

**THE EFFECTS OF GENETIC MANIPULATION ON THE STRESS RESPONSE AND  
DISEASE RESISTANCE OF COHO SALMON**

by

**ESTHER JHINGAN**

B.Sc., University of British Columbia, 1995

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF**

**MASTER OF SCIENCE**

in

**THE FACULTY OF GRADUATE STUDIES**

(Department of Animal Science)

We accept this thesis as conforming  
to the required standard

**THE UNIVERSITY OF BRITISH COLUMBIA**

November 1998

© Esther Jhingan, 1998

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Animal Science

The University of British Columbia  
Vancouver, Canada

Date November 28, 1998

## ABSTRACT

Genetic manipulation is widely used for the improvement of agricultural organisms. However, alterations of the genome could also result in unintentional changes, and could perhaps alter the performance of the organism. Performance traits such as sensitivity to stressors and to pathogens are strongly influenced by genetic information, and could therefore be affected by alterations of the genome. In this thesis, I tested the hypothesis that genetic manipulation alters the stress response and disease resistance of fish. Specifically, the studies examined the effects of the insertion of a growth hormone (GH) gene construct and triploidy on coho salmon (*Oncorhynchus kisutch*).

In this study, the GH transgene did not alter the physiological or cellular stress responses to a sudden heat shock, relative to those of non-transgenic fish. Exposure of the diploid fish to a bacterial pathogen (*Vibrio anguillarum*) appeared to reveal differences in disease resistance between different transgenic lines. The first filial generation of GH transgenic fish, which were also a more rapidly growing line, appeared to be more susceptible to disease relative to non-transgenic fish. The second filial generation of GH transgenic fish, which were a slower growing transgenic line, were equally or more resistant than the non-transgenic fish. Disease resistance was compromised by triploidy, and presence of the GH transgene in triploid fish did not appear to further modify the triploid's resistance to the pathogen.

## TABLE OF CONTENTS

	Page
ABSTRACT .....	ii
TABLE OF CONTENTS .....	iii
LIST OF FIGURES .....	v
ACKNOWLEDGEMENTS .....	vii
DEDICATION .....	viii
 1. <u>GENERAL INTRODUCTION</u> .....	 1
1.1 STRESS RESPONSE IN FISH .....	1
1.2 DISEASE RESISTANCE IN FISH .....	3
1.3 GENETICS OF STRESS RESPONSE AND DISEASE RESISTANCE .....	4
1.4 PERFORMANCE OF GENETICALLY MANIPULATED ORGANISMS .....	5
1.5 FRAMEWORK OF THESIS .....	6
 2. <u>GENERAL MATERIALS AND METHODS</u> .....	 11
2.1 FISH .....	11
2.2 SAMPLING PROTOCOL .....	13
2.3 IDENTIFICATION OF TRANSGENIC FISH .....	13
2.4 MEASUREMENTS OF THE STRESS RESPONSE .....	14
2.5 MEASUREMENT OF THE IMMUNE SYSTEM .....	16
 3. <u>EFFECTS OF GH TRANSGENE ON THE STRESS RESPONSE OF COHO SALMON</u> .....	 18
3.1 INTRODUCTION .....	18
3.2 MATERIALS AND METHODS .....	19
3.3 RESULTS .....	22
3.4 DISCUSSION .....	35

4. <u>THE DISEASE RESISTANCE OF TRIPLOID AND DIPLOID COHO SALMON</u> <u>TRANSGENIC FOR GH</u> .....	38
4.1 INTRODUCTION .....	38
4.2 MATERIALS AND METHODS .....	39
4.3 RESULTS .....	41
4.4 DISCUSSION .....	46
 5. GENERAL DISCUSSION .....	 48
6. BIBLIOGRAPHY .....	51

## LIST OF FIGURES

	Page
<b>Figure 1.1</b> Simplified illustration of stress responses in a fish and possible outcomes. ....	8
<b>Figure 1.2</b> Simplified diagram of physiological stress response in a fish exposed to a stressor. ....	9
<b>Figure 1.3</b> Simple diagram of components of the immune system in fish. ....	10
<b>Figure 2.1</b> Picture of an agarose gel showing samples containing (+) and lacking (-) the GH construct. ....	17
<b>Figure 2.2</b> Western blot, showing control and heat shocked samples.....	17
<b>Figure 3.1</b> Plasma cortisol and glucose in control and handled F2 MTGH1 transgenic coho salmon and their non-transgenic siblings following a 5 minute handling stressor.....	23
<b>Figure 3.2</b> Plasma cortisol and glucose in F2 MTGH1 transgenic coho salmon and their non-transgenic siblings after pooling treatment groups (handled and control) due to lack of cortisol or glucose response to handling. ....	24
<b>Figure 3.3</b> Gill lysozyme activity in F2 MTGH1 transgenic coho salmon and in their non-transgenic siblings. ....	25
<b>Figure 3.4</b> Effects of a 2-hour 9 °C heat shock on plasma cortisol and glucose levels in F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls).....	28
<b>Figure 3.5</b> Effects of a 2-hour 9 °C heat shock on hsp70 expression in the gills and liver of F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls). ....	29
<b>Figure 3.6</b> Effects of a 2-hour 9 °C heat shock on hsp70 expression in the kidney and muscle of F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls). ....	30
<b>Figure 3.7</b> Effects of a 2-hour 9 °C heat shock on hsp70 expression in the red blood cells of F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls). ....	31

- Figure 3.8** Increase in hsp70 expression (relative to control values) following a 2 hour 9°C heat shock in the gill and liver of F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls).....32
- Figure 3.9** Increase in hsp70 expression (relative to control values) following a 2 hour 9°C heat shock in the kidney and muscle of F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls).....33
- Figure 3.10** Increase in hsp70 expression (relative to control values) following a 2 hour 9°C heat shock in the red blood cells of F1 H3GH1 transgenic coho salmon, their smaller non-transgenic siblings (age controls) and fish of similar size (size controls).....34
- Figure 4.1** Percent cumulative mortality in F1 MTGH1 transgenic coho salmon and in non-transgenic fish of similar size, after an immersion challenge with *Vibrio anguillarum*. ....43
- Figure 4.2** Plasma lysozyme activity in F1 MTGH1 transgenic coho salmon and in non-transgenic fish of similar size 7 days after an immersion challenge with *Vibrio anguillarum*. ....44
- Figure 4.3** Percent cumulative mortality in diploid and triploid F2 MTGH1 transgenic coho salmon and in their non-transgenic siblings, after a low dose and high dose immersion challenge with *Vibrio anguillarum*. ....45

## ACKNOWLEDGEMENTS

This work was made possible by grants to G.K. Iwama from the Canadian Bacterial Diseases Network and Natural Sciences and Engineering Research Council of Canada. I would like to thank G.K. Iwama and R.H. Devlin for their guidance, their love of science is inspirational, and their enthusiasm contagious. I greatly appreciate the work of R.H. Devlin and his lab in providing the transgenic fish. I would also like to thank the members of my supervisory and examining committee, T. P.T. Evelyn, E.M. Donaldson and D.D. Kitts.

Thanks to Rob Forsyth, Matt Vijayan, Robyn Roome, Ellen Teng, Paige Ackerman, and John Morgan for their valuable advice and helpful discussions. Thanks to Ellen Teng and Robyn Roome for their support and encouragement.



## DEDICATION

"Kind words can be short and easy to speak but their echoes are truly endless."

-Mother Theresa

I would like to dedicate this thesis to my parents, Oscar and Norma Martinez, as well as to my husband, Jim Jhingan, for their unconditional support and faith. Their love and encouragement, along with that of my sister, Abril, and brother, Constantino, is greatly treasured. Thanks for the kind words you've given to me over the years. Thanks to Leo for always making sure that we appreciate the little things in life, and treasure every moment.

## 1. GENERAL INTRODUCTION

Genetic manipulation plays a key role in the modification of agricultural organisms, including fish. Selection and domestication have been used for centuries to improve agricultural strains, but more recently, advances in biotechnology have increased the variety of methods available to achieve genetic manipulation. Genetic manipulation has the potential to cause unintentional phenotypic changes in the genetically modified organism (GMO) in addition to the targeted phenotypic changes. Changes in tolerance to pathogens and stress, as well as changes in appetite, reproduction and behaviour could result in significant alterations of the GMO's performance, and alter the ecological impact of any escaped GMOs. Similarly, the performance of the GMOs in culture could also be affected. Studies on the effects of genetic manipulation such as transgenesis and triploidy on the performance of fish are limited, therefore, in this study, I conducted experiments to determine if genetic manipulation has had an impact on the ability of fish to respond to stress and to cope with pathogens.

### 1.1 **STRESS RESPONSE IN FISH**

Fish may experience stress, be it from natural challenges such as escaping from predators, or from aquacultural practices such as handling. We have all experienced stress, nevertheless, it is still difficult to define it. A definition that is used by some researchers is that given by Brett (1958), in which he describes stress as "a state produced by an environmental factor which extends the normal adaptive responses of an animal, or which disturbs the normal functioning to such an extent that the chances of survival are significantly reduced". As the fish attempts to compensate for the effects imposed by a stressor, several changes such as physiological and cellular responses

take place which will enable the fish to deal with or avoid the stressor (Figures 1.1 and 1.2).

The physiological or generalized stress response in fish can be considered in terms of the level of biological organization that it involves, and is accordingly divided into primary, secondary and tertiary responses (reviewed by Wedemeyer *et al.*, 1990). The primary response involves changes in the neuroendocrine and endocrine systems. Once the stressor is perceived, the neuroendocrine and endocrine systems respond by increasing the release of catecholamines and corticosteroids into the circulation. Changes in tissue and blood follow (secondary response), such as the increase in blood glucose levels that provide the fish with more energy. If the stressor is too severe or prolonged, or if the fish is continually challenged, the compensatory changes may become maladaptive and impair the health of the fish. For example, the increase of plasma corticosteroids can become maladaptive and negatively impact essential functions such as growth, reproduction, and immunocompetence (see review by Barton and Iwama, 1991), affecting the individual and possibly the population (tertiary response).

In addition to physiological responses, most stressors can also elicit a cellular stress response in which heat shock proteins (hsps) are induced. This response appears to be universal, as heat shock proteins are one of the most highly conserved group of proteins and their induction occurs in all organisms tested so far (review by Iwama *et al.*, 1998). Hsps occur constitutively in the cell and perform essential functions in protein synthesis, assembly and translocation, as well as in the regulation of protein function. Hsps stabilize unfolded proteins and prevent incorrect protein interactions. As certain stressors can cause proteins to unfold and misfold, hsp synthesis is dramatically increased in response to stressors to perform functions such as the prevention of

protein aggregation and aiding in the refolding of unfolded proteins, helping to protect the cell from damage.

## 1.2 DISEASE RESISTANCE IN FISH

Disease occurs in all organisms and ecosystems, with or without the influence of man, and can act as a major selective force in evolution. Disease can have adverse consequences as it can impact the performance of fish, as well as lead to mortalities.

Several factors are involved in determining the outcome of an encounter with pathogens. The ability of the pathogen to cause disease is intrinsically affected by its genetic composition, as well as being affected by the dose, duration and route of entry of the pathogen (LaPatra *et al.*, 1989). Similarly, the ability of the host to resist disease is to a large extent determined genetically, but is also affected by age, size, and by biological processes such as smoltification, reproduction, stress and state of the immune system (review by Hedrick, 1998). Previous exposure to the pathogen can also increase the resistance of the host to the pathogen due to the immunological memory of the adaptive immune system. Changes in the environment (oxygen, pH, temperature) can also affect the occurrence of disease, as these changes can have favourable or detrimental effects on pathogens and hosts.

Changes in environment, pathogen or host can shift the balance of their relationships and favour disease. Nevertheless, the immune system of the host plays an important role in determining the outcome. The immune system of fish consists of two arms of protection, one is non-specific in nature, whereas the other recognizes specific pathogens (Figure 1.3).

### 1.3 GENETICS OF STRESS RESPONSE AND DISEASE RESISTANCE

The way that an organism responds to challenges such as stressors and pathogens is largely determined by its genome. A genetic basis for the response to stress has been suggested by studies revealing differences in the primary and secondary responses among different species of fish (Fevolden *et al.*, 1991), as well as between different stocks within coho salmon (McGeer *et al.*, 1991) and chickens (Edens and Siegel, 1975). Genetic variation in disease resistance is also well documented. Researchers have found differences in disease resistance between different salmonid species, between different stocks of coho salmon (review by Chevassus and Dorson, 1990), as well as between and within breeds of a variety of farm animals (review by Müller and Brem, 1991).

The fact that performance traits such as the above are strongly influenced by genes has allowed humans to improve economically important traits in farm animals. For years, genetic manipulation has been achieved through breeding programs. Now, advances in molecular biology are opening new doors in the field of genetic manipulation.

Major improvements in the performance of agricultural organisms could be achieved by transgenesis; that is, the insertion of recombinant DNA into the genome of an organism. The use of transgenic technologies would allow us to accelerate the pace of the genetic improvement of a specific trait, which was previously limited by the relatively slow pace of selective breeding. To date, however, the only gene transfer that has successfully improved the phenotype of fish is the transfer of the GH gene (Du *et al.*, 1992; Devlin *et al.*, 1994).

In order to minimize interactions between escaped transgenic and wild fish, transgenic fish to be used for production would have to be sterile (Donaldson and

Devlin, 1996). One of the most practical methods of producing sterile fish is the induction of triploidy- that is, the production of fish with three sets of chromosomes, which generally renders the fish sterile (Benfey, 1991). As a result, the production of transgenic fish may also require another genetic manipulation- one involving chromosome numbers.

#### 1.4 PERFORMANCE OF GENETICALLY MANIPULATED ORGANISMS

An important aspect of genetically manipulated organisms that must be considered is the overall cost of the expression of the desired phenotype on the organism, that is, what are the overall effects (in addition to changes on the desired phenotype) of the alteration of the genome.

The first insertion of recombinant DNA into an animal dates back to 1980 (Gordon *et al.*, 1980). Since then, transgenesis has been performed on many other organisms. An extensive amount of research in the field of transgenesis has involved plants, where various genes for disease resistance, insect resistance and stress tolerance have been successfully transferred (reviewed by Snow and Morán Palma, 1997). The concern that transgenic crops may invade natural habitats has brought about studies of the performance of transgenic plants. These studies have found that transgenes conferring resistance to herbicides do not affect the plants' overall performance (measured by fecundity and survival; Crawley *et al.*, 1993; Bergelson, 1994).

Most of the research on transgenic animals has involved the insertion of the GH gene. Although insertion of the GH gene successfully increases growth rates in mice, it does not appear to alter growth in livestock animals such as pigs and sheep (review by Pursel and Rexroad, 1993). Other effects of the transgene included health problems

such as joint pathology in pigs and sheep, and reproductive and endocrine abnormalities, as well as an overall decrease in the life-span in mice (reviews by Pursel and Rexroad 1993; Bartke *et al.*, 1994). GH transgenes have successfully increased growth rates in fish (Du *et al.*, 1992; Devlin *et al.*, 1993), but they have also resulted in the expression of adverse phenotypes. Growth abnormality similar to that seen in mammals with acromegaly has been observed in coho (Devlin *et al.*, 1995) and Atlantic salmon (Hew *et al.*, 1995) transgenic for the GH construct. Also, an inferior swimming capacity was noted in GH transgenic coho salmon relative to fish of similar size (Farrell *et al.*, 1997).

The health of the fish also appears to be affected by manipulation of chromosome numbers. In general, triploid salmonids tend to survive more poorly and show reduced or equal growth relative to their diploid counterparts (Utter *et al.*, 1983; Galbreath *et al.*, 1994; Withler *et al.*, 1995), except in salmonid hybrids, where the reverse may be true (review by Chevassus and Dorson, 1990). It also appears that triploidy may render the fish more susceptible to the stress of sea water transfer under certain conditions, compared to diploid fish (Johnson *et al.*, 1986).

## 1.5 FRAMEWORK OF THESIS

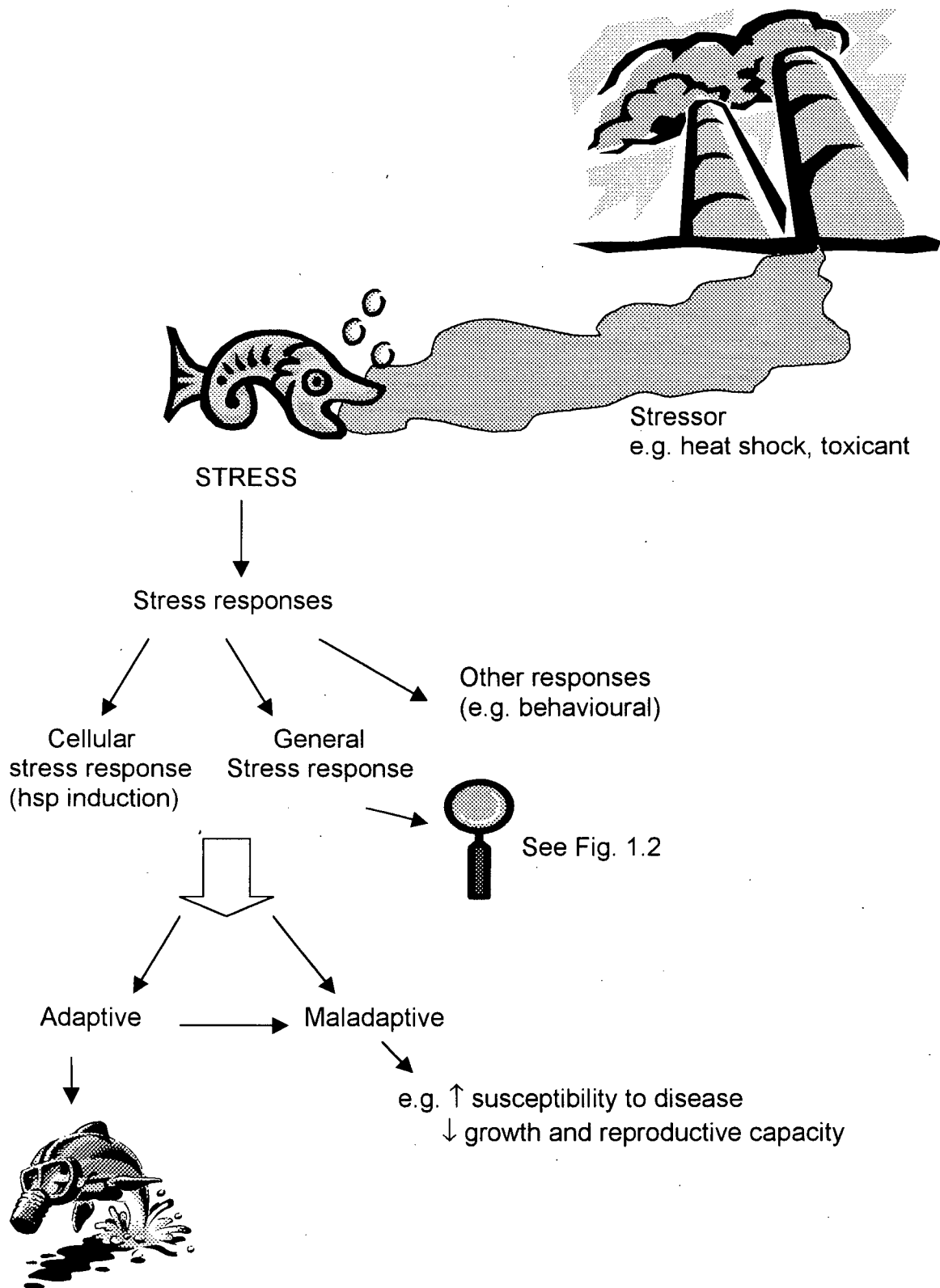
In this thesis, the impact of genetic manipulation on the sensitivity of fish to stress and pathogens was studied.

The physiological and cellular stress responses of fish containing the GH transgene were compared to those of their non-transgenic siblings of similar size. When transgenic fish had outgrown their non-transgenic siblings, older fish of similar size were also included as an additional control group to control for size. Cortisol and glucose are commonly assessed in fish as indicators of the primary and secondary stress response,

respectively (Wedemeyer *et al.*, 1990), and were therefore measured following exposure to two different stressors (a temperature and a handling stressor). Heat shock proteins in gill, liver, kidney and skeletal muscle tissue, as well as in red blood cells, were measured after the heat stress in order to assess the cellular stress response.

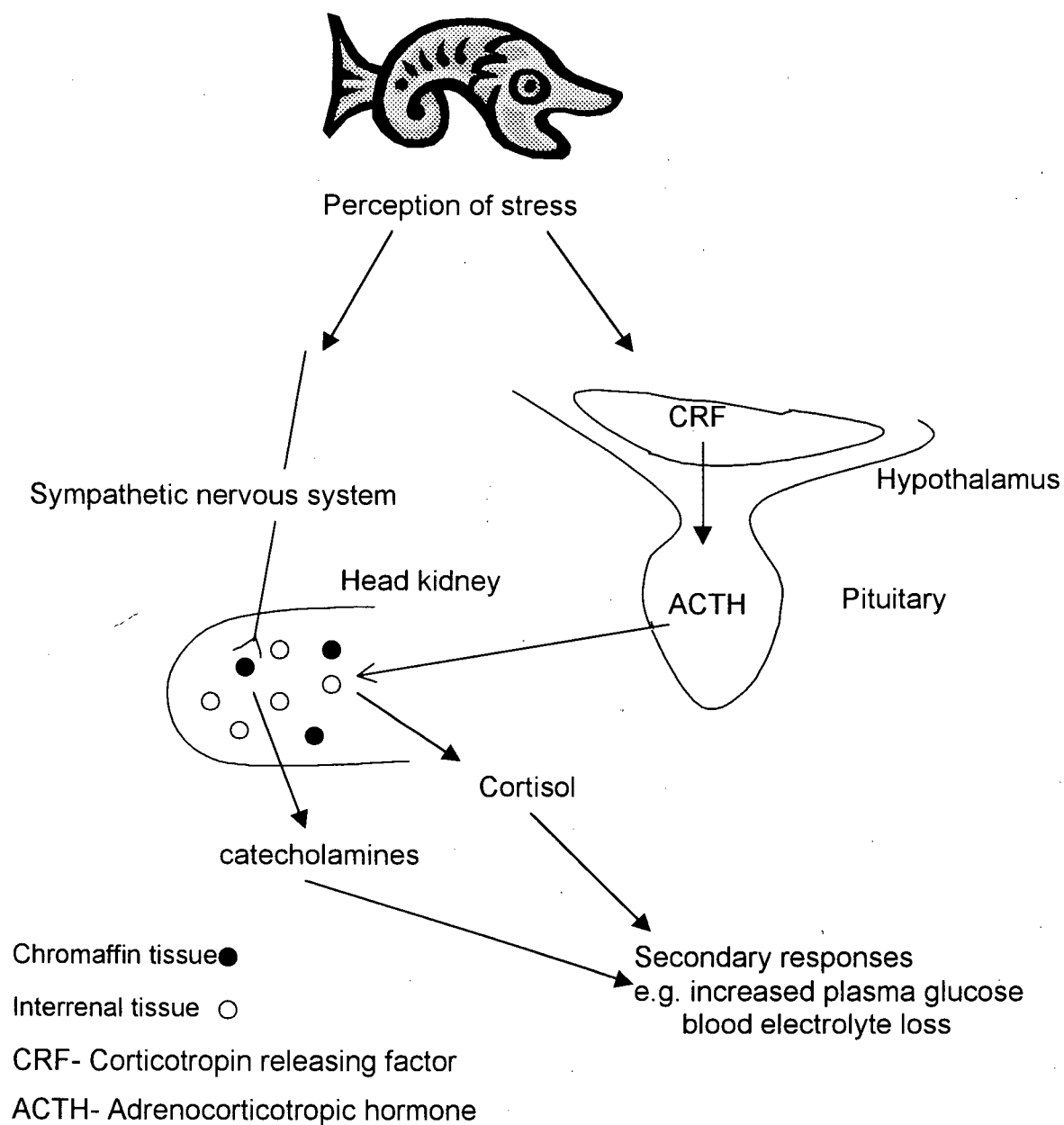
Fish were also exposed to a bacterial pathogen (*Vibrio anguillarum*) to determine if genetic manipulation, in this case the presence of GH transgene and/or triploidy, had any impact on their disease resistance. Disease resistance was measured by determining survival after a challenge with a bacterial pathogen.





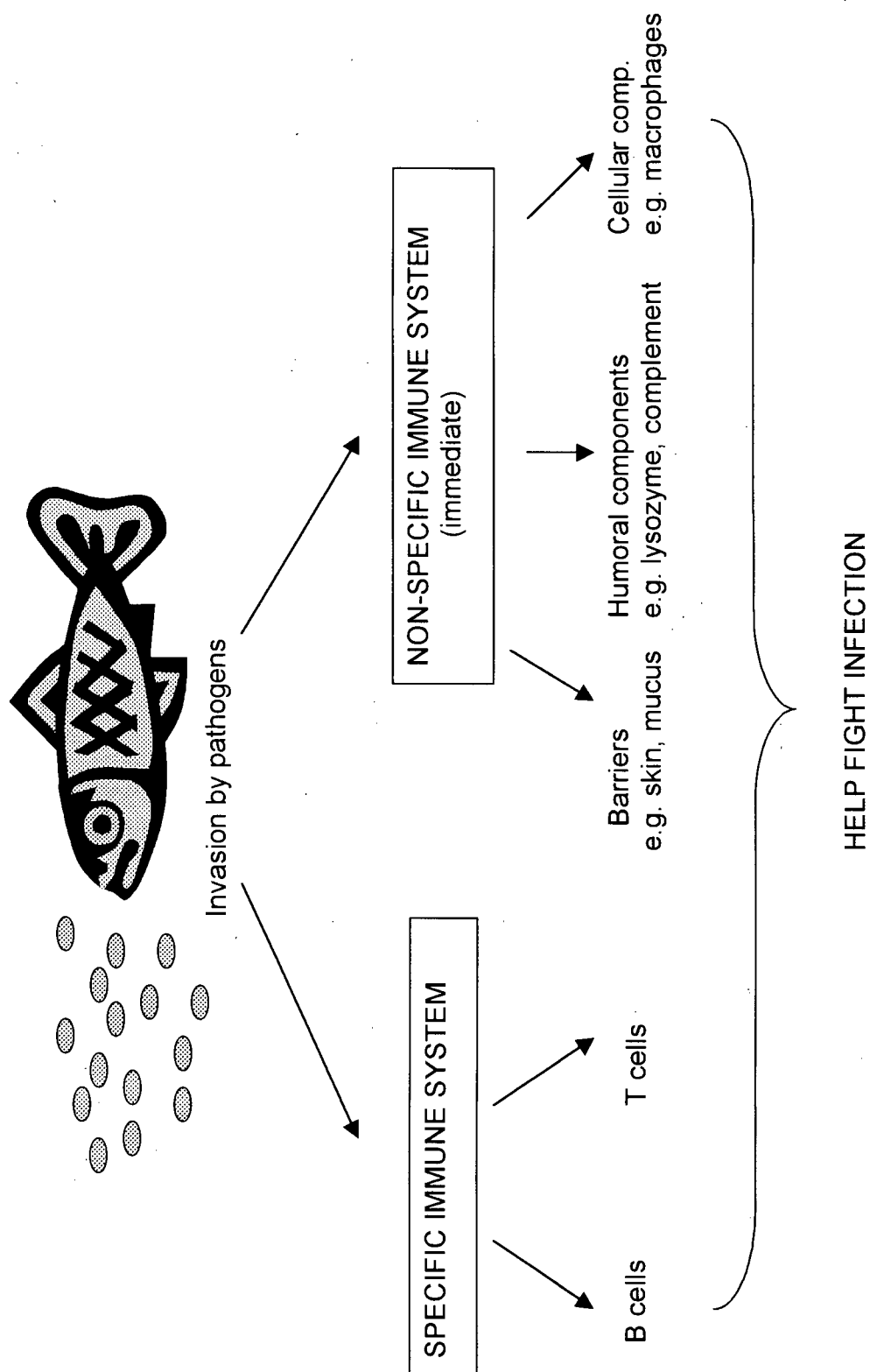
**Figure 1.1**

Simplified illustration of stress responses in fish and possible outcomes.



**Figure 1.2**

Simplified diagram of physiological stress response of a fish exposed to a stressor.



**Figure 1.3**  
Simple diagram of components of immune system in fish.

## 2. GENERAL MATERIALS AND METHODS

### 2.1 FISH

The West Vancouver Laboratory and Chehalis River Hatchery (Department of Fisheries and Oceans, Canada) provided transgenic and control coho salmon, respectively. The male founder fish (parental generation) in this thesis were transgenic coho salmon that had been produced by microinjecting a GH construct into fertilized eggs immediately after fertilization (Devlin *et al.*, 1994). The sockeye salmon GH constructs consisted of the GH-1 gene fused to either the metallothionein-B promoter (pOnMTGH1 transgenic fish), or to the histone-3 promoter (pOnH3GH1 transgenic fish).

Fish were kept in 90 L tanks supplied with dechlorinated Vancouver City water, and were fed commercial feed once daily to satiation under natural photoperiod.

**F1 pOnMTGH1 transgenic salmon.** First filial generation (F1) MTGH1 transgenic coho salmon were produced at the West Vancouver Laboratory facilities. A male founder MTGH1 transgenic coho salmon was used to fertilize the eggs from a wild female from the Chehalis River hatchery. Control fish were older non-transgenic coho salmon of size similar to transgenic fish. All fish were held in fresh water to avoid possible prior contact with *Vibrio anguillarum*. On July 26 1996, when F1 MTGH1 transgenic fish and control fish were approximately the same size ( $37.9 \pm 8.12$  g and  $28 \pm 7.26$  g, respectively), they were subjected to a disease challenge (for more information, see Chapter 4).

**F1 pOnH3GH1 transgenic salmon.** F1 H3GH1 transgenic coho salmon were produced at the West Vancouver Laboratory facilities. A male founder H3GH1 transgenic coho salmon was used to fertilize the eggs from a wild female from the Chehalis River hatchery. Control fish were their smaller, non-transgenic siblings (age controls), and older coho salmon of similar size (size controls). On October 17, 18 and

19, 1996, size controls, transgenic fish and age controls were subjected to a heat shock challenge (see Chapter 3), and their cortisol and glucose response to stress, as well as cellular stress response (heat shock protein expression) were measured.

**F2 pOnMTGH1 triploid and diploid transgenic salmon.** Second filial generation (F2) MTGH1 transgenic coho salmon were produced at the West Vancouver Laboratory facilities. An F1 male MTGH1 transgenic coho salmon was used to fertilize the eggs from wild females from the Chehalis River hatchery. Half of the fertilized eggs were pressure shocked 30 minutes after fertilization in order to obtain triploids (as described by Benfey *et al.*, 1988). The remaining half of the eggs were left to develop as diploid fish. Control fish were the triploid and diploid non-transgenic siblings of the transgenic fish. Triploid and diploid fish were maintained in separate tanks, but transgenic and non-transgenic fish were raised communally. Transgenic fish were identified only after the termination of the experiments.

On June 16, 1997, diploid and triploid F2 MTGH1 transgenic and non-transgenic fish ( $3.11 \pm 2.31$  g and  $2.5 \pm 1.43$  g, respectively for diploid and triploid fish) were subjected to a disease challenge. Fish were assumed to be fully immunologically mature at this time, as they reach this status once they attain 0.5 g in size (Tatner and Horne, 1983) (Although immune functions are strongest in fish of 3.0g and larger (Amend and Johnson, 1981). On August 7, 1997, diploid transgenic ( $10.28 \pm 5.16$  g) and non-transgenic fish ( $4.26 \pm 2.09$  g) which were not used in the disease challenge were subjected to a handling stressor (chasing with a dip net for 5 min), and their stress response was measured (see Section 2.4 for details).

## 2.2 SAMPLING PROTOCOL

The sampling protocol for all studies consisted of removing 3 fish from a tank and anaesthetizing them in a solution of 100 mg/L of aminobenzoic acid ethyl ester (MS 222, Sigma Chemical Co., St Louis, MO) buffered with an equal weight of sodium bicarbonate. The fish were bled immediately by caudal puncture, using a syringe coated with sodium heparin (heparinized). In small fish (less than 100 g), blood was collected by severing the caudal peduncle and collecting the blood in heparinized hematocrit tubes. Blood was centrifuged and the plasma obtained was stored at -50°C for future assays. Liver, kidney, gill, muscle and red blood cell samples were stored at -50°C. Fish from the same tank were sampled as quickly as possible to prevent the sampling procedure itself from elevating cortisol levels. Approximately 6 fish were sampled at each sampling point.

## 2.3 IDENTIFICATION OF TRANSGENIC FISH

In the experiments using F2 MTGH1 fish, presence of the GH transgene was detected by Polymerase Chain Reaction (PCR). In all other experiments, transgenic fish were distinguished from their non-transgenic siblings by their larger size.

**DNA Extraction.** Caudal fin samples (preserved in ethanol) were digested in Proteinase K buffer (200 ng/mL Proteinase K, 10 mM Tris, 10 mM EDTA, and 1% SDS) at 37°C overnight. DNA was extracted by a salt extraction (5 M NaCl) followed by a PCI (phenol:chloroform:isoamyl, 50:50:1) extraction. The DNA was precipitated in isopropanol, and washed in 70% ethanol before being dissolved in a Tris-EDTA buffer (10 mM Tris, 1mM EDTA). DNA was stored at 4°C until day of PCR.

**Polymerase Chain Reaction (PCR).** The GH construct, when present, was amplified by PCR. Briefly, PCR's were performed in 48  $\mu$ L of Cetus buffer containing 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM  $MgCl_2$ , 25 pmoles of MT and GH1 primers, and 0.2 mM dNTP's. Samples were analyzed by gel electrophoresis in 1% agarose gels.

## 2.4 MEASUREMENTS OF THE STRESS RESPONSE

Components of the primary and secondary stress responses were measured (plasma cortisol and lucose, respectively). A component of the cellular stress response was also measured (hsp70).

**Cortisol.** Plasma cortisol concentration was determined by using a commercially available  $^{125}I$  radioimmunoassay kit (Incstar Corporation, MN) based on competitive binding principles (Foster and Dunn, 1974).

**Glucose.** Plasma glucose concentration was determined enzymatically by the glucose oxidase method (Sigma Diagnostics, MO). The enzymatic reactions involved in this assay result in the production of a dye with an absorbance maximum at 505 nm. The intensity of the colour was measured in a spectrophotometer and the intensity obtained was used to determine glucose concentration of the sample, as the intensity of the colour produced is directly proportional to the concentration of glucose in the sample (over the range of glucose concentrations examined).

**Western blotting (hsp70).** Tissues were sonicated for 5-25 sec (depending on tissue) in a hypotonic solution containing Tris-HCl (100mM), 0.1% sodium dodecyl sulphate (SDS) and protease inhibitors (5  $\mu$ M Leupeptin, 1.5  $\mu$ M Aprotinin, 5  $\mu$ M Pepstatin and 5 mM PMSF). The homogenate was then added to an equal volume of 2x Laemmli's sample buffer and boiled for 5 min (in the case of RBC and muscle samples, the homogenate was centrifuged at 13000 rpm for 1.5 min and the supernatant was used

instead). Duplicate aliquots of homogenate (or supernatant) were used to determine protein concentrations using a BCA protein assay kit (Sigma Diagnostics).

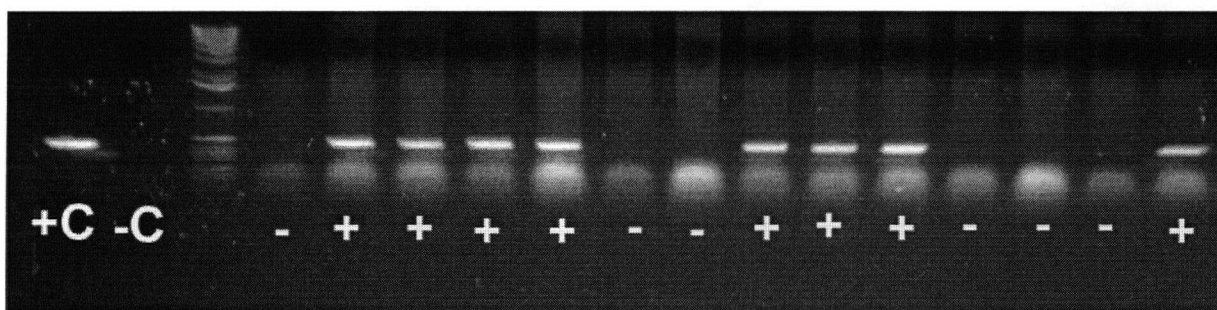
Proteins were separated by SDS- PAGE (SDS- polyacrylimide gel electrophoresis) according to the Laemmli buffer system (Laemmli, 1970), in 0.75mm thick gels. Equal amounts of protein were loaded (15-20  $\mu$ g per lane, depending on tissue), and a control sample was loaded onto each gel to allow for better comparisons between gels.

Strips of the gels containing proteins in the 70-kda range were cut out and transferred (Semi-dry transfer apparatus, Bio-Rad™) to a single nitrocellulose membrane (0.2  $\mu$ m pore size, Bio-Rad). The membrane was then blocked (2% skim milk, 0.5%  $\text{NaN}_3$ ) and dried between filter papers until the following day, at which time the blot was blocked further. The membrane was incubated for 1 h with the primary antibody, a polyclonal rabbit anti-trout hsp 70 antibody (provided by E.P.M. Candido, raised as described by Forsyth *et al.*, 1997) which recognizes both the constitutive and inducible forms of hsp70. The membrane was then incubated for 1 h with the secondary antibody (goat anti-rabbit antibody conjugated to alkaline phosphatase (GAR-AP, Gibco-BRL), and incubated for 1 –15 min with substrate (Nitroblue tetrazolium, 330 mg/L and 5-bromo-4-chloro-3-indolyl phosphate, 165 mg/L in alkaline buffer) as described by Blake *et al.* (1984). The intensity of the hsp70 bands was determined using a ScanJet II p (Hewlett Packard) and SigmaGel software (Jandel Scientific, CA, USA). Hsp70 expression was expressed in units relative to a positive control sample, which was run on every gel.



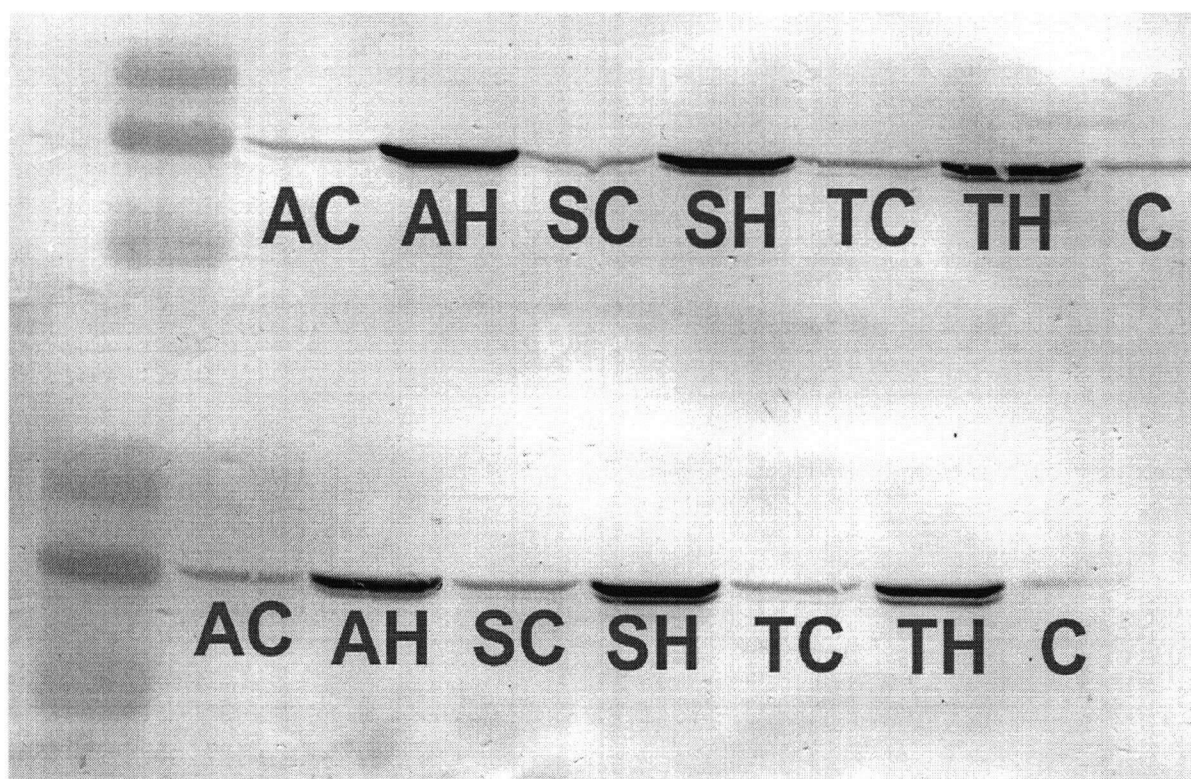
## 2.5 MEASUREMENT OF IMMUNE FUNCTION

**Lysozyme.** Gill and plasma lysozyme activity was measured by using the lysoplate method introduced by Osserman and Lawlor (1966). Gill tissue was homogenized (1:4 w/v) in phosphate buffer (0.06M, pH 6.0), whereas plasma was used undiluted. The homogenate was centrifuged and the supernatant stored at -20°C. Agarose (0.5%) plates were prepared, containing the lysozyme sensitive *Micrococcus lysodeikticus* (60µg/mL, Sigma Chemical Co.) and 0.02M NaCl in phosphate buffer (0.06M, pH6.0). Samples and standards were deposited (in triplicate, in separate plates) in 3mm wells in the lysoplates. Standards used were a range of concentrations (150 to 15,000 µg/mL) of hen egg white lysozyme (HEWL, Sigma Chemical Co.). The activity of the HEWL (under same conditions as lysoplate assay) was determined by the turbidometric assay (Parry *et al.*, 1965; modified by Grinde, 1989). The lysoplates were incubated in a moist chamber at room temperature for 17 h, and the diameter of the clearance zones around the wells was measured with callipers. Lysozyme activity was determined by linear regression, using the lysozyme activity determined for the HEWL standards.



**Figure 2.1**

Picture of an agarose gel showing samples containing (+) and lacking (-) the GH gene construct, along with positive (+C) and negative (-C) controls.



**Figure 2.2**

Western blot, showing control (Age control, AC; size control, SC; and transgenic control, TC) and heat shocked (AH, SH, and TH) liver samples from fish sampled 48 hours after heat shock. To allow for better comparisons between gels, hsp70 was expressed relative to a control sample (C) which was run on every gel. Only 2 out of the 6 gels transferred are shown.

### 3. EFFECTS OF THE MTGH1 TRANSGENE ON THE STRESS RESPONSE OF COHO SALMON

#### 3.1 INTRODUCTION

Growth hormone therapy has been experimentally used as a method of increasing fish growth rates and reducing the length of the production cycles (review by McLean and Donaldson, 1993). Although it effectively increases growth rates and even increases feed conversion efficiency (Gill *et al.*, 1985), its administration, via implant or repeated injections is labour intensive as well as stressful to the fish. An alternative method to increase growth rates in fish is to produce fish that are genetically engineered to have higher growth rates, a trait that could then be passed on to their progeny, removing the need to treat all fish to be cultured. Transgenic technologies have been used to develop fast growing fish using a GH gene construct (Du *et al.*, 1992; Devlin *et al.*, 1994). These fast growing transgenic fish would be extremely valuable for commercial culture, as they can attain market size in a shorter period of time. Unfortunately, the overall effects of chronically elevated GH levels on the physiology of the fish are unknown. It is therefore important to determine what effects a GH construct may have on the transgenic fish's performance.

An aspect of vital importance to the culture of fish is their sensitivity to stress, as stress can negatively impact the performance of the fish (see General Introduction). Links between the pituitary somatotropes (which produce GH) and the HPI (hypothalamic-pituitary-interrenal) axis of fish have been suggested by findings that stress can alter GH levels (Pickering *et al.*, 1991). Similarly, increased GH levels have been reported to increase the stress response in mice transgenic for GH (Cecim *et al.*, 1991) and has also been shown to increase the *in vitro* sensitivity of the interrenal to

ACTH in coho salmon (Young, 1988). The purpose of this study, therefore, was to determine what effects the expression of the GH transgene can have on the stress response of fish. Fish were subjected to a sudden heat shock or to a handling stressor, and indicators of stress, such as cortisol and glucose were measured. In the heat shock experiment, heat shock proteins were also measured.

### 3.2 MATERIALS AND METHODS

#### a) Handling Stressor

**Fish.** Transgenic fish in this study were F2 MTGH1 transgenic coho salmon ( $10.28 \pm 5.16$  g) (see General Materials and Methods for more details). Control fish were their non-transgenic siblings that were approximately the same size ( $4.26 \pm 2.09$  g) at the time of the experiment. Groups of 26 fish (transgenic fish and their non-transgenic siblings) were transferred to eleven 90-L tanks and were acclimated for at least one week before the handling disturbance. Transgenic and non-transgenic fish were kept in the same tank, and transgenic fish were identified only after the termination of experiment when caudal fin samples were analyzed for presence of the GH transgene by PCR (see General Materials and Methods). Tanks were separated based on the treatment they would receive (handling or no handling) and the time they would be sampled (0h, 1h, 3h, 6h or 24 h after handling). Fish were not fed on sampling days.

**Handling Stressor.** The handling disturbance consisted of chasing the fish with a dip net for 5 min (Vijayan *et al.*, 1994). Experimental groups were handled at 9 a.m., whereas control groups were not handled. Sampling times were 0h, 3h, 6h, and 24 h after handling.

**Sampling.** Details of the sampling procedure are described in the General Materials and Methods. Briefly, fish were anaesthetized, and blood samples and gill samples were taken. Plasma and gill samples were stored at  $-50^{\circ}\text{C}$  until assayed.

**Physiological parameters.** Plasma cortisol and glucose concentrations were measured as described in the General Materials and Methods.

**Immunological parameters.** Gill lysozyme activity was measured by the lysoplate method, as described in the General Materials and Methods.

**Statistical Analysis.** A two-way Analysis of Variance (ANOVA), using group (transgenic or non transgenic) and treatment (handled or non handled) as factors, was used to detect significant differences ( $p < 0.05$ ) in plasma cortisol and glucose levels, as well as in lysozyme activity. However, since there was no treatment effect on the cortisol or glucose responses, treatment groups within a group were pooled, and one-way ANOVA tests were performed to detect group effect. When differences were found, Student-Newman-Keuls tests were used to identify the different group. When the data were not normal or did not have equal variance, values were log-transformed prior to being analyzed.

#### b) Heat shock

**Fish.** Transgenic fish in this study were F1 H3GH1 transgenic coho salmon ( $75.1 \pm 15.61$  g) (see General Materials and Methods for more details). Control fish were their smaller, non-transgenic siblings ( $10.3 \pm 3.01$  g), and older fish of similar size ( $49.6 \pm 10.59$  g). Groups of 20 fish were transferred to six 90-L tanks and were acclimated for at least three weeks before the heat shock experiment. Water temperature was  $13 - 14^{\circ}\text{C}$  at the time of the experiment. Fish from different groups (transgenic fish, size controls and age

controls) were divided into different tanks, and groups were further divided into control and heat-shocked groups. Fish were not fed the day prior to the heat shock challenge or sampling.

**Heat Shock Challenge.** The heat shock challenge consisted of transferring the fish to tanks at 23°C for a two-hour period. Control (sham challenged) fish were transferred to tanks at 13.5°C. Following the two hours at the specified temperature, the fish were transferred back to their original tanks (13.5°C) and sampled 1h, 48h and 3 weeks after the heat shock.

**Sampling.** Each group was heat shocked on a different day, so that each group could be sampled at approximately the same time of the day. Details of the sampling procedure are described in the General Materials and Methods. Briefly, fish were anaesthetized, and blood samples, as well as gill, liver, kidney and muscle samples were taken. Blood samples were centrifuged, and plasma and red blood cells were stored separately. All samples were stored at -50°C until assayed.

**Stress indicators.** Plasma cortisol and glucose concentrations were measured as described in the General Materials and Methods.

**Heat Shock Proteins.** Hsp70 expression was measured in the gill, liver, kidney, muscle and red blood cells by western blotting (see General Materials and Methods for details).

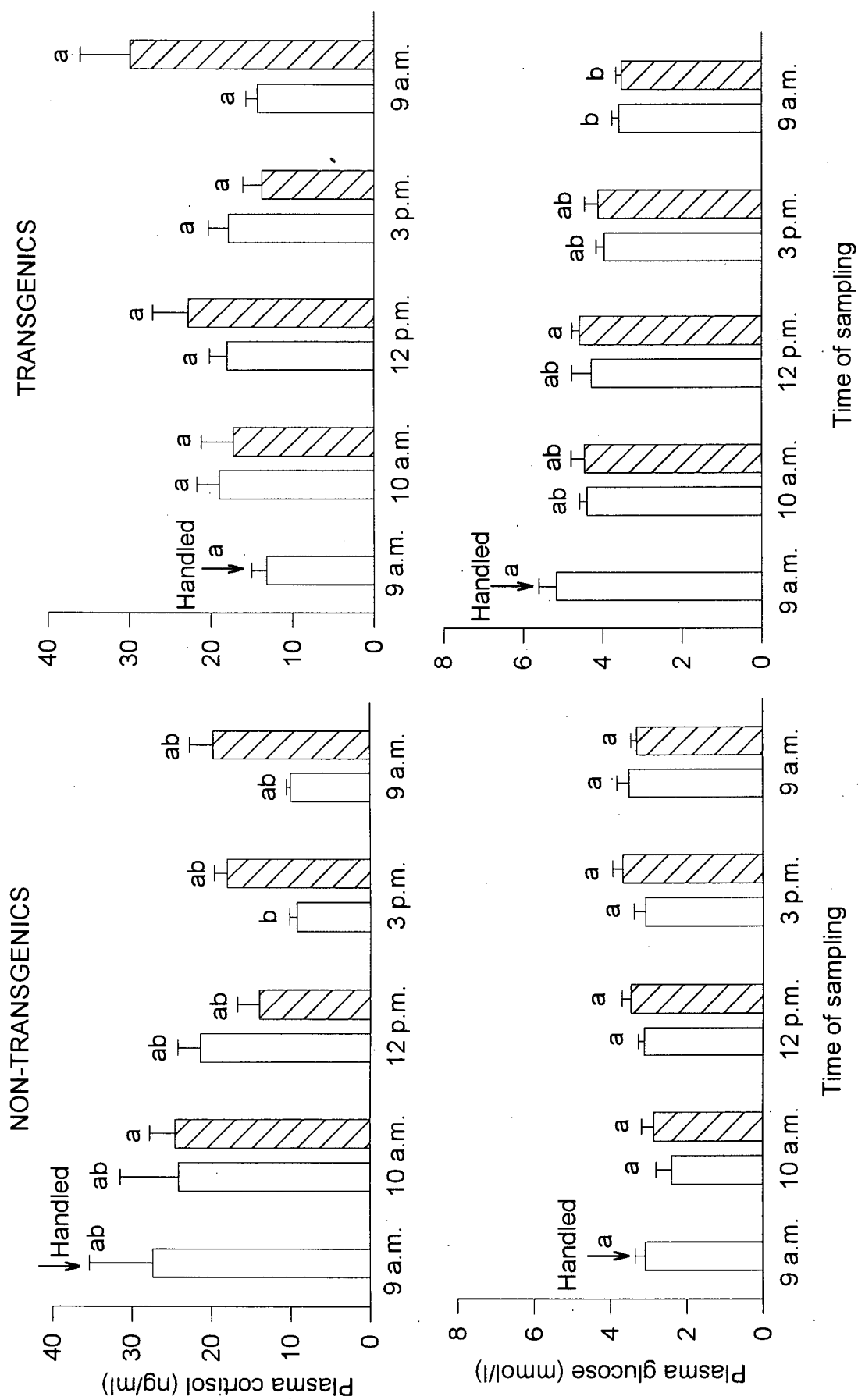
**Statistical Analysis.** A two-way Analysis of Variance (ANOVA), using group (transgenic, size control or age control) and treatment (control or heat shocked) as factors, was used to detect significant differences ( $p < 0.05$ ) in plasma cortisol, glucose and hsp70 expression. When differences were found, Student-Newman-Keuls tests were used to identify the different group. When the data were not normal or did not have equal variance, values were log-transformed prior to being analyzed.

### 3.3 RESULTS

#### a) Handling Challenge

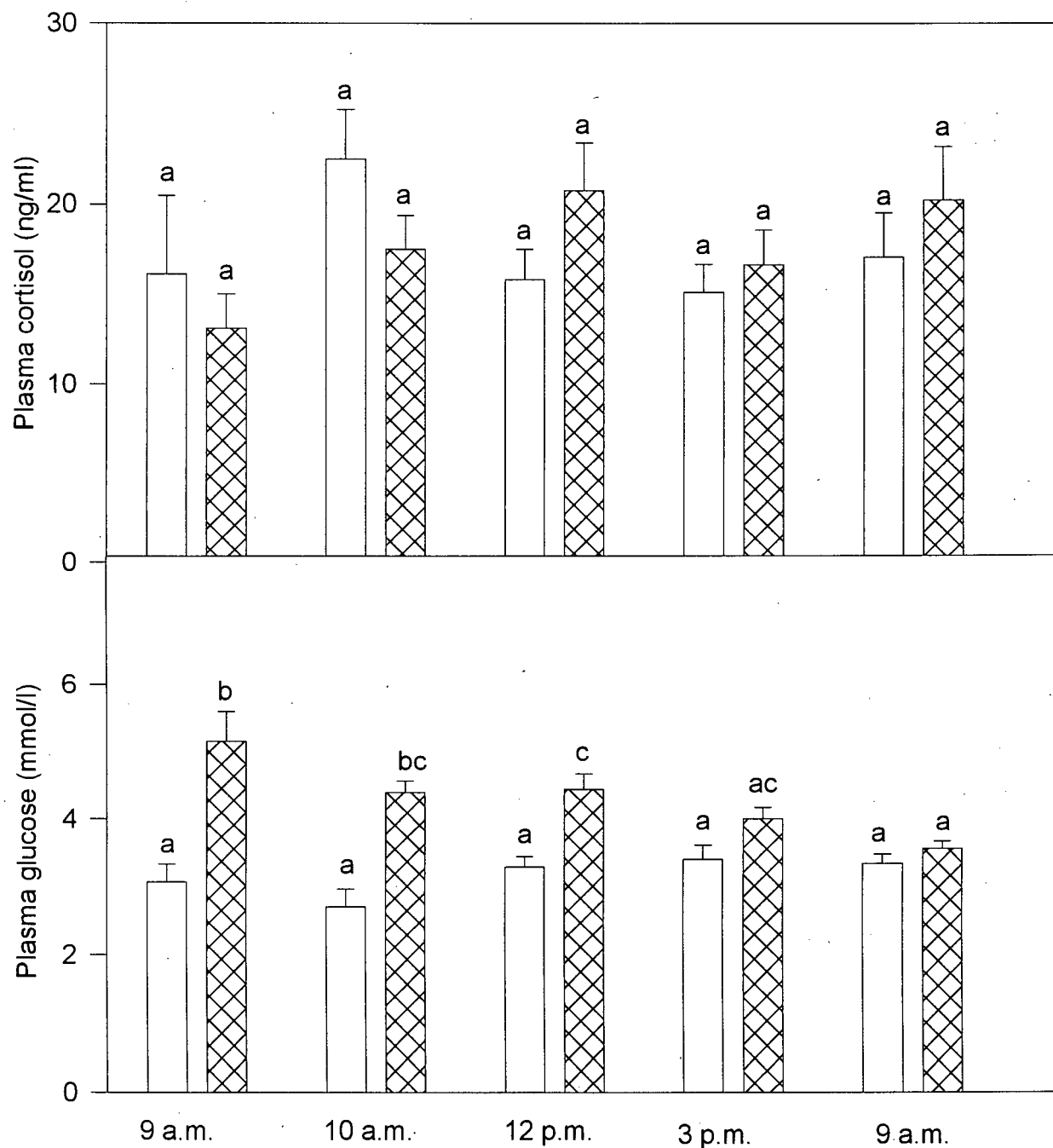
A 5-min handling stressor (chasing) failed to elicit a cortisol or glucose response in both transgenic fish and their non-transgenic siblings (Figure 3.1). Similarly, Wedemeyer *et al.* (1972) reported a lack of significant pituitary-interrenal activation in response to acute handling stress. Due to the lack of a cortisol and glucose response, handled and non-handled groups were pooled, and results are presented simply as physiological and immunological parameters.

Plasma cortisol levels did not differ between transgenic and non-transgenic fish (Figure 3.2a). Blood glucose levels, on the other hand, tended to be higher in transgenic fish relative to their non-transgenic siblings (Figure 3.2b). Gill lysozyme levels tended to be lower in transgenic fish (Figure 3.3).



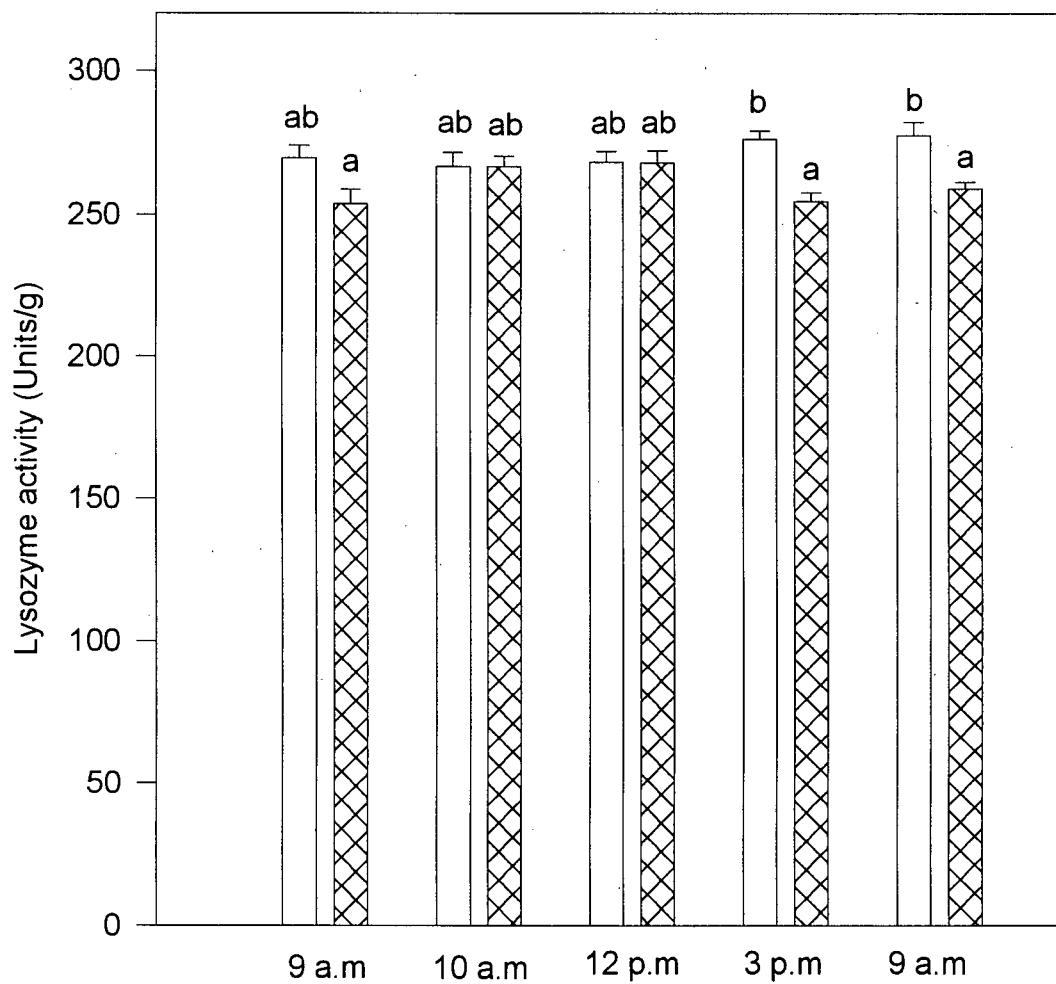
**Figure 3.1**  
 Plasma cortisol and glucose in control (□) and handled (▨) F2 MTGH1 transgenic coho salmon and their non-transgenic siblings following a 5 min handling stressor. Letters denote significant differences ( $p < 0.05$ ). Mean  $\pm$  S.E.,  $n=6$ .





**Figure 3.2**

Plasma cortisol and glucose in F2 MTGH1 transgenic coho salmon (▨) and their non-transgenic siblings (□) after pooling treatment groups (handled, non-handled) due to lack of cortisol or glucose response to handling. Mean ± S.E., n=6. Letters denote significant differences (p<0.05).



**Figure 3.3**

Gill lysozyme activity (per gram of tissue) in F2 MTGH1 transgenic coho salmon (▨) and in their non-transgenic siblings (□).

Letters denote significant differences ( $p < 0.05$ ). Mean + S.E.,  $n = 6$ .

## b) Heat Shock

In order to determine the temperature to use for the heat shock challenge, an experiment was performed where 2 transgenic fish and 2 non-transgenic fish were immersed in 25°C baths. The temperature tolerance appeared to be lower in the transgenic fish, as they appeared more distressed and died after a few minutes. As a result, I decided to use a temperature of 23°C (below the apparently lethal temperature) for the heat shock.

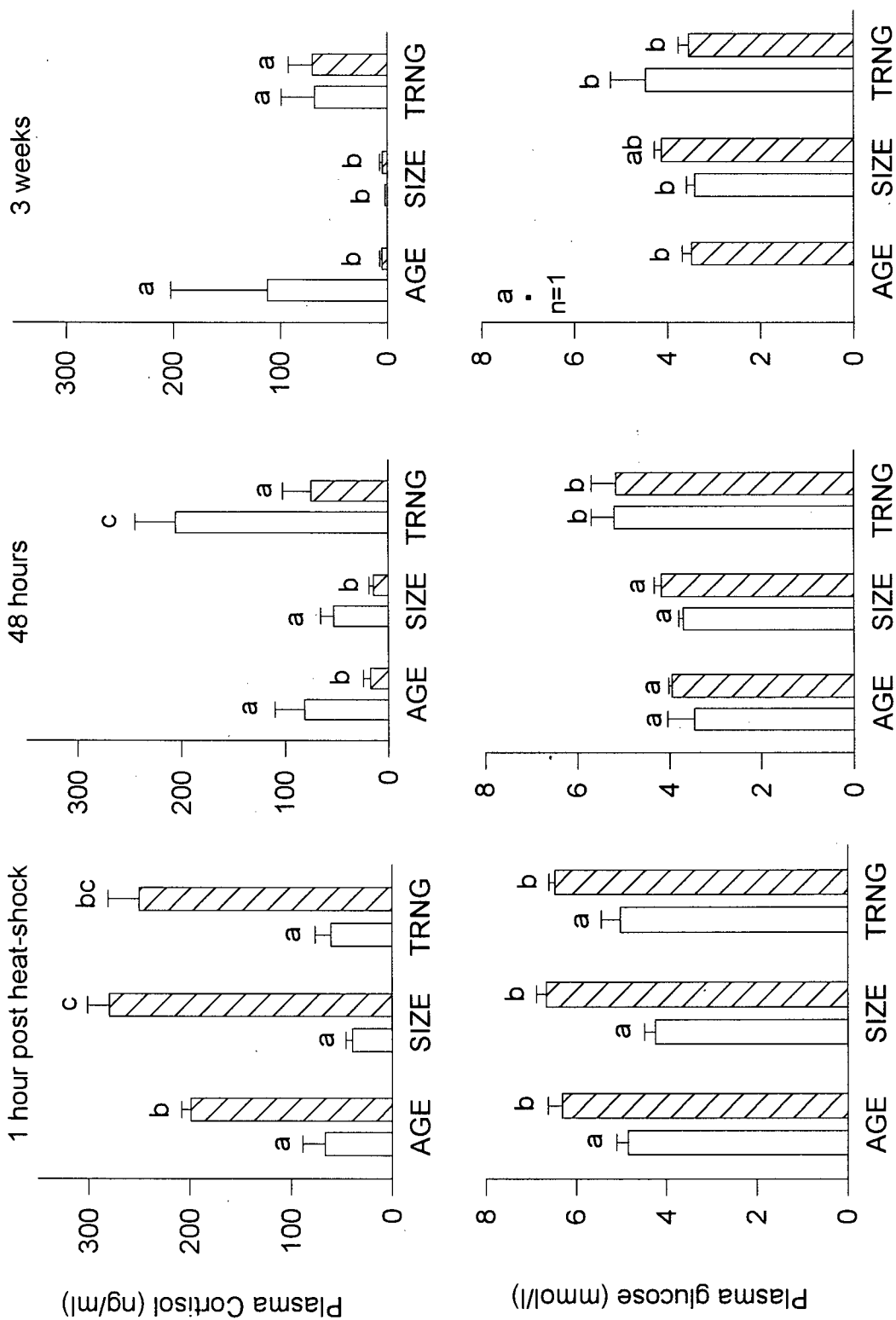
The heat shock appeared to result in a significantly more severe stress response, as indicated by a higher cortisol and glucose response 1 h after heat shock, relative to handling alone (Figures 3.4a and b). The cortisol and glucose response did not appear to be affected by the transgene. After a 1 h recovery, plasma cortisol and glucose levels were significantly greater in heat shocked fish, but were not different between transgenic and non-transgenic fish (Figure 3.4a). There was, however, a significant difference in plasma cortisol levels between the two non-transgenic groups 1 h after exposure to the heat shock, as the size controls had a greater cortisol increase than the age controls. This difference in cortisol response may have occurred due to low oxygen levels experienced by size controls, as one of the two air supplies failed during the heat shock, however, heat shocked transgenic fish experienced a similar drop in oxygen levels.

48 h after recovery, cortisol and glucose levels had decreased back to resting levels, except in sham challenged fish, which continued to have elevated cortisol levels. At this sampling time, transgenic fish had higher plasma glucose levels compared to the non-transgenic fish. By 3 weeks after recovery, plasma cortisol in fish of all groups was at resting levels (except in sham challenged age controls, which continued to experience elevated cortisol levels), and transgenic fish showed significantly higher

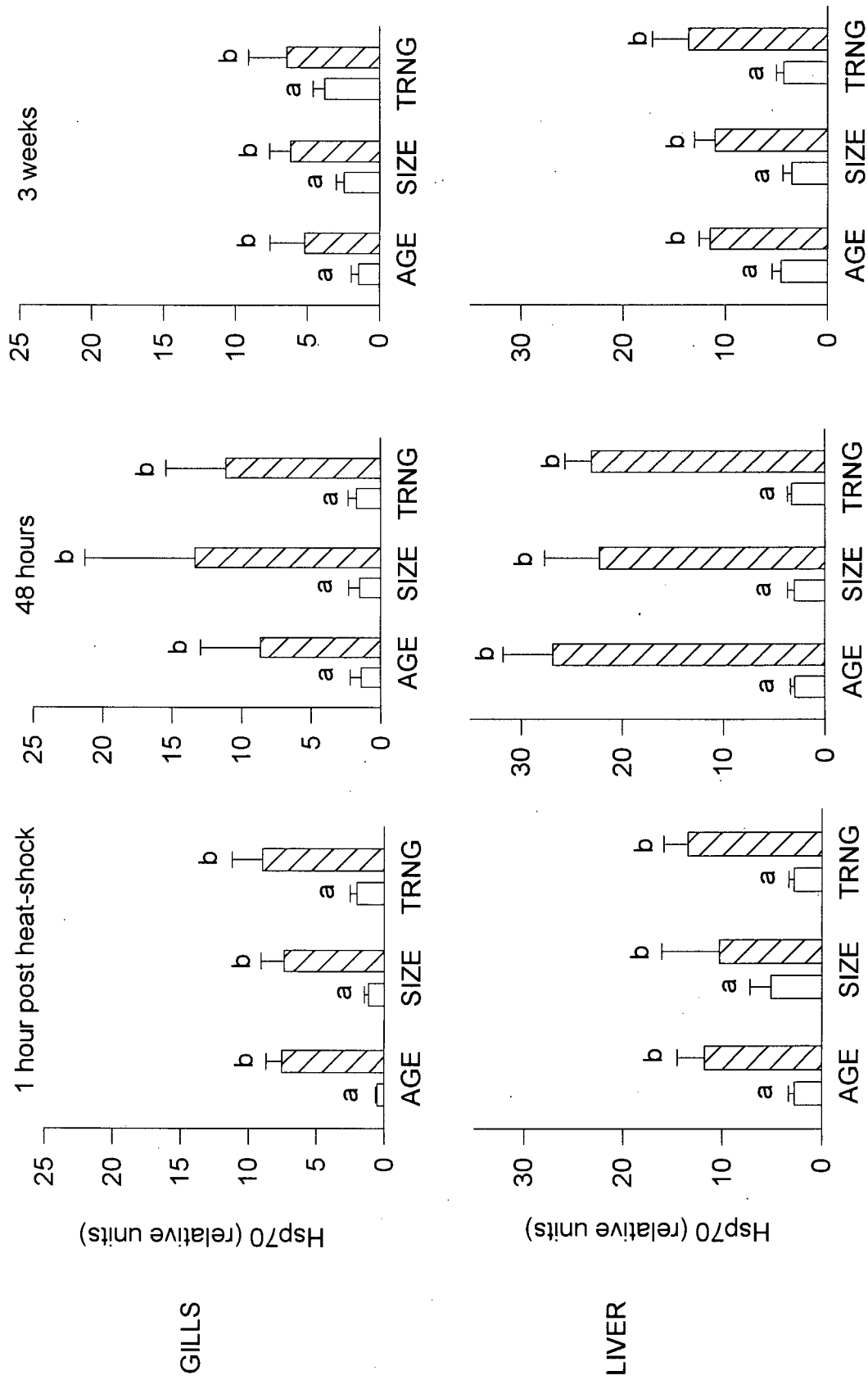
resting cortisol levels than the non-transgenic controls ( $p < 0.05$ ). At this sampling time, resting plasma glucose levels in transgenic fish were not different from those of non-transgenic fish.

Background hsp70 expression (measured by Western Blotting- see General Materials and Methods) in the kidney was significantly lower in age control fish relative to transgenic fish (Figure 3.5a). Background expression of hsp70 in the remaining tissues sampled did not differ between groups (Figures 3.5b-3.7).

In order to account for differences in background hsp70 expression, increases in the hsp70 expression of heat shocked fish were reported as values relative to those of their non heat shocked cohorts (Figures 3.8-3.10). Timing of hsp70 induction was tissue specific. Hsp70 induction was seen in the gills and liver of all fish (Figure 3.5) after a 1 h recovery, and at this time, the level of induction in the gills was greatest in age control fish (Figure 3.8a). Hsp70 expression in the gills and liver of all heat shocked fish remained elevated 48 h after the heat shock. Induction of hsp70 in the other tissues sampled (kidney, skeletal muscle and red blood cells) was not apparent until the second sampling time (48h; Figures 3.6 and 3.7). The level of induction in muscle tissue was greatest in age control fish relative to size controls and transgenic fish. 3 weeks after the heat shock, hsp70 levels had returned to normal in all tissues, except in the liver and gills.

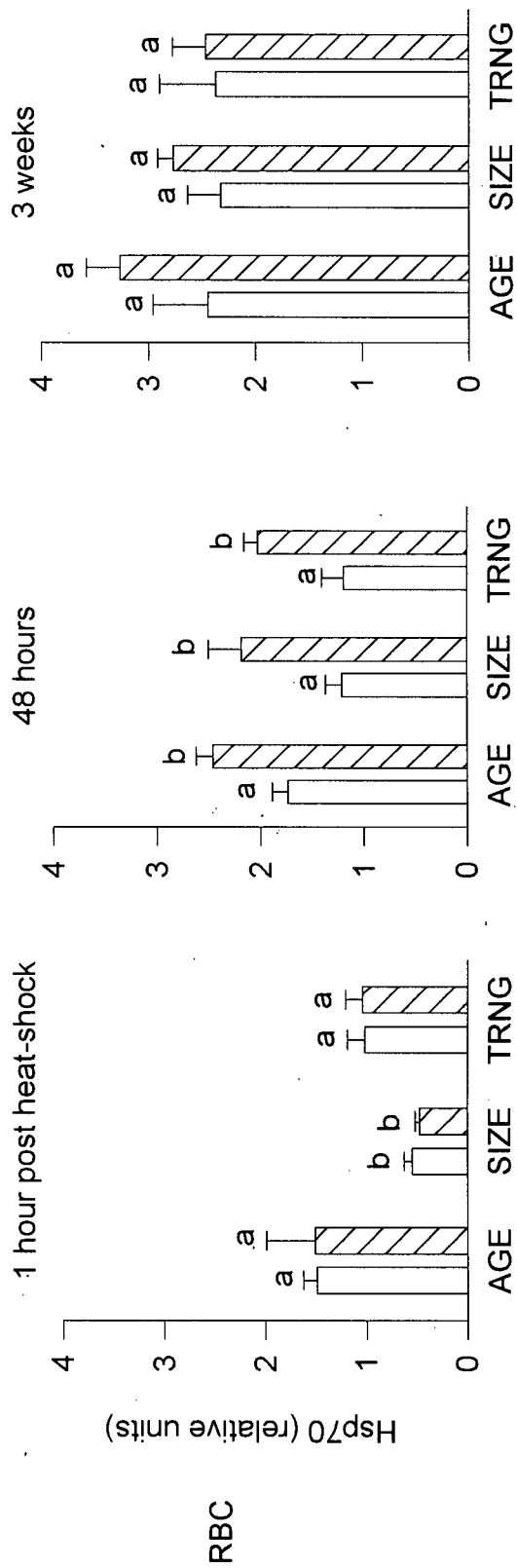


**Figure 3.4**  
 Effects of a 2 hour 9 °C heat shock on plasma cortisol (A) and glucose (B) levels in F1 H3GH1 transgenic coho salmon (TRNG), their smaller non-transgenic siblings (AGE) and fish of similar size (SIZE). (Non-heat shocked □ versus heat shocked ▨ ) Letters denote significant differences ( $p < 0.05$ ). Mean  $\pm$  S.E.,  $n = 6$ .

**Figure 3.5**

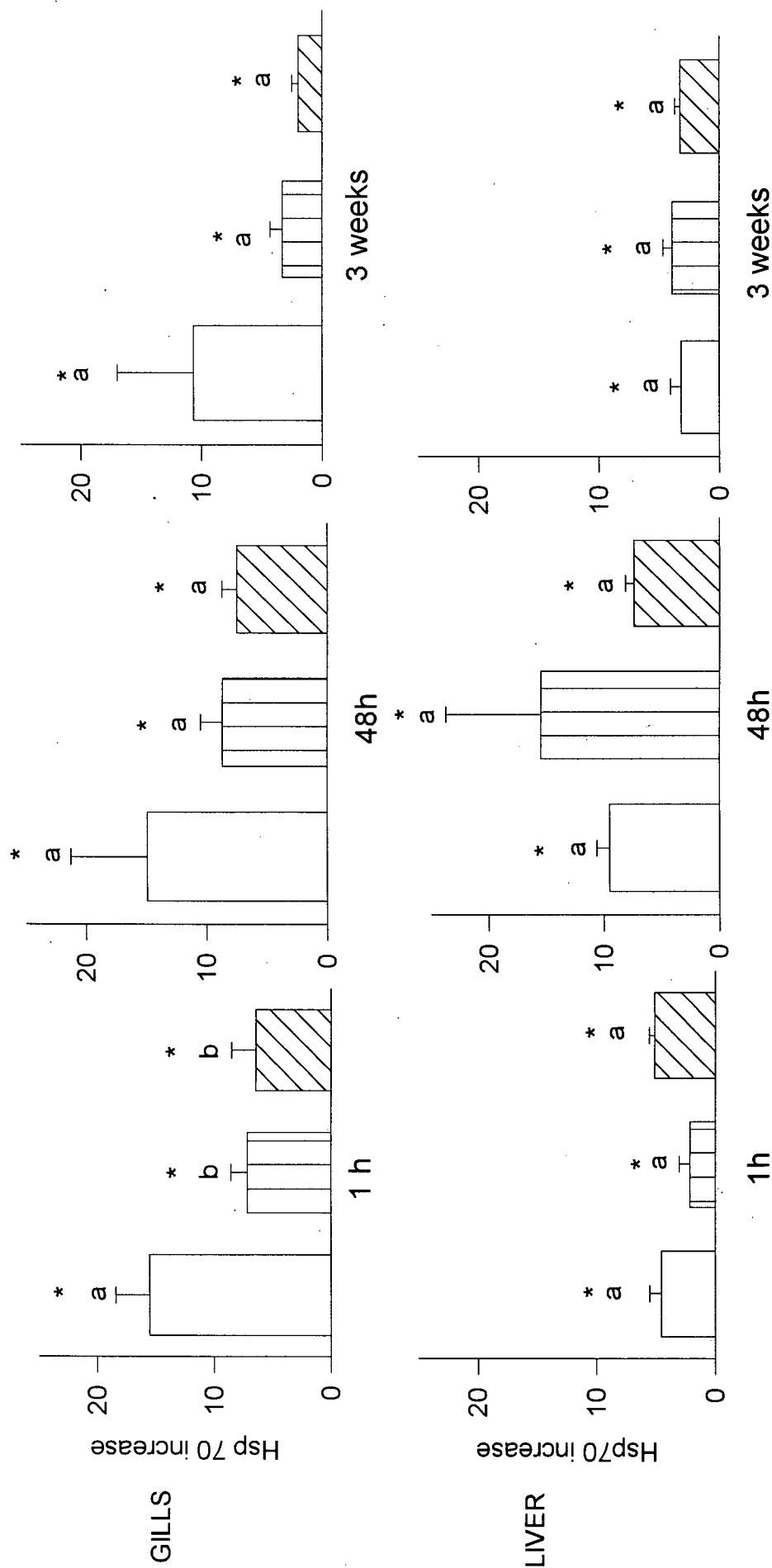
Effects of a 2 hour 9 °C heat shock on hsp70 expression in the gills and liver of F1 H3GH1 transgenic coho salmon (TRNG), their smaller non-transgenic siblings (AGE) and fish of similar size (SIZE). (Non-heat shocked □ versus heat shocked ▨ ) Letters denote significant differences ( $p < 0.05$ ). Mean  $\pm$  S.E.,  $n = 6$ .

**Figure 3.6**  
Effects of a 2 hour 9 °C heat shock on hsp70 expression in the kidney and muscle of F1 H3GH1 transgenic coho salmon (TRNG), their smaller non-transgenic siblings (AGE) and fish of similar size (SIZE). (Non-heat shocked □ versus heat shocked ▣ )  
Letters denote significant differences ( $p < 0.05$ ). Mean  $\pm$  S.E.,  $n = 6$ .



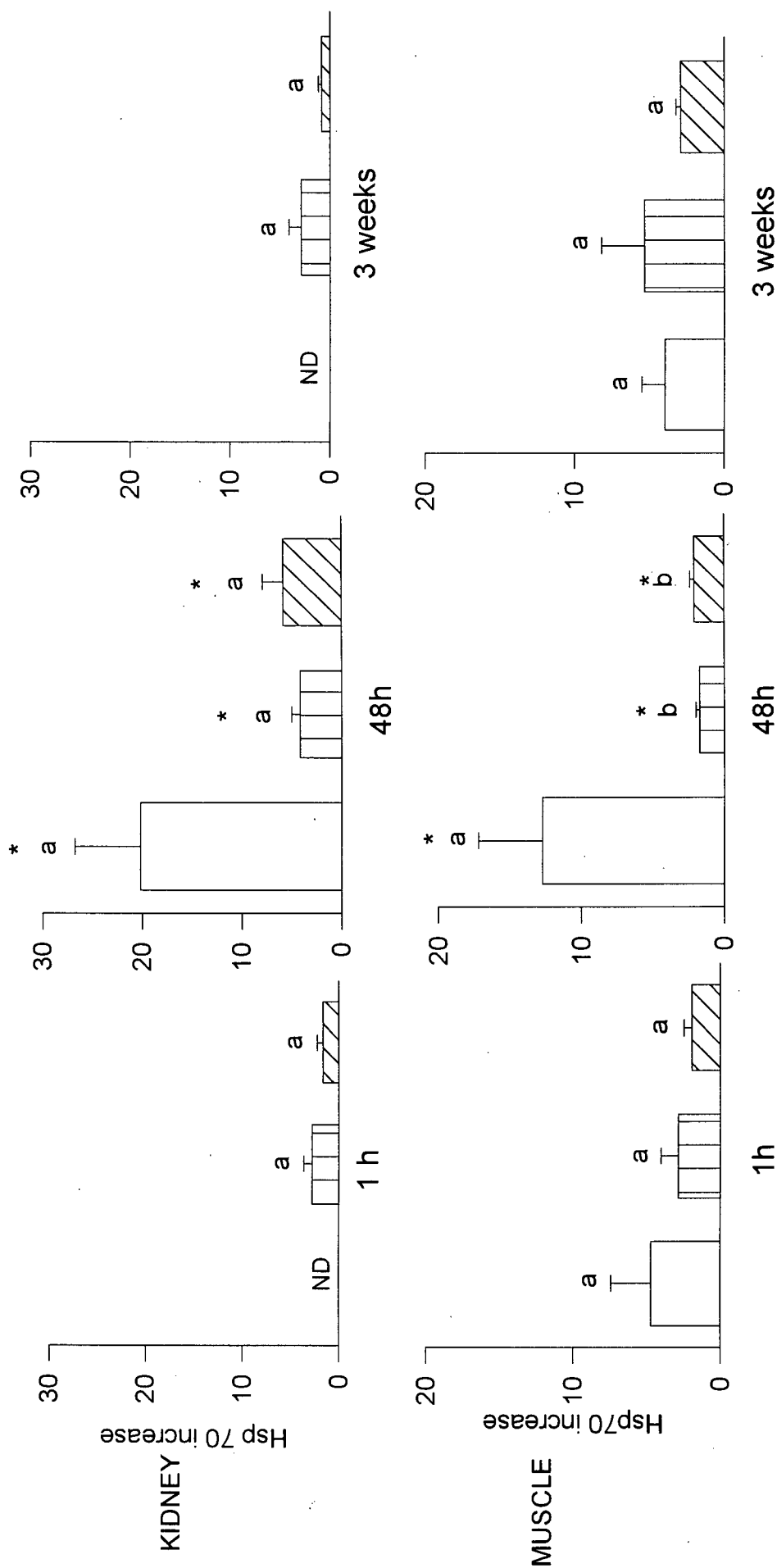
**Figure 3.7**  
Effects of a 2 hour 9 °C heat shock on hsp70 expression in the red blood cells of F1 H3GH1 transgenic coho salmon (TRNG), their smaller non-transgenic siblings (AGE) and fish of similar size (SIZE). (Non-heat shocked □ versus heat shocked ▨ ) Letters denote significant differences ( $p < 0.05$ ). Mean  $\pm$  S.E.,  $n = 6$ .





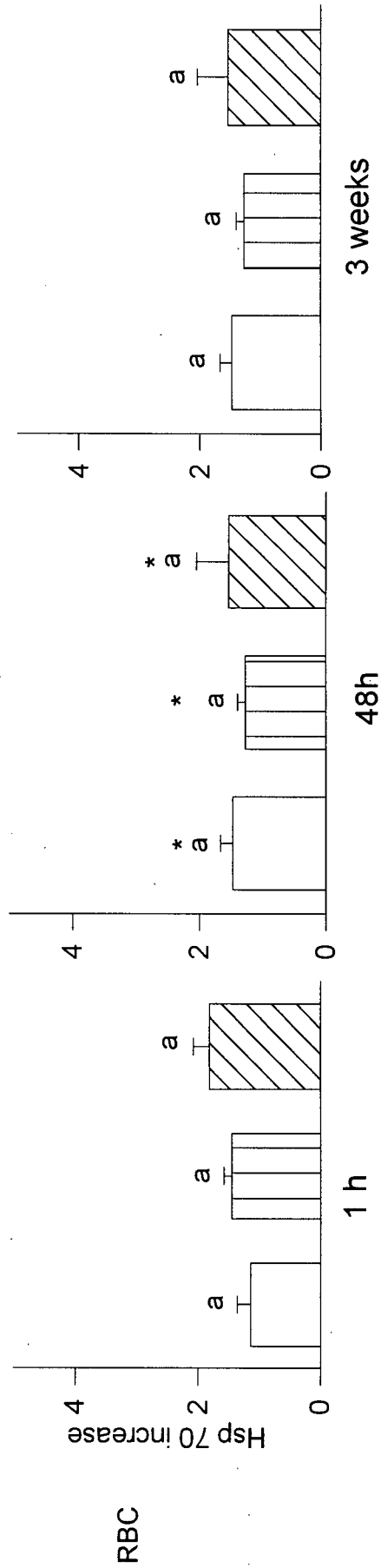
**Figure 3.8**

Increase in hsp70 expression (fold, relative to control values) following a 2 hour 9°C heat shock in the gills and liver of F1 H3GH1 transgenic coho salmon (▤), their non-transgenic siblings (age controls □) and fish of similar size (size controls ▨). Mean ± S.E., n=6. Asterisks denote hsp70 induction. Different letters denote significant ( $p < 0.05$ ) differences.



**Figure 3.9**

Increase in hsp70 expression (fold, relative to control values) following a 2 hour 9°C heat shock in the kidney and skeletal muscle of F1 H3GH1 transgenic coho salmon (hatched), their non-transgenic siblings (age controls) and fish of similar size (size controls). Asterisks denote hsp70 induction. Different letters denote significant ( $p < 0.05$ ) differences. Mean  $\pm$  S.E.,  $n=6$ .



**Figure 3.10**

Increase in hsp70 expression (fold, relative to control values) following a 2 hour 9°C heat shock in red blood cells of F1 H3GH1 transgenic coho salmon (■), their non-transgenic siblings (age controls□) and fish of similar size (size controls▨). Mean±S.E., n=6. Asterisks denote hsp70 induction. Different letters denote significant (p<0.05) differences.

### 3.4 DISCUSSION

#### Basal Physiology

Most studies on the effects of GH on basal glucocorticoid levels point to a stimulatory effect of GH on basal glucocorticoid levels, whether GH levels were increased by a transgene (Cecim *et al.*, 1991), by injection (Cheung *et al.*, 1988) or by a GH secreting tumor (Coyne *et al.*, 1981). Basal cortisol levels were also found to be increased in GH transgenic individuals in the heat shock study, but it could not be established whether this was due to the transgene or to an increase in cortisol brought about by smoltification (Barton *et al.*, 1985), as some transgenic fish were smolting. In F2 MTGH1 transgenic fish, however, no differences in basal cortisol levels were found when compared to their non-transgenic siblings.

Basal glucose levels were not affected in H3GH1 transgenic fish. However, in F2 MTGH1 transgenic fish, basal glucose levels tended to be higher relative to non transgenic fish. These results fail to clarify the already uncertain effect of chronically elevated levels of GH on plasma glucose. Studies on GH transgenic mice have found that generally plasma glucose in GH transgenic animals can be maintained at normal levels by the concurrent elevation of insulin (review by Bartke *et al.*, 1994), whereas some acromegalics can suffer from high plasma glucose levels (possibly as a result of insulin resistance; Wasada *et al.*, 1997).

Although GH has been found to activate rainbow trout macrophages (Sakai *et al.*, 1996), and increased lysozyme activity has been associated with macrophage activation (Secombes and Fletcher, 1992), basal lysozyme activity was generally found to be lower in MTGH1 transgenic fish than in non-transgenic fish.

### Stress Response

Previous studies on the effects of GH on the stress response suggest that elevated levels of GH may increase sensitivity to stress. A study performed on MTGH mice found that transgenic mice had an increased cortisol response to ether stress (Cecim *et al.*, 1991). Similarly, rats containing a GH secreting tumour have been found to have an increased cortisol response (Coyne *et al.*, 1981), and coho salmon injected with GH have an increased interrenal sensitivity to ACTH (hormone which stimulates cortisol synthesis) *in vitro* (Young, 1988). It is possible that the lack of an effect on the cortisol response in this study may have been due to the use of a weaker transgene promoter (histone), relative to the metallothionein promoter used by Cecim *et al.* (1991). In the present study, the transgene did not appear to have an effect on the stress response, as the cortisol and glucose responses did not differ between transgenic and non-transgenic fish. It should be noted, however, that although some transgenic fish were undergoing a change in pigmentation indicative of smolting, they did not exhibit the increased cortisol response typical of smolts (Barton *et al.*, 1985). These findings could therefore suggest that in this study the GH transgene may have at the very least not increased their sensitivity to stress.

Effects of the GH transgene on heat shock proteins have not been studied. Constitutive hsp70 expression was not affected by the transgene. The mortalities which I observed in a preliminary heat shock (25°C) would appear to indicate that transgenic fish have a lower temperature tolerance. However, the level of induction of hsp70 did not differ between transgenic and non-transgenic fish, therefore indicating that the extent of cellular damage due to the heat shock was not different between the two. On the other hand, induction did differ between the age controls and the larger fish (size controls and transgenic fish), as age controls had a higher hsp70 induction in the gills

and muscle. These results could indicate a different treatment effect of the heat shock on different body sizes. The smaller fish (age controls) probably experienced a higher heating rate compared to the large fish (size controls and transgenic fish) due to their greater surface to volume ratio, relative to the larger fish (transgenic fish and size controls). Rats experiencing a high heating rate have been reported to have a greater hsp70 induction relative to animals exposed to a lower heating rate (Flanagan *et al.*, 1995).

These findings suggest that the GH transgene did not affect the cellular stress response in this study.

#### 4. THE DISEASE RESISTANCE OF TRIPLOID AND DIPLOID COHO SALMON

##### TRANSGENIC FOR GH

##### 4.1 INTRODUCTION

Animal studies have uncovered that relationships exist between the endocrine and immune systems. Corticosteroids have immunosuppressive effects (review by Barton and Iwama, 1991). Similarly, GH has been found to play a key role in the immunity of animals. Although mainly produced by pituitary somatotropes, GH is also produced by cells of the immune system (Weigent *et al.*, 1988). In mammals, GH has been found to stimulate the proliferation of cells of the immune system and the killing capacity of macrophages (review by Auernhammer and Strasburger, 1995). Similar effects are found in fish, as GH also stimulates phagocytes (Sakai *et al.*, 1996) and non-specific cytotoxic cells (Kajita *et al.*, 1992) in rainbow trout. Further evidence of the importance of GH in immunity was provided by Edwards *et al.* (1991), who found that removal of the pituitary increased the susceptibility of mice to a lethal bacterial infection, and that GH treatment restored resistance back to normal.

Although it is clear that GH is essential for immunity, chronic elevations of GH may not have the same positive effects. Pigs and mice transgenic for GH experience kidney and liver problems, and consequently have a lower life expectancy (Wolf *et al.*, 1993). Naturally occurring abnormalities in GH levels do not reflect the importance of GH on the immune system, as GH deficient humans do not suffer from increased disease susceptibility (reviews by Auernhammer and Strasburger, 1995; Saito *et al.*, 1996). Similarly, the oversecretion of GH seen in acromegalics does not affect their disease resistance (Kotzmann *et al.*, 1994). It appears that in these cases, the immune system may not be affected, or may be able to compensate for the endocrine status.

The effect of the GH transgene on the health of fish has not been previously studied, and it was therefore my objective to determine whether the transgene would affect the health of the fish, as previously seen in transgenic pigs and mice. As discussed previously (General Introduction), transgenic fish for use in culture would most likely have to be sterilized via triploidy. Therefore, in addition to studying the effects of the transgene on the disease resistance, the effect of triploidy must also be determined. Studies on triploid coho and Atlantic salmon have so far indicated that their survival rates are lower than those of diploid fish (Utter *et al.*, 1983; Galbreath *et al.*, 1994; Withler *et al.*, 1995), but a recent study on rainbow trout found no effect of triploidy on the resistance to diseases such as vibriosis, IHN (Infectious Hematopoietic Necrosis), or furunculosis (Yamamoto and Iida, 1995a).

In order to study the effects of the GH transgene as well as those of triploidy on disease resistance, triploid and diploid transgenic and non-transgenic fish were exposed to the bacterial pathogen *Vibrio anguillarum*, and mortalities were recorded.

## 4.2 MATERIALS AND METHODS

**Fish.** Diploid F1 MTGH1 transgenic coho salmon ( $37.9 \pm 8.12$  g) and diploid ( $3.11 \pm 2.31$  g) and triploid ( $2.5 \pm 1.43$  g) F2 MTGH1 transgenic coho salmon were used in two separate disease challenge tests. Control fish were non-transgenic fish of similar size (diploid and both diploid and triploid, respectively; see General Materials and Methods for more details). In the first experiment ( $10.6 \pm 0.3^\circ\text{C}$ ), transgenic and non-transgenic fish were kept in different tanks, whereas in the second experiment ( $13.9 \pm 0.3^\circ\text{C}$ ), separation was based on ploidy (and transgenic fish were identified only at the termination of the experiment). Groups were further separated into *Vibrio anguillarum*



challenged tanks and sham challenged tanks. Fish were not fed the day prior to the disease challenge or sampling.

**Pathogen.** *Vibrio anguillarum* (the causative agent of vibriosis) was isolated from the kidney of a juvenile coho salmon that had died of vibriosis. The pathogen was grown for 20 hours at room temperature on plates of trypticase soy agar (TSA, Difco Laboratories) with 1.5% NaCl. Colonies were suspended in cold, sterile peptone (0.1% w/v) saline (0.85% w/v), and the suspension's turbidity was adjusted to 1 O.D. at 540 nm. Dilutions were prepared with the estimate that a 1 O.D.<sub>540</sub> suspension had  $10^9$  *Vibrio* cells/ml. Actual challenge doses were determined by plating dilutions of the 1 O.D. suspension and counting the number of colony forming units after 16 h.

**Immersion Challenge.** Fish were immersed in a 3-L solution of peptone (0.1% w/v) saline (0.85% w/v) containing the appropriate dose of *Vibrio anguillarum*. Control (or sham challenged) fish were placed in a similar bath, except for the addition of the pathogen. The bath immersions were of a 20-minute duration, and the solutions were aerated.

**Sampling.** Fish from the F1 MTGH1 group were lethally sampled (for lysozyme activity) 7 days after the disease challenge (6 fish from each group).

Details of the sampling procedure are described in the General Materials and Methods. Briefly, fish were anaesthetized, and blood samples were taken. Plasma samples were stored at -50°C until assayed.

**Immunological parameter.** Plasma lysozyme activity was measured by the lysoplate method, as described in the General Materials and Methods.

**Mortalities.** The experiment was ended after at least five days of zero mortalities. Mortalities were collected once daily and 30% of the daily mortalities were autopsied to confirm that death was due to vibriosis. Fish were considered to have died of vibriosis if

colonies grown from kidney samples were raised, round, cream coloured, smooth and did not produce pigments, and if gram stained smears contained many gram negative short comma shaped rods.

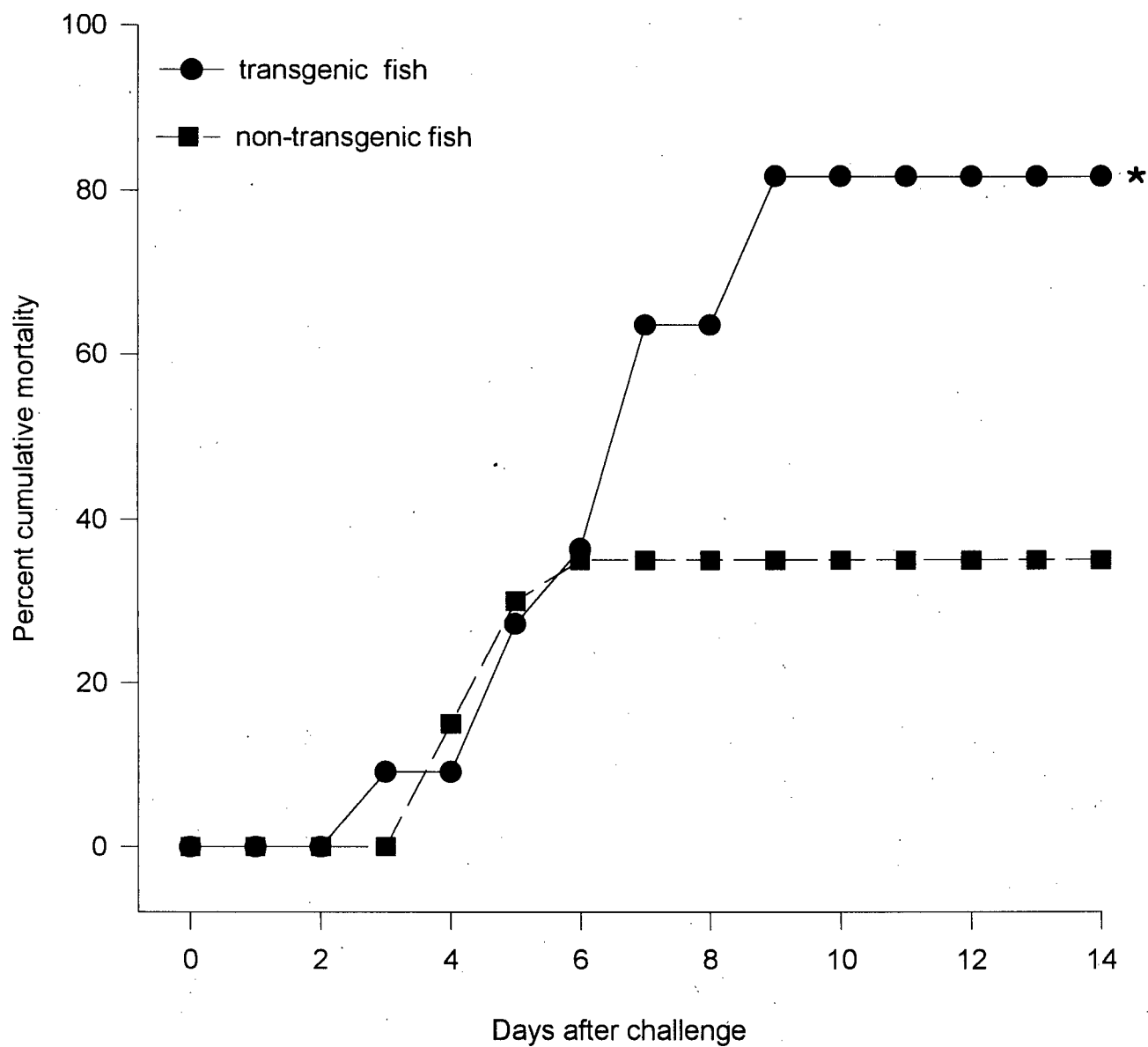
**Statistical Analysis.** Differences in cumulative mortalities (at end of experiment) were analyzed by Chi-square analysis ( $p < 0.05$ ). In the experiment where ploidy was an additional variable, different groups were identified using a Tukey-type test for proportions.

### 4.3 RESULTS

In the first disease challenge test, transgenic fish suffered higher cumulative mortalities when exposed to an immersion challenge with *Vibrio anguillarum* (Figure 4.1). Even though the small sample size reduced the power of the test (0.56), the difference in cumulative mortalities was found to be significant ( $p = 0.03$ ). 14 days after the challenge, 82% of the transgenic fish had died of vibriosis, compared to 35% of non-transgenic fish of similar size. No mortalities occurred in the sham challenged fish. Mortalities for the transgenic fish began on day 3 and finished on day 10, whereas for the non-transgenic fish, they began one day after, and finished 4 days before. Basal lysozyme activity was not different between transgenic and non-transgenic fish. The *Vibrio anguillarum* challenge significantly elevated plasma lysozyme activity in transgenic fish, but not in non-transgenic fish (Figure 4.2). In *Vibrio anguillarum* challenged fish, the plasma lysozyme activity was almost twice as high in transgenic fish compared to non-transgenic fish, but this was probably a reflection of the diseased status of the fish sampled. At the time of sampling, mortalities had already finished for non-transgenic fish, but were still ongoing for transgenic fish. Therefore, the non-transgenic fish sampled were probably recovered or resistant, whereas some of the transgenic fish

may have been fighting the infection (increases in lysozyme activity can occur during an infection- Moyner *et al.*, 1993).

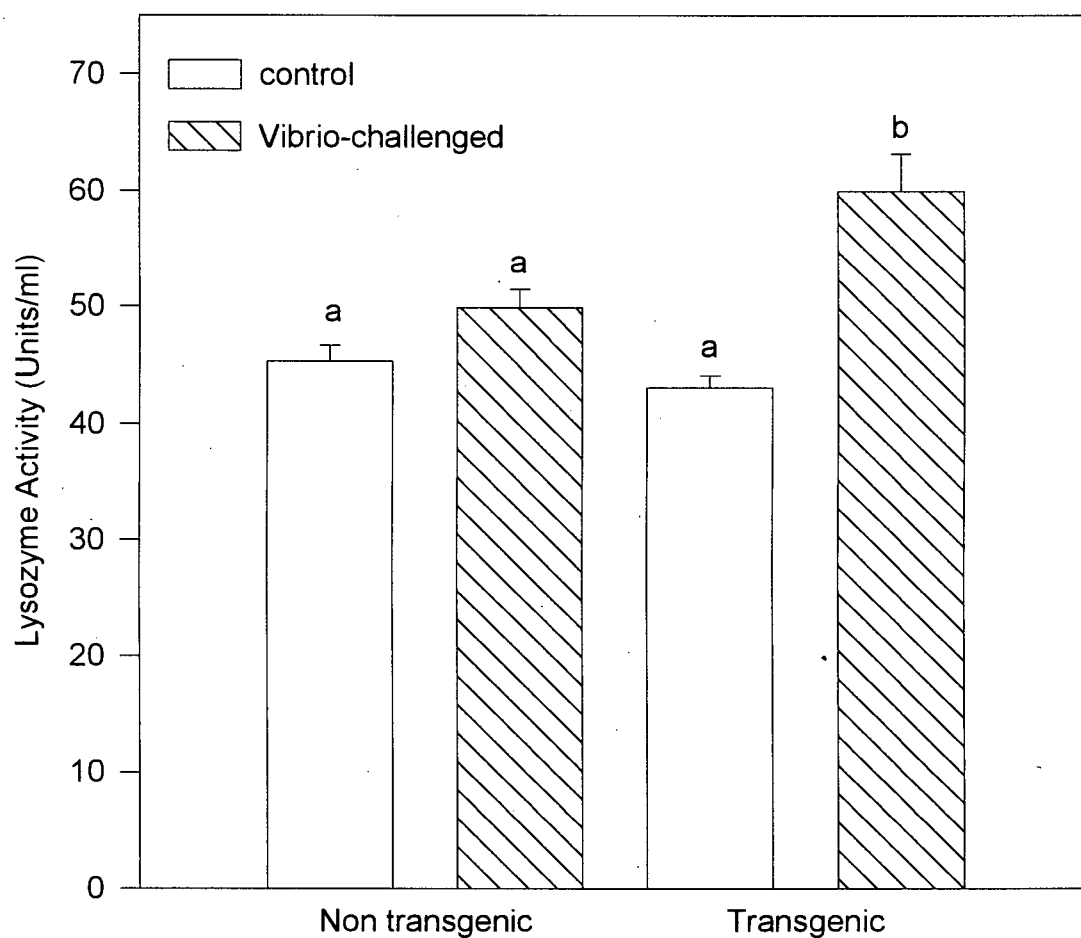
In the second disease challenge, both triploid and diploid transgenic fish exposed to a low dose of *Vibrio anguillarum* ( $10^6$  cfu/ml; Figure 4.3) performed better (85% and 43% mortalities, respectively) than their non-transgenic counterparts (95% and 59% mortalities, respectively). When the dose of the pathogen was increased tenfold, however, transgenic fish performed differently (Figure 4.3); cumulative mortalities in diploid transgenic fish significantly increased from 43% to 67% when the pathogen dose was increased tenfold, whereas mortalities in their non-transgenic diploid siblings remained unchanged. Similarly, mortalities in triploid transgenic fish increased from 85% to 100% with a 10-fold increase in pathogen dose, whereas mortalities in non-transgenic triploids actually decreased from 95% to 85%. In general, triploid fish experienced higher mortalities than diploid fish in both the low and high dose immersion challenges with *Vibrio anguillarum*.



**Figure 4.1**

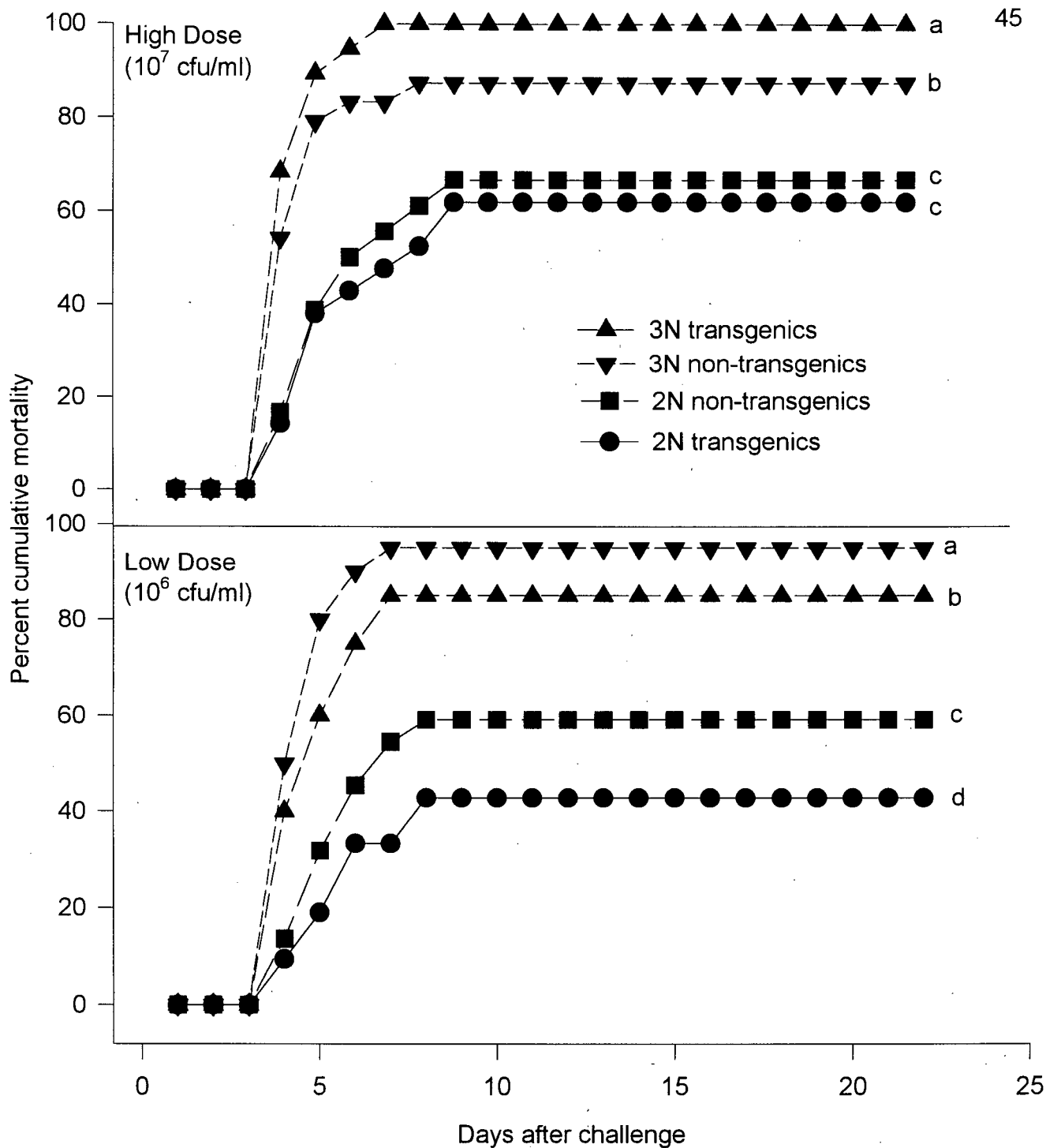
Percent cumulative mortality in F1 MTGH1 transgenic coho salmon and in non-transgenic coho salmon of similar size, after an immersion challenge with *Vibrio anguillarum* ( $10^8$  cfu/ml). n= 11 and 20, respectively.

\* represents a significant ( $p < 0.05$ ) difference in cumulative mortality at the end of the experiment (14 days after challenge).



**Figure 4.2**

Plasma lysozyme activity in F1 MTGH1 transgenic coho salmon and in fish of similar size 7 days after an immersion challenge with *Vibrio anguillarum* ( $10^8$  cfu/ml).  $n=7$ . Different letters denote significant differences ( $p<0.05$ ).



**Figure 4.3**

Percent cumulative mortality in diploid and triploid F2 MTGH1 transgenic coho salmon and their non-transgenic siblings after a low dose or a high dose immersion challenge with *Vibrio anguillarum*. n= 107.

Different letters denote significantly different ( $p < 0.05$ ) cumulative mortalities at the end of the experiment (21 days after challenge).

#### 4.1 DISCUSSION

Mice and coho salmon containing the GH transgene have been documented to have a shorter life span (Wolf *et al.*, 1993; Steger *et al.*, 1993; Devlin *et al.*, 1995). In this study, diploid F1 MTGH1 transgenic fish were found to be less resistant to vibriosis than non-transgenic fish of similar size, however, due to the low power of the experiment, we should interpret this result with caution. The transgene did not appear to impair the disease resistance of diploid F2 MTGH1 transgenic fish, as they were equally or more resistant than their non-transgenic siblings, depending on whether they were exposed to a high or low pathogen dose. Transgenesis appeared to increase the sensitivity of the fish to changes in pathogen dose, as a tenfold increase in pathogen dose resulted in a higher increase of mortalities in transgenic than in non-transgenic fish. However, the lack of replicate tanks does not allow me to exclude the possibility that the apparent difference in the sensitivity to dose was caused by a tank effect.

Although lysozyme is a bactericidal enzyme, high basal lysozyme activity has been found to be positively correlated to high disease susceptibility in Atlantic salmon (Lund *et al.*, 1995) and chinook salmon (Balfry, 1997). The lower basal lysozyme activity seen in the diploid F2 MTGH1 transgenic fish in the previous chapter therefore reinforces the possibility that the transgene may have conferred increased disease resistance. However, basal lysozyme activity in the F1 MTGH1 transgenic fish was not found to be different from that of non-transgenic fish, although between-family differences could have masked any differences in lysozyme activity in the F1 MTGH1 experiment, as transgenic and non-transgenic fish were derived from different families.

Although the experiments were not designed to allow for a comparison between disease challenges, the differences in lysozyme activity and susceptibility to vibriosis

between diploid F1 and F2 MTGH1 transgenic fish, as well as the differences in the growth of the two transgenic lines (F2's were  $10.28 \pm 5.16$  g by August, whereas F1's were already  $37.9 \pm 8.12$  g by July) could reflect a differential impact of the transgene on different transgenic lines or generations. The differences in lysozyme, disease resistance and growth could also be due other factors such as environmental variables. Nonetheless, it appears that in certain lines of diploid GH transgenic fish, the transgene does not impair disease resistance.

The effect of triploidy was a lot clearer, as triploid fish were consistently more susceptible to vibriosis than their diploid siblings. The increased susceptibility of triploid fish could be attributed to the overall decrease in the number of immune cells (Small and Benfey, 1987), as cell number is decreased to maintain organ sizes in the face of increased cell volume (due to higher DNA content). A decrease in the number of immune cells may affect the ability to successfully protect the body from pathogens, and perhaps contributes to the impaired disease resistance seen in triploid fish. However, a previous study did not detect any differences in the susceptibility of triploid rainbow trout to disease (vibriosis, furunculosis or IHN; Yamamoto and Iida, 1995a), and found no differences in components of their non specific immune system (complement and neutrophils; Yamamoto and Iida, 1995b), relative to diploid rainbow trout. The transgene did not appear to further affect the health of triploid fish, as triploid transgenic fish performed better than their non-transgenic cohorts in the low dose challenge, but worse in the high dose challenge. This study suggests that triploidy could reduce any health benefits provided by the GH construct, as triploidy affected the disease resistance of both transgenic and non-transgenic fish.



## 5. GENERAL DISCUSSION

The goal of this thesis was to determine the influence of genetic manipulation on the performance of fish, specifically on the stress response and disease resistance. The results obtained from the experiments suggest that manipulation of ploidy can impair fish health, and that insertion of a GH construct may not always affect the performance of transgenic fish.

Genetically manipulated organisms have a remarkable ability to compensate for the changes caused by the genetic alteration, and can usually maintain a relatively normal physiology, as has been exemplified by transgenic mice overexpressing vasopressin ( a hormone which limits the water content and volume of urine) which were able to maintain normal urine production (Miller *et al.*, 1993). Similarly, it appears that the GH transgenic fish in this study were able to maintain a relatively normal physiology in spite of GH overexpression and possible, but unknown pleiotropic effects of the transgene. This thesis did not attempt to identify the mechanisms by which homeostasis was maintained in the face of increased GH production, but they could include changes in the regulation of active levels of GH, such as down-regulation of GH receptors and increased GH binding proteins.

The stress response was studied in transgenic fish containing a GH gene construct under the control of a histone promoter. Neither the physiological stress response nor the cellular stress response to a heat shock were affected by the transgene. However, since the stress response was only studied in H3GH1 transgenic fish, it cannot be generalized that any GH construct would have a similar lack of effect on the stress response of fish. Transgenic mice carrying a stronger promoter (metallothionein) appear to experience a heightened stress response (Cecim *et al.*,

1991). Perhaps the use of a weak promoter such as the histone promoter resulted in a lower expression of the GH, therefore limiting the effects of GH on the stress response.

Disease resistance was studied in two lines of transgenic fish: F1 and F2 transgenic fish containing a GH gene construct under the control of a metallothionein promoter. Transgenic animals carrying a GH construct appear to have shorter life spans, as transgenic mice appear to age prematurely (Wolf *et al.*, 1993; Steger *et al.*, 1993), and some transgenic coho salmon carrying GH constructs have been reported to suffer high mortalities by one year of age (Devlin *et al.*, 1995). However, this thesis suggests that disease resistance may not always be impaired by the GH gene construct. Disease resistance appeared to be impaired only in F1 transgenic fish, which were from a faster growing line, whereas the F2 transgenic fish (a slower growing line) were found to be equally or even more disease resistant than their non-transgenic siblings. There are a number of factors that may have contributed to the difference in susceptibility to vibriosis between F1 and F2 transgenic fish, such as differences in temperature or uncontrollable environmental factors. Nonetheless, there remains the possibility that the difference in susceptibility could be based on differences between transgenic lines or generations.

As different transgenic founder fish will experience different levels of growth stimulation (due to factors such as differences in copy numbers of the transgene, different insertion locations; Devlin *et al.*, 1995), each transgenic line should be evaluated separately. Similarly, the phenotype of different generations have the potential to differ, for example, differences will exist between founder and F1 transgenic fish, as the transgene is expressed mosaically in founder fish, and expressed in every cell in their transgenic progeny (Devlin *et al.*, 1995).

Manipulation of ploidy impaired the disease resistance of fish in this study, as triploid coho salmon showed a lower disease resistance to vibriosis relative to their diploid siblings. Triploid fish, in spite of having increased cell volume, manage to maintain normal organ size by decreasing cell numbers (Swarup, 1959). However, this compensation leads to its own problems, as the overall decrease in red blood cells can lead to a decreased amount of hemoglobin in the blood of Atlantic salmon (Benfey and Sutterlin, 1984). Similarly, reduced numbers of leukocytes in triploid fish (coho and Atlantic salmon; Small and Benfey, 1987) could perhaps lead to decreased ability to ward off pathogens, resulting in the increased susceptibility to disease reported in this study and many others (Utter *et al.*, 1983; Galbreath *et al.*, 1994; Withler *et al.*, 1995). However, triploidy does not always appear to impair the health of fish, as the susceptibility of rainbow trout to vibriosis, furunculosis or IHN does not seem to be affected by triploidy (Yamamoto and Iida, 1995a). Further studies are needed to elucidate the cause of the impaired health seen in many triploid fish in order to achieve a method of reproductively containing transgenic fish without jeopardizing their health.

Although the studies performed in this thesis lacked replication due to the valuable nature of the transgenic fish, the following conclusions can be drawn:

1. Overexpression of GH may not always result in impaired performance, as measured by sensitivity to stress and pathogens.
2. The performance of transgenic fish may differ between different lines of fish, therefore the performance of each transgenic line should be assessed independently.
3. Manipulation of ploidy can result in an increase in susceptibility to disease.

6. BIBLIOGRAPHY

- Amend, D.F. and Johnson, K.A. (1981).  
Current status and future needs of *Vibrio anguillarum* bacterins. *International Symposium on Fish Biologics: Serodiagnostics and Vaccines*, Developm. Biol. Standard. Leetown, U.S.A. **49**: 403-417.
- Auernhammer, C. J. and Strasburger, C.J. (1995).  
Effects of growth hormone and insulin-like growth factor I on the immune system. *European Journal of Endocrinology*, **133** : 635-645.
- Balfry, S. K. (1997)  
Ph.D. thesis, pp. 150, University of British Columbia, Vancouver, Canada.
- Bartke, A., Cecim, M., Tang, K., Steger, R. W., Chandrashekar, V. and Turyn, D. (1994).  
Neuroendocrine and reproductive consequences of overexpression of growth hormone in transgenic mice. *Proceedings of the Society for Experimental Biology & Medicine*, **206** : 345-359.
- Barton, B. A., Schreck, C. B., Ewing, R. D., Hemmingsen, A. R. and Patino, R. (1985).  
Changes in plasma cortisol during stress and smoltification in coho salmon, *Oncorhynchus kisutch*. *General and Comparative Endocrinology*, **59** : 468-471.
- Barton, B. A. and Iwama, G. K. (1991).  
Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, **1** : 3-26.
- Benfey, T. J. and Sutterlin, A. M. (1984).  
The haematology of triploid landlocked Atlantic salmon, *Salmo salar* L. *Journal of Fish Biology*, **24** : 333-338.
- Benfey, T.J., Bosa, P.G., Richardson, N.L. and Donaldson, E.M. (1988).  
Effectiveness of a commercial-scale pressure shocking device for producing triploid salmonids. *Aquacultural Engineering* **7**: 147-154.
- Benfey, T. J. (1991).  
The physiology of triploid salmonids in relation to aquaculture. *Canadian Technical Report of Fisheries and Aquatic Sciences*, **1789** : 73-80.
- Bergelson, J. (1994).  
Changes in fecundity do not predict invasiveness: a model study of transgenic plants. *Ecology*, **75** : 249-252.
- Blake, M.S., Johnston, K.H., Russell-Jones, G.J. and Gotschlich, E.C. (1984).  
A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on western blots. *Analytical Biochemistry*, **136** : 175-179.

Brett, J. R. (1958).

Implications of assessment of environmental stress, H.R. MacMillan Lectures in Fisheries, University of British Columbia, Vancouver, Canada.

Cecim, M., Ghosh, P. K., Esquifino, A. I., Began, T., Wagner, T. E., Yun, J. S. and Bartke, A. (1991).

Elevated corticosterone levels in transgenic mice expressing human or bovine growth hormone genes. *Neuroendocrinology*, **53** : 313-316.

Cheung, A., Hall, T. R. and Harvey, S. (1988).

Stimulation of corticosterone release in the fowl by recombinant DNA-derived chicken growth hormone. *General and comparative endocrinology*, **69** : 128-132.

Chevassus, B. and Dorson, M. (1990).

Genetics of resistance to disease in fishes. *Aquaculture*, **85** : 83-107.

Coyne, M. D., Alpert, L. C., Harter, K. C. and Nunez, A. (1981).

Effect of the growth hormone-secreting tumor StW5 on pituitary and adrenal gland function in rats. *Hormone Research*, **14** : 36-46.

Crawley, M. J., Hails, R. S., Rees, M., Kohn, D. and Buxton, J. (1993).

Ecology of transgenic oil seed rape in natural habitats. *Nature*, **363** : 620-623.

Donaldson, E.M. and Devlin, R.H. (1996).

Uses of biotechnology to enhance production. In: *Principles of salmonid culture*. W. Pennell and B.A. Barton (eds). Elsevier, Amsterdam, pp 969-1020.

Devlin, R. H., Yesaki, T. Y., Biagi, C. A., Donaldson, E. M., Swanson, P. and Chan, W. K. (1994).

Extraordinary salmon growth. *Nature*, **371** : 209-210.

Devlin, R. H., Yesaki, T. Y., Donaldson, E. M. and Hew, C. L. (1995).

Transmission and phenotypic effects of an antifreeze/GH gene construct in coho salmon (*Oncorhynchus kisutch*). *Aquaculture*, **137** : 161-169.

Du, S.J., Gong, Z., Fletcher, G.L., Shears, M.A., King, M.J., Idler, D.R., and Hew C.L. (1992).

Growth enhancement in transgenic Atlantic salmon by the use of an "all fish" chimeric growth hormone gene construct. *BioTechnology* **10** : 176-181.

Edens, F. W. and Siegel, H. S. (1975).

Adrenal responses in high and low ACTH-response lines of chickens during acute heat stress. *General and Comparative Endocrinology*, **25** : 64-73.

Edwards, C. K. I., Yunger, L. M., Lorence, R. M., Dantzer, R. and Kelley, K. W. (1991).

The pituitary gland is required for protection against lethal effects of *Salmonella typhimurium*. *Proceedings of the National Academy of Science USA*, **88** : 2274-2277.

- Farrell, A. P., Bennett, W. and Devlin, R. H. (1997). Growth-enhanced transgenic salmon can be inferior swimmers. *Canadian Journal of Zoology*, **75** : 335-337.
- Fevolden, S. E., Refstie, T. and Roed, K. H. (1991). Selection for high and low stress response in Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, **95** : 53-65.
- Flanagan, S. W., Ryan, A. J., Gisolfi, C. V. and Moseley, P. L. (1995). Tissue-specific HSP70 response in animals undergoing heat stress. *American Journal of Physiology*, **268** : R28-32.
- Foster, L. B. and Dunn, R. T. (1974). Single-antibody technique for radioimmunoassay of cortisol in unextracted serum or plasma. *Clinical Chemistry*, **20** : 365-368.
- Forsyth, R.B., Candido, E.P.M., Babich, S.L. and Iwama, G.K. (1997). Stress protein expression in coho salmon with bacterial kidney disease. *Journal of Aquatic Animal Health*. **9** : 18-25.
- Galbreath, P. F., St.Jean, W., Anderson, V. and Thorgaard, G. H. (1994). Freshwater performance of all-female diploid and triploid Atlantic salmon. *Aquaculture*, **128** : 41-49.
- Gill, J.A., Sumpter, J.P., Donaldson, E.M., Dye, H.M., Souza, L., Berg, T., Wypych, J. and Langley, K. (1985). Recombinant chicken and bovine growth hormones accelerate growth in aquacultured juvenile pacific salmon *Oncorhynchus kisutch*. *Bio/Technology* **3**: 643-646.
- Gordon J.W., Sxcangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddie, F.H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proceedings of the National Academy of Science USA*, **77** (12):7380-7384.
- Grinde, B. (1989). Lysozyme from rainbow trout (*Salmo gairdneri*) as an antibacterial agent against fish pathogens. *Journal of Fish Diseases*, **12** : 95-104.
- Hedrick, R. P. (1998). Relationships of the host, pathogen, and environment: Implications for disease of cultured and wild fish populations. *Journal of Aquatic Animal Health*, **10** : 107-111.
- Hew, C. L., Fletcher, G. L. and Davies, P. L. (1995). Transgenic salmon: tailoring the genome for food production. *Journal of Fish Biology*, **47** (Supplement A) : 1-19.
- Iwama, G.K., Thomas, P.T., Forsyth, R.B and Vijayan, M.M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries*, **8** : 35-56.

- Johnson, O. W., Dickhoff, W. W. and Utter, F. M. (1986).  
Comparative growth and development of diploid and triploid coho salmon,  
*Oncorhynchus kisutch*. *Aquaculture*, **57** : 329-336.
- Kajita, Y., Sakai, M., Kobayashi, M. and Kawauchi, H. (1992).  
Enhancement of non-specific cytotoxic activity of leucocytes in rainbow trout  
*Oncorhynchus mykiss* injected with growth hormone. *Fish & Shellfish Immunology*, **2** :  
155-157.
- Kotzmann, H., Koller, M., Czernin, S., Clodi, M., Svoboda, T., Riedl, M., Boltz-Nitulescu,  
C. and Luger, A. (1994).  
Effect of elevated growth hormone concentrations on the phenotype and functions of  
human lymphocytes and natural killer cells. *Neuroimmune interactions*, **60**
- Laemmli, U. K. (1970).  
Cleavage of structural proteins during the assembly of the head of bacteriophage T4.  
*Nature*, **227** : 680-685.
- LaPatra, S. E., Fryer, J. L., Wingfield, W. H. and Hedrick, R. P. (1989).  
Infectious hematopoietic necrosis virus (IHNV) in coho salmon. *Journal of Aquatic  
Animal Health*, **1** : 277-280.
- Lund, T., Gjedrem, T., Bentsen, H. B., Eide, D. M., Larsen, H. J. S. and Roed, K. H.  
(1995).  
Genetic variation in immune parameters and associations to survival in Atlantic salmon.  
*Journal of Fish Biology*, **46** : 748-758.
- McGeer, J. C., Baranyi, L. and Iwama, G. K. (1991).  
Physiological responses to challenge tests in six stocks of coho salmon (*Oncorhynchus  
kisutch*). *Canadian Journal of Fisheries and Aquatic Sciences*, **48** : 1761-1771.
- McLean E. And Donaldson, E.M. (1993).  
The role of growth hormone in the growth of poikilotherms. In: *The endocrinology of  
growth , development, and metabolism in vertebrates*, Schreibman, M.P., Scanes, C.G.  
and Pang P.K. (eds). Academic Press, San Diego, CA, pp 43-71
- Miller, M., Kawabata, S., Wiltshire-Clement, M., Reventos, J. and Gordon, J. W. (1993).  
Increased vasopressin secretion and release in mice transgenic for the rat arginine  
vasopressin gene. *Neuroendocrinology*, **57** : 621-625.
- Moyner, K., Roed, K., Sevatdal, S. and Heum, M. (1993).  
Changes in non-specific immune parameters in Atlantic salmon, *Salmo salar* L., induced  
by *Aeromonas salmonicida* infection. *Fish & Shellfish Immunology*, **3** : 253-265.
- Müller, M. and Brem, G. (1991).  
Disease resistance in farm animals. *Experientia*, **47** : 923-936.

- Osserman, E.F. and Lawlor, D.P. (1966).  
Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *Journal of Experimental Medicine*, **124**: 921-951.
- Parry, R.M., Chandau, R.C. and Shahani, R.M. (1965).  
A rapid and sensitive assay of muramidase. *Proceedings of the Society for Experimental Biology & Medicine*. **119**: 384-386.
- Pickering, A. D., Pottinger, T. G., Sumpter, J. P., Carragher, J. F. and Le Bail, P. Y. (1991).  
Effects of acute and chronic stress on the levels of circulating growth hormone in the rainbow trout, *Oncorhynchus mykiss*. *General and comparative endocrinology*, **83** : 86-93.
- Pursel, V. G. and Rexroad, C. E. J. (1993).  
Status of research with transgenic farm animals. *Journal of Animal Science*, **71** (Supplement 3) : 10-19.
- Saito, H., Inoue, T., Fukatsu, K., Ming-Tsan, L., Inaba, T., Fukushima, R. and Muto, T. (1996).  
Growth hormone and the immune response to bacterial infection. *Hormone Research*, **45** : 50-54.
- Sakai, M., Kobayashi, M. and Kawauchi, H. (1996).  
In vitro activation of fish phagocytic cells by GH, prolactin and somatolactin. *Journal of Endocrinology*, **151** : 113-118.
- Secombes, C. J. and Fletcher, T. C. (1992).  
The role of phagocytes in the protective mechanisms of fish. *Annual Review of Fish Diseases*, **2** : 53-71.
- Small, S. A. and Benfey, T. J. (1987).  
Cell size in triploid fish. *The Journal of Experimental Zoology*, **241** : 339-342.
- Snow, A. and Moran Palma, P. (1997).  
Commercialization of transgenic plants: potential ecological risks. *BioScience*, **47** : 86-96.
- Steger, R. W., Bartke, A. and Cecim, M. (1993).  
Premature ageing in transgenic mice expressing different growth hormone genes. *Journal of Reproduction and Fertility*, **46** (Suppl.) : 61-75.
- Swarup, H. (1959).  
Effect of triploidy on the body size, general organization and cellular structure in *Gasterosteus aculeatus*(L.). *Journal of Genetics*, **56** : 143-155.



- Tatner, M. F. and Horne, M. T. (1983).  
Susceptibility and immunity to *Vibrio anguillarum* in post-hatching rainbow trout fry, *Salmo gairdneri* Richardson 1836. *Developmental and Comparative Immunology*, **7** : 465-472.
- Utter, F. M., Johnson, O. W., Thorgaard, G. H. and Rabinovitch, P. S. (1983).  
Measurement and potential applications of induced triploidy in Pacific salmon. *Aquaculture*, **35** : 125-135.
- Vijayan, M., Pereira, C. and Moon, T. W. (1994).  
Hormonal stimulation of hepatocyte metabolism in rainbow trout following an acute handling stress. *Comparative Biochemistry and Physiology*, **108C** : 321-329.
- Wasada, T., Aoki, K., Sato, A., Katsumori, K., Muto, K., Tomonaga, O., Yokoyama, H., Iwasaki, N., Babazono, T., Takahashi, C., Iwamoto, Y., Omori, Y. and Hizuka, N. (1997).  
Assessment of insulin resistance in acromegaly associated with diabetes mellitus before and after transphenoidal adenomectomy. *Endocrine Journal*, **44** : 617-620.
- Wedemeyer G. (1972).  
Some physiological consequences of handling stress in the juvenile coho salmon (*Oncorhynchus kisutch*) and steelhead trout (*Salmo gairdneri*). *Journal Fisheries Research Board of Canada*, **29** (12):1780-1783.
- Wedemeyer, G. A., Barton, B. A. and McLeay, D. J. (1990).  
Stress and Acclimation. In : *Methods for fish Biology*, Schreck, C.B. and Moyle, P.B. (eds). American Fisheries Society, Bethesda, MD, pp 451-489.
- Weigent, D.A., Baxter, J.B., Wear, W.E., Smith, L.R., Bost, K.E., and Blalock, J.E. (1988).  
Production of immunoreactive growth hormone by mononuclear leukocytes. *FASEB J.*, **2**: 2812-2818.
- Withler, R. E., Beacham, T. D., Solar, I. I. and Donaldson, E. M. (1995).  
Freshwater growth, smolting, and marine survival and growth of diploid and triploid coho salmon (*Oncorhynchus kisutch*). *Aquaculture*, **136** : 91-107.
- Wolf, E., Kahnt, E., Ehrlein, J., Hermanns, W., Brem, G. and Wanke, R. (1993).  
Effects of long-term elevated serum levels of growth hormone on life expectancy of mice: lessons from transgenic animal models. *Mechanisms of Ageing and Development*, **68** : 71-87.
- Yamamoto, A. and Iida T. (1995a).  
Susceptibility of triploid rainbow trout to IHN, furunculosis and vibriosis. *Fish Pathology* **30** (2) : 69-70.
- Yamamoto, A. and Iida T. (1995b).  
Non-specific defense activities of triploid rainbow trout. *Fish Pathology* **30** (2) : 107-110.

Young, G. (1988).

Enhanced response of the interrenal of coho salmon (*Oncorhynchus kisutch*) to ACTH after growth hormone treatment in vivo and in vitro. *General and Comparative Endocrinology*, **71** : 85-92.