HSP70 RNA EXPRESSION

IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) CLONAL LINES

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of PILAR HEREDIA-MIDDLETON find it satisfactory and recommend that it be accepted.

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Abstract

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Several review articles have been published on the role of heat shock proteins, and their link to temperature in fish and other organisms. Heat shock protein 70 (70KDa) is the most commonly-expressed protein in response to thermal stress, and its expression has been associated with differences in thermal habitats. This thesis presents a general background and review of heat shock proteins, followed by a review of aquatic and marine organisms' response to thermal stress. In addition, I described the heat shock response to thermal stress of *Oncorhynchus mykiss* rainbow trout clonal lines; developed from individuals from different habitats. The experiment characterized response of three clonal lines, Arlee, OSU and Whale Rock. The expression patterns of hsp70 RNA from blood were quantified using real-time PCR. The hsp70 RNA expression patterns of these clonal lines were found to be significantly different. The most striking result was the lack of any detectable response by the Arlee line. Both Whale Rock and OSU hsp70 expression patterns showed a maximum induction temperature at 22°C; Whale Rock

expression was the highest in this study. There do not appear to have been any previous studies on relative quantification of hsp70 expression of blood in aquatic organisms or fish clonal lines.

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DEDICATION

This thesis is dedicated to my husband, Jonathan, and my two little boys, Curran and Owen.

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CHAPTER 1

HEAT SHOCK PROTEINS EXPRESSION IN FISH: AN INTRODUCTION & REVIEW

Several review articles have been published on the role of heat shock proteins, and their link to temperature and metal exposures in fish and other organisms (Ackerman 2000, Basu 2002, Iwama 1998 & 2004, Hofmann 1999). Heat shock protein 70 (70KDa) is the most commonly expressed protein in response to thermal stress and its expression has been implicated to differences in thermal habitats (Buckley 2001). The goal of this thesis is to characterize the heat shock response of *Oncorhynchus mykiss* rainbow trout clonal lines developed from individuals from different thermal habitats. The heat shock response will be characterized by the expression patterns of the hsp70 total RNA in the three clonal lines. It's important to understand protein function and characteristic expression patterns and variation under thermal stress. In this chapter I will review what is known about heat shock proteins. First, a general background will be presented, followed by reviews of studies with different organisms, and lastly what is known about heat shock response in fish.

BACKGROUND ON HEAT SHOCK PROTEINS

Heat shock proteins (hsps) are molecular chaperones which are essential to the cell. Chaperones assist proteins in folding, fidelity, and translocation. These molecular chaperones protect vital cellular functions by inhibiting faulty interactions that may produce pointless structures or processes (Ellis 1993, Maresca and Lindquist 1991). They are the cell's response to stress. Their expression has been closely linked with temperature since the 1930's, when they were discovered in the salivary glands of *Drosophila* fruit flies exposed to heat stress. They are called heat shock proteins for their initial link to high temperatures in *Drosophila*. Since the mid-1930's there have been numerous reports about animals' response to thermal stress that lead

to changes in phenotypes. In Himalayan rabbits, if local surface temperature is at, or lower than, 25°C a white rabbit would develop black ears, nose and paws. If the white rabbit is reared at temperature above 30°C the rabbits are all white (Klug and Cummings 2000).

By the 1940's it was observed that once hsps were induced other proteins disappeared. Then researchers began finding the hsps outside the salivary gland cells, such as in the brain, Malphigian tubules, and wing imaginal discs of *Drosophila*. It was not until the 1970's that definitive evidence associating hsps to the chromosomal puffs; purified mRNA hybridized to the puffs translated to specific hsps when added to *in vivo* systems. Heat shock responses were found in cells and tissues in chicken, *E.coli*, yeast, and plants. Heat shock genes were eventually cloned in eukaryotes and nucleotides sequences became available. In these sequences there was a high degree of similarity in certain hsps groups among different diverse organisms. Hsps expression increases as a response to heat shock in the same manner in many organisms from bacteria to humans (Hofmann 1999; Maresca and Lindquist 1991). Among the hsps, Hsp70 is the form most frequently induced by thermal stress (inducible form). Hsp70 is the most highly conserved of all the hsps; the DNA sequence is more than 50% similar among bacteria, yeast and *Drosophila*. Homology in the DNA sequences increases even further at the amino acid level between the fruitfly (*Drosophila melanogaster*) and *H. sapiens*, and between *Drosophila melanogaster* and *E.coli* to approximately 70% (Sørensen 2003).

The family of molecular chaperones include Hsp100, Hsp90, Hsp70, dnaJ (Hsp40), immunophilins, Hsp60, small heat shock proteins, and components of the steroid aporeceptor complex (Song *et al.* 2001). Hsp90, Hsp70, and Hsp60 are highly involved in cytoprotection.

Unstressed normal cells synthesize heat shock proteins in considerable amounts, i.e., they are constitutive (Petersen and Lindquist 1990, Maresca and Lindquist 1991, Currie *et al.* 1997), thus performing their chaperone functions in the cells. Parsell (1993) altered the number of copies of the gene, with additional wild-type copies or with antisense genes. These experiments resulted in different rates of thermotolerance in flies; high rates on the flies with extra copies and lower rates in the flies with antisense genes. The experiments demonstrated the importance of hsp70 in thermotolerance. Studies of whole embryo flies revealed that extra copies of hsp70 provided a faster response to high temperatures and thermotolerance, but no increases in survival to adulthood. In addition, Feder *et al.* (1999) found that unnecessary expression of hsp70 retards growth in tissue culture cells and whole organisms. Therefore, there are significant limitations to hsp70 expression.

Research in yeast (reviewed by Lund, 1991) shows that hsp70 can be inhibited or enhanced by co-chaperones. Co-chaperones or accessory proteins help hsp70 in its chaperoning functions, and are necessary because hsp70 alone doesn't refold proteins efficiently. In yeast, DnaJ and GrpE are co-chaperones, and these are involved in the ATP, ADP/ATP exchange rates of DnaK (the equivalent of hsp70 in yeast). In eukaryotes, Bag1, Hip, Chip, and Hop are the co-chaperones that may be involve in regulating hsp70 in some manner or other similarly to yeast.

The general response in *Drosophila* is (Krebs and Bettencourt, 1999):

- Under extreme stress hsp70 is expressed in high levels. With hyperthermia, but not extreme stress, the response is much lower than under extreme stress.
- No expression of hsp70 eliminates thermotolerance.
- Too much hsp70 expression can reduce thermotolerance.

- Tolerant *Drosophila* flies usually express more hsp70, they survive better under stress conditions, but do worse under normal conditions
- Individuals of the same line, with relatedness among individuals similar to full siblings, are more alike in thermotolerance than individuals from different lines.
- In genetically variable lines the induction of thermotolerance correlates to hsp70 expression.

Hsp70 expression and thermotolerance varies during development. At the pupal and adults stages *Drosophila* produce less hsp70 than when young and hsp70 levels weren't correlated to their thermotolerance. Pupae and young fruitflies are much more thermotolerant than later stages in development. The benefits of hsp70 expression vary with development. Hsp70 is beneficial early in the embryonic stage, early pupa, and early adulthood only (Figure 1 in Krebs and Bettencourt, 1999). Moreover, thermotolerance varied with the amount of hsp70 levels (basal levels). Feder (2003) described the variation in heat shock response observed in *Drosophila*, and classified the sources of variation into different areas in the genome that may be involved in regulating the response, but are "not mutually exclusive." These areas are: instability of the proteome upstream of the heat shock response, variation in the hsp coding sequence, hsp gene copy number, cis-regulatory elements, trans-regulatory factors, co-chaperones, co-factors, and variations in chaperone targets. The relative roles of these areas in regulation of the response are still unclear.

The induction of heat shock proteins under thermal stress arrests other cellular functions or causes malfunctioning, thus prolonged exposure is detrimental. These proteins are present

and expressed in various tissues and cells in different amounts depending on the magnitude of the heat shock, the duration, and the thermal history of the organism.

VARIATION IN ORGANISMAL RESPONSE

The general response to thermal stress observed in many aquatic organisms has three main traits: different expression of hsp70 isoforms, different activation and maximum temperatures, and basal levels. These traits have been observed in mussels (Hofmann and Somero 1996, Buckley et al 2001, Franzellitti et al. 2005), oysters (Piano et al. 2002, Hamdoun et al. 2003), nemertians (Okazaki et al. 2001), sea-urchins (Osovitz and Hofmann 2005), Prawns (Selvakumar 2005), marine snails (Tomanek and Somero 1999), and cnidarians (Rossi and Snyder 2001).

Some organisms show genetically driven responses (not altered by environmental stress) while others seem to be influenced by a combination of environment and genetics. Tomanek and Somero (1999) found genetic differences between congeneric marine snails (genus *Tegula*) in their enhanced, maximum induction, and inactivation synthesis. All four species of marine snails showed the same temperature inactivation for hsp70 (33°C, for reference see their Figure 5), regardless of acclimation temperature. Furthermore, the maximum temperature and activation temperatures didn't fluctuate much from control temperatures. It was concluded that thermotolerance was not modified by acclimation, adding that heat shock is energetically costly, and could lead to decreases in fitness. On the other hand, Hofmann and Somero (1996) found differences in response to thermal stress from two congeneric blue mussels (*Mytilus*) after

acclimation to the same temperature for eight weeks. The northern species *M. trossulus* (residing in a colder range of temperatures) showed a lower induction temperature, and higher levels of hsp70 overall, than its southern *M. galoprovincialis* (warmer) relative. It is worth emphasizing the fact that both of these investigations were on done congeneric species. Three of the four marine snails were collected from the same location (Pacific Grove, California). On the other hand, the congeneric mussels were collected from two latitudinal distinct areas; Garrison Bay, Washington and La Jolla, California. Buckley et al. (2001) showed similar results. They investigated the *M. trossulus* (northern species) induction temperature as a function of its recent thermal history. The comparison of its induction temperature (from winter (February) to summer (August), and field versus laboratory acclimated individuals) showed cold-acclimated organisms with more sensitivity to higher temperatures than warm-acclimated organisms. These are similar results to the earlier study by Hofmann on congeneric individuals. Individuals from the same species acclimated to different thermal ranges showed the same response as those individuals from the field, higher sensitivity to thermal stress when acclimated or accustomed to cold temperatures.

In oysters we see some differences in response as well. Piano (2002) investigated the *Ostrea edulis* response to thermal stress. The investigation demonstrated changes in hsp69 expression in gill and mantle tissues. Induction was found 4 hours post-exposure at temperatures of ≥38°C, 23°C higher than controls, and maximum induction in the gills and mantle were 24 and 48 hours post-exposure respectively. Alternatively, *Crassostrea gigas* thermal response showed limits that correlated with the changes in the inducible hsp70, and heat shock cognate protein 70 (hsc70) control levels were correlated with increases in ambient temperature.

Summer versus winter control levels of hsp70 mRNA showed non-significant differences, while the hsp70 protein levels were significantly different. Their investigation also showed that acclimation clearly helped the oysters handle thermal stress and increased survival. But they found "no extension in the upper thermal limits for survival" when comparing survival between summer and winter (see their Figure 8). This indicates some genetic basis in the upper thermal limits for this species. Hsc70 levels were associated with moderate increases in upper thermal limits of survival. The importance of hsc70 was also indicated in a study with the Mediterranean mussel. Investigations by Franzellitti (2005) revealed that hsc70 played an important role in cytoprotection in long term thermal stress, while the hsp70 seemed to have protective functions in acute short term exposures.

HSF1 increases are detected just before any increases in hsp70, and the connection between hsp70 and HSF1 is explained by the classical negative feedback loop model described by Morimoto (1993) and based on the proteotoxidity model. The proteotoxidity model is based on the observations that denatured or foreign proteins induce heat shock proteins. Hsp70 is highly expressed after heat shock treatment, reacting and mediating the further production of hsp70. Under no stress, hsp70 is bound to the HSF1 in the cytosol in an inactive form, possibly as a multi-protein aggregate (Morimoto 1993). When a heat shock occurs there is a denaturing of proteins in the cytosol. These denaturing proteins seize the hsp70 leading to the disruption of the hsp70-HSF1 complex and dissociation. Then HSF1 trimerizes, translocates to the nucleus (increases in HSF1 would be detectable) and binds to the HSE. The HSF1-HSE complex remains inactive until stress-induced phosphorylation begins. This is followed by the normal transcription and translation processes that lead to the expression of the hsp70. Thus, levels of

hsp70 in the cytosol are said to be responsible for the dissociation of the HSF1-HSE complex. HSF1 levels would increase before any increases in hsp70 RNA or protein. The negative feedback loop refers to the levels of hsp70 in the cytosol; a lack of hsp70 in the cytosol when demand is high (due to the thermal stress) would trigger the heat shock response.

Hofmann (1999) reviewed several heat shock responses in marine organisms and found seasonality in the expression levels and acclimation temperatures. Hofmann's study concluded "the interpretation here is that the animals are turning on the hsps genes at a later point following heat stress", showing the plasticity of hsp expression. Warm-acclimated organisms show higher basal levels and thermotolerance. Therefore, it has been concluded that induction temperature and heat shock differences are dependent on the thermal history of the organism.

THE HEAT SHOCK REPONSE IN FISHES

The hsp response in fish is comparable to that in other model organisms such as *Drosophila*, yeast and *E. coli*, providing evidence of the conservative nature of these proteins. The stress response in fish has been described as having three levels, primary, secondary and tertiary (Iwama et. al 1998). The primary responses are the neuro-endocrine and endocrine responses that are characterized by the rapid release of hormones into the circulation such as adrenalines, Cortisol, and catecholamines. There is evidence suggesting that there is a complex relationship between stress hormones and hsps (Basu *et al*. 2001 and 2003, Ackerman *et al.* 2000).

The secondary response is biochemical and physiological; representing the change in the blood chemistry in response to the hormone releases such as the production of glucose. The tertiary stress response is the decrease in growth, disease resistance, reproduction potential, smolting, and swimming performance with the consequent effects on population abundance and diversity. Fish are ectothermic (poikilothermic) organisms, which makes them very sensitive and susceptible to small changes in water temperature and chemistry. Consequently, a tremendous amount of research has been done in the last few years on heat shock protein's effect at the organism and cellular levels.

Several researchers have contributed important information on the function of hsps in fish. Their studies have been done using cell lines (cloned cells are capable of dividing, immortal lines), primary cell cultures (cells recently removed from the animal or tissue; maintaining the function or characteristics of the cell or tissue in vivo), and whole organisms (Iwama, *et al.* 1998). Overall, these proteins are expressed at higher than normal levels after thermal stress and hsp70 seems to be the most prominent protein expressed. Different fish species and different populations of the same species differ in their tolerance to stressors (Iwama, *et al.* 1999). Studies on cell lines (Kong *et al.* 1996) have several advantages; they are relatively easy to maintain, are uniform, plentiful, and have shown increased expression of different hsps (of various KDa sizes) due to temperature and metal exposure. Kong *et al*. (1996) examined the expression patterns of hsps in fish CHSE-214 cells (Chinook salmon embryonic cells) exposed to heat shock. The experiment exposed the cells to heat shock treatments at 24°C ranging from a few minutes to up to 30 hours. The analysis was done by SDS-PAGE with ^{35}S, Western and Northern blot analysis. The results showed expression patterns similar to those in other species, withhsp70 the major

polypeptide induced. But, in the long term heat shock treatment, the cells also induced two other proteins (40KDa and 45Kda). These proteins were absent, or in low amounts, in the recovery period (at normal temperature) and after short-term exposures. Hsp70 synthesis peaked in the first 2 hours and then quickly decreased; as this protein decreased hsp42 began to increase in the cell. It seems that these two heat shock proteins (hsp70 and hsp42) are expressed under different regulatory mechanisms; hsp42 continues high expression as long as the stress is present.

Ojima (2005b) observed significant induction of five hsps from *O. mykiss* RTG-2 cells. These were hsp70a, hsp70b, hsc70a, hsc70b, and hsp47, with the most obvious increases in hsp70a and hsp70b. The fact that he observed increases in hsc70 is new, since in most other investigations hsc70 was unresponsive to acute thermal stress. This could be due to the sensitivity of the technique (real-time RT-PCR) used.

Currie *et al.* (2000) examined the response to stress in the blood[a] and tissues of the rainbow trout (*O. mykiss*). The purpose was to compare the hsp70 mRNA levels between tissues and red blood cells *in vivo* and *in vitro*. In addition, the acclimation effects on the hsps expression levels were also investigated. Rainbow trout acclimated to 10°C were exposed to an increased temperature of 25°C (upper lethal limits) and held there for 3 hours. The fish were then returned to 10°C for 1 hour. The blood, heart, liver, brain, and the red and white muscle were collected and tested by Northern Blots. No hsp70 expression was observed under control conditions except for the blood hsp70 expression level which was apparent. Overall, under thermal stress every sample showed an increased mRNA level for both hsp30 and hsp70.

[a] Fish red blood cells are nucleated

Currie *et al.* (2000 and 1997) concluded that hsp70 mRNA expression levels can be accurately measured in erythrocytes and clearly reflected the expression levels seen in the tissues.

In the *in vitro* thermal acclimation experiments *O. mykiss* (500-1000g) was acclimated to two different temperatures, 5°C and 17°C, for a period of two months. Blood samples collected after the acclimation period of two months were given a heat shock of 20, 22.5, 25, and 27°C for 90-120 minutes. As expected, the hsp70 mRNA basal levels were higher in the warm-acclimated treatment than the cold treatment. Hsp70 mRNA levels increased dramatically in the warm-acclimated fish after a three degree temperature increase (20°C) while in the cold acclimated fish induction was at 25°C. This indicates a decreased induction temperature of 5°C from the cold to the warm acclimated fish. The hsp30 mRNA basal levels were barely detected prior to the heat shock. After the heat shock, the hsp30 mRNA levels increased by 40 fold in the cold-acclimated fish and by 20 fold in the warm-acclimated fish. The data showed no effect on the induction temperature of both proteins in the cold treatment. The cold-acclimated fish had an induction temperature for hsp70 (25°C) higher than the warm-acclimated fish (20°C). Thus, cold-acclimated fish blood showed an increased induction temperature for hsp70 and higher levels of hsp30 mRNA.

Acclimation or Thermal History

Most researchers have found that warm-acclimated fish tend to have higher induction temperatures. For example, Dietz and Somero showed (Iwama, 1999) that in warm-acclimated goby fish (genus *Gillichthys*) the 90 kDa hsp induction temperature increased. On the other

hand, Koban (Iwama, 1999) showed that acclimated catfish hepatocytes didn't show any changes in induction temperature. Whole organism studies such as Lund *et al.* (2002) showed that hsp70 mRNA induction was around 22°C-25°C in field and lab experiments. Miramachi Atlantic Salmon (*Salmo salar*) parr were collected from local rivers and brooks; where summer temperature was 16°C. The fish were held at 16°C in tanks, and a cold control tank was held at 7.5°C before heat shock treatments. The heat shock treatment temperatures were 22°C, 25°C, and 28°C and the fish was kept at this temperature for 1 to 2 hours before samples were collected (white and red muscle, bone and skin). Analysis was done by SDS-PAGE and Northern blotting with the use of ^{32}P probes. The results showed RNA levels were increased by 6-8 times (25°C and 28°C; a 10°C plus difference). Moreover, the lab experiments showed a greater increase in hsp70 RNA levels than field studies; the field hsp70 levels were only significantly elevated at 27°C. Temperatures in the small brooks in the summer can be very warm, and in fact the hsps were found to be elevated for 29 days per year in these organisms. Both (field and lab) organisms showed that threshold induction temperatures for this species are 5-10°C above their normal, which is consistent with other studies. Lab versus field induction differences may be due to the ability of the fish to find crevices or pools and refuges (day-night temperatures fluctuations) from the high temperatures. Another possibility would be that field animals had already experienced some natural heat shock and were therefore acclimated to changes in temperatures.

Hightower *et al.* (1999) looked at the heat shock responses of congeneric tropical and desert fish species. The treatments exposed the fish and cultured cells to their lethal temperature of 41°C. Analyses using SDS-PAGE showed that hsp70 threshold temperature (33°C) seems to

be linked to the most frequently selected temperature by the fish. On the other hand hsp30 was linked to high temperatures rarely selected by the fish; it was noted that perhaps these proteins had different modes of expression. This may explain the differences in expression seen in the Currie *et al.* (2000) study mentioned previously. Hightower also showed that one isoform of hsp70 (isoform 3) was strongly associated with high levels of thermal resistance. The higher the levels of hsp70 (all isoforms) in the fish, the higher the survival rate was at high temperatures. The authors noted that there may be several modes of acquiring thermal resistance by these tropical species.

The importance of acclimation on the heat shock response has been noted by many. Iwama (1998) reviewed stress protein expression in fish. In this review, Mazur *et al.* (Iwama *et al.*, 1998) observed no increase of hsp70 in red blood cells from cutthroat trout in the first hour after heat shock, but significant increases five hours later. Iwama reasoned that this result may be due to "sufficient" hsp70 in the cytosol at the time of the heat shock, thus resulting in some tolerance. In other words, high expression of this protein in the cytosol rendered the cell less susceptible and more tolerant to heat stress. It seems that there must be a threshold temperature when the stress response is triggered and it is this triggering of the expression that seems dependent on the environment.

TECHNIQUES FOR DETECTION OF HEAT SHOCK PROTEINS

Heat shock proteins can be studied at a variety of levels to answer certain questions: at the protein level (what does the hsp gene encode?), RNA level (where and when is the hsp gene

expressed?), and at the transcription and regulation levels (how is it regulated or activated?). The technique used depends on the question or research goal, the equipment available, the expertise available, the available funding and cost. There are several books on methods for proteins[b], RNA[c], and DNA studies that can be easily found in most libraries, many of the same procedures and precautions used in heat shock proteins are generic on this subject.

For protein studies, the most common methods are Western blotting and SDS-PAGE. Two dimensional SDS-PAGE is more powerful than one dimensional SDS-PAGE, but both of these techniques have been well standardized; many companies sell kits and reagents, thus it is relatively inexpensive. SDS-PAGE is used in conjunction with other methods such as Western blotting. Western blotting lets you visualize the heat shock protein bound to a protein-specific antibody in a membrane surface. Primary polyclonal antibody Rabbit anti HSP70 is followed up by a secondary antibody such as a goat anti rabbit IgG (Sigma Company). There are several commercially available rainbow trout antibodies to heat shock proteins. PMSF inhibitor of serine Proteases (leupeptin; 10µg/ml) is also highly recommended. The analysis relies on a comparison between the sample and positive controls for band intensity. Usually a scanner is used to semi-quantify the bands and their expression, which can be costly. Western blotting has been used as the main technique to detect the hsps. Thus, there are many protocols already

[b] For protein polyacrylamide gels and loading buffers see Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

[c] For RNA extraction and isolation the most common method is by P. Chomczynski and N. Sacchi, Single-step Method of RNA isolation by Acid Guanidium Thiocyanate- phenol-chloroform extraction. Analytical Biochemistry 162, 156-159 (1987).

available, and since the protein is highly conserved the need for species-specific antibody is low. The main concern with this technique is possible degradation by the proteases, a difficulty associated with all proteins.

For studies focusing on where and when the hsp gene is expressed RT-PCR, Q (quantitative) real time PCR, and Northern blotting can be used. RT-PCR and Q real-time PCR use little RNA, while Northern blotting requires a substantial amount (10µg per lane). Northern blots are used for RNA samples separated in a gel. The RNA is transferred to a membrane and hybridized with labeled (^{32}P) probes specific to hsps. The need for 10µg of RNA makes the technique useless for larval or embryonic stages of development, where the amount of starting material is small. The results are semi-quantitative since the analysis, like that for Western Blots, is based on a comparison with controls. Real-Time Q-PCR is a technique that enables the initial amount of the template to be quantified in real-time in a reaction; this method can therefore quantify the enhanced expression of heat shock protein RNA levels. Real-Time Q-PCR has applications in quantifying mRNA expression, genomic DNA or viral copy number, transgene copy number, allelic differences, and microarray data. Real-time Q-PCR works by measuring the product as it is amplified (in real-time) while still in the exponential growth part of the curve. By knowing the product amount in real-time (at a particular cycle), one can extrapolate the initial amount of template before the reaction began. It works by attaching a fluorescence probe to the target sequence, resulting in an increase of fluorescence as the product accumulates; the real-time thermocycler machine measures the fluorescence. Real–Time Q-PCR gives absolute quantification which relies on signal intensities representing the expression levels of the gene and is compared to a standard curve.

Real-Time Q-PCR is a very good alternative to other methods (RT-PCR, Northern Blots, etc.) when quantification is essential. RT-PCR and Northern Blots are sensitive, easily duplicated and accurate techniques to measure RNA levels. With RT-PCR large amounts of tissue, cells or blood are not required, so this method may be useful for juvenile or young stages that are more susceptible to changes or that may have greater susceptibility to effects on survival. All these techniques could enable the detection of very small differences in RNA levels. On the other hand, Real-Time Q-PCR is new, more expensive than RT-PCR (you need special thermocyclers, reagents, probes, etc.) and requires several controls. Another concern for this technique is the use of housekeeping genes for internal controls. Dheda (2004) found that several commonly used housekeeping genes vary slightly between samples (i.e. ß-actin, GAPDH). Even though these difficulties exist, the technique appears to be a very accurate way to detect low levels of RNA expression.

Microarrays (cDNA) for salmonids have been recently produced by Rise *et al.* (2004). The cDNA microarray is based on the cDNA libraries of several salmonids and reported 80,388 ESTs from five salmonids: Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), lake whitefish (*Coregonus clupeaformis*), all belonging to the family Salmonidae. Microarrays are expensive and they need both a special scanner and computer programs to analyze the data. On the other hand, the advantages of microarrays are many. This technique would enable us to see what other genes are expressed under thermal stress, and the degree of expression in the whole organism and/or specific tissues. Podrabsky and Somero (2004) used the microarray technology

to study the killifish *Austrofundulus limnaeus*. They observed changes in gene expression in response to long term acclimation to constant temperatures (20, 26, and 36°C); in addition to fluctuating temperatures of 20°C to 37°C. The small heat shock proteins appear more active when temperatures fluctuated while hsp70 and hsp90 responded to "strongly to chronic high temperatures." They hypothesized that perhaps HMGB1 (high motility group b1) proteins may act as a modulator of transcription, due to its roles in assembly of transcription initiation; a "global sensor" of thermal stress.

The recent advances in fish research inform us that we are still in the early stages of exploring the role of heat shock proteins and the triggering mechanisms that enable some fish to tolerate high temperatures better than others. It is well understood that acclimation plays a significant role in the induction of these proteins and that different organisms react to stress in the same manner by the induction of hsp70. What is still not clear is how this response is initiated, or why induction occurs at different temperatures regardless of the species' evolutionary preference. There are other questions that need to be addressed as well. What is the role of hsps in natural selection and speciation? How many genes control thermotolerance? Why do some species and/or individuals show differences in isoforms expressed? Why does restraining stress increase hsp levels in mice, but not in fish? New techniques such as microarrays may bring better understanding of the gene-gene, protein-protein and protein-gene interactions and the overall role the environment plays in gene expression.

It is important to examine the expression of these proteins in whole organisms as opposed to cell lines or cell cultures, because the responses vary from species to species. Moreover,

laboratory conditions are very different than field; both conditions should be observed whenever possible. There are fish clonal lines that have been develop from populations of hatchery individuals whose thermal habitats are different. The assumption would be that fish inhabiting areas whose water temperature ranges are relatively cold would express heat shock proteins differently (perhaps showing a lower induction temperature) than fish inhabiting warm water temperature ranges. Hsp expression would increase as the stress level increases in the fish. Do rainbow trout clonal lines developed from distinct thermal histories express hsp70 differently?

In the next chapter the results from the first quantification by real-time PCR of hsp70 total RNA in rainbow trout (*Oncorhryncus mykiss*) clonal lines will be discussed. The chapter has been written as ready for publication at a peer-reviewed journal; therefore the format follows the journal requirements.

CHAPTER 2

HSP70 RNA EXPRESSION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) CLONAL LINES

INTRODUCTION

Fish and other aquatic animals are exposed to a range of seasonal and daily temperatures. Salmon and trout living in small tributaries or even large rivers sometimes encounter very high temperatures with summer water temperatures as high as 25°C (Quinn 2005). They prefer cold temperatures in the range of 4°C to 15°C. Since fish are poikilothermic organisms; their internal temperature doesn't vary more than 1°C from their surroundings. Consequently, their need to respond quickly and efficiently to environmental changes in water quality (such as temperature) is great. One way to monitor stress responses related to temperature effects in fishes is by their heat shock protein expression levels.

Expression of heat shock proteins has been linked to increased immunoresponse to bacteria (Steward et al. 2001, Basu et al. 2002), increased development rate (Quinn 2005 –see page 148, Thomason 1996), neurological diseases (Morimoto and Santoro 1998), reduced metabolic condition in steelhead trout (Viant et al. 2003), age of fish (Murtha et al. 2003), and reproductive delays, among other physiologically important responses. Moreover, prolonged exposure to thermal stress is detrimental in all organisms and can lead to lethal or irreversible consequences (Song et al. 2001). These proteins are present and expressed in various tissues and cells in different amounts depending on the magnitude and duration of the heat shock and the thermal history of the organism (Iwama et. al. 1998 and 1999). The inducible isoform of heat shock protein 70 (a 70KDa protein; Hsp70) is frequently induced by thermal stress, and is the most highly conserved of all the heat shock proteins. Hsp70 cDNA sequences of rainbow trout revealed a 79% homology to those found in Drosophila melanogaster and S. cerevisiae (Kothary et al. 1984). Recent investigations of fish have used both wild and/or outbred laboratory-raised

organisms, and cell lines. Clonal lines developed from different thermal habitats would provide information on the differences and similarities in response to thermal stress, and may reflect their original thermal ranges. Clonal lines of individuals are genetically identical (isogenic; see Young et al. 1996). The goal of this thesis is to answer the question: do differences exist in hsp70 RNA expression among clonal lines developed from individuals of varying thermal ranges?

Most hsp70 studies in fishes have shown increased levels of hsp70 mRNA and protein as a result of the heat shock treatments (Kong et al. 1996, Currie et al. 1997 and 2000, Hightower et al. 1999, Lund et al. 2002, Ojima et al. 2005a and 2005b). Other studies have shown that some species of fish appear to have little heat shock response or subtle increases in Hsp70. The Atlantic cod (Gadus morhua) seems to express very little change in Hsp70 when exposed to their upper thermal limits (Zakhartsev et al. 2005). Place and Hofmann (2005) found that two Antarctic fish (Trematomus bernacchii and Pagothenia borchgrevinki) had lost their heat shock response (insignificant changes of hsp70); while a phylogenetically distant species (Lycodichthys dearboni) still conserved the ability to respond.

A number of studies have brought to light how little we know about the control of this response; is the controlling factor genetics, the environment or a combination of both? The variation in response is complicated by the fact that in some organisms thermal response is affected by the recent thermal history. The role acclimation has on the induction temperature for hsp70 RNA and protein expression has been investigated in fish and other organisms in recent years (Currie et al. 2000, Buckley and Hofmann 2002). Buckley &and Hofmann (2002)

observed in the goby (Gillichtys mirabilis) that warmer-acclimated organisms increase induction temperature as the acclimation temperature increases. On the other hand, experiments by Currie et al. (2000) on rainbow trout showed the opposite; warmer-acclimated red blood cells showed a lower induction temperature than cold acclimated cells. Little is known about the genetic basis for variation in regulation of heat-shock response between individuals or species. Recent reviews by Iwama (1998) and Basu (2002) have emphasized the importance of more research to try to understand the variation of heat shock response observed among tissues, species, and thermal histories of fishes and other aquatic organisms.

This study seeks to evaluate relative quantification of hsp70 RNA expression in red blood cells from rainbow trout (Oncorhynchus mykiss) clonal lines in response to acute thermal stress. Relative quantification of hsp70 total RNA by real-time PCR is a sensitive and precise method for detection of stress (Ginzenger 2002, Deprez et al. 2002, Bustin et al. 2005). Some investigators have previously used red blood cells for hsp70 studies and shown that it can be a non-lethal and reliable method (Currie 1997 and 2000, Lund 2003, for a review see Iwama 1998). The measurement of hsp70 in blood is also a very practical way to measure stress in either the field or in aquaculture facilities. These clonal lines are homozygous and developed from different source populations whose thermal ranges are somewhat different. Their original populations were exposed to different thermal regimes. The recently-developed Whale Rock, California line originated from the most southerly location, near San Luis Obispo, California. It may have been exposed to the warmest temperatures and therefore it could show some phenotypic differences in thermal response.

There do not appear to have been any previous studies on relative quantification of hsp70 expression in the blood (only on cell lines; see both Ojima et al. 2005 publications) or using fish clonal lines. Moreover, there have been very few studies on hsp70 on fish blood (Currie et al. 1997 & 2000 showed that blood was a reliable non lethal method to detect hsp70), and none have quantified the expression using real-time PCR. Therefore this experiment had several challenges to overcome: a new technique, small quantities of blood, limited number of individuals and clonal line availability.

MATERIALS AND METHODS

Fish

All homozygous clonal lines used in this study were obtained from the Washington State University fish hatchery (see Parson and Thorgaard (1985) for details on the production of clonal lines). The three clonal lines utilized were: Arlee a domesticated line (YY) originally developed from fish from the Arlee hatchery in Montana; OSU, an all female domesticated line (XX) developed from fish from Oregon State University, and Whale Rock (YY) which comes from Whale Rock Reservoir near San Luis Obispo, California and is a semi wild recently-developed line (2001). Most hatchery stocks of rainbow trout are derived from populations from the McCloud River, a tributary of the Sacramento River in California (Behnke 2002). These lines have been grown at Washington State University trout hatchery, under similar conditions (kept at 13°C at the time of collection), and they were all approximately one year old at the time the experiment was carried out.

Blood Collection and Experimental Design

The experiment was carried out over three (3) days: Day 1, Day 2, and day 3 (following Day 2). Blood (~100 µl) was collected from the heart on one (1) fish per clonal line per day, with a 1cc syringe with approximately 100 µl (volume was adapted as necessary to equal the amounts of blood) physiological saline solution (PSS[d]; as described in Currie et al. 1999) with 20 mmol/L EDTA. All materials used were sterile and RNAse free when necessary. Immediately after the collection, the blood was very gently mixed by hand with the PSS, and stored on ice.

Blood was transferred to labeled 1.5 ml centrifuge tubes in the laboratory and volumes noted. The blood was centrifuged at 450 x g for one to two minutes; the supernatant (plasma) was discarded and an equal volume of PSS added. 10µl of blood cell suspension (5µl blood and 5 µl PSS) was distributed into labeled sterile 1.5 ml centrifuge tubes (three per temperature), and 40 µl of PSS (kept at13°C) was added. Water baths were prepared earlier for each of the corresponding temperature treatments (13°C, 16°C, 18°C, 20°C, 22°C, and 24°C). Blood was acclimated for 30 minutes in the control temperature bath (13°C). Then, a set of nine centrifuge tubes was removed from control temperature and placed in the corresponding treatment water bath. Heat shock lasted 45 minutes; samples were then returned to the 13°C control temperature. Preliminary data on real-time-PCR using SYBR Green had shown that a 45 minutes heat shock was sufficient to obtain an increase in hsp70 RNA expression at 24°C. The time it took from blood collection to start of exposure experiments was less than one (1) hour.

[d] The physiological saline solution (PSS) consist of: 124.1 mmol/L NaCl, 5.1 mmol/L KCl, 1.9 mmol/L $MgSO_4$, 1.5 mmol/L Na_2HPO_4, 11.9 mmol/L $NaHCO_3$, 1.1 mmol/L $CaCl_2$, 5.6 mmol/L glucose, and 20 mmol/L EDTA to prevent clotting.

mRNA expression has been shown to reach significant levels within one hour of heat shock in lobster (Chang 2005), fish CHSE-214 cells (Kong. et al. 1996), and rainbow trout RTG-2 cells (Ojima 2005a and 2005b). In the brain, liver, gills, and gonads of mature zebrafish, expression increased after treatments ranging from minutes (Murtha et al. 2003) to one hour (Råbergh et al. 2000). Heat shock samples were centrifuged at 450g for one minute, supernatant removed, and TRIzol® Reagent added (1000 µl per sample; following the manufacturers protocol (GibcoBRL life technologies protocol, cat #15596-026). All samples (n=54) were stored at 4°C or on ice, then at -80°C until the end of the experiment.

RNA Isolation

Total RNA isolation was done following TRizol® Reagent protocol (Invitrogen; based on the method developed by Chomczynski and Sacchi in 1987). To ensure the removal of DNA, an additional step of Phenol-Chloroform extraction (at a ratio of 5:1) was done. This additional step was done after the addition of chloroform (in TRizol® protocol), centrifugation at 10,000 RPM for 15 minutes, and the aqueous phase (RNA) was transferred to a new 1.5 ml centrifuge tube. An equal amount of 5:1 phenol chloroform to the aqueous phase (RNA) volume was added. The Phenol and Chloroform were added directly to each centrifuge tube, no pre-mixing was done[e]. The samples were gently mixed by hand, and centrifuged at 10,000 RPM for 15 minutes. The RNA in the aqueous phase was removed and placed in a new centrifuge tube. This additional step was follow with chloroform as in the instructions of the TRIzol® reagent

[e] If the aqueous phase was 500 µl, then 416 µl phenol plus 84 µl of chloroform was added to each sample.

protocol. The final RNA pellet was dissolved into 30 µl of RNAse free water. All samples were stored at -80°C.

RNA Concentration

The concentration of RNA was determined spectrophotometrically by measuring the absorbance at A260 nm. RNA with the concentration of 40 µg per ml has an optical density of 1 at A260[f]. At least three readings of equivalent concentration were done per sample, to confirm quantification.

cDNA Synthesis

Reverse transcriptase synthesis of cDNA was done with 0.4µg of RNA per reaction[g], and following the protocol of superscript II RT PCR by Invitrogen (cat#18064-022). I used 0.5µl or 100 units of Superscript ™ II RT instead of the 200 units recommended. The cDNA was dissolved in RNAse free water to a final volume of 100 µl.

Real-time-PCR

Real-time-PCR is a very good alternative to other methods (e.g. RT-PCR, Northern Blots) because it doesn't require a large volume of blood, can remove cortisol (Sathiyaa et al.

[f] O.D. reading x ((40 µg/ 1000 µl)/ 1 O.D. unit) x dilution factor = concentration in µg/ µl.

[g] (Final Volume x Final concentration) / (Initial concentration of RNA)= 1(µl) x 1(µg/µl) / (concentration), adjusted amount of µl used accordingly.

2001), is an accurate quantification (based on real-time) and can be duplicated easily. This technique enables the detection of very small differences in hsp RNA expression.

Real-time PCR works by measuring the product in the exponential growth part of the curve as it is amplified. By knowing the product amount in real-time (at a particular cycle number) one can extrapolate the initial amount of template before the reaction began. It works by attaching a fluorescence probe or adding a fluorescence dye to the target primer sequence, being amplified, resulting in an increased fluorescence as the product accumulates; the real-time thermocycler machine captures the fluorescence. A cycler threshold (Ct) value is measured and plotted at a time when the sample fluorescence is significantly higher than the background fluorescence. The Ct value is the cycles required to obtain the fluorescence necessary to reach the threshold and it is directly proportional to the amount of template. Ct values are the average of triplicates for both target and control genes, all results are normalized to the control gene, called ΔCt. Each sample ΔCt value was normalized to OSU 13°C ΔCt values; which gives the ΔΔCt values[h]. This last step of normalization was done for two reasons. The first reason was to be able to compare relative values of hsp70 between clonal lines, and the second was to normalize to one mean GAPDH value. GAPDH Ct values were found to vary slightly with temperature (see Figure 1); although preliminary data using another clonal line with conventional PCR had shown otherwise. There are some concerns on the use of housekeeping genes for internal controls (Dheda et al. 2004) GAPDH showed no differences among varying

[h] Ct of hsp70 – Ct of GAPDH = ΔCt per sample; ΔCt per sample - ΔCt OSU 13°C sample = ΔΔCt

temperatures, or individuals (n=2) on preliminary experiments (data not shown). The sensitivity of real-time PCR may reveal differences undetectable in RT-PCR.

TaqMan® Universal PCR master mix by Applied Biosystems (ABI catalog number 4304437) was the selected system for real-time-PCR (fluorogenic 5´ nuclease chemistry); I used a two step Real-time-PCR for RNA quantification. The real-time-PCR protocol used was ABI-TaqMan PCR protocol #PN 04304449, and User Bulletin #2 by ABI (both available from ABI web site). The probes were designed according to the ABI recommendations using Primer Express (ABI User Bulletin#2, and for additional information see M. Tevfik Dorak web site http://dorakmt.tripod.com/genetics/realtime.html). We selected GAPDH as our internal control based on our conventional PCR experience and preliminary SYBR green Real-time PCR studies on another clonal line (Hot Creek).

The TaqMan® MGB probe for hsp70 sequence that utilized was 5'-AAGGGCCGGCTCAG-3', fluorogenic labeled 6-FAM, and the TaqMan® MGB probe for GAPDH sequence was 5'-TTCGGCATCGAGGAGG-3' labeled with VIC. The hsp70 forward primer was 5'-CAAGATCACCATCACCAACG-3' and the reverse primer was 5'-AGGATGATGCACAGAGGGAG-3'; amplicon size was 108 base pairs. The GAPDH forward primer was 5'-GTAATGCATCTTGCACGACT-3' and the reverse primer was 5'-AGAACATCATCCCTGCCTC-3'; amplicon size was 187 base pairs. The sequences for Hsp70 and GAPDH amplicons were compared to those in the NCBI BLAST home page (http//:www.ncbi.nlm.nih.gov/BLAST/), and TIGR (http://www.tigr.org/tdb/tgi/index.shtml) and confirmed to be as expected.

The multiplex master mix primer final concentrations for hsp70 and for GAPDH were optimized and used at 200nM for both forward and reverse primers. The probe's final concentrations for both genes were 25nM and final reaction volume was 50 µl. The PCR universal thermal cycling conditions were 50 °C UNG activation step, a 95 °C AmpliTaq Gold enzyme activation, and 40 cycles of 95 °C denaturation and 64°C anneal and extension steps. Real-time-PCR was performed using ABI Prism 7300 thermocycler (Applied Biosystems). All samples were run in triplicate and in every 96 well plates three (3) random samples (chosen prior to runs, one per clonal line) were repeated as controls to quantify plate run variation. All reagents were the same manufacturer's lot numbers and mixed just prior to addition to the master mixes. The cDNA was 1/3 diluted in RNAse free water and 5 µl of this was added to each reaction. Real-time PCR was performed on two of the three aliquots taken from the individual fish.

Real-time-PCR Optimization

Relative quantification can be performed as either a standard curve or comparative method (see Applied Biosystems User Bulletin#2). The relative comparative method uses arithmetic formulas to generate the same results as the standard curve method. To use this method we first established the efficiencies of both genes (see Table 1). A 100% efficient reaction will yield a 10-fold increase in PCR amplicon every 3.32 cycles during the exponential phase of amplification ($\log_2 10 = 3.3219$). We found efficiencies for GAPDH to be 100.68% and for hsp70 it was 98.24% (2.44% differences) in the singleton reactions. The estimation of the

efficiency (E) of a real-time PCR assay can be calculated by the following formula: $E = (10^{-1/\text{slope}} - 1) \times 100$.

RESULTS

Relative expression values of mean hsp70 normalized to the OSU 13°C ΔCt values shows three different patterns of expression in these clonal lines (see Figure 2). Whale Rock clones show a steady increase of expression as temperature increases, reaching its maximum expression levels at 22°C (T_{max}); with a nearly 1.5 times expression increase from the control. The Whale Rock expression pattern shows a decline of hsp70 relative expression at 24°C. This suggests that Whale Rock may be a very sensitive organism to changes in temperature, and that 24°C may be its upper thermal limits. OSU expression pattern shows a decreased expression at 16°C to 18°C, and increases past control levels at 20°C. The increase is maintained until 24 °C, the maximum temperature reached on these expression measurements. OSU and Whale Rock T_{max} is at 22°C (with a 1.27 and 2.48 expression levels respectively; see Table 2). These data agree with most investigations on trout and salmon; that the induction temperatures for these organisms lies in the upper 20's °C. Arlee, on the other hand, shows no response to acute thermal stress. Basal levels of expression in all three lines show slight differences, but these were found to be non- significant ($P > 0.05$).

To establish the differences between the clonal lines we used a multi-group ANOVA Analysis of Variance with significant levels of $P < 0.05$ (see Table 3). Significant differences between the three clonal lines were found at 18°C ($P < 0.0153$) and at 22°C ($P < 0.0115$). No significant differences were found between the dates of treatment.

ANOVA analysis of variance between the clonal lines' ΔΔCt values shows slightly different results (see Table 4). Here, the P values are significantly ($P < 0.05$) different in the control temperature (13°C) and at 18°C, 22°C, and 24°C. Moreover, when comparing each line to each other for significant differences ($P < 0.05$), statistical analysis showed that Arlee was the clonal line most significantly different from OSU and Whale Rock at 18° C and at 22°C.

DISCUSSION

The hsp70 RNA expression in response to thermal stress by the Arlee, OSU and Whale Rock clonal rainbow trout lines were significantly different. This contradicts the assumption that as a conserved protein, hsp70 would show low variation in expression pattern within a species. However, it agrees with many investigations reflecting the plasticity of expression in thermal response found among aquatic organisms.

The most striking and important result was the lack of any detectable heat shock response by the Arlee clonal line. Arlee showed no increase in hsp70 RNA levels at any of the temperatures tested. The temperature difference between the controls and maximum temperature of exposure was 11°C. This maximum temperature of 24 °C has been shown to induce hsp70 RNA expression in rainbow trout (Currie et al. 2000), brook trout Salvelinus fontinalis (Lund et al. 2003), Atlantic salmon Salmo salar (Lund et al. 2002), and in the preliminary experiments on clonal and outbred rainbow trout (data not shown here).

The lack of response in the hsp70 RNA expression patterns to acute heat shock could result from a lack of hsp70 gene activation, RNA transcription (Currie et al. 1997) or higher Hsp70 protein levels in the cytosol, among many possibilities[i]. The activation of production of Hsps begins with the heat shock or stressor, a higher than normal temperature (>5° C). When a heat shock occurs there is a denaturing of proteins in the cytosol. The high temperatures, the accumulation of denaturing proteins, and aggregates of non-native proteins unbind Hsp70 from HSF1[j]. The free HSF1 is activated, trimerized, and translocates to the nucleus; where it concentrates during heat shock (Jolli et al. 1997). HSF1 activation may also be dependent on the acclimation temperature of the organism; as shown in the goby Gillichthys mirabilis (Buckley and Hofmann 2002). When the HSF1 is triggered by the heat shock, phosphorylation takes place, activating the heat shock element (HSE) in the promoter area and followed by gene expression. There are no introns in the induced form of the gene and thus the response is immediate; the non-induced form or constitutive form of this gene (hsc) contains introns (Zafarullah et al., 1992). The sensitivity to thermal stress of the Whale Rock and OSU lines may show that accumulation of denatured proteins is occurring in the cytosol, and the HSF1 has been triggered. Hofmann (1999) pointed-out that perhaps the changes in HSF1 levels affect the activation of hsp genes. There is also evidence that hsp70 bound to HSF1 in the cytosol may be involved in the release or deactivation of the HSF1 bound to HSE. Airaksinen (2003), agreeing with Hofmann's research, shows that the zHSF1 isoforms are involved in the specific response to

[i] Hsp70 as a chaperone is one of the many proteins and factors that are involve in the mechanism cytoprotection and protein rescuing.

[j] There are four different kinds of HSF in eukaryotes, and according to Morimoto (1993) and Jolli et al. (1997), it is HSF1 that binds to the heat shock element (HSE).

heat shock of the zebrafish, Danio rerio. HSF1 has been shown to alter embryonic growth in female mice ($hsf1^{-/-}$) who lack this protein (Christians et al. 2000). Christians' study shows the importance of hsf1 for development, bringing into question the role of HSF1 in the development of rainbow trout. The activation of the HSE followed by the normal transcription and translation processes is what leads to the expression of the Hsp70. It is the presence of Hsp70 in the cytosol in addition to changes in HSF1 (levels, location, and state) that may trigger further induction and response.

Lack of heat shock response has been seen in other species recently. Place and Hofmann (2005) found no significant changes in hsp70 induction in two closely-related Antarctic fish species. These species have lost their ability to induce hsp70, and this loss was attributed to changes in the expression of hsp70 gene from an inducible state to a constitutive state. The changes in expression may occur when the heat shock response is no longer necessary for the organism. Hsp70 expression is costly for organisms, reducing resources for growth, rapid development, and longevity (Sørensen 2003). Hormones have also been shown to affect the expression of hsps (hsp70 and hsp90); maybe through their association with the glucocorticoid receptor (Basu et al. 2001 and 2003, Sathiyaa et al. 2001). In this experiment, plasma was removed as soon as the blood was collected. However, the blood was not tested for cortisol before or after experimentation.

The increases in hsp70 RNA expression at 18°C and at 22°C in the Whale Rock line were unexpected. This semi-wild clonal line was recently (2001) developed from a strain whose range may have encountered relatively warm temperatures during the summer months (central

California). Therefore, it was assumed that the expression pattern would resemble that of organisms acclimated to thermal stress; higher temperature tolerance or extension of the upper thermal limits. The trend between exposure to thermal stress and higher acclimation had been reported for other aquatic and marine organisms (Hofmann 1999, Halpin et al. 2002), such as marine snails (Tomanek and Somero 1999), mussels (Hofmann and Somero 1996), and oysters (Piano et al. 2002, Hamdoun et al. 2003). However, Currie et al. (2000) saw lower induction temperature with warm acclimation in an in vitro study with red blood cells. In Currie's study the warm-acclimated cells showed a lower induction temperature than cold-acclimated cells (5 °C lower than cold acclimated). The researchers suggested that perhaps the warm acclimation may alter protein stability, increasing the accumulation of misfolded and denatured proteins in the cytosol. This accumulation may trigger the increased expression of hsps. The Whale Rock individuals show the highest basal levels (although not significantly) of hsp70 RNA and its induction temperature, which was lower than expected, resembles the results of warm-acclimated in vitro cells by Currie et al. (2000). In this experiment, all clonal lines had been grown under the same conditions; therefore induction and T_{max} seem to be an intrinsic response. Krebs and Bettencourt (1999) thought that higher basal levels in Drosophila represent the cell's clues for possible environmental changes. Nakano and Iwama (2002) saw higher hsp70 basal levels in the liver of the field tidepool sculpin; which experiences the highest range of temperatures. This would seem consistent with high basal levels of Whale Rock individuals in the present study.

As the first observations of hsp70 RNA expression in these clonal lines, it may be too early to have complete understanding of the observations. Their significant difference, in relative Hsp70 RNA levels, suggests that Whale Rock is more sensitive to changes in water

temperature than domesticated OSU and Arlee lines. Arlee shows no induction of expression of hsp70 RNA under the conditions in this study. A consistent lack of response by Arlee would confirm the results, and this line could be used in crosses to other lines to identify the specific loci responsible for its lack of response. Both genetics and environment may play a combined role in the thermal response seen in these rainbow trout clonal lines. To continue Hsp70 expression studies in response to thermal stress with other clones from distinct habitats, in addition to HSF1 and initial hsp70 proteins basal levels, may help understanding the complexity of the heat shock response.

TABLES

Table 1: Real-time PCR efficiencies (10^(1/Slope)-1)

	GAPDH singleton Rxn	Hsp70 singleton Rxn	GAPDH multiplex Rxn	Hsp70 multiplex Rxn
Slope =	-3.3058	-3.3648	-3.4598	-3.3676
10^(1/slope) -1 =	1.007	0.982	0.946	0.981
(10^(1/s)1)*100 =	100.68%	98.24%	94.55%	98.13%

Table 2: Relative Quantification of hsp70 expression ($2^{-\Delta\Delta Ct}$ Values)

Temperature (°C)	WR	Arlee	OSU
13	1.22	0.47	1.00
16	1.27	0.25	0.70
18	1.75	0.32	0.80
20	1.83	0.33	1.23
22	2.48	0.33	1.26
24	2.14	0.37	1.22

Table 3: ANOVA analysis of Hsp70 levels between clonal lines.

3 fishes per clonal line (n=3)

Temperature (°C)	hsp70 means	d.f	F-value	P
13	0.91	2	3.15	0.1508
16	0.82	2	3.42	0.1359
18	0.99	2	14.15	0.0153
20	1.29	2	2.29	0.2177
22	1.46	2	16.63	0.0115
24	1.36	2	3.21	0.15

Table 4: ANOVA analysis of ΔΔCt values between Clonal lines

Temperature (°C)	ΔΔCt means	d.f	F-value	P
13	0.51	2	7.56	0.0438
16	0.73	2	3.20	0.1481
18	0.39	2	14.98	0.0139
20	0.14	2	5.25	0.0761
22	-0.01	2	10.09	0.0274
24	-0.02	2	10.04	0.03

FIGURES

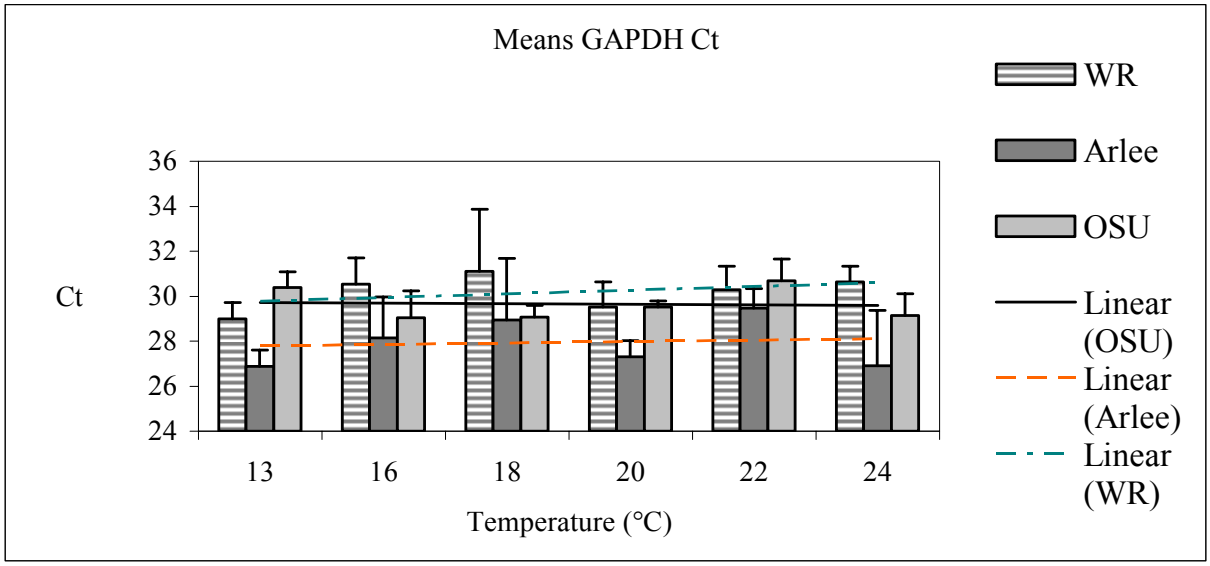

Figure 1: GAPDH Ct mean values ± S.D. (error bars) for the three clonal lines (3 individuals per clonal line; total n=9). Temperature is in degrees Celsius. Lines represent the regression line of the values per clonal line.

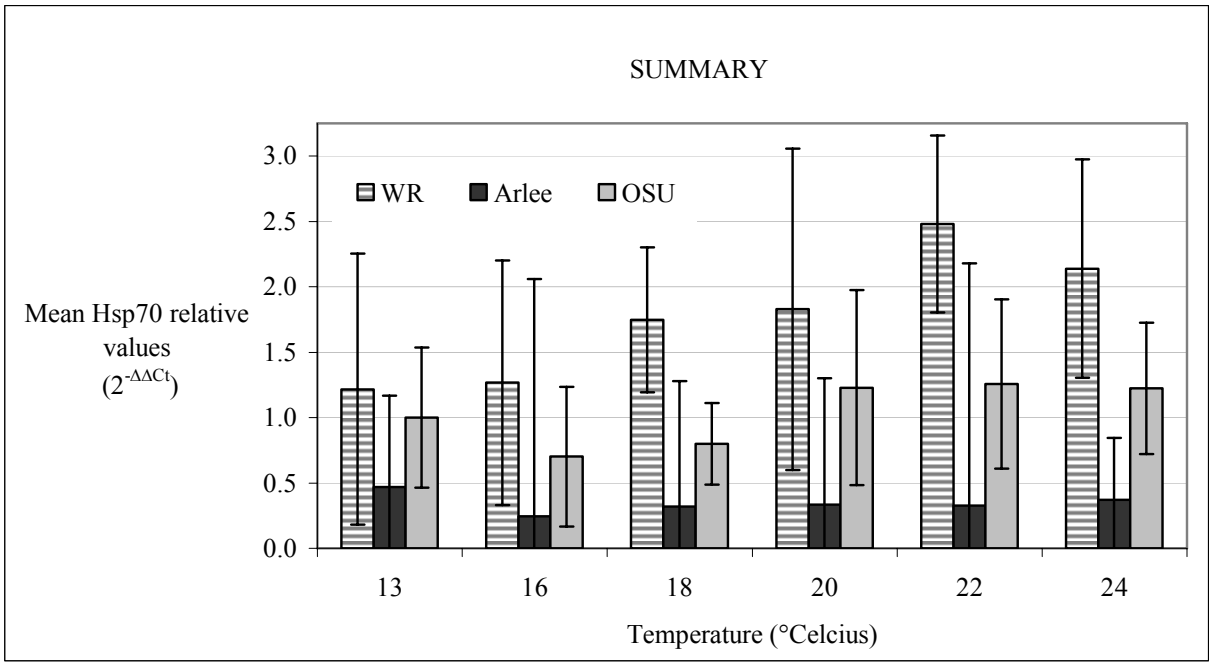

Figure 2: Relative expression values of the three clonal lines (Whale rock (WR), Arlee, and OSU) mean hsp70 normalized to ΔCt values of OSU 13°C. Bars represent the C.I. (95%) of the ΔΔCt values. Significant differences ($P<0.05$) were found at 18°C and at 22°C.

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