitable combination tested in the optimisation was used. Equal primer concentrations may be optimal for the p38 MAPK assay. Because of ambiguity in the optimisation efforts it was not made use of for experimental RT-qPCR analyses.

Exon-exon junction spanning in the EF1Aa and EF1Ab primers were indicated by Olsvik *et al.* by alignment with zebra fish sequences (7). HSP70 and p38 MAPK primers were computationally predicted to span an exon junction with NCBI primer blast tool. This should limit the impact of genomic DNA contamination.

EF1Ab RWD primer had to be added in excess, this corresponds to a known mismatch between the primers who were designed for *Salmo salar* (GCF_000233375.1) and the *Oncorhynchus tshawytscha* (GCF_002872995.1) genome, which is the parent organism of CHSE-214 cells and may stack with other effects. To use this analysis on Atlantic salmon tissue samples primer concentrations must be optimised for the new assay.

Probe optimisation

Increasing probe additions did not seem to influence the ΔRF (delta raw fluorescence) which describes the relationship between background fluorescence and signal amplitude. This indicates that addition of probe beyond the limiting concentrations to obtain a good signal strength will not increase accuracy or precision. For the EF1Aa assay 200nm/µl probe was added as opposed to the 100nm/µl to ensure adequate amplitude of the raw fluorescence signal strength. The lower signal response from the EF1Aa assay may be caused by many different factors such as probe design, fluorophore quantum yield and

efficiency or even probe-target affinity. Inhibition due to excess primer or probe could be investigated further, if it is necessary to account for this imprecision in further studies.

4.4 Lysis techniques

Three different methods for cell lysis were evaluated and the technique most conducive to our lab workflow was selected for use in the thermal stress experiment. The methods were: 1. direct lysis in cell dish with buffer RLT and freezing, 2. cell removal with cell scraper and fixation in RNAlater, and 3. collection with trypsin and centrifugation to remove media.

For RNA extraction it was deemed important to minimise the period from removing the cell dishes from the controlled incubator environment and until fixation. To this end the most efficient methods add lysis buffer or fixation agent (RNAlater) directly to the dish, instead of lifting the cells from their growth surface with trypsin. Addition of lysis buffer after the removal of media and freezing in -80˚C is the fastest workflow. This method does not allow the samples to be kept in storage for long after freezing, because RNA degradation still occur during storage. According to the reference literature of the manufacturer addition of RNAlater preservation agent to the well and cell collection with a cell scraper allows samples to be transferred to tubes and kept at 4°C for up to 30 days before it is necessary to isolate RNA (*Appendix 18*). To minimise hands on time during the 24-hour time series, the latter method was selected for use in the thermal stress experiment. The lysis buffer-freezing method is optimal for quick sample collection during optimising and initial experiments, as one can quickly move to cDNA synthesis.

4.5 Sources of bias

Variable cell counts

Cell densities needs to be approximately equal across samples. A variation in cell density may introduce confounding factors to the findings. This would be expression changes independently of heat induced changes. As cells run out of space and resources to grow, the proportion of cells in active growth is reduced and transcriptional changes occur. If this happens during an experiment, erroneous conclusions may be drawn. By ensuring that all cell populations are in log-phase, these sources of errors are avoided. It was expected, as well as observed, that induced thermal stress would arrest cell proliferation. When this occurs while the cells are in log-phase, it is ensured to be due to heat shock and not crowding. Before stress induction, near-equal cell density was ensured in all experimental dishes. Cell density was re-estimated before harvesting and observed arrested cell proliferation in most dishes.

Template

Template concentrations should be verified and noted at one point in the workflow to ensure inhibitory amounts of template are not added. Because each assay has its own individual system of affinities and melting points, near inhibitory cDNA additions may cause inhibition in one assay but not others. Initial optimisation demonstrated this effect as the template amount was inhibitory. This was easily fixed by limiting the template amount.

An LOD was not established as cDNA availability or target abundance are not constraining factors in the assay. Positive detection of EF1Aa by addition of 6.25pg of cDNA was obtained during optimising analyses. Findings indicate that cDNA addition should be kept between 10pg and 400pg/µl. The standardised protocol for obtaining RNA from CHSE-214 cells yield good results (*Appendix 12, 19*).

cDNA measurement by qubit fluorometer is relatively imprecise and all pipetting steps necessary to dilute cDNA are relatively inaccurate because widely disparate volumes are mixed together. The "normalised" and presumed equal template additions may therefore wary considerably. If normalising cDNA concentration induces more imprecision than the isolation and synthesis workflow the dilution step should be excluded.

The Ct values of reference genes and assumed differentially expressed genes, scale in parallel with increasing template. This parallel change in Ct value does not affect the ΔCt calculation. Additional protocols that are optimised for the assay in question should yield cDNA concentrations in the qPCR reaction that does not inhibit the amplification. To obtain accurate results, each experimental analysis always needs to be independently normalised. All these arguments suggest that standardising template additions is not conductive to robust quantitation of mRNA in this study.

Ct threshold

In this thesis the computationally proposed Ct threshold was used for the majority of samples. In some assays it was marginally elevated above the proposed value. If these Ct thresholds weren't elevated a "no Ct" result was given upon exporting data. This error occurred very selectively for row 10 or 8, some RT-qPCR plates did not experience this error at all. The "no Ct" result occurred even though the amplification plots demonstrated successful amplification in all affected samples. Results obtained from row 10 and 8 did not deviate from comparable analyses or their technical duplicates. Due to this it was proposed that it most likely was a software bug.

Omission of common quality insurance steps

DNase digestion was not performed following RNA isolation. This step was omitted due to experimental conditions eliminating the necessity.

A No-RT control is a control run with RNA instead of cDNA to account for or detect genomic contamination. If the No-RT control is positive, steps should be taken to eliminate genomic DNA (41). Such controls were omitted because genomic DNA contamination should not affect the assays with exon-exon spanning primers.

A no amplification control is a control where the polymerase is omitted. This type of control is used to assess the stability or degradation rate of the probe. It can also serve as a baseline for background noise (41). This type of control was not deemed necessary for this study. The Aria-MX software determines background signal levels and corrects for the stability of fluorescence probe by calculating a background normalisation factor and correcting for the probe degradation slope when plotting the delta RF plots.

Neither an RNA integrity number nor a 260/280 absorption value was obtained due to time constraints. All samples were treated equally through the entire workflow, thus proportions of stress gene mRNA to reference gene mRNA should not be affected by partial RNA degradation. If degradation happened it should present equally across the sample range.

Width of data series

The maximum width of our data series is four points, pairwise technical duplicates of two biological parallels. Due to time and material constraints the data series are not complete. Calculations of expression changes in presumed differentially expressed genes is restricted by the availability of suitable reference gene measurements (*Table 4*). p38 MAPK data is further restricted by failed analyses of one biological duplicate from T_0-T_7. The restricted width of all series allows for a minimal amount of mathematical manoeuvrability. A few measurement errors could be conclusively excluded, but several suspected outliers could not be excluded in good faith. In a wider data series, the impact of such minor outliers would disappear in the averaging process. Because of the incomplete data, the significance of our findings drops sharply for all time points after T_7. This could weaken any conclusions drawn from data later in the experiment, however no significant expression changes are indicated by the data collected from T_8 and later.

Delay of HSP70 peak

The expression of HSP70 peaked at 2-4 hours, instead of the immediate peak described by other researchers (27). This may be explained by the time it takes to heat cell dishes up to 26˚C. Back of the envelope thermodynamics calculations show that the media did not reach 26˚C before an hour after incubation (*Appendix 1*). This delay can be avoided by preheating media, and then changing the 20˚C media to the preheated at the start of incubation. This does however complicate logistics. It is far simpler to place all well-plates in the 26˚C incubator simultaneously and to be aware of the cause of the delay.

4.6 Normalisation

Two reference genes were used to calculate relative expression change, and validation of expression stability of the chosen reference genes was performed. It may require a full study to isolate candidate reference genes from a larger pool. This study made use of previously identified reference genes in Atlantic salmon (6, 7). It made the process of verifying those findings shorter and less involved.

The previous description of EF1Aa and EF1Ab rank the latter as more stable (6, 7). This is supported by our own findings which show a two-fold expression change between the genes after T_8. Only two reference-genes were measured, and from the presented data, it cannot be conclusively demonstrated that EF1Ab is expressed with greater stability than EF1Aa. Why studies keep finding a skewing of the stability in favour of EF1Ab, is not entirely clear (6, 7). It is possible that between the two paralogues, EF1Aa experiences more flux in expression due to it being recruited to more non-canonical roles of the EF1 complex.

Time points T_5, T_6, T_7 show higher than normal standard deviance in Ct values in EF1Aa (*Table 5*). Multiple concurrent measurement errors in T_7 is what lead to excluding all data from this time point from further analyses. These are the only time points where the standard deviation reaches 1.0 or greater, peaking at 2.9 (EF1Aa C T_7, Table 5). These numbers indicate that the conflicting data in this study are most likely due to methodical error.

Vandesompele J. *et al.* argues strongly for including multiple reference genes (20). If the protocols in this study are adapted for use in other sample material, there are only some indication that EF1Ab would remain the most stable reference gene (20). They propose that three geometrically averaged reference genes should be used in expression studies.

The reference genes used in this study are validated reference genes for expression studies of Atlantic salmon, as described by Olsvik *et al.* (7). Caution must be exercised in any case and the validation performed by Olsvik *et al.* did not test stability during thermals stress. Independent research implicates the role of the EF1A complex in stress related roles and this involvement may cause its expression to differ under stress conditions. This thesis' findings are adequate for validation of EF1Aa and EF1Ab as reference genes. To gain any valuable insights on the stability of EF1Aa relative to EF1Ab would require broader and complete data sets of both parallels.

4.7 Interactions between HSP70 and EF1Aa.

During heat shock there seems to be a transient pause in translation elongation (42). Vera *et al.* finds that EF1Aa plays a role in the heat shock response in mammals (43). In the study, it was found that EF1Aa recruits HSF1 to HSP70's promoter and activates

transcription. It then stabilises and facilitates transport of HSP70 mRNA from nucleus to ribosomes (43). The cells in the study consisted of human and mouse cell lines and not piscine or salmonids. It still may raise questions of EF1Aa's role in heat shock response in ectomorphs, and its suitability as a reference gene in thermal stress studies. It would be prudent to re-evaluate the choice of reference genes to not include EF1Aa. Expanding the amount of reference genes to minimise the possible impact of EF1Aa instability is also an option.

Using mRNA as an indicator of cellular adaption

Change in gene expression does not always correlate strongly with change in protein level. To use an mRNA quantitation to infer protein abundance, the mRNA – protein relationship should be qualified. To do this, direct measurement of the protein must be made as well. Discrepancies and uncertainties with this relationship were described in 1998 by Gygi *et al*. stating that for true understanding of the biological systems in question a proteomic approach is also necessary (44).

Transcriptomic analyses are favoured over proteomic ones due to convenience. Measuring relative or absolute quantities of mRNA is simple and reproducible, when compared to the measuring of protein. Correlation between transcript and protein abundance can be as low as 40% (45, 46). The correlation seems to be affected by several processes on different levels of translation and these may be influenced by different cell states (47,48). There has been observed a delay in protein synthesis, with accumulation of ribosomes at the 5' end of the open reading frame, due to a transient pausing of the translation machinery in heat shock. (42, 48). This makes protein abundance from mRNA measurements impossible without proteomic studies (48).

A majority of studies on the correlation of mRNA to protein abundance have all been done in steady or normal state cells, instead of making a time series (42, 44-46, 48). Liu *et al.* did review long-term and short-term state stages, and steady state cells (47). Here the findings indicated at bulk-level and approximated steady states, that mRNA levels dictate protein levels. They found that during stress response or other highly dynamic phases, post-transcriptional processes are affected. Leading to stronger deviation from ideal correlations (47). Other research has found corroborating data supporting the idea that mRNA may increase during stress response without a corresponding increase in protein levels. Where cold adapted cells experienced a large increase in HSP70 proteins during heats stress while warm adapted cells had an increase in mRNA but not protein (27).

Since there is uncertainty with the mRNA to protein abundance, a genomic expression analysis will only give data on mRNA expression. To gain insights into actual protein expressions, under the conditions analysed in this thesis, proteomic analysis is advised.

4.8 Magnitude of expression change

A heat shock response lasting approximately 7 hours was measured with the upregulation of HSP70 was observed in the CHSE-214 cells upon incubation at 26°C. The swift return to normal levels and continued growth at incubation for 24 hours or more indicate successful adaption to the new temperature.

The HSP70 expression change in salmonid cells during heat shock has been investigated by end point PCR and quantitated with visual inspection (27). This quantitation method allows researchers to estimate large expression level changes, whereas RT-qPCR fold expression change analysis can allow for much finer resolution. The observed 70-fold increase in abundance of the HSP70 mRNA is higher than the generally reported expression changes from RT-qPCR studies. While expression changes at this magnitude are not uncommon, it is unusual to analyse them with RT-qPCR. Most gene expression analysis studies attempt to investigate changes that are too small to be analysed with end point PCR. The application of RT-qPCR for relative quantitation of HSP70 expression during heat shock is like searching for an aircraft carrier with a metal detector.

Because the experimental temperatures tangent the upper bound of the growth area it is unlikely that the cells can adapt to the temperature without permanent changes in protein levels. After adaption to 26°C HSP70 mRNA returns to pre-stress levels, however that does not mean that chaperone activity returns to the baseline level, nor that intracellular conditions concerning protein life span and stability remains unaltered. It would seem HSP70 mRNA is useful as a marker for recently initiated stress, but it may not be as useful to detect chronic stress.

The p38 MAPK expression changes are not significant, $p \geq 0.10$ (*Appendix 1*). This seems to indicate that any theoretical involvement of p38 MAPK in sub lethal heat shock is on the protein level. It would be interesting to see if this relationship changes with higher temperatures.

4.9 Fish health implications

Previous research indicates cell mortality when salmon are exposed to higher temperatures than what was used in this study, Poppe *et al.* describes tissue damage and cell differentiation changes in the gills and nasal cavities of live salmon exposed to temperatures of 28-34°C for 30 seconds (4). These findings suggest that temperatures of around 30°C are sufficiently high to cause lasting harm to exposed epithelial cells. Tissue damage from hyperthermia will induce prolonged stress and this could be a contributing factor to poor fish health.

The swift return of HSP70 mRNA to normal levels and indications of continued growth at incubation for 24 hours or more indicate successful adaption to the new temperature. This

indicates that the upper threshold conductive to good fish health in farmed salmon may lie around 26-28˚C.

4.10 Further research

The EF1Aa and EF1Ab assays as well as the HSP70 assay were optimised and applicable for high throughput research. However, the p38 MAPK assay needs further optimisation. Its target has no known mismatches in the *Oncorhynchus tshawytscha* genome and should yield high efficiency upon successful optimisation. The observed results of analysing p38 MAPK with excess RWD primer is a less than optimal amplification slope. Genetic drift in the CHSE-214 genome, or unintended interactions with other PCR components are possible explanations but it should be possible to establish a good protocol without resorting to any steps beyond running an extensive primer optimisation table.

Before the data is used for any real-world applications it is necessary to perform a clinical proteomic study of HSP70 and p38 MAPK. This is to confirm that changes in expression levels are real changes in protein abundances. Such a study can in turn be of use during investigations of thermal stress in live salmon. To make use of the findings in this study, it is necessary to investigate the impact of short-term thermal stress at temperatures below 28-34°C, previously described to cause tissue damages in live salmon, and to determine if these temperatures are sufficiently high to successfully delouse salmon.

Such studies could improve upon industry practices and help to understand the long-term physiological impacts stresses have upon fish development. Better fish health would increase yields and improve profit, which would allow for further development of the aquaculture industry.

As p38 MAPK is strongly implicated in the promotion of apoptosis it would be an interesting further avenue of research to investigate the expression changes of p38 MAPK when salmonid cells are exposed to higher temperatures. The nature of p38 MAPK transcription regulation when cells are exposed to lethal temperatures should be established because the differences between a survivable heat shock and a lethal heat shock in transcription patterns is more useful than the upregulation of HSP70 to indicate the fish health outcome of thermic delousing.

5 Conclusions

CHSE-214 cells appear to be a viable model organism for *in vitro* studies of gene expression patterns in heat stress. Data obtained from this gene expression analysis study indicate that EF1Aa and EF1Ab are suitable reference genes, despite the possible downregulation of EF1Aa, as indicated by external sources and our own data. Fold expression change for the reference genes are between 1.187- and 0.382. HSP70 expression levels serve as a strong indicator of recently induced heat stress, a fold expression change of 75 was observed. There is a lack of data regarding the expression patterns of p38 MAPK, this novel study indicates no significant transcriptional regulation at 26°C. It is possible that p38 MAPK transcription does not change unless cells experience distress.

6 Appendix

1. Gene expression data
2. EF1α mRNA
3. Final RT-qPCR plate setup
4. HSP70 mRNA
5. Optimisation plate setup
6. P38 mRNA
7. PerfeCta® Multiplex qPCR SuperMix, Low ROX™
8. Primer and probe HSP70 salmon info
9. Primer and probe p38 MAPK salmon info
10. Primer optimisation info
11. Probe optimisation info
12. Protocols
13. qScript® cDNA Synthesis Kit
14. Qubit dsDNA HS Assay user manual
15. Qubit RNA HS Assay user manual
16. Report RT-qPCR T0-T5 b stress 4.4.19, graph example
17. RNA concentration of all samples
18. RNAlater® Handbook
19. RNeasy® Mini Handbook
20. VWR Taq DNA Polymerase Master Mix

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