# THE CELLULAR HEAT SHOCK RESPONSE AND THERMOTOLERANCE OF FISHES

by

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## Abstract

The relationship between the cellular heat shock response and thermotolerance was examined in fishes. The first series of experiments was conducted on fishes from various latitudes whose body and water temperatures range from –1.7 °C in Antarctica to 37 °C in the sub-tropical ocean. Although all fishes synthesized 70-kDa heat shock protein (hsp70) under a heat stress, the induction temperature for hsp70 was strongly correlated with the habitat temperature of the species. A higher hsp70 mRNA level at a near-lethal temperature in the Antarctic fish, *Trematomus bernacchii*, was not associated with a higher level of hsp70. A part of the translational pathway from hsp70 mRNA to hsp70 might have been modified in this species during adaptation to extreme cold. Thus, they might not be able to increase the level of hsp70, at least, immediately after heat shock.

I chose the intertidal cottids as model animals to examine the relationship between the level of hsp70 and the thermotolerance of fish in more detail. The tidepool sculpin (*Oligocottus maculosus*) is known to be distributed over a wide range of the intertidal zone including upper tidepools where it can experience wider temperature extremes than in lower tidepools that are usually occupied by the fluffy sculpin (*O. snyderi*). The lethal temperature and the induction temperature for liver hsp70 were higher in the tidepool sculpin, while changes in hsp70 levels were more thermally sensitive in the fluffy sculpin. The relationship between hsp70 mRNA and hsp70 levels in those sculpins under heat shock imply that the cellular concentration of hsp70 may be controlled at the translational level in the tidepool sculpin, and at the transcriptional level in the fluffy sculpin. Higher viability under a severe heat shock at 28 °C was observed in the tidepool sculpin in its natural habitat than in the same species which was acclimated at constant 10 °C for 2 weeks in the laboratory. This result indicates that the fluctuating environment in the intertidal zone may enhance the resistance to heat in this species.

Overall results from this study indicate that, although the cellular heat shock response is conserved in various species of fishes, its function as well as the range of functional temperature seems to have been modified during the adaptation to the habitat temperature of the species. Studies in the intertidal sculpins imply that a relatively small difference in the habitat temperature can cause these modifications.

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# List of Abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| ATP | adenosine triphosphate |
| B.C. | British Columbia |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| BSA | bovine serum albumin |
| DDW | double distilled water |
| DEPC | diethylpyrocarbonate |
| DNA | deoxyribonucleic acid |
| DO | dissolved oxygen |
| EDTA | ethylenediamine-tetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| ENSO | El Niño/Southern Oscillation |
| h | hour |
| HHW | highest high water |
| hsp | heat shock protein |
| kDa | kilo Dalton |
| LDH | lactate dehydrogenase |
| LLW | lowest low water |
| MDH | malate dehydrogenase |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| mRNA | messenger ribonucleic acid |
| MS-222 | tricaine methanesulfonate |

| | |
|---|---|
| NBT | nitro blue tetrazolium |
| PCR | polymerase chain reaction |
| PPi | pyrophosphate |
| ppt | parts per thousand |
| rRNA | ribosomal ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| SE | standard error |
| SSC | sodium chloride and sodium citrate |
| TBS | tris-buffered saline |
| TTBS | tween-20 tris-buffered saline |

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# General Introduction

In this thesis, I examined the role of the cellular heat shock response in the thermal adaptation of fishes with different ranges of thermal tolerances. Environmental temperature can affect directly the biochemical reactions of molecules, cellular processes and function of tissues of ectothermic animals (Logue *et al.* 1995). Whole body metabolism, activity levels, spawning, development and growth of animals are also influenced by temperature. Therefore, environmental temperature is likely an important factor in determining the behavior and the geographic distribution of animals. In fact, Stefan *et al.* (1995) demonstrated that the presence or absence of fishes in the 3,002 Minnesota lakes are well predicted by temperature and dissolved oxygen level.

An animal has a range of temperatures in which its biological and biochemical processes perform normally. While various physiological, biochemical and molecular factors likely play important roles in determining the range of thermal tolerance of the species, genetically fixed stability of cellular proteins may play one of the most important key roles in establishing the range of optimal temperature for cellular biochemical reactions, thus, tissue functions and thermal tolerance of the whole body of the organism. It is known that the homologous proteins for stenothermal and eurythermal species are tolerant of narrow and wide ranges of temperature, respectively (Somero *et al.* 1996). For example, the barracuda (*Sphyraena* spp.) from the north temperate habitat where the temperature ranges from 15 to 22 °C, has both the thermolabile and the thermostable forms of cytoplasmic malate dehydrogenase (cMDH), while the species from tropical niches, where the habitat temperatures range from 22 to 30 °C, has only the thermostable form of cMDH (Graves and Somero 1982; Lin and Somero 1995a). Thermal properties of enzymes are well correlated with habitat temperatures, or body temperatures, of ectothermic animals. Thermal stability of lactate dehydrogenase-A (LDH-A) was examined in various

ectothermic animals with body temperatures ranging from –1.86 °C in Antarctic fish (*Pagothenia borchgrevinki*) to 47 °C in the desert iguana (*Dipsosaurus dorsalis*) (Somero *et al.* 1996). LDH-A of the Antarctic fish unfolds at the lowest temperature, while that of the desert iguana was the most heat resistant. Species' habitat temperatures are also reflected in the kinetic properties of LDH-A. The Michaelis-Menten constant ($K_m$) of pyruvate for orthologous homologues of LDH-A were similar among various species at their optimal temperatures, i.e. at –1.9 °C in Antarctic fish (*Trematomus centronotus*), at less than 20 °C in Cascade frog (*Rana cascadae*), at 14 to 22 °C in the barracuda (*Sphyraena idiastes*), at 9 to 38 °C in the eurythermal goby (*Gillichthys seta*) and at 30 to 47 °C in the desert iguana (*D. dorsalis*) (Somero 1995; Somero *et al.* 1996). The functional thermal range of LDH-A was also in agreement with the environmental temperature of species in those studies.

It has been well demonstrated that the cellular proteins of animals may have been highly sensitive to changes in environmental temperatures during evolution, resulting in the fine-tuning of the function and properties of those cellular proteins to habitat temperatures. Thus, there are different thermal stability points in homologous proteins (e.g. LDH) among different species. There are numerous studies showing that a difference of only a few degrees in habitat temperature may be enough to cause differences in the functional and structural properties of cellular proteins (see review by Somero 1995). It has also been reported that the amount of change in protein sequences required to modify the thermal sensitivity of a protein is very small; a few changes in amino acid sequence can enhance protein thermal stability (Fields and Somero 1997; Jaenicke 1991; Matthews 1987). For example, even a single amino acid change is enough to change LDH stability (Fields and Somero 1998; Somero *et al.* 1996). These studies indicate that alterations in structure and function of cellular proteins to adapt to habitat temperature could have been achieved by a minor modification of protein sequence during evolution, and that

structures of cellular proteins are finely selected to maintain appropriate stabilities and functions at physiological temperatures of animals (Jaenicke 1991; Somero 1995).

There are two choices for animals when they are exposed to temperatures outside of their physiological range; to escape and find a more suitable temperature, or to wait until the ambient temperature becomes suitable. The behavioral regulation in the first choice may be possible for animals in most occasions in nature. If the animals must stay and experience the changes in temperature, then the physiological and morphological adaptations can take place only when the changes in temperature take place at a rate that is slow enough. However, rapid changes in temperature can prevent the normal function of cellular proteins; the decrease in temperature may fatally reduce the speed of biochemical reactions while an increase in temperature can denature cellular proteins. These effects on the functions of the cellular proteins by rapid increase or decrease in temperature can threat the survival of animals when they occur in various parts of the body, and are fatal especially in the synaptic components of the central nervous system, thus collapsing neural communications throughout the whole body.

Recently, issues of global warming and global climate change have become serious considerations. Global temperature increases in the range of 2 to 4 °C are expected over the next half century (Somero and Hofmann 1997; Southward *et al.* 1995). According to the Government of British Columbia (B.C.), Canada (Environmental Trends in British Columbia 2000), the average rate of warming around the world over the past century has been 0.3-0.6 °C. They reported that coastal B.C. has warmed at about the same rate as the global average, however, interior B.C. has warmed at twice the rate of the average; more than 1 °C over the past century. The realistic impact of this scale of temperature shift on animals in nature is unknown. This scale of temperature change can affect significantly the physiological and ecological states of animals, especially when those changes in temperature occur near the upper thermal limits of animals (Wuebbles *et al.* 1999). Even though the rate of increase in yearly average temperature

is slow, addition of a few degrees on the maximum daytime temperature during summer can be fatal for many ectothermic animals, if the maximum daytime temperature at present is already close to their lethal temperature. There are many reports on the effects of climate warming on animals. The growth rate, population size, distribution range, and reproduction can be changed by a warming environmental temperature in plankton (Southward *et al.* 1995); in invertebrates (Hogg and Williams 1996; Rooney *et al.* 1996; Watt 1992); and in vertebrates, including fish (Keleher and Rahel 1996; Lehtonen 1996; Welch *et al.* 1998). Grant and Grant (1993) reported that the evolutionary selective pressure on the morphology of Darwin's finches on the Galapagos Islands was influenced by El Niño events; small beak size was favored after a severe El Niño, which changed the food supply of the island. They suggested that global warming might enhance the effect of El Niño on these evolutionary changes in that bird. Such reports imply that a global climate change can affect a variety of organisms both directly and indirectly and both in long-term and in the short-term. It is also possible that the magnitude of impact can be different between species with different thermal sensitivities. Thus, prediction of effects of global warming on animals is not simple. While we need an extensive assessment of the ecological impact which global warming has on animals, the mechanism underlying the determination of thermal tolerances of animals should be understood to predict potential changes in the physiology, metabolism, and behavior of animals associated with environmental temperature increase.

It is well documented that all organisms from bacteria to humans respond to various physical, chemical, and biological stressors, including increasing temperature, by synthesizing a group of highly conserved proteins known as stress proteins or heat shock proteins (hsps) (for reviews, see Craig and Gross 1991; Feder and Hofmann 1999; Hightower 1991; Iwama *et al.* 1998 and 1999; Parsell and Lindquist 1993). The primary functions of hsps are, as molecular chaperones, assisting in the folding and translocation of newly synthesized proteins under

unstressed conditions, as well as in the repair of damaged proteins that are denatured and destabilized by stressors (Beckmann *et al.* 1990; Ellis 1987; Hartl 1996; Hightower 1993; Martin *et al.* 1992; Sadis and Hightower 1992; Zimmermann 1998). Therefore, hsps assist other cellular proteins to function appropriately both in normal and in stressed states. Under stress, many proteins undergo a common unfolding pattern resulting in the formation of a molten globule intermediate which is considered to trigger the synthesis of hsps (Freeman *et al.* 1999; Santoro 2000). The cellular stress response is the term used to describe the production of new stress-inducible proteins that help to confer the resistance of an organism against environmental stressors. The cellular heat shock response is one of the stress responses induced by heat stress (Hightower 1993).

Many studies have provided direct evidence that hsps play a critical role in the thermostability of proteins. Sanchez and Lindquist (1990) have shown that the hsp104 gene-deleted yeast (*Saccharomyces cerevisiae*) was less thermotolerant compared with the wild type. The upper thermal limit of the rat embryo fibroblast significantly declined when the function of hsp70 (70-kDa hsp) was inhibited by needle injected monoclonal hsp70 antibody (Riabowol *et al.* 1988). *Escherichia coli* into which a gene encoding a small hsp (16.9-kDa hsp from rice) was introduced, showed higher lethal temperature and less denatured proteins under a heat shock compared with the control *E. coli* which did not have the gene of the small hsp introduced (Yeh *et al.* 1997). DnaK (hsp70 of *E. coli*) protected LDH-A purified from the three-spine stickleback (*Gasterosteus aculeatus*) from inactivation by heat (Zietara and Skorkowski 1995). Prändle *et al.* (1998) confirmed that the transgenic plant, *Arabidopsis thaliana*, with a newly found heat shock factor gene having a critical role for expression of hsps, conferred thermotolerance. Finally, the transgenic nematodes (*Heterorhabditis bacteriophora*) with the *Caenorhabditis elegans* heat-inducible hsp70 gene showed higher survival rate (>90%) with heat treatment than the wild-type strain (2-3%) (Hashmi *et al.* 1998). The stability and normal function of each protein molecule

that is protected by hsps, is vital for the health of cells, and consequently for the tissues and the whole body. These functions of hsps are believed to be universal, although the expression of hsps is different between various organisms.

The function of hsps, especially hsp70, is now well understood, and correlations between the expression of hsps and the resistance to stress in animals as well as the correlations between the threshold for hsp expression and levels of stress that animals naturally undergo, are well established by various field and laboratory experiments (see Feder and Hofmann 1999). For example, induction of hsps by the cellular stress response can be observed in algae with an upper thermal limit close to 5 °C, as well as in hyperthermophilic bacteria where the optimal growth temperature is near 100 °C (Gross 1998). Furthermore, the number and amount of hsps synthesized under stress depend on the nature and the magnitude of the stressor. Therefore, it has been suggested that hsps could be useful indicators of stressed states in animals (Bradley 1990; Dunlap and Matsumura 1997; Hightower 1993; Ryan and Hightower 1996; Sanders and Martin 1993). However, there are still ambiguous questions that should be answered; 1) is the denaturation of cellular proteins one of main causes of death both in stenothermal and eurythermal species? 2) are the fundamental function and importance of hsps the same between stenothermal and eurythermal species? and 3) if cellular proteins of eurythermal species are thermally stable, are heat-inducible hsps less important in those species? Judging from the correlation between the expression of hsps and thermotolerance of animals, hsps are now generally considered to play an important role in the determination of thermotolerance in animals. However, it is still uncertain whether the difference in the magnitude of hsp functions is directly responsible for the range of thermotolerance, or is the thermotolerance of an animal determined by other factors, such as the genetically determined thermal stability of cellular proteins. In other words, it may be possible that "the expression of hsps by stress" is highly conserved in all organisms, but the importance of their functions may be reduced or enhanced depending on the

nature of the other cellular proteins in the organisms. To answer those questions, it would be desirable to compare changes in hsp levels between stenothermal and eurythermal animals when they are exposed to stressful levels of temperature changes.

Various classes of hsps have been found. The functions of all of hsps are not yet clear. Nevertheless, the mechanisms of some of these hsps as molecular chaperones have been well studied (see review byYamashita 1997). Hsp70 and hsp60 can stabilize newly synthesized proteins including those in translation, assist in folding of those proteins with ATP-dependent mechanisms and prevent the formation of misfolded protein structures (Ellis 1987; Gething and Sambrook 1992; Hartl 1996; Morimoto *et al.*1990 and 1994; Rothman 1989). Hsp70 is found in the cytosol, mitochondria and endoplasmic reticulum, while hsp60 is exclusively found in the mitochondria (Hartl 1996). Hsp90 is known to have a function of molecular chaperone through interactions with the steroid hormone and Ah receptors and tyrosinekinases (Hutchison *et al.* 1992; Perdew 1988; Sanchez *et al.* 1990; Smith *et al.* 1990). Hsp28 is found in cytoplasm as assemblies of more than 500-kDa, and prevents denaturation and aggregation of cellular proteins under stresses (Arrigo and Welch 1987; Das and Surewicz 1995; Jakob *et al.* 1993). I chose hsp70 as an indicator of the stress in my experiment since this hsp is the most studied in the all hsp family proteins. In addition, hsp70 is considered to play a major and essential role in the protein metabolism of cells, since it is usually expressed at high levels in various tissues of animals and is highly conserved from bacteria to higher vertebrates (Craig and Gross 1991). Hsp70 belongs to a multigene family and at least one form is constitutively expressed in unstressed cells (heat shock cognate = hsc70), while one or more isoforms are expressed only under stress (hsp70). They have an essential role in protein folding, translocation, and the degradation of misfolded proteins under both stressed and non-stressed states of the cells. Hsp70 functions in an ATP-dependent manner; binding and releasing hydrophobic segments of an unfolded polypeptide chain in an ATP-hydrolytic reaction cycle, in which binding results in the

stabilization of the unfolded state of polypeptide, while releasing may serve to progress the folding pathway (Hartl 1996).

I used various fishes for my study, since fishes are extremely diverse in their thermal physiology. There are about 20,000 different species of fish over the globe occupying thermal niches ranging from –1.8 °C at the poles to 45 °C in geothermal springs (Goldspink 1995). Most fish, except some warm-bodied fish such tunas and blue marlin (Carey *et al.* 1971; Dewar *et al.* 1994; Dickson 1994), are considered aquatic ectotherms unable to regulate or maintain body temperature by physiological means; as a result their body temperatures fluctuate with that of the ambient temperatures (Jobling 1997). It is well known that fish have their physiological optima within their thermal environment (Beitinger and Fitzpatrick 1979; Jobling 1981; Kellogg and Gift 1983; Magnuson and Beitinger 1978; Magnuson *et al.* 1979). Therefore, the range of normal physiological temperatures and the threshold temperatures for heat injury can be extremely diverse among fishes. Thus, comparing the physiological and the biochemical properties among fishes with different thermal tolerances could be a good strategy to study thermal adaptation in ectothermic animals in general.

Numerous reports on the cellular heat shock response in fish are summarized in the review by Iwama *et al.* (1998). Most of the observations have been made using fish cell lines (see Iwama *et al.* 1998), primary cultures of hepatocytes (Koban *et al.* 1987; Norris *et al.* 1995; White *et al.* 1994), and renal proximal tubule cells (Brown *et al.* 1992). However, a relatively small number of studies have been reported on the whole body of the animal (Iwama *et al.* 1999). Therefore, the relationship between the expression of hsp70 and the range of the organismal thermotolerance of fish has not been comprehensively examined, yet.

Thus, in this project, I compared the levels of hsp70 between various tissues of fishes from different latitudes, across which water and body temperature can range from –1.7 °C in Antarctic areas to 37 °C in the tidepools of a sub-tropical ocean; I examined the relationship between the

cellular heat shock response and thermal resistance of these fishes. I also compared in more detail the lethal temperature and the cellular heat shock responses between three sculpins, two from the intertidal zone and one from the subtidal zone of temperate region. These sculpins are evolutionarily closely related, but show different vertical distribution patterns in the intertidal zone, which may be related to different ranges of water temperatures tolerated by those fishes. Therefore, these sculpins can be a good model to assess how small differences in the habitat temperature are related to the pattern of the cellular heat shock response in fish. Finally, I examined the effects of thermal history and the physiological state on the induction of thermotolerance of the tidepool sculpin, which has the highest thermotolerance of those three sculpins. Although hsp70 levels in various tissues were measured in the experiments in Chapter 1, the liver hsp70 levels were mainly examined in the rest of the experiments for 3 reasons; 1) liver is an important site for protein, glucose and fat metabolism, and it is often considered representative of the metabolic response of the whole body to stress; 2) liver is one of the major sites of high protein turn-over; and 3) there are many published reports of the cellular stress response in primary cultured hepatocytes.

# Chapter 1

# Cellular heat shock responses in fishes: Extreme examples from various latitudes

## Introduction to Chapter 1

In this chapter, expression of hsp70 in fishes from Antarctica, the sub-tropical Pacific Ocean and the tropical Amazonian river, in which body temperatures can range from −1.7 °C to 37 °C, was compared. The cellular heat shock response, including the expression of hsps, is ubiquitous among tested model organisms from bacteria to mammals (Feder and Hofmann 1999; Iwama *et al.* 1999). Although, the term "ubiquitous" is often used for this phenomenon, the expression pattern of hsps, such as the number of hsps and the temperature for the induction under a heat stress, varies among species. As mentioned in General Introduction, fish are extremely diverse in their thermal niches, thus, also in their optimal temperatures and ranges of thermal tolerances. Since the stability of cellular proteins is generally correlated with the stenothermal and eurythermal nature of the species (Somero *et al.* 1996), the cellular heat shock response can also be affected by the range of thermal tolerance. The question, as to which part of the cellular heat shock response has been conserved and which part has been modified, and how that modification has been reflected in the difference in thermotolerances of fishes should be answered. Comparing the pattern of the cellular heat shock response between fishes with different thermal tolerances may add useful information regarding the evolution of the cellular heat shock response in response to variations in habitat temperature.

## Section 1-1. Cellular heat shock responses and seasonal changes in hsp70 levels in various tissues of Okinawan fishes: The effects of El Niño

### Introduction

I tested sub-tropical fishes as one of the extreme examples of warm-acclimatized fish. Fish in the sub-tropical ocean of Okinawa, Japan, generally experience 20 °C as the minimum water temperature in winter and 30 °C as the maximum in summer. There are two possibilities concerning the cellular heat shock response of a warm-acclimatized fish. The first possibility is that they may induce the general heat shock response as observed in other temperate fishes, while the second possibility is, if their body proteins are inherently stable to the heat stress, clear induction of hsps may not be observed by a heat shock. The first objective of this study was to confirm which response is more prevalent in these sub-tropical fishes.

The second objective of this study was to assess the effects of heat stress on sub-tropical fish. It is highly possible that the upper thermal limit of Okinawan fishes is near the habitat temperature, especially during a summer when the ocean temperature becomes high. Somero and Hofmann (1997) suggested that global temperature changes, such as a 2 to 4 °C global warming scenario over the next half century, could bring significant impacts on physiological and ecological states of animals, especially when these changes in temperature occur near the upper thermal limits of organisms. The El Niño/Southern Oscillation (ENSO) that occurred during 1997 to 1998 was unusually large (Izaurralde *et al.* 1999), which resulted in the warmest year in at least the last 150 years (Wilkinson *et al.* 1999). El Niño is generally characterized by warm ocean temperatures, whereas La Niña is defined by cold ocean temperatures in the Equatorial Pacific. Since some aquatic animals live close to their thermal limits, an increase of only a few degrees in the ocean temperature could threaten their survival (Logue *et al.* 1995). There are numerous reports of coral damage during the ENSO of 1998 (Wilkinson *et al.* 1999). Three to 5 °C above normal water temperatures were often recorded in the Indian Ocean during

1998, which resulted in bleaching and subsequent massive death of corals in this region. Severe coral bleaching also was observed during 1998 around Andaman Islands in India (Ravindran *et al.* 1999), and in the Great Barrier Reef in Australia (Berkelmans and Oliver 1999). Although there are many reports documenting high coral mortality associated with high ocean temperatures, indicating potential threat of ongoing global warming to coral health (Atwood *et al.* 1992; Fagoonee *et al.* 1999; Kushmaro *et al.* 1997; Roberts 1993; Sebens 1994; Winter *et al.* 1998), the effects of warm ocean temperature on fish have not been examined as extensively as in corals. Thus, in this section, I examined the threshold induction temperature for hsp70 in sub-tropical fishes in Okinawa, as well as the effect of a warm ocean temperature, caused by ENSO in the summer of 1998, on levels of hsp70 in various tissues of these fishes.

## Materials and Methods

### *Heat shock experiments in the reef fish and the tidepool fish*

The reef fish, the five-banded damselfish (*Abudefduf vaigiensis*), and the intertidal goby (*Bathygobius cocosensis*) were collected near the Sesoko Station, Tropical Biosphere Research Center, The University of the Ryukyus, on the Sesoko Island, Okinawa, Japan in June 1998. The reef fish were caught by hook and line, while the tidepool fishes were collected by dip nets from the tidepools. Thirty fish were collected for each species. Both fishes were acclimated at 28 °C, which was the average ocean temperature of early summer in Okinawa, for 2 weeks. After gill and liver tissues were sampled from 10 fish in each species as control samples, the rest of the fishes were transferred to 33 or 38 °C. Ten fish in each species were transferred to each temperature. Two hours after transfer, fishes were returned to the acclimation temperature, and allowed to recover for 4 h. Fish were then anesthetized with high-dose MS-222 (200mg/L), and the gill and the liver tissues were sampled. Tissue samples were frozen immediately on dry ice and stored at –80 °C until analysis.

*Recovery time*

The length of recovery time after a heat shock treatment was determined by the time required for hsp70 synthesis. Preliminary tests showed that the level of hsp70 in the liver tissue of the rainbow trout peaked between 3 h and 24 h after heat shock. Shorter recovery times (3-6 h) were chosen for the experiments in Chapter 1 because at that time, the survival of fishes long time after a severe heat shock had not been confirmed. Recovery times of 20-24 h were used in the experiments with the intertidal sculpins in the later Chapters because the survival of those sculpins at 24 h, even after a severe heat shock, had been well established in the preliminary tests.

*Seasonal fish collection and tissue sampling*

To examine the effects of the warm ocean temperature in the summer of 1998 on the stress response of fish in Okinawa, 4 to 8 of the reef fish (*A. vaigiensis*), and 2 to 8 of the intertidal species inhabiting tidepools (the black-lined blenny, *Istiblennis lineatus*) were collected in August 1998, February 1999 and in August 1999 near the Sesoko Station. Another intertidal species (the goby, *B. cocosensis*) were collected 6 each in August 1998 and in February 1999, as well. Collected fish were anesthetized in high-dose MS-222 (200mg/L) and were immediately killed by cutting the nerve chord followed by being frozen in liquid nitrogen. Frozen fish samples were shipped on dry ice to The University of British Columbia (UBC), Vancouver, B.C., Canada, and tissue samples (brain, gill, liver and skeletal muscle) were taken from frozen fish, re-frozen immediately on dry ice, and stored at –80 °C until analysis.

*Monitoring the surface ocean temperature*

Seasonal fluctuations of the surface ocean temperature have been monitored twice a day at 08:30 and 13:00 for the last 10 years in front of the Sesoko Station (26° 37' 56" N, 127° 51' 59" E) as the Record of Coastal Observation at Sesoko Station, Tropical Biosphere Research Center,

The University of the Ryukyus, Okinawa, Japan. The water temperature was monitored by two standard thermometers, and the average value was recorded. The relationship between the ocean temperature and the cellular stress responses of the fishes described above was examined.

### *Sample treatments for SDS-PAGE*

Tissue samples from the five-banded damselfish and the intertidal fishes were homogenized with a sonicator (Vibra Cell, Sonic and Materials Inc., USA) in a homogenizing buffer (0.1% SDS [w/v], 0.02mg/mL PMSF, 0.25mg/mL EDTA, 1μg/mL pepstatin A, 1μg/mL leupeptin and 1μg/mL aprotinin in 100mM Tris-HCl buffer, pH 7.5), with a 10mg tissue to 100μL buffer ratio. Homogenates were then centrifuged at 7600×g for 3 min. One hundred to 400μL of the supernatant were transferred to a tube containing the same volume of 2×Laemmli's sample buffer (4% SDS [w/v], 20% glycerol [v/v], 10% β-mercaptoethanol [v/v] and 0.0025% bromophenol blue [w/v] in 0.5M Tris-HCl buffer, pH 6.8) (Laemmli 1970) for SDS polyacrylamide gel electrophoresis (SDS-PAGE). The rest of the supernatant was transferred to an empty tube for the protein assay. Samples for SDS-PAGE were then boiled in a heatblock for 3 min. The total protein content of samples was determined by the BCA method (Smith *et al.* 1985). The same method for the sample treatment for SDS-PAGE was used in all of experiments in subsequent chapters.

### *Western blot analysis for total hsp70*

The following method was adapted from Forsyth *et al.* (1997) for Western blotting. For SDS-PAGE, samples were diluted with 1×Laemmli's buffer to yield a concentration of 1mg protein/mL, and 20μL (20μg of total protein) of diluted samples were loaded on acrylamide gels to separate proteins depending on their molecular weights by SDS-PAGE. Acrylamide

concentrations of 12% for the separating gels and 4% for the stacking gels were used. After the proteins were separated by SDS-PAGE at 75V for 15 min and at 150V for 1 h, the separated proteins were transferred to 0.2μm pore size nitrocellulose membranes (Bio-Rad, USA) at 17V for 30 min with transfer buffer (48mM Tris, 39mM glycine, 20% methanol [v/v] and 0.0375% SDS [w/v], pH 9.2) using a semi-dry transfer apparatus (Bio-Rad, USA). Transferred membranes were blocked in 2% skim milk (w/v) in Tween-20 Tris-buffered saline, TTBS (17.4mM Tris-HCl, 2.64mM Tris Base, 0.5M NaCl and 0.05% Tween-20 [v/v]) with 0.05% sodium azide (w/v) for more than 1 h, then rinsed once and soaked in TTBS for 5 min. Membranes were then soaked in the primary antibody (rabbit IgG for rainbow trout hsp70/hsc70 [70-kDa heat shock cognate=constitutive form of hsp70]) at a 1:3000 dilution in 2% skim milk for 1 h. After 3 washes in TTBS for 5 min each, membranes were soaked in the secondary antibody (goat anti rabbit IgG) at a dilution of 1:3000 in TTBS for 1 h. After 3 washes in TTBS for 5 min each, and one wash in TBS for 5 min to remove the Tween-20, membranes were developed in a NBT (333μg/mL) / BCIP (167μg/mL) solution in alkaline phosphatase buffer (0.1M Tris-HCl, 0.1M NaCl and 21mM $MgCl_2$, pH 9.5) for 5 min. Bands on membranes were scanned and the intensities were quantified with Sigma-Gel software (Jandel Scientific, USA). The level of hsp70 in a coho salmon (*Oncorhynchus kisutch*) liver sample, which was loaded on each gel at the same volume, was used to standardize sample loading. The same method for Western blot analysis was used in all experiments in subsequent chapters.

That the primary antibody reacts with both hsp70 and hsc70, and does not differentiate between inducible hsp70 and constitutive hsp70. Thus, detection of increase in the inducible hsp70 may have been difficult if the constitutive level of the species was high enough to mask the change in the level of the inducible form. If several isoforms of the hsp70 family proteins were clearly separated by the electrophoresis, as was the case in the experiments in the sculpins (Section 3-1), it would have been possible to detect the change in the level of each isoform of the

hsp70 family.

### *Statistical analyses*

To discern significant differences among groups from different treatment temperatures in each species, One way Analysis of Variance (ANOVA) or the Kruskal-Wallis ANOVA on ranks was used, followed by the Student-Newman-Keuls Test (all pairwise multiple comparison procedures). A significance level of $p \leq 0.05$ or $p \leq 0.001$ was used for all tests, and the appropriate P-value was noted in each case. Two way ANOVA was applied for the data from the time course experiments to examine time effects (compared with the initial value) and treatment effects (compared with the control value). Since the levels of hsp70 were shown as relative values to the control value of the coho salmon liver sample, an arcsin square root transformation was applied to those values prior to ANOVA. All of these statistical analyses were applied to the data sets in all of experiments in subsequent chapters.

## Results

### *Cellular heat shock responses in the reef fish and the tidepool fish*

Five out of 10 of the reef fish (the five-banded damselfish) were dead within 5 min after transfer to 38 °C from 28 °C, while all of the tidepool fish (the goby) were alive at 38 °C over the entire experiment. All fishes of both species survived a transfer to 33 °C.

The tidepool fish showed a significantly higher level of gill hsp70 only at 38 °C (Fig. 1-1). There were no significant changes in liver hsp70 levels at any temperatures in either species (Fig. 1-2). The overall levels of hsp70 were higher in the tidepool fish compared with the reef fish both in gill and liver tissues.

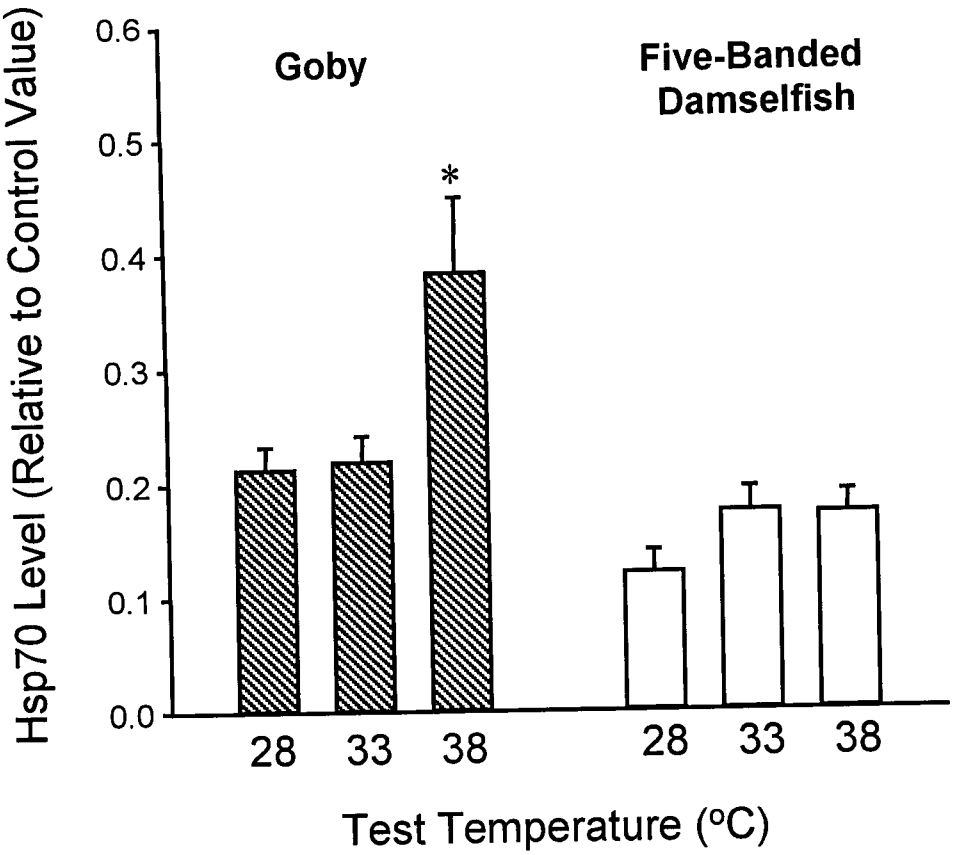


**Figure 1-1.** Hsp70 levels in gill tissues from the tidepool fish (the goby; *B. cocosensis*) and the reef fish (the five-banded damselfish; *A. vaigiensis*) at 28, 33 and 38 °C. Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=10 for the tidepool fish and n=5-10 for the reef fish). * indicates significant difference compared with the value at 28 °C ($p \leq 0.05$).

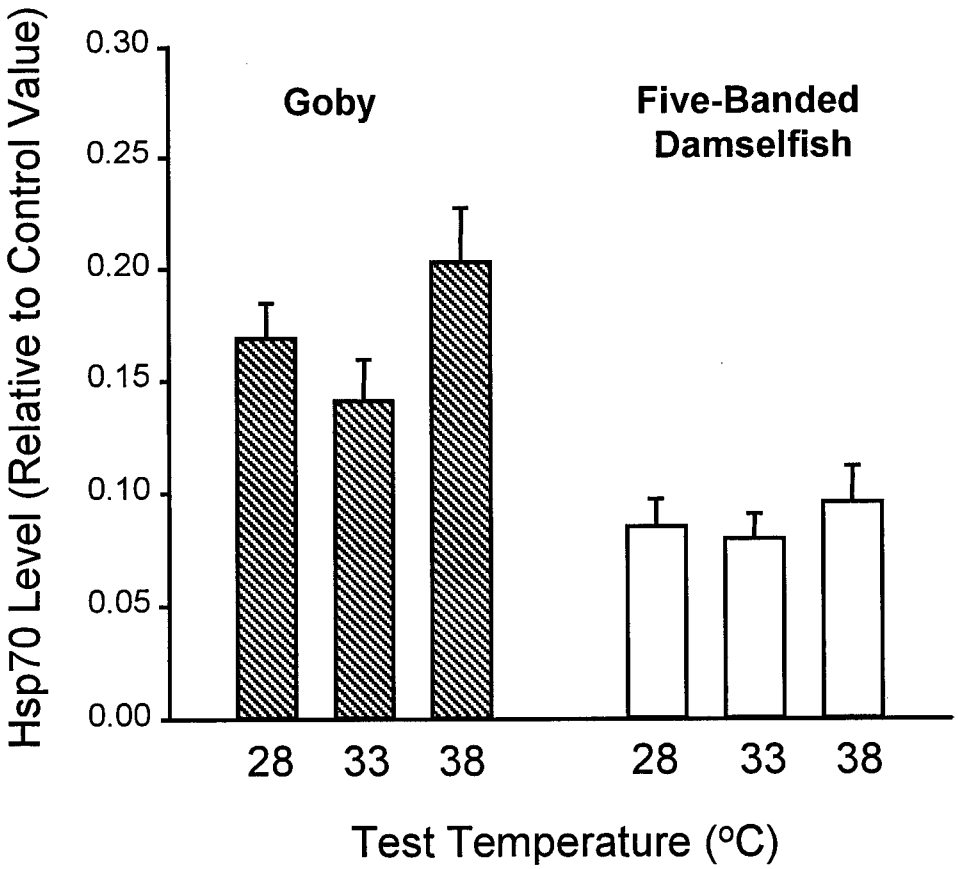


**Figure 1-2.** Hsp70 levels in liver tissues of the tidepool fish (the goby; *B. cocosensis*) and the reef fish (the five-banded damselfish; *A. vaigiensis*) at 28, 33 and 38 °C. Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=10 for the tidepool fish and n=5-10 for the reef fish).

*Seasonal changes in the surface ocean temperature*

Figure 1-3 shows the fluctuations of the surface ocean temperature for the last 10 years near Sesoko Island, Okinawa, Japan. The surface ocean temperature in winter corresponded well with the events of El Niño or La Niña. It can be estimated from this graph that the five-banded damselfish, a reef fish, experienced 32.1 °C as the highest temperature in the summer of 1998; 19.8 °C as the lowest temperature in the winter of early 1999; and 30.5 °C as the highest temperature in the normal summer of 1999. The tidepool fishes (the goby and the black-lined blenny) would have experienced higher water temperatures in the summer, and lower temperatures in the winter than the five-banded damselfish, since the water temperature in a tidepool at low tide rapidly increases in summer and decreases in winter to greater extents compared to the ocean temperature. In fact, 36.5 °C was recorded in a tidepool with fishes on Sesoko Island on a sunny day in June 1998.

*Seasonal changes in hsp70 levels in the reef fish and the tidepool fishes*

Figure 1-4 shows the levels of hsp70 in the brain, gill, liver and muscle tissues in the reef fish, the five-banded damselfish, in August 1998 (summer 1998), February 1999 (winter) and August 1999 (summer 1999). In the liver, the levels of hsp70 were drastically reduced in the summer of 1998 compared to other seasons. In the muscle tissue, hsp70 levels were significantly lower in the winter than in the summers.

Figure 1-5 shows the level of hsp70 in various tissues of the black-lined blenny. Levels of hsp70 were significantly lower in the gill tissues in the summer of 1998 compared with the winter and the summer of 1999. Figure 1-6 shows levels of hsp70 in various tissues of the goby. Levels of hsp70 in the liver tissue was significantly lower in the summer of 1998 compared to the winter. There was no reduction in levels of gill hsp70 in the summer of 1998 as observed in the black-lined blenny.

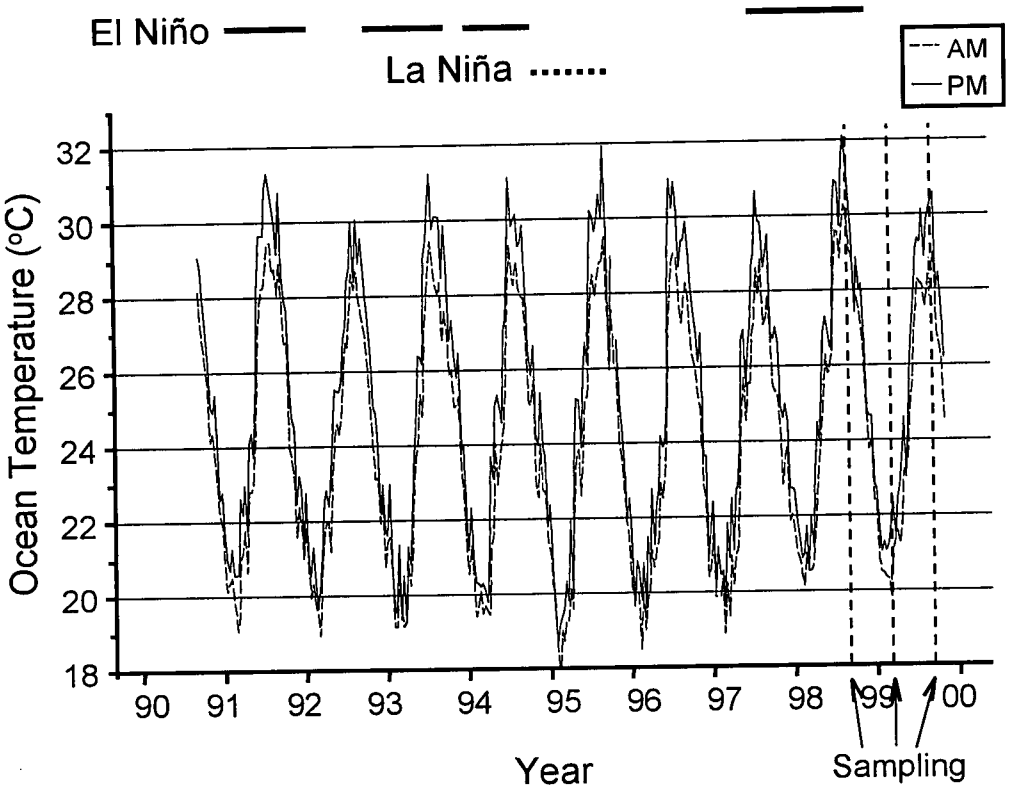


**Figure 1-3.** Seasonal changes in the surface ocean temperature near Sesoko Island, Okinawa, Japan, monitored for last 10 years. The dash line in the graph shows temperatures monitored in mornings (08:30), while the solid line is temperatures monitored in afternoons (13:00). The sampling times of the fishes are shown by arrows on the X-axis. El Niño and La Niña events reported by NOAA (National Oceanic and Atmospheric Administration) are shown by horizontal bars on the top of the graph.

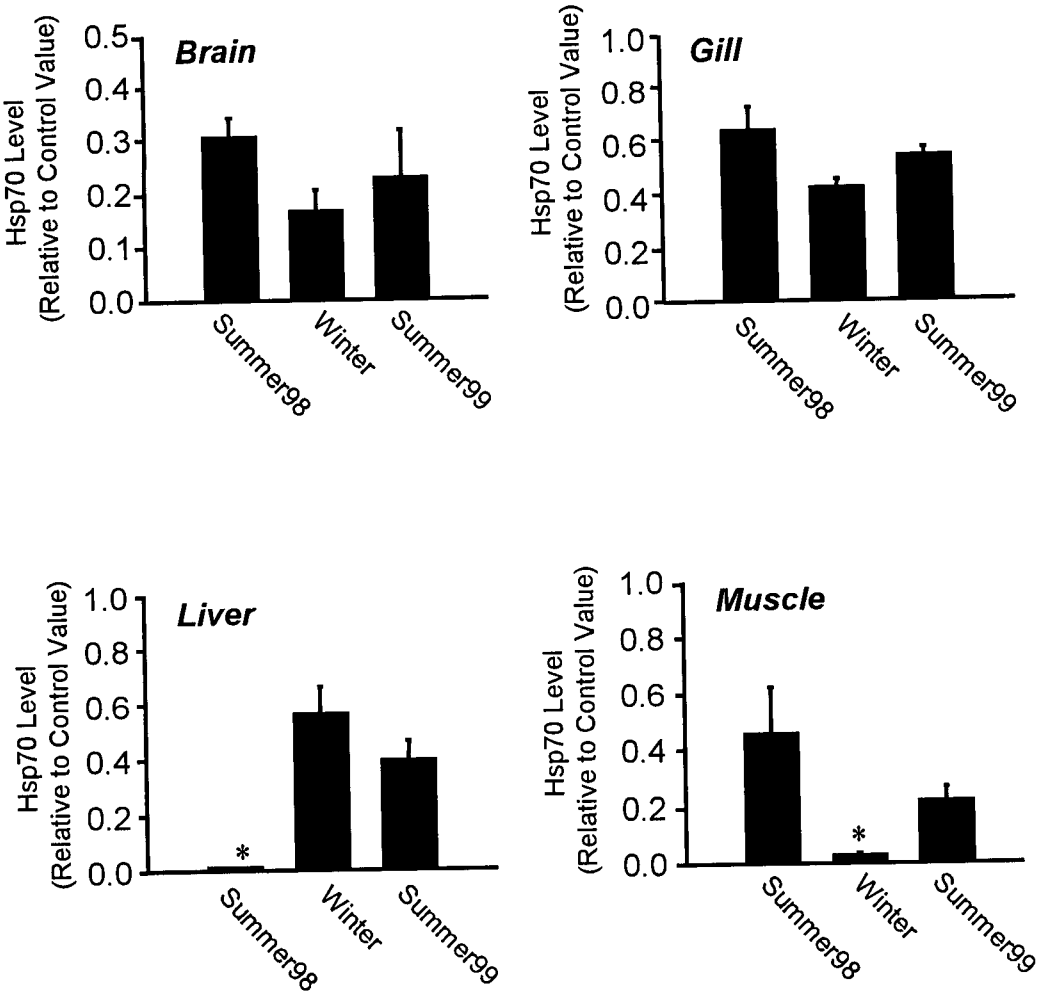


**Figure 1-4.** Hsp70 levels in the brain, gill, liver and muscle tissues of the five-banded damselfish (*A. vaigiensis*) sampled in August 1998 (Summer98), February 1999 (Winter) and August 1999 (Summer99). Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=4-8). * indicates significant difference in the level of hsp70 between the groups of the fish from different sampling times ($p \leq 0.05$).

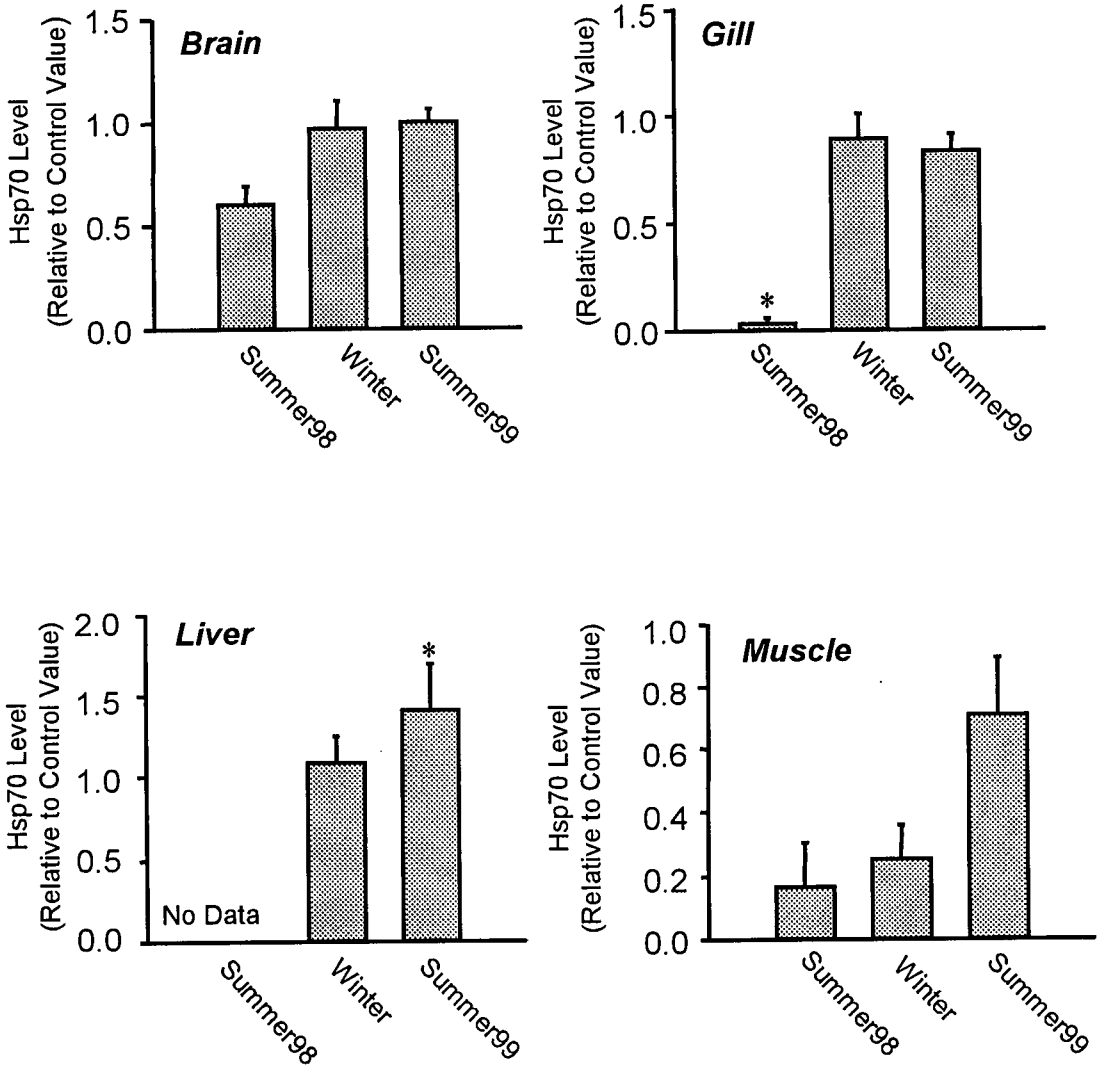


**Figure 1-5.** Hsp70 levels in the brain, gill, liver and muscle tissues of the black-lined blenny (*I. lineatus*) sampled in August 1998 (Summer98), February 1999 (Winter) and August 1999 (Summer99). Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver (Mean±SE, n=2-8). * indicates significant difference in the level of hsp70 between the groups of the fishes from different sampling times ($p \leq 0.05$).

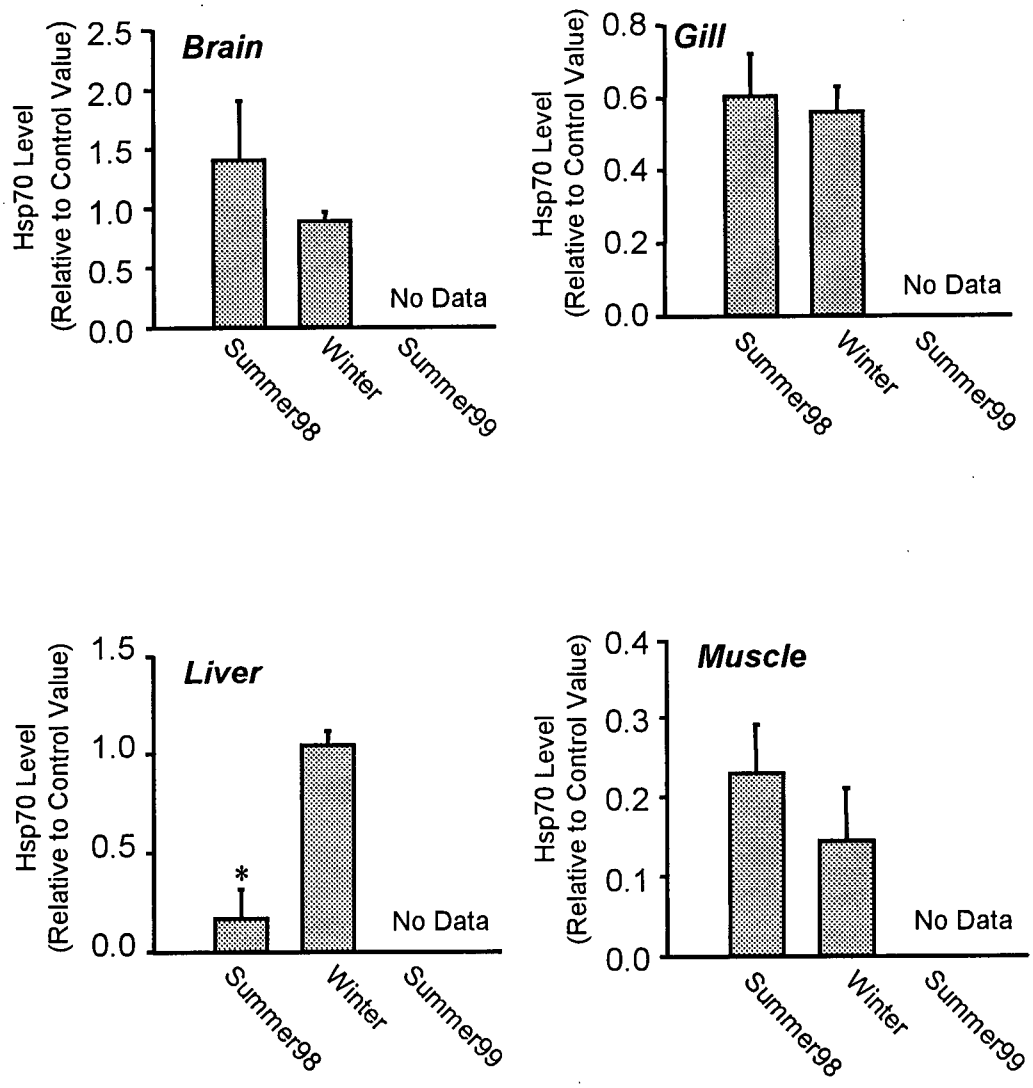


**Figure 1-6.** Hsp70 levels in the brain, gill, liver and muscle tissues of the goby (*B. cocosensis*) sampled in August 1998 (Summer98) and February 1999 (Winter). Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver (Mean±SE, n=6). * indicates significant difference in the level of hsp70 between the groups of the fishes from different sampling times ($p \leq 0.05$).

## Discussion

In the heat shock experiments, the goby showed a higher resistance to heat stress than the five-banded damselfish when exposed to an increase of 10 °C (28 °C to 38 °C). In the intertidal zone, the water temperature of a tidepool can fluctuate dramatically by the combination of tidal cycle and time of the day (Horn *et al.* 1999). In addition, the water temperature in a tidepool can be significantly higher in summer (Green 1971a) and lower in winter (Nakamura 1976a) than the ocean temperature during a low tide. I observed a water temperature of 36.5 °C in a tidepool in the tested area when the ocean temperature was about 30 °C (field observation). Thus, it is more than possible that the goby have a higher upper thermal limit than the five-banded damselfish.

Levels of hsp70 were tissue specific, especially in the goby; a significantly higher ($p \leq 0.05$) hsp70 level in the gill tissue was observed at 38 °C in the goby (Fig. 1-1). However, this tendency was not clear in the liver tissue (Fig. 1-2). It may be noteworthy that the overall levels of hsp70 were higher in the goby than in the five-banded damselfish at all temperatures (Fig. 1-1 and Fig. 1-2), which may be related to higher thermal tolerance of the goby than the five-banded damselfish.

The function of the liver tissue of the reef fish, related to protein turnover and repair, might be, at least partly, attenuated during summer of 1998, judging from a depression of hsp70 in this tissue (Fig. 1-4). Hsp70 levels in various tissues of two tidepool fishes in the summer of 1998 and in the winter showed inter-species difference, even though they were collected from the same tidepools (Fig. 1-5 and Fig. 1-6). Severe reduction of hsp70 in the gill tissues of the black-lined blenny in the summer of 1998 compared to the other seasons (Fig. 1-5) indicates that the cellular protein function and the turnover of the gill tissue of this fish might have been severely disturbed. Liver hsp70 levels were also significantly lower in the summer of 1998 compared to the winter in the goby (Fig. 1-6). However, since no sample of this species was available for the

summer of 1999, it is not clear whether the reduction of liver hsp70 in the summer of 1998 was caused by the ENSO event or by seasonal effects.

Inhibited protein turnover rates in the gill and the liver tissues of the rainbow trout by a 2 °C increase during the peak summer water temperature has been reported by Reid *et al.* (1995 and 1998). Animals synthesize hsps in response to elevated temperature. But when temperature increases too high, synthesis of hsps may be reduced. For example, the intertidal mussel (*Mytilus trossulus*) shows a sharp attenuation of protein synthesis, except hsps, at 30 °C, and fully blocked protein synthesis, including hsps, at 35 °C (Hofmann and Somero 1996a; Somero and Hofmann 1997). This metabolic depression can happen in the natural environment, when the mussel is emerged during a low tide (Hofmann and Somero 1995). It is possible that the same kind of metabolic depression or attenuation of hsp70 synthesis might have happened in liver and gill tissues of the five-banded damselfish and of the black-lined blenny, respectively, during the summer ENSO of 1998.

It is also possible that hypoxia in tidepool water caused a metabolic depression in the gill tissue of the black-lined blenny, resulting in depression of hsp70 levels in that tissue in the summer of 1998. It is known that transcription of many genes is suppressed under hypoxia, and the magnitude of increase in hsp70 by heat stress is affected by oxygen availability. Currie *et al.* (1999) demonstrated that inhibition of oxidative phosphorylation suppressed the increase in hsp70 levels in rainbow trout red blood cells heat-shocked at 25 °C. They showed as well that the transcription of hsc70 (70-kDa heat shock cognate) of those cells increased at 25 °C compared to 10 °C in air, but not in a hypoxic condition with 100% nitrogen. Since tidepools in Sesoko Island are formed on the coral bed which does not have much vegetation, organisms in those tidepools may experience chronic hypoxia during the daytime; increased metabolism due to heat would increase consumption of $O_2$ by the organisms within those tidepools as well as reduce $O_2$ content of the water. Therefore, the possibility should not be excluded that the warmer

water temperature in the summer of 1998 enhanced a hypoxic state in the tidepools which downregulated the transcription of proteins, including that of hsp70 genes, in the black-lined blenny.

Even though the effects of warm ocean temperature on tropical fish have not been studied as extensively as it has been in corals, there is some evidence showing physiological damage can occur in fish during El Niño years. Growth reductions of otoliths, coinciding with a strong El Niño event during 1982~1983, were reported in the widow and the yellowtail rockfish (*Sebastes ntomelas* and *S. flavidus*, respectively) inhabiting the coast of central and northern California (Woodbury 1999). Effects of El Niño also were observed in warm-acclimatized tropical fishes. Forty-one of 44 coral reef fishes (Pomacentridae) in the Galapagos Archipelago had a check in their otoliths due to a major change in environmental and/or growth conditions that corresponded to the timing of 1982~1983 El Niño (Meekan *et al.* 1999). During that year, the islands were surrounded by tropical waters of 25 to 28 °C at the maximum. The upper thermal limit of tropical fish might be near the habitat temperature, especially during summer when the ocean temperature becomes considerably higher. The fish in Sesoko Island in Okinawa experienced water temperatures of 32 °C during the summer of 1998 (Fig. 1-3), and probably even higher in tidepools. Even though no mortality as in corals was observed in fishes in Sesoko Island during the summer of 1998, these extremely high habitat temperatures during that summer might have exceeded the physiological thermal range of fishes inhabiting this area.

In summary, a reef fish (the five-banded damselfish) and a tidepool fish (the black-lined blenny) inhabiting the sub-tropical ocean of Okinawa had different patterns of hsp expression in the summer of 1998 coinciding with a strong El Niño, compared to the normal summer of 1999. Judging from the severe reductions of hsp70 levels in some tissues, some fish could have been stressed by prolonged warm ocean temperatures in the summer of 1998. It is noteworthy that the surface ocean temperature of the region in the summer of 1998 was only about 1 °C warmer than

in normal years (Fig. 1-3). Therefore, the results of the present study indicate that relatively small increases in ocean temperature could affect the cellular stress response of fishes.

## Section 1-2. The cellular heat shock response in Antarctic fish

### Introduction

It is well documented that the cellular heat shock response, including the expression of hsps, is a ubiquitous feature of the cell responding to heat stress among all tested organisms from bacteria to higher vertebrates (Feder and Hofmann 1999; Iwama *et al.* 1998). Along with the understanding of the close relationship between the expression of the heat shock response and the heat resistance of animals, another simple question arises; do Antarctic fish in the Southern Ocean which has undergone a reduction in temperature from 20 °C to –1.8 °C over last 55-60 million years show the general heat shock response?

In contrast to tropical fishes, there are fishes inhabiting the Antarctic marine environment in which water temperature is near the freezing point of seawater. Not only is it cold, but also the water temperature of the Antarctic marine environment has been extremely stable over the past 20 million years (Carratù *et al.* 1998). At the Oligocene-Miocene boundary, about 25 to 22 million years ago, the Antarctic marine environment became isolated with the opening of the Drake Passage and with the formation of a circumpolar hydrographic barrier (Bargelloni and Lecointre 1998). In this unique and isolated environment, the biochemical and physiological mechanisms of Antarctic marine organisms have been modified during evolution with a strong and continuous selective pressure for exclusive adaptation to low temperature. At present, some Antarctic fishes live at –1.9 °C with seasonal water temperature changes of 0.2 °C (Eastman 1993; Somero 1991). Although Antarctic fish show some general physiological heat shock responses such as increases in haematcrit, plasma osmolarity and $Cl^-$ concentration (Franklin *et al.* 1991); and increases in heart rate and plasma catecholamines (Forster *et al.* 1998), there are

no comprehensive studies of the cellular heat shock response in Antarctic fishes. Therefore, I measured hsp70 and its mRNA levels in the liver tissue of *Trematomus bernacchii* exposed to various temperatures. *T. bernacchii* is a common and stenothermal notothenioid fish in Antarctica not known to occur in waters of more than 1 to 2 °C (Norman 1940). Somero and DeVries (1967) reported that the upper incipient lethal temperature of this species is about 6 °C, which is the lowest upper lethal temperature reported for any organism. Based on this, I decided to heat shock *T. bernachii* at 3 and 7 °C, which could be considered as mild and severe heat shocks for this species, respectively.

## Materials and Methods

### *Fish*

A common Antarctic fish, *T. bernacchii*, was collected near the Japanese Antarctic Base (69° 20' S, 39° 34' E) on November 12th 1999. Twenty fish were caught by hook and line from holes made in the sea ice, under which the depth of water was about 5 to 10m. Caught fish were kept in a cooler box with ocean water of –1.7 °C. It took 10 min to collect 20 fish by 4 researchers of the 40th Japanese Antarctic Research Expedition. Then fish were transferred to the Japanese Antarctic Base. Transfer took less than 30 min. The water temperature in the cooler box was kept at –1.7 °C during transfer by adding blocks of sea ice. Since 5 out of the 20 fish were dying when they arrived at the Base, the remaining 15 fish were used for the following heat shock experiment.

### *Heat shock experiment and liver sampling*

Four and 5 fish were directly transferred to 3 °C and 7 °C, respectively. Six fish were also transferred to –1.7 °C as the control. Three fish in –1.7 °C were sampled for liver tissues at 1 h after transfer, since they had died by then. The rest of the fish in all groups were kept for 2 h at

their respective temperatures, and then returned to –1.7 °C for recovery for 4 to 6 h. For the liver tissue sampling, fish were immediately killed by cutting the spinal nerve chord followed by the excision of the liver tissue. All sampling procedures were conducted in a cold room kept at 3 °C. Liver tissues were kept at –85 °C in 15mL tubes until transferred to the laboratory at UBC, Vancouver, B.C., Canada. The tissue samples were also kept at less than –80 °C during the transfer from the Japanese Antarctic Base to UBC, and stored at –80 °C until analysis for hsp70 by Western blotting (for the methods of Western blot analysis, see Materials and Methods in Section 1-1) and hsp70 mRNA by Northern blotting.

*Northern blot analysis for liver hsp70 mRNA*

*RNA Isolation.* Tissue samples were homogenized with a polytron homogenizer (Kinematica, Switzerland) in a homogenizing buffer (1.9M guanidium thiocyanate, 12mM sodium citrate, 0.24% N-lauroyl sarcosine [w/v], 9.5mM sodium acetate and 47.6% citrate buffer saturated phenol [v/v]) at a ratio of 2mL/100mg tissue. Then, 200μL of chloroform was added to each sample and the aqueous layer was transferred to another tube after centrifugation at 9700×g for 20 min at 4 °C. Each sample was mixed with 1mL isopropanol, and then kept on ice for 15 min, and thereafter centrifuged at 9700×g for 20 min at 4 °C. After aspiration of the liquid, the pellet was rinsed in 1mL of 75% ethanol and centrifuged at 9700×g for 20 min at 4 °C. The pellet was then dissolved in DEPC (diethylpyrocarbonate)-treated water (1mL/1L DDW) at a ratio of 3μL/1mg liver tissue. RNA quantity (μg/mL) and purity of the isolated RNA solutions were calculated from absorbencies of each sample at 260nm and 280nm.

*Electrophoresis and Electrotransfer.* An aliquot of isolated RNA solution containing 15μg RNA was transferred to a separate tube, dried with a speed vacuum, and mixed with loading buffer

containing 16.7% formaldehyde (v/v), 20mM MOPS (3-[N-morpholino] propanesulfonic acid) buffer (20mM MOPS, 0.3M sodium acetate and 50mM EDTA), 5% glycerol (v/v) and 0.4% bromophenol blue (w/v) in DEPC-treated water. Samples were then loaded on 1% agarose gels (1% agarose [w/v], 20mM MOPS buffer, 1.1% formaldehyde [v/v] and 0.0075% ethidium bromide [v/v] in DEPC-treated water). Gels were run in 20mM MOPS buffer at 20V for 20h. After 4 washes in DEPC-treated water for 5 min each, RNA was transferred to membranes (GeneScreen Hybridization Transfer Membrane, Biotechnology Systems, USA) between Whatman #3 filter papers in a transfer buffer (20mM Tris-HCl, 10mM sodium acetate and 0.5mM EDTA) at 33V (0.8Amp) for 3 h. The transferred RNA was cross-linked on membrane in an UV crosslinker (Hoefer Scientific Instruments, USA). Membranes were kept in a dark place between filter papers until they were used for Northern hybridization.

*Northern Hybridization.* Hybridization was carried out at 65 °C for >16 h in hybridization buffer containing 1M NaCl, 0.1% Na-PPi (w/v), 1% SDS (w/v), 10×Denhardt solution (2g ficoll, 2g polyvinylpyrrolidone and 2g BSA in 1L) in 0.05M Tris-HCl (pH 7.5), and 100mg/mL of calf thymus DNA (Sigma Co., diluted in sterile DDW and denatured by boiling). The hybridization probe was a fragment from rainbow trout hsp70 DNA amplified by PCR (Polymerase Chain Reaction) which hybridizes with both hsp70 mRNA and hsc70 mRNA. After hybridization, membranes were washed 4 times for 15 min each at 65 °C in washing buffer containing 0.1% SDS (w/v) in 2×SSC (0.3M NaCl and 30mM sodium citrate in DEPC treated water). Membranes were exposed in a storage phosphor screen (Molecular Dynamics, USA) for 24 h. Bands were detected with a phosphor imager and band intensities were quantified with the associated software for Molecular Dynamics system (Molecular Dynamics, USA). β-actin mRNA levels, measured with a human β-actin cDNA probe (Clontech, USA), were supposed to

be used to standardize RNA loading. However, the β-actin cDNA probe did not hybridize well with β-actin mRNA of the Antarctic fish. Therefore, 28S rRNA levels were used instead.

## Results

The polyclonal antibody against rainbow trout hsp70 and rainbow trout hsp70 cDNA probe showed high cross reactivities with *T. bernacchii* hsp70 and hsp70 mRNA in Western and Northern blot analyses, respectively. Liver hsp70 levels did not change significantly at –1.7, 3 and 7 °C (Fig. 1-7). On the other hand, hsp70 mRNA levels in the liver tissue of this species were significantly higher at 7 °C compared with –1.7 °C and 3 °C (Fig. 1-8).

## Discussion

In this study, levels of both hsp70 and its mRNA were measured for the first time in the whole-body heat shock experiment of Antarctic fish. The first study related to the cellular heat shock response in Antarctic fishes was conducted by Maresca *et al.* (1988). They examined the presence of hsp70 genes by Southern blot analysis with *Drosophila* hsp70 gene probe in three species of Antarctic fishes, eurythermal *Notothenia rossii*, stenothermal *T. bernacchii*, and extremely cold adapted ice fish, *Chionodraco kathleene*. The authors demonstrated that all the species examined may have more than one copy per genome of hsp70-like DNA sequence. They also examined transcription of hsp70 gene by Northern blot analysis with *Drosophila* hsp70 gene probe in a culture of the spleen tissue from *N. rossii* incubated at 0, 5, 8 and 12 °C for 3 h. Although they did not quantify band intensities of hsp70 mRNA due to the low stringency experimental condition, they concluded that hsp70 gene is transcribed at all higher temperatures with 8 °C at the maximum. More recently, Carratù *et al.* (1998) measured the levels of hsp70 mRNA in cultured spleen of *T. bernacchii* incubated at –1.2 ($C_4$) or 5 °C for 4 h. They showed that levels of transcription at 5 °C increased about 50% compared to the initial level ($C_0$) and

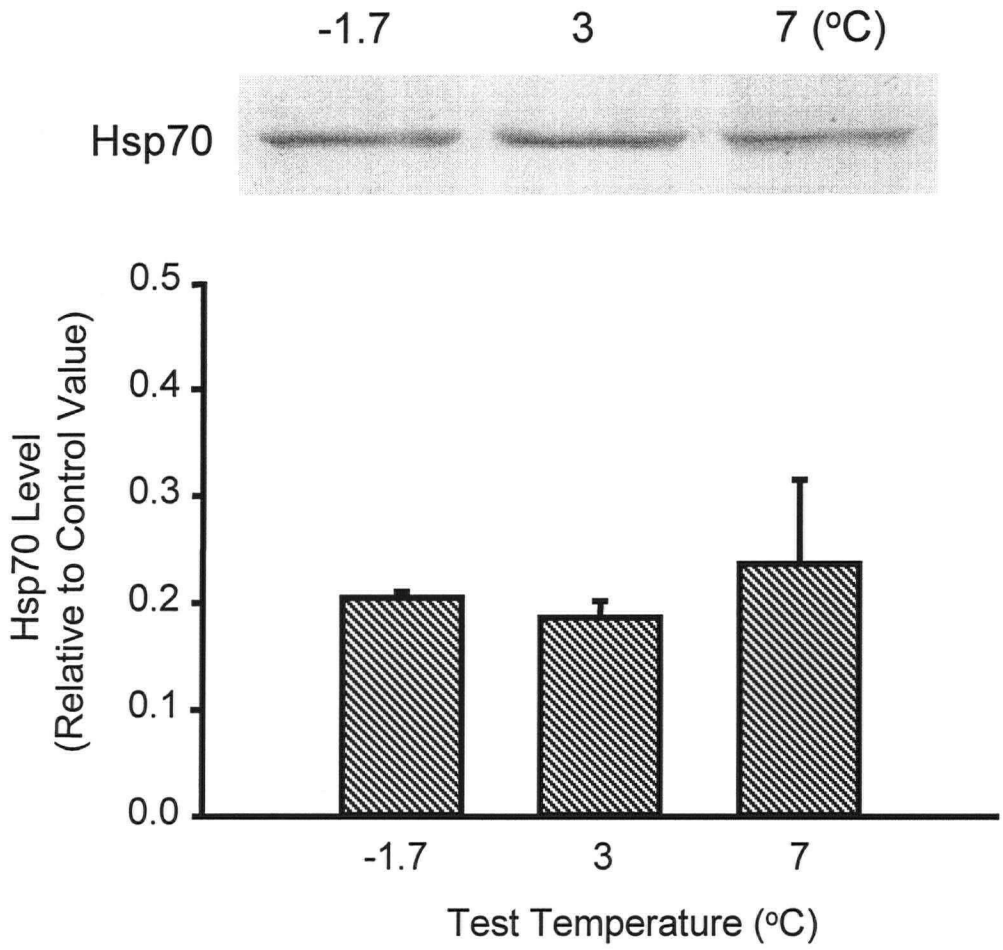


**Figure 1-7.** The upper panel shows hsp70 by Western blot analysis in liver tissuesof *T. bernacchii* at –1.7, 3 and 7 °C (the result from the same individual is shown in the upper panel of Figure 1-8). The lower graph is levels of hsp70 based on band intensities. Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=4-6).

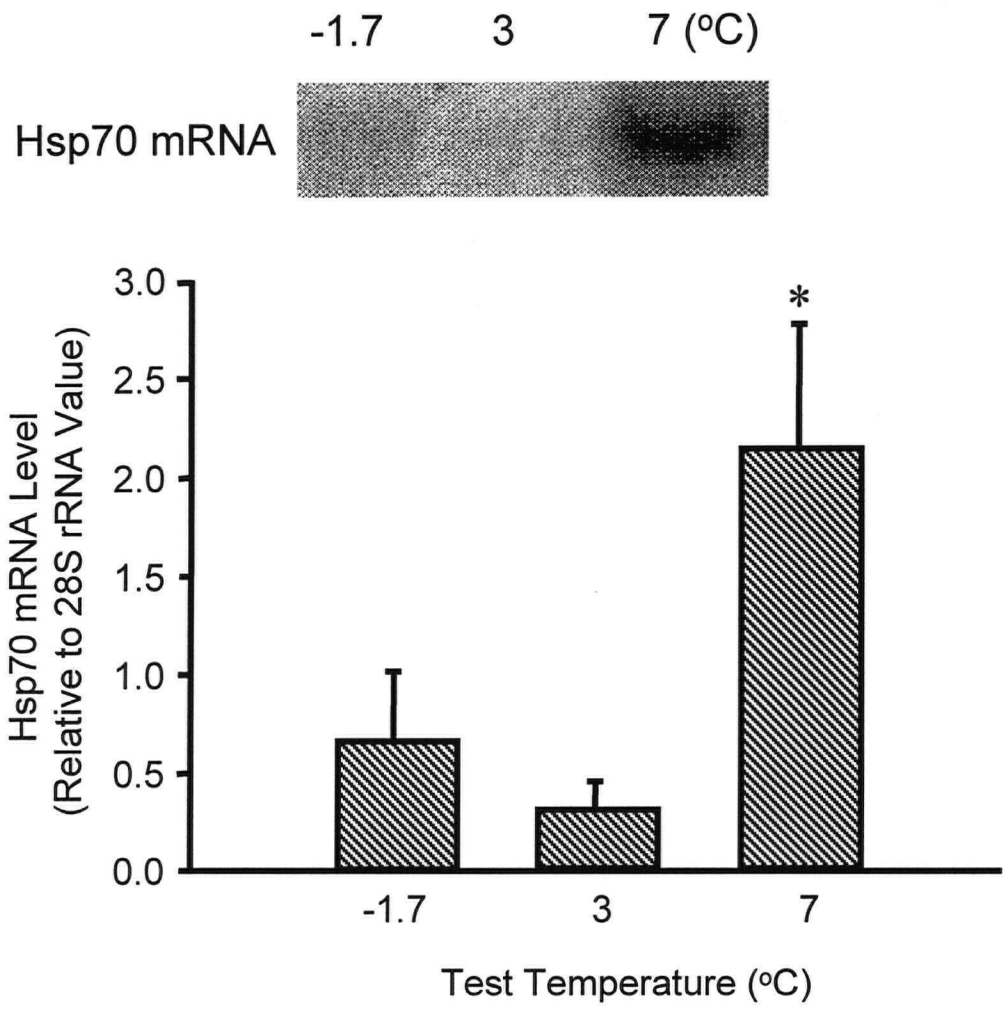


**Figure 1-8.** The upper panel shows hsp70 mRNA by Northern blot analysis in liver tissues of *T. bernacchii* at –1.7, 3 and 7 °C (the result from the same individual is shown in the upper panel of Figure 1-7). The lower graph is levels of hsp70 mRNA based on band intensities standardized by corresponding values of the 28S rRNA levels (Mean±SE, n=4-6). * indicates significant difference compared with the control value at –1.7 °C ($p \leq 0.05$).

10% compared to $C_4$. Judging from a 15% increase in hsp70 mRNA levels in $C_4$ compared with $C_0$, they concluded that handling processes for cultured spleen was stressful enough to induce an increase in hsp70 transcription at –1.2 °C and masked some of the heat shock response at 5 °C. Although there are possibilities of improvement in the experimental protocols of these studies, they showed that Antarctic fish have hsp70 DNA which is activated at higher temperatures. However, Somero *et al.* (1998) found no evidence of any hsp inductions in the gill, brain, liver, spleen and muscle tissues of *T. bernacchii* exposed to 6 or 9 °C for 2 h and allowed to recover for 1 h in an *in vivo* experiment. The authors suggested that it was the first case in which the heat shock response did not occur in an animal, even though the tissues of *T. bernacchii* contained constitutively expressed molecular chaperones as in other species. The authors concluded that the capacity of increased chaperone synthesis in response to thermal stress may have been lost in *T. bernacchii* during adaptation to extremely cold and stable thermal conditions.

My results from Western blot analysis for liver hsp70 of *T. bernacchii* were in agreement with Somero *et al.* (1998); the levels of hsp70 did not change among the groups of fish exposed to –1.7, 3, and 7 °C (Fig. 1-7). However, the hsp70 mRNA levels were significantly higher at 7 °C compared with both –1.7 and 3 °C (Fig. 1-8). Note that results from the same fish are presented in the band images of Figure 1-7 and Figure 1-8. The fish at 7 °C had more than 10 times higher hsp70 mRNA level than that of the fish at –1.7 °C. Nevertheless the hsp70 level of that fish at 7 °C was not significantly higher compared to the level of the fish at –1.7 °C (see upper panels of Fig. 1-7 and Fig. 1-8). There are three possibilities for the inconsistency between hsp70 and hsp70 mRNA levels in my study. The first possibility is that hsp70 DNA in *T. bernacchii* was transcribed by heat shock, but those transcripts were not translated, possibly because the fish lost the ability to translate stress-induced hsps during the long-term adaptation to a cold marine environment. The second possibility is that the turnover rate of hsp70 also increased at 7 °C, so that the increase of hsp70 production due to the higher hsp70 mRNA

production at that temperature was masked by the degradation of this protein. The third possibility is that 4-6 h recovery after heat shock was not long enough for that species to synthesize hsp70, even though it was confirmed by Somero *et al.* (1998) that 1 h recovery was enough to induce hsp70 in *G. mirabilis*. Indeed, Carpenter and Hofmann (2000) found an increase in hsp70 levels in various tissues of *T. bernacchii* kept at 4 °C for 21 days. This species may show the cellular heat shock response after heat stress, but very slowly.

In summary, I demonstrated the cellular heat shock response in a stenothermal Antarctic fish, *T. bernacchii*, on the transcriptional level but not on the translational level at least within several hours after a heat shock treatment. Studies in Antarctic fishes are insufficient to allow us understanding of the mechanisms by which the heat shock response of those fishes may have been lost. There is a broad scope of research in the future to examine the evolutionary aspects of the cellular heat shock response of Antarctic fishes.

## Section 1-3. The cellular heat shock response in a tropical fish in Brazil

### Introduction

The Amazon is another unique environment characterized by a tropical climate with high precipitation. The Amazon basin is comprised of rivers, lakes, small streams, beaches, floodplain areas and flooded forests, producing a complex aquatic landscape (Val and de Almeida-Val 1995). The water temperature of the large water systems is constant over the region ranging from 29±1 °C to 30± 1 °C throughout the year, while that of small lakes and ponds show large diurnal oscillations (Sioli 1984). The fish fauna of the Amazon basin is far richer than that of any other river system, and the Amazon ichthyofauna has representatives of almost all groups of freshwater fishes (Val and de Almeida-Val 1995). Since the biochemical and the physiological mechanisms of these fishes are highly specialized for warm and thermally-stable environments, they can be a good example of fish inhabiting the opposite extreme from

Antarctic fishes with respect to the adaptation to various habitats. How these Amazonian fishes respond to a higher temperature than their habitat temperature has not been reported. In addition, since Amazonian fish are warm-adapted, a low temperature can also be a serious problem for their survival. Kent *et al.* (1988) reported that the channel catfish (*Ictalurus punctatus*) and the green sunfish (*Lepomis cyanellus*) showed increased protein content and concentration in the liver and the heart tissues when the acclimation temperature was gradually reduced (<1 °C/h) from 25 °C to 15 °C. Yamashita *et al.* (1996) found newly synthesized 70-kDa protein induced by a cold shock in the cultured rainbow trout cell line, RTG-2, when the culturing temperature was shifted from 22 °C to 4 °C. Agrawal and Srivastava (1976) demonstrated that levels of white blood cells and thrombocytes increased and decreased, respectively, in a tropical fish (*Colisa fasciatus*) after 2 min cold shock at 2 °C. These reports show that fish respond to a cold stress at the cellular protein level and at physiological level. Thus, in this section, I examined effects of a heat shock, as well as a cold shock, on hsp70 levels in a common Amazonian fresh water fish, the tambaqui (*Colossoma macropomum*). This fish inhabits the main rivers, flood forests, as well as lakes connected to the rivers of the Amazon. In lakes, this species experiences average temperatures of 28 °C up to 37 °C during the summer, with chronic hypoxia. The oxygen content of the water can decrease close to 0% sat. at least for a short period of the day in those lakes (personal communications: Dr. A.L. Val, INPA and Dr. R. Huet de Salvo Souza, IBAMA).

## Materials and Methods

Forty-two tambaqui (*C. macropomum*) were raised at the fish hatchery of the Brazilian Ministry of the Environment (IBAMA), Pirrasununga, Brazil. Fish were acclimated at 26 °C, which was the normal water temperature of the hatchery for more than 2 months before being used in the experiments. After sampling gill, heart and liver tissues from 6 fish as the 0 h samples, 12 fish were transferred to 36 °C as the heat-shocked group, and 12 other fish were

transferred to 18 °C as the cold-shocked group. One hour after transfer, fish in both groups were returned to the acclimation temperature, and allowed to recover. Fish were then sampled for the gill, heart and liver tissues at 1 h and 4 h during the recovery period. Six fish each were sampled from the heat-shocked group and the cold-shocked group in each sampling. Another six fish, kept at 26 °C over the experiment, were sampled at 1 h and at 4 h sampling as the control. Tissue samples were frozen immediately with liquid nitrogen, transferred to UBC on dry ice, and stored at –80 °C until analysis by Western blotting (for the methods of Western blot analysis, see Materials and Methods in Section 1-1).

## Results

Figure 1-9 shows the changes in liver, gill and heart hsp70 levels by a +10 °C (26 °C to 36 °C) heat shock or a –8 °C (26 °C to 18 °C) cold shock. A tissue dependent response was observed in the heat or the cold shock. Significantly higher levels of hsp70 were observed at 4 h after the heat shock in the gill tissue compared with the control value at 4 h. The cold shock did not show any effects on hsp70 levels in this tissue.

The levels of hsp70 showed a general increase temporarily both by heat shock and cold shock in the heart tissue of the tambaqui. However, the levels decreased again at 4 h in both groups. These changes were clearer in the heat-shocked group than in the cold-shocked group; the levels of heart hsp70 were significantly lower at 4 h compared with the control value at 4 h in the heat-shocked group. There was no clear change in liver hsp70 levels after the heat shock and the cold shock. There was no significant change in levels of hsp70 in all tissues compared with the respective initial levels after the heat shock or the cold shock.

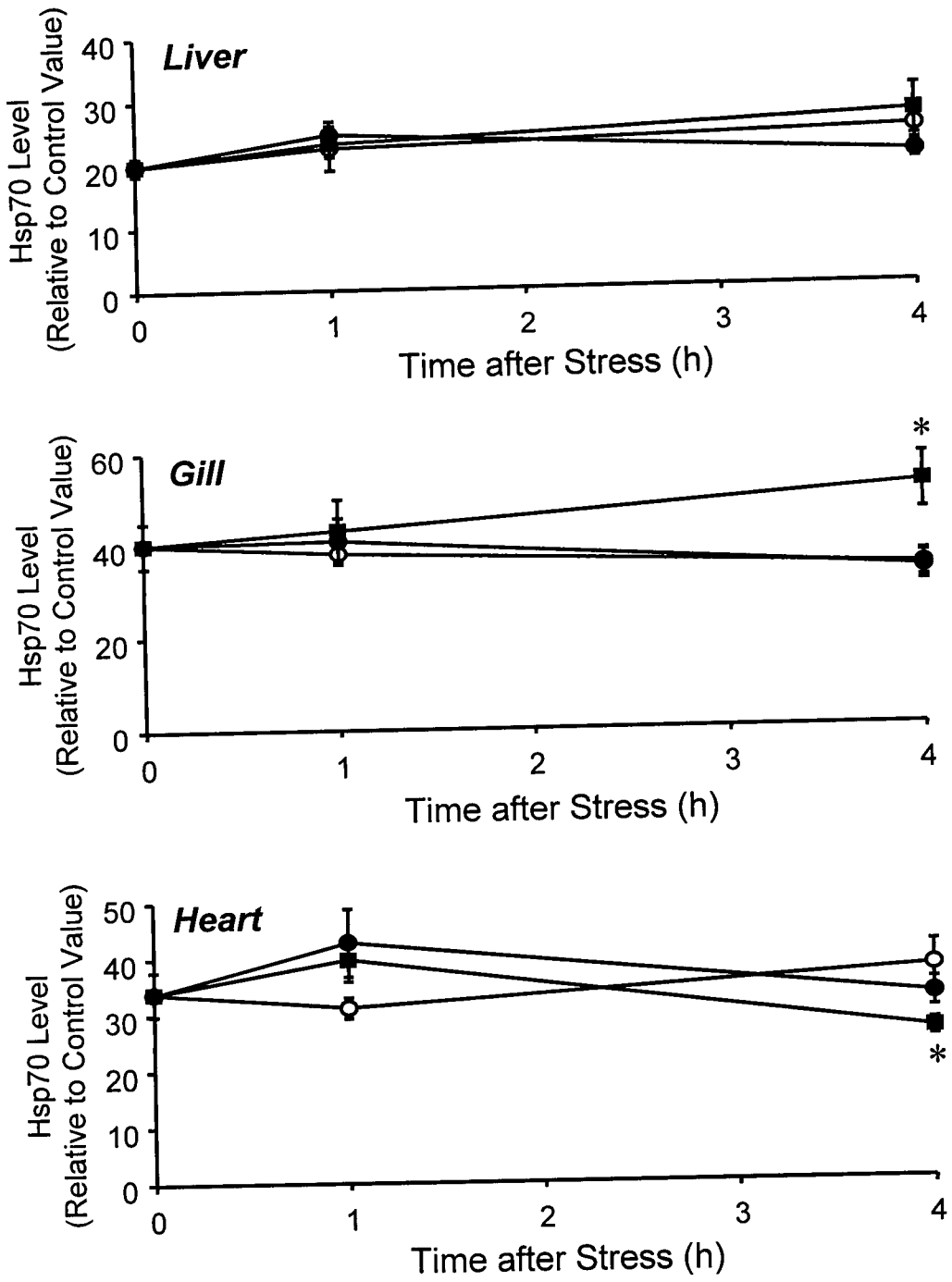


**Figure 1-9.** Changes in levels of hsp70 in the liver, gill and heart tissues of the tambaqui (*C. macropomum*) after the heat shock (■) or the cold shock (●). (○) indicates the control values. Hsp70 values are shown in relative values based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=10). * indicates significant difference in the level of hsp70 compared with the control value at the time ($p \leq 0.05$). There was no significant changes in levels of hsp70 compared with the initial value in any tissues after heat shock or cold shock.

## Discussion

The cellular heat shock and cold shock responses were tissue specific in the tambaqui (Fig. 1-9). There were no clear changes in hsp70 levels in the liver tissue by either the heat shock or the cold shock. The level of hsp70 was increased only by the heat shock in the gill tissue, while that of the heart tissue was temporarily increased, but decreased 4 h after the heat shock and the cold shock. Kent *et al.* (1988) found that the liver tissue of the channel catfish (*I. punctatus*) showed an approximately two-fold increase in liver mass, cell size, total protein and total enzyme activity by transfer from 25 °C to 15 °C, which resulted in a protein hypertrophy. It may be noteworthy that the heart tissue of the catfish has also shown increase in mass and total protein by a cold acclimation, but protein concentrations do not change, in their study. If a protein hypertrophy occurred in the liver tissue of the cold-shocked tambaqui in this study, changes in hsp70 levels might be masked by increases in the total protein level.

Recent evidence indicates that the adaptation to high temperature may have analogies with cold adaptation (Carratù *et al.* 1998). Soto *et al.* (1999) demonstrated that a small hsp (hsp17.5) of the chestnut (*Castanea sativa*) enhanced survivability of *E. coli* both at 50 °C (heat shock) and 4 °C (cold shock). In the present study, the expression patterns of hsp70 after the heat shock and cold shock had the same tendency in the heart tissue of the tambaqui; the level of heart hsp70 tended to increase at 1 h, but to decrease at 4 h after the heat shock or the cold shock compared with the control value at the respective time. It is possible that hsp70 may have a common or similar function both in the heat-shocked and the cold-shocked situations in tambaqui tissue.

The tambaqui is known to have high resistance to both heat and hypoxia that are common problems encountered in its habitat, such as a fresh water lakes in the Amazon basin (Val and de Almeida-Val 1995). They can survive at 40 °C and have more thermostable LDH compared with other Amazonian species (de Almeida-Val and Val 1993; de Almeida-Val *et al.* 1990, 1991 and 1992). The cellular enzyme properties of this species are also insensitive to increasing

temperature; the $K_m$ value of LDH for pyruvate was not affected by temperature, and $Q_{10}$ of this enzyme was very small both in skeletal muscle and the heart of the tambaqui (Val and de Almeida-Val 1995). Val and de Almeida-Val (1995) suggested that biochemical and physiological regulation occurs in Amazonian fish more frequently in response to changes in environmental oxygen level, which may frequently occur in their habitats. For example, the tambaqui can even change the shape of its lip by extending it within 2 h in severe hypoxia to improve oxygen uptake from oxygen-rich surface water (Val and de Almeida-Val, 1995). The small $Q_{10}$ observed in the tambaqui indicated that this species cannot sustain a high metabolic rate at any temperature (Somero 1969). Acclimatization to low oxygen levels that frequently occur may be related to the ability of this fish to sustain a low metabolic rate at high temperatures, which is beneficial in a common aspect of Amazonian water system; high water temperature with low oxygen levels.

The tambaqui is known to induce a series of LDH isozymes by activation of "silent genes" that are not expressed at normal temperatures when they are acclimated at 20 °C; it was reported that this species has a significant amount of redundant DNA that has been made nonfunctional because they are no longer needed by the organism (Val and de Almeida-Val 1995). Since the temperature of the Amazon basin was once 18 to 20 °C during the glacial period (Bigarella and Ferreira 1985), some genes, functional at a low temperature, may have lost their expression during the interglacial period when the temperature increased to that of the present. Some of those genes can be activated, like the silent gene of LDH, if the temperature becomes low enough, even though the product proteins may not be functional any longer. In this study, I measured only hsp70. However, there may be other proteins induced by a cold-shock in this species. Studies of cold-shock inducible proteins in the tambaqui may be a productive approach to understanding the degeneration of cold adaptation in this species during evolution.

## Discussion of Chapter 1

General cellular heat shock responses were observed in all fishes tested in this chapter. However, the patterns of those inductions seem different between species. For example, a sub-tropical fish had a significantly higher hsp70 level at more than 30 °C, while the cold acclimated Antarctic fish did not show any clear increases in hsp70 by increases in temperature, even though they had significantly higher hsp70 mRNA levels at a near-lethal high temperature. The general cellular heat shock response seems likely to have been conserved among various species of fishes, but its properties may have been modified within species during adaptation to their respective habitat temperature. The underlying mechanism that determines the induction temperature for hsp70, and the thermal stability of hsp70 itself would be interesting to compare between these fishes in future studies.

Some sub-tropical fishes in Okinawa showed a high sensitivity to a relatively small change in ocean temperature (Section 1-1). The water temperature, especially in tidepools, can be near the lethal temperature range of inhabiting fishes, especially during the summer. A global climate change, which can increase the ocean temperature by a few degrees within relatively short time, would have a significant impact on those fishes, if it happens near their lethal temperatures.

Both the tambaqui and the five-banded damselfish are warm water fishes. However, the environments they inhabit are very different in physical and chemical water qualities. Beside the difference in salinity (the five-banded damselfish is in the ocean while the tambaqui is in fresh water aquatic system), the most extraordinary difference between the habitats of these species may be oxygen levels. The oxygen level of the ocean can be always well-saturated by the mixing action of turbulence and current, while most tropical floodplains in the Amazon experience permanent or periodical hypoxia (<50% DO), and diurnal fluctuations in oxygen levels are amplified in small lakes (anoxic at night and 250% DO during day) (Val and de Almeida-Val 1995; Junk *et al.* 1997). When animals are exposed to high temperatures, their

metabolism is generally up-regulated which may be related, at least partly, to increased hsp functions to repair damaged cellular proteins. On the other hand, when animals are exposed to severe hypoxia, their metabolism as well as the most of gene transcriptions are down-regulated. How the Amazonian fishes cope with this double-stress of high temperature and low oxygen is not known. The function of hsps and the regulation of production of these proteins at high temperatures with (the tambaqui) and without (the five-banded damselfish) adaptation to hypoxia can be a good example of evolutionary modification of hsp functions during the environmental adaptation of animals.

In conclusion, I had a strong impression that increase in the level of hsp70 by a heat shock is highly conserved response among various species of fishes. However, the pattern of the expression may have been modified within species during evolution and adaptation to the respective habitat temperature. For example, Antarctic *T. bernacchii* did not have a higher hsp70 level even at a near-lethal high temperature at which the hsp70 mRNA level significantly increased compared with the level at −1.7 °C, indicating a part of the translational pathway of hsp70 may have been lost in this species during the adaptation to extreme cold temperatures. The induction temperature for hsp70 was generally in agreement with the habitat temperature of the species, which supports the possibility of a close relationship between the function of hsp70 and the thermal tolerance of the species.

# Chapter 2

# Cellular heat shock responses in tidepool and fluffy sculpins in the natural environment

## Introduction to Chapter 2

Expression of hsp70 in liver tissues of two intertidal sculpins, the tidepool sculpin (*Oligocottus maculosus*) and the fluffy sculpin (*O. snyderi*), are compared in this chapter. These sculpins show different vertical distribution in the intertidal zone, which may relate to different habitat temperatures that these fishes experience in nature.

The intertidal zone is a unique environment between the land and the ocean. The combination of the tidal cycle and the time of day can bring rapid changes in the physical and chemical factors in the water of isolated tidepools (Horn *et al.* 1999). Changes in weather can make those changes in the water quality of tidepools unpredictable. Temperature and salinity of isolated tidepools can fluctuate dramatically as a result of isolation, precipitation and evaporation during a daytime low tide. Oxygen levels can rise during a period of photosynthesis by algae during daytime, but can drop considerably during a period of respiration at night (Davenport and Woolmington 1981). In spite of these harsh environmental conditions, rocky intertidal habitats are often highly productive and rich in nutrients and organisms. These organisms have met these challenging environments with unique physiological, biochemical and ecological mechanisms (Horn *et al.* 1999). These organisms are often eurythermal and sometimes have very high heat resistances. For example, extreme heat tolerance was shown by the intertidal goby, *Gillichthys seta*, whose body temperature can range from 8 to 40 °C due to both seasonal and daily changes in water temperature (Dietz and Somero 1992), and by intertidal mussels, *Mytilus trossulus*, whose body temperature can range from 10 to 35 °C during a day in summer (Hofmann and Somero 1995). Expression of hsp70 in these intertidal animals was investigated by Hofmann

and Somero (1995) as well. However, there is still a wide scope for further studies in the cellular heat shock response of intertidal fishes, especially in natural environments.

I chose the tidepool sculpin and the fluffy sculpin for the experimental animals in my thesis, since they have a well-studied background in ecology, but have not been studied with respect to their thermal physiology. The tidepool sculpin and the fluffy sculpin are common congeneric cottid fishes on the west coast of North America, distributed between the Gulf of Alaska and mid-California (Green 1971a; Morris 1960 and 1962). Belonging to the same genus, they are evolutionarily closely related. They are also similar in morphology, diet, and ecology (Nakamura 1971). They both inhabit tidepools in the intertidal zone during a low tide. While their distributions can overlap, it also has been shown that they generally have different distribution patterns during a low tide. The tidepool sculpin inhabits a wide range of the intertidal zone from lower to upper tidepools, but tends to aggregate in the upper and the middle tidepools. The fluffy sculpin prefers lower tidepools or the subtidal zone (Green 1971a; Nakamura 1976a and b). The upper tidepools have a larger fluctuation in water temperature, with higher average temperatures compared with lower tidepools (Green 1967; Nakamura 1976a). Other physical and chemical factors in the tidepools beside water temperature, such as salinity and oxygen levels, can induce a stress response and affect hsp70 levels in the cells of fish (Currie *et al.* 1999; Kojima *et al.* 1996). However, water temperature seems to be the most important factor in determining the different distribution ranges of these sculpins. In fact, higher resistance to warmer temperatures in the tidepool sculpin compared to the fluffy sculpin has been shown (Nakamura 1970 and 1976a). Therefore, I focused on the effects of temperature on heat shock responses of these sculpins in my study. In addition, in spite of those accumulating data in ecological and behavioral aspects of these species, physiological aspects of thermal resistances of these species have not been studied. Thus, the tidepool and the fluffy sculpin can be good models to study effects of habitat temperature on the cellular heat shock response of fishes.

## Section 2-1. Field monitoring of water qualities in tidepools, and field observations of cellular heat shock responses in sculpins

### Introduction

To compare the responses of tidepool and fluffy sculpins to changes in water temperature, I examined cellular heat shock responses of these sculpins in the field. It has already been shown that organisms inhabiting the intertidal zone can be highly sensitive to temperature. The intertidal mussel, *M. trossulus*, had higher ubiquitin and hsp70 levels in the intertidal zone than in a submerged location (Hofmann and Somero 1995). They also had seasonal changes in those levels, when the animal experienced changes of the body temperature from 10 to 35 °C in August, and 7.4 to 15.4 °C in February (Hofmann and Somero 1995; Roberts *et al.* 1997). The longjaw mudsucker, *G. mirabilis*, possess two isozymes of cytosolic malate dehydrogenase (cMDH) of which one is more thermostable than the other (Lin and Somero 1995b). The ratio of one to the other showed seasonal changes; winter fish had a higher percentage of thermolabile cMDH than summer fish. Furthermore, Stillman and Somero (1996) showed that the respiratory physiology and cellular biochemical properties of crabs (*Petrolisthes* spp.) were more stable under thermal stress and aerial exposure in species from the upper intertidal zone where the habitat temperature was as high as 31 °C, than in species from the lower intertidal zone where the maximum habitat temperature was around 16 °C.

Such studies indicate that the difference in habitat temperature, caused by geographic distribution or seasonal cycle, may be involved in the regulation of cellular protein production and its function in animals inhabiting the intertidal zone. In the present study, levels of hsp70 in liver tissues of tidepool and fluffy sculpins were compared in the field to examine the cellular heat shock response in their natural habitats. In order to know the thermal conditions that those sculpins experience in natural tidepools in more detail, I monitored changes in the water

temperature, as well as other water qualities such as salinity and oxygen level, in tidepools in the rocky intertidal zone of B.C., Canada, which these sculpins inhabit.

## Materials and Methods

### *Long term monitoring of water temperature in tidepools by data loggers*

Changes in water temperatures in a lower, middle and upper tidepool on Ross Islets, near Bamfield Marine Station, Bamfield, British Columbia (B.C.), Canada, were monitored by data loggers for 17 days from June 30$^{th}$ to July 16$^{th}$ of 1999. One data logger for the lower and the upper tidepools, and 2 data loggers for the middle tidepool were bolted on the rocks, so that the data loggers stayed under the surface of water during a low tide. Accumulated data in the data loggers were downloaded to a computer and analyzed by Boxcar Pro software.

### *Changes in water quality in a selected tidepool during a low tide*

Water temperature, salinity and oxygen concentration of a tidepool were monitored in October 1997 on First Beach, and in July 1998 on Pachena Beach near Bamfield, B.C. Temperature and oxygen level were monitored with the OxyGuard Handy Mk III Portable DO Meter (Point Four Systems Inc., Canada), while salinity was measured with a temperature-compensated portable refractometer (Cambridge Instrument Inc., USA). These variables were measured at various points in one tidepool, as well as in tidepools over the course of the low tide.

### *Seasonal changes in liver hsp70 in the tidepool sculpin*

The tidepool sculpin (*O. maculosus*) was sampled from several tidepools on Lions Bay, Vancouver, B.C., Canada, every month from August 1998 to July 1999. Collection of fish was conducted around the date of a full moon of each month. Time of collection was dependent on the time of LW on the day (generally in early afternoon during summer and in early morning

during winter). Six to 7 fish were collected from tidepools every month. Fish were caught by dip nets, and killed immediately in the field by cutting the nerve chord following anesthetization by an overdose of MS-222 (500mg/L). Temperature and oxygen level were monitored with the OxyGuard Handy Mk III Portable DO Meter (Point Four Systems Inc., Canada), and salinity was measured with a temperature-compensated portable refractometer (Cambridge Instrument Inc., USA) in the ocean and the tidepools, from which the fish were collected, on the day of sampling. Fish samples were then transferred to the laboratory at UBC to sample the liver tissue for the measurement of hsp70 by Western blotting. The liver tissue was kept in a 1.5mL microcentrifuge tube and stored at -80 °C until analysis. All liver samples for a whole year were analyzed together to eliminate inter-analysis variation.

*Hsp70 expression in tidepool and fluffy sculpins during a low tide*

Tidepool and fluffy sculpins (*O. snyderi*) were sampled from lower tidepools every 1 h from 07:45 for 4 h until 11:45 during a low tide in July 1999 on Wizard Islet near Bamfield Marine Station, Bamfield, B.C. The weather on that day was rainy and cloudy with sun breaks, and the LLW (Lowest Low Water) was at 09:49. Even though the HHW (Highest High Water) of that day was about 15:00, the lower tidepools, from which the fish were sampled, started connecting to the ocean at 11:55. Two to 6 fish for each species were sampled for liver tissues at each sampling time. Liver samples were frozen immediately with liquid nitrogen in the field, and stored at –80 °C until analysis by Western blotting. Changes in water temperature in the tidepools from which these fishes were collected were monitored with a temperature-oxygen meter (OxyGuard Handy Mk III Portable DO Meter, Point Four Systems Inc. Canada).

*Hsp70 expression in the tidepool sculpin during low and high tides*

The tidepool sculpin was sampled from an upper tidepool every 1 h from 12:30 until 16:30 during a low tide on July 21st 1999 on Wizard Islet near Bamfield Marine Station, Bamfield, B.C. It was sunny and the LLW was at 13:40. Six fish were caught and the liver tissue was collected at each sampling time in the field. Tissue samples were frozen immediately in liquid nitrogen in the field. After the 5th sampling at 16:30, 48 fish were collected from the same tidepool, and transferred to the stock tank in the station. The water temperature in the stock tank was 11 °C. Sampling was continued from the stock tank from 18:00 on July 21st to 10:00 on July 22nd to examine the changes in liver hsp70 level during the submerged period, which was supposed to happen in nature. The HHW was at 20:00. Sampling times for the submerged period were at 18:00, 20:00, 22:00 and 24:00 the first day, and at 04:00, 08:00 and 10:00 the second day. Liver samples were frozen immediately with liquid nitrogen. Tissue samples both from the field and the laboratory were stored at –80 °C until analysis for hsp70 by Western blotting (for the methods, see Materials and Methods in Section 1-1). The changes in water temperature that the fish experienced over the whole sampling were monitored by a data logger.

## Results

*Changes in the water temperature in tidepools*

The changes in water temperatures in the lower, middle and upper tidepools are shown in Figure 2-1. These graphs show that the range of temperature fluctuation was dramatically different depending on the position of the tidepool in the intertidal zone, as well as the position in a tidepool, i.e., surface or bottom of the middle tidepool. The scale of changes in the water temperature was also dependent on the weather, which means the highest temperature in a tidepool during a low tide is unpredictable for the organisms in the tidepool.

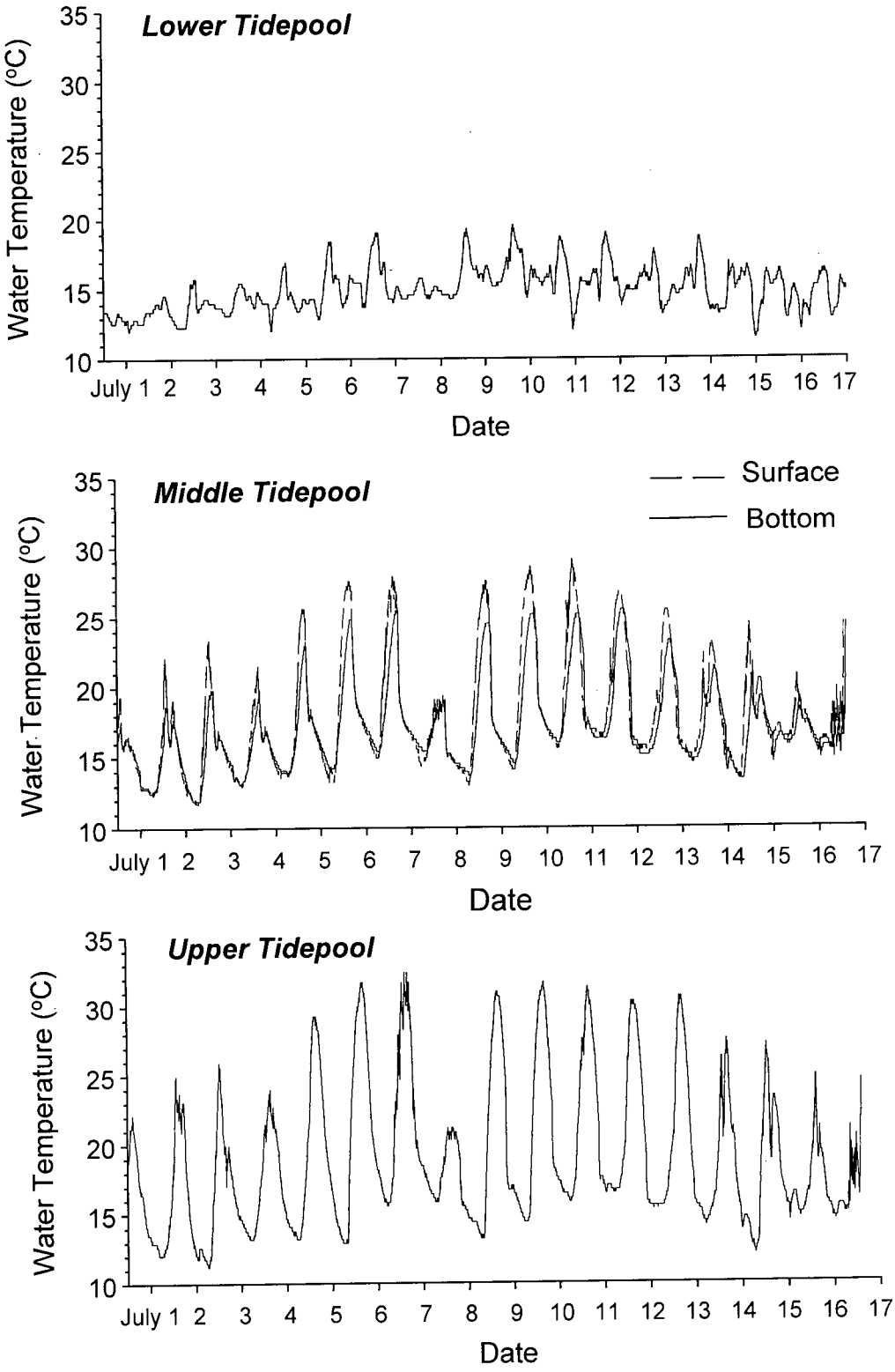


**Figure 2-1.** Changes in water temperatures in the lower, middle and upper tidepools on Ross Islet, Bamfield, B.C., Canada, measured by data loggers during the summer of 1999. In the middle tidepool, temperatures at the bottom (solid line) and at the surface (dashed line) of one tidepool were monitored.

*Changes in the water quality of a tidepool during a low tide*

Figure 2-2 shows the time-dependent changes in temperature, salinity and oxygen concentration in a tidepool on First Beach, Bamfield on October 11th 1997. The size of the tidepool was 70cm wide, 80cm long and 6cm at the deepest. The fluctuation in water quality in that tidepool was representative of tidepools of the area during a low tide. Even larger fluctuations can occur in some upper tidepools, especially when it is sunny in the summer. Aggregations of juvenile tidepool sculpins were observed in that tidepool. It was sunny with an air temperature of 12.7 °C during measure events. The time of the lowest low water (LLW) was at 14:30 and the surface ocean salinity was less than 15 ppt because of a heavy rain the day before. Figure 2-2 shows a rapid increase in water temperature concomitant with a supersaturation of oxygen in the isolated tidepool during that low tide.

*Difference in the water quality depending on the position of one tidepool*

Figure 2-3 shows a picture of a tidepool and temperature, oxygen concentration and salinity at various positions of that tidepool on Pachena Beach, Bamfield in July 14th 1998. All values were monitored at 09:25. It was a mostly cloudy day and the time of the LLW was at 10:55. The size of the tidepool was 226cm wide, 310cm long and 35cm at the deepest. The surface of the tidepool was covered with eelgrass (*Zostera marina*) and surf grass (*Phyllospadix* spp.). Numerous tidepool sculpins and fluffy sculpins, together with other organisms, such as the mosshead sculpin (*Clinocottus globiceps*), the Californian mussel (*M. californianus*), the giant green anemone (*Anthopleura xanthogrammica*) and the purple shore crab (*Hemigrapsus nudus*) were observed in that tidepool. Figure 2-3 shows large differences in water quality, especially in oxygen concentration, depending on the position and the depth within that tidepool.

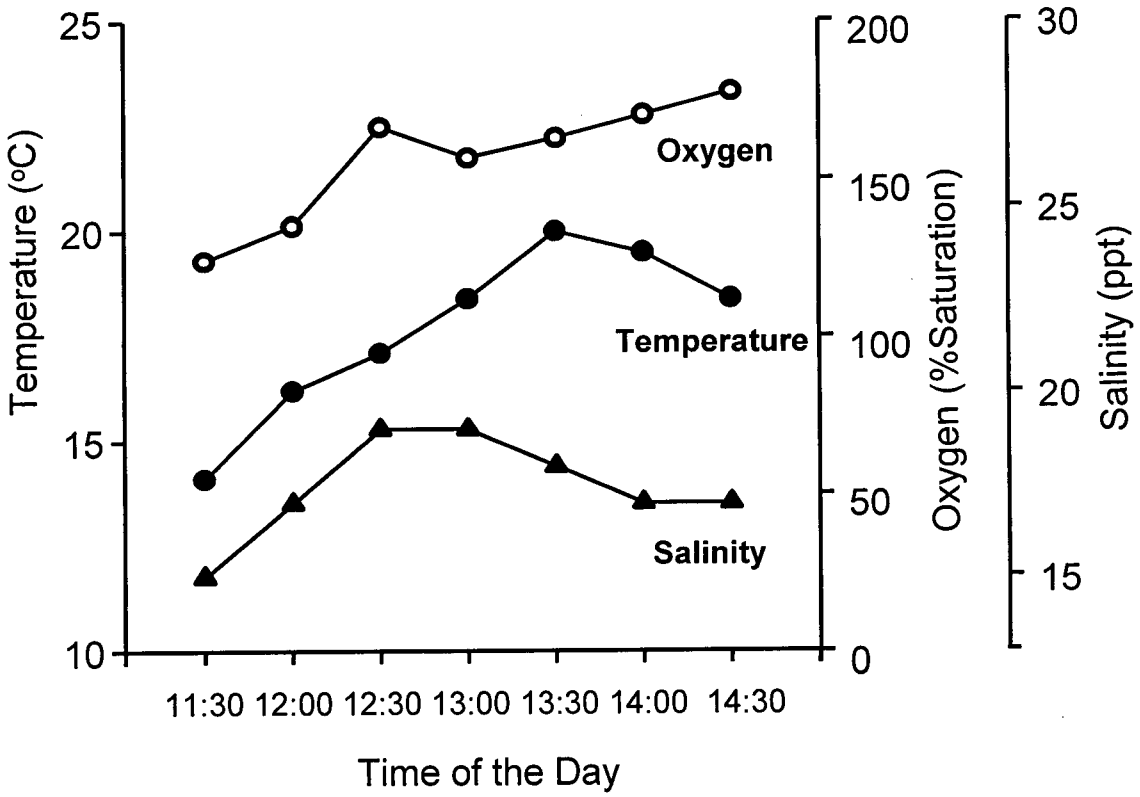


**Figure 2-2.** Changes in water temperature (°C), oxygen concentration (% saturation) and salinity (ppt) in a tidepool over the time course of a day. These variables were monitored on a sunny day in October 1997 at First Beach, Bamfield, B.C., Canada.

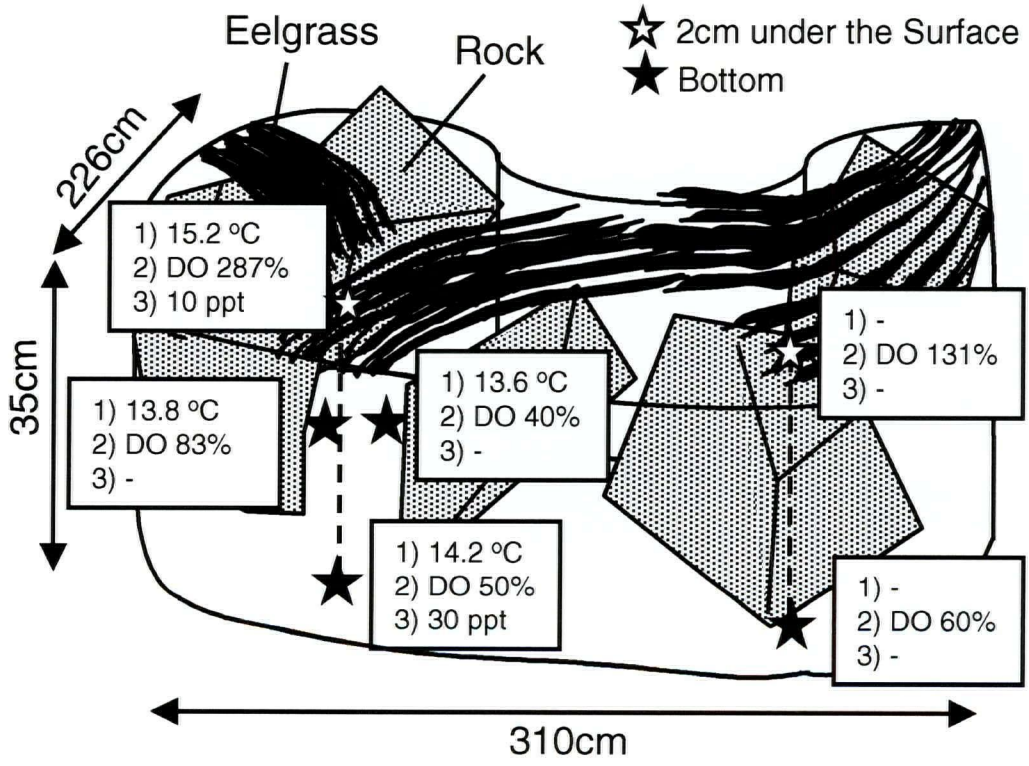


**Figure 2-3.** A picture of a tidepool and a 3-dimensional diagram representing that tidepool on Pachena Beach, Bamfield, B.C., Canada. ★ indicates the site of monitoring and attached numbers are temperature (°C), oxygen concentration (% sat.) or salinity (ppt) at that site. Factors were all monitored at 09:25 in a morning of a cloudy day in July 1998. It was shown that the water quality of a tidepool varied dramatically depending on the position within that tidepool. Generally, water layers of [low temperature/ low oxygen / high salinity] at the bottom and [high temperature / high oxygen / low salinity] at the surface of that tidepool were formed after the isolation of the tidepool, if there was no mixing of water by factors such as a heavy rain.

*Seasonal changes in liver hsp70 levels in the tidepool sculpin*

Temperature of the ocean, air and of the water in a tidepool on Lions Bay, B.C. changed seasonally (Fig. 2-4). The difference in the water temperature in the tidepool between summer and winter was large. Even though tidepool sculpins were observed in this tidepool through the year from August 1998 to July 1999, the size of population of this species in the intertidal zone of Lions Bay changed markedly; the number observed in tidepools declined sharply from fall to winter until early spring. Aggregations of juvenile tidepool sculpins were observed in tidepools on Lions Bay in April, and the population of adult tidepool sculpin grew toward the summer. The level of hsp70 in liver tissue of the tidepool sculpin from Lions Bay did not change significantly through the year (Fig. 2-5).

*Changes in liver hsp70 levels in tidepool and fluffy sculpins during a low tide*

Figure 2-6 shows changes in liver hsp70 levels for 4 h during a low tide in tidepool and fluffy sculpins, and changes in water temperatures of the lower tidepools from which the fishes were sampled. The level of liver hsp70 in the tidepool sculpin did not significantly change during a low tide. On the other hand, the hsp70 level in the liver tissue from the fluffy sculpin increased during the low tide, and became significantly higher at 4 h compared with the level at the beginning. Although the level of liver hsp70 in the tidepool sculpin did not change during the low tide, levels of liver hsp70 in the tidepool sculpin were significantly higher than those in the fluffy sculpin in earlier period of the low tide.

*Changes in liver hsp70 levels in the tidepool sculpin during low and high tides*

Neither significant changes nor clear patterns correlated with the tidal cycle or changes in the water temperature were observed in levels of liver hsp70 in tidepool sculpins collected from an upper tidepool (Fig. 2-7).

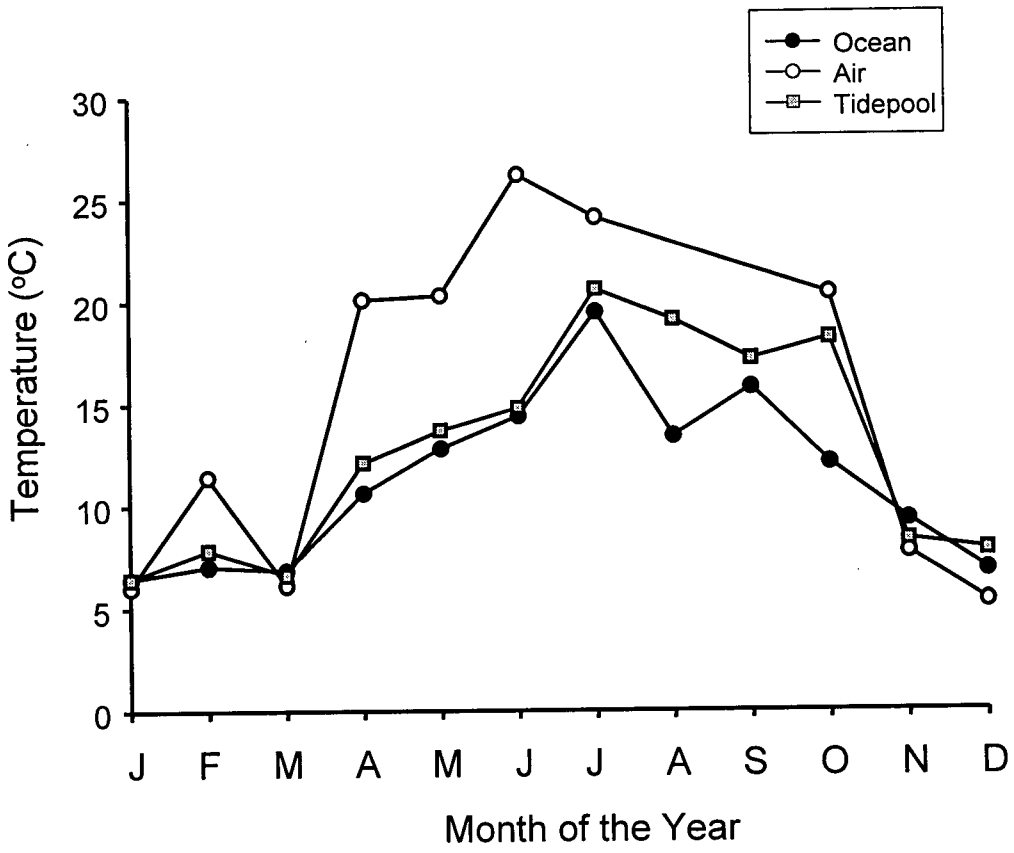


**Figure 2-4.** Seasonal changes in temperatures of the ocean, air and water in the tidepool, from which the tidepool sculpin was collected for liver hsp70 measurements. These temperatures were monitored at Lions Bay, Vancouver, B.C. every month.

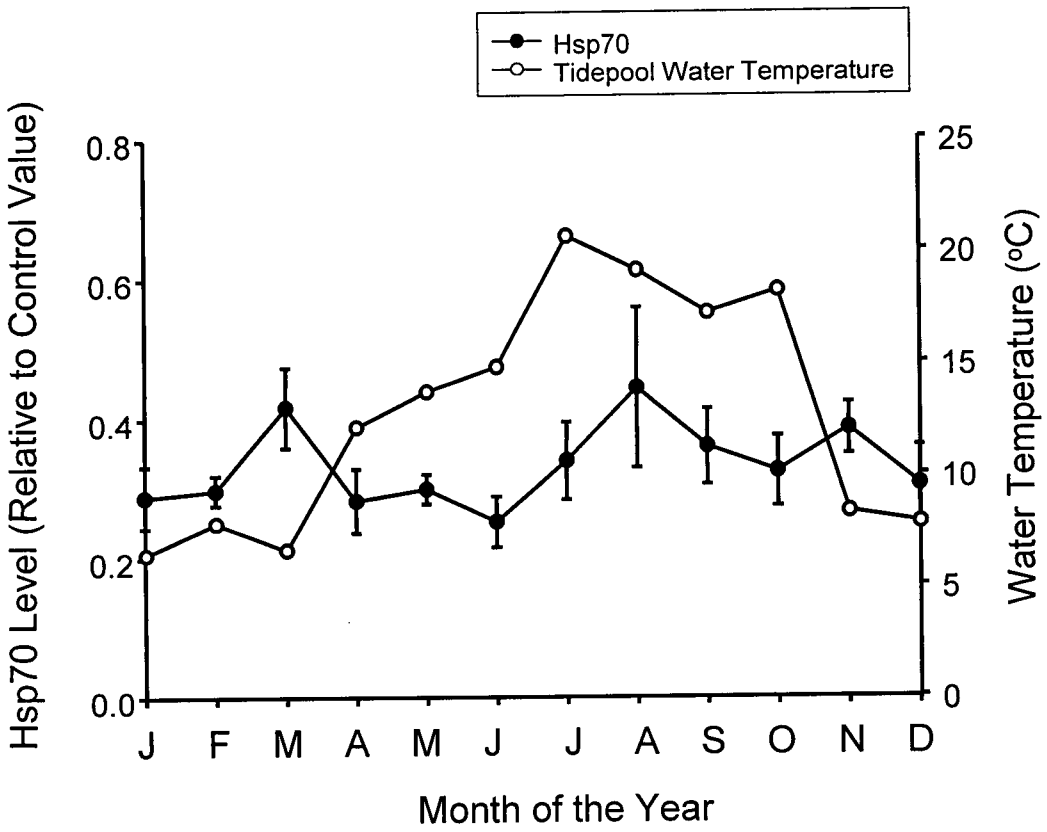


**Figure 2-5.** Seasonal changes in liver hsp70 levels in the tidepool sculpin and the water temperature in the tidepool from which the tidepool sculpin was collected for hsp70 measurements. Fish were collected at Lions Bay, Vancouver, B.C., Canada, every month. There was no significant change in levels of liver hsp70 in the tidepool sculpin in any month of the year (Mean±SE, n=6).

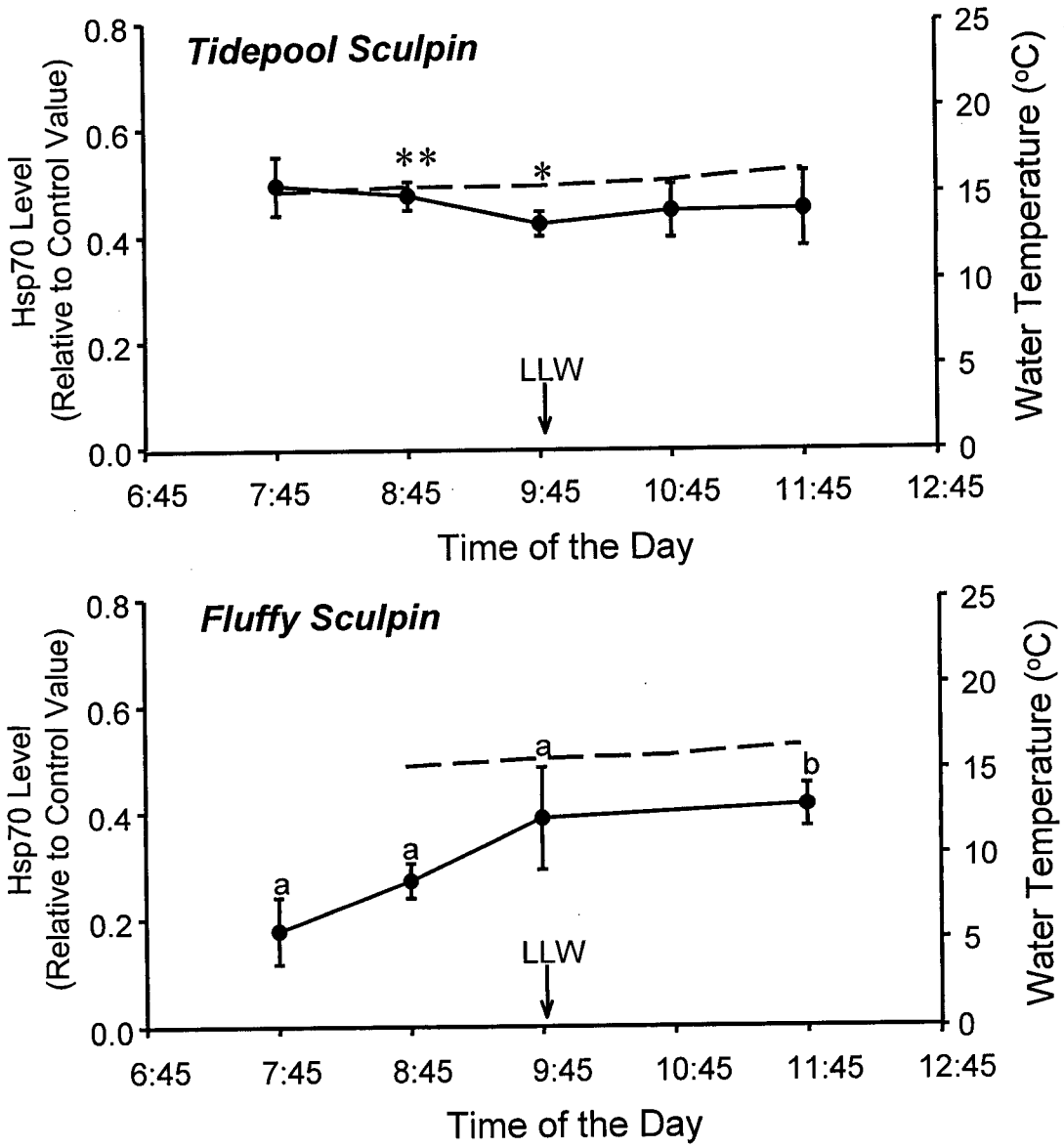


**Figure 2-6.** Changes in liver hsp70 levels in the tidepool and the fluffy sculpins during a low tide. Dash lines indicate the changes in water temperatures of the tidepools, from which the fishes were collected every hour for 4 h. The vertical arrow shows the time of LLW (lowest low water) on that day. Different alphabet letters indicate significant difference compared to the level at the beginning (Mean±SE, n=6, $p \leq 0.05$), while * shows significant difference in liver hsp70 levels between tidepool and fluffy sculpins sampled at the same time (*; $p \leq 0.05$, **; $p \leq 0.001$). There were no significant changes in levels of liver hsp70 in the tidepool sculpin during the low tide, while the level of liver hsp70 in the fluffy sculpin became significantly higher at the end of the low tide compared with the level at the beginning. Levels of liver hsp70 were significantly higher in the tidepool sculpin than in the fluffy sculpin in earlier period of low tide.

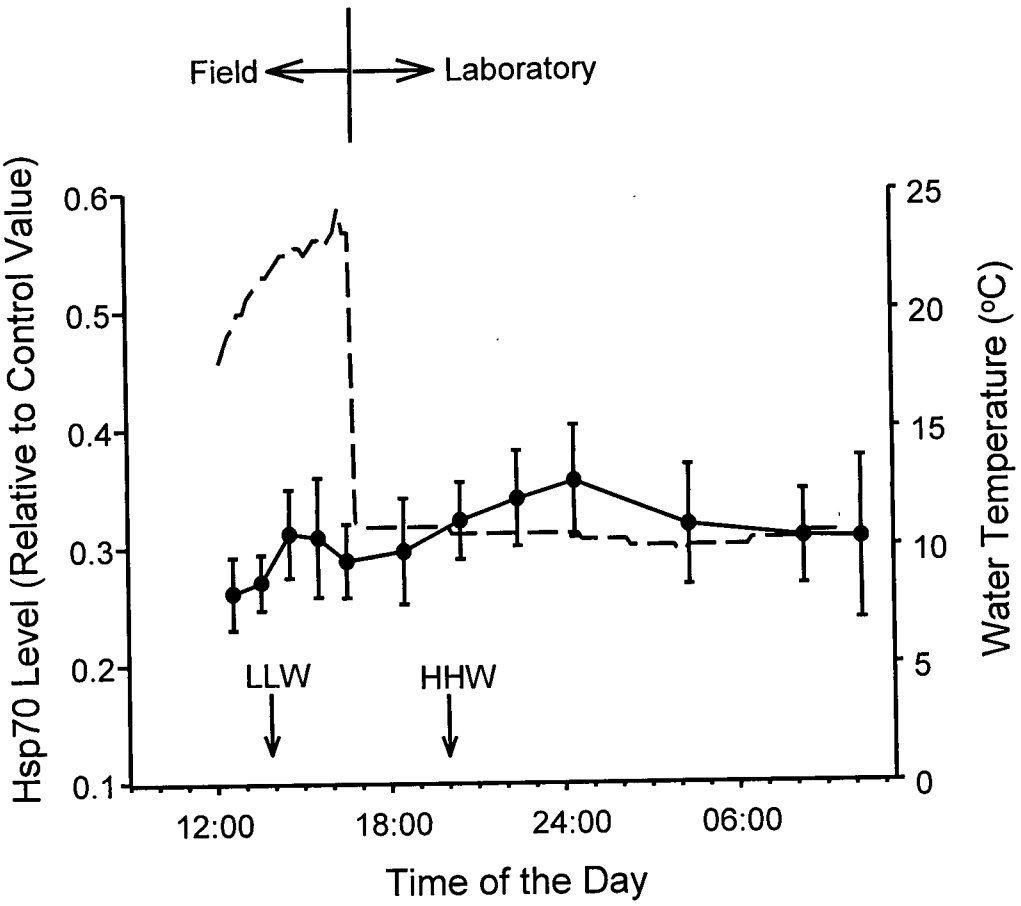


**Figure 2-7.** Changes in liver hsp70 levels in the tidepool sculpin during a low and a high tides (Mean±SE, n=6). Dash line indicates the changes in the water temperature that the fish experienced during the sampling both in the field and in the laboratory. The arrows show the time of LLW (lowest low water) and HHW (highest high water) of the day. The vertical line above the graph indicates the time that tidepool sculpins were transferred to the laboratory from the field for sampling during a high tide. There were no significant changes in the liver hsp70 level of the tidepool sculpin over a low or a high tide period.

## Discussion

The range of fluctuations in water temperature was different dependent on the position of tidepools (Fig. 2-1). The highest and the lowest temperatures on July 6$^{th}$ were 13.5 and 18.8 °C for the lower tidepool, 15.2 and 25.5 °C for the middle tidepool (bottom), and 15.8 and 32.5 °C for the upper tidepool. The surface of the middle tidepool was 15.1 for the lowest and 27.9 °C for the highest, which was 2-2.5 °C higher than those at the bottom of the tidepool. Obviously, fish may experience a different range of change in water temperature during a low tide depending on the position of their habitat tidepool. The tidepool sculpin was found both in upper and lower tidepools. Numerous tidepools formed on Ross Islet were predominantly occupied by tidepool sculpins. On the other hand, fluffy sculpins were hardly found, and only a few of them were in a very low tidepool on this Islet. The same tendencies in the distribution and the population size of these sculpins were observed on Wizard Islet, as well. In our field observations, it was found that not only the range of distribution, but also the size of population in the intertidal zone during a low tide are very different between these species. Together with former reports (Green 1971a; Morris1962; Nakamura 1976a), our field observations strongly imply that the tidepool sculpin could have a higher thermal tolerance than the fluffy sculpin.

Different cellular heat shock responses during a low tide between tidepool and fluffy sculpins were shown in this study. The level of liver hsp70 in the tidepool sculpin in a lower tidepool did not change over a low tide, while that of the fluffy sculpin significantly increased during the low tide (Fig. 2-6). There were no clear changes in liver hsp70 levels of the tidepool sculpin in the upper tidepool correlated with the tidal cycle and changes in the water temperature (Fig. 2-7). The level of liver hsp70 in the tidepool sculpin may not be affected by the tidal cycle or temperature fluctuations both in lower and upper tidepools. An increase in levels of liver hsp70 in the fluffy sculpin in a lower tidepool during a low tide, with relatively mild temperature

fluctuations, indicating that the cellular heat shock response of this species may be influenced by changes in temperature more than in the tidepool sculpin.

A strong correlation has been reported between the cellular heat shock response and thermotolerance in intertidal animals. Dietz and Somero (1992) showed a different threshold induction temperature for hsp90 between two intertidal gobiid fishes, *G. mirabilis* and *G. seta*. Hofmann and Somero (1996a) demonstrated interspecific differences in the hsp70 induction between two intertidal mussels, *M. trossulus* and *M. galloprovincialis*, northern and southern species, respectively. Sanders *et al.* (1991) found different hsp induction patterns in two species of *Collisella* limpets that occupy different intertidal habitats. These reports support the hypothesis that distribution patterns of animals in the intertidal zone can affect expression of hsps, so that the tidepool and the fluffy sculpins may have different cellular heat shock responses.

In contrast to the results from the tidepool sculpin (Fig. 2-7), Hofmann and Somero (1996b) reported clear patterns of changes in levels of hsp70 and ubiquitin conjugates associated with a high or a low tide in the intertidal mussel, *M. trossulus*. In the intertidal mussel, a significant decrease in the hsp70 level was observed during a low tide when they were exposed, while it was restored during a high tide when they were submerged again. The authors concluded that the mussel decreased, or completely depressed its body metabolism during a low tide when its body temperature became more than 40 °C, which resulted in degradation of stored hsp70 during a low tide. During a high tide, when the mussel was covered by the cool water again, it restored normal metabolism and started hsp70 synthesis again. The tidepool sculpin may have a different cellular heat shock response from the mussel in nature, since this fish stays active even during a low tide. Metabolism and synthesis of hsps might not be depressed during a low tide in the tidepool sculpin. Thus, no clear depression was observed in the liver hsp70 level of this species during a low tide (Fig. 2-7).

The level of liver hsp70 in the tidepool sculpin did not change significantly through a year in this study (Fig. 2-5). Fader *et al.* (1994) showed clear seasonal changes in hsp70 expression in stream fishes (*Pimephales romelas*, *Salmo trutta*, *Ictalurus natalis* and *Ambloplites rupestris*). These fishes showed the lowest levels of hsp70 occurring in winter, followed by a high level in spring, a significant decrease in summer and in fall. It may be possible that those fish had more distinct seasonal changes in hsp70 because they are more stenothermal, and more sensitive to changes in water temperatures than the tidepool sculpin. However, Hofmann and Somero (1995), and Roberts *et al.* (1997) also reported seasonal changes in hsp70 and ubiquitin levels in the eurythermal intertidal mussels (*M. californianus* and *M. trossulus*). Again, during winter, intertidal mussels might reduce their whole-body metabolism, resulting in reduction of hsp70 and ubiquitin expressions as well. On the other hand, tidepool sculpins in tidepools were relatively active during winter too, probably because their activity must be restored as soon as the tide becomes high. A cold shock is also known to induce the 70-kDa cold shock protein in fish (Yamashita *et al.* 1996). Judging from the fact that the tidepool sculpin can be exposed to a water temperature of 1.5 °C in a tidepool during winter (Nakamura 1976a), these fish may need to express a relatively high amount of molecular chaperones to keep themselves active in severe, near-zero coldness in winter.

Levels of liver hsp70 in the tidepool sculpin in the lower tidepool were significantly higher than in the fluffy sculpin when liver hsp70 levels did not change in the tidepool sculpin, but increased in the fluffy sculpin during a low tide (Fig. 2-6). Therefore, the tidepool sculpin had a higher basal level of liver hsp70 which did not change, while the fluffy sculpin had a lower basal level of hsp70 at the beginning of a low tide which increased during a low tide. Ulmasov *et al.* (1992) found that lizards inhabiting the Middle Asia deserts had a higher content (2- to 5-fold) of hsp70-like proteins at normal physiological temperatures than species from Northern regions. The authors proposed a general rule of correlation between the thermal habitat of a given species

and the amount of hsp70 at normal temperature. Therefore, the higher basal hsp70 levels in the liver tissue of the tidepool sculpin than in the fluffy sculpin may be related to a wider range of habitat temperature and a higher thermal tolerance of the tidepool sculpin than the fluffy sculpin.

In summary, I demonstrated different cellular heat shock responses in the natural environment between closely related intertidal sculpins with different vertical distribution patterns in the intertidal zone. Overall observations showed that the tidepool sculpin had a higher capacity of basal hsp functions and less sensitivity to changes in water temperatures compared to the fluffy sculpin. These features of the cellular heat shock response in the tidepool sculpin may help them to live in the upper intertidal with temperature extremes.

## Section 2-2. Field transfer experiments: Are distribution patterns of tidepool and fluffy sculpins related to thermotolerances?

### Introduction

In this study, I transplanted the tidepool and the fluffy sculpins from lower tidepools to an upper tidepool, and measured the changes in levels of liver hsp70. Even though different ranges of distributions in the intertidal zone have been observed between these sculpins in former reports (Green 1967; Green 1971a; Morris 1960 and 1962; Nakamura 1970, 1976a and b), as well as in this study (Section 2-1), there is no direct evidence showing that distributions of these sculpins are influenced by their thermal tolerances.

Homing behavior is known in many tidepool fishes; they move out of their tidepool at a high tide, yet can be found in the same tidepool on several subsequent low tides (Gibson 1999). Homing behavior also was found both in the tidepool sculpin (Craik 1981; Green 1971b; Khoo1974) and in the fluffy sculpin (Yoshiyama *et al.* 1992). Williams (1957) suggested that the homing behavior of intertidal fishes is a mechanism by which shallow water fishes of rocky shore avoid being left by the tide in an unfavorable situation at low tide, such as a tidepool that

disappears through sub-surface drainage. In addition, evidence for the ability of fish to remember the home-pool and to come back to it, rather than the regular occupation of selected small home range around the home-pool, has been provided by the return of fish after displacement. Gibson (1999) reviewed the homing ability of many intertidal fishes, and the tidepool and the fluffy sculpins were both among the group of fishes that have remarkably high ability of homing. The tidepool sculpin could return to its home-pool after displacement of more than 100m, and the knowledge for homing seemed to be remembered by this species for months (Green 1971b; Khoo 1974). On the other hand, the fluffy sculpin was known to return to its home-pool after 76m displacement, and the memory used for homing seemed to last up to a month (Yoshiyama *et al.* 1992). These reports suggest that both the tidepool and the fluffy sculpins have strictly determined "home-pools", which may be selected by these fishes, probably according to their physical and chemical preferences in the intertidal environment.

Assuming from the distribution patterns of these sculpins, the selection of the home-pool by the tidepool sculpin is apparently not limited by potentially limiting physical and chemical factors such as temperature and salinity, while that of the fluffy sculpin is strongly influenced by its preference for plant cover, such as the eelgrass (*Zostera marina*) that is dense in lower and middle tidepools, together with its inability to resist high temperatures (Nakamura 1976a and 1976b). To confirm if the heat resistance is an important factor for these sculpins to select their home-pools, I examined levels of stress in these sculpins when they were transferred to an upper tidepool with a higher temperature than in the lower tidepool, which they had originally selected.

## Materials and Methods

### *Transplanting tidepool and fluffy sculpins*

A transfer experiment was conducted at Wizard Islet, Bamfield, B.C., Canada in July 1999. It was sunny, and the LLW was at 11:58. In this experiment, 10 tidepool sculpins and 5 fluffy

sculpins were transferred separately in small cages at 13:00 from their lower tidepools to the control tidepool (the control group), which was similar in the position and the size with their respective home pools, or to the upper tidepool (the upper tidepool group), of which position was higher in the intertidal and the size was smaller than their home-pools. The control tidepool in the transfer experiment was carefully chosen so that it was similar to the home tidepools of both species in position and size, and thus, the rate of increase in the water temperature in the control tidepool during a low tide was also similar to that in the home-pools (data not shown). Thus, only the potential stress by transfer to an unfamiliar tidepool, if there was any, should be considered for those fishes transferred to the control tidepool. Therefore, the difference in the liver hsp70 level between the control and the upper tidepool can be considered as the effects of the change in the water temperature. One hour after transfer, fish were separately transferred to the stock tank of Bamfield Marine Station in a cooler box with cool seawater (15 °C), and recovered for 20 h at 11 °C. Liver samples were taken from the fish after the recovery and stored at –80 °C until analysis for hsp70 by Western blotting (for methods, see Materials and Methods in Section 1-1). Changes in water temperatures that these sculpins experienced in the control and the upper tidepools were monitored by data loggers.

## Results

### *Effects of transfer on the level of liver hsp70*

The temperature of the upper tidepool was about 5 °C higher than the control tidepool over the experiment in the field (Fig. 2-8). The level of hsp70 in the liver tissue of the tidepool sculpin was same between the control and the upper tidepool groups (Fig. 2-8). On the other hand, the liver hsp70 level of the fluffy sculpin in the upper tidepool group was significantly higher than that of the control group. The constitutive level of liver hsp70 in the control tidepool was significantly higher in the tidepool sculpin than in the fluffy sculpin.

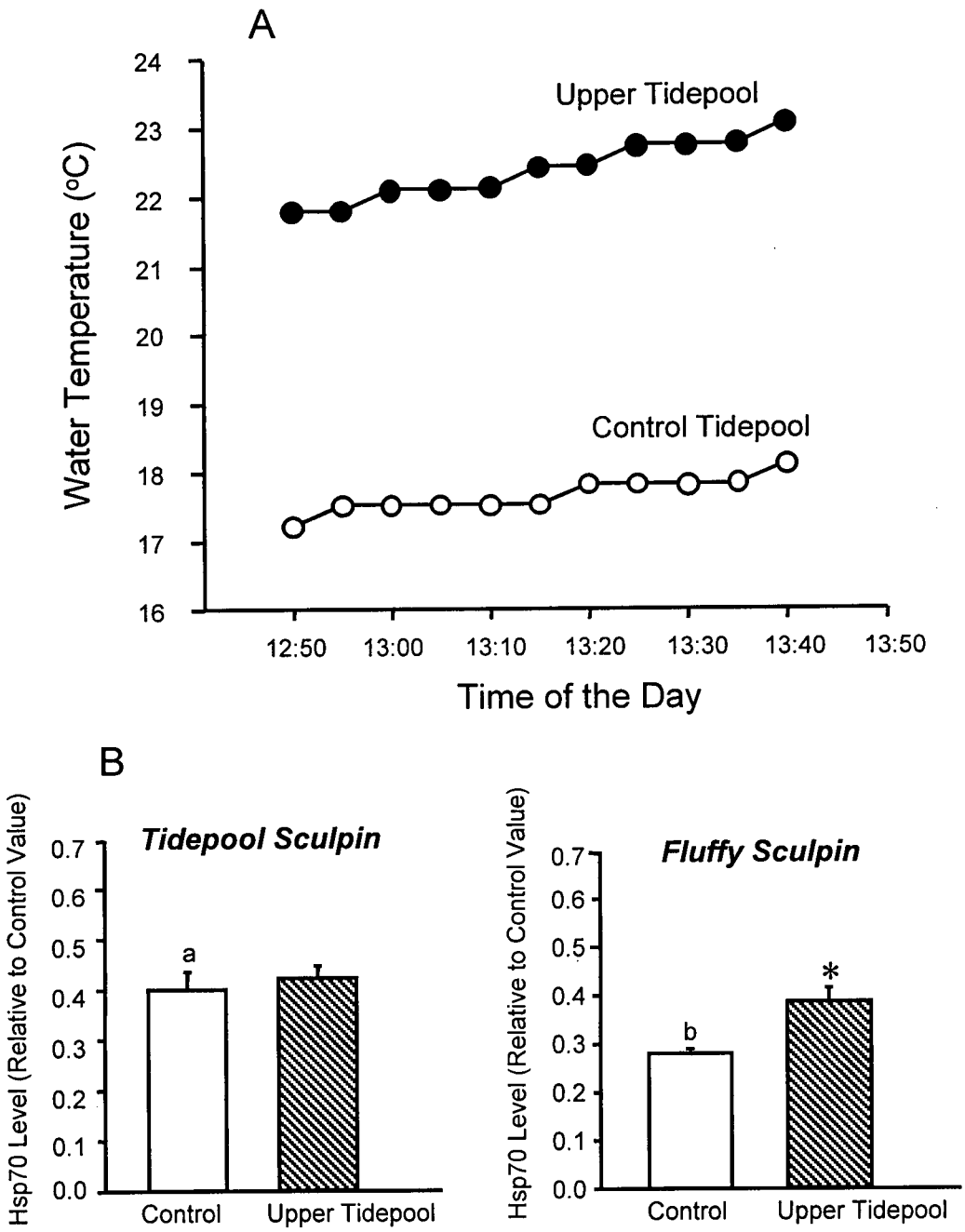


**Figure 2-8.** The upper graph (A) shows changes in water temperatures during the transfer experiment in the control and the upper tidepools, to which the fishes were transferred. The lower graphs (B) show levels of liver hsp70 in the tidepool and the fluffy sculpins transferred to the control and the upper tidepools (Mean±SE, n=5-10). * indicates significant difference between the control and the upper tidepool ($p \leq 0.05$). Different alphabet letters indicate significant difference between the tidepool and the fluffy sculpins ($p \leq 0.05$).

## Discussion

The tidepool sculpin, transferred to the upper tidepool, did not show any changes in the liver hsp70 level compared with the group transferred to the control tidepool. The fluffy sculpin transferred to the upper tidepool had significantly higher liver hsp70 levels than the group transferred to the control tidepool (Fig. 2-8).

An aggregation of tidepool sculpins, but no fluffy sculpin, in the upper tidepool used in the present experiment, was originally observed. Therefore, the upper tidepool used in this experiment was within the range of distribution of the tidepool sculpin, but it was probably outside the distribution range of the fluffy sculpin. Judging from the expression of hsp70, the tidepool sculpin found in the lower tidepool was not stressed by transfer to the upper, warmer tidepool, probably because being in the upper tidepool was normal for this species. The fluffy sculpin had a higher liver hsp70 level in the upper tidepool, probably because going to that upper part of the intertidal zone was not normal for this species.

It might be noteworthy that the tidepool sculpin had a relatively high liver hsp70 in the control tidepool, and it was significantly higher than that of the fluffy sculpin (Fig. 2-8). Significantly higher basal hsp70 levels in the tidepool sculpin compared with the fluffy sculpin were observed in the time course sampling from lower tidepools, as well (Fig. 2-6). However, the levels of liver hsp70 in the tidepool sculpin did not change either during a low tide (Fig. 2-6) or by transfer to warm water (Fig. 2-8). These observations imply that this species may always have a high store of hsp70, regardless of the water temperature in the tidepool or the position in the intertidal zone. It also means that the tidepool sculpin may be adapted to be less sensitive to temperature changes which may be related to the capacity of this species to occupy the wide range of the intertidal zone. In contrast to the tidepool sculpin, the fluffy sculpin had lower basal liver hsp70 levels in the control tidepool, and showed a significant increase in the liver hsp70 level after transfer to the upper tidepool, indicating that upper tidepools may be more stressful

for this species than lower tidepools. These results lead me to hypothesize that patterns of cellular heat shock responses can be, at least partly, related to the differences in selection of home-pools and in the range of vertical distributions during a low tide between tidepool and fluffy sculpins.

## Discussion of Chapter 2

Different cellular heat shock responses were shown between the tidepool and the fluffy sculpin in this research. The tidepool sculpin showed an insensitive response, while the fluffy sculpin showed a sensitive response in the level of liver hsp70 to changes in water temperatures in tidepools during a low tide. At the same time, the tidepool sculpin had consistently higher levels of liver hsp70 than the fluffy sculpin (Fig. 2-6 and 2-8). Judging from the remarkable difference in the range of fluctuation and the difference in the highest value of the water temperature between the lower and the upper tidepools during a low tide (Fig. 2-1), it seems possible that the heat resistance may be one of the keys that separate these sculpins into different distribution ranges. As well, the thermal sensitivity of the cellular heat shock response may be related to the determination of heat resistance in the tidepool and the fluffy sculpins.

In conclusion, the data in this study provided a positive clue for the direct influence of the cellular heat shock response in the thermally-dependent distribution of fish in nature. A large possibility of future studies is left in this direction of studies.

# Chapter 3

## Heat shock experiments in sculpins with different thermotolerances

### Introduction to Chapter 3

In this chapter, I focused on the difference in the mechanism of hsp70 expression between tidepool and fluffy sculpins, as well as the sailfin sculpin (*Nautichthys oculofasciatus*), by a series of experiments in the laboratory. From the field studies, I demonstrated that the tidepool sculpin could have a wider range of thermotolerance than the fluffy sculpin (Chapter 2). My findings in the previous chapter also indicate that different patterns of hsp70 expression between these sculpins may be involved in their different thermal tolerances.

Although the cellular heat shock response is a phenomenon widely studied, relatively few studies of the regulatory mechanisms of the cellular heat shock response have been conducted (Dietz 1994). While many scientists have been eager to understand how organisms work, less attention has been paid to studying how animals came to be the way they are (Mongold *et al.* 1996). In the study of evolutionary adaptation to temperature, the majority of information has been obtained from comparative studies between populations or species occupying different thermal niches. If the trait measured is temperature-dependent, it can be important for the temperature adaptation of animals. Therefore, examining the cellular heat shock response between evolutionarily closely related species is a valuable method to study the relationship between the role of hsps and determination of species' range of thermal tolerance. Hofmann and Somero (1996a) compared hsp70 and ubiquitin levels between two intertidal mussels. The northern species, *M. trossulus*, is distributed from Alaska to central California and the southern species, *M. galloprovincialis*, is distributed from central California to Baja California. They reported that *M. trossulus* was more thermally sensitive and had higher amounts of hsp70 and ubiquitin than *M. galloprovincialis*, which suggested that *M. trossulus* was more susceptible to

reversible (indicated by hsp70 level) and irreversible (indicated by ubiquitin level) protein damages than *M. galloprovincialis*. They also documented that the threshold induction temperature for hsp70 was correlated with thermal niches of these species, i.e., 23 and 25 °C for *M. trossulus* and *M. galloprovincialis*, respectively. Dietz and Somero (1993) examined the levels and the induction temperature of hsp70 and hsp90 between the brain, gill, and liver tissues of four marine fishes, the buffalo sculpin (*Enophrys bison*), the speckled sanddab (*Citharichthys stigmaeus*), the English sole (*Parophrys vetulus*) and the Pacific staghorn sculpin (*Leptocottus armatus*). They found that even though the constitutive levels of hsp70 and hsp90 varied widely among different tissues from an animal, overall threshold induction temperatures for these hsps varied little among tissues, and concluded that the threshold temperature for hsps may reflect the recent thermal exposure of the individual fish, as well as the thermal history of the species during its evolution. Ulmasov *et al.* (1992) demonstrated a direct correlation between the characteristic temperature of the ecological niche of a species and the amount of hsp70-like proteins in the cell at normal temperature in nine lizards collected from various thermal niches. These reports strongly indicate that the habitat temperature of the species may be very important for the evolution of the cellular heat shock response.

The tidepool sculpin and fluffy sculpin seem like good models to assess the influence of environmental temperature on the expression of hsps, since they are evolutionarily close and are similar in their ecological and morphological characters, except in the range of the vertical distribution in the intertidal zone which may bring different habitat temperatures to them. By measuring both hsp70 and hsp70 mRNA levels in the tidepool and the fluffy sculpins, controls of hsp70 expressions at the transcriptional and the translational levels in those sculpins were examined in this chapter.

## Section 3-1. Lethal temperatures and cellular heat shock responses in tidepool, fluffy and sailfin sculpins at various temperatures

### Introduction

The relationship between the lethal temperature and the cellular heat shock response of the tidepool sculpin (*O. maculosus*), fluffy sculpins (*O. snyderi*), and sailfin sculpin (*N. oculofasciatus*), was investigated in this section. The sailfin sculpin is known to occur only in the subtidal zone during a low tide, and thus, it could have a lower heat resistance than tidepool and fluffy sculpins.

For the study of the thermal tolerance of an animal, the resistance to heat stress should be clearly defined. In spite of the well-established correlation between habitat temperature and the induction temperature for hsps in these studies, the lethal temperature has been rarely examined in any animals, probably because of the difficulty in definition of "lethal temperature".

For the upper lethal temperature of Antarctic fishes, Somero and DeVries (1967) used the temperature that the resistance time of the fish at that temperature acutely dropped. To compare the temperature tolerance between the tidepool and the fluffy sculpin, Nakamura (1976a) also used the average resistance times of these sculpins at various temperatures. In this study, I defined the lethal temperature as the temperature which can kill the fish quickly (within 3 to 10 min), since I had found in my preliminary tests that these sculpins had clear threshold temperatures, below which they survived at least for 2 h, but above which they died quickly. The correlation between the lethal temperature, level of hsp70 and range of vertical distribution in the intertidal zone was investigated between those three species of sculpins in this section.

### Materials and Methods

*Fish*

Tidepool sculpins were collected from Popham Island, Vancouver, B.C. in June 1998 with dip nets. Fish were transferred to stock tanks at The University of British Columbia, Vancouver, B.C. Fish were kept under a photo regime of 12 h L:12 h D at 10 °C for more than 4 weeks until they were used in experiments.

Fluffy sculpins were collected from tidepools near Bamfield Marine Station, Bamfield, B.C. in July 1998 and transferred to the outside stock tanks of the station. They were kept at 12 °C under natural photoperiod for at least 2 weeks. Both species were fed with live mussels each day.

Sailfin sculpins were reared by Dr. Jeffrey Marliave at the Vancouver Aquarium, Vancouver, B.C. The fish had been hatched in the aquarium in April 1999, and kept at 9 °C until used in the experiment in February 2000. Fish were kept under a photo regime of 24 h L, and fed with frozen krill each day.

*Heat shock experiments*

During the heat shock experiments, tidepool, fluffy and sailfin sculpins were transferred directly to higher temperatures from their respective acclimation temperatures of 10, 12 and 9 °C. Tidepool sculpins were transferred to 10, 15, 18, 20, 23, 25, 28 and 30 °C. Fluffy sculpins were transferred to 12, 15, 18, 22, 25 and 28 °C. Sailfin sculpins were transferred to 9, 15, 18, 20, 22, 24 and 26 °C. For all species, 6 fish were transferred to each temperature. Control fish for the potential effects of handling stress were transferred to their respective acclimation temperatures. Feeding was stopped 24 h before any transfers. Two hours after transfer, fish were returned to their respective acclimation temperatures for 22 to 24 h before any sampling. Liver and brain tissues were sampled for Western blot analysis (for methods, see Materials and Methods in Section 1-1). Tissue samples were stored in 1.5 mL microcentrifuge tubes at -80 °C until analysis.

*Silver staining*

For SDS-PAGE, liver and brain samples were diluted with 1×Laemmli's buffer to yield a concentration of 1mg protein/mL, and 20μL (20μg total protein) of diluted samples were loaded on acrylamide gels to separate proteins depending on their molecular weights by SDS-PAGE (for methods, see Materials and Methods of Section 1-1). Liver and brain samples at 10, 18, 25 and 28 °C for the tidepool sculpin, at 12, 18, 25 and 28 °C for the fluffy sculpin, and at 9, 18, 22, and 24 °C for the sailfin sculpin were examined. Acrylamide concentrations of 12% for the separating gels and 4% for the stacking gels were used. Gels were run at 75V for 15 min and 150V for 1 h. Gels were stained with a silver staining kit (Bio-Rad, USA). Stained gels were dried between cellophane sheets for storage.

*Separating isoforms of hsp70 family*

To separate the isoforms of the hsp70 family of proteins, all procedures in Western blot analysis (see Materials and Methods of Section 1-1) were kept the same, except for the running time of the electrophoresis gels. Gels were run at 75V for 15 min and at 150V for 3 h. Liver and brain samples at 10, 18, 25 and 28 °C for the tidepool sculpin, at 12, 18, 25 and 28 °C for the fluffy sculpin, and at 9, 18, 22 and 24 °C for the sailfin sculpin were examined. With this procedure, 2 to 3 isoforms of the hsp70 family were successfully separated in liver and brain samples from all sculpins.

## Results

*Mortality under heat shock treatments*

Table 1 shows the number of fish that survived after a heat shock for 2 h at the various temperatures. There were no mortalities in the tidepool sculpin at any temperatures from 10 to

**Table 1.** The numbers of survived fish after 2 h at various temperatures. Fish were transferred directly from 10 °C for the tidepool sculpin, 12 °C for the fluffy sculpin, and 9 °C for the sailfin sculpin. N=6 at start for all species. The number in parentheses indicates the time (min), within which all fish in the group died.

| Tidepool Sculpin | | Fluffy Sculpin | | Sailfin Sculpin | |
|---|---|---|---|---|---|
| Temperature (°C) | Number of Fish Survived | Temperature (°C) | Number of Fish Survived | Temperature (°C) | Number of Fish Survived |
| 10 | 6 | 12 | 6 | 9 | 6 |
| 15 | 6 | 15 | 6 | 15 | 6 |
| 18 | 6 | 18 | 6 | 18 | 6 |
| 20 | 6 | | | 20 | 6 |
| | | 22 | 6 | 22 | 6 |
| 23 | 6 | | | | |
| | | | | 24 | 0 (60 min) |
| 25 | 6 | 25 | 6 | | |
| | | | | 26 | 0 (10 min) |
| 28 | 6 | 28 | 0 (7 min) | | |
| 30 | 0 (3 min) | | | | |

28 °C. Nevertheless, all the fish began swimming abnormally immediately following direct transfer to 30 °C, and died within 3 min at that temperature. The fluffy sculpin survived all temperatures from 12 to 25 °C, but died at 28 °C. All the fluffy sculpins died within 3 to 7 min after direct transfer to 28 °C. There was no mortality in the sailfin sculpin at any temperatures from 9 to 22 °C. However, all fish died within 1 h at 24 °C, and within 10 min at 26 °C. Therefore, the tidepool sculpin had a lethal temperature of about 2 °C and 4 °C higher than the fluffy sculpin and the sailfin sculpin, respectively.

*Changes in protein profiles by heat shock treatments*

The protein profiles of liver and brain tissues from the tidepool, the fluffy, and the sailfin sculpins by silver staining were shown in figure 3-1. It shows that there was a higher amount of a 90-kDa protein at 25 °C in liver tissues of the tidepool sculpin. Furthermore, Figure 3-1 shows new proteins observed in the liver tissues of tidepool sculpin at all temperatures at a higher level than in the fluffy sculpin. There was a protein which was expressed at a high level at all temperatures in brain tissue of the fluffy sculpin, but was expressed strongly only at 18 °C in the tidepool sculpin.

*Total hsp70 levels at different temperatures*

Significantly higher hsp70 was observed in the liver tissue of the tidepool sculpin only at 28 °C compared with other temperatures as shown in Figure 3-2. The levels of hsp70 in the liver tissue of the fluffy sculpin were significantly higher at 22 °C and 25 °C compared with lower temperatures. Therefore, the threshold induction temperature for hsp70 in the tidepool sculpin was 28 °C, while it was 22 °C in the fluffy sculpin. No significant changes were observed in levels of liver hsp70 in the sailfin sculpin at any temperatures. There were no significant changes in the level of brain hsp70 at any temperatures in all sculpins (Fig. 3-3).

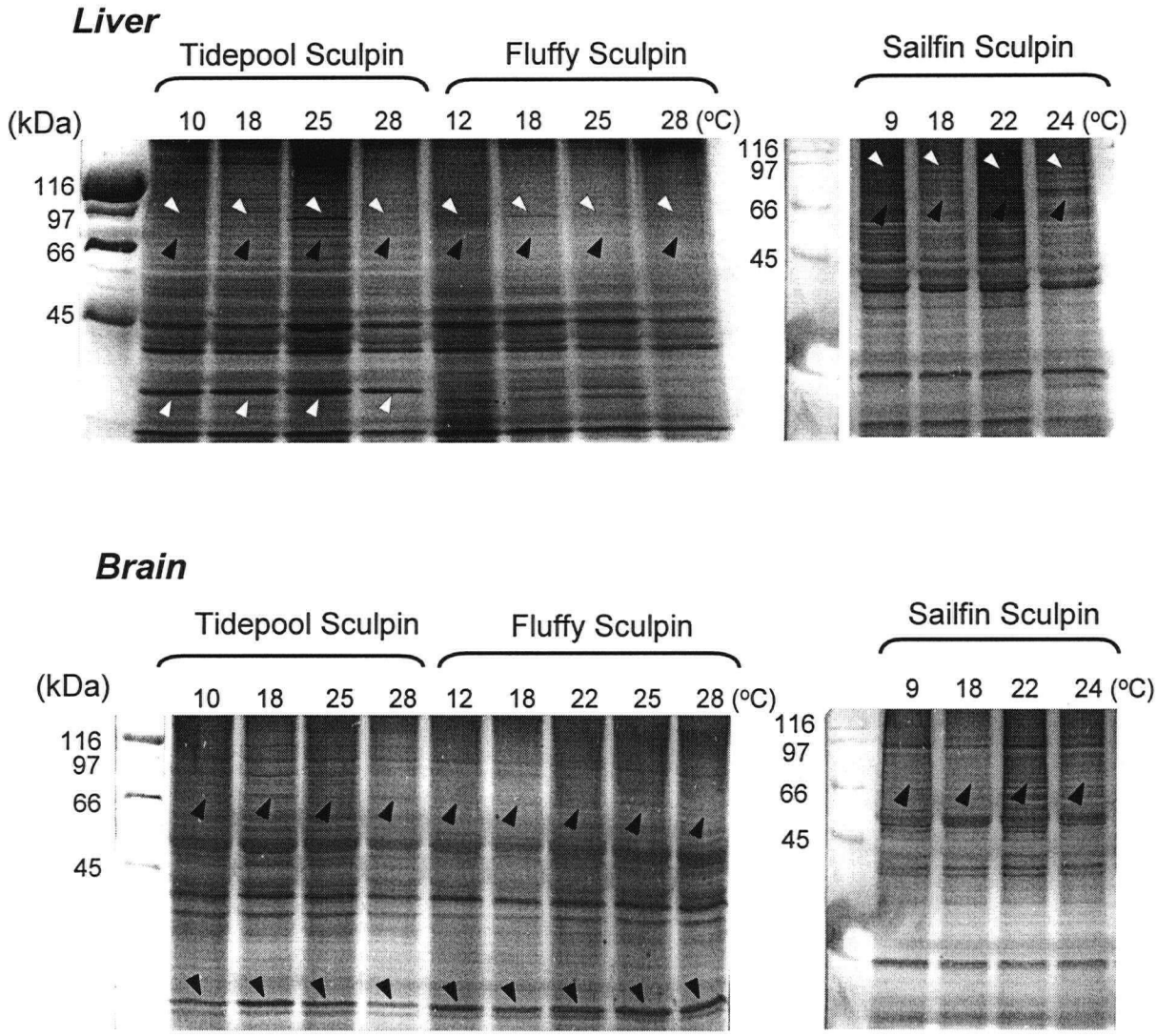


**Figure 3-1.** Cellular protein profiles by silver staining in liver and brain samples of the tidepool and the fluffy sculpins at various temperatures. A heat-inducible protein with a molecular weight of about 70-kDa is shown with (▲) both in liver and brain tissues. (▽) shows another heat-inducible protein found in the liver tissue with a molecular weight of about 90-kDa. That protein was observed to be at higher levels at 25 °C in the liver tissue of the tidepool sculpin. (△) shows a protein which was higher in liver tissues of tidepool sculpin than in the fluffy sculpin at the all temperatures tested. There was a protein (▼) which was observed at the same level at all temperatures in the brain tissue of the fluffy sculpin, while the level of that protein seemed to be affected by temperature in the tidepool sculpin; the level at 18 °C was higher compared with other temperatures.

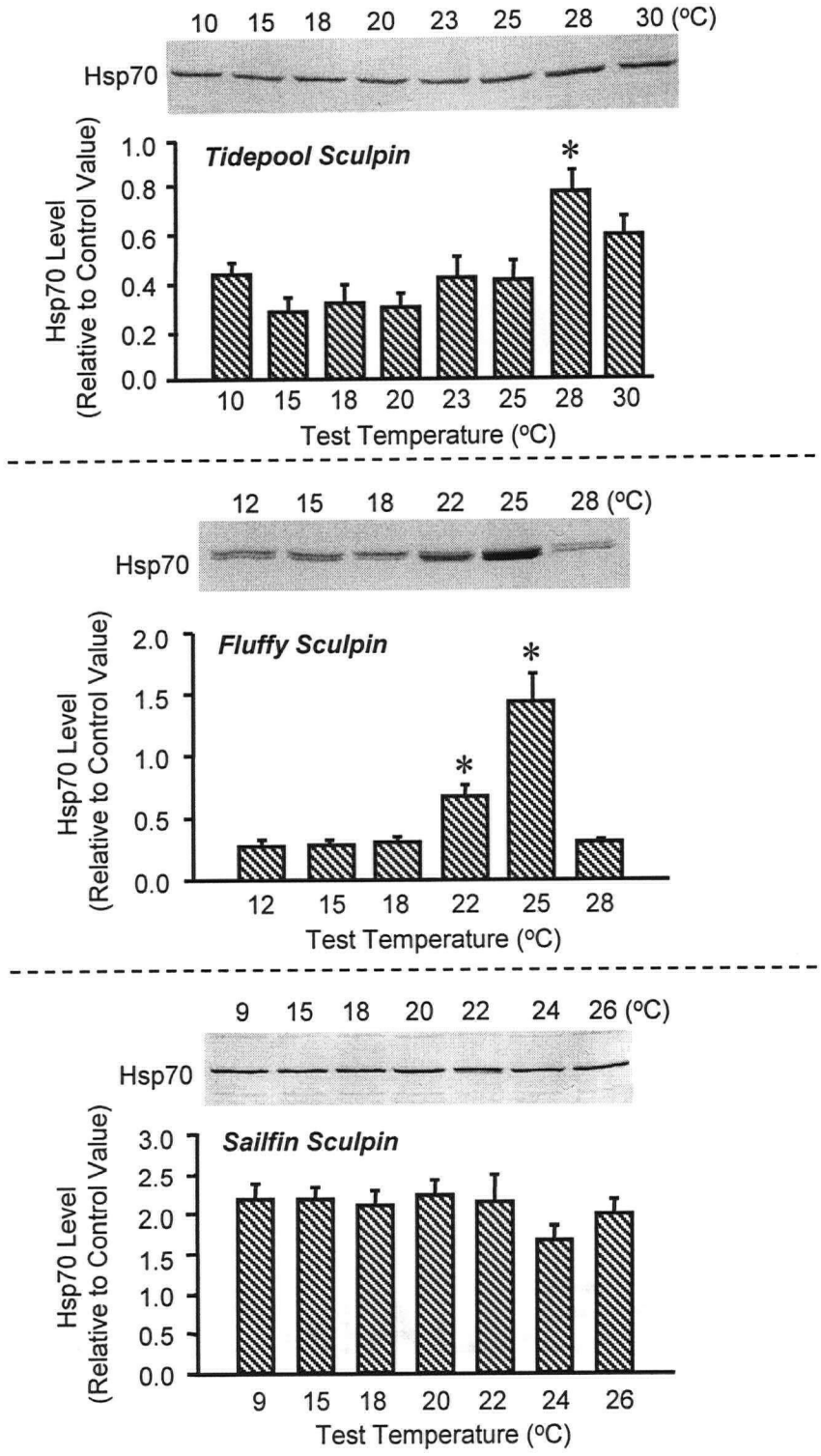


**Figure 3-2.** Total hsp70 by Western blot analysis in liver tissues of the tidepool, fluffy and sailfin sculpins at various temperatures. The graphs are levels of hsp70 based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=6). * indicates significant difference compared with the hsp70 level at the acclimation temperature (10 °C for the tidepool sculpin, 12 °C for the fluffy sculpin, and 9 °C for the sailfin sculpin, $p \leq 0.05$).

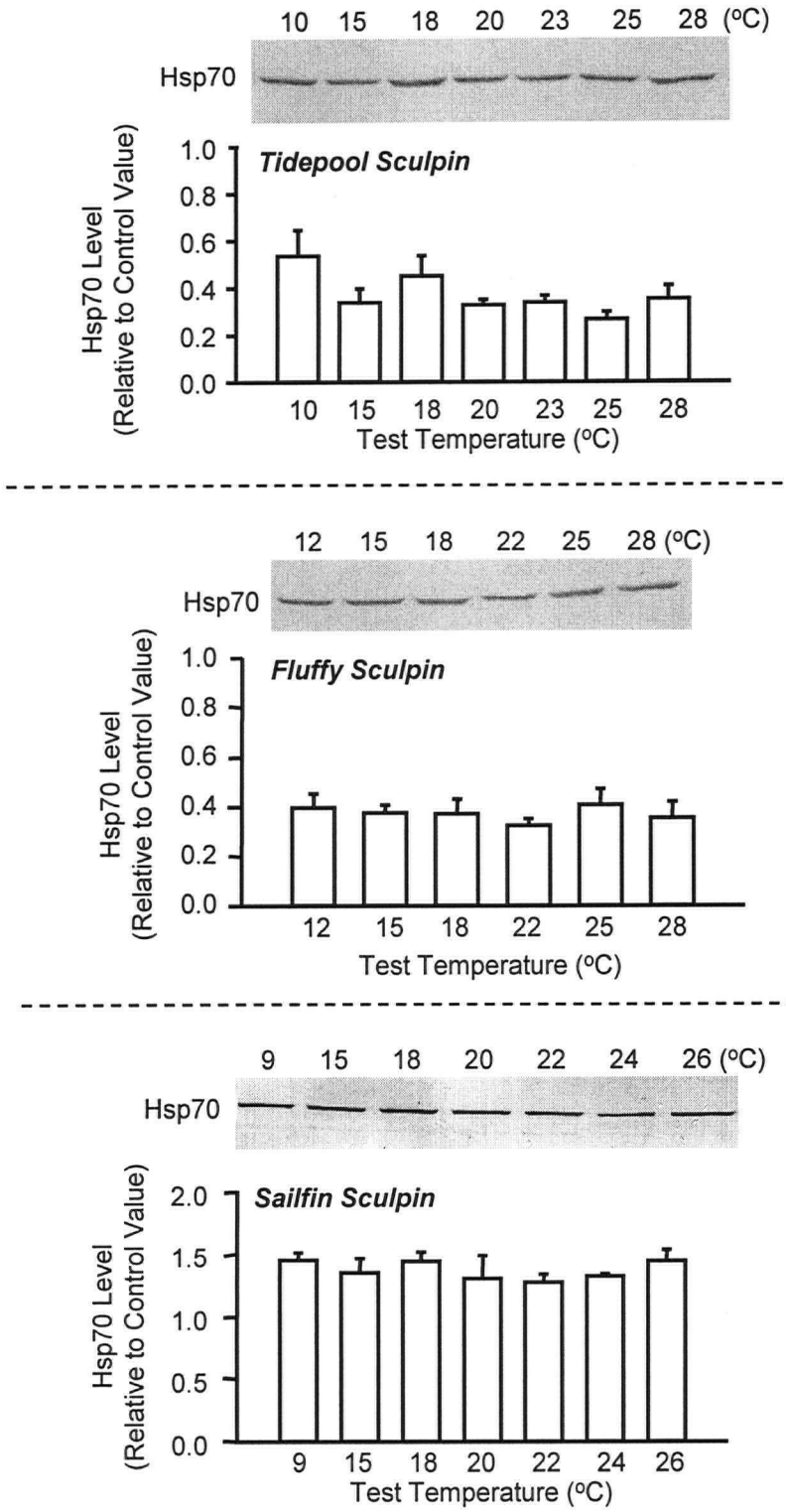


**Figure 3-3.** Total hsp70 by Western blot analysis in brain tissues of the tidepool, fluffy and sailfin sculpins at various temperatures. The graphs are levels of hsp70 based on band intensities standardized with the level of hsp70 in a coho salmon liver sample (Mean±SE, n=6). There was no significant change in brain hsp70 levels of all species at any tested temperatures.

*Isoforms of the hsp70 family at different temperatures*

Figure 3-4 shows the separation of isoforms belonging to the hsp70 family in liver tissues of tidepool, fluffy and sailfin sculpins by Western blot analysis. In liver tissues, isoform-A was observed at all temperatures in the tidepool sculpin, while isoform-B was observed only at 28 °C. Two isoforms (C and D) were observed at all temperatures in liver tissue of the fluffy sculpin. At 25 °C, the third isoform (E) was observed in the fluffy sculpin. Levels of isoform-A did not change significantly in liver tissue of the tidepool sculpin at any temperature. Even though isoform-C and isoform-D were expressed at all temperatures in liver tissue of the fluffy sculpin, levels of both isoforms were significantly higher at 25 °C, especially the level of isoform-D, which was 7-fold higher at that temperature, compared to the level at the acclimation temperature. In addition, isoform-E was observed only at 25 °C. Thus, the total level of the proteins belonging to the hsp70 family in liver tissue was 6-fold higher at 25 °C in the fluffy sculpin, while it was only 2-fold higher at 28 °C in the tidepool sculpin when compared with the basal levels at respective acclimation temperatures (10 °C for the tidepool sculpin and 12 °C for the fluffy sculpin) (Fig. 3-2). There was one isoform (F) that was observed at the same level in the liver tissue of the sailfin sculpin at all temperatures tested except at 22 °C. At 22 °C, isoform-G was slightly observed beside isoform-F. Isoform-F did not result in a significant increase in the total hsp70 level in the liver tissue of the sailfin sculpin at 22 °C (Fig. 3-2). In brain tissues, isoform-a in the tidepool sculpin, or isoform-c in the fluffy sculpin was observed at all tested temperatures (Fig. 3-5). There was only one form of hsp70 observed at the same level in the brain tissue of the sailfin sculpin at all temperatures. The levels of isoform-a in the tidepool sculpin did not change at any temperatures, while isoform-b was observed only at 28 °C in the brain tissue of the tidepool sculpin. The levels of isoform-c were similar at all temperatures, while isoform-d was observed at a low level only at 25 °C in the brain tissue of

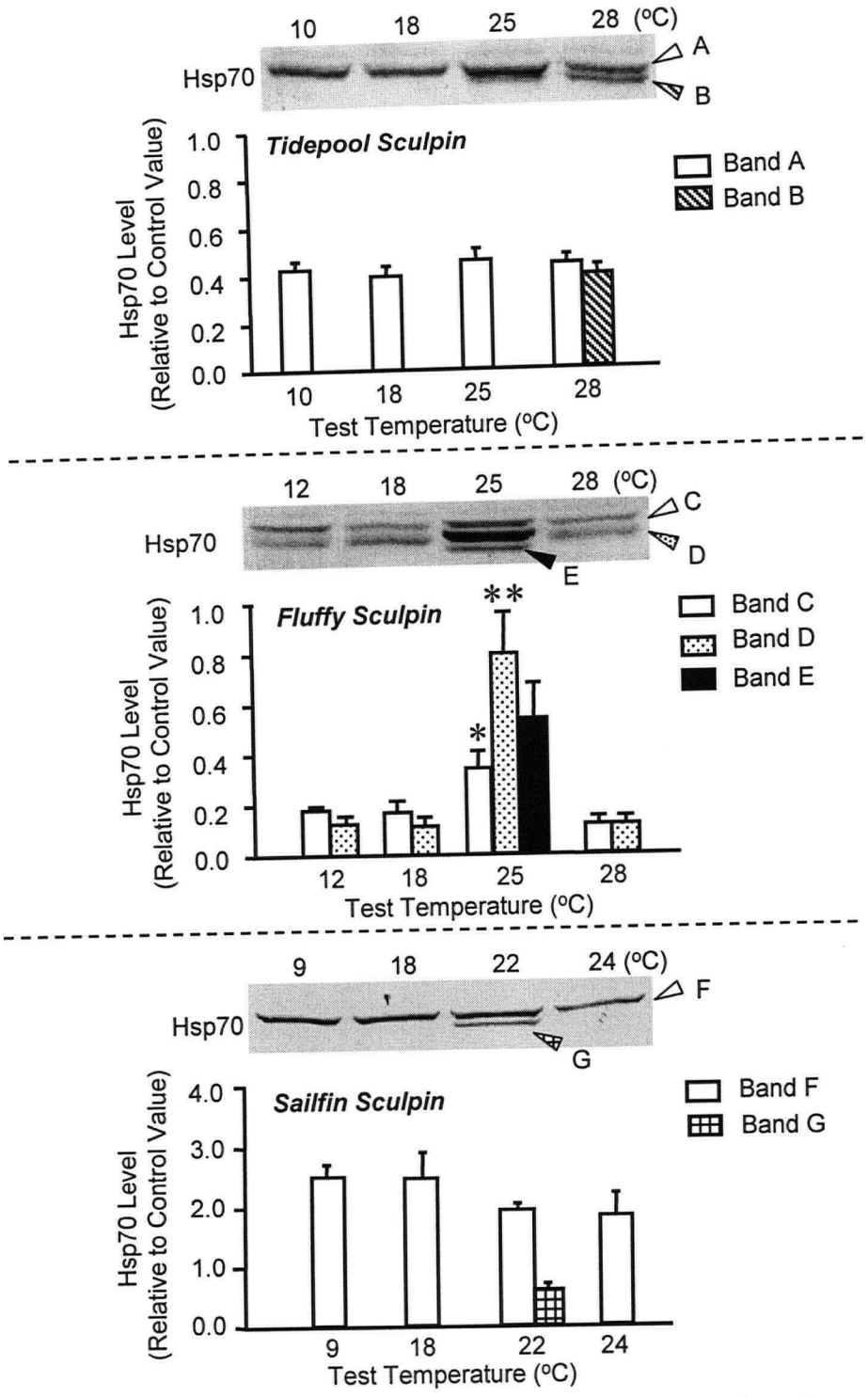


**Figure 3-4.** Isoforms of the hsp70 family by Western blot analysis in liver tissues of the tidepool, fluffy and sailfin sculpins at various temperatures. Levels of isoforms observed in liver tissues of the tidepool sculpin (isoforms-A and -B), fluffy sculpin (isoforms-C, -D and -E), and sailfin sculpin (isoforms-F and -G) are shown separately in graphs (Mean ± SE, n=6). Levels of isoforms were based on band intensities standardized with the level of hsp70 in a coho salmon liver sample. * ($p \leq 0.05$) and ** ($p \leq 0.001$) indicate significant difference compared with the level at the acclimation temperatures (10 °C for the tidepool sculpin, 12 °C for the fluffy sculpin and 9 °C for the sailfin sculpin).

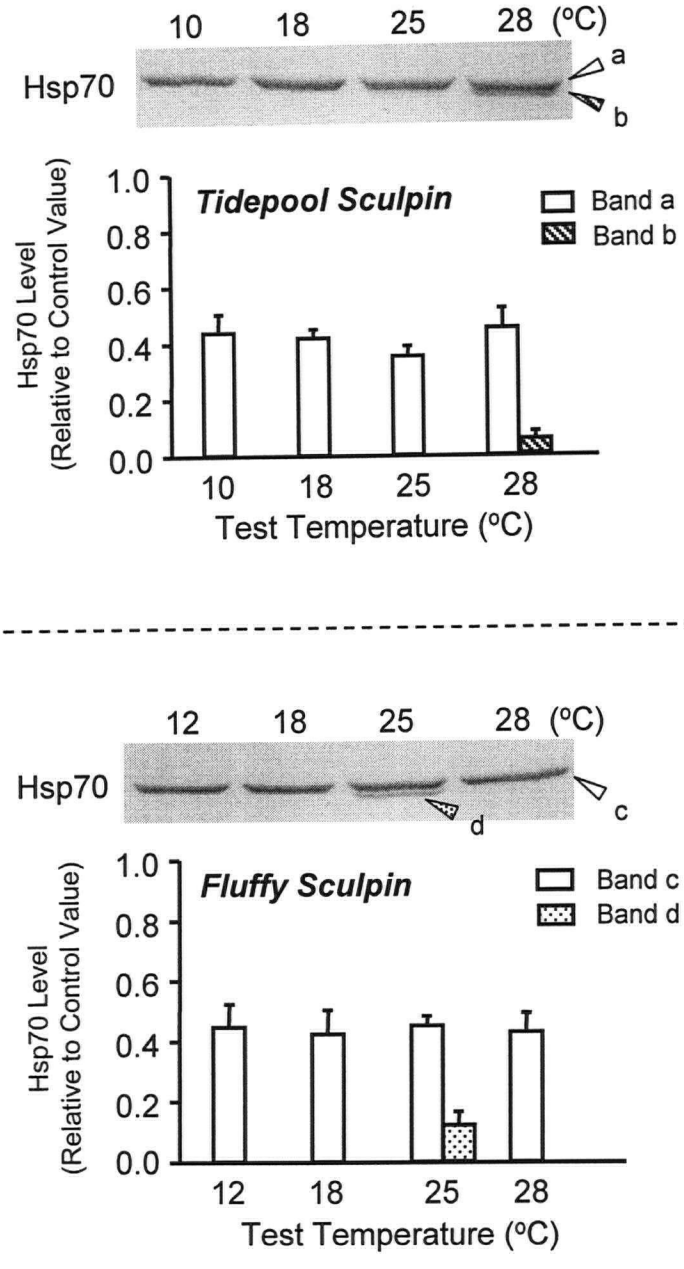


**Figure 3-5.** Isoforms of hsp70 family by Western blot analysis in brain tissues of the tidepool and the fluffy sculpins at various temperatures. There is only one form of hsp70 observed in the brain tissue of the sailfin sculpin. Therefore, it is not shown here. Levels of isoforms observed in brain tissues of the tidepool sculpin (isoforms-a and -b) and the fluffy sculpin (isoforms-c and -d) are shown separately in lower graphs (Mean $\pm$ SE, n=6). Levels of isoforms were based on band intensities standardized with the level of hsp70 in a coho salmon liver sample. There was no significant changes in levels of constitutive hsp70 in brain tissues of both species at any tested temperatures.

the fluffy sculpin. The heat-inducible hsp70 (isoforms-b or –d) did not result in significantly higher levels of total hsp70 in brain tissues of either species (Fig. 3-3).

## Discussion

Mortality tests demonstrated that the lethal temperature of the tidepool sculpin was 30 °C, while that of the fluffy sculpin was 28 °C, and that of the sailfin sculpin was 26 °C (Table 1). All were in agreement with vertical distribution patterns of these sculpins in the intertidal zone. The threshold induction temperature for hsp70 in the liver tissue was in agreement with the lethal temperature in the tidepool and the fluffy sculpins (Fig. 3-2), but not in the sailfin sculpin. There was no clear increase in liver hsp70 levels at any tested temperatures in the sailfin sculpin. A significantly higher hsp70 level in the liver tissue was observed at only 2 °C lower than the lethal temperature in the tidepool sculpin, while it was about 6 °C lower than the lethal temperature in the fluffy sculpin. Induction temperatures for the heat-inducible hsp70 in liver tissues of the three sculpins were correlated with their lethal temperatures, i.e., 28 °C for the tidepool sculpin, 25 °C for the fluffy sculpin, and 22 °C for the sailfin sculpin. No clear induction temperatures for total hsp70 were observed in brain tissues of any sculpins, yet the induction temperatures for the heat-inducible hsp70, 28 °C for the tidepool sculpin and 25 °C for the fluffy sculpin, were correlated with their lethal temperatures (Fig. 3-3 and Fig. 3-5). There was no inducible form of hsp70 in the brain tissue of the sailfin sculpin. These results support the possibility that heat shock proteins may have an important role in determining some aspects of thermal tolerances in fishes.

Different patterns of isoforms of the hsp70 family were observed between liver tissues of those sculpins in this study (Fig. 3-4). Temperature-dependent variations in the expression of hsp70 isoforms have also been reported among several strains of desert Poeciliid fishes collected from the northern desert area and from the southern tropical area of Mexico (Norris *et al.* 1995;

White *et al.* 1994). In those studies, constitutive isoforms were identical among species, while heat-inducible isoforms were very polymorphic; each species expressed 5 to 7 inducible isoforms. In addition, species adapted to the desert environment had a lower variety of inducible isoforms than the tropical species. They concluded that the migration of an ancestral fish from the thermally stable southern tropical streams to the northern desert streams with large temperature fluctuations may have resulted in some loss of hsp diversity during their evolution. Our results in liver tissues of the tidepool and the fluffy sculpins are consistent with those observations. Only one heat-inducible form of hsp70 was observed in the tidepool sculpin that lives in the upper tidepools with a wider fluctuating temperature relative to the lower tidepool (Fig. 3-4). In contrast, one heat-inducible isoform and two heat-sensitive isoforms were found in the fluffy sculpin that lives in lower tidepools or the subtidal zone with relatively stable temperature regimes. One inducible form of hsp70 (B) was observed only at 28 °C, while the constitutive form (A) was expressed at the same level at all temperatures in the tidepool sculpin (Fig. 3-4). For species experiencing long term and regular habitat temperature changes due to the seasonal cycle or migration, the possession of multiple isoforms with different optimal temperatures may be beneficial. However, for the tidepool sculpin, which experiences hourly and unpredictable changes of the water temperature, the induction of different isoforms of inducible hsp70 according to the ambient temperature might be energetically expensive. A small number of proteins, that are functional over a wide range of temperatures, may be more beneficial for the species like the tidepool sculpin, than expressing a large number of isoforms with different thermal optima. On the other hand, three isoforms of the hsp70 family were observed in the liver tissue of the fluffy sculpin. Isoform-C and -D were observed at relatively low level at all temperatures except at 25 °C. The level of those isoforms were significantly and 2-fold and 6-fold higher at 25 °C, respectively, compared with the levels at 12 °C, along with the other isoform-E which was observed only at 25 °C, resulting in the highest total hsp70 level at 25 °C in

the fluffy sculpin (Fig. 3-2). Our field observation showed that the fluffy sculpin was rarely found in tidepools in which the water temperature exceeded 23 °C. To inhabit the potentially severe intertidal environment, the fluffy sculpin may develop behavioral strategies to select tidepools which do not become warmer than the threshold induction temperature for hsp70 of this species.

The results from the sailfin sculpin are, however, against these theories. There were only 2 isoforms of hsp70, one of which was observed slightly only at 22 °C, in the liver tissue of the sailfin sculpin, even though this species inhabits the subtidal zone which is more thermally stable compared with the intertidal zone. This species also showed insensitivity in the level of liver hsp70 to increases in water temperature. There are two possibilities concerning this inconsistency. The first possibility is that the water temperature in the subtidal zone is stable enough for this species to lose the ability to synthesize higher amounts of hsp70 when they are subjected to a higher temperature. The other possibility is that because the sailfin sculpin used in this study was hatched and raised in the aquarium at a constant temperature in a stock tank, they might not respond to a heat stress as they may do in nature. Experiments with the wild stock of this species may be necessary to confirm which possibility is more likely.

Different patterns of hsps among different tissues of one species were observed in fishes such as the killifish (*Fundulus heteroclitus*, Koban *et al.* 1991), the fathead minnow (*Pimephales promelas*, Dyer *et al.* 1991), and in several marine fishes such as the buffalo sculpin (*Enophrys bison)*, the speckled sanddab (*Citharichthys stigmaeus*), the English sole (*Parophrys vetulus*), and the Pacific staghorn sculpin (*Leptocottus armatus*) (Dietz and Somero 1993). In the present study, patterns of hsp70 levels at various temperatures were different between liver and brain tissues in all sculpins; the level of isoforms in the tidepool sculpin, or the level and the number of isoforms in the fluffy sculpin and in the sailfin sculpin, were reduced in brain tissues compared with liver tissues (Fig. 3-4 and Fig. 3-5). One constitutive hsp70 was observed at all

temperatures, while one inducible hsp70 was induced only at 28 °C both in the liver and the brain tissues of the tidepool sculpin. Nevertheless, the level of the inducible form at 28 °C in the brain tissue was much less than that in the liver tissue. Only two isoforms of hsp70 were observed in the brain tissue of the fluffy sculpin, while three isoforms were observed in the liver tissue of this species dependent on the temperature. One isoform (c) was observed in the brain tissue, yet two isoforms (C and D) were observed at all temperatures in liver tissue of the fluffy sculpin. At 25 °C, the other isoform (d) was expressed at low level in the brain tissue, while isoform-C, -D and the other isoform (E) were observed at high levels in the liver tissue of the fluffy sculpin. There was only one form of hsp70 in the brain tissue of the sailfin sculpin, which had 2 isoforms (F and G) at 22 °C in the liver tissue. Dyer *et al.* (1991) also reported that the smallest numbers of hsps were synthesized in the brain tissue of the fathead minnow. They also documented the highest induction temperature for stress response in the brain tissue compared with gill and muscle tissues. They hypothesized that the limited capacity of the brain tissue to induce hsps was related to the thermal limits of organismal survival. My results were consistent with their observations in terms of the lower capacity of the brain to induce the general heat shock response. Nevertheless, my data did not demonstrate the clear correlation between the heat shock response in the brain tissue and the lethal temperature of the whole organism. The tidepool sculpin had a higher lethal temperature than the fluffy sculpin and sailfin sculpin, even though the brain tissues of these species did not show any clear difference in their capacity to increase hsp70 levels at high temperatures (Fig. 3-3). There might be different mechanisms other than the general heat shock responses in brain tissues of these sculpins to protect the central nervous system at high temperatures.

There is another possibility to explain the lack of the cellular heat shock response in the brain tissue in my study. This may have been caused by a region-specific hsp70 expression in the brain. Bechtold *et al.* (2000) successfully demonstrated the association of stress-inducible

hsp70 with pre- and postsynaptic elements in the rat brain after 1 h heat shock at 42 °C. However, they also showed that this phenomenon was more significant in the cerebellum than in the forebrain. In my experiment, the whole brain tissue from individual fish was homogenized to measure hsp70 level. Since the cerebellum of fish brain occupies a relatively small part of the whole brain, the significant response of this region to heat shock treatments might have been masked by the lower responses in the other parts of the brain.

In summary, I observed a general agreement between lethal temperatures and cellular heat shock responses of three sculpin species. Significantly higher levels of hsp70 were found only at 28 °C in the liver tissue of the tidepool sculpin, which was only 2 °C lower than the lethal temperature for this species. An increased level of hsp70 was found at 22 °C in the liver tissue of the fluffy sculpin, which was about 6 °C lower than its lethal temperature. The constitutive form of hsp70 was observed at all temperatures in both species. However, the range of temperatures, which is functional for constitutive hsp70, may be different between these species. Further studies both in the laboratory and the field are necessary to determine whether the differences in heat shock responses observed in the present study were related to differences in thermal tolerance, and whether such responses affect habitat selection in these intertidal fishes.

## Section 3-2. Effects of chronic higher temperatures on the basal levels of hsp70 in tidepool and fluffy sculpins

### Introduction

The thermal properties of tidepools in the intertidal zone are unique compared with the main body of the ocean. The water temperature of a tidepool fluctuates constantly and is rarely stable (Green 1971a; Nakamura1976a). Our data also showed that the water temperatures in upper, middle and lower tidepools repeated an increase and a decrease approximately every 24 h, even though the range of fluctuation in the water temperature was different between the tidepools

(Fig. 2-1). In other words, even though fish in a tidepool are exposed to a high temperature, such as more than 30 °C during a daytime low tide, this temperature will not be sustained for a long time because of the combination of the tidal cycle and the daily cycle of air temperature. Therefore, the intertidal fish may have adapted to a wide fluctuation of water temperatures rather than to a constant high temperature, i.e. they can show resistance to a high temperature for hours, but not for days, as likely happens in a natural tidepool.

To test this hypothesis, I examined the response of the tidepool and the fluffy sculpins to a long-term heat shock treatment that lasted from 6 h to 2 weeks. I also measured the changes in liver hsp70 levels over the course of the heat shock to examine if the speed of hsp70 inductions in these sculpins under heat stresses is applicable to the life in the intertidal zone.

## Materials and Methods

To examine if high acclimation temperatures lead to high basal hsp70 levels in fish, and how long fish take to shift the state of cellular heat shock response adjusted to the new, high temperature, time course samplings were conducted after tidepool and fluffy sculpins were transferred to higher temperatures from the original acclimation temperatures. The tidepool sculpin was acclimated at 10 °C, and the fluffy sculpin was acclimated at 12 °C for 10 to 14 days in 250L stock tanks. Then fish were transferred to 22 and 28 °C for the tidepool sculpin, and 22 and 25 °C for the fluffy sculpin. These two temperatures were chosen for each species as mild and severe temperatures.

### *Time course sampling at 22 °C in the tidepool sculpin*

After sampling 6 fish from the stock tank as the 0 h sample, 66 fish were transferred to a glass tank with the water at a temperature of 22 °C. Another 66 fish were transferred to a glass tank at 10 °C as the control group to exclude the effects of general handling stresses. Fish were

sampled from both glass tanks at 0.5, 1, 2, 3, 6, 12, 24 h, 2, 4 days, 1 week and 2 weeks after transfer. Six fish were sampled at each sampling point from both groups.

*Time course sampling at 28 °C in the tidepool sculpin*

After sampling 9 fish from the stock tank as the 0 h sample, 23 fish were transferred to 28 °C. Fish were sampled at 0.5, 1, 3 and 6 h after transfer. Six fish each were sampled at 0.5 and 1 h, while 9 and 2 fish were sampled at 3 and 6 h, respectively.

*Time course sampling at 22 °C in the fluffy sculpin*

After sampling 5 fish from the stock tank as the 0h sample, 30 fish were transferred to 22 °C, and 10 fish were transferred to 12 °C as the control group. Five fish were sampled at 1, 3, 6, 12, 24 h and 4 days after transfer in the 22 °C group. As well, 5 fish were sampled at 6 h and 4 days after transfer in the control group.

*Time course sampling at 25 °C in the fluffy sculpin*

Three fish were sampled from the stock tank as the 0 h sample. Fifteen fish were then transferred to 25 °C. Fish were sampled at 1, 3 and 6 h after transfer. Five fish were sampled at each sampling point.

In all experiments, the tanks were covered with black plastic bags to reduce the amount of disturbance to the fishes. Liver samples were taken from fish and stored at -80 °C until analysis for hsp70 by Western blotting (for methods, see Materials and Methods in Section 1-1).

## Results

*Changes in liver hsp70 at 22 and 28 °C in the tidepool sculpin*

Figure 3-6 shows the changes in liver hsp70 levels in the tidepool sculpin after being transferred to higher temperatures (22 and 28 °C) from the acclimation temperature (10 °C). In the tidepool sculpin, there was no clear change in hsp70 levels at any time after transfer to 22 °C from 10 °C over 2 weeks compared to the level at the beginning. However, the level at 22 °C was significantly higher compared to the control level at 2 weeks. Since some fish started dying at 2 h or 3 h after transfer to 28 °C, and then most of the fish died at 6 h, the sampling was stopped at 6 h after the transfer to that temperature. There was no significant change in the level of hsp70 in the tidepool sculpin at 28 °C, although relatively low liver hsp70 levels were observed at 0.5 and 1 h after transfer to 28 °C compared with the original level at 0 h, and they returned to the original level at 3 h.

*Changes in liver hsp70 at 22 and 25 °C in the fluffy sculpin*

The levels of hsp70 in the liver tissue of the fluffy sculpin significantly increased after transfer to 22 °C from 12 °C (Fig. 3-7). The level was significantly higher at 6 h compared with the control level at 6 h, and sustained the same level until the end of the experiment at 4 days after transfer. However, liver hsp70 levels of the fluffy sculpin at 22 °C did not significantly change compared with the initial level at any time after transfer. The liver hsp70 level of this species also increased when it was transferred to 25 °C from 12 °C (Fig. 3-7). The level of hsp70 at 25 °C became significantly higher than the original level at 6 h after transfer, when the fish started dying and the sampling was stopped.

## Discussion

The tidepool sculpin did not show significant changes in levels of liver hsp70 at the sublethal temperature (22 °C) over 2 weeks compared with the level at the beginning (Fig. 3-6). Liver hsp70 levels of this species did not become significantly higher than the control level until

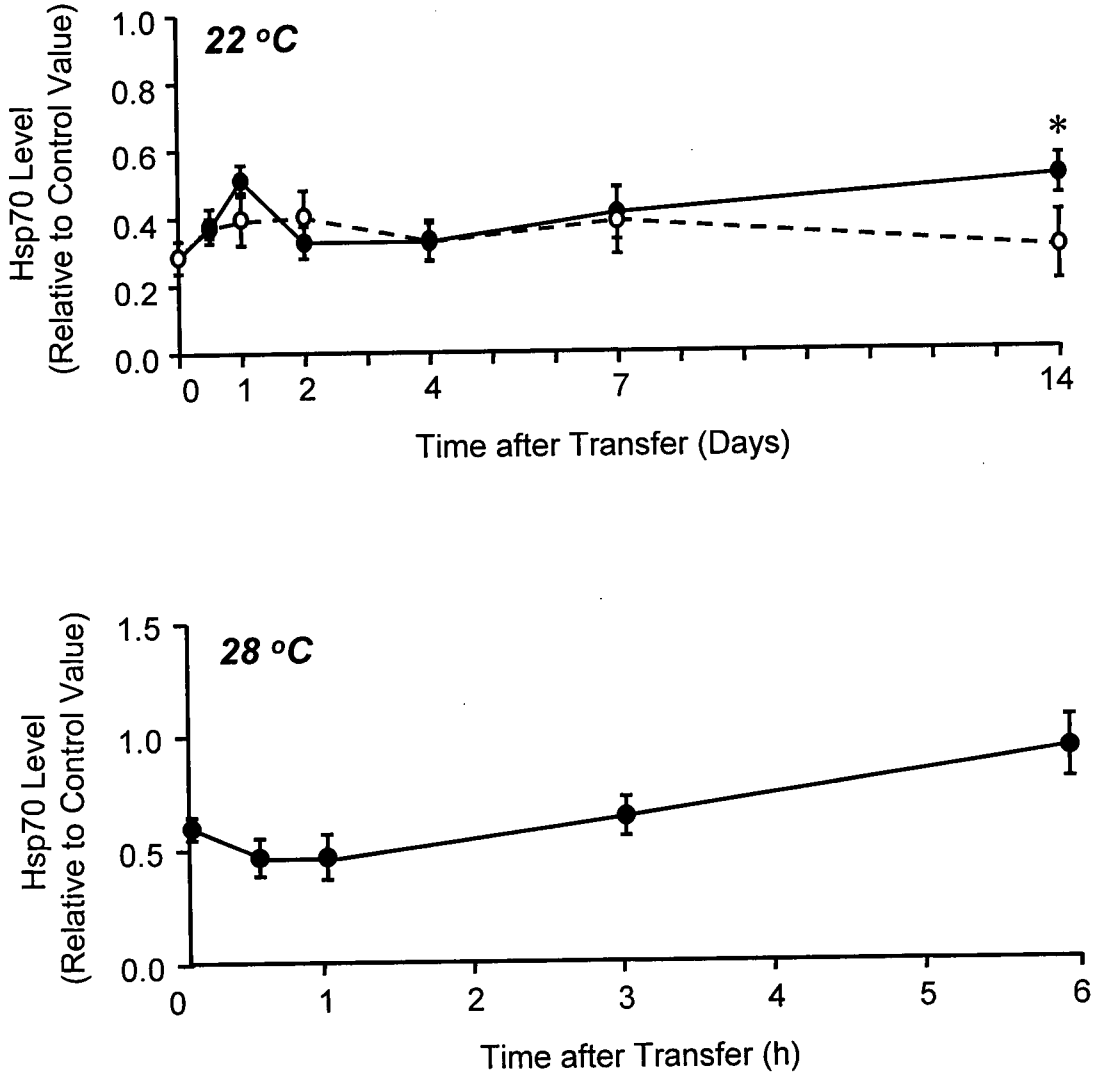


**Figure 3-6.** Black circles show changes in liver hsp70 levels in the tidepool sculpin after transfer to 22 °C or 28 °C from 10 °C (Mean±SE, n=2-9). White circles in the upper graph indicate changes in the control group which was transferred to 10 °C. * indicates significant difference compared with the control level at the same time ($p \leq 0.05$). There was no significant change compared with the level at the beginning in levels of liver hsp70 in the tidepool sculpin at any time after transfer to 22 °C or 28 °C.

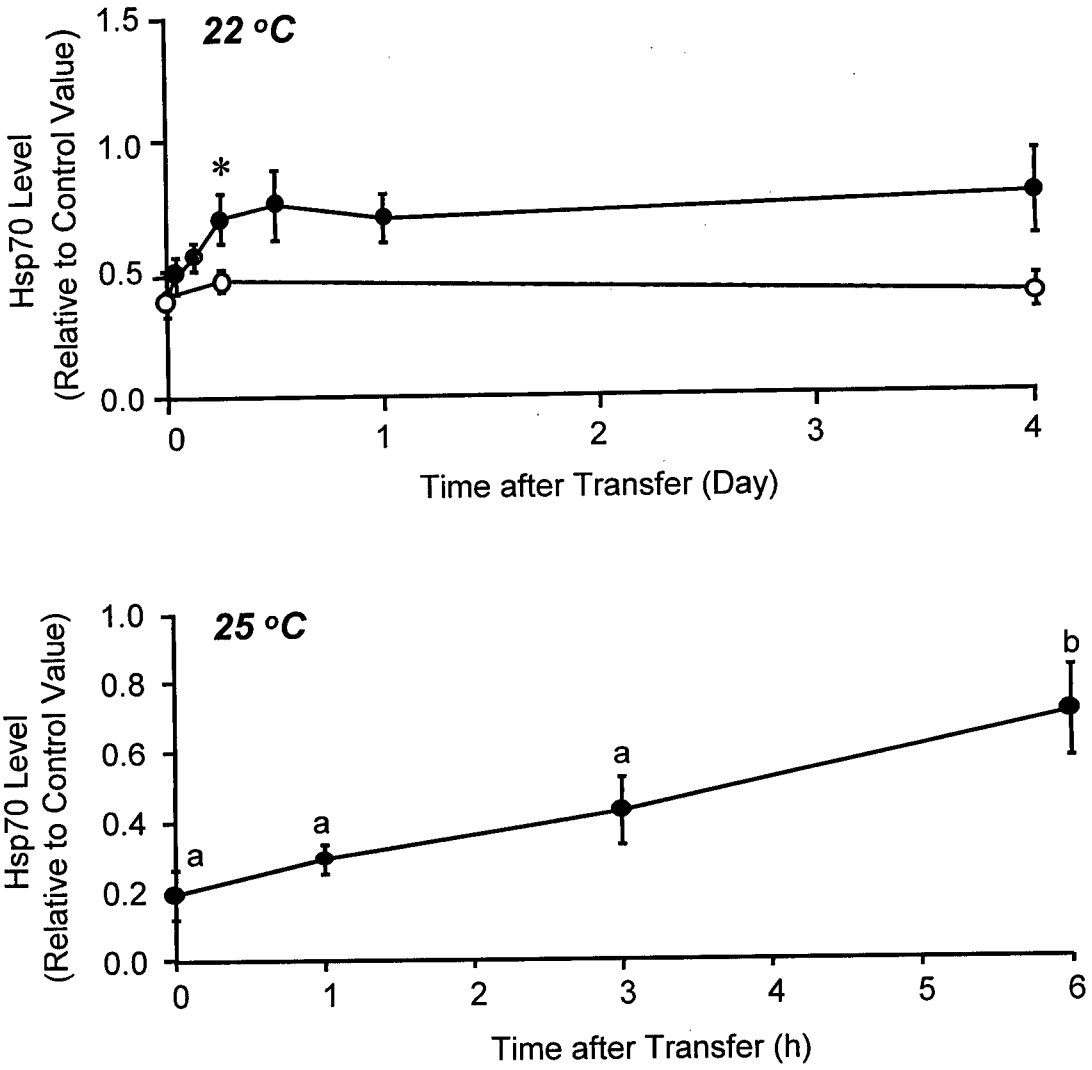


**Figure 3-7.** Black circles show changes in liver hsp70 levels in the fluffy sculpin after transfer to 22 °C or 25 °C from 12 °C (Mean±SE, n=3-5). White circles in the upper graph indicate changes in the control group which was transferred to 12 °C. * indicates significant difference compared with the control value, while different alphabet letters show significant difference compared with the value at the beginning ($p \leq 0.05$). The level of liver hsp70 in the fluffy sculpin was significantly higher at 6 h after transfer to 22 °C compared to the control value at that time and sustained the same level at least for 4 days. The liver hsp70 levels became also significantly higher at 25 °C compared to the value at the beginning at 6 h after transfer when the fish in this group started dying.

2 weeks after transfer. Judging from their feeding behavior, as well as no mortality during the experiment, 22 °C did not seem very stressful for the tidepool sculpin. This is supported by the fact that I kept the tidepool sculpin in a glass aquarium with the water temperature ranging from 20 to 21 °C for more than 3 months, without any abnormal behavior or feeding problems. There were no clear increase in liver hsp70 levels in the tidepool sculpin at 28 °C, which was only 2 °C lower than its lethal temperature. Nevertheless, not all the fish tested could survive more than 6 h at that temperature. It may be noteworthy that this sculpin had a significantly higher amount of liver hsp70 when heat shocked at 28 °C for 2 h and allowed to recover at 10 °C for 22 h (Fig. 3-2). However, when they were continuously kept at 28 °C for up to 6 h in this study, the levels of liver hsp70 tended to decrease during the first 1 h (Fig. 3-6). These results indicate that the significantly higher liver hsp70 in the tidepool sculpin by a 2 h heat shock at 28 °C might be synthesized while they were recovering from the heat shock. If they were not allowed to recover from the heat shock, they would not able to induce higher hsp70.

The fluffy sculpin had significantly higher levels of liver hsp70 at the sublethal temperature (22 °C) at 6 h after the transfer compared with the control level at that time, and sustained that level at least for 4 days (Fig. 3-7). In contrast to the tidepool sculpin at 28 °C, this species also had significantly higher liver hsp70 at 25 °C, which was 3 °C lower than the lethal temperature of this species. The levels of hsp70 in the liver tissue of the fluffy sculpin at 25 °C were more than 2-fold greater than that at 22 °C, when the fish was allowed to recover at 12 °C after a 2 h heat shock (Fig. 3-2). In this study, levels of liver hsp70 were the same between at 22 °C and at 25 °C at 6 h after transfer when the fish at 25 °C started dying. Again, it is possible that higher hsp70 levels might be yield in the liver tissue of the fluffy sculpin during the recovery after the severe heat shock at 25 °C.

Both in the 2 h heat shock in Section 3-1 and in the chronic heat shock in this section, the fluffy sculpin was more thermally sensitive than the tidepool sculpin in terms of the expression

of hsp70. Newly synthesized hsp70 may not have an important role in the tidepool sculpin both under the mild (22 °C) and severe (28 °C) heat stresses, since this species did not have higher liver hsp70 levels at these temperatures, except when they were returned to the acclimation temperature (10 °C) after the 2 h heat shock at 28 °C. In nature, this species may not need to have a high hsp70 level under most low tide conditions, as was already suggested in the field observations (Chapter 2). Even when they are exposed to a severely high temperature of more than 28 °C, which often occurs in nature (Fig. 2-1), they may be able to survive it without the help of hsps, since it is likely that the water temperature will decrease within several hours before it threatens their survival. On the other hand, change in levels of liver hsp70 in the fluffy sculpin showed a higher sensitivity to increases in water temperature than in the tidepool sculpin. However, this species hardly ever occurs in a tidepool where the water temperature can become more than 23 °C (Chapter 2). This species may select a tidepool which is not thermally stressful for them.

In summary, the results in this study, together with the results from the previous sections indicate that the tidepool sculpin may adapt to the intertidal environment with potential thermal stresses in more time-dependent fashion, while the fluffy sculpin may adapt in more space-dependent fashion. Repeats in increases and decreases in the water temperature of a tidepool may provide natural heat shock and recovery, especially for the tidepool sculpin, that allows them to sustain relatively high basal hsp70 levels (cf. Fig. 2-6 and Fig. 2-8).

## Section 3-3. Regulation in hsp70 production: Transcription of hsp70 mRNA in tidepool and fluffy sculpins

### Introduction

I investigated the transcriptional regulation of hsp70 induction between tidepool and fluffy sculpins. It may help us to understand the different expression processes of hsp70 between these

sculpins under acute thermal stresses. In spite of the abundance of data supporting the key role of hsps in the thermotolerance of animals (Mosser *et al.* 1986; Mosser and Bols 1988), the regulation of hsp induction under heat stress is less understood. The level of hsp demonstrates only the net amount of this protein as the result of production and degradation of the hsp. Therefore, even though levels of liver hsp70 in the tidepool sculpin were the same at 10 and 25 °C as shown in the former section of this chapter (Fig. 3-2), it could be possible that both the production and degradation of hsp70 increased at 25 °C, so that the net amount of hsp70 did not change at that temperature. Thus, examining production and clearance of hsps is important in understanding the behavior and regulation of these proteins under heat stresses.

By comparing the levels of mRNA and that product protein, I can assume if the expression of hsp70 is controlled by transcription (mRNA and protein levels are correlated) or translation (mRNA and protein levels are not correlated). There are several reports investigating the changes in hsp70 and its mRNA in fishes. The synthesis of hsp70 was correlated with the accumulation of hsp70 mRNA in chinook salmon (*Oncorhynchus tshawytscha*) embryonic (CHSE) cells (Gedamu *et al.* 1983; Misra *et al.*1989) and cultured fibroblasts of the rainbow trout (*O. mykiss*) (Kothary and Candido 1982), indicating that the control of hsp70 production may be at the transcriptional level in these species. Koban *et al.* (1991) demonstrated that the synthesis of hsp70 in erythrocytes of heat shocked killifish, *Fundulus heteroclitus*, was not affected by inhibition of transcription with actinomycin-D, and concluded that pre-existing hsp70 mRNA was enough to induce hsp70; thus the induction of hsp70 might be controlled at the translational level in these cells. The different patterns of the relationship between hsp70 and hsp70 mRNA levels shown in these studies may be attributed to the experimental protocol or different functions between embryonic cells, fibroblasts, and red blood cells. It may be also possible that the regulatory point may depend on the eurythermal or stenothermal nature of the

species; the chinook salmon and the rainbow trout are relatively stenothermal, while the killifish is eurythermal.

There are relatively few studies that compared expression patterns of hsp mRNA between species with different thermal tolerances. Therefore, in this study, I measured the levels of hsp70 mRNA in the tidepool and the fluffy sculpins subjected to various temperatures, and examined the relationship with the level of hsp70 measured in Section 3-1.

## Materials and Methods

### *Hsp70 transcription*

For Northern blotting, fish were heat shocked in the same way as samples for Western blotting, but only at 10, 18, 25 and 28 °C for the tidepool sculpin, and at 10, 18 and 25 °C for the fluffy sculpin. Livers were sampled with an autoclaved surgical kit, and kept at -80 °C until analysis. All the procedures were carried out in RNase-free conditions. For detailed methods for Northern blotting analysis, see Materials and Methods of Section 1-2. For this experiment, sample loading was standardized by β-actin levels measured with a human β-actin DNA probe.

## Results

### *Hsp70 mRNA levels at different temperatures*

Figure 3-8 shows hsp70 mRNA levels in the tidepool and the fluffy sculpin liver samples at various temperatures. Levels of liver hsp70 mRNA did not change significantly in the tidepool sculpin. The hsp70 mRNA level was significantly higher at 25 °C than at lower temperatures in the fluffy sculpin liver tissue.

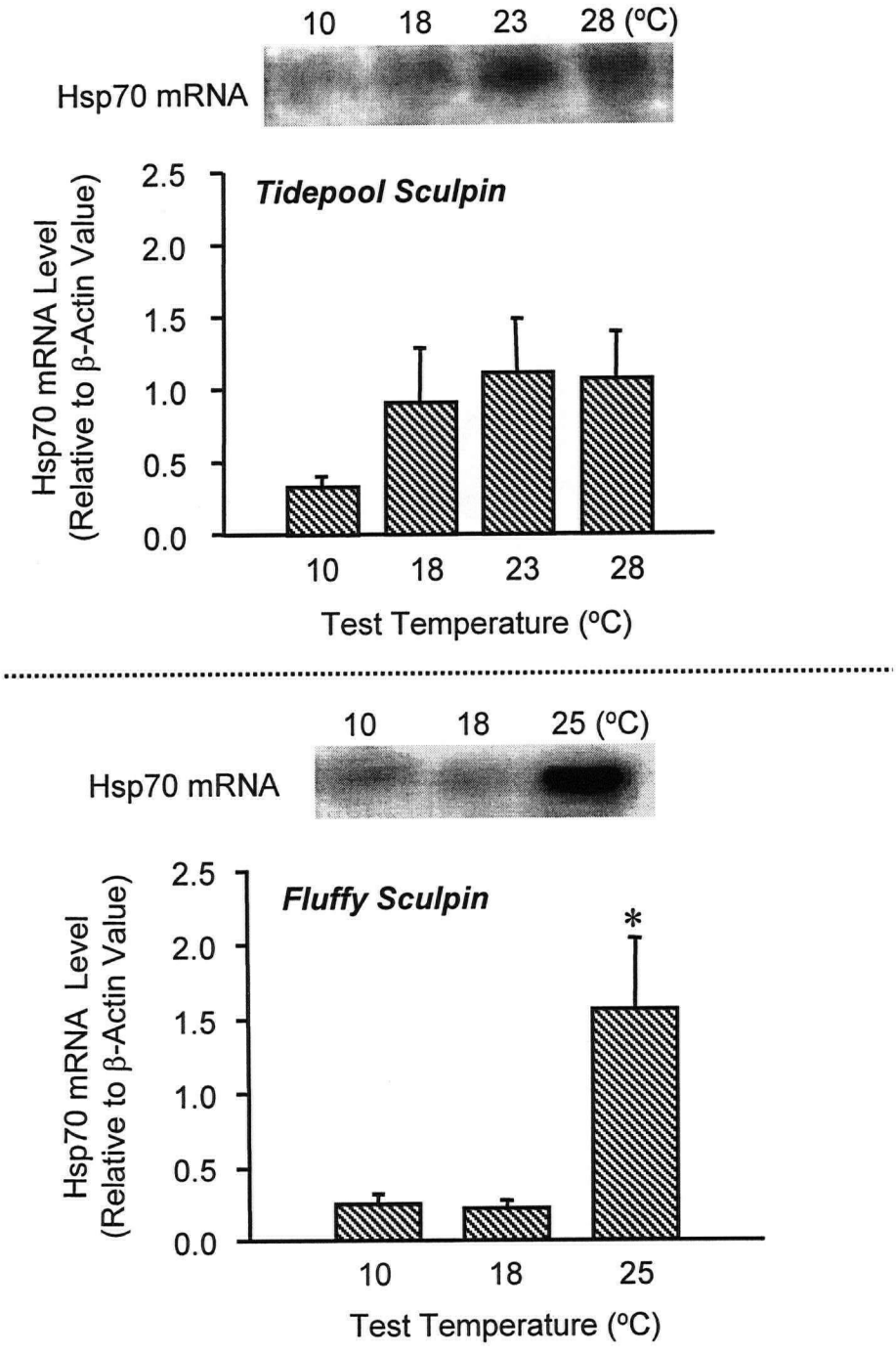


**Figure 3-8.** The upper panel shows hsp70 mRNA by Northern blot analysis in liver tissues of the tidepool and the fluffy sculpins at various temperatures. Lower graphs are based on band intensities standardized by corresponding value of the β-actin mRNA levels (Mean±SE, n=6). There were no statistically significant differences between groups of the tidepool sculpin heat shocked at different temperatures. Liver hsp70 mRNA level was significantly higher at 25 °C compared with 10 and 18 °C in the fluffy sculpin(*; $p \leq 0.05$).

## Discussion

Hsp70 mRNA levels in the liver tissue corresponded to total hsp70 levels in the fluffy sculpin (Figs. 3-2 and 3-8), indicating the direct effect of hsp70 gene transcription on elevated hepatic hsp70 levels in this species. Hsp70 mRNA levels in liver tissues of the tidepool sculpin did not significantly change because of temperature, while levels of hsp70 were significantly higher at 28 °C (Figs. 3-2 and 3-8). Both hsp70 induction, as well as degradation, may have been increased at 18 and 23 °C in liver tissue of the tidepool sculpin, so that no changes in the net hsp70 level would have been observed. Another possibility is that the constitutive level of hsp70 mRNA may be kept relatively high in the tidepool sculpin liver tissue, while the translation to hsp70 may not occur at 18 and 23 °C. Thus, it is indicated that the level of hsp70 in the tidepool sculpin may be controlled at the translational level.

It is known that the expression of hsp70 mRNA after heat shock occurs within a relatively short period. When cultured rainbow trout cells (RTG-2) were placed at 28 °C, hsp70 mRNA concentration increased dramatically during the first 2 h, whereas when cells were heat-shocked at 28 °C for 1 h and allowed to recover at 22 °C, the level of hsp70 mRNA returned to the control level within 3 to 4 h (Kothary *et al.* 1984). Gedamu *et al.* (1983) showed that the expression of hsp70 mRNA after heat shock occurs within 30 min to 4 h in chinook salmon embryo cells. Gross and Watson (1998a) also reported a marked decrease in hsp70 mRNA and hsp104 mRNA 30 min after a heat shock, followed by an increase 60 to 90 min after the heat shock in yeast, *Saccharomyces cerevisiae*. In this study, elevated hsp70 mRNA levels were observed in the liver tissue of the fluffy sculpin even at 24 h after the heat shock. However, it is possible that more significant changes in hsp70 mRNA levels in those sculpins responding to heat stress may have been seen if tissue samples were taken within a much shorter period. Therefore, changes in hsp70 mRNA levels along a time course after heat shock treatments should be measured to observe the heat shock response at the mRNA level in more detail. Determination of the hsp70

turnover rate, both at the protein level and at the mRNA level, may also be necessary for further understanding the underlying regulatory processes of the hsp70 expression in those sculpins.

In summary, the cellular level of hsp70 in the liver tissue may be controlled at the translational level in the tidepool sculpin, while it may be controlled at the transcriptional level in the fluffy sculpin, because the level of hsp70 mRNA in the liver tissue corresponded with the expression of hsp70 only in the fluffy sculpin but not in the tidepool sculpin. More detailed studies in transcriptional and translational regulations during both the heat shock and the recovery states in the tidepool and the fluffy sculpins are necessary to understand the different regulatory mechanisms of hsp70 expression under heat stresses between these sculpins.

## Discussion of Chapter 3

The tidepool sculpin had a higher lethal temperature and hepatic hsp70 induction temperature than the fluffy sculpin (Table 1, Fig. 3-2). The tidepool sculpin had a higher liver hsp70 level only at the higher temperature (28 °C), while the fluffy sculpin had a higher level of liver hsp70 at lower temperatures (22-25 °C). The induction temperature for liver hsp70 in the tidepool sculpin was only 2 °C lower than its lethal temperature while that of the fluffy sculpin was 6 °C lower than its lethal temperature. During chronic exposure to a high temperature, the fluffy sculpin had significantly higher liver hsp70 levels within 6 h at 22 °C and at 25 °C (Fig. 3-7), while the tidepool sculpin did not show any increases in liver hsp70 until 2 weeks after transfer to 22 °C, and at any time after transfer to 28 °C (Fig. 3-6). Recently, Krebs (1999) found the same tendency in three species of fruit flies in genus *Drosophila*; *D. mojavensis*, the desert species, is more thermotolerant than *D. melanogaster* and *D. simulans*, and shows a higher level of hsp70 only at higher temperatures. He also found that, in contrast to *D. melanogaster*, *D. mojavensis* did not require a large quantity of hsp70 in advance of the stress to achieve the high thermotolerance (Krebs and Feder 1997, Krebs 1999). He suggested the possibility that in some species, the expression of hsp70 prior to the heat stress might be necessary to protect their own hsp70 induction pathway. The thermal stability of the hsp induction pathway should be compared between the tidepool and the fluffy sculpin to confirm whether the suggestion by Krebs (1999) can explain why hsp70 expression follows the lethal temperature more closely in the tidepool sculpin than in the fluffy sculpin.

Judging from the relationships between the transcriptional and the translational expressions of hsp70 in liver tissues of the tidepool sculpin and the fluffy sculpin, I pointed to a likelihood in the Discussion of Section 3-3 that the level of hsp70 may be regulated at the translational level in the tidepool sculpin, while it may be regulated at the transcriptional level in the fluffy sculpin. However, as I already mentioned, this possibility has to be carefully confirmed by a more

detailed examination in the hsp70 gene transcription of these sculpins, since the regulation of hsp expression seems to be a complex process. Behaviours of hsps and hsp mRNAs under heat stress are in contrast to those of other cellular proteins and mRNAs. When cells of the fruit fly, *D. melanogaster*, are transferred from 25 to 37 °C, the translation of pre-existing mRNAs is repressed more than 95% within 10 min, while newly transcribed hsp mRNAs are translated with very high efficiencies (Lindquist 1980). During the recovery, hsp synthesis is repressed and normal protein synthesis is restored. The repression of hsp70 synthesis is accompanied by the selective degradation of its mRNA, while pre-existing mRNAs are stable during both heat shock and recovery (Petersen and Lindquist 1988). Petersen and Lindquist (1988) also demonstrated that hsp70 mRNA from *D. melanogaster* is inherently unstable with a half-life of 15 to 30 min at normal temperature, but stabilized under the heat shock, probably by some changes in the physiology of heat-shocked cells that inactivates, or allows the message to bypass, the degradation pathway for hsp70 mRNA at the normal temperature. The change in the turnover rate of hsp70 mRNA in human leukemic cells before and after the heat shock was in agreement with those reports in *D. melanogaster*; hsp70 mRNA in these cells was known to have a half-life of 2 h at normal temperature, while the half-life increased to more than 7 h after the heat shock (Mivechi *et al.* 1992). Edington and Hightower (1990) reported longer half-life of hsp itself after a heat shock; the half-life of hsp23 in secondary cultures of chicken embryo cells increased from 2 h at the normal temperature (37 °C) to 13 h at 44 °C. These reports strongly indicate the existence of more than one form of regulation that contributes to adequate accumulation of hsps in cells under heat stress, and degradation of hsps during recovery, i.e. regulation in transcription of hsp gene, stabilization of hsp mRNAs as well as stabilization of hsps.

In conclusion, the intertidal sculpins studied in this chapter had lethal temperatures and induction temperatures for liver hsp70 that were correlated with their vertical distribution in the intertidal zone. Although the lethal temperatures of the tidepool and the fluffy sculpins differed

by only 2 °C, they showed a larger difference in induction temperature of the cellular heat shock response; the fluffy sculpin had 6 °C lower hsp70 induction temperature in the liver tissue than did the tidepool sculpin. Judging from lethal temperatures, fluffy sculpins could live occupy wider range of the intertidal zone, however they do not indeed. Therefore, it may be more likely that different habitat selection between these sculpins would be related to the induction temperature of the cellular heat shock response than the lethal temperature of the species. The evolution of cellular heat shock responses of animals may have been extensively influenced by thermal conditions in the intertidal zone where the temperature is one of the major challenges for inhabiting animals.

# Chapter 4

## Some factors that may affect the thermotolerance of fish: The tidepool sculpin

### Introduction to Chapter 4

It is generally believed that hsps play important roles in determination of thermal tolerances of animals (see review by Feder and Hofmann 1999). On the other hand, changes in surrounding temperatures also can affect whole body metabolism and physiological states of animals. Not only the synthesis of hsps, but also other metabolic and morphological changes can occur during a heat shock. Therefore, a multilevel mechanism with many factors may be operating in concert for the protection and survival of heat-shocked cells (Gross and Watson 1998b). The physiological and biochemical processes that stimulate the induction of hsps are still unclear. However, Currie *et al.* (1999) suggested that changes in energy metabolism may have potential effects on hsp function and expression, since stressful conditions often disturb energy metabolism and, thus, change cellular ATP concentrations, which influence the accumulation of denatured cellular proteins as well as ATP-dependent pathways of hsp functions. Therefore, studies of the relationship between the expression of hsp70 and physiological and metabolic states under heat stress in an animal seem important to understand how stress responses at various levels operate for an organism's adaptation to heat stress. Here I examined the effects of the thermal history, which may influence the physiological state of an animal, on the strength of thermotolerance and the rate of increase in hsp70 levels under a heat stress in the liver tissue of the tidepool sculpin. I also examined the relationship between the heat shock response and changes in other physiological stress variables, such as the plasma cortisol level and the oxygen consumption rate, under heat stresses.

## Section 4-1. Importance of the tidal cycle for the tidepool sculpin to keep a higher thermal tolerance in nature

### Introduction

Through the experiments on the tidepool and the fluffy sculpins, I found some inconsistencies between the results from the field and the laboratory experiments. First, no tidepool sculpins survived for more than 3 min after direct transfer to 30 °C (Table 1). However, I observed several tidepool sculpins swimming actively in a natural tidepool in which the water temperature was 29.3 °C. Second, the tidepool sculpin had significantly higher constitutive levels of liver hsp70 than the fluffy sculpin in a natural tidepool with water temperatures of 17-18 °C (Fig. 2-8). Nevertheless, there were no clear differences between the levels of liver hsp70 in the tidepool and the fluffy sculpins heat shocked at 18 °C in the laboratory (Fig. 3-2).

There are major differences in the thermal environments that those sculpins experienced between the field and the laboratory. The primary difference between these two situations is the stability in water temperature. In nature, the tidepool sculpin experiences continuous changes in water temperature and in other water qualities, such as salinity and the oxygen concentration, caused by the tidal cycle and weather changes (e.g. rain). In the laboratory, fish were kept in a stock tank at constant 10 °C. In addition, water temperature in a tidepool does not increase suddenly during a low tide in nature as it did in the heat shock experiment in the laboratory, even though the fish in a tidepool in nature often experience a rapid decrease in water temperature at a high tide (see Figure 2-1). Physiological and biochemical adjustments likely occur during the gradual temperature changes in nature, and these may allow fish to adapt to the higher temperatures at a low tide compared to the direct transfer in the laboratory experiment. However, it is unknown whether the fluctuating, but gradually changing, water temperature in a natural tidepool contributes to a higher hsp70 levels and the higher thermal tolerance of the tidepool sculpin compared to those observed in the laboratory. To test this hypothesis, I compared the

heat resistance and the level of liver hsp70 between two groups of the tidepool sculpin; one group was acclimated at constant 10 °C in the laboratory while the other group was collected from a tidepool just before the heat shock treatment.

## Materials and Methods

### *Effects of tidal rhythm on the thermotolerance of the tidepool sculpin*

To examine whether the natural tidal rhythm has any effect on the tidepool sculpin to sustain a high level of cellular hsp70 in nature, I conducted the following experiment. Nineteen tidepool sculpins were acclimated at 10 °C for 2 weeks before being used in the experiment in July 1999 to eliminate the tidal rhythm (acclimated group=A). Another group of 19 fish were collected as the not-acclimated group (NA) from a tidepool on Wizard Islets, Bamfield, B.C., Canada and was kept at 10 °C less than 24 h until used in the experiment.

After sampling liver tissues from 8 fish in each group, the remaining 11 fish for the A group and 11 fish for the NA group, were separately transferred to 28 °C to compare the viability between the acclimated and natural groups at that temperature. The viability test was stopped at 4 h after transfer, since all the fish in the A group died. Then liver samples were taken from the fish in both groups. Liver tissues were immediately frozen with liquid nitrogen, and stored at -80 °C until Western blot analysis for hsp70 (see Material and Methods of Section 1-1 for the details of Western blot analysis).

## Results

### *Effects of tidal rhythm on viability and induction of hsp70 in the tidepool sculpin*

The viability at 28 °C was clearly different between the A group and the NA group (Fig. 4-1). Fish in the A group started dying at 2 h after transfer, and all fish in this group had died at 3.5 h after transfer to 28 °C from 10 °C. Only one fish died in the NA group at 4 h after the

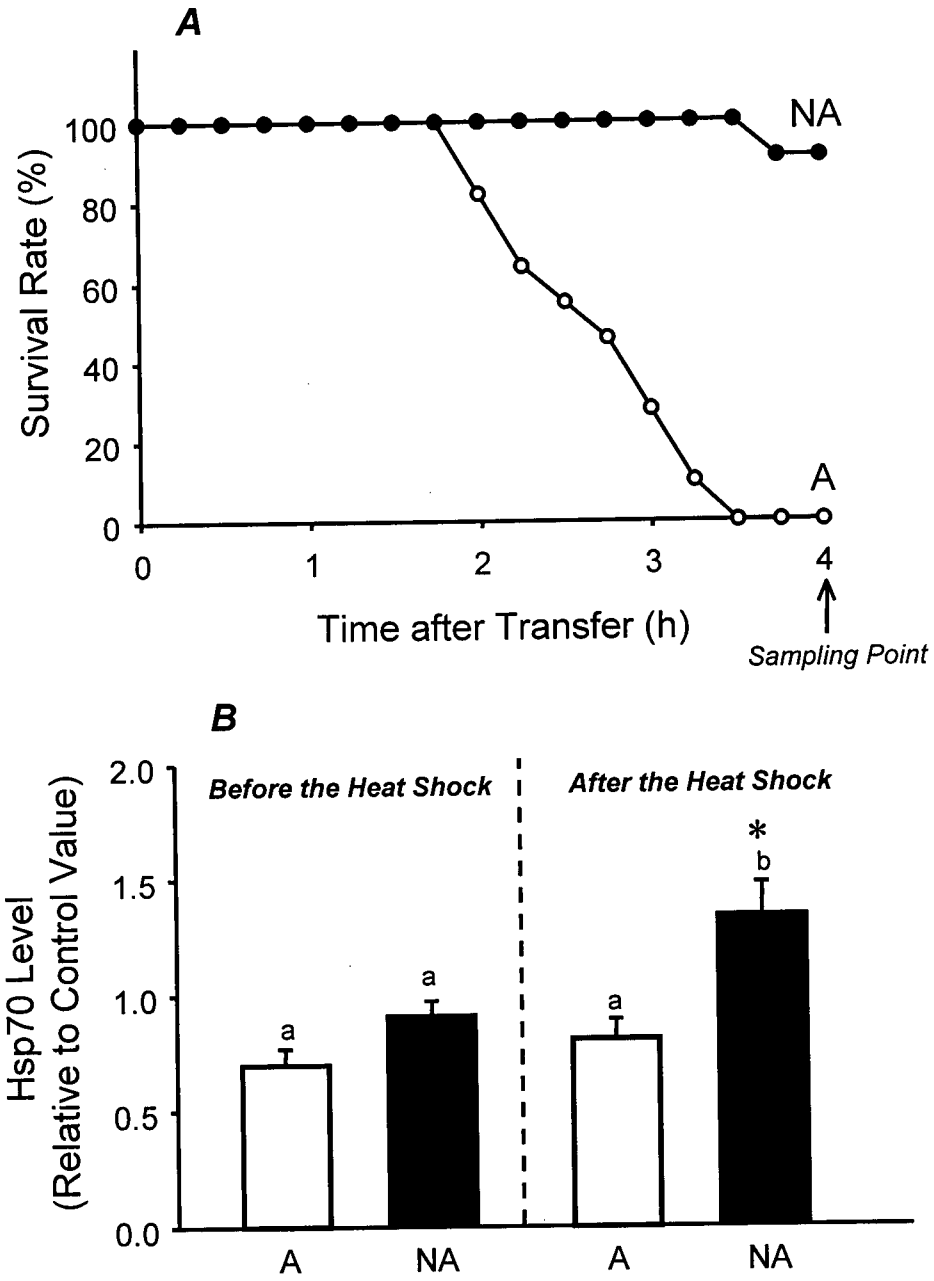


**Figure 4-1.** The upper graph (A) shows the survival rate of the tidepool sculpin after transfer to 28 °C. Black circles indicate the survival rates of the NA (Not Acclimated) group, which was collected from a natural tidepool 22 h before the experiment. White circles are survival rates of the A (Acclimated) group, which was acclimated at constant 10 °C for 2 weeks before the experiment. The lower graph (B) is levels of liver hsp70 in the A (white bar) and the NA (black bar) groups of the tidepool sculpin before and after the heat shock at 28 °C (Mean±SE, n=8-11). * indicates significant difference between before and after the heat shock ($p \leq 0.05$). Different alphabet letters mean significant difference between A and NA groups ($p \leq 0.05$). Significantly higher level of liver hsp70 was observed after the heat shock only in the NA group.

transfer. The level of hsp70 in the liver tissue before the heat shock at 28 °C was not significantly different between the A and the NA groups. The liver hsp70 level became significantly higher within 4 h after transfer to 28 °C in the NA group, while that of the A group did not change with the heat shock. As a result, the level of hsp70 in liver tissue was significantly higher in the NA group compared with the level in the A group after the heat shock.

## Discussion

There were clear differences both in the time for resistance at 28 °C and the level of liver hsp70 after the heat shock between these 2 groups of the tidepool sculpin (Fig. 4-1). The tidepool sculpin, which had been living up to 24 h before the experiment in the natural fluctuating temperature, had a longer resistance time at 28 °C compared with the ones acclimated at constant 10 °C. The NA group of the tidepool sculpin had higher liver hsp70 levels after the heat shock treatment compared with the level before the heat shock, while levels of liver hsp70 of the A group did not change before and after the heat shock. These results indicate that the tidepool sculpin in nature could have higher thermal tolerance, and had a higher level of hsp70 faster than the same species acclimated at constant temperature in the laboratory. The process for hsp70 synthesis might have started in the NA group, but not in the A group when they were heat shocked. Measurement of hsp70 mRNA levels in those groups may be useful to explain this question.

Induced thermotolerance, or thermoprotection, is a term that has been given to a process in which a previous experience of a moderately high temperature induces hsps. This not only enhances thermotolerance at higher temperatures that would be lethal without the first mild heat shock, but also confers resistance to other stressors, or *vice versa*. Thermoprotection has been observed in various organisms from yeast to mammals both in cultured cells and whole organisms (see review by Parsell and Lindquist 1994). Since animals in the intertidal zone

experience changes in various physical and chemical environmental factors, this mechanism is likely providing the protection needed to inhabit that potentially stressful environment. Being in a natural tidepool with continuously fluctuating temperature, salinity and oxygen levels, the tidepool sculpin may express constitutively higher amounts of hsp70 and may have higher amounts of hsp70 mRNA to increase the rate of hsp70 induction, resulting in their higher resistance to temperature.

It was shown that the induction temperature for hsps increased when the acclimation temperatures of the goby, *G. mirabilis* (Dietz 1994), and of the medaka, *Oryzias latipes* (Oda *et al.* 1991), were increased also. It has been previously shown also that acclimation to a high temperature led to an elevated basal level of hsp72 in the rat (Maloyan *et al.* 1999). These results support the likelihood that the threshold temperature of hsp production may not be programmed exclusively genetically, but that it may be modified by the recent thermal history of the organism. These modifications in hsp induction pathways may allow organisms to accumulate adequate amount of hsps to survive when exposed to various stressors.

In this study, I demonstrated that the different thermal tolerances correlated with the rate of increase in the hsp70 level among the same species dependent on the given thermal environments. The possibility should be carefully considered that the results from the series of heat shock experiments in the acclimated sculpins in Chapter 3 could be different if the fishes were not acclimated at a constant temperature. In this study, I hypothesized that a potentially harsh, variable environment in nature may be rather beneficial for the inhabiting animals to maintain higher resistance to stressors. This hypothesis should be elucidated in future studies to understand true abilities of animals to cope with stressors in their natural habitats.

## Section 4-2. Oxygen consumption rate, plasma cortisol and liver ubiquitin levels in the tidepool sculpin at various temperatures

## Introduction

Hsps comprise a relatively large part of the total cellular proteins. In *E. coli*, more than 20 % of all cellular polypeptides belong to this class of proteins under a severe heat stress (Morimoto *et al.* 1994). Gross and Watson (1998b) demonstrated that approximately 10% of the total mRNA were either upregulated or downregulated in *S. cerevisiae* after heat shock. Since protein synthesis (Pannevis and Houlihan 1992), as well as the molecular chaperone function of hsps (Martin *et al.* 1992), can be energetically costly, changes in the synthesis of cellular proteins, including hsps, could alter cellular metabolism. If these changes occur in various tissues of an animal, it can affect the whole-body metabolism. In fact, changes in environmental temperature can affect whole-body metabolism and physiological states of ectothermic animals, as indicated by general and classic stress indicators such as plasma cortisol levels, oxygen consumption rates, and plasma glucose levels. However, interactions between the cellular stress response and changes in those stress indicators have not been studied well. To expand the understanding of the relationship between the cellular heat shock response and other physiological and metabolic stress responses, I examined the relationship between liver ubiquitin levels, plasma cortisol levels and whole body oxygen consumption rates of the tidepool sculpin at various temperatures. Cortisol plays a major role in the regulation of hydromineral balance and metabolism in fish, and is often used as an indicator of stress (Wendelaar Bonga 1997), while ubiquitin is a molecular chaperone, which has been known to have an important role in the irreversible degradation of damaged proteins (Varshavsky 1997).

Ubiquitin is a small 76-residue protein. While ubiquitin-dependent pathways have been known to play major roles in various biological processes including cell differentiation, the cell cycle, embryogenesis, apoptosis, signal transduction, DNA repair, transmembrane and vascular transport, and stress response and functions of the nervous system, many of those pathways involve the degradation of ubiquitin-conjugated, damaged proteins in an ATP-dependent fashion

by cytoplasmic non-lysosomal proteases (see reviews by Parsell and Lindquist 1993; Varshavsky 1997). The importance of ubiquitin-dependent abnormal protein degradations was shown in HepG2 cells, a human hepatocarcinoma cell line (Fisher *et al.* 1997); *Saccharomyces cerevisiae* (Lee *et al.* 1996); and in cadmium resistance in *S. cerevisiae* (Jungmann *et al.* 1993). Hofmann and Somero (1995) successfully used the level of ubiquitin conjugates as the biomarker for protein damage in the intertidal mussel, *M. trossulus*, under heat stress. Therefore, I also used the level of ubiquitin conjugates in the liver tissue from the tidepool sculpin as the indicator of irreversible protein denaturation under various strengths of heat stresses.

## Materials and Methods

### *Measurements of oxygen consumption rates*

The oxygen consumption rate of individual tidepool sculpins was measured using a double-jacket glass chamber designed by Iwama (UBC). Each fish was acclimated for 2 h in the inside chamber (2L) filled with sea water, and the water was kept at 10 °C by recirculating freshwater of this temperature through the outside chamber surrounding the inside chamber. Oxygen consumption of the fish was then measured with a Strathkelvin Model 781 oxygen meter and microelectrode (Strathkelvin Instrument, Glasgow, Scotland) for 1 h. Measurements were conducted at 10, 15, 18, 20, 23, 25 and 28 °C. Each fish was used only for one measurement; oxygen consumption rates of 6 fish were measured at each temperature; therefore 42 tidepool sculpin were used in all. Oxygen consumption rate was calculated as $mgO_2/kg/h$ for each fish.

### *Sampling of plasma and liver tissues, and plasma cortisol measurements*

After the tidepool sculpin was acclimated at 10 °C for 4 weeks, the fish was transferred directly to 10, 15, 18, 20, 23, 25, and 28 °C from the acclimation temperature. Six fish were transferred to each temperature. Feeding was stopped 24 h before any transfers. Two hours after

transfer, fish were returned to 10 °C for 22 to 24 h before sampling. Blood samples were taken using hematocrit tubes, centrifuged at 7600×g for 5 min and plasma samples were collected for the cortisol assay. Liver tissues also were sampled for ubiquitin measurements, and frozen immediately on dry ice in 1.5 mL microcentrifuge tubes. Both plasma and liver tissue samples were stored at -80 °C until analysis. The level of plasma cortisol was measured using a cortisol ELISA (enzyme-linked immunosorbent assay) kit (Medicorp Co, USA).

*Dot blot analysis for liver ubiquitin levels*

The ubiquitin level in the liver tissue of the tidepool sculpin was measured by dot blotting using an antibody raised against mouse ubiquitin (StressGen, B.C., Canada). Liver samples were homogenized and prepared the same way as for SDS-PAGE to make 1mg protein/mL concentration (see Materials and Methods in Section 1-1). Then 10 μL of each sample was directly applied on the 0.2μm pore size nitrocellulose membrane (Bio-Rad, USA). After the membrane was dried between filter papers at room temperature for 2 h, the membrane was blocked in 2% skim milk in TTBS for more than 1 h, then rinsed once and soaked in TTBS for 5 min. Membranes were then soaked in the primary antibody (rabbit IgG for mouse ubiquitin) at a 1:500 dilution in 2% skim milk for 1 h. After 3 washes in TTBS for 5 min each, membranes were soaked in the secondary antibody (goat anti rabbit IgG) at a dilution of 1:3000 in TTBS for 1 h. After 3 washes in TTBS for 5 min each, and one wash in TBS for 5 min to remove the Tween-20, membranes were developed in a NBT / BCIP solution in alkaline phosphatase buffer for 5 min. Spots on membranes were scanned and the intensities were quantified with Sigma-Gel software (Jandel Scientific, USA).

## Results

*Correlation between plasma cortisol levels and oxygen consumption rate*

Changes in plasma cortisol levels in tidepool sculpins at various temperatures were positively correlated with those in oxygen consumption rates (Mean±SE, n=6, r=0.786, p=0.025). Figure 4-2 shows mean oxygen consumption rates and the plasma cortisol levels of the tidepool sculpin measured at various temperatures from 10 to 28 °C. The oxygen consumption rate was significantly higher at 23 °C, while the plasma cortisol level was significantly higher at 28 °C compared with the respective values at 10 °C (Mean±SE, n=6, $p \leq 0.05$). The variance in the oxygen consumption rate became larger at higher temperatures (25 and 28 °C) when some of tested fish were severely stressed, especially at 28 °C.

*Correlation between the liver ubiquitin level and the oxygen consumption rate*

In contrast with the plasma cortisol level, the liver ubiquitin level showed a significant negative correlation with the oxygen consumption rate in the tidepool sculpin (Mean±SE, n=6, r=-0.750, p=0.038). Figure 4-3 shows the changes in liver ubiquitin levels and associated oxygen consumption rates at various temperatures. The level of total ubiquitin in the liver tissue of this species became significantly lower at 25 °C compared to the level at 10 °C (Mean±SE, n=6, $p \leq 0.05$).

## Discussion

In this study, whole body oxygen consumption rates at various temperatures were significantly and positively correlated with plasma cortisol levels, and negatively correlated with liver ubiquitin levels (Fig. 4-2 and Fig. 4-3). Davis and Schreck (1997) also showed a significant correlation between oxygen consumption rates and plasma cortisol levels in juvenile coho salmon (*Oncorhynchus kisutch*) in response to moderate handling stress, but no correlation after

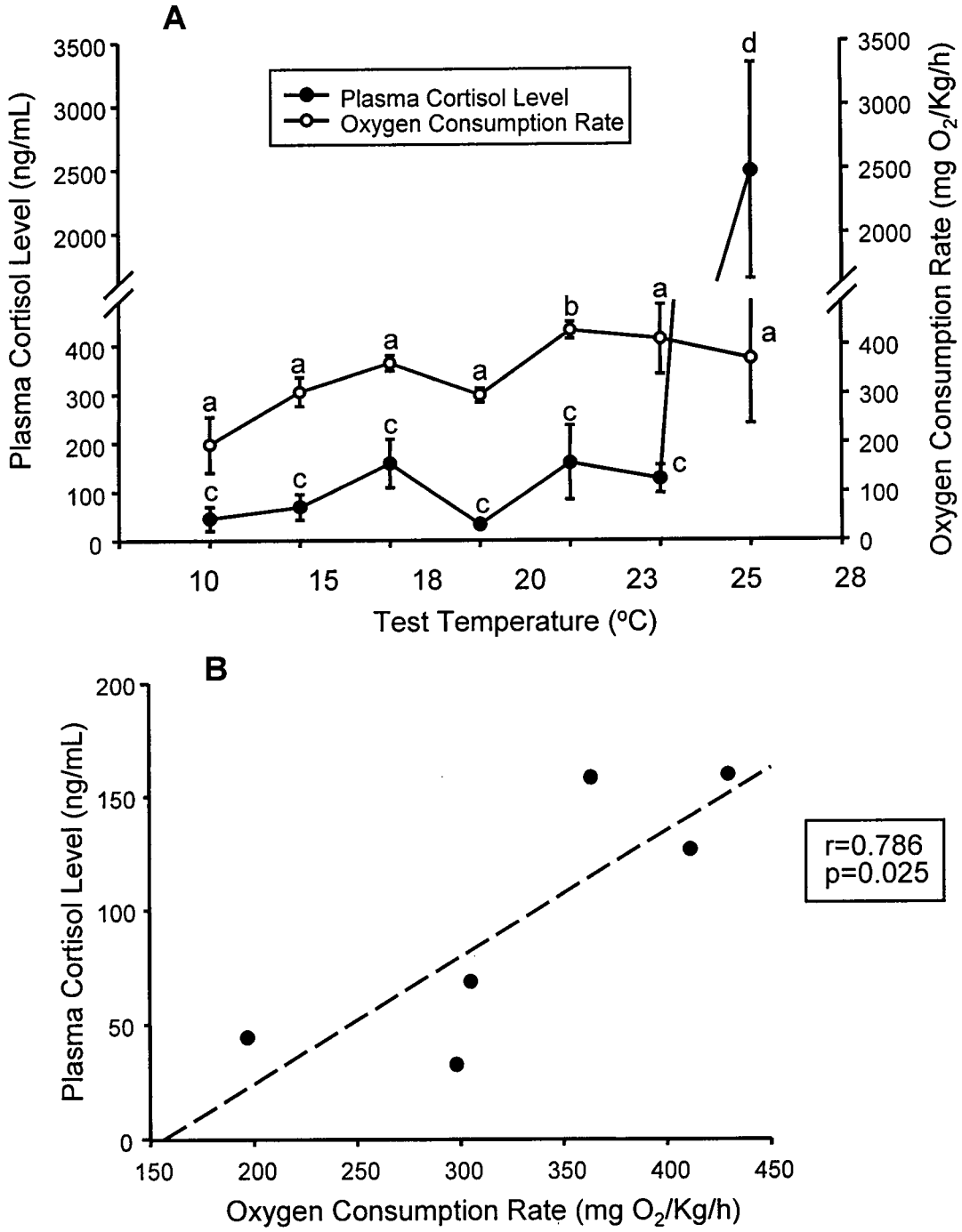


**Figure 4-2.** Black circles indicate levels in plasma cortisol, while white circles are the oxygen consumption rate of the tidepool sculpin measured at various temperatures from 10 to 28 °C (Mean±SE, n=6) (A). Different alphabet letters indicate significant difference compared to the level at 10 °C ($p \leq 0.05$). There was a positive correlation between the plasma cortisol level and the oxygen consumption rate ( r=0.786, p=0.025) (B).

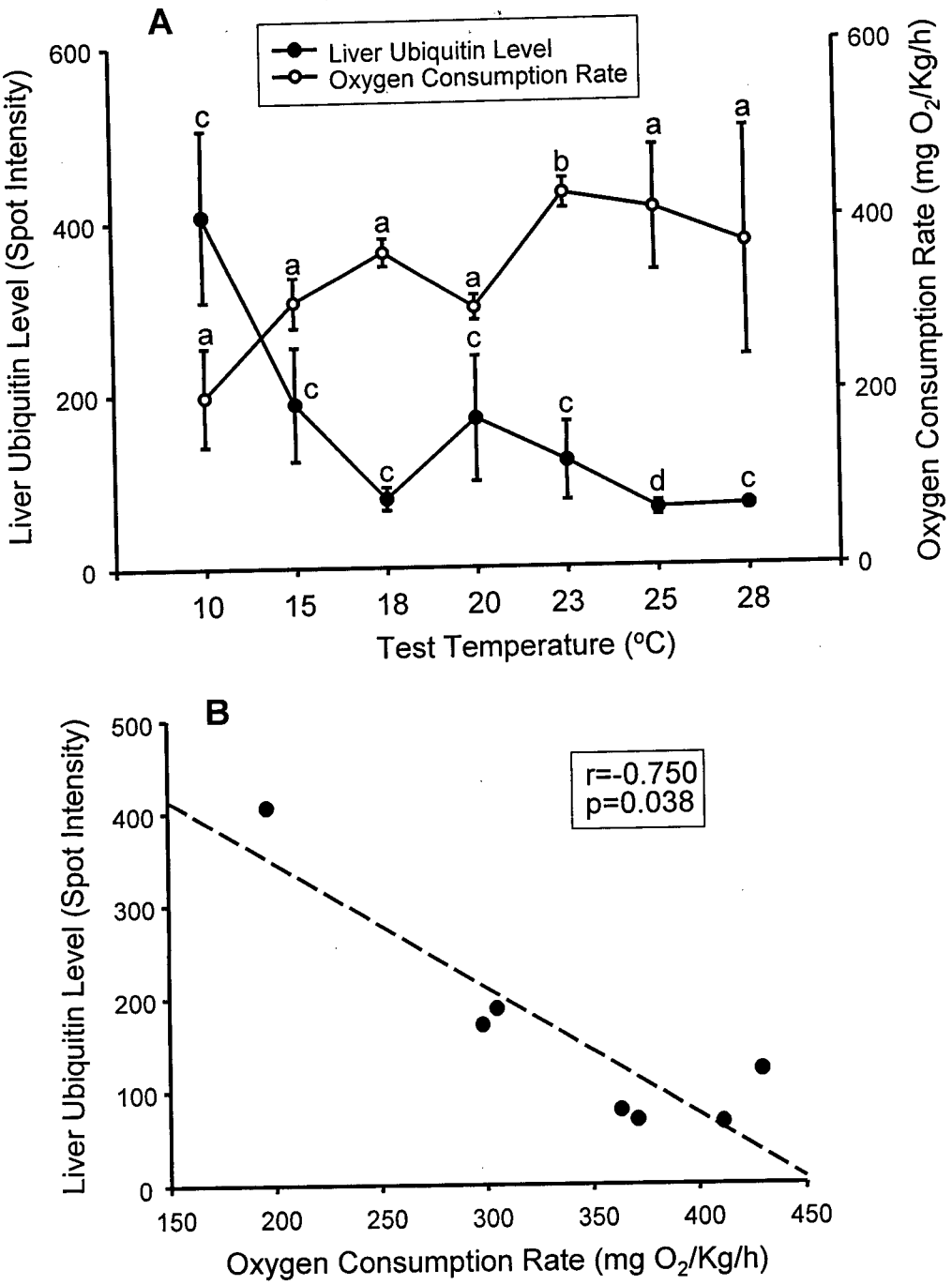


**Figure 4-3.** Black circles show liver ubiquitin levels, while white circles indicate the oxygen consumption rate of the tidepool sculpin measured at various temperatures from 10 to 28 °C (Mean±SE, n=6) (A). Different alphabet letters indicate significant difference compared to the level at 10 °C ($p \leq 0.05$). There was a negative correlation between the liver ubiquitin level and oxygen consumption rate in the tidepool sculpin (r=-0.750, p=0.038) (B).

a severe handling stress. They suggested that cortisol might have an influence on oxygen consumption only in a moderate disturbance, however, cortisol might not have major effects on metabolic rate, since implantation of cortisol did not change the oxygen consumption rate of the juvenile coho salmon.

No clear correlation was found between plasma cortisol level and ubiquitin conjugate levels in tissues (gill, brain and muscle) of the blue mao mao (*Scorpis violaceus*) after confinement stress (Ryan *et al.* 1995). However, they suggested the possibility that ubiquitin has a major role in the physiology of this fish, since the overall levels of ubiquitin conjugate and free ubiquitin in that species were very high; about 10 times and 3 times higher than those reported in the rat, respectively. Wing and Goldberg (1993) proposed that glucocorticoids activate the function of ubiquitin in damaged protein degradation. In contrast to their report, there was no significant correlation between levels of liver ubiquitin and plasma cortisol in this study (data not shown). Differences in experimental protocol and species may account for the different results. Apparently more comprehensive examinations are necessary in future studies.

Hand (1998) reported that protein degradation via the ubiquitin-dependent pathway is depressed under anoxia or aerobic acidosis in the brine shrimp (*Artemia franciscana*) embryo. Whole body oxygen consumption rate and the liver ubiquitin level in the tidepool sculpin were negatively correlated in our study. Especially under the severe heat shock at 25 and 28 °C, the levels of liver ubiquitin were considerably lower than the original level at 10 °C. The fish could have been experiencing minor acidosis or hypoxic condition because of high water (body) temperature and high oxygen demand, especially at 28 °C. However, it is not clear whether the ubiquitin-dependent pathway due to anoxia or acidosis observed in the brine shrimp embryo can be generalized among various organisms, since the brine shrimp embryo is extremely tolerant of prolonged oxygen depression. As a result, brine shrimp may have a unique mechanism for survival under severe anoxia. Yet, it is highly possible that the energetic state of an organism

can affect the function of ubiquitin, since the protein degradation pathway by ubiquitin is ATP dependent. Examination in the relationship between the function of ubiquitin and the energetic state of the animal is essential to confirm that possibility.

## Discussion of Chapter 4

In Section 4-1, I demonstrated that the expression of liver hsp70 and the thermal resistance were different within a species depending on their recent thermal history. No direct evidence of different physiological states between these two groups of the tidepool sculpin were shown in this study. Nevertheless, my results imply that the physiological state of an animal can be an important factor to determine the capacity of its thermotolerance under heat stress. The process of the organismal heat shock response, from the detection of changes in temperature to the cellular heat shock response, is not fully understood. After an animal senses changes in environmental temperature, there could be complex processes, including metabolic and neuro-endocrine modifications in tissues and the whole body of an animal, before a response at the cellular level occurs. Therefore the physiological state of animals at the time they experience changes in temperature, i.e., how much the consequent processes in the whole-body heat shock response is "ready" to induce hsps in the cells, may be important to determine the speed and the magnitude of the cellular heat shock response. In addition, the status of the process could be affected by the recent thermal history of the animal, which is supported by the results in Section 4-1.

The interactions between the cellular heat shock response and metabolic and endocrinological states of animals have not been well studied. Only recently, has work in this field been reported. Deane *et al.* (1999) reported that injection of growth hormone (GH) and prolactin (PRL) significantly reduced the levels of liver hsp70 as well as hsp70 mRNA in the silver seabream (*Sparus sarba*), but cortisol injection had no effect on those levels. The consequence of downregulation in hepatic hsp70 by GH and PRL on the physiology of this fish is unknown. Liver hsp70 level did not change in rainbow trout after handling stress when the plasma cortisol level was significantly elevated (Vijayan *et al.* 1997). However, depression of heat-induced hsp70 induction by cortisol was observed in the primary culture of rainbow trout

hepatocytes, suggesting the possibility that higher levels of cortisol could inhibit hsp70 synthesis by binding with cortisol receptors in the cytosol (Iwama *et al.* 1999).

The reports shown above are sometimes inconsistent, probably because of different experimental protocols. Knowledge in this field of study is still far from sufficient to obtain a clear conclusion of the effects of physiological states on the cellular heat shock response of animals. Nevertheless, as shown in my study, as well as by Deane *et al.* (1999), the neuro-endocrine state may affect hsp dynamics. Thus, the relationships between the cellular and the whole body stress responses, as well as between those stress responses and the stress tolerance of the organism, could be an important topic of study in the future.

## General Discussion

The general cellular heat shock response was found in all fishes examined in this study, except in the Antarctic fish in which it was shown that some parts of the process of the general cellular heat shock response may have been lost during the adaptation to extreme cold (Section 1-2). Although there are tissue-specific differences in general, the induction temperatures for hsp70 were different among species. However, they were in general agreement with the upper lethal temperatures or the range of habitat temperatures of the species. Therefore, although expression of hsps are always found in various organisms subjected to heat stresses, the magnitude and the process of the cellular heat shock response may have been modified within species during the course of adaptation to their habitats.

The fundamental problem in cells which both cold-adapted and tropical fishes experience when they are subjected to temperatures that are higher than their physiological temperature is likely the destabilization and denaturation of cellular proteins. Since synthesis of hsp70 is triggered by aggregation of abnormal proteins (Freeman *et al.* 1999; Santoro 2000), differences in threshold temperatures for higher hsp70 levels between species observed in this study may be determined simply by the temperature that triggers the denaturation of cellular proteins, which is different depending on the stenothermal and eurythermal nature of fishes. Results of this study has lead me to two possibilities: 1) the general mechanism of the cellular heat shock response is similar and conserved among species, and species-specific expression of hsps is caused by the properties of other cellular proteins; or 2) the cellular heat shock response has been also modified by habitat temperatures. Probably, one or both of these is possible, depending on the case. In most of temperate and warm-adapted fishes, the importance and the range of temperatures for hsp function may be dependent on the genetically determined thermal stability of cellular proteins; if cellular proteins are stable over a wide range of temperature, hsps may not have major role except at very high temperatures near the lethal temperature of the species. On the

other hand, the hsp expression pathway itself may have been modified in the Antarctic fish which likely has not been required the synthesis of heat-inducible hsps for a long time during adaptation to the extreme cold, resulting in no clear increase in the level of liver hsp70 at least within 4-6 h after a severe heat shock (Section 1-2). There is no direct evidence for these speculations at the moment. The relationship between the temperature which triggers the denaturation of cellular proteins and the temperature for hsp70 induction, as well as the thermal stability of hsp70 itself, should be compared between these species to answer these questions.

In point of fact, there are many reports suggesting that the importance of hsps and their operating pathways may be different among species. In spite of accumulating data indicating that hsps have an important role in thermal tolerance of organisms, there is a significant number of contradicting reports. There are many reports about yeast showing that the synthesis of hsps is not required for the induction of thermotolerance. For example, a normal thermotolerance of the yeast, *Saccharomyces cerevisiae*, was induced under the suppression of protein synthesis by cycloheximide (Hall 1983; Watson *et al.* 1984), in mutants unable to induce hsps (DeVirgilio *et al.* 1991; Smith and Yaffe 1991), and in yeasts where no hsps were induced (Barnes *et al.* 1990; Coote *et al.* 1991). Borrelli *et al.* (1996) demonstrated that thermotolerance was induced in Chinese hamster ovary (CHO) cells without synthesis of hsps. These inconsistencies about the importance of hsps in the heat resistance of organisms might be caused by different protocols in the experiments and by different types of hsps measured. However, it also can be attributed to different functions and importance of hsps in thermal adaptation among organisms as mentioned in the previous paragraph. Even though the importance of hsps are now well established in many organisms, the diversity of their functions and mechanisms also should be thought-out in future studies.

As mentioned in the Discussion of Section 3-3, the level of hsps could be regulated at various points. I measured only total hsp70 levels in this study. Therefore, there is no

information about the effects of heat shock treatments on the induction and degradation of hsp70 in this study. A high level of hsp70 can be caused by a high production rate with the same or lower degradation rate, or the same production rate with a lower degradation rate. Even though the level of hsp70 did not change, there are three possibilities: 1) no change in production and degradation rates; 2) a higher production rate with a higher degradation rate; and 3) a lower production rate with a lower degradation rate. Therefore, a significantly higher level of hsp70 in my results does not necessarily mean an increase in synthesis of that protein occurred. The level of newly synthesized hsp70 by a heat shock treatment is important to know in order to understand the regulation of hsp70 production under heat stress. Measurement of the incorporation rates of L-[$^{35}$S]methionine into newly synthesized hsp70 could be a good method to estimate the net production rate of this protein.

The rate of increase in the cellular hsp concentration may be different depending on pre- or post-transcriptional regulation. Transcriptional regulation can take a longer time to affect new hsp levels compared to translational regulation. Heat shock experiments on fish hepatocyte primary cultures using actinomycin-D as a transcriptional inhibitor, and cycloheximide as a translational inhibitor may reveal whether transcription of hsp70 DNA or translation of hsp70 mRNA is more responsible for the induction of hsp70 synthesis in the species. It is yet known whether the different regulatory pathways of the cellular level of hsp are related to the stenothermal or eurythermal nature of an animal. Not only the function of hsps, but also the regulation of the cellular hsp level under heat stress is need to be examined to understand the diversity in the cellular heat shock response relating to the different habitat temperature of the animals.

The relationship between upper lethal temperature and the induction temperature for hsp70 could be altered depending on the recent thermal history of the animal as mentioned in the Discussion of Section 4-1. Yet many fish showed species-specific lethal and induction

temperatures for liver hsp70 that were within a very narrow temperature range (~2 °C) in the laboratory. For example, tidepool sculpins survived at 28 °C at least for several hours, but instantly died at 30 °C when they were acclimated at 10 °C (Table 1). It seems that the lethal temperature of the species is ruled by the threshold for temperature-dependent chemical and physical changes in cells such as denaturation of cellular proteins. Therefore, the magnitude of effects on fish brought about by a few degrees increase in water temperature can be dramatically different between when it happens well below the lethal temperature and when it happens at a temperature near the lethal temperature of the species. Significant effects of a relatively small increase in water temperature on fishes were shown in Section 1-1 as sharp reductions of hsp70 levels in some tissues of sub-tropical fishes by prolonged warmer ocean temperatures in the summer of 1998 associated with El Niño event.

As shown in Chapter 4, thermotolerance seems to be different not only among species, but also within a species dependent on the physiological state and the developmental stage of the animal. It is well known that the thermal tolerance of the yeast, *S. cerevisiae*, varied with growth phase (Plesset *et al.* 1987). Somero *et al.* (1996) proposed that the high thermal stability of LDH-A from the adult frog, *Rana cascadae*, may reflect temperature conditions encountered by the tadpole, since the adult frog probably does not experience temperatures of more than 20 °C at high elevations in the Cascade Mountains of the Pacific Northwest region of the United States of America, while the tadpole of this species is known to be subjected to water more than 20 °C in shallow ponds. DuBeau *et al.* (1998) demonstrated protection produced by a mild heat shock treatment against osmotic resistance in the Atlantic salmon (*Salmo salar*). However, they stated that the protection was observed only during parr-smolt transformation when the fish experience a dramatic physiological and morphological change from the freshwater adapted state to the sea water adapted state. I often observed small tidepool sculpins in higher, smaller tidepools with very high temperatures. I also observed that small tidepool sculpins showed a higher resistance

to severe heat shock at 28 °C than large tidepool sculpins in the laboratory (data not shown). These observations indicate that juvenile tidepool sculpins have a higher heat resistance compared with adult tidepool sculpins. However, I did not find any correlations between body weight and the level of liver hsp70 in the tidepool sculpin (data not shown). Intra-species changes in the range of thermotolerance dependent on its developmental stages may be influenced more by the physiological and the metabolic states of an animal regulated by hormonal controls rather than functions of hsps which may be related to the acute response to heat stress. As discussed in Chapter 4, various metabolic and neuro-endocrine changes can occur in fish when water temperature changes. Although hsps are believed to play a major role in thermotolerance of an animal, apparently hsps are not exclusive. The physiological processes surrounding the cellular heat shock response may be involved in the determination of the thermotolerance of an animal, and in the functions of hsps. Some of the inconsistencies in hsp functions shown in the previous paragraph may be explained by the effects of different physiological states of the animal. The relationships between the function of hsps and the physiological states of an animal are important to understand the whole body mechanism of thermotolerance of the animal.

Thermoprotection or cross protection could be a realistic strategy for stress resistance in animals in nature, especially in habitats with a number of potential stressors. As explained in the Discussion in Section 4-1, thermoprotection or induced thermotolerance is the term for the process in which a treatment with sublethal temperature increases survival at the lethal temperature of the species, whereas cross protection refers to induced tolerance against stressors by prior exposure to non-homologous stressors. There are numerous studies demonstrating thermoprotection in various species from invertebrates to mammals. However, most of these studies have been conducted in cultured cells from mammals (Kampinga *et al.* 1994; Michel *et al.* 1994); in fish, such as cultured cell line from the medaka (*Oryzias* spp., Arai *et al.* 1994); and the

primary cultures of renal proximal-tubule cells from the winter flounder (*Pleuronectes americanus,* Brown *et al.* 1992). At the organismal level, it was shown recently in the Pacific oyster (*Crassostrea gigas*) that exposure to sublethal temperature (37 °C) for 1 h dramatically enhanced the survival at 43 °C which had been determined as the lethal temperature of that species (Shamseldin *et al.* 1997); that induced thermotolerance was retained for at least 2 weeks (Clegg *et al.* 1998). Organismal thermoprotection was also confirmed in the sea urchin (*Paracentrotus lividus*) embryo (Giudice *et al.* 1999). It may be noteworthy that the efficiency of thermoprotection was dependent on the developmental stages of this species; the embryo heated at 31 °C after hatching became resistant to a second treatment at 35 °C, while the embryo heated at the same temperature before hatching did not show any induced thermotolerance and degenerated. In those studies, induced thermotolerance was positively correlated with the amount of hsp70 produced during the pretreatment at sublethal temperatures. Since animals experience more than one temperature in nature, thermoprotection may be a realistic mechanism for their survival at severe environmental temperatures. For example, Gehring and Wehner (1995) found a relatively high amount of hsp70 in the brain tissues of a desert ant (*Cataglyphis* spp.) incubated at 25 °C, and the level did not significantly change at higher temperatures, even though the body temperature of the ant can be more than 50 °C while they are actively foraging under the midday sun on the Saharan sand surface. They also found that the desert ant taken from their nest at 30 °C had a similar level of brain hsp70 as found at 25 °C in the laboratory, and suggested the possibility that the desert ant might precondition themselves in the nest prior to the exposure to extremely high temperatures outside of the nest.

There also are many reports of cross protection with various combinations of stressors. When the rat was exposed to 41 °C as a mild heat shock, protection in heart tissue from oxidative injury induced by $H_2O_2$ perfusion was significantly increased compared with rats without the heat pretreatment (Bornman *et al.* 1998). The higher protection by mild heat shock was

associated with higher expression of hsp70 during the subsequent stress in their study. The heat resistance of the proteins also corresponded with elevated levels of hsp70 by pretreatments with sodium arsenite, ethanol, and diamide (Kampinga *et al.* 1995). Wiegant *et al.* (1999) demonstrated that the rat hepatoma cell line, Reuber H35, expressed higher survival rates at its lethal temperature (43.5 °C) when it was treated with low-dose chemicals such as arsenite, cadmium, mercury, lead, copper, menadione and diethyleithiocarbamate. It may be noteworthy that those chemicals did not show any effects on the level of hsp70 and the thermotolerance of cells when they were applied on the control cells grown at 37 °C. However they enhanced induction of hsp70, as well as the thermotolerance of the cells, when they were applied on cells pretreated with a mild-heat shock (42 °C). A similar result was reported in the fruit fly, *D. melanogaster* (Minois *et al.* 1999). The fly, previously kept at 3 or 5×g had higher resistance to heat and higher hsp70 levels than the fly kept at 1×g (terrestrial gravity). Again, the fly kept at the hypergravity did not show any changes in the level of hsp70, but had a higher level of hsp70 only when they were subjected to a heat shock. They concluded that even though the hypergravity did not enhance the translation of hsp70, the expression process of this protein might have been initiated in the hypergravity. This may have happened with the tidepool sculpin in Section 4-1. All of the tidepool sculpin did not show significant differences in the levels of liver hsp70 before the heat shock. However, the tidepool sculpins not-acclimated to the constant temperature had higher hsp70 levels after the heat shock and higher heat resistance than the fish acclimated to the constant temperature (Fig. 4-1).

In the intertidal zone, fish are naturally exposed to changes in a number of physical and chemical factors. Thus, the fish inhabiting the intertidal zone can be a good model for the *in vivo* study of such protective processes in nature, that have been well established in *in vitro* laboratory experiments. There has been no direct evidence demonstrating thermoprotection and cross protection to be realistic processes in this intertidal fish to date. However, judging from the data

that tidepool sculpins acclimatized to the natural intertidal habitat had a higher thermal resistance and faster induction of hsp70 compared with the same species acclimated to a constant laboratory condition, being in the natural fluctuating environment seems to enhance the capacity of resistance to heat stress in this species. As well, as shown in Section 4-2, metabolic and physiological changes can occur in the tidepool sculpin with temperature changes. By being subjected to continuously fluctuating water temperature in a tidepool, the processes of those physiological and metabolic adjustments in fish may be kept activated, so that the fish may be able to adapt to a new temperature relatively easily.

In summary, I showed generally a strong correlation between the lethal temperature, induction temperature for hsp70 and habitat temperature in various fishes whose water and body temperatures can range from –1.7 °C in Antarctica to close to 37 °C in a tidepool of the sub-tropical ocean. These results indicate that, even though the importance of hsps in the heat resistance of animals has been widely established, their functions and the regulatory mechanisms for expression may be modified during the adaptation to a specific habitat temperature of the species. I also demonstrated that a relatively small difference in habitat temperatures could be reflected in the cellular heat shock response, thus, in the lethal temperature of the three intertidal sculpins. In addition, each of those species showed a lethal temperature and an induction temperature for hsp70 within a very narrow range. These results indicate that, in spite of a considerably wide range of thermal tolerance in these sculpins, a very small increase in water temperature could affect a significant impact; especially if it happens at a temperature near their lethal temperatures. In fact, an impact of warmer ocean temperatures due to a strong El Niño on cellular heat shock responses of sub-tropical fishes was demonstrated in Section 1-1. Animals have adapted to a wide variety of thermal environments in almost every part of this planet. Therefore, the mechanisms underlying thermal adaptation can also be extremely diverse among organisms. The expression of hsps is ubiquitous among organisms. However, the processes

surrounding the expression of hsps and the function of these important chaperones are obviously not simple or uniform. As shown in Chapter 4, the speed and the amount of hsp70, as well as the associated induction of thermotolerance can be different even within a species dependent on the physiological states. As well, the whole body metabolism and neuro-endocrine signals may have influences on the cellular energetic and physiological states, thus, the production and the function of hsps. Continuous studies with good models, representative of various thermal environments, are important to understand the diversity in thermal physiology of animals, which may be essential for the realistic estimation of impacts by phenomena such as global warming on the physiology and the behavior of animals.

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