CARDIORESPIRATORY MODIFICATIONS, AND LIMITATIONS, IN GROWTH HORMONE TRANSGENIC ATLANTIC SALMON (Salmo salar)

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Cardiorespiratory Modifications, and Limitations, in Growth Hormone Transgenic Atlantic Salmon (*Salmo salar*)

By

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Abstract

GH transgenic Atlantic salmon transgenic grew 3.6x faster than non-transgenic controls, and most aspects of their cardiorespiratory system were upregulated. For example, they had greater resting and post-stress catecholamine levels, higher post-stress haemoglobin concentrations, increased maximum heart performance, and elevated aerobic enzyme activities. However, they had a higher routine metabolic rate without a change in maximum metabolic rate (MO_{2max}), resulting in a decreased metabolic scope, and subsequently a lowered critical swimming speed. Overall, this study supports the theory of symmorphosis, in that most aspects of the transgenic's cardiorespiratory system are upregulated to accommodate the additional demands of higher growth and/or activity. However, the transgenics MO_{2max} appears to be limited by their gill surface area, which was not upregulated. Further, it provides information on their physiology that can be used to: 1) understand their needs in an aquacultural setting; and 2) infer the possible ecological consequences of their escape.

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1.0 Introduction

1.1 Relevance of Study

Cultured Atlantic salmon yields have more than tripled in the last decade, owing in part to a greater knowledge of husbandry practices, better feeds, and better quality strains (ASF 2003). With time to market-weight being a major factor that determines profitability, faster growing fish are necessary if the industry is to thrive while providing an affordable product. Genetic modification has been recently used as an innovative way to achieve higher growth rates. This technique creates fish that contain an extra copy of the growth hormone gene, and they can display dramatically accelerated growth rates (~2-10-fold) compared to that of non-transgenic salmon (Du *et al.* 1992; Devlin 1994; Stokstad 2000). Although several studies have examined the biology of growth hormone (GH) transgenic salmon, information is often conflicting due to the use of different strains, controls, and species (coho, *Oncorhynchus kisutch*, and chinook, *Oncorhynchus tshawytscha*, salmon have also been genetically modified).

1.2 Previous Findings

Enhanced growth is accompanied by an increased demand for oxygen to supply the aerobic pathways that support growth and protein deposition (Hogendoorn 1983) and the fish's basal functions. Elevated routine metabolism (MO_2) in GH transgenic (or growth hormone treated) fish has been observed in previous studies (Stevens *et al.* 1998;

Cook *et al.* 2000b; Herbert *et al.* 2001), but few studies have gone into any depth to examine the ways in which these fish obtain oxygen and transport it throughout their bodies. Stevens and Sutterlin (1999) found that transgenic salmon have a greater gill surface area that presumably allows for enhanced oxygen uptake from the water. Further, GH enhanced salmon have been shown to differ in tissue enzyme activities (Blier *et al.* 2002), blood characteristics (Cogswell *et al.* 2002), and physical activity levels (Abrahams and Sutterlin 1999; Herbert *et al.* 2001). These apparent adaptations of their physiological and biological systems lead one to suspect that most of the steps in the oxygen utilization pathway have been upregulated to a certain extent.

1.3 Application of the Theory of Symmorphosis

A universal upregulation of the transgenic's metabolic systems would be in agreement with the theory of symmorphosis; a theory which states that all of an animal's systems have just enough structural capacity to be energetically optimal at an intermediate level between the highest and lowest strains they face (Weibel 2002). Pörtner (2002) found evidence that temperature acclimation can be used as a good model of symmorphosis, as organisms maintain their capacity for oxygen delivery at an average level between that of the highest and lowest temperatures which they encounter. Thus, if this theory holds true, GH transgenic salmon should have upregulated (although not necessarily equally) all aspects of their cardiorespiratory system to meet the higher metabolic demands placed on them. The findings of Stevens and Sutterlin (1999) and Stevens *et al.* (1998) suggest that this may be occurring in GH transgenic Atlantic

salmon, as they had a gill surface area 1.24 times that of the non-transgenic controls but an oxygen uptake of 1.6 times, with no explanation for the discrepancy. Clearly, other aspects of oxygen uptake, transport, or utilization must have been enhanced to account for this difference.

1.4 Oxygen Demand and Swimming Performance

This study was designed to examine the GH transgenic's capacity to obtain and transport oxygen, and the efficiency with which oxygen is utilized. Perhaps the most interesting and important variable examined was routine MO_2, as this parameter is a measure of whether GH transgenic salmon have an inherently higher metabolic rate. Past studies have found divergent data with respect to routine MO_2, and report either no difference (Leggatt *et al.* 2003) or an elevated rate of oxygen consumption (Stevens *et al.* 1998; Herbert *et al.* 2001; Lee *et al.* 2003). The data collected in these studies, however, is not easy to compare due to differences in the species used, and variability in experimental design and methodology. Studies have been done on young fish (~10g, Herbert *et al.* 2001), and adult fish (over 2kg, Lee *et al.* 2003), and have used holding temperature (Stevens *et al.* 1998) or age (Lee *et al.* 2003) to size-match groups. Some authors have also drawn conclusions based on comparing GH transgenic fish with those given doses of exogenous growth hormone (Herbert *et al.* 2001). Finally, testing has been done on both coho and Atlantic salmon, two species that belong to different genera (*Oncorhynchus* and *Salmo*, respectively) and can have different life histories. Therefore,

past studies tended to draw conclusions based on unique protocols, with inconsistencies in experimental design, making comparative data interpretation difficult.

GH transgenics (or GH implanted fish) have an increased level of spontaneous activity as compared to controls (Abrahams and Sutterlin 1999; Herbert *et al.* 2001), which could elevate oxygen consumption (Fry 1971), and result in an overestimation of routine MO_2. Herbert *et al.* (2001) corrected for this by taking oxygen consumption measurements only when the fish were in a stationary position, with only minimal fin movements. When this was done, no significant difference in oxygen consumption was evident between GH treated and control fish. Measurements of metabolism are further complicated by the fact that transgenic fish have an increased appetite, and eat more than non-transgenic individuals (Cook 1999; Cook *et al.* 2000b; Leggatt *et al.* 2003). This results in an increase in the total amount of oxygen utilized for digestion (specific dynamic action, SDA). Therefore, it has also been suggested that the greater routine MO_2 reported in previous studies may just be a function of an increase in feeding and activity. The current study controlled for differences in activity and SDA between the groups by taking routine MO_2 measurements only on food deprived fish (fasted for 48 hours prior to testing) while they were stationary and made no large, energetically costly, movements.

Maximum performance, in terms of maximum oxygen consumption (MO_{2max}) and critical swimming speed (U_{crit}), also provides a means for testing differences between GH transgenic and non-transgenic fish. Previous studies have report that transgenic fish have a lower (by 11%, Lee *et al.* 2003), higher (by 60%, Stevens *et al.* 1998), or no difference

(McKenzie *et al.* 2003) in MO_{2max} when compared to non-transgenic controls. There is also conflicting data with regards to U_{crit}, with studies finding that GH transgenic salmon can be inferior (Farrell *et al.* 1997; Lee *et al.* 2003) or equivalent swimmers (Stevens *et al.* 1998) as non-transgenics. These divergent findings could be due to the different experimental protocols and the different species of transgenic salmon used. The current study is able to provide further insight into why the transgenic salmon's performance may differ from the controls, as in-depth enzymatic, haematological, and cardiac analyses were done concurrently with the swimming tests.

1.5 Enzyme and Tissue Analyses

One curious trait of Atlantic salmon is their tendency to exhibit a bimodal size distribution during their first winter of life; with one group ceasing feeding and stopping growth, while the other continues to feed and grow (Thorpe 1977; Thorpe *et al.* 1982; Nathanailides and Stickland 1996). This results in fish that are either fast-growers, or slow-growers. This situation was artificially reproduced here, using the GH transgenics as the fast-growers and non-transgenics as the slow-growers. This allowed for the comparison of aerobic mitochondrial enzyme activity in fish that were affected only by endogenous physiological factors, rather than having them being manipulated by environmental changes. It is the function of these mitochondrial enzymes to provide the energy needed to fuel the fish's growth and metabolism, and it was hypothesised that the enhanced growth of GH transgenic Atlantic salmon may provide enough of a selective pressure to upregulate their aerobic enzyme activity.

Cytochrome-C oxidase (CCO) is one of these enzymes, and plays a key role in aerobic mitochondrial metabolism, as it functions as the final step in the electron transport chain. The activity of this enzyme can be used as a measure of total fish metabolism (Goolish & Adelman 1987), correlates well with oxygen consumption rates in different tissues (Simon and Robin 1971), and has been shown to increase in fast growing fish (Houlihan *et al.* 1993). Citrate synthase (CS), a key Kreb's Cycle enzyme, is another candidate for growth rate induced upregulation. A study by Blier *et al.* (2002) found no difference in CS in the white muscle and gills of GH transgenic coho salmon, and concluded that enzyme activities in these tissues weren't related to the high growth rates in GH transgenics. However: 1) white muscle has a low aerobic capacity and may not be as affected by an increase in oxygen usage as more aerobic tissues; and 2) although the gills have a high protein turnover that requires a constant supply of oxygen for protein synthesis, Stevens and Devlin (2000) suggested that increased gill surface area may be the primary method of upregulation in the gills, not an increase in enzyme activity. Therefore, it is possible that other, highly aerobic tissues (e.g. the heart and red muscle) may exhibit growth-dependent alterations in CS and/or CCO activity to allow for increased energy demand.

Enzymes can also be a useful tool to test symmorphosis as they are costly to produce, are needed in fairly high amounts, and their activities are closely regulated by organisms. For example, enzymes have been shown to increase their activity to compensate for thermal gradients within tissues (Fudge *et al.* 2001). Moreover, the

theory of symmorphosis would suggest that an increase in the activity of key aerobic enzymes is needed to fuel a higher growth rate.

1.6 Blood Oxygen Transport Capability

Another parameter in the cardiorespiratory system that is a good candidate for growth rate dependent upregulation is the oxygen transport capacity of the blood. As the blood moves through the capillaries of the gill lamellae, the amount of oxygen extracted from the water is largely dependent on the oxygen binding affinity of haemoglobin, the number of erythrocytes, and the concentration of haemoglobin. An increase in the oxygen carrying capacity of the GH transgenic salmon's blood, mediated by increased haematocrit and/or haemoglobin concentration, could allow for enhanced oxygen uptake and transport. Therefore, haematological measurements of GH transgenic and control fish were performed to determine whether GH transgenic Atlantic salmon exhibited any differences in blood oxygen transport properties.

To date, only one study has looked for differences in the blood composition of transgenic Atlantic salmon as compared to controls (see Cogswell *et al.* 2002). These authors found that transgenic salmon had smaller and more numerous erythrocytes, and theorized that two factors may have caused these differences: 1) elevated growth hormone levels lead to an increase in insulin-like growth factor, resulting in a faster cell cycle, and thus smaller cells; or 2) the decrease in cell volume (and subsequent increase in the surface area to volume ratio) is an adaptive mechanism to deal with the increased oxygen needs of the transgenic fish. The latter theory does have some merit, as previous

studies have linked a higher metabolic rate to a decrease in erythrocyte cell volume (Glomski *et al.* 1992). Other haematological factors that may aid the transgenics in acquiring and transporting more oxygen, such as a greater haematocrit and haemoglobin concentration, were also assessed in this study.

1.7 Stress Hormones

Increases in the levels of plasma stress hormones (e.g. cortisol and catecholamines) can be a problem in commercial aquaculture, because GH levels are depressed in stressed fish, and fish can lose energy stores due to increased activity (avoidance) or through the cessation of feeding (Pickering 1981). The aquacultural environment provides a host of potential stressors for tank-reared fish, such as handling, crowding, and competition for food. It is important to know how stressed fish are in order to determine ways to reduce its negative affects, and thus achieve maximum growth. Repeated stress over time can have adverse affects on growth, reproduction, and resistance to disease (Wendelaar Bonga 1997), all of which are factors that hinder production and decrease profits. Therefore, knowing how fish respond to environmental perturbations can lead to innovations that may help reduce stress and increase productivity. This was the reasoning behind measuring plasma stress hormone levels in GH transgenic Atlantic salmon, as stress is negatively correlated to growth rate and deserves attention if one is to truly assess the growth performance of fish.

The stress response in fish is a function of both external stimuli and how a fish perceives and reacts to these events. The most obvious response to external stresses is

avoidance, whereby the fish tries to escape from a particular stressor into an area where it is no longer affected by it. In order to do this, the fish needs to quickly mobilize energy reserves in the form of stored carbohydrate, lipids, and proteins. Catecholamines are released into the blood stream from the chromaffin cells in response to many situations that require increased oxygen transport or increased energy mobilization (Wendelaar Bonga 1997). They initiate the catabolism of energy stores in various tissues through activation of cell surface receptors (Donaldson 1981; Pickering *et al.* 1991). Also, they cause erythrocytes to be released from the spleen (Nilsson and Grove 1974; Kita and Itazawa 1989; Perry and Kinkead 1989), and regulate erythrocyte pH to control oxygen binding capacity (Nikinmaa *et al.* 1984; Primmett *et al.* 1986). Both of these haematological changes allow more oxygen to be transported during times of high demand. Cortisol is released from the interrenal tissue (contained in islets around the posterior cardinal vein) (Pickering 1981), and has been shown to be beneficial in carbohydrate metabolism (Brown 1993). Thus, it allows for a mobilization of energy stores that give the fish the ability to escape quickly, or the stamina to stay and fight.

The increased GH production in GH transgenic salmon raises an important question: i.e. how do elevated GH levels affect plasma stress hormone levels? Although it is has been shown that GH and cortisol have an inverse relationship in Atlantic salmon (McCormick *et al.* 1998), there are also data that suggest a positive relationship exists between these two hormones (Dufour *et al.* 2000; McCormick 2001). Thus, this study measured both the levels of catecholamines (epinephrine and norepinephrine) and cortisol to determine whether GH transgenic Atlantic salmon have an altered stress response.

1.8 Heart Morphometrics and Performance

Finally, the potential contribution of the heart to the GH transgenic salmon's increased growth rate was examined. To my knowledge, only two studies have examined the hearts of transgenic fish as compared with non-transgenic conspecifics. One study, by Pitkanen *et al.* (2001), measured the DNA concentration (a parameter used to measure cell number) in several muscle tissues in growth enhanced Arctic char (*Salvelinus alpinus*). They found that the genetically modified fish had a significantly greater mass specific heart size (by 20-32%) and a higher DNA concentration (indicating more cells). Although cardiac performance wasn't measured in their study, it can be inferred that the transgenic Arctic char's heart would have a greater pumping capacity due to its greater size. In contrast, McKenzie *et al.* (2003) found no difference in the size of transgenic tilapia hearts as compared to their non-transgenic counterparts. The cause of the discrepancy between studies is not known, but species and size differences, the type and makeup of the transgene, and environmental variables may all play a role.

Many studies have shown that the heart is extremely plastic and can adapt to meet additional demands placed on it (see Gamperl and Farrell 2004 for a review). Fish kept at low temperatures have a greater heart size to compensate for the increase in blood viscosity that occurs in cold water (Goolish 1987; Graham and Farrell 1992; Driedzic *et al.* 1996). Also, levels of GH and testosterone increase as fish mature, and these hormones have been shown to have a significant stimulating effect on heart size and pumping capacity (Franklin and Davie 1992; Yang *et al.* 1999). Hearts get larger during maturation to ensure that adequate amounts of oxygen are available to meet the demands

of reproduction and the stressful events that come with it (i.e. fighting conspecifics, digging a redd, etc.) (Franklin and Davie 1992). Interestingly, Fleming *et al.* (1996) found that domesticated salmon's hearts were smaller than wild salmon's despite having higher GH levels (Fleming *et al.* 2002). This, however, is most likely caused by the much higher levels of physical activity that wild fish have over their tank-reared counterparts, physical training can increase heart size and results in an enhancement of cardiac function (Davison 1989; Farrell *et al.* 1991). Thus, it is likely that heart size and function have also been upregulated in the GH transgenic Atlantic salmon to meet the increased metabolic demands associated with elevated growth rates, as well as increased levels of activity and SDA as compared to their non-transgenic counterparts.

The current study determined whether the above statement is true by measuring ventricle size (Relative Ventricular Mass, RVM) and several indices of resting and maximum *in situ* cardiac performance [stroke volume (S_v), cardiac output (Q), heart rate (f_H), and myocardial power output (P_o)]. These measurements allowed for a comprehensive comparison of heart performance in GH transgenic salmon vs. controls, and gave a clear picture of how the hearts of salmon from both groups performed under normal and stressful conditions.

1.9 Overall Purpose of the Study

Based on the need for more data on the physiology of GH transgenic salmon, the study outlined above was conducted to provide a detailed and accurate picture of how these fish differ from non-transgenic conspecifics. This is the most comprehensive

examination of the cardiorespiratory system of GH transgenic fish to date, and identifies how the cardiorespiratory system of post-smolt fish has adapted to accelerated growth, and/or activity, as compared with size-matched non-transgenic conspecifics. Further, the transgenic salmon act as a novel model to test the theory of symmorphosis, as they may demonstrate how an animal upregulates various body systems in order to cope with an increase in physical demand. Ultimately. this research will allow for a better understanding of the physiology of GH transgenic Atlantic salmon, and provide valuable information that can be used to maximize their production in an aquaculture setting. In addition, it is my opinion that these experiments will reveal the inherent strengths and weaknesses of post-smolt GH transgenic Atlantic salmon, and infer the extent to which they may compete/interact with natural populations if they escape into the wild.

2.0 Materials and Methods

2.1 Experimental Fish

In this document, fish referred to as 'transgenics' are the fifth generation of the EO-1α transgenic strain of Atlantic salmon that were created in 1989 by injecting fertilized eggs with a chimeric growth hormone (GH) gene construct (opAFP-GHc2) consisting of an ocean pout (*Macrozoarces americanus*) antifreeze protein gene promoter linked to chinook salmon growth hormone cDNA (Du *et al.* 1992). The EO-1α transgenics strain contains a single copy of the transgene (Fletcher personal communication). This transgene and its rapid growth phenotype have exhibited stable Mendelian inheritance over 6 generations to date (Fletcher *et al.* 2004). Control salmon were unmodified individuals from the same Saint John River strain as the transgenics that were one year older (ages were approximately 20 months and 8 months post-fertilization at the time of testing).

All fish were reared from the smolt stage in seawater (32ppt) in a 6 m diameter by 1.5 m deep tank at 10°C, with seasonally ambient photoperiod (at the Ocean Sciences centre St. John's, NL). They were fed (with high energy dry extruded salmon feed, Corey Feed Ltd.) by automatic feeder seven times daily. This regime allowed the controls and transgenics to grow at 0.30 and 1.03 % day^{-1}, respectively, over the experimental period (~December 2002 to September 2003). One week prior to each experiment, the test fish were removed from the 6 m diameter tank and placed in a 2 m diameter, by 1.5 m deep, holding tank, which received the same water and photoperiod as the large tank.

This allowed for the separation of the transgenic and non-transgenic salmon, and for ease of capture with minimal stress on the animals.

When performing these types of experiments one must take into account the inherent differences between individuals with respect to a range of biological factors. For example, Dickson and Kramer (1971) showed that factors such as sexual maturity, temperature, and season have a marked effect on a fish's metabolism. Therefore, each experiment compared size-matched control and transgenic fish (500-1300 g, depending on the particular experiment), and they were all tested at the same temperature (10°C). In order to size-match the transgenic and control fish, the transgenics were approximately 1 year younger than the controls. The use of two different year classes was also done in other studies (Farrell *et al.* 1997; Stevens and Devlin 2000), and although this results in fish of two different life histories, it is still preferable to using temperature to retard the transgenic's growth rates (as in Stevens *et al.* 1998 and Cook *et al.* 2000a&b). Temperature alone can cause significant variance in metabolism since many physiological variables in fish change by 2–3 fold with each 10°C alteration in temperature (Q_{10} 2-3). Even though the controls are a year older, this age difference did not appear to affect their degree of sexual maturity. Most fish showed no signs of sexual characters (e.g. no egg sac), and those that did were only in the early stages of development (i.e. they were immature).

All salmon used for the experiments were tested in order to confirm whether they were transgenic or non-transgenic by polymerase chain reaction (PCR) using adipose fin tissue (Figure 1). A buffer containing 10 mM Tris (pH 8.0), 50 nM KCl, 1.5 mM $MgCl_2$,

and 0.1% Triton X-100 was used for all PCR analyses. The primers used to detect the transgene construct were 2653-GCT-CTT-CAA-CAT-CGC-GGT-CA and 654-ATA-TGG-AGC-AGC-TTC-AGG-AC. Samples were analyzed by electrophoresis using a 2% agarose gel and stained with ethidium bromide, then visualized by exposure to UV light and photographed.

2.2 Metabolic Physiology and Swimming Performance

2.2.1 Experimental Design

Swimming tests were done using an 81 litre Blazka-type swim-tunnel respirometer (University of Waterloo, Biotelemetry Institute, Waterloo, ON). The front of this respirometer contained a plastic grid that created uniform water flow (Taylor and McPhail 1985), and was covered with black plastic that provided the fish with a dark refuge (this minimized stress due to external stimuli). The rear of the tunnel was fitted with a plastic grid as well as electrified (<5V) stainless steel bands to prevent the fish from resting on the grid. Aerated seawater (10°C) was supplied to the respirometer at 2 L min^{-1} by a submersible pump (Little Giant Pump Co., Oklahoma City, OK) that was placed in a 270 L, aerated, reservoir whose temperature was regulated by a 3/4 horsepower heater/chiller (Memorial University of Newfoundland Technical Services, St. John's NL). Initially, measurements of water oxygen content were recorded for 20

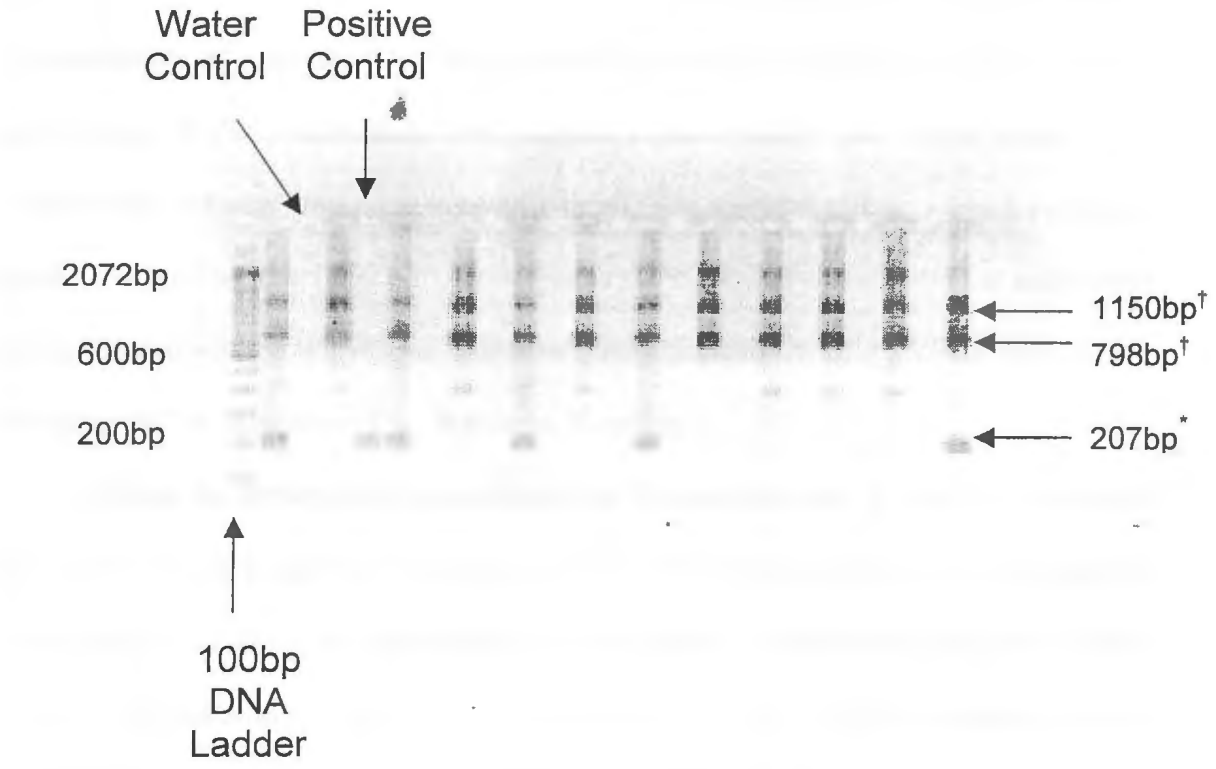


Figure 1. Identification of transgenic and control Atlantic salmon using polymerase chain reaction (PCR). All samples were analyzed by electrophoresis using a 2% agarose gel and visualized with ethidium bromide. A water control was run to ensure that no exogenous genetic material was present in the samples and a positive control indicated the position of the transgene. * Presence of a band (207bp) indicates a sample is from a transgenic Atlantic salmon. † Banding representing the endogenous Atlantic salmon GH genes, GH1 (1150bp) and GH2 (798bp).

minutes while the tunnel was closed, and contained no fish. This procedure allowed for correction of the fish's metabolism due to bacterial respiration and photosynthesis. Measurements of routine MO_2, MO_{2max}, and U_{crit} were then performed on 8 fish from each group. Water temperature and oxygen content within the swim tunnel were continuously measured by pumping water through an external circuit using a peristaltic pump (Masterflex model 7523-20, Cole Palmer), and oxygen consumption at each speed was measured with a WTW Oxi 340 meter in conjunction with a CellOx 325 oxygen electrode (WTW Instrument Co., Weilheim, Germany).

Before the respirometry experiments, the 8 transgenics and 8 controls were fasted for 2 days. Their length was measured, and they were then placed in the respirometer approximately 12-16 hours before testing to allow them to acclimate to the tunnel, and to recover from handling. All fish were initially given a 30 minute conditioning swim (water velocity 0-2 BL s^{-1}) to accustom them to swimming in the tunnel and to changes in water velocity (Jain *et al.* 1997). During the remainder of the acclimation period, the respirometer was constantly flushed with aerated seawater, and a current of 0.5 BL s^{-1} was maintained.

2.2.2 Measurements of Critical Swimming Speed and Metabolism

After the 12-16 hour acclimation period, the swimming performance of individual fish was tested using a standard procedure known as the U_{crit} test (Hammer 1995). This test involves increasing water velocity in small increments until the fish is totally fatigued, and stops swimming. After measuring the routine metabolism of each fish at

0.5 BL s^{-1}, water velocity in the tunnel was increased by 0.25 BL s^{-1} every 20 minutes until the fish was no longer capable of swimming (Farrell *et al.* 1997). The critical swimming speed was then calculated using the equation:

$$U_{crit} = U_f + (t_f / t_i \times U_i)$$

Where: U_f is the water velocity of the last completed increment, t_f is the time spent at the last water velocity increment, t_i is the time period spent at each water velocity increment (20 minutes), and U_i is the water velocity increment (0.25 BL s^{-1}) (Brett 1964). Because the cross-sectional area of the fish was greater than 10% of the swim-tunnel's cross-sectional area, U_{crit} values were corrected for solid blocking effects using the formula of Bell and Terhune (1970):

$$U_F = U_T (1 + \epsilon_s)$$

Where: U_F is the corrected swimming speed, U_T is the water velocity in the tunnel without a fish, and ϵ_s is the fractional error due to solid blocking. ϵ_s was defined for each fish by:

$$\epsilon_s = \tau\lambda (A_o/A_T)^{1.5}$$

Where: τ is a factor dependent on the swim chamber's cross sectional area (cross sectional area/tunnel length), λ is the shape factor for the test object (λ= 0.5 BL body thickness^{-1}), A_o is the cross sectional area of the fish (obtained by bisecting the fish at its

widest point and taking a digital image (using a Coolpix 2500 digital camera, Nikon USA, Melville, NY) and analyzing it using the digital imagining software Matrox Inspector 3.0 (Matrox Electronic Systems Ltd., Dorval, Qc)), and A_T is the cross sectional area of the swim chamber.

Oxygen uptake was measured at every velocity increment throughout the experiment. Routine MO_2 (mg O_2 kg^{-1} hr^{-1}) was measured when the fish was exposed to a 0.5 BL s^{-1} current and making minimal movements. Standard oxygen consumption (mg O_2 kg^{-1} hr^{-1}) was obtained from a semi-log plot of swimming speed (BL sec^{-1}) and routine MO_2 (LOG mg O_2 kg^{-1} hr^{-1}), and using the derived linear regression to extrapolate back to 0 BL sec^{-1}. MO_{2max} (mg O_2 kg^{-1} hr^{-1}) represents the highest oxygen consumption that the fish was able to achieve. Absolute metabolic scope was calculated by subtracting standard MO_2 from MO_{2max}, and factorial metabolic scope was calculated by dividing MO_{2max} by standard MO_2.

After all metabolic and swimming tests were complete, the fish were sacrificed using cerebral percussion, and their mass, depth and width (both taken at a point immediately anterior to the dorsal fin), opercular length (distance from the tip of their nose to the most distal end of the opercula), and caudal peduncle depth (maximum depth of the caudal peduncle) were measured using a set of callipers. The gill arches were removed from the left side of each fish and placed in cold (4°C) 9:1 formalin and stored in a fridge until gill surface area measurements were done. Caudal fin surface area was obtained by taking a digital photograph of the caudal fin (using a Coolpix 2500 digital camera) and analysing it using digital image analysis software (Matrox Inspector 3.0).

2.2.3 Data/Statistical Analysis

Routine MO_2, standard MO_2, and MO_{2max}, as well as U_{crit} and absolute and factorial scope, were compared between groups using an ANCOVA (Sigmaplot, SPSS Inc., Chicago, IL) For analyses of oxygen consumption, and metabolic scope, the fish's mass was used as the covariate. For U_{crit}, the fish's length was used as the covariate. To minimize variability due to allometric growth of morphometric characters the data was transformed (log_{10}) and regressed against the logarithm of fork length. The resultant slope was then used to standardize the data using the equation of Ihssen *et al.* (1981):

$$M_I = M_o \times (L/L_o)^b$$

where M_I is the transformed character, M_o is the observed character, L is the grand sample mean body length of females, L_o is the observed body length, and b is the common within-groups slope obtained from the analysis of covariance of the regression plot (Reist 1986). A significance level of $p<0.05$ was used for all analyses, and all data in the tables, figures, and text are means ± S.E.

2.3 Muscle Enzyme Activity and Protein Content

2.3.1 Tissue Collection and Storage

Fish were sacrificed with a sharp blow to the head and samples of red, white, and ventricular muscle were quickly removed, placed in cryovials, and immediately frozen in

liquid nitrogen. However, before the ventricle was frozen, it was washed with saline and blotted dry in order to remove any remaining red blood cells from the lumen. Samples were stored in a -80°C freezer until assays were performed.

2.3.2 Protein Content Measurement

The protein concentration of each tissue was measured using the Coomassie Plus Protein Assay Reagent protocol and reagents (Pierce USA). This procedure is based on the change in absorbance between 465nm and 595nm when Coomassie Blue G-250 (Pierce USA) binds to proteins in an acidic solution. Tissue protein content was obtained by comparing the absorbance values for all samples to a standard curve created from serial dilutions of a known concentration of BSA (Pierce USA).

2.3.3 Enzyme Assays

The following procedure for determining enzyme activity was adapted from Fudge *et al.* (2001). Tissue samples were homogenized in ice-cold 50 mM imidazole buffer (1:20 wt/vol) (for pH see values below). Enzyme activity was measured in the supernatant at 10°C using a spectrophotometer at wavelengths of 412 nm and 550 nm for CS and CCO, respectively. For the CCO assay, a stock CCO solution was made in phosphate buffer and excess ascorbic acid was added as a reducing agent. This solution was dialyzed against several changes of potassium buffer to remove excess ascorbic acid. The final CCO concentration in the cuvette was 0.1 mM. The assay conditions were as follows. CCO (E.C. 1.9.3.1): 10 mM K_2HPO_4/KH_2PO_4 buffer at pH 7.6 for the heart and

pH 8.0 for the red and white muscle, with the reaction being initiated with 20 μL of supernatant for the heart and red muscle, and 30 μL for the white muscle (ε=18.5, reduced-oxidized). CS (E.C. 4.1.3.7): 75 mM Tris, 0.25 mM DTNB, 0.4 mM acetyl CoA (pH 7.0), 0.5 mM oxaloacetate, with the reaction initiated by 10 μL of supernatant in all tissues (DTMB ε=13.6) The final volume in the cuvettes was 1 ml. Activities were optimized for each tissue and enzyme (for pH and all component concentrations) prior to actual testing using a separate group of transgenic and control salmon from the same strain.

2.3.4 Data/Statistical Analysis

Enzyme activities were compared between fish by means of a two-way ANOVA (fish and tissue) assuming equal variances using Sigmaplot (SPSS Inc., Chicago, IL). A significance level of $p<0.05$ was used for all analyses.

2.4 Gill Surface Area

2.4.1 Tissue Collection and Analysis

The concepts and measurement procedures described in Stevens and Sutterlin (1999) and Hughes (1984) were used to measure gill surface area. They have, however, been modified to utilize digital imaging tools. These imaging tools allow for a highly accurate measurement of the gill surface area, as a larger sample size can be obtained due to the ease of acquiring data as compared with previous methods. Thus, this larger

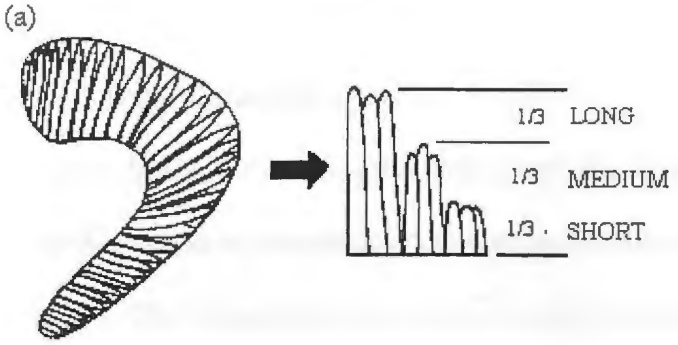
sample size eliminates the assumptions of gill component size and shape that are evident in older methods (Hughes 1990). The gill samples were taken from each fish used in the oxygen consumption and swimming experiments in order to directly determine the relationship between gill surface area and oxygen consumption.

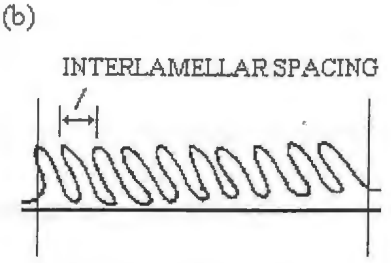
The 4 gill arches from the left side of each fish were removed and preserved in cold (4°C) 9:1 formalin. Hughes (1984) noted that there is unavoidable shrinkage during fixation. To correct for this, the extent of shrinkage was determined by extracting five individual filaments of different initial sizes, placing them in water on a cavity slide and measuring filament length and lamellar area using the methodology described below. The water was then replaced with 9:1 formalin, and the slide covered and refrigerated. Further measurements were made after 24 hours and 72 hours to determine the degree of shrinkage. The shrinkage was found to be <1.5% for both filament length and lamellar spacing in both the transgenics and controls after 24 hours, and 2% after 72 hours. All subsequent measurements were corrected to account for this shrinkage.

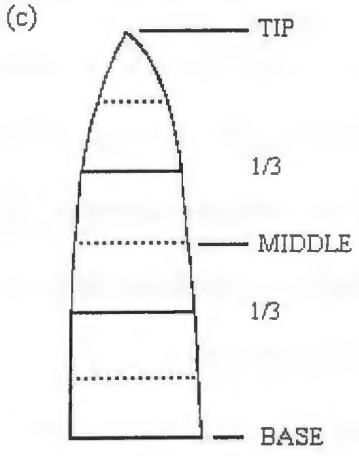
The biometry of the preserved gill tissue was determined by taking advantage of the digital image analysis software Matrox Inspector 3.0. In order to calibrate the system, a stage micrometer was used. Images were captured with a light microscope (Wild Makroskop model M420, Wetzlar, Germany) linked to a digital camera (Pixera PVC 100, Los Gatos, CA), and a desktop PC using Pixera Studio (I-Cube, Rowland Heights, CA). The lengths of all gill filaments, on all 4 arches from one side of the fish, were measured from base to tip, taking into account the fixation-associated curvature. These were then summed to obtain the total filament length (L). The filaments of each arch were then

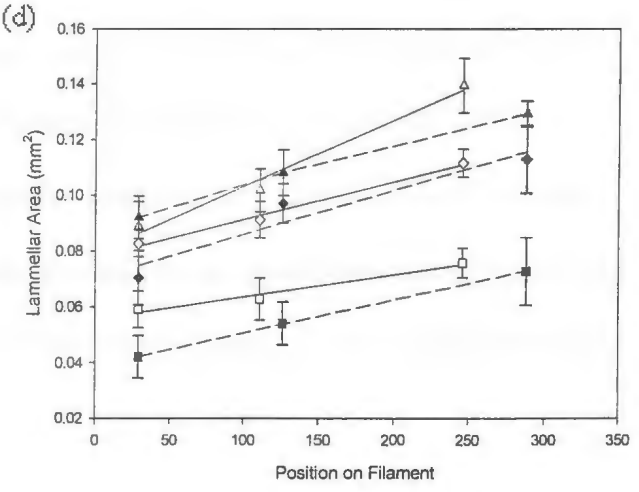
divided into three groups according to length: short, medium, and long (a subjective scale based on the lengths of the shortest and longest filaments) (Figure 2a). A medium sized filament from each of the three groups was selected, and the interlamellar spacing was obtained by measuring the distance covered by 10 adjacent lamellae and dividing that measurement by 10. This gave the average spacing between each lamella (Figure 2b). Since lamellar area is greater at the base of the filament (due to a greater width), lamellae were sampled from all regions of the filament to obtain an accurate area measurement. A small sample of lamellae (2-4) were dissected from the base, middle, and tip (bottom 3rd, middle 3rd, and top 3rd) of the filament, taking care to obtain lamella that were an average size for that particular region (Figure 2c). They were then spread flat on a glass slide in order to obtain digital images. Their area was then measured using the Matrox Inspector 3.0 computer program, which calculates the area of the digital image by counting the number of pixels contained within its perimeter. After the measurements were made on one side of the filament, the filament was turned over and measurements were repeated for the other side. Once the lamellar areas at all three positions were measured, from all three filament length categories, a linear equation was created in order to interpolate the areas of the remaining lamellae on the filament. These equations were a function of the length of the filament, the mean area of a lamella in each particular filament section, and the lamellar spacing (Figure 2d). Interpolated lamellar areas were then summed and multiplied by two to obtain the bilateral surface area of the lamella, and then multiplied by two again to obtain the area of lamellae on both sides of the filament. Each filament's lamellar area was calculated individually using a spreadsheet program (Microsoft Excel

Figure 2. Method for measuring the surface area of a gill. (a) The filaments of each arch were divided into three groups according to length: short, medium, and long. (b) Interlamellar spacing was obtained by measuring the average distance covered by 10 adjacent lamellae and dividing that measurement by 10. (c) Lamellar area was obtained from a subset of lamella from the base, middle, and tip regions (bottom 3rd, middle 3rd, and top 3rd) of the filament. The measured lamella were taken from the middle of each region (dotted lines) to get an average size. (d) A linear equation was created to interpolate the areas of the remaining lamellae on the filament. These equations were created based on the length of the filament, the mean area of a lamella at each position on the filament (lowest numbered position being the tip and highest being the base), and the lamellar spacing. This was done for large (▲), medium (♦), and small (■) filaments from both the transgenic (solid lines, open symbols) and controls (dashed lines, closed symbols). Error bars represent ± 1 standard error (p<0.05).


(a)
1/3 LONG
1/3 MEDIUM
1/3 SHORT


(b)
INTERLAMELLAR SPACING


(c)
TIP
1/3
MIDDLE
1/3
BASE


(d)
Lammellar Area (mm²)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
50
100
150
200
250
300
350
Position on Filament

spreadsheet program (Microsoft Excel 2000, Microsoft Corporation, Redmond, Washington) and then summed to give the entire lamellar area of the arch. The arch's areas were then summed, and multiplied by two, to account for both sides of the fish to ultimately get the fish's total gill surface area.

2.4.2 Data/Statistical Analysis

In order to reduce the type-1 error in this experiment, it was necessary to correct the *p*-value to accommodate for what are known as comparisonwise and experimentwise errors. The comparisonwise error is the probability that the whole comparison will erroneously declare a significant difference when, in fact, none exists (Kuehl 1994). The experimentwise error rate is the probability that a significant difference will appear somewhere in the entire set of comparisons, when in fact this is not the case (Kuehl 1994). One must take into account the experimentwise error because, in this ANCOVA analysis, statistical analyses were performed on each step used to determine gill surface area (e.g. filament length, interlamellar spacing, lamellar etc.), and therefore a source of type-1 error can be introduced at any step within the calculation. The adjusted *p*-value (α_c) can be obtained using the equation:

$$\alpha_c = 1 - (1 - \alpha_e)^{1/n}$$

Where: n is the number of tests, and α_e is the set experiment-wise *p*-value of 0.05. Thus, in this particular experiment, the equation is:

$$\alpha_c = 1 - (1 - 0.05)^{1/16} = 0.0032$$

In order to standardize the gill areas for the mass of the fish, each individual was scaled (either up or down depending on the fish's mass) to 1 kg and its gill morphometric values were adjusted accordingly. The gill areas from the controls and transgenics were then compared using the corrected *p*-value (0.0032) by means of an ANCOVA, with body weight as the covariate. A comparison of gill surface area to maximum (data not shown) and standard oxygen consumption (Figure 5) was made between the two groups. Regression analysis was performed to determine whether there was a correlation between gill area and oxygen uptake, making sure to compare the oxygen uptake and gill surface area from the same fish. All statistical analyses were performed using Sigmaplot (SPSS Inc., Chicago, IL).

2.5 Haematological Parameters and Stress Hormone Measurements

2.5.1 Blood Sampling

Transgenic and control salmon (n=8) were anaesthetized in seawater containing 0.1 g L^{-1} of MS-222. They were then placed in a supine position on a surgical sponge, and anaesthesia was maintained by constantly irrigating their gills with oxygenated seawater containing MS-222 (0.05 g L^{-1}) maintained at 8-10°C. A cannula (PE 50; 0.965 mm outer diameter, 0.58 mm inner diameter) was then inserted into the dorsal aorta, to allow for blood sampling, according to the method of Smith and Bell (1964). Thereafter, the fish was placed in a black Perspex box (40 cm long x 10 cm wide x 10 cm deep) that received 10°C seawater at a rate of 1 L min^{-1} from an aerated seawater reservoir. After a

48-hour recovery period, a resting sample of blood (0.3 ml) was taken from each fish and used for the various haematological procedures described below. Haematocrit was measured in triplicate by collecting blood in 20 µl capillary tubes, and centrifuging them in a haematocrit centrifuge for 30 seconds. Haemoglobin concentration was measured using the cyanomethaeglobin method (Cogswell *et al.* 2002); where 8 µl of blood was added to 2 ml of Drabkin's reagent and its absorbance (at 540 nm) corresponds to its haemoglobin content (as read from a standard curve). Erythrocyte size and circularity were determined using the blood smear technique described by Cogswell *et al.* (2002). Blood smears were prepared and 12 erythrocytes · blood smear^{-1} were analysed for cell perimeter, optical surface area, and circularity (a measure of how round the cell is). The measurements were made using a light microscope (Wild Makroskop M420) linked to a digital camera (Pixera PVC 100), and a desktop PC using Pixera Studio (I-Cube) and digital image analysis software (Matrox Inspector 3.0). Mean corpuscular haemoglobin concentration (MCHC) was determined as in Sadler *et al.* (2000) using the equation:

$$\frac{\text{Haemoglobin Concentration (g dl}^{-1}\text{) x100}}{\text{Haematocrit (\%)}}$$

A further 1 ml of blood was drawn from the fish, placed in a centrifuged tube and spun for 30 seconds at 6000 rpm. 550 µl of plasma was then placed in a cryovial (containing 20 µl EDTA and 20 µl glutathione) and quickly frozen in liquid nitrogen. This plasma was used to measure resting catecholamine levels, while the remaining plasma was frozen in another cryovial to be used for the measurement of resting cortisol levels.

The fish was then subjected to a 45 second net stress and returned to the black box. Blood for haematological measurements was immediately sampled as described above, and a further 1 ml was taken and spun for 30 seconds at 6000 rpm. Again, 550 µl of plasma was obtained from the centrifuged blood, placed in a cryovial (prepared as above), and frozen to measure post-stress catecholamine levels. Thirty minutes later, 300 µl of blood was drawn from the fish and centrifuged (as above), and 100 µl of plasma was frozen in a cryovial to measure post-stress cortisol levels.

The extraction of catecholamines (epinephrine and norepinephrine) from the plasma involved their binding to alumina at a neutral to alkaline pH, and their elution at an acidic pH (< 3.0). Catecholamines were extracted by combining 200-500 µl of plasma, 28 mg of aluminum oxide, 50 µl of DHBA (45 µmol l^{-1}), 50 µl of 0.1M $HClO_4$ (perchloric acid), and enough Tris/$Na_2EDTA{\bullet}2H_2O$ buffer (1.5 mol l^{-1} Tris, $Na_2EDTA{\bullet}2H_2O$ 0.06 mol l^{-1}, pH 8.6) to get a final volume of 1 ml. Samples were then shaken and centrifuged at 7000 rpm for 2 minutes and the plasma was aspirated from the alumina. The alumina was then washed twice with 500 µl of Milli-Q water (pH 7.0) (with 5 min of shaking and centrifugation in between), and aspirated to near dryness. Finally, 250 µl of perchloric acid (pH ≅ 1.0) was added to the tube, the tube was shaken for two 2 minute periods (separated by 5 min.), and the tube was centrifuged at 7000 rpm for 1 minute. This final procedure removed the bound DHBA and catecholamines from the alumina, and 100 µl of the perchloric acid solution was then injected into the HPLC for the quantification of DHBA, epinephrine, and norepinephrine. The extraction procedure

was conducted in a room with controlled temperature (7±1 °C), and the samples were kept on ice, and in the dark, during the extraction procedure and between injections.

Separation and measurement of the catecholamines in perchloric acid was performed using a Bioanalytical Systems reverse phase high performance liquid chromatography system (HPLC) (BAS) (480 series, model MR 9245, Bioanalytical System Inc., USA). This system consisted of a PM 80 pump, an LC epsilon electrochemical detector (model E5), and an electrochemical cell (LC44 dual glassy carbon working electrode with an Ag/AgCl reference electrode (3 M NaCl-gel)). A manual injection valve (model 7125) equipped with a 50 µl loop was used for injection of samples into the column (2.0 mm ID x 10 cm ODS, 3 µm pore size, model MF 8954). The mobile phase was composed of 94.5 g mol^{-1} MCAA (monochloroacetic acid), 372.2 g mol^{-1} Na_2EDTA $2H_2O$ (thylenediminetetraactic acid), 41.05 g mol^{-1} CH_3CN (acetonitrile, methyl cyanide), 232.3 g mol^{-1} SOS, and 10M NaOH (sodium hydroxide). The output from the detector was collected and compare with standards using a computer running ChromGraph Control and ChromGraph Report version 2.30 software (2001, Bioanalytical System Inc.).

Plasma cortisol concentrations were measured using a Coat-a-Count® Cortisol radioimmunoassay (RIA) (Diagnostics Products Limited, Los Angeles, CA). The assay works on the principle of competition between ^{125}I-labeled cortisol and plasma cortisol for antibody sites. The antibody is immobilized to the wall of a polypropylene tube, and by simply decanting the supernatant, the antibody fraction of the radiolabelled cortisol can be isolated. The tubes were counted in a gamma counter (Packard Autogama 5650,

Canberra Packard, Concord, ON), and calculations of cortisol concentrations were made using RiaSmart™, RIA/QC Software (Packard Instruments, Concord, ON) and a cortisol standard curve. The kit was stored in a refrigerator at 2-8°C prior to use, and the assay was done at room temperature.

2.5.2 Data/Statistical Analysis

Resting and post-stress haematocrit, haemoglobin, erythrocyte morphometrics, and stress hormone levels were compared between groups and between resting and post-stress values by means of a two-way repeated measures ANOVA (Sigmaplot, SPSS Inc.). A significance level of $p<0.05$ was used for all analyses.

2.6 Heart Size and Maximum Cardiac Performance

2.6.1 Surgery and Experimental Protocols

Transgenic and control salmon (n=8 and 7, respectively) were anaesthetized using 0.1 g L^{-1} MS-222, and placed ventral side up on a surgical sponge platform where their gills were continuously irrigated with 10°C oxygenated seawater containing 0.5 g L^{-1} MS-222. The fish were then injected with 0.5 ml of heparinized saline (100 i.u. ml^{-1}) (Sigma Chemical Co., St. Louis, MO) via the caudal vein to prevent blood clotting. The pectoral and pelvic fins were removed, and the body cavity was then exposed through a mid ventral incision. Both the stomach and intestines were tied off with braided umbilical tape (Baxter Healthcare Corporation, Deerfield, IL) at a position inferior to the liver, to

prevent blood flow to the gastrointestinal tract. The isolated part of the digestive tract was then removed.

An input cannula (stainless steel; 2.0 mm outer diameter, 1.5 mm inner diameter) was tied into the hepatic vein (using 3-0 silk sutures, American Cyanamid Company, Pearl River, NY) that leads into the sinus venosus. Perfusion with oxygenated saline (10°C) (containing 10 nM adrenaline, see recipe below) was started immediately after the cannula was inserted, and the height of the perfusate bottle relative to the heart was set to ensure adequate cardiac output during surgery. Several gill arches were then cut to prevent excess pressure build up in the heart, and the lower jaw was removed. The remaining gill arches were removed, and the isthmus was cut between the 2nd and 3rd gill arches to expose the ventral aorta in cross-section. An output cannula (stainless steel; 1.5 mm outer diameter, 1.3 mm inner diameter) was then tied into the ventral aorta with 1 USP silk (American Cyanamid Company).

To prevent loss of perfusate through the back of the heart, the ducts of Cuvier were tied off. To accomplish this, a 1 USP silk suture (American Cyanamid Company) was passed at a sharp angle from the corner of the opercular cavity to the exposed muscle of the abdominal wall, and then threaded through the oesophagus and back into the oral cavity. This suture was tightened until a fin twitch occurred (due to the crushing of the vagus nerve), indicating that the duct of Cuvier was tied off. After tying off both ducts of Cuvier, the fish was bisected at a point immediately posterior to the pelvic fins and transferred to the temperature controlled *in situ* heart apparatus (Farrell *et al.* 1986).

Once placed in the *in situ* apparatus, the input cannula was attached to an adjustable constant-pressure head that was used to manipulate atrial filling pressure, and the output cannula was connected to tubing whose height could be adjusted to control end-diastolic pressure. The heart was perfused with physiological saline (recipe below) from temperature controlled (10°C) water-jacketed bottles. To prevent excessive cardiac work while the input pressure was being set to a physiological relevant resting cardiac output (~16 ml min^{-1} kg^{-1}) (Kiceniuk and Jones 1977), and to let the heart recover from surgery, output pressure was maintained at 20-30 cm H_2O. Subsequently, output pressure was raised to 50 cm H_2O, a level comparable to *in vivo* arterial pressure (Kiceniuk and Jones 1977). After allowing the heart to stabilize at an output pressure of 50 cm H_2O for 15 minutes, resting cardiac performance values were taken. Then, maximum cardiac output (Q_{max}) was determined by increasing input pressure from the height required to achieve resting cardiac output to 1.5 cm, and then in a stepwise fashion (0.5 cm increments) to 6.5 cm H_2O (Figure 3). Power curves were then obtained for the *in situ* heart by lowering output pressure to 30 cm H_2O, and increasing output pressure by 10 cm increments until the heart could no longer pump (or a height of 100 cm was reached) (Figure 3). The time spent at each level of input or output pressure was just long enough to allow cardiac performance to stabilize, approximately 30 seconds.

Input and output pressures were measured using Gould (P23 ID, Oxnard, CA) and Grass (PT300, Warwick, RI) pressure transducers, respectively, and cardiac output was measured with a 2-N flow probe in conjunction with a T206 flow meter (Transonic Systems Inc., Ithaca, NY). Input and output pressures were corrected to account for the

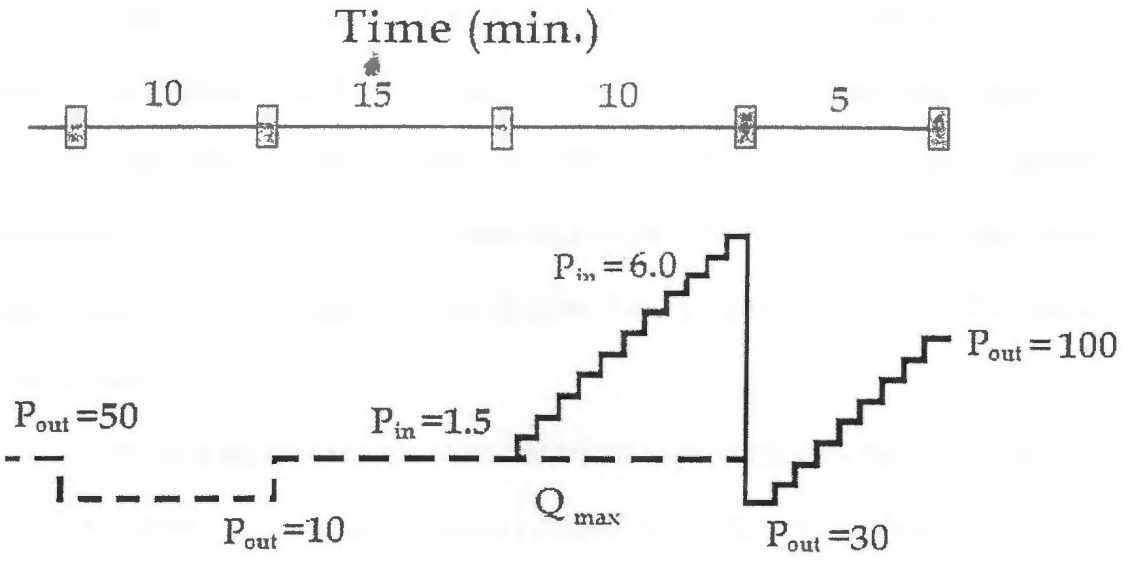


Figure 3. Experimental protocol for measuring Q_{max} and power output of the Atlantic salmon heart. The dashed line represents the end-diastolic pressure developed by the ventricle, determined by adjusting the height of the output pressure head. P_{out} was normally set to a physiologically realistic value of 50 cm H_2O; however a sub-physiological level of P_{out} (20-30 cm H_2O) was used at the start of the protocol to let the heart recover from surgery. The first set of steps identify the maximum cardiac output test (Q_{max}), where P_{in} was raised sequentially from 1.5 cm to 6.0 cm, while the second set of steps identify the myocardial power test where output pressure was raised from 30 cm H_2O to 100 cm H_2O, while P_{in} remained at 6.5 cm H_2O.

effect of the cannulas' resistance between the point of measurement and the heart (using the predetermined calculations in Faust (2001)), and the pressure transducers were calibrated daily against a static column of water, where zero pressure (0 cm H_2O) was equal to the saline level in the experimental bath. Pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system (BIOPAC Systems Inc., Santa Barbara, CA), and the acquired signals were stored and analyzed using Acknowledge Software (Biopac Systems Inc.) installed on a 300 MHz Macintosh G3 computer.

After each experiment, the heart was tested to ensure that no leaks were present. This was done by: 1) clamping the input perfusate line with a pair of haemostats and ensuring cardiac output fell to zero; and 2) raising the output tube while the input line was clamped, and ensuring no significant backflow occurred. No leaks were found in any of the heart preparations. The hearts were then dissected from the fish, and the chambers were separated, blotted dry, and weighed. The compact myocardium of the ventricle was then separated from the spongy by dissection, and each was weighed individually.

2.6.2 Experimental Solutions

Hearts were perfused with physiological marine teleost saline during the surgery and during the experimental period containing (in mM): 181.3 NaCl, 5.0 KCl, 2.30 CaCl x $2H_2O$, 1.99 $MgSO_4$ x $6H_2O$, 2.58 TES Acid, 7.33 Sodium TES base, and 5.55 dextrose. (Gamperl *et al.* Unpublished). These chemicals were obtained from Fisher Scientific (Fair Lawn, NJ), with the exception of the TES salt and adrenaline, which were

purchased from Sigma Chemical Co. (St. Louis, MO). The TES buffer system was used to simulate the buffering capacity of salmon plasma, and the normal change in blood pH with temperature ($\Delta pKa/dT$ = 0.016 pH units $^{o}C^{-1}$) (Keen *et al.*, 1993). Adrenaline (10nM) was added to the perfusate to ensure the long-term viability of the *in situ* heart (Graham and Farrell 1989). The solution was continuously gassed with oxygen during both surgery and while the *in situ* measurements were being made. The pH of the surgical saline was approximately 7.76 at 12°C.

2.6.3 Data/Statistical Analysis

Cardiac function was continuously monitored throughout the experiment by measuring cardiac output (ml $min^{-1}kg^{-1}$), input pressure (P_{in}) and output pressure (P_{out}). Although data was continuously collected, cardiac function was only analysed at specific intervals during each experiment. Resting cardiac function [P_{in}, f_H (beats min^{-1}), Q_{rest}, and S_V] was measured prior to the Q_{max} test. Maximum cardiac function was quantified by measuring Q_{max}, f_H, S_V, and P_O. Heart rate was calculated by measuring the number of systolic peaks during a 20 second interval, then multiplying by 3. Stroke volume (ml kg^{-1} $beat^{-1}$) and P_O (mW g $ventricle^{-1}$) were calculated as:

Stroke Volume (S_V) = Cardiac output (ml $min^{-1}kg^{-1}$)/ Heart rate (beats min^{-1})

Myocardial Power Output (P_O) = ($Q \cdot (P_{out}-P_{in}) \cdot a$)/Ventricular mass (g)

Where: P_{out} and P_{in} are output and input pressures (in cm H_20) respectively, and a= 0.098 (mW sec ml^{-1} cm H_2O^{-1}) is the conversion to milliwatts. Data were analyzed between groups by means of a one-way ANOVA (p<0.05) (Sigmaplot, SPSS Inc.).

Heart chamber masses were compared between control and transgenic salmon by means of an ANCOVA with body mass as the covariate. Relative ventricular mass (RVM; mass of ventricle/body mass x 100) and Ventricle:Atrium and Ventricle:Bulbus mass ratios were compared between control and transgenics salmon using a one-way ANOVA (p<0.05) (Sigmaplot, SPSS Inc.).

3.0 Results

3.1 Morphometrics

Transgenic and control salmon were similar in fork length, opercular length, caudal peduncle depth, and caudal fin surface area (Table 1a).

3.2 Metabolism and Swimming Performance

Figure 4 shows the oxygen consumption rate of transgenic and control salmon at all swimming speeds. Both the routine MO_2 and standard MO_2 were significantly greater in the transgenics (p=0.03 for both), exhibiting an 21% and 25% elevation over that of the controls, respectively (Table 1b). However, MO_{2max} was not significantly different between groups (p=0.42). The higher standard MO_2, coupled with the unchanged MO_{2max}, resulted in the transgenics having a significantly lower absolute scope (by 18%) and factorial metabolic scope (by 29%). U_{crit} was significantly greater in the control salmon (p=0.039), as they swam 11% faster than the transgenics. No correlation was found between U_{crit} and red muscle enzyme activity (data not shown).

3.3 Gill Morphometry

The gills of the transgenic and control fish showed no obvious differences in morphology when viewed by the naked eye or under a dissecting microscope. Table 2 shows the mean values for a number of gill parameters scaled to 1 kg (see pg 27), which allows for comparison of the transgenic and control fish's gill areas independent of body

Table 1. Physical characteristics, oxygen consumption, metabolic scope, and critical swimming speed for transgenic and control Atlantic salmon at 10°C. Values represent means ± 1 standard error. * indicates a significant difference ($p<0.05$). (a) Morphometric measurements for fish used in the oxygen consumption and swimming experiments. (b) Routine, standard, and maximum oxygen consumption (mg O_2 kg^{-1} hr^{-1}), absolute and factorial metabolic scope, and U_{crit} (BL sec^{-1}) for transgenic and control Atlantic salmon.

(a)

	Control (n=8)	Transgenic (n=8)	Trans/Con Ratio	*p*-value
Mass (g)	884 ± 86	828 ± 40	0.94	0.56
Fork Length (cm)	56.5 ± 3.7	56.1 ± 2.0	0.99	0.92
Depth (cm)	10.8 ± 0.7	10.6 ± 0.3	0.98	0.68
Opercular Length (cm)	8.9 ± 0.7	8.8 ± 0.4	0.98	0.61
Peduncle Depth (cm)	4.2 ± 0.4	4.2 ± 0.2	0.99	0.97
Caudal Fin Area (cm^2)	47.1 ± 6.9	52.8 ± 3.5	1.12	0.24

(b)

Oxygen Consumption (mg O_2 kg^{-1} hr^{-1})	Control (n=8)	Transgenic (n=8)	Trans/Con Ratio	*p*-value
Standard	46.4 ± 2.1	58.1 ± 4.4	1.25	0.03*
Routine	64.5 ± 3.9	78.2 ± 4.7	1.21	0.03*
Maximum	418.2 ± 18.6	379.5 ± 25.3	0.91	0.42
Absolute Scope	373.0 ± 18.8	306.2 ± 19.2	0.82	0.03*
Factorial Scope	9.13 ± 0.49	6.51 ± 0.58	0.71	0.004*
U_{crit} (BL sec^{-1})	2.2 ± 0.1	2.0 ± 0.1	0.91	0.04*

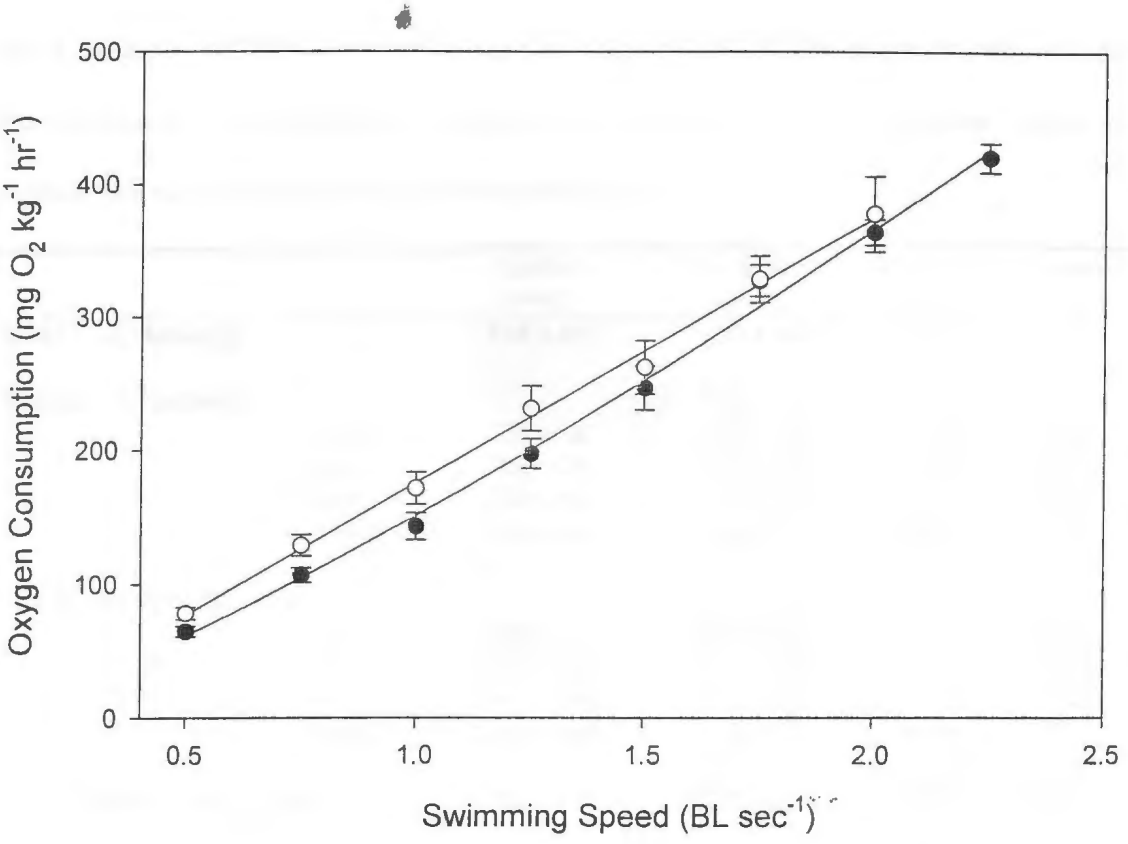


Figure 4. Mean standard oxygen consumption (mg O_2 kg^{-1} hr^{-1}) of transgenic (open circles, $y = -9.88 + 174.92x + 9.17x^2$) and control (closed circles, $y = -18.27 + 147.49x + 21.95x^2$) Atlantic salmon at various swimming speeds in a Blazka swim tunnel. Values represent means ± 1 standard error (n=8). Fitted lines represent a linear regression (R^2=0.99).

Table 2. Gill morphometric parameters for transgenic and control Atlantic salmon. Values shown are means ± 1 standard error, and all data were compared using an ANCOVA with body mass as the covariate (except for body mass which was compared using a one-way ANOVA, $p<0.05$). Significance for the ANCOVA was determined using the calculated comparisonwise *p*-value (see methods section). Filament length and number are values for the left side of the body only.

		Control (n=8)	Transgenic (n=8)	Trans/Con Ratio	*p*-value
Mean Body Mass (g)		884 ± 86	828 ± 40	0.97	0.56
Number of Filaments					
	Arch1	251 ± 19	256 ± 15	1.02	0.86
	Arch2	268 ± 20	276 ± 13	1.03	0.73
	Arch3	274 ± 19	267 ± 13	0.97	0.77
	Arch4	260 ± 26	240 ± 12	0.92	0.51
Length of Filaments (mm)					
	Arch1	2380 ± 87	2600 ± 89	1.14	0.26
	Arch2	2669 ± 121	2911 ± 86	1.13	0.20
	Arch3	2580 ± 159	2672 ± 45	1.10	0.35
	Arch4	2135 ± 166	2032 ± 76	0.99	0.10
Total Filament Length (mm)		9765 ± 416	10216 ± 247	1.08	0.35
Lamellar Density (# mm^{-1})		26.6 ± 3.5	22.8 ± 3.2	0.86	0.25
Number of Lamellae (Entire Fish)		306570 ± 21845	285129 ± 13175	0.93	0.41
Area of Lamella (mm^2) (One Side)					
	Large Base	0.144 ± 0.011	0.195 ± 0.001	1.36	0.01
	Large Middle	0.113 ± 0.013	0.111 ± 0.002	0.98	0.94
	Large Tip	0.094 ± 0.002	0.101 ± 0.001	1.07	0.71
	Medium Base	0.107 ± 0.002	0.136 ± 0.002	1.27	0.16
	Medium Middle	0.113 ± 0.001	0.121 ± 0.001	1.07	0.60
	Medium Tip	0.069 ± 0.002	0.081 ± 0.001	1.17	0.64
	Small Base	0.124 ± 0.001	0.115 ± 0.001	0.93	0.60
	Small Middle	0.071 ± 0.001	0.107 ± 0.001	1.51	0.01
	Small Tip	0.046 ± 0.001	0.078 ± 0.001	1.69	0.11
Gill Area (Entire Fish) (mm^2 g^{-1})		143.37±10	134.8±6.0	0.94	0.49

mass. No significant differences were found in the filament length or total gill surface area with respect to arches 1, 2, or 3, however arch 4 had significantly shorter filaments in both the transgenics ($p<0.0001$) and controls ($p=0.059$) (being 26% and 17% shorter than the average filament lengths from the remaining arches, respectively). Arch 4 also had a significantly smaller ($p<0.0001$) total surface area than the other arches. Lamellae at the base of the filaments had a greater surface area ($p<0.001$) than those at the distal ends, and this variation was due to the filament being wider at the base.

Transgenic salmon had a consistently (though not significantly) greater lamellar area at most of the positions on the filaments (Table 2), and marginally longer average filament lengths on each arch (except arch 4). These differences were contrasted by the controls having a slightly higher lamellar density than the transgenic fish. These morphological differences offset each other, and resulted in similar total mass specific gill surface areas between transgenic and control salmon (143.3±10.1 mm^2 g^{-1} and 134.8±6.8 mm^2 g^{-1}, respectively) ($p=0.49$). Further, no relationship between standard (Figure 5) or maximum oxygen consumption (data not shown) and gill surface area were evident.

3.4 Stress Hormones

Resting cortisol levels were not significantly different between the control and transgenic salmon (Table 3). Both the transgenic and control salmon showed a significant increase in post-stress cortisol when compared to their resting values (by 35% and 51%, respectively). However, post-stress cortisol levels in the control salmon were

significantly higher (by 28%) than in the transgenics. Resting and post-stress norepinephrine levels were significantly higher in the transgenics than in the controls (by 2.6x and 1.8x, respectively) (p=0.004), with both groups exhibiting a significant post-stress response as compared to their resting values (2.1x and 3.0x, respectively). Both the transgenic and control salmon had significantly greater post-stress epinephrine responses as compared to their resting values (by 3.6x and 3.7x, respectively), with the transgenics having a 1.67x higher post-stress plasma concentration than controls (p=0.03). Finally, the transgenic salmon had a significantly greater (by 108%) total resting and total post-stress (by 70%) catecholamine response as compared to the controls.

3.5 Haematological Parameters

The control salmon's erythrocytes had a significantly greater perimeter (by 3%) and compactness (by 8%) than the transgenics' (Table 4). These differences, however, may have limited biological significance as there was no difference found in the erythrocyte's optical surface area (p=0.10). No significant differences were found for haematocrit when comparing fish types, or pre/post-stress samples (Table 4). The control fish's blood exhibited a limited haemoglobin stress response, with post-stress levels <4% higher than pre-stress levels. Although the transgenic salmon had a significantly greater post-stress haemoglobin concentration than the control salmon (by 12%), this was not a statistically significant increase over that of their resting value (p=0.075). No difference was found in MCHC, either between the transgenics and controls, or when pre/post values were compared. These minimal differences in blood parameters following the 45

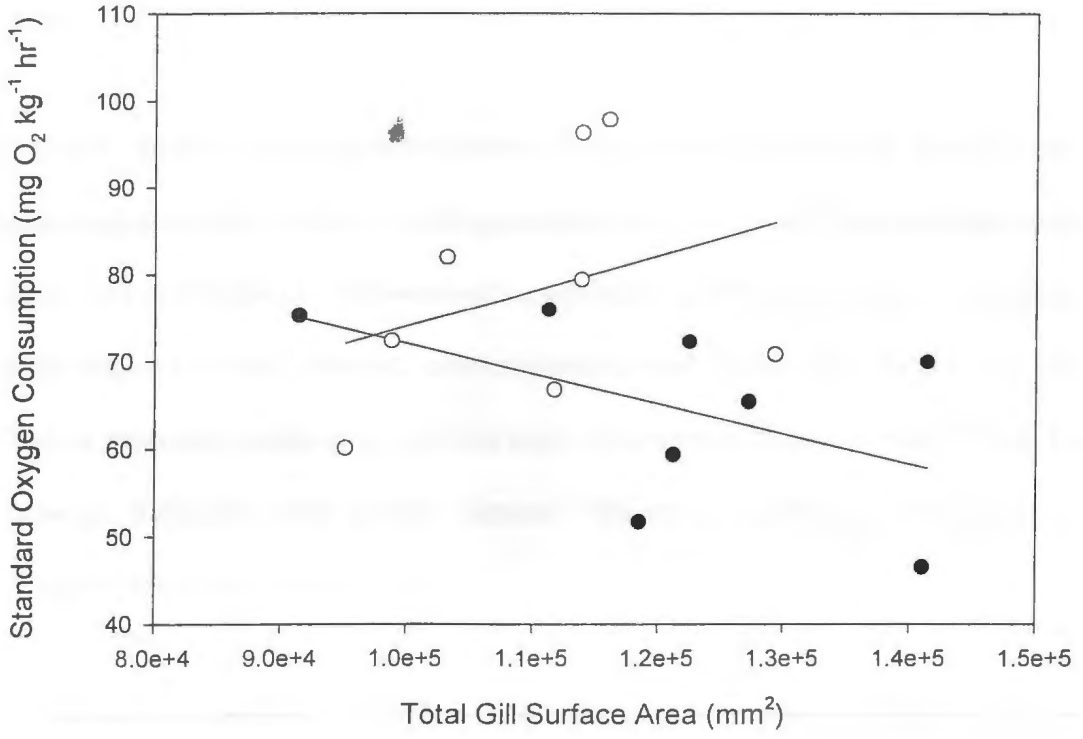


Figure 5. Relationship between standard oxygen consumption (mg O_2 kg^{-1} hr^{-1}) and total gill surface area (mm^2) for transgenic (open circles, $y = 0.0004 + 33.4x$, $R^2=0.11$, $p=0.67$) and control (closed circles, $y = -0.0003 + 0.106.25x$, $R^2=0.25$, $p=0.55$) Atlantic salmon. Fitted lines are linear regressions.

Table 3. Resting and post-stress plasma cortisol and catecholamine levels in transgenic and control Atlantic salmon. Resting measurements were taken 48h after cannulation and black box confinement. Post-stressed catecholamine levels were taken immediately after a 45 second net stress, whereas post-stressed cortisol levels were taken 30 minutes later. Values represent means ± 1 standard error. *indicates a significant difference ($p<0.05$) between transgenic and control salmon, †indicates a significant difference ($p<0.05$) between resting and stressed fish.

		Control (n=8)	Transgenic (n=8)	Trans/Con Ratio	*p*-value
Cortisol (ng ml^{-1})	Rest	12.1 ± 1.7	11.6 ± 2.3	0.95	0.86
	Stress	24.7 ± 2.3†	17.8 ± 1.3†	0.72	0.02*
Epinephrine (nM)	Rest	3.3 ± 0.6	5.8 ± 1.7	1.76	0.17
	Stress	12.3 ± 2.1†	20.6 ± 2.8†	1.67	0.03*
Norepinephrine (nM)	Rest	1.7 ± 0.3	4.3 ± 0.8	2.53	0.02*
	Stress	5.0 ± 0.8†	8.9 ± 0.7†	1.78	0.004*
Total Catecholamines (nM)	Rest	4.9 ± 0.9	10.2 ± 2.1	2.08	0.04*
	Stress	17.4 ± 2.9†	29.6 ± 3.4†	1.70	0.02*

Table 4. Erythrocyte morphometrics and haematological parameters for transgenic and control Atlantic salmon. Morphometrics were measured using optical techniques, with compactness being a function of the area and perimeter of the erythrocyte (values farther from 1 indicate a more oblong shape). All values represent means ± 1 standard error. *indicates a significant difference ($p<0.05$) between transgenic and control salmon, †indicates a significant difference ($p<0.05$) between resting and post-stressed samples.

Morphometric Parameter		Control (n=7)	Transgenic (n=8)	Trans/Con Ratio	*p*-value
Optical Surface Area (mm^2)		129.7 ± 2.0	125.5 ± 2.0	0.97	0.10
Perimeter (mm)		45.2 ± 0.4	44.0 ± 0.4	0.97	0.02*
Compactness		1.3 ± 0.01	1.2 ± 0.01	0.92	0.02*
Blood Parameter					
Haematocrit	Resting	29.8 ± 1.6	30.6 ± 2.0	1.02	0.79
(%)	Stress	33.3 ± 2.9	31.5 ± 1.9	0.95	0.66
Haemoglobin	Resting	6.2 ± 0.2	6.6 ± 0.2	1.06	0.24
($g\ dl^{-1}$)	Stress	6.4 ± 0.2	7.3 ± 0.3	1.14	0.04*
MCHC	Resting	21.4 ± 1.4	22.8 ± 1.7	1.07	0.63
	Stress	20.7 ± 1.6	23.9 ± 1.2	1.15	0.18

second net stress suggest that this type of stressor was not severe enough to elicit any major haematological changes in these fish.

3.6 Enzyme activity and Protein Content

In both groups, protein content was found to be greatest in the white muscle, followed by the red muscle, and then the heart muscle (ranging from 147.8 to 118.1 units · g protein^{-1}) (Table 5). No difference was found in protein content of the heart or the red muscle when fish groups were compared. However the transgenics' white muscle had a significantly greater protein content (by 4%) than the controls. CS and CCO activity were greatest in the heart (~0.2 and 0.190 units · g protein^{-1}, respectively), while white muscle had the lowest activity (~0.010 and 0.018 units · g protein^{-1}, respectively), and the red muscle was intermediate (~0.07 and 0.15 units · g protein^{-1}, respectively). The transgenic hearts had a significantly greater CS activity, both in terms of activity per wet mass (data not shown), and per gram of protein (p=0.0002 and p=0.00039, respectively). Although, no difference in CS activity was observed in the white or red muscle, the CCO activity was significantly higher in the transgenics' red muscle, on both a per-gram wet mass (data not shown) and per-gram protein basis (p=0.018 and p=0.007, respectively).

Table 5. Protein content (mg · g wet tissue^{-1}) and CS and CCO enzyme activity (units · g protein^{-1}, at 10°C) in heart, red, and white muscle tissue from transgenic and control Atlantic salmon. Values represent means ± 1 standard error. *indicates a significant difference (p<0.05) between transgenic and control salmon. Protein content was highest in the white muscle>red muscle>heart (p<0.001), and both enzyme's activities were highest in the heart>red muscle>white muscle (p<0.001).

	Control (n=8)	Transgenic (n=8)	Trans/Con Ratio	p-value
Protein				
Heart	118.1 ± 0.3	118.4 ± 0.3	1.01	0.52
Red	134.7 ± 0.5	133.8 ± 0.3	0.99	0.16
White	141.9 ± 0.7	147.8 ± 0.4	1.04	<0.0001*
CS activity				
Heart	0.117 ± 0.002	0.129 ± 0.002	1.10	<0.0001*
Red	0.071 ± 0.002	0.069 ± 0.001	0.97	0.34
White	0.011 ± 0.001	0.010 ± 0.001	0.91	0.16
CCO activity				
Heart	0.190 ± 0.003	0.192 ± 0.003	1.01	0.73
Red	0.145 ± 0.002	0.152 ± 0.001	1.05	0.007*
White	0.017 ± 0.000	0.019 ± 0.000	1.12	0.21

3.7 Heart Morphometrics and Performance

The hearts of the transgenics had a greater RVM (by 29%) than the control salmon (Table 6), while the atrium and bulbus masses showed no significant differences between groups. The relative amount of compact myocardium was also not different between the groups, and there was no correlation between the amount of compact myocardium and body mass (data not shown). When the mass of the atrium and bulbus, relative to the ventricle, was compared between groups, no significant differences were found. However the transgenic salmon tended to have a greater ratio (suggesting a larger ventricle compared to the rest of the heart) in terms of both the atrium and the bulbus (p=0.1 and 0.054, respectively).

Resting *in situ* input pressures were subambient, and, although there was a 36% difference in average resting input values between groups, a high degree of variation made this difference insignificant (Table 7). Heart rates were significantly greater in the transgenic salmon than in the controls, with resting and maximum heart rates being 14% (or approximately 11 BPM) and 7% (or approximately 5 BPM) higher, respectively. The transgenic fish were able to develop a significantly greater maximum cardiac output (by 18%, when scaled with body mass), however, this difference was not evident when calculated with respect to ventricular mass. Maximum stroke volumes were not significantly different between the transgenics and controls, either per gram body mass

Table 6. Heart chamber morphometrics in transgenic and control Atlantic salmon. Ventricle mass was compared using the relative ventricular mass (RVM) measurement and also by using an ANCOVA with body mass as a covariate (Ventricle mass (g)). 'Ventricle:Atrium' is the ratio of ventricular mass to atrial mass, and 'Ventricle:Bulbus' is the ratio of the ventricular mass to the bulbus' mass. Values represent means ± 1 standard error. *indicates a significant difference ($p<0.05$).

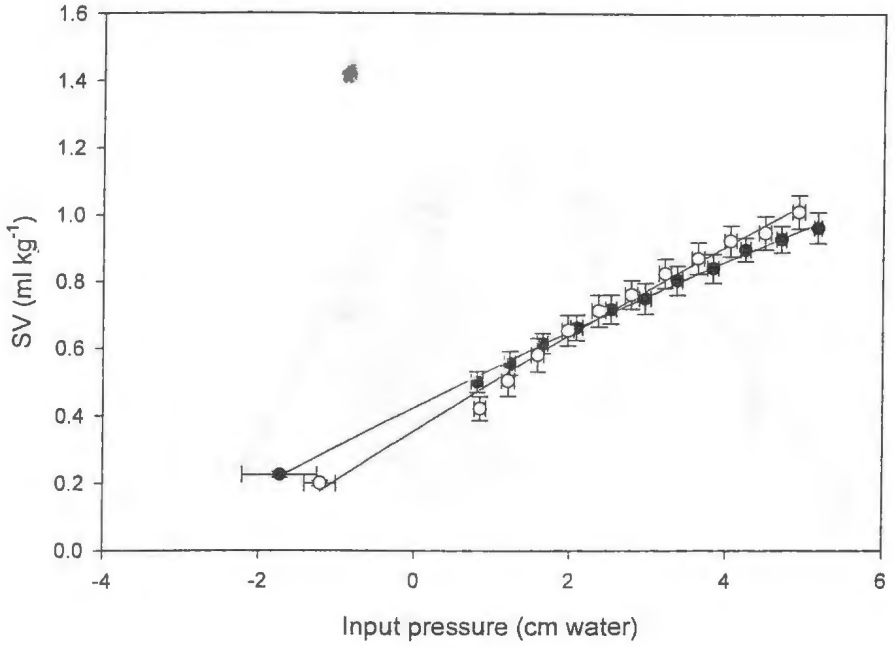
	Control (n=8)	Transgenic (n=8)	Trans/Con Ratio	*p*-value
Body Mass (g)	595 ± 21	577 ± 21	0.97	0.63
Ventricle Mass (g)	0.410 ± 0.15	0.517 ± 0.02	1.26	<0.0001*
RVM	0.069 ± 0.002	0.089 ± 0.002	1.29	<0.0001*
Atrium Mass (g)	0.098 ± 0.01	0.103 ± 0.01	1.05	0.54
Bulbus Mass(g)	0.120 ± 0.01	0.113 ± 0.01	0.94	0.77
Ventricle:Atrium Mass	4.4 ± 0.3	5.16 ± 0.3	1.17	0.10
Ventricle:Bulbus Mass	3.64 ± 0.4	4.8 ± 0.5	1.32	0.06
% Compact Myocardium	44.8 ± 1.0	46.5 ± 1.1	1.04	0.45

Table 7. Resting heart rate and input pressure, and maximum cardiac performance, in transgenic and control Atlantic salmon. Resting P_{in} was set to achieve a physiological resting cardiac output of approximately 16 ml min^{-1} kg^{-1}. Values represent means ± 1 standard error. *indicates a significant difference ($p<0.05$).

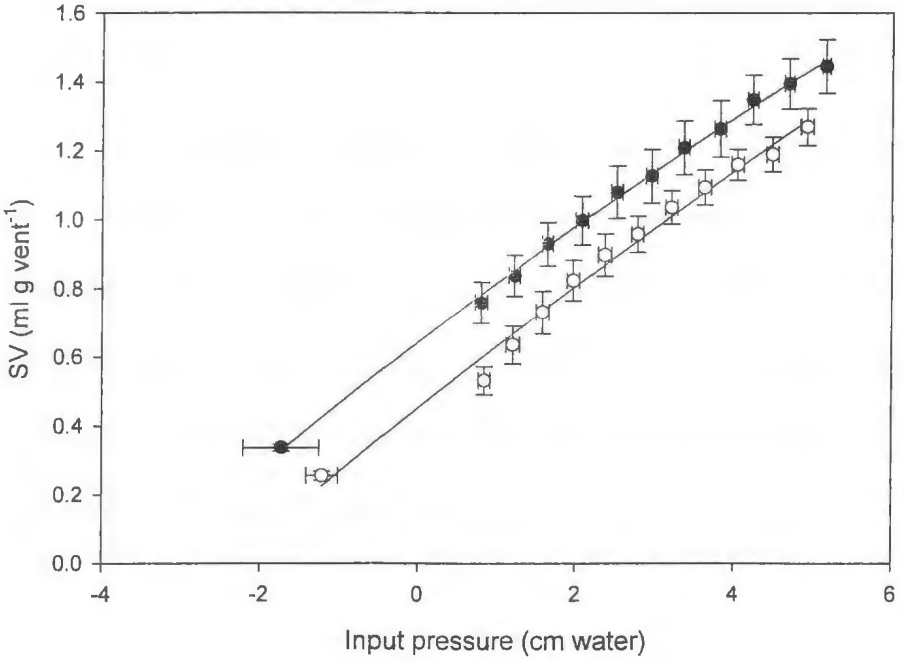
	Control (n=8)	Transgenic (n=7)	Trans/Con Ratio	*p*-value
Resting				
P_{in} (cm Water)	-1.51 ± 0.60	-0.97 ± 0.25	0.64	0.35
Heart Rate (BPM)	73 ± 2	84 ± 3	1.14	0.007*
Maximum				
Q (ml min^{-1} kg^{-1})	63.8 ± 1.9	75.5 ± 2.8	1.18	0.005*
SV (ml kg^{-1})	0.93 ± 0.03	1.03 ± 0.05	1.11	0.16
Heart Rate (BPM)	69 ± 1	74 ± 2	1.07	0.04*
Q (ml min^{-1} g $vent^{-1}$)	96.0 ± 4.4	95.7 ± 1.9	0.99	0.95
SV (ml g $vent^{-1}$)	1.4 ± 0.09	1.3 ± 0.04	0.94	0.28
Power (mW g $vent^{-1}$)	9.69 ± 0.41	9.67 ± 0.5	0.99	0.98

Figure 6. The effect of increased input pressure (in cm water) on maximum *in situ* stroke volume (ml · kg^{-1} and ml · g $ventricle^{-1}$) of transgenic and control Atlantic salmon hearts. (a) Transgenic salmon are represented by open circles ($y = 0.36 + 0.14x - 0.002x^2$, $R^2=0.98$) and controls are represented by closed circles ($y = 0.43 + 0.11x - 0.002x^2$, $R^2=0.99$). (b) Transgenic salmon are represented by open circles ($y = 0.45 + 0.18x - 0.003x^2$, $R^2=0.99$) and controls are represented by closed circles ($y = 0.64 + 0.17x - 0.003x^2$, $R^2 =0.99$). Curves were fitted with second order regressions. Error bars represent ± 1 standard error ($p<0.05$).

(a)


SV (ml kg-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-4
-2
0
2
4
6
Input pressure (cm water)

(b)


SV (ml g vent-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-4
-2
0
2
4
6
Input pressure (cm water)

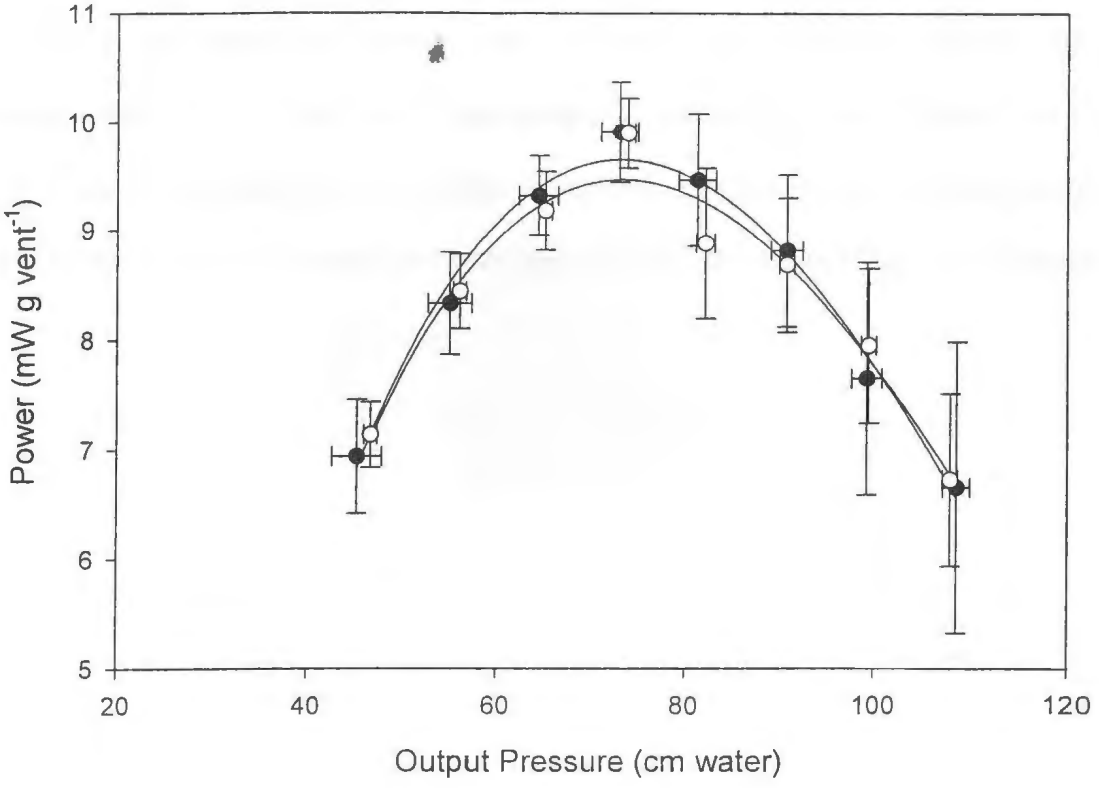


Figure 7. The effect of increased output pressure (in cm water) on myocardial power output (mW · g ventricle^{-1}) of *in situ* transgenic (open circles, $y = -13.2 + 0.71x - 6.91x^2 + 1.83x^3$, $R^2=0.95$) and control (closed circles $y = -14.2 + 0.75x - 7.2x^2 + 1.83x^3$, $R^2=0.98$) Atlantic salmon hearts. Hearts were left at the P_{in} at which Q_{max} was obtained while P_{out} was being manipulated. Curves were fitted with a second order regression. Error bars represent ± 1 standard error ($p<0.05$).

(Table 7 and Figure 6a) or per gram ventricle (Table 7 and Figure 6b). However, the transgenics tended to have lower ventricle-specific stroke volumes due to their larger ventricles and higher heart rates. Maximum power output was not different between groups (control: 9.69 mW g $vent^{-1}$, and transgenic: 9.67 mW g $vent^{-1}$) (Table 7 and Figure 7). Further, maximum power was achieved at similar output pressures in both groups (73 cm H_2O), and the relationships between power output and output pressure were identical.

4.0 Discussion

4.1 Overview of Study

It is well documented that exercise training increases heart function (Farrell *et al.* 1990; Farrell *et al.* 1991), swimming performance (Bainbridge 1962; Davison and Goldspink 1977; Mcfarlane and McDonald 1999), growth rate (Houlihan and Laurent 1987) and fuel efficiency (Lauff and Wood 1997), and alters the stress response (Woodward 1983), by applying an energetic pressure to fish. Thus, the fish is forced to upregulate its body's systems in response to the added demands of exercise (see Gallaugher *et al.* 2001). My hypotheses at the beginning of this research were: 1) that GH transgenic Atlantic salmon, under the influence of the additional GH gene, are being "metabolically trained"; and 2) this constant metabolic pressure associated with enhanced growth results in an upregulation of their cardiorespiratory system to cope with the additional stress placed on it.

This study shows that numerous aspects of the transgenic salmon's cardiorespiratory system are upregulated. For instance, they have a 29% larger heart with an 18% greater capacity to pump blood (Tables 6 and 7). Their blood can carry more oxygen due to its significantly greater post-stress haemoglobin content and smaller erythrocytes (Table 4). The greater activity of the aerobic enzymes CCO and CS in the red and heart muscle, respectively (Table 5), may increase the transgenic salmon's ability to generate energy through oxidative metabolism. Finally, their stress hormone levels have been altered through changes in cortisol, epinephrine, and norepinephrine (Table 3),

a modification that may affect how they react to environmental stressors and adjust other hormone-mediated physiological functions.

Overall, the upregulation of the GH transgenic's cardiorespiratory system appears to be in agreement with the theory of symmorphosis. However, the "pressure" of the transgene, and the associated metabolic upregulation, was not enough to change all physiological aspects of these fish. Gill surface area did not increase in the transgenic salmon, and this lack of response appears to be limiting their maximum performance. This may account for their lower U_{crit}, unchanged MO_{2max}, and subsequently lower metabolic scope (Table 1). Thus, even though most of their cardiorespiratory system was upregulated, a complete elevation, as suggested by symmorphosis, was not achieved. It is known that the addition of a transgene can have pleiotropic affects on an animal's systems (Berkowitz and Kryspin-Sorensen 1994). However, I consider it highly unlikely that the transgene's effect would be evident in all other systems tested and not in the gills; especially since the gills are responsible for 87% of the oxygen uptake in salmonids (Kirsch and Nonnotte 1977). Other possible reasons for this lack of change in gill surface area are discussed in detail beginning on page 66.

This study builds on the work of other authors who measured metabolic function in GH transgenic fish, and helps to clear up issues about previous studies due to possible methodological problems. First, a major concern regarding previous studies was that the transgenic fish had skeletal and muscular deformities (Farrell *et al.* 1997; Ostenfeld *et al.* 1998). This resulted in these fish not being in the same physiological condition or health as the non-transgenics, and thus comparisons were ambiguous. This may have been

associated with the presence of several copies of the transgene. However, the strength of the promoter, the sight of incorporation, or the makeup of the gene construct itself could also have lead to the physiological defects found in these lines. Second, routine MO_2 was often measured on groups of fish, rather than on individuals (Stevens *et al.* 1998; Cook *et al.* 2000a, b). This likely resulted in elevated MO_2 levels in the transgenics due to their higher rates of activity and more aggressive nature (Abrahams and Sutterlin 1999; Devlin *et al.* 1999). These two confounding factors were eliminated in this study, as my transgenic salmon were from a stable line with only one additional copy of the GH gene (Fletcher *et al.* 2001) and showed no signs of gross morphological defects, and all metabolic tests were done on individuals whose activity level was controlled. Given the comprehensive nature of this study, the fact that the influence of extraneous environmental variables were minimized (by rearing the fish in a common tank), and that the effects of morphological anomalies and differential activity rates were removed, I believe this study provides an accurate picture of how the presence of an additional GH gene affects post-smolt Atlantic salmon physiology. Further, this study acts as a well controlled model for symmorphosis, given that the only variable changed between groups is the addition of a single GH gene.

4.2 Metabolic Rate and U_{crit}

4.2.1 Routine Metabolic Rate

The routine MO_2 of my control Atlantic salmon is similar to values reported for other salmonids (56-61 mg O_2 kg^{-1} hr^{-1}) (Hughes and Saunders 1970; Ultsch *et al.* 1980; Gallaugher *et al.* 2001). However, the transgenic salmon had a significantly greater routine metabolic rate (1.2x) as compared to the controls. This magnitude of difference between the transgenics and controls is in agreement with Lee *et al.* (2003), who found an elevated routine MO_2 of a 1.21x in adult transgenic coho salmon. However, it is in contrast to Stevens *et al.* (1998) and Cook *et al.* (2000b) who found a ~1.7x increase, and Leggatt *et al.* (2003) who report that control coho salmon had higher routine metabolic rates. The greater difference found by Stevens *et al.* (1998) and Cook *et al.* (2000b) was likely due to their fish being smaller, as the disparity in growth rates between GH transgenics and non-transgenics tends to be greater when they are young (<100 g) (Margaret Shears, personal communication). Further, they used groups of approximately 50 fish in a common respirometer to obtain routine MO_2. The more active and aggressive transgenics (Abrahams and Sutterlin 1999; Herbert *et al.* 2001; Leggatt *et al.* 2003) would likely move around the tank more, a situation that would lead to much higher oxygen consumption rates than true routine levels. Also when fish are brought into the lab from the wild (as in Leggatt *et al.* 2003) they tend to be very skittish (Bob Devlin, personal communication) and this can greatly affect their oxygen consumption rates, and consequently the validity of comparisons between groups. Finally, confounding factors

such as time of day (De Silva *et al.* 1986; Waller 1992), sex, and season have been shown to affect metabolic measurements (Dickson and Kramer 1971), and their possible influence cannot be eliminated when comparing studies.

With a body of evidence showing that transgenic fish have an increased activity and greater appetite and SDA as compared with controls (Abrahams and Sutterlin 1999; Cook *et al.* 1999; Herbert *et al.* 2001; Leggatt *et al.* 2003), one must be careful when comparing oxygen uptake measurements. Greater spontaneous activity cannot be a factor in the transgenic fish's increased routine MO_2 found in this study. Only minimal pectoral fin movements and occasional tail beats were made by the fish, with them resting on the bottom of the tunnel throughout most of the 20 minute routine MO_2 measurement. In addition, the salmon used in these studies were fasted for 2 days prior to testing, a period twice that required for this species to complete digestion at 7°C (Sveier *et al.* 1999). Further, Leggatt *et al.* (2003) showed that 48 hours after the start of fasting transgenic and non-transgenic coho salmon did not have a significantly different resting oxygen uptake. Thus, this research demonstrates that the higher routine MO_2 is a genuine physiological trait of these post-smolt GH transgenic salmon, not due to differences in spontaneous activity or SDA.

Several physiological changes have been noted in transgenic fish that may support their inherently greater routine MO_2. These include a 2.2x greater intestinal surface area (Stevens and Devlin 1999), a greater amount of red muscle (Hill *et al.* 2001), and a greater white muscle protein content (Hill *et al.* 2001; this study). An elevated aerobic capacity is necessary to facilitate increased protein synthesis (Mathers *et al.* 1992), thus a

higher protein turnover rate and greater oxygen consumption are clearly linked (Goolish and Adelman 1987; Waterlow and Millward 1989; Houlihan 1991). Since protein is energetically more costly to synthesize than any other body constituent (Hommes 1980) and white muscle accounts for 70% of body mass and 40% of the body's protein synthesis (Fauconneau and Arnal 1985; Houlihan *et al.* 1988), it is likely that the higher protein synthesis (and/or possibly a greater turnover rate) in the transgenics' white muscle (evidenced by its higher protein content, this study) is contributing to their significantly increased routine MO_2. Transgenic salmon grow faster, and this may have contributed to their elevated routine MO_2, as the two have also been linked in a variety of fish species (Pauly 1998; Galarowicz and Wahl 2003). This higher growth rate, and the greater protein synthesis rate associated with it (Couture *et al.* 1998), would exacerbate the effect that the transgenics' more proteinacious muscle has on oxygen requirement, and result in an even greater increase in routine MO_2 over the slower growing controls.

4.2.2 Maximum Metabolic Rate and Metabolic Scope

The MO_{2max} values for the transgenic and control Atlantic salmon were not different, and agree with those for other salmonids (350-375 mg O_2 kg^{-1} hr^{-1} at similar temperatures) (Kiceniuk and Jones 1977; Alsop and Wood 1997; Burgetz *et al.* 1998). This agrees with the findings of McKenzie *et al.* (2003) who found no difference in MO_{2max} in transgenic tilapia when compared to non-transgenic controls, but contrasts Lee *et al.* (2003) and Stevens *et al.* (1998) who report an 11% lower and 60% higher MO_{2max} in transgenic coho and Atlantic salmon, respectively. This discrepancy between studies

is most likely due to methodological differences. For instance, Lee *et al.* (2003) used large (>2 kg) tank reared transgenic coho salmon and compared them to ocean-ranched coho salmon that were sampled during their spawning migration. These ocean-ranched "controls" would be expected to have a higher MO_{2max} than the tank-reared transgenics, given the physiological upregulation associated with the migration, prey capture, predator avoidance, etc., of the prolonged ocean portion of their life cycle. Thus, environmental factors, rather than direct effects of the transgene, most likely lead to the lower MO_{2max} exhibited by their transgenics. Further, Stevens *et al.* (1998) used considerably smaller fish (~10 g), making direct comparisons to the current study difficult. For instance, smaller fish eat more per gram body mass, a difference that can be 2-5 fold between 30g and 650g (Silverstein and Freeman 2001; Damsgard *et al.* 1999). This greater food intake, and thus SDA, could have caused their MO_{2max} to increase to accommodate the consistently large oxygen demand associated with large meals. Fish feeding at high rates also exhibit a greater degree of energetically costly foraging activity (Beamish 1964; Smit 1965; Brett and Zala 1975; Jobling 1994; Krohn and Boisclair 1994), which may also have lead to an elevated MO_{2max}. Finally, transgenic fish have an increased ability to process food (Cook 2000a). This, when coupled with the smaller salmon's larger food intake (Cook *et al.* 1999; Cook *et al.* 2000a), would result in them having to allocate a proportionately greater amount of oxygen for digestion and would therefore need an elevated MO_{2max} to still maintain enough metabolic capacity for other processes. Even though McKenzie *et al.* (2003) used smaller fish as well (~70g), comparisons are confounded by the effect of their ~25°C water temperature's on digestion and activity.

The higher routine MO_2, and unchanged MO_{2max}, resulted in the transgenic salmon having a 29% lower scope for activity than controls. A depressed metabolic scope (by 19%) was also found by Lee *et al.* (2003) in transgenic coho salmon, while McKenzie *et al.* (2003) showed no difference in scope between transgenic and non-transgenic tilapia (due to concomitant increases in routine MO_2 and MO_{2max}). The adult transgenic salmon's lower scope for activity in this study (and in Lee *et al.* 2003) results in them having less oxygen to carry out physiological processes (e.g. activity, digestion, reproduction, etc.). Fish must be able to multitask (or budget) their oxygen between these demanding processes (Pauly 1998). Pauly (1998) calculated energy budgets for 17 species of fish based on their metabolic, growth, and activity rates. He concluded that fish have to "choose" to allocate the limited oxygen they have to fuel either a higher growth rate or greater performance. Thus, with their higher standard MO_2, the transgenics appear to be favouring growth, at the expense of maximum performance (as evidenced by their lower U_{crit}).

4.2.3 Critical Swimming Speed

U_{crit} values for the transgenic and control Atlantic salmon (2.0-2.2 BL sec^{-1}) agree well with those of similar sized salmonids (2.13-2.66 BL sec^{-1}, respectively) (Gallaugher *et al.* 2001; Dunmall and Schreer 2003). The transgenic salmon used in this study, however, had a 9% lower U_{crit} than the control fish. A lower U_{crit} was also shown by Farrell *et al.* (1997) and Lee *et al.* (2003) for GH transgenic coho salmon (by 37% and 22%, respectively), and by Stevens *et al.* (1998) for GH transgenic Atlantic salmon

(p=0.09). All studies used size-matched (length and mass) GH transgenic and control salmon (approximately 14 cm and 25 g in Stevens *et al.* (1998), 20 cm and 100 g in Farrell *et al.* (1997), and 55 cm and 2000 g in Lee *et al.* (2003)), and in all cases control fish outperformed the transgenics (4.8 vs. 4.1 BL s^{-1}, 3.4 vs. 2.13 BL s^{-1}, and 1.6 vs. 1.25 BL s^{-1}, in each study, respectively). Based on these data, it appears that a lower maximum swimming speed is characteristic of GH transgenic salmonids. In contrast, however, McKenzie *et al.* (2003) found that transgenic tilapia achieved a similar U_{crit} as their non-transgenic counterparts. This illustrates that species differences may exist.

The decreased swimming ability of the transgenics could be the result of several factors, including changes in muscle and skeletal properties, inadequate circulation, or environmental effects. Transgenic salmon have been shown to have shortened opercula (Farrell *et al.* 1997), lower condition factor (Stevens *et al.* 1998), allometric compression (Lee *et al.* 2003), and cranial and caudal peduncle abnormalities (Ostenfeld *et al.* 1998), which could lead to poor swimming performance (Ostenfeld *et al.* 1998). However, the transgenics in this study exhibited none of these features, eliminating morphological differences as a cause of their lower U_{crit}. Pörtner (2002) states that circulation and ventilation are the primary limiting factors associated with oxidative metabolism. Therefore, a decreased performance would be expected if a fish's circulatory system is not able to adequately supply its body with oxygen. However, circulatory shortfalls are an unlikely cause of the transgenics' lower U_{crit}, as they have enhanced *in situ* heart performance (Table 7), higher catecholamine levels (Table 3), and slightly elevated haematological parameters (Table 4). Finally, a lack of training from being held in high

density, low volume, tanks was suggested by Lee *et al.* (2003) as the cause of poorer swimming performance in their transgenic salmon. This conclusion makes sense, as their "control" fish were returning from the wild where they were exposed to natural environmental conditions such as fast river/ocean currents, turbulence, and drastically different feeding and inter/intra-species interactions. There was no difference in life history between the controls and transgenics in the present study, as both groups were reared in the same tank from the point of salt water transfer as smolts to the time of testing. Having eliminated these factors as possible causes for the transgenic salmon's decreased swimming performance, the ultimate cause(s) are unknown at this point. However, the unchanged gill surface area may be a clue to what is limiting these fish (see below).

4.3 Gill Surface Area

Gill surface area was not different between the transgenic and control salmon in this study, and their gill areas (134 and 143 $mm^2\ g^{-1}$, respectively) agree with reported values on similar sized salmonids (120-197 $mm^2\ g^{-1}$) (Hughes 1980; Palzenberger and Pohla 1992; Pauly 1998). Stevens and Devlin (2000) also observed no difference in gill surface area between adult transgenic coho salmon and their non-transgenic counterparts. In contrast, however, Stevens and Sutterlin (1999) showed that GH transgenic Atlantic salmon smolts had a 1.6x greater gill surface area as compared with non-transgenic controls. There are several possible reasons why the presence of the GH transgene caused the upregulation of mass-specific gill area in younger transgenic Atlantic salmon,

but not in adults. For instance, smaller fish have higher mass specific-oxygen requirements than larger fish: due to the allometric relationship between body mass and MO_2 in fishes (Rodnick *et al.* 2004), and a higher SDA and greater foraging activity, as discussed earlier. Also, the Atlantic salmon smolts used by Stevens and Sutterlin (1999) had a 70% greater routine MO_2 compared to that of their controls, a value much greater than the 20% increase measured in my adult fish. When these two factors are combined, it suggests that the younger fish had a greater pressure on their cardiorespiratory system to supply their bodies with oxygen as compared to the adults. Therefore, a larger mass-specific gill surface area may be required for the younger fish, whereas an upregulation would not be needed in older fish. Finally, the young salmon used by Steven's and Sutterlin (1999) were freshwater pre-smolts, as opposed to the adult, saltwater, fish used here (and by Stevens and Devlin (2000)). Numerous studies have demonstrated that a variety of changes occur in gills after saltwater transfer; these include changes in ion flux (Mancera and McCormick 1999; Pelis and McCormick 2001; Arnesen *et al.* 2003), chloride cell proliferation (Perry 1998; Pelis and McCormick 2001), and gill membrane thickening (Perry 1998). These structural alterations allow the fish to osmoregulate in saltwater, however, they may do so at the expense of oxygen uptake (Perry 1998). In light of the above considerations, and the fact that environmental variables (such as water oxygen levels, crowding, etc.) can affect gill formation during ontogeny, direct comparison of gill surface areas of my fish with those of Stevens and Sutterlin (1999) may be inappropriate.

4.4 Enzyme Activity

CS and CCO enzyme activities in heart, red, and white muscle measured in this study are consistent with activities reported by other authors on similar species (2.1-28 μmol activity min^{-1} g wet $mass^{-1}$, depending on tissue, when Q_{10} and temperature corrected) (Philip *et al.* 1975; Pelletier *et al.* 1995; Nathanalides 1996; Cordiner and Egginton 1997; Leonard *et al.* 2001). Transgenic salmon had a higher CCO activity in their red muscle, and a higher CS activity in their heart muscle, as compared with control salmon. In contrast, there were no changes in white muscle enzyme activity, a finding that agrees with Blier *et al.* (2002) who studied GH transgenic coho salmon. Since white muscle makes up approximately 70% of a fish's total protein, this lack of a change in white muscle aerobic capacity suggests that the increased enzyme activity in the heart and red muscle were not growth related. The findings of this study, however, are in contrast to a number of studies performed on other species that have found positive associations between CS/CCO and growth rate in white muscle (largemouth bass, Goolish and Adelman 1987; saithe, Mathers *et al.* 1992; cod, Pelletier *et al.* 1993 and Pellitier *et al.* 1995). Thus, it is apparent that growth rate and aerobic enzyme activities can be correlated, but it is not a universal phenomenon, possibly owing to differential growth rates, tissues, and species differences across studies.

If growth rate was not the driving force behind the enzymatic upregulation observed in the transgenic's heart and red muscle, it is likely that higher physical activity (Abrahams and Sutterlin 1999; Herbert *et al.* 2001; Leggatt *et al.* 2003) was involved. Both the heart and red muscle are aerobic tissues, and their use would be higher with

even modest increases in actively level; potentially resulting in an elevated aerobic capacity of their enzymes. Further, the lack of change in the transgenics' white muscle is not surprising, as white muscle is only recruited at swimming speeds greater than 80% of U_{crit} (Jones 1982; Burgetz *et al.* 1998). Although exercise training can increase white muscle enzyme activity (Farrell *et al.* 1991), it occurs at a much higher physical activity level than these tank-reared fish are most likely to achieve on a daily basis.

Several physiological and biochemical factors may explain why both CS and CCO were not concurrently upregulated in the heart and red muscle. For instance, CCO is a rate-limiting enzyme in the electron transport chain (ETC), while CS is not rate limiting in the Kreb's Cycle (Pellitier *et al.* 1993). Therefore, simultaneous increases in CS and CCO activity are not necessary to allow for an increase in ATP production or protein synthesis that accompanies higher growth/activity. This may explain why CS activity was not increased in the red muscle. Moreover, the larger heart size of the transgenics may explain why CS activity, but not CCO activity, was upregulated in the cardiac tissue. Else and Hulbert (1985) determined a high level of cardiac demand was met by an increase in heart size and cardiac mitochondrial volume. Since CS is found in the mitochondrial matrix (volume dependent), and CCO is found in the ETC of the mitochondrial membrane (membrane dependent) (Pelletier *et al.* 1995), a larger mitochondrial volume could result in a greater CS concentration, with no change in CCO. However, histological tests would have to be done to determine if the transgenics did indeed have a greater cardiac mitochondrial volume.

The enzymatic modifications in the transgenics raise an important question: Are they caused by higher growth rate, or a result of exercise training due to their greater activity level? There is support for both a higher growth rate (Goolish and Adelman 1987; Mathers *et al.* 1992; Nathanailides and Stickland 1996; Couture *et al.* 1998; Pörtner 2002) and a greater activity (Johnston and Moon 1980; Farrell *et al.* 1989; Farrell *et al.* 1991) as causes of increased enzyme activity. However, not all tissues or enzymes act the same way with respect to either stimulus, and species differences exist (Nathanailides 1996). Therefore, it is difficult to determine the exact cause for these enzymatic changes without further experimentation under conditions that control for growth and/or activity. Further, with the relatively small differences in enzyme activity between groups the biological relevance of this altered activity may be questionable. However, when looked at in light of the theory of symmorphosis, the upregulation of the transgenic's enzyme activity could be acting in concert with the other physiological changes found in these fish to cause an increase in their metabolic function.

4.5 Haematology

The slightly smaller perimeter (by 3%) and more compact nature of the transgenics' erythrocytes (Table 4) are in line with a previous study done on transgenic Atlantic salmon by Cogswell *et al.* (2002). They found that transgenic salmon had smaller erythrocytes (by 5%) and suggested that this may be due to factors including higher growth rate and increased activity levels. Although the differences shown in both studies are statistically significant, it is, however, questionable whether these small

changes translate into biologically relevant elevations in oxygen transport capability or circulatory function (especially since there was no change in optical surface in this study).

There was no post-stress elevation in haemoglobin concentration, haematocrit, or MCHC in either the transgenic or control salmon. It was expected that the haematocrit and haemoglobin concentration would increase after the stressful event, as higher catecholamine levels cause red blood cells to be released by the spleen (Nilsson and Grove 1974; Kita and Itazawa 1989; Perry and Kinkead 1989). However, it is probable that the relatively small increases in post-stress catecholamine levels were not enough to elicit any changes in the haematological properties in these fish.

4.6 Cardiac Morphometry and Performance

Relative ventricular mass (RVM) (0.07-0.09%) and maximum cardiac output (64-76 ml min^{-1} kg^{-1}) of my control Atlantic salmon was similar to that of other salmonids (Farrell *et al.* 1996). However, the *in situ* hearts of the transgenic salmon exhibited marked increases in maximum cardiac output (18%) and RVM (19%) as compared with the controls. The larger heart agrees with Pitkanen *et al.* (2001), who found an increase in RVM (by 38%) in GH transgenic Arctic char (*Salvelinus alpinus*), but contrasts the study of McKenzie *et al.* (2003), where GH transgenic tilapia exhibited no difference in heart size as compared to size matched non-transgenic conspecifics. An increased heart size/performance in the transgenic salmon would be advantageous for transporting oxygen to growing (Graham and Farrell 1989; Franklin and Davie 1992; Clark and

Rodnick 1998) and hard working (Farrell *et al.* 1991) skeletal muscle. How the increase in heart size influenced cardiac output is not directly obvious, as SV (in ml kg^{-1} or ml g $ventricle^{-1}$) was not elevated in the transgenics (p=0.16 and p=0.26, respectively). However, the larger RVM would have allowed the transgenics to maintain stroke volume (negating the negative staircase effect or the limitations on ventricular filling, Farrell *et al.* 1996) at their higher heart rates (by 7%); enabling them to achieve a significantly greater *in situ* maximum cardiac output. In light of these changes in cardiac size/performance, it is apparent that the adult Atlantic salmon heart is plastic, and can grow to accommodate the additional demands placed on it that result from alterations in their physiology/activity due to the transgene. This plasticity is consistent with studies showing heart size in salmonids is influenced by training (Farrell *et al.* 1988; Farrell *et al.* 1991), cold acclimation (Goolish 1987; Graham and Farrell 1989; Rodnick and Sidell 1997), and maturation (Franklin and Davie 1992).

It is unclear what factor(s) caused the upregulation of the transgenics' heart size and performance, however several possibilities exist. Direct stimulation of heart size by elevated GH levels is unlikely, as Fleming *et al.* (1996) found that domestic Atlantic salmon had smaller hearts than their wild counterparts even thought they possessed high plamsa GH levels (Fleming *et al.* 2002). However, demands on the heart can be affected by: 1) growth rate (Goolish 1987; Goolish and Adelman 1987); 2) exercise training and activity level (Davie and Farrell 1991; Farrell *et al.* 1991; Gallaugher *et al.* 2001); and 3) feed intake (Axelsson *et al.* 2000). The transgenics did have a higher growth rate (by 3.6x) than the controls, and probably had a greater activity as well (Abrahams and

Sutterlin 1999; Herbert *et al.* 2001; Leggatt *et al.* 2003). Further, since post-prandrial cardiac output and gut blood flow have been shown to increase (Axelsson *et al.* 2000), it is also possible that the greater food intake of the transgenics salmon (Cook *et al.* 2000a,b) increased cardiac output and RVM due to the demands of a greater SDA. In order to determine the relative contributions of these factors, further experiments controlling for growth, activity, and/or SDA between control and transgenic fish are needed.

One surprising result related to cardiac function/morphology was the lack of an increase in % compact myocardium, even though RVM and cardiac output increased. Previous studies have shown: 1) that increased RVM is associated with, and elevated cardiac demand causes, an enhancement of the compact myocardial layer (Santer and Greer Walker 1980; Graham and Farrell 1992); and 2) that thickening of this layer is required to maintain stroke volume (ml g ventricle^{-1}) as ventricular volume increases (Law of Laplace). However, there are two explanations as to why no change in the compact layer was observed here. First, no change in stroke volume (ml g ventricle^{-1}) was recorded. Second, is possible that the transgenic salmon had a decreased vascular resistance, which would offset the effect of the higher cardiac output on blood pressure, and thus decrease the amount of work performed by the heart. However, no data currently exists to support this latter hypothesis.

A particularly interesting finding was the elevated intrinsic *in situ* heart rate of the transgenic fish. The transgenic salmon's greater heart rate may be the result of several factors. For instance, adrenaline (10nM) was used during the *in situ* cardiac performance

experiments, and it is unknown whether there were any changes in the transgenics' myocardial *B*-adrenergic/signal transduction systems caused by the effect of the transgene or the greater GH production itself. Adrenaline causes heart rate to increase (Farrell *et al.* 1991), and it is possible that changes in adrenaline receptor density/affinity could have elevated the transgenic salmon's sensitivity to exogenous adrenaline. Although such a finding has not been found in fish, rat studies have shown that *B*-receptor affinity can be heightened by GH injection (Iwasaki *et al.* 1982; Popova *et al.* 1990). Also, the greater food intake of the transgenics could have affected heart rate, as both feed-restricted cod and sturgeon fed lipid reduced diets (Genge *et al.* 2004 and Angisola *et al.* 1996, respectively) exhibit significantly lower *in situ* heart rates than control animals. This feeding-related effect on heart rate may be due to alterations in membrane composition of the heart's pacemaker cells, thus affecting their rates of excitability/firing. Finally, it is unclear whether the results of these *in situ* experiments translate to the *in vivo* situation, where the heart is affected by both cholinergic and adrenergic nervous stimulation (Laurent *et al.* 1983).

4.7 Stress Hormones

Resting cortisol levels (11.6 and 12.1 ng ml^{-1}) were not different between the transgenics and controls, and were similar to those reported by several other studies on salmonids (~10 ng ml^{-1}) (see the review by Gamperl *et al.* 1994; Ackerman *et al.* 2000). The lack of a significant difference between the groups is, however, in contrast to the work of Jhingan *et al.* (2003), who found transgenic coho salmon had 40% higher resting

cortisol levels than non-transgenic controls. It is unclear why the findings of the current study are inconsistent with theirs, but it is most likely due species/strain or handling differences, or the position or type of transgene used.

The cortisol stress response of these fish (~2-fold increase) was low compared to other studies, where 10-100 fold post-stress increases have been observed in a variety of fish species (see reviews by Barton and Iwana 1991, and Gamperl *et al.* 1994). At present, I have no explanation for this finding. However, I am certain that the values obtained in this study accurately reflect *in vivo* cortisol levels in these Atlantic salmon. Measurements of cortisol in this study were run concurrently with samples from other marine species (Costa *et al.* 2004), and these fish exhibited cortisol concentrations that were within the range of expected values. The type/degree of stressor can also be eliminated as a cause of the low stress response, as Ackerman *et al.* (2000) also used a 45 second net stress on rainbow and cutthroat trout, and found a 10-fold increase in cortisol levels over resting values. The control salmon did, however, have a significantly greater (by 28%) post-stress cortisol response as compared with the transgenics, suggesting that the transgenic salmon may have a blunted stress response. The transgenics' reduced cortisol response may be explained by the work of Boujard and Leatherland (1992), who found that resting cortisol and GH levels were negatively correlated in rainbow trout. However, this explanation does not fit with the findings of a number of other studies. For instance, Nielsen *et al.* (1994) showed a concurrent increase in GH and cortisol in exercised rainbow trout. Further, GH injection studies suggest that GH increases interrenal function in coho salmon, resulting in elevated plasma cortisol levels (Higgs *et*

al. 1977; Young 1988). Clearly more work is needed to determine why these transgenic Atlantic salmon had a diminished cortisol stress response, and what physiological consequences this may have. The latter question is particularly relevant since Shrimpton *et al.* (1995) showed that higher GH levels increase cytosolic corticosteroid receptor concentration; a response that may compensate for reduced post-stress cortisol levels.

Resting plasma epinephrine and norepinephrine levels in the present study (3.3-5.8 nM) are similar to those of other confined salmonids (rainbow trout) (range 1.0-3.9 nM) (Perry *et al.* 1987; Tang and Boutilier 1988; Fievet *et al.* 1990; Reid and Perry 1991). Although resting epinephrine values were not significantly different between groups, resting norepinephrine, and total catecholamine, values were significantly greater in the transgenic salmon (by 2.5-fold and 2.1-fold, respectively) (Table 3). Further, the transgenic salmon's post-stress catecholamine levels were significantly higher than the controls, in terms of epinephrine, norepinephrine, and total catecholamine levels. As with cortisol, the post-stress catecholamine response of the transgenic and control salmon after the 45-second net stress was much lower than that reported by other studies (where 5 to 140-fold increases occurred) (Barton and Iwana 1991; Gamperl *et al.* 1994). This lack of response could be due to a number of reasons (e.g. species/strain differences, handling, stressors used, etc.). Irrespective of the absolute post-stress levels, the transgenics clearly have higher resting catecholamine levels, and exhibit a stronger catecholamine stress response. This may have been induced by a downregulation in the transgenics ability to utilize catecholamines through a decrease receptor affinity or number, but further testing would be needed to determine if this is the case. Their

elevated catecholamine levels, however, could be the result of higher growth and/or activity, or a stimulatory effect of the additional GH gene on the chromaffin tissue itself. Although I am unaware of any other data relating GH and catecholamine levels in salmon, there is support for the former hypothesis in fishes. Gamperl *et al.* (1988) suggested that performance training increases catecholamine levels in rainbow trout.

The elevated resting and post-stress catecholamine levels in the transgenic salmon would enhance numerous physiological functions. For instance: 1) Catecholamines dilate the afferent branchial artery, while constricting the efferent branchial artery (Booth 1979; Nilsson 1986; Butler *et al.* 1989; Wendelaar Bonga 1997). This results in recruitment of dormant lamellae, thereby increasing effective gill surface area. 2) They increase ventilatory frequency (Randall and Taylor 1991; Perry *et al.* 1991). 3) Elevated catecholamine levels cause the release of erythrocytes from the spleen (Nillson and Grove 1974; Kita and Itazawa 1989; Perry and Kinead 1989) and increase the affinity of haemoglobin for oxygen (Nikinmaa 1992a; Nikinmaa 1992b). 4) They have positive chronotropic and inotropic effects on the heart (Farrell 1991), and higher catecholamine levels support cardiac function under a number of conditions (e.g. increased activity, growth, and hypoxia). 5) They maintain (or elevate) plasma glucose levels by increasing glycogenolysis and gluconeogenesis in the liver (Ottolenghi *et al.* 1984; Mommsen *et al.* 1988; Perry *et al.* 1988; Wright *et al.* 1989). 6) Finally, they are involved in the mobilization of lipids, carbohydrates, and proteins during times of growth and starvation (Sumpter *et al.* 1991). It is also possible that the increased catecholamine levels influenced growth rate since studies have found that *B*-agonists may increase growth in

finfish (see Mustin and Lovell 1993; Mustin and Lovell 1995 vs. Moccia *et al.* 1998; Vadenburg *et al.* 1998). The latter possibility suggests that the elevated GH levels also indirectly stimulate growth.

The relatively low post-stress cortisol and catecholamine levels found in these Atlantic salmon suggests that they are a low-stress lineage, and would presumably be a good candidate for commercial aquaculture. A low-stress strain would be beneficial to producers in several ways. A muted stress response can result in increased food conversion ratios, as unstressed fish do not waste excessive energy performing activities related to stress avoidance (Vijayan and Leatherland 1988; Olvera *et al.* 2001). The fish would also be less susceptible to disease, as high cortisol levels have been show to suppress immune function (Wendlaar Bonga 1997). This would keep populations strong due to lower mortality and morbidity. Thus, when these beneficial effects are combined, they would lead to increased production and profitability, the ultimate goal for the producer.

4.8 Conclusions/ Implications of This Study

Although this comprehensive study provides a wealth of information on the physiology of GH transgenic Atlantic salmon, it has created numerous questions that require further experimentation. Perhaps the most pressing issue is a basic one: Is increased growth rate or a higher activity level responsible for the upregulation of many aspects of the GH transgenic Atlantic salmon's cardiorespiratory system? Irrespective of the ultimate cause, we have shown that GH transgenic Atlantic salmon had a greater need

for oxygen at rest, and that they have alterations in heart morphology and performance, haematological parameters, enzyme activities, and the stress hormone response. Given the number of modifications in the transgenics' cardiorespiratory system, our data largely supports the theory of symmorphosis, suggesting that they are compensating for metabolic changes using a "whole animal" approach.

Perhaps the most interesting finding is that the metabolic capacity of the adult GH transgenic Atlantic salmon appears to be limited by their gill surface area; a situation that is not evident in smaller transgenic salmon (Stevens and Sutterlin 1999). However, more research is needed to better understand how gills grow during ontogeny and how they may limit growth/performance. Finally, when their decreased metabolic scope and swimming performance are coupled with a potentially depressed immune system (Jhingan *et al.* 2003) (data on coho salmon) and a weakened anti-predator response (Sundström *et al.* 2003; Sundström *et al.* 2004) it appears that the addition of the GH transgene does not affect the Atlantic salmon's ability to out-compete wild populations any more than fish currently under intensive aquaculture.

6.0 Bibliography

Ackerman, P.A., Forsyth, R.B., Mazur, C.F., and Iwama, G.K. 2000. Stress hormones and the cellular stress response in salmonids. Fish Physiol. Biochem. **23(4)**: 327-336.

Abrahams, M.V., and Sutterlin, A. 1999. The foraging and anti-predator behaviour of growth enhanced transgenic Atlantic salmon. J. Anim. Behav. **58**: 933-942.

Alsop, D.M., and Wood, C.M. 1997. The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. **200**: 2337-2346.

Arnesen, A.M., Toften, H., Agustsson, T., Stefansson, S.O., Handeland, S.O., and Bjornsson, B.T. 2003. Osmoregulation, feed intake, growth and growth hormone levels in 0+ Atlantic salmon (*Salmo salar* L.) transferred to seawater at different stages of smolt development. Aquaculture. **222(1-4)**: 167-187.

Atlantic Salmon Federation (ASF). 2003. www.asf.ca. Website accessed Feb 1, 2003.

Axelsson, M., Thorarensen, H., Nilsson, S. 2000. Gastrointestinal blood flow in the red Irish lord, *Hemilepidotus hemilepidotus*: long-term effects of feeding and adrenergic control. J. Comp. Physiol. B. **170**: 145-152.

Bainbridge, R. 1962. Training, speed and stamina in trout. J.Exp. Biol. **39**: 129-153.

Barton, B.A., and Iwama, G.K. 1991. Physiological changes in fish from stress in aquaculture with emphasis on the responses and effects of corticosteroids. Annu. Rev. Fish. Dis. **1**: 3-26.

Beamish, F.W.H. 1964. Influences of starvation on standard and routine oxygen consumption. Trans. Am. Fish. Soc. **98**: 103-107.

Bell, W.H., and Terhune, L.D.B. 1970. Water tunnel design for fisheries research. Fish. Res. Board. Can. Tech. Rep. No. 195.

Berkowitz, D.B., and Kryspin-Sorensen, I. 1994. Transgenic fish: Safe to eat? A look at the safety considerations regarding food transgenics. Biotech. 12(3): 247-252.

Blier, P.U., Lemieux, H., and Devlin, R.H. 2002. Is the growth rate of fish set by digestive enzymes or metabolic capacity of the tissues? Insight from transgenic coho salmon. Aquaculture. **209**: 379-384.

Booth, J.H. 1979. The effect of oxygen supply – epinephrine and acetylcholine on the distribution of blood flow in trout gills. J. Exp. Biol. **83**: 31-9.

Boujard, T., and Leatherland, J.F. 1992. Circadian pattern of hepatosomatic index, liver glycogen and lipid content, plasma non-esterified fatty acid, glucose, T sub(3), T sub(4), growth hormone and cortisol concentrations in *Oncorhynchus mykiss*) held under different photoperiod regimes and fed using demand-feeders. Fish Physiol.Biochem.**10** (**2**): 111-122.

Brett, J.R. 1964. The respiratory metabolism and swimming performance of young sockeye salmon. J. Fish. Res. Board. Can. **21**: 1183-1226.

Brett, J.R., and Zala, C.A. 1975. Daily patterns of nitrogen excretion and oxygen consumption of sockeye salmon (*Oncorhynchus nerka*) under controlled conditions. J. Fish. Res. Bd. Can. **32**: 2479-2486.

Brown, J. A. 1993. Endorcrine responses to environmental pollutants, *In* Fish Ecophysiology (Rankin J.C, and Jensen, F.B. eds). Chapman & Hall. New York.

Burgetz, I.J., Rojas-Vargas, A., Hinch, S.G., and Randall, D.J. 1998. Initial recruitment of anaerobic metabolism during sub-maximal swimming in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. **201**: 2711-2721.

Butler, M., Axelsson, M., Ehrenstrom, F., Metcalfe, J.D., and Nilsson, S. 1989. Circulating catecholamines and swimming performance in cod, *Gadus mohua*. J.Exp. Biol. **141**: 377-387.

Clark, R.J., and Rodnick, K.J. 1998. Morphometric and biochemical characteristics of ventricular hypertrophy in male rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. **201**: 1541-1552.

Cogswell, A.T., Benfey, T.J., and Sutterlin, A.M. 2002. The hematology of diploid and triploid transgenic Atlantic salmon (*Salmo salar*). Fish. Physiol. Biochem. **24**: 271-277.

Cook, J.T. 1999. Bioenergetics of growth hormone transgenic Atlantic salmon (*Salmo salar*) reared under simulated aquacultural conditions. MSc Thesis, Atlantic Veterinary College, University of Prince Edward Island, Canada. pg.138.

Cook, J.T., McNiven, M.A., Richardson, G.F., and Sutterlin, A.M. 2000a. Growth rate, feed digestibility and body composition of growth enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture. **188(1-2)**: 15-32.

Cook, J.T., Sutterlin, A.M., and M.A. McNiven. 2000b. Effects of food deprivation on oxygen consumption and body composition of growth enhanced transgenic Atlantic salmon (*Salmo salar*). Aquaculture. **188**: 47-63.

Cordiner, S. and Egginton, S. 1997. Effects of seasonal temperature acclimatization on muscle metabolism in rainbow trout, *Oncorhynchus mykiss*. Fish Physiol. Biochem. **16(4)**: 333-343.

Costa, I., Pertersen, L., and Gamperl, A.K. 2004. The metabolic physiology and stress response of North Atlantic teleost species. Canadian Society of Zoologists Conference 2004.

Couture, P., Dutil, J.D., and Guderley, H. 1998. Biochemical correlates of growth and condition in juvenile Atlantic cod (*Gadus morhua*) from Newfoundland. Can. J. Fish. Aquat. Sci. **55(7)**: 1591-1598.

Damsgard, B., Arnesen, M.A., and Jobling, M. 1999. Seasonal patterns of feed intake and growth of Hammerfest and Svalbard Arctic charr maturing at different ages. Aquaculture **171**: 149-160.

Davie, P.S., and Farrell, A.P. 1991. Coronary and luninal circulation of the heart. Can. J. Zool. **69**: 1993-2001.

Davison, W. 1989. Training and its effects on teleost fish. Comp. Biochem. Physiol. **94**: 1-10.

Davison W., and Goldspink, G. 1977. The effect of prolonged exercise on the lateral musculature of brown trout. J. Exp. Biol. **74**: 115-122.

De Silva, C.D., Premawansa, S., and Keembiyahetty, C.N. 1986. Oxygen consumption in *Oreochromis niloticus* (L.) in relation to development, salinity, temperature and time of day. J. Fish Biol. **29(2)**: 267-277.

Devlin R.H., Yesaki, T.Y., Biagi, C.A., Donaldson, E.M., Swanson, P., and Chan, W.K. 1994. Extraordinary growth in salmon. Nature. **371**: 209-210.

Devlin, R.H., Johnsson, J.I., Smailus, D.E., Biagi, C.A., Joensson, E., and Bjoernsson, B.T. 1999. Increased ability to compete for food by growth hormone-transgenic coho salmon *Oncorhynchus kisutch* (Walbaum). Aquacult. Res. **30(7)**: 479-482.

Dickson, I.W., and Kramer, R.H. 1971. Factors influencing scope for activity and active standard metabolism of rainbow trout (*Salmo gairdneri*). J. Fish. Res. Board Can. **28**: 587-596.

Donaldson, E.M. 1981. The pituitary-interrenal axis aws an indicator of stress in fish. *In* Stress and fish (A.D. Pickering eds.). Academic Press. New York/London. pg. 11-47.

Driedzic, W.R., Bailey, J.R., and Sephton, D.H. 1996. Cardiac adaptations at low temperature in non-polar teleost fish. J. Exp. Zoo. **275**: 186-195.

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 construct. Biotechnology. **10**: 176-181.

Dufour, S., Huang, Y.S., Rousseau, K., Sbaihi, M., Le Belle, N., Vidal, B., Marchelidon, J., Querat, B., Burzawa-Gerard, E., Chang, C. F., and Schmitz, M. 2000. *In* Puberty in teleosts: New insights into the role of peripheral signals in the stimulation of pituitary gonadotropins. Reprod. Physiol. Fish. pg. 455-461.

Dunmall, K.M., and Schreer, J.F. 2003. A comparison of the swimming and cardiac performance of farmed and wild Atlantic salmon, *Salmo salar*, before and after gamete stripping. Aquaculture. **220(1-4)**: 869-882.

Farrell, A.P., Bennett, W., and Devlin, R.H. 1997. Growth-enhanced transgenic salmon can be inferior swimmers. Can. J. Zool. **75**: 335-337.

Farrell, A.P., Gamperl, A.K., Hicks, M.T., Shiels, H.A., and Jain, K.E. 1996. Maximum cardiac performance of rainbow trout (*Oncorhynchus myskiss*) at temperatures approaching their upper lethal limit. J. Exp. Biol. **199**: 663-672.

Farrell, A.P., Hammons, A.M., Graham, M.S., and Tibbits, G.F. 1988. Cardiac growth in rainbow trout, *Salmo gairdneri*. Can. J. Zool. **66(11)**: 2368-2373.

Farrell, A.P., Johansen, J.A., Steffensen, J.F., Moyes, C.D. 1990. Effects of exercise training and coronary ablation on swimming performance, heart size, and cardiac enzymes in rainbow trout, *Oncorhynchus mykiss* . Can. J. Zool. **68(6)**: 1174-1179.

Farrell, A.P., Johansen, J.A., and Suarez, R.K. 1991. Effects of exercise-training on cardiac performance and muscle enzymes in rainbow trout, *Oncorhynchus mykiss*. Fish Physiol. and Biochem. **9(4)**: 303-312.

Farrell, A.P., MacLeod, K.R., and Chancey, B. 1986. Intrinsic mechanical properties of the perfused rainbow trout heart and the effects of catcecholamines and extracellular calcium under control of acidotic conditions. J. Exp. Biol. **125**: 319-345.

Farrell, A.P., Small, S., and Graham, M.S. 1989. Effect of heart rate and hypoxia on the performance of a perfused trout heart. Can. J. Zool. **67(2)**: 274-280.

Fauconneau, B., and Arnal, M. 1985. In vivo protein synthesis in different tissues and the whole body of rainbow trout (*Salmo gairdneri* R.). Influence of environmental temperature. Comp. Biochem. Physiol. A. **82(1)**: 179-187.

Faust, H.A. 2001. *In* Myocardial hypoxia tolerance and preconditioning in rainbow trout (*Oncorhynchus mykiss*): functional and biochemical assessment of myocardial damage. Portland State University (Master's thesis). pg. 106.

Fievet, B., Caroff, J., and Motais, R. 1990. Catecholamine release controlled by blood oxygen tension during deep hypoxia in trout: effects on red cell Na/H exchanger activity. Resp. Physiol. **79**: 81-90.

Fleming, I.A., Agustsson, T., Finstad, B., Johnsson, J.I., Bjornsson, B.T. 2002. Effects of domestication on growth physiology and endocrinology of Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. **59(8)**: 1323-1330.

Fleming, I.A., Jonsson, B., Gross, M.R., Lamberg, A. 1996. An experimental study of the reproductive behaviour and success of farmed and wild Atlantic salmon (*Salmo salar*). J. Appl. Ecol. **33(4)**: 893-905.

Fletcher, G.L., Goddard, S.V., Shears, M.A., Sutterlin, A., and Hew, C.L. 2001. Transgenic salmon: potential and hurdles. *In* Proceedings OECD symposium on "Molecular Farming". (Toutant, J.P., and Balazs, E. eds.). pg. 57-65.

Fletcher, G.L, Shears, M.A., Yaskowiak, E.S., King, M.J., and Goddard, S.V. 2004. Gene Transfer: Potential to Enhance the Genome of Atlantic Salmon for Aquaculture. Aust. J. Exper. Agricult. In Press, October 2004.

Franklin, C.E., and Davie, P.S. 1992. Sexual maturity can double the heart mass and cardiac power output in male rainbow trout. J. Exp. Biol. **171**: 139-148.

Fry, F.E.J. 1971. The effect of environmental factors on the physiology of fish. *In* Fish Physiology VI. (Hoar, W.S., and Randall, D.J. eds.) Academic Press. New York. pg. 1-98.

Fudge, D.S., Ballantyne, J.S., and Stevens, E.D. 2001. A test of biochemical symmorphosis in a heterothermic tissue: bluefin tuna white muscle. Am. J. Physiol. R. **280**: 108-114.

Galarowicz, T.L., and Wahl, D.H. 2003. Differences in growth, consumption, and Metabolism among walleyes from Different Latitudes. Trans. Am. Fish. Soc. **132(3)**: 425-437.

Gallaugher, P.E., Thorarensen, H., Kiessling, A., and Farrell, A.P. 2001. Effects of high intensity exercise training on cardiovascular function, oxygen uptake, internal oxygen transport and osmotic balance in chinook salmon (*Oncorhynchus tshawytscha*) during critical speed swimming. J. Exp. Biol. **204(16)**: 2861-2872.

Gamperl, A.K., Bryant, J., and Stevens, E.D. 1988. Effects of a sprint training protocol on growth rate, conversion efficiency, food consumption and body composition of rainbow trout, *Salmo gairdneri* Richardson. J. Fish . Biol. **33**: 861-870.

Gamperl, A.K., Vijayan, M.M., and Boutilier, R.G. 1994. Epinephrine, norepinephrine, and cortisol concentrations in cannulated seawater-acclimated rainbow trout

(*Oncorhynchus mykiss*) following black-box confinement and epinephrine injection. J. Fish. Biol .**45(2)**: 313-324.

Glomski, C.A., Tamburlin, J., and Chainani, M. 1992. The phylogenetic odyssey of the erythrocyte. II. Fish, the lower vertebrate experience. Histol. Histopathol. **7**: 133-139.

Goolish, E.M. 1987. Cold-acclimation increases ventricle size of carp, *Cyprinus carpo*. J. Therm. Biol. **12**: 203-206.

Goolish. E. M., and Adelman, I.R. 1987. Tissue-specific cytochrome oxidase activity in largemouth bass: the metabolic costs of feeding and growth. Physiol. Zool. **60**: 454-464.

Graham, M.S., and Farrell, A.P. 1989. The effects of temperature acclimation and adrenaline on the performance of a perfused trout heart. Physiol. Zool. **62**: 38-61.

Graham, M.S., and Farrell, A.P. 1992. Environmental influences on cardiovascular variables in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J. Fish. Biol. **41**: 851-858.

Hammer, C. 1995. Fatigue and exercise tests with fish. Comp. Biochem. Physiol. **112**: 1-20.

Herbert, N.A., Armstrong, J.D., and Bjornsson, B.TH. 2001. Evidence that growth hormone-induced elevation in routine metabolism of juvenile Atlantic salmon is a result of increased spontaneous activity. J. Fish. Biol. **59**: 754-757.

Higgs, D.A., Fagerlund, U.H.M., McBride, J.R., Dye, H.M., and Donaldson, E.M. 1977. Influence of combinations of bovine growth hormone, 17α-methyltestosterone, and L-thyroxine on growth of yealing coho salmon (*Oncorhynchus kisutch*) Can. J. Zool. **55**: 1048-1056.

Hill, J.A., Kiessling, A., and Devlin, R.H. 2000. Coho salmon (*Orcorhyncus kitsutch*) transgenic for a growth hormone gene construct increased rates of muscle hyperplasia and detectable levels of differential gene expression. Can. J. Fish. Aquat. Sci. **57**: 939-950.

Hogendoorn, H. 1983. Growth and production of the African catfish. Bioenergetic relations of body weight and feeding level. Aquaculture. **35**: 1-7.
Hommes, F.A. 1980. The energy requirements for growth. A re-evaluation. Hutr. Metab. **24**: 110-13.

Houlihan, D.F. 1991. Protein turnover in ectotherms and its relationship to energetics. *In* Advances in comparative and environmental physiology. vol. 7 (R. Gilles eds.). Springer-Verlag. Berlin. pg. 1-43.

Houlihan, D.F., Hall, S.J., Gray, C., and Noble, B.S. 1988. Growth rates and protein turnover in Atlantic cod, *Gadus morhua*. Can. J. Fish. Aquat. Sci. **45(6)**: 951-964.

Houlihan, D.F., and Laurent, P. 1987. Effects of exercise training on the performance, growth, and protein turnover of rainbow trout (*Salmo gairdneri*).
Can. J. Fish. Aquat. Sci. **44(9)**: 1614-1621.

Houlihan, D.F., C. Stanley, and A. Foster. 1993. Biochemical correlates of growth rate in fish. *In* Fish Ecophysiology (Rankin, J.C.R., and Jenson, F.B. eds.). Chapman & Hall. London. pg. 45-67.

Hughes, G.M. 1984. Measurement of gill area in fishes: Practices and problems. J. Marine Biol. Assoc. U. K. **64**: 637-655.

Hughes, G.M. 1990. On Different methods available for measuring the area of gill secondary lamellae of fishes. J. Mar. Biol. Ass. U.K. **70**: 13-19.

Hughes, G.M., and R.L., Saunders. 1970. Responses of the respiratory pumps to hypoxia in the rainbow trout (*Salmo gairdneri*). J. Exp. Biol. **53**: 529-545.

Ihssen, P.E., Evans, D.O., Christie, W.J., Reckahn, J.A., DesJardine, R.L. 1981. Life history, Morphology, and electrophoretic characteristics of five allopatric stocks of lake whitefish (*Coregonus clupeaformis*) in the Great Lakes region. Can. J. Fish. Aquat. Sci. 38(12): 1790-1807.

Iwasaki, M., Kobayashi, M., Ohgaku, S., Maegawa, H., and Shigeta, Y. 1982. Effect of L-dopa administration on insulin binding: Possible role of growth hormone in regulation of insulin receptor affinity. Horm. Metab. Res. **14(7)**: 342-345.

Jain, K.E., Hamilton, J.C., and Farrell, A.P. 1997. Use of ramp velocity test to measure critical swimming speed in rainbow trout, *Oncorhychus mykiss*. Comp. Biochem. Physiol. **126**: 161-179.

Jhingan, E., Devlin, R.H., and Iwama, G.K. 2003. Disease resistance, stress response and effects of triploidy in growth hormone transgenic coho salmon. J. Fish Biol. **63(3)**: 806-823.

Jobling, M. 1994. *In* Fish Bioenergetics. Chapman & Hall. New York.

Jones, D.R. 1982. Anaerobic exercise in teleost fishes. Can. J. Zool. **60**: 1131-1134.

Johnston, I.A., and Moon,T.W. 1990. Endurance exercise training in the fast and slow muscles of a teleost fish (*Pollachius virens*). J. Comp. Physiol. **135(2)**: 147-156.
Keen, J.E., Vianzon, D.M., Farrell, A.P., and Tibbits, G.F. 1993. Thermal acclimation alters both the adrenergic sensitivity and adrenorecptor density of trout cardiac tissue. J. Exp. Biol. **81**: 27-47.

Kiceniuk, J.W., and D.R., Jones. 1977. The oxygen transport system in trout (*Salmo gairdneri*) during sustained exercise. J. Exp. Biol. **69**: 169-179.

Kirsch, R., and Nonnotte, G. 1977. Cutaneous respiration in three freshwater teleosts. Respir. Physiol. **29(3)**: 339-354.

Kita, J., and Itazawa, Y. 1989. Release of erythrocytes from the spleen during exercise and splenic constriction by adrenaline infusion in the rainbow trout. Jap. J. Ichthyol. **36**: 48-52.

Krohn, M., and Boisclair, D. 1994. The use of stereo-video system to estimate the energy expenditure of free-swimming fish. Can. J. Fish. Aquat. Sci. **51**: 1119-1127.

Kuehl, R.O. 1994. *In* Statistical principles of research design and analysis. Wadsworth Publishing. Belmont. pg. 686.

Laurent, P., Holmgren, S., and Nilsson, S. 1983. Nervous and humoral control of the fish heart: structure and function. Comp. Biochem. Physiol. A. **76(3)**: 525-542.

Lee, C.G., Devlin, R.H., and Farrell, A.P. 2003. Swimming performance, oxygen consumption and excess post-exercise oxygen consumption in adult transgenic and ocean-ranched coho salmon. J. Fish. Biol. **62(3)**: 753-766.

Leggatt, R.A., Devlin, R.H., Farrell, A.P., and Randall, D.J. 2003. Oxygen uptake of growth hormone transgenic coho salmon during starvation and feeding. J. Fish Biol. **62(5)**: 1053-1066.

Leonard, J.B.K., and McCormick, S.D. 2001. Metabolic enzyme activity during smolting in stream- and hatchery-reared Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. **58(8)**: 1585-1593.

Mancera, J.M., and McCormick, S.D. 1999. Influence of cortisol, growth hormone, insulin-like growth factor I and 3,3',5-triiodo-L-thyronine on hypoosmoregulatory ability in the euryhaline teleost *Fundulus heteroclitus*. Fish Physiol. Biochem. **21(1)**: 25-33.

Mathers, E.M., Houlihan, D.F., and Cunningham, M.J. 1992. Nucleic acid concentrations and enzyme activities as correlates of growth rate in saithe *Pollachius virens*: growth rate estimates of open-sea fish. Mar. Biol. **112**: 363-369.

McCormick, S.D., Shrimpton, J.M., Carey, J.B., O'Dea, M.F., Sloan, K.E., Moriyama, S., and Bjornsson, B.T., 1998. Repeated acute stress reduces growth rate of Atlantic salmon parr and alters plasma levels of growth hormone, insulin-like growth factor I and cortisol Aquaculture. **168(1-4)**: 221-235.

McCormick, S.D. 2001. Effects of Growth Hormone and Cortisol on Na super(+)-K super(+)-2Cl super(-) Cotransporter Localization and Abundance in the Gills of Atlantic Salmon. Gen. Comp. Endocrinol. **124(2)**: 134-143.

McFarlane, W.J., and McDonald, G. 1999. *In* Improving sprint performance of juvenile rainbow trout through selection and training. Fish Performance Studies. pg. 131-134.

McKenzie, DJ., Martinez, R., Morales, A., Acosta, J., Morales, R., Taylor, E.W., Steffensen, J.F., and Estrada, M.P. 2003. Effects of growth hormone transgenesis on metabolic rate, exercise performance and hypoxia tolerance in tilapia hybrids. J. Fish Biol. **63(2)**: 398-409.

Moccia, R.D., Gurure, R.M., Atkinson, J.L., and Vandenberg, G.W. 1998. Effects of the repartitioning agent ractopamine on the growth and body composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fed three levels of dietary protein. Aquacult. Res. **29(9)**: 687-694.

Mommsen, T.P., Walsh, P.J., Perry, S.F., and Moon, T.W. 1988. Interactive effects of catecholamines and hypercapnia on glucose production in isolated trout hepatocytes. Gen. Comp. Endocrinol. **70(1)**: 63-73.

Mustin, W.G., and Lovell, R.T. 1993. Feeding the repartitioning agent, ractopamine, to channel catfish (*Ictalurus punctatus*) increases weight gain and reduces fat deposition. Aquaculture. **109(2)**: 145-152.

Mustin, W.G., and Lovell, R.T. 1995. Dietary protein concentration and daily feed allowance influence response of channel catfish, *Ictalurus punctatus* (Rafinesque), to ractopamine. Aquacult. Nutr. **1(1)**: 21-26.

Nathanailides, C. 1996. Metabolic specialization of muscle during developments in cold-water and warmwater fish species exposed to different thermal conditions.Can. J. Fish. Aquat. Sci. **53(9)**: 2147-2155.

Nathanailides C., and N.C., Stickland. 1996. Activity of cytochrome *c* oxidase and lactate dehydrogenase in muscle tissue of slow growing (lower modal group) and fast growing (upper modal group) Atlantic salmon. J. Fish. Biol. **48**: 549-551.

Nielsen, M.E., Boesgaard, L., Sweeting, R.M., McKeown, B.A., and Rosenkilde, P. 1994. Plasma levels of lactate, potassium, glucose, cortisol, growth hormone and triiodo-L-thyronine in rainbow trout (*Oncorhynchus mykiss*) during exercise at various levels for 24 h. Can. J. Zool. **72(9)**: 1643-1647.

Nikinmaa, M., Cech, J.J., and McEnroe, M. 1984. Blood oxygen transport in stressed striped bass (*Morone saxatilis*): role of beta-adrenergic responses. J. Comp. Physiol. B. **154**: 365-369.

Nilsson, S. 1986. Control of gill blood flow. *In* Fish Physiology: Recent advances (Nilsson, S. and Holmgren S. eds.). Croom Helm. London. pg. 87-101.

Nilson, S., and Grove, G.D. 1974. Adrenergic and cholinergic innervation of the spleen of the cod: *Gadus morhua*. Eur. J. Pharmacol. **28**: 135-43.

Nikinmaa, M. 1992a. How does environmental pollution affect red cell function in fish? Aquat. Toxicol. **22**: 227-238.

Nikinmaa, M. 1992b. Membrane transport and control of hemoglobin oxygen affinity in nucleated erythrocytes. Physiol. Rev. **72**: 301-321.

Olvera, M.A., Lara, M., Guzman, B.E., and Lopez, W.G. 2001. Effect of the use of probiotics on the growth of tilapia *Oreochromis niloticus* reared under stress conditions. Aquaculture 2001: Book of Abstracts. pg. 497.

Ostenfeld, T.H., McLean, E., and Devlin, R.H. 1998. Transgenesis changes body and head shape in Pacific salmon. J. Fish Biol. **52(4)**: 850-854.

Ottolenghi, C., Puviani, A.C., Baruffaldi, A., and Brighenti, L. 1984. Epinephrine effects on carbohydrate metabolism in catfish, *Ictalurus melas* . Gen. Comp. Endocrinol. **55(3)**: 378-386.

Palzenberger, M., and H. Pohla. 1992. Gill surface area of water-breathing freshwater fish. Rev. Fish Biol. Fish. **2**: 187-216.

Pauly, D. 1998. Tropical fishes: patterns and propensities. J. Fish Biol. A. **53**: 1-17.

Pelis, R.M., and McCormick, S.D. 2001. Effects of Growth Hormone and Cortisol on Na super(+)-K super(+)-2Cl super(-) Cotransporter Localization and Abundance in the Gills of Atlantic Salmon. Gen. Comp. Endocrinol. **124(2)**: 134-143.

Pelletier, D., Guderley, H., Dutil, J-D. 1993. Does the aerobic capacity of fish muscle change with growth rates? Fish Physiol. Biochem. **12(2)**: 83-93.

Pelletier, D., Blier, P.U., Dutil, J.D., and Guderley, H. 1995. How should enzyme activities be used in fish growth studies? J. Exp. Biol. **198(7)**: 1493-1497.

Perry, S.F. 1998. Relationships between branchial chloride cells and gas transfer in freshwater fish. Comp. Biochem. Physiol. A. **119(1)**: 9-16.

Perry, S.F., and Kinkead, R. 1989. The role of catacholamine sin regulating arterial oxygen content during acute hypercapnic acidosis in rainbow trout (*Salm gairdneri*). Resp. Physiol. **77**: 365-378.

Perry, S.F., Kinkead, R., and Fritsche, R. 1991. Are circulating catecholamines involved in the control of breathing by fishes? Rev. Fish. Biol. Fish. **2**: 65-83.

Perry, S.F., Malone, S., and Ewing, D. 1987. Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). Can J. Zool. **65(4)**: 896-902.

Perry, S.F., Verbost, P.M., Vermette, M.G., and Flik, G. 1988. Effects of epinephrine on branchial and renal calcium handling in the rainbow trout. J. Exp. Zool. **246(1)**: 1-9.

Philip, A.R., Newsholme, E.A., and Zammit, V. 1975. Activities of citrate synthase and NAD+-linked and NADP+-linked isocitrate dehydrogenase in muscle from vertebrates and invertebrates. Biochem J. **154**: 689-700.

Pickering, A.D. 1981. Adrenergic responses to stress in fish. *In* Stress and fish. Academic Press. Toronto. pg. 48-75.

Pickering, A.D., Pottinger, T.G., 1995. Biochemical effects of stress. *In* Biochemistry and Molecular Biology of Fishes, 5. Environmental and Ecological Biochemistry. (Hochachka, P.W., and Mommsen, T.P. eds.). Elsevier. Amsterdam, pg. 349–379.

Pitkanen, T.I., Xie, S.Q., Krasnov, A., Mason, P.S., Molsa, H., and Stickland, N.C. 2001. Changes in tissue cellularity are associated with growth enhancement in genetically modified Arctic char (*Salvelinus alpinus* L.) carrying recombinant growth hormone gene. Mar. Biotechnol. **3**: 188-197.

Popova, J., Robeva, A., and Zaharieva, S. 1990. Muscarinic receptor activity change after prolonged treatment with growth hormone and somatostatin. Comp. Biochem. Physiol. **96(1)**: 119-123.

Pörtner, H.O. 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. Comp. Biochem. Physiol. **132**: 739-761

Primmett, R., Randall, D.J., Mazeaud, M., and Boutilier, R.G. 1986. The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow trout (*Salmo gairdneri*) during exercise. J. Exp. Biol. **122**: 139-148.

Randall, D.J., and Taylor, E.W. 1991. Evidence of a role for catecholamines in the control of breathing in fishes. Rev. Biol. Fish. Biol. **1**: 139-57.

Reid, S.D., and Perry, S.F. 1991. The effects and physiological consequences of raised levels of cortisol on rainbow trout (*Salmo mykissi*) erythrocyte B-adrenoreceptors. J. Exp. Biol. **158**: 217-240.

Reist, J. D. 1986. An empirical evaluation of coefficients used in residual and allometric adjustment of size covariation. Can. J. Zool. **64**: 1363-1368.

Rodnick, K.J., Gamperl, A.K., Lizars, K.R., Bennett, M.T., Rausch, R.N., and Keeley, E.R. 2004. Thermal tolerance and metabolic physiology among redband trout in south-eastern Oregon. J. Fish. Biol. **64**: 310-335.

Rodnick, K.J., and Sidell, B.D. 1997. Structural and biochemical analysis of cardiac ventricular enlargement in cold-acclimated striped bass. Am. J. Physiol. **273**: 252-258.

Sadler, J., Wells, R.M.G., Pankhurst, P.M., and Pankhurst, N.W. 2000. Blood oxygen transport, rheology and haematological responses to confinement stress in diploid and triploid Atlantic salmon, *Salmo salar*. Aquaculture. **184(3-4)**: 349-361.

Santer, R.M., and Greer Walker, M. 1980. Morphological studies on the ventricle of teleost and elasmobranch hearts. J. Zool. Lond. **190**: 259-272.

Shrimpton, J.M., Devlin, R.H., McLean, E., Byatt, J.C., Donaldson, E.M., and Randall, D.J. 1995. Increases in gill cytosolic corticosteroid receptor abundance and saltwater tolerance in juvenile coho salmon (*Oncorhynchus kisutch*) tested with growth hormone and placental lactogen. Gen. Comp. Endocrin. **98**: 1-15.

Silverstein, J.T., and Freeman, D.W. 2001. Feed intake of large and small catfish in mixed size populations. Aquaculture 2001: Book of Abstracts. pg. 593.

Simon, M. M., and Robin, E. D. 1971. Relationship of cytochrome oxidase activity to vertebrate total and organ oxygen consumption. Int. J. Biochem. **2**: 569-573.

Smit, H. 1965. Some experiments on the oxygen consumption of goldfish (*Carassius auratus* L.) in relation to swimming speed. Can J. Zool. **43**: 623-633.

Smith, L.S., and Bell, G.R. 1964. A technique for prolonged blood sampling in free swimming salmon. J. Fish. Res. Bd. Can. **21**: 711-717.

Stevens, E.D., and Devlin, R.H. 2000. Gill morphology in growth hormone transgenic Pacific coho salmon *Onchorhynchus kisutch*, differs markedly from that of GH transgenic Atlantic salmon. Environ. Biol. Fish. **58**: 113-117.

Stevens, E.D., Sutterlin, A.M., and Cook, T. 1998. Respiratory metabolism and swimming performance in growth hormone transgenic Atlantic salmon. Can. J. Fish. Aquat. Sci. **55**: 2028-2035.

Stevens, E.D., and Sutterlin, A. 1999. Gill morphometry in growth hormone transgenic Atlantic salmon. Environ. Biol. Fish. **54**: 405-411.

Stokstad, E. 2002. Engineered fish: Friend or foe to the environment. Science. **297**: 1797-1799.

Sumpter, J.P., Le Bail, P.Y., Pickering, A.D., Pottinger, T.G., and Carragher, J.F. 1991. The effect of starvation on growth and plasma growth hormone concentrations of rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrin. **83(1)**: 94-102.

Sundström, L. F., Devlin, R.H., Johnsson, J.I., and Biagi, C.A. 2003. Vertical position reflects increased feeding motivation in growth hormone transgenic coho salmon (*Oncorhynchus kisutch*). Ethology. **109**: 701-712.

Sundström, L. F., Lohmus, M., Johnsson, J.I., and Devlin, R.H. 2004. Growth hormone transgenic salmon pay for growth potential with increased predator mortality. Proc. R. Soc. Lond. B (Suppl.).

Sveier H., Wathne E., and Lied E. 1999. Growth, feed and nutrient utilisation and gastrointestinal evacuation time in Atlantic salmon (*Salmo salar*): the effect of dietary fish meal particle size and protein concentration. Aquaculture. **180**: 265-282.

Tang, Y., and Boutilier, R.G. 1988. Correlation between catecholamine release and degree of acidotic stress in trout. Am. J. Physiol. R. **255**: 395-399

Taylor, E.B., and McPhil, J.D. 1985. Variation in burst and prolonged swimming performance among British Columbia populations of coho salmon, *Oncorhynchus kisutch*. Can. J. Fish. Aquat. Sci. **42**: 2029-2033.

Thorpe, J.E. 1977. Bimodal distribution of length of Atlantic salmon (*Salmo salar*, L.) Under artificial rearing conditions. J. Fish. Biol. **11**: 175-184.

Thorpe, J.E., Talbot, C., and Villarreal, C. 1982. Bimodality of growth and smolting of Atlantic salmon *Salmo salar*, L. Aquaculture. **28**: 123-132.

Vandenberg, G.W., and Moccia, R.D. 1998. Growth performance and carcass composition of rainbow trout, *Oncorhynchus mykiss* (Walbaum), fed the beta -agonist ractopamine. Aquacult. Res. **29(7)**: 469-479.

Vijayan, M.M., and Leatherland, J.F. 1988. Effect of stocking density on the growth and stress-response in brook charr, *Salvelinus fontinalis*. Aquaculture. **75(1-2)**: 159-170.

Ultsch, G.R., M.E., Ott, and N. Heisler. 1980. Standard metabolic rate, critical oxygen tension, and aerobic scope for spontaneous activity of trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) in acidified water. Comp. Biochem. Physiol. A. **67**: 329-335.

Waller, U. 1992. Factors influencing routine oxygen consumption in turbot, Scophthalmus maximus . J. Appl. Ichthyol. **8(1-4)**: 62-71.

Waterlow, J.C. and Millward, D.J. 1989. Energy cost of turnover of protein and other cellular constituents, *In* Energy Transformations in Cells and Organisms (Wieser, W. and Gnaiger, E. eds.), Gerg Thieme. Stuttgart. pg. 277-82.

Weibel, E.R. 2002. *In* Symmorphosis: On form and function in shaping life. Harvard University Press. New York.

Wendelaar Bonga. S.E. 1997. The stress response in fish. Physiol. Rev. **77(3)**: 591-625.

Woodward, J.J. 1983. The effects of prolonged physical exercise on plasma catecholamines and the stress response of rainbow trout (*Salmo gairdneri*).Diss. Abst. Int. Pt. B. Sci. & Eng. **43(10)**: 88.

Wright, P.A., Perry, S.F., and Moon, T.W. 1989. Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. J. Exp. Biol. **147**: 169-188.

Yang, B., Greene, M., and Chen, T.T. 1999. Early embryonic expression of the growth hormone family protein genes in the developing rainbow trout, *Onchorynchus mykiss*. Molec. Repro. Devolop. **53**: 127-134.

Young, G. 1988. Enhanced response of eh interregnal of coho salmon (Oncorhynchus kisutch) to ACTH after growth hormone treatment *in vivo* and *in vitro*. Gen. Comp. Endocrinol. **71**: 85-92.

Zhang, W., Zhang, L., and Lin, H. 1996. Effects of catecholamines on growth hormorne (GH) secretion in grass carp (*Ctenopharyngodon idellus*). Acta. Hydrobiol. Sin. **20(4)**: 340-344.