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Cardiorespiratory modifications, and limitations, in post-smolt growth hormone transgenic Atlantic salmon *Salmo salar*

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Summary

In recent years, there has been a great deal of interest in how growth hormone (GH) transgenesis affects fish physiology. However, the results of these studies are often difficult to interpret because the transgenic and non-transgenic fish had very different environmental/rearing histories. This study used a stable line of size-matched GH Atlantic salmon (*Salmo salar*) that were reared in a shared tank with controls (at 10°C, for ~9 months) to perform a comprehensive examination of the cardiorespiratory physiology of GH transgenic salmon, and serves as a novel test of the theory of symmorphosis. The GH transgenic salmon had a 3.6× faster growth rate, and 21 and 25% higher values for mass-specific routine and standard oxygen consumption ($\dot{M}_{O_2}$), respectively. However, there was no concurrent increase in their maximum $\dot{M}_{O_2}$, which resulted in them having an 18% lower metabolic scope and a 9% reduction in critical swimming speed. This decreased metabolic capacity/performance was surprising given that the transgenics had a 29% larger heart with an 18% greater mass-specific maximum *in situ* cardiac output, a 14% greater post-stress blood haemoglobin concentration, 5–10% higher red muscle and heart aerobic enzyme (citrate synthase or cytochrome oxidase) activities, and twofold higher resting and 1.7× higher post-stress, catecholamine levels. However, gill surface area was the only cardiorespiratory parameter that was not enhanced, and our data suggest that gill oxygen transfer may have been limiting. Overall, this research: (1) shows that there are significant metabolic costs associated with GH transgenesis in this line of Atlantic salmon; (2) provides the first direct evidence that cardiac function is enhanced by GH transgenesis; (3) shows that a universal upregulation of post-smolt (adult) GH transgenic salmon cardiorespiratory physiology, as suggested by symmorphosis, does not occur; and (4) supports the idea that whereas differences in arterial oxygen transport (i.e. cardiac output and blood oxygen carrying capacity) are important determinants of inter-specific differences in aerobicity, diffusion-limited processes must be enhanced to achieve substantial intra-specific improvements in metabolic and swimming performance.

Key words: transgenic, symmorphosis, heart, oxygen transport, critical swimming speed, cardiovascular function, stress, catecholamines, cortisol, Atlantic salmon, *Salmo salar*.

Introduction

Growth hormone (GH) transgenesis has recently been used as an innovative way to achieve higher growth rates in fish aquaculture. This technique introduces a growth hormone (GH)-promoting transgene into the fish's DNA, resulting in fish with dramatically accelerated growth rates (~two to tenfold) as compared with non-transgenic conspecifics (Du et al., 1992; Devlin, 1994; Martinez et al., 1999; Stokstad, 2002). Accompanying this enhanced growth is an increased demand for oxygen to supply the aerobic pathways that support it, and various studies have reported increases in resting/routine metabolism (Stevens et al., 1998; Cook et al., 2000b; Lee et al., 2003), and/or changes in one or more aspects of the cardiorespiratory physiology (Stevens and Sutterlin, 1999; Stevens and Devlin, 2000; Blier et al., 2002; Cogswell et al., 2002; McKenzie et al., 2003) of GH transgenic fishes. However, it has been difficult to construct a clear picture of how GH trangenesis affects fish physiology because of the use of different strains, size *versus* age-matched controls, and especially experimental groups with different rearing/environmental histories. Thus, we performed the most comprehensive examination of the cardiorespiratory physiology and performance capacity of GH transgenic fish (measuring routine metabolic rate, metabolic capacity, critical swimming speed [U_{crit}], gill surface area, haematocrit and haemoglobin levels, erythrocyte morphometrics, stress hormone levels, *in situ* cardiac performance and tissue oxidative enzyme activities) to date, and identified how the GH

transgenic Atlantic salmon's cardiorespiratory system has adapted to accelerated growth, and/or increased activity, as compared with size-matched non-transgenic conspecifics. Further, because differences in rearing environment/environmental history have been a criticism of earlier studies on the effects of GH transgenesis on salmon physiology (e.g. Lee et al., 2003; Leggatt et al., 2003), we reared control (non-transgenic) and GH transgenic fish in a common tank from the point of salt water transfer as smolts (i.e. for >9 months). This allowed for a more accurate picture of how ectopic expression of GH affects the metabolic, exercise and stress physiology of salmonids.

When the research conducted so far on GH transgenic salmon is combined, the data suggest that many of the steps in the oxygen uptake, transport and utilization pathway have been upregulated to support their increased growth rates. A universal upregulation of the cardiorespiratory system of GH transgenics would be in agreement with the theory of symmorphosis, which predicts that the size of the parts in a system must be matched to overall functional demand, and that animals must provide their complex systems with a functional capacity that can cope with the highest expected functional demands (Weibel et al., 1991; Weibel, 2002). Indeed, Stevens and Sutterlin (Stevens and Sutterlin, 1999) suggest that this may be occurring in GH transgenic Atlantic salmon, as they reported that transgenic Atlantic salmon juveniles have a gill surface area 1.24× that of non-transgenic controls, and an oxygen uptake 1.7-fold higher.

The GH transgenic salmon is a unique model for testing the theory of symmorphosis, and investigating how animals upregulate aspects of their oxygen uptake–transport–utilization pathway to cope with increases in physiological and metabolic demand. Thus, an additional goal of this research was to examine how GH transgenesis affects the structural and/or functional capacity of several steps in the oxygen uptake–transport–utilization pathway. In this study we measured numerous physiological parameters, but in the context of the theory of symmorphosis as it relates to the capacity of the fish to deliver and utilize oxygen we were specifically interested in: (1) gill surface area; (2) blood haematocrit and haemoglobin concentration; (3) heart morphometrics and maximum *in situ* cardiac function; (4) tissue aerobic enzyme activities; and (5) maximum, post-stress, catecholamine levels. Although the latter measurement, in particular, does not fit with the strict definition of symmorphosis as it relates to the cardiorespiratory system, many argue that the original definition of symmorphosis is too rigid, and Weibel (Weibel, 2002) himself states that "*what counts in an integrated system such as the animal body is good integration of the parts to make a successful whole*". Catecholamines significantly enhance the functioning of numerous elements of the fish cardiorespiratory system (Randall and Perry, 1992), and thus including catecholamines in the examination of symmorphosis as it relates to the functional capacity of this system is appropriate.

Materials and methods

Fish referred to as 'transgenics' are fifth generation EO-1α transgenic Atlantic salmon (*Salmo salar* L.) 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 transgenic strain contains a single copy of a transgene with a rapid growth phenotype and has exhibited stable Mendelian inheritance over six 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 (32 ppt) in a common 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, Canada), and were implanted with pit tags (Avid Identification Systems Inc., CA, USA) so that individuals could be identified. They were fed (with high energy dry extruded salmon feed; Corey Feed Mills Ltd., NB, Canada) by an automatic feeder seven times daily. This regime allowed the controls and transgenics to grow at 0.30 and 1.03% per day, respectively, over the experimental period (approx. 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.

To confirm whether fish were transgenic or non-transgenic, polymerase chain reaction (PCR) was performed using adipose fin tissue. A buffer containing 10 mmol l^{-1} Tris (pH 8.0), 50 nmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 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 2654-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 (Fig. 1).

Metabolic physiology and swimming performance

Fish metabolism and swimming performance (at 10°C) were measured using a 81 l Blazka swim-tunnel respirometer (University of Waterloo, Biotelemetry Institute, Waterloo, ON, Canada). This swim-tunnel was covered with black plastic to provide the fish with a darkened refuge, and the rear of the tunnel was fitted with electrified (<5 V) stainless steel bands to prevent the fish from resting on the rear grid of the tunnel. Water temperature and oxygen content within the respirometer were measured continuously by pumping water through an external circuit containing a custom flow chamber and a galvanic oxygen electrode (Model CellOx 325 WTW Inc.,

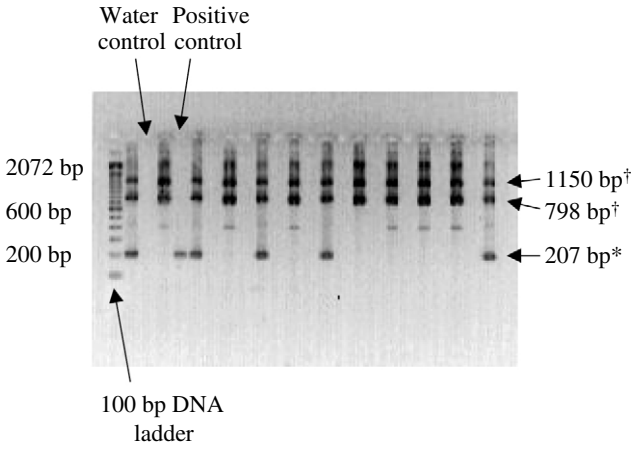

Fig. 1. Identification of GH transgenic and control Atlantic salmon using polymerase chain reaction (PCR). All samples were analyzed by electrophoresis using a 2% agarose gel and visualized with ethidium bromide. A water control was run to ensure that no exogenous genetic material was present in the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

Weilheim, Germany) that was connected to an oxygen meter (Model Oxi 340, WTW Inc.).

Before the respirometry experiments, all fish were fasted for 2 days. Their length (*BL*=body length) was then measured, and they were placed in the respirometer approximately 12–16 h before testing to allow them to acclimate to the tunnel, and to recover from handling. All fish were initially given a 30 min 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.

After the acclimation period, swimming capacity was measured using a standard U_{crit} test (Hammer, 1995). Routine metabolism ($mg\ O_2\ kg^{-1}\ h^{-1}$) was measured for 20 min at a speed of $0.5\ BL\ s^{-1}$, a speed at which the fish made minimal movements. Water velocity was then increased by $0.25\ BL\ s^{-1}$ every 20 min, with oxygen consumption measured for 15 min, starting 5 min after the desired speed was reached. This procedure was repeated until the fish was no longer capable of swimming, and could not separate itself from the back grid of the respirometer. Critical swimming speed was calculated using the equation:

$$U_{crit} = U_f + (t_f/t_i U_i)\ , \quad (1)$$

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 (20 min), and U_i is the water velocity increment ($0.25\ BL\ s^{-1}$) (Brett, 1964). If the cross-sectional area of the fish was greater than 10% of the cross-sectional area of the swim-tunnel, U_{crit} values were corrected for solid blocking effects using the formula of Bell and Terhune (Bell and Terhune, 1970).

Standard oxygen consumption ($mg\ O_2\ kg^{-1}\ h^{-1}$) was obtained from a semi-log plot of swimming speed ($BL\ s^{-1}$) *versus* log $\dot{M}_{O_2}$ ($mg\ O_2\ kg^{-1}\ h^{-1}$), and using the derived linear regression to extrapolate back to $0\ BL\ s^{-1}$. $\dot{M}_{O_2,max}$ ($mg\ O_2\ kg^{-1}\ h^{-1}$) was measured as the highest oxygen consumption that each fish achieved. Absolute metabolic scope was then calculated by subtracting standard $\dot{M}_{O_2}$ from $\dot{M}_{O_2,max}$, and factorial metabolic scope was calculated by dividing $\dot{M}_{O_2,max}$ by standard $\dot{M}_{O_2}$.

After all metabolic and swimming measurements were complete, the fish were sacrificed using cerebral concussion, 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. The gill arches were then removed from the left side of each fish, placed in cold 9:1 formalin, and stored at (4°C) until gill surface area was measured. Caudal fin surface area was obtained by taking a digital photograph of the caudal fin using a Coolpix 2500 digital camera (Nikon USA, Melville, NY, USA) and analysing it using Matrox Inspector 3.0 (Matrox Electronic Systems Ltd., Dorval, QC, Canada).

Routine $\dot{M}_{O_2}$, standard $\dot{M}_{O_2}$, and $\dot{M}_{O_2,max}$, as well as U_{crit} and absolute and factorial scope, were compared between groups using an ANCOVA (Sigmaplot, SPSS Inc., Chicago, IL, USA). For analyses of oxygen consumption, and metabolic scope, the mass of the fish was used as the covariate. For U_{crit}, the length of the fish was used as the covariate. To minimize variability due to the allometric growth of morphometric characters the data were 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. (Ihssen et al., 1981):

$$M_1 = M_o(L/L_o)^b\ , \quad (2)$$

where M_1 is the transformed character, M_o is the observed character, L is the grand sample mean body length of all fish, 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.

Muscle enzyme activity and protein content

Fish from each group (N=8) were sacrificed with a sharp blow to the head and samples of red and white muscle were quickly removed and immediately frozen in liquid nitrogen. Before the ventricle was frozen, however, it was washed with saline and blotted dry in order to remove any blood from the lumen. All samples were stored at –80°C until assays were performed. Protein concentration in each tissue was measured using the Coomassie Plus Protein Assay Reagent protocol and reagents (Pierce Biotechnology Inc., Rockford, IL, USA). The

following procedure for determining enzyme activity was adapted from Fudge et al. (Fudge et al., 2001). Tissue samples were homogenized in ice-cold 50 mmol l^{-1} imidazole buffer (1:20 w/v) (for pH see below), and enzyme activity was measured in the supernatant at 10°C using a spectrophotometer and wavelengths of 412 nm and 550 nm for citrate synthase (CS) and cytochrome *c* oxidase (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 then dialyzed against several changes of potassium buffer to remove excess ascorbic acid. The final CCO concentration in the cuvette was 0.1 mmol l^{-1}, and assay conditions were as follows. CCO (EC 1.9.3.1): 10 mmol l^{-1} 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 (EC 4.1.3.7): 75 mmol l^{-1} Tris, 0.25 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid (DTNB), 0.4 mmol l^{-1} acetyl CoA (pH 7.0), 0.5 mmol l^{-1} oxaloacetate, with the reaction initiated by 10 μl of supernatant in all tissues (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.

Enzyme activities were compared between groups using a two-way ANOVA (fish and tissue) assuming equal variances (tested with an *F*-test) using Sigmaplot (SPSS Inc., Chicago, IL, USA). A significance level of $P<0.05$ was used for all analyses.

Gill surface area

The concepts and measurement procedures described in Stevens and Sutterlin (Stevens and Sutterlin, 1999) and Hughes (Hughes, 1984) were used to measure gill surface area. They were, however, modified to utilize digital imaging tools. Further, 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 four gill arches from the left side of each fish were removed and preserved in cold (4°C) 9:1 formalin. Shrinkage was found to be <1.5% for both filament length and lamellar spacing in both the transgenics and controls after 24 h, and 2% after 72 h. All subsequent measurements were corrected to account for this shrinkage.

The biometry of the preserved gill tissue was determined using digital image analysis software (Matrox Inspector 3.0, Matrox Electronic Systems Ltd.). Images were captured with a light microscope (Wild Makroskop model M420, Wetzlar, Germany) linked to a digital camera (Pixera PVC 100, Los Gatos, CA, USA), and a desktop PC using Pixera Studio (I-Cube, Rowland Heights, CA, USA). The lengths of all gill filaments, on all four 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). Then, a medium sized filament from each of the three groups was selected, and the interlamellar spacing obtained by measuring the distance covered by 10 adjacent lamellae. This gave the average spacing between each lamella.

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 third, middle third, and top third) of the filament, taking care to obtain lamella that were an average size for that particular region. They were then spread flat on a glass slide, digital images were obtained of both sides, and areas were determined using Matrox Inspector 3.0. Once the lamellar areas at all three positions were measured, from all three filament length categories, a linear equation was created 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. 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, and summed to give the entire lamellar area of the arch. The area of all the arches were then summed, and multiplied by two, to account for both sides of the fish.

In order to reduce the type-1 error, it was necessary to correct the *P* value to accommodate for comparisonwise and experimentwise errors (Kuehl, 1994). The adjusted *P* value (α_c) was obtained using the equation:

$$\alpha_c = 1 - (1-\alpha_e)^{1/N}, \quad (3)$$

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 was:

$$\alpha_c = 1 - (1-0.05)^{1/16} = 0.0032 . \quad (4)$$

In order to standardize the gill areas for the mass of the fish, each individual was scaled to 1 kg, and its gill morphometric values adjusted accordingly. The gill areas from the controls and transgenics were then compared using the corrected *P* value (0.0032) by means of an analysis of covariance (ANCOVA), with body mass as the covariate. A comparison of gill surface area to routine and maximum oxygen consumption was then made by fitting linear regressions to the data. All statistical analyses were performed using Sigmaplot (SPSS Inc.).

Haematological parameters and stress hormone measurements

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) was then inserted into the dorsal aorta, to allow for blood sampling, according to the method of Smith and Bell (Smith and Bell, 1964). Thereafter, fish were placed in a black Perspex box (40 cm long×10 cm wide×10 cm deep) that received 10°C seawater at a rate of 1 l^{-1} min^{-1} from an aerated seawater reservoir. After a 48 h recovery period, a resting blood sample (0.3 ml) was taken from each fish, and various haematological parameters were measured. Haematocrit was measured in triplicate by collecting blood in 20 μl capillary tubes, and centrifuging them for 2 min. Haemoglobin concentration was measured using the cyanomethaemoglobin method, and erythrocyte optical surface area, perimeter and circularity were determined using blood smears (Cogswell et al., 2002). These measurements were made using a light microscope (Wild Makroskop model M420) and a desktop PC running Pixera Studio (I-Cube) and the digital image analysis software Matrox Inspector 3.0. Mean corpuscular haemoglobin concentration (MCHC) was determined as in Sadler et al. (Sadler et al., 2000).

A further 1 ml of blood was drawn from the fish, placed in a centrifuged tube and spun for 30 s at 15,600 ***g***. 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, and the remaining plasma was frozen for measurement of resting cortisol levels.

Each fish was then netted, held in the air for 45 s, and returned to their black box. Blood for haematological measurements was immediately sampled as described above, and a further 1 ml was taken and spun for 30 s at 15,600 ***g***. Again, 550 μl of plasma was obtained from the centrifuged blood, placed in a cryovial, and frozen to measure post-stress catecholamine levels. 30 min 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 plasma catecholamines epinephrine (EPI) and norepinephrine (NE) were measured using high performance liquid chromatography (HPLC, Bioanalytical Systems Inc, Lafayette, IN, USA) with electrochemical detection (+650 mV) after extraction with alumina (BAS LCEC Application Note 14). Separation of the catecholamines was performed on a reverse phase column (ODS, 3.0 mm i.d.×10 cm long, 3 μm pore size; model MF 8954), using an aqueous mobile phase (containing per litre: 7.088 g of monochloroacetic acid, 186.1 mg $Na_2EDTA{\cdot}2H_2O$, 15 ml acetonitrile and 32.3 mg sodium octyl sulphate, pH 3.00–3.05) pumped at a flow rate of 1 ml min^{-1}. EPI and NE plasma concentrations were calculated relative to the NE/EPI synthetic standards (75 ng ml^{-1} NE, 75 ng ml^{-1} EPI), and with DHBA (3,4-dihydroxybenzylamine) as an internal standard. Recoveries from the alumina, determined on standards, were in the order of 60–70%, and were used in the determination of individual plasma CA concentrations. Plasma cortisol concentrations were measured using a commercial Coat-A-Count® cortisol radioimmunoassay (RIA) kit (Diagnostic Products Corporation, Los Angeles, CA, USA).

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

Cardiac performance

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 anaesthetic (0.5 g l^{-1} MS-222). The fish were injected with 0.5 ml of heparinized saline (100 i.u. ml^{-1}) (Sigma Chemical Co., St Louis, MO, USA) *via* the caudal vein, and an *in situ* heat preparation was obtained as described by Farrell et al. (Farrell et al., 1986; Farrell et al., 1989).

Once placed in the *in situ* heart 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, the height of which could be adjusted to control end-diastolic pressure. The heart was then perfused with physiological saline (recipe below) from temperature controlled (10°C) water-jacketed bottles. Output pressure was initially maintained at 2–3 kPa to let the heart recover from surgery, and to prevent excessive cardiac work while the input pressure was being set to a physiologically relevant resting cardiac output (16 ml min^{-1} kg^{-1}) (Kiceniuk and Jones, 1977). Subsequently, output pressure was raised to 5 kPa, a level comparable to *in vivo* arterial pressure (Kiceniuk and Jones, 1977). After allowing the heart to stabilize for 15 min., resting cardiac performance measurements were recorded. Then, maximum cardiac output was determined by increasing input pressure from the height required to achieve resting cardiac output to 0.15 kPa, and then in a stepwise fashion (0.05 kPa increments) to 0.60 kPa (Fig. 2). Finally, power curves were obtained by lowering output pressure to 3.0 kPa, and increasing output pressure in 1.0 kPa increments until the heart could no longer pump (or a height of 10 kPa) (Fig. 2). The time spent at each level of input or output pressure was just long enough to allow cardiac performance to stabilize, approximately 30 s.

Input and output pressures were measured using Gould (P23 ID, Oxnard, CA, USA) and Grass (PT300, Warwick, RI, USA) pressure transducers, respectively, and cardiac output was measured with a 2N flow probe in conjunction with a T206 flow meter (Transonic Systems Inc., Ithaca, NY, USA). Input and output pressures were corrected to account for cannula resistance between the point of measurement and the heart (using predetermined calculations from Faust, 2001), and the pressure transducers were calibrated daily against a static column of water. Pressure and flow signals were amplified and filtered using a Model MP100A-CE data acquisition system

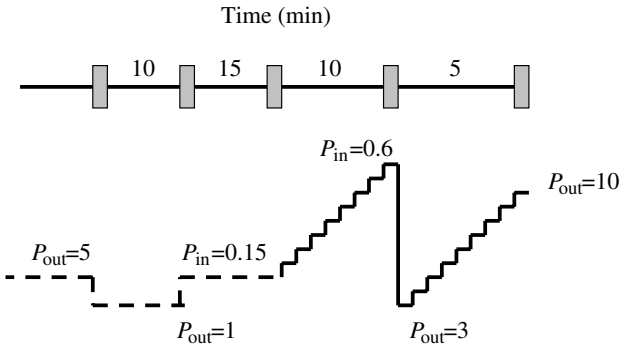

Fig. 2. Experimental protocol for measuring $\dot{Q}_{max}$ and power output of the *in situ* Atlantic salmon heart. The broken line represents the end-diastolic pressure developed by the ventricle, determined by adjusting the height of the output pressure head. P_{out} was normally set to a physiologically realistic value of 5 kPa; however a sub-physiological level of P_{out} (2–3 kPa) was used at the start of the protocol to let the heart recover from surgery. The first set of steps indicate the maximum cardiac output test ($\dot{Q}_{max}$), where P_{in} was raised sequentially from 0.15 kPa to 0.6 kPa, whereas the second set of steps indicate the myocardial power test where output pressure was raised from 3 kPa to 10 kPa, while P_{in} remained at 0.60 kPa.

(BIOPAC Systems Inc., Santa Barbara, CA, USA), and the acquired signals were stored and analyzed using Acknowledge Software (Biopac Systems Inc., Santa Barbara, CA, USA), 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 clamping the input perfusate line with a pair of haemostats and ensuring cardiac output fell to zero, then raising the output tube and ensuring no significant backflow occurred. The hearts were then dissected from the fish and the chambers separated, blotted dry, and weighed. The compact myocardium of the ventricle was then separated from the spongy myocardium by dissection, and each was weighed separately.

Experimental solutions

Hearts were perfused with physiological marine teleost saline during the surgery and during the experimental period. This saline (pH 7.76 at 12°C) contained (in mmol l^{-1}): 181.3 NaCl, 5.0 KCl, 2.30 $CaCl_2.H_2O$, 1.99 $MgSO_4.6H_2O$, 2.58 TES acid, 7.33 sodium Tes base, and 5.55 dextrose. These chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA), with the exception of the TES salt and adrenaline, which were purchased from Sigma Chemical Co. (St Louis, MO, USA). The TES buffer system was used to simulate the buffering capacity of salmon plasma (Keen et al., 1993). Epinephrine (10 nmol l^{-1}) was added to the perfusate to ensure the long-term viability of the *in situ* heart (Graham and Farrell, 1989). The saline was continuously gassed with oxygen during both surgery and while the *in situ* measurements were conducted.

Cardiac function was continuously monitored by measuring input pressure (P_{in}) and output pressure (P_{out}), cardiac output ($\dot{Q}$, ml min^{-1} kg^{-1}), heart rate (f_H, beats min^{-1}), and stroke volume (V_S, ml kg^{-1} and ml g $vent^{-1}$). Although data were continuously collected, cardiac function was only analysed at specific intervals during each experiment. Resting cardiac function ($\dot{Q}_{rest}$, f_H, and V_S) was measured prior to the $\dot{Q}_{max}$ test. Maximum cardiac function was quantified by measuring $\dot{Q}_{max}$, f_H, V_S and power output (P_0) . Heart rate was calculated by measuring the number of systolic peaks during a 20-s interval, and V_S (ml kg^{-1} $beat^{-1}$) and P_0 (mW g $ventricle^{-1}$) were calculated as follows:

$$V_S = \dot{Q} / f_H \quad (5)$$

$$P_0 = \dot{Q} \bullet (P_{out} - P_{in}) \bullet a / M_v , \quad (6)$$

where P_{out} and P_{in} are output and input pressures (in cm H_2O) respectively, M_v is ventricle mass and a=0.098 (mW s ml^{-1} cm^{-1} H_2O) is the conversion to milliwatts. Statistical differences between groups were identified using a one-way ANOVA (P<0.05) (Sigmaplot, SPSS Inc.), with the exception of heart chamber masses which were compared by means of an ANCOVA with body mass as the covariate.

Results

The transgenic and control salmon were similar in body mass, fork length, depth, opercular length, caudal peduncle depth and tail surface area (Table 1A).

Routine $\dot{M}_{O_2}$ and standard $\dot{M}_{O_2}$ were 21% and 25% greater, respectively, in the transgenic salmon than in the controls. However, $\dot{M}_{O_2,max}$ was not different between groups (P=0.42) (Fig. 3; Table 1B). The higher standard $\dot{M}_{O_2}$, coupled with the unchanged $\dot{M}_{O_2,max}$, resulted in the transgenics having significantly lower absolute (by 18%) and factorial metabolic scopes (by 29%). These differences in metabolic scope were reflected in the U_{crit} measurements, where transgenic salmon swam 9% slower than the controls (approx. 100 and 93 cm s^{-1}, respectively).

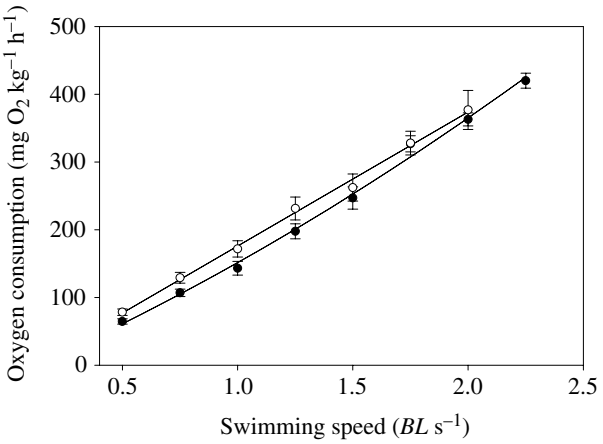

Fig. 3. Oxygen consumption of transgenic (open circles, y=–9.88+174.92x+9.17x^2) and control (closed circles, y=–18.27+147.49x+21.95x^2) Atlantic salmon at various swimming speeds. Values are means ± 1 standard error (N=8).

Table 1. *Physical characteristics (A), and oxygen consumption, metabolic scope and critical swimming speed (B) of GH transgenic and control Atlantic salmon at 10°C*

	Control	Transgenic	Trans/Con ratio	*P* value
A				
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	0.76
Opercular length (cm)	8.9±0.7	8.8±0.4	0.98	0.79
Peduncle depth (cm)	4.2±0.4	4.2±0.2	0.99	0.89
Tail area (cm^2)	47.1±6.9	52.8±3.5	1.12	0.44
B				
Oxygen consumption (mg O_2 kg^{-1} h^{-1})				
Routine	64.5±3.9	78.2±4.7	1.21	0.03*
Standard	46.4±2.1	58.1±4.4	1.25	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 s^{-1})	2.2±0.1	2.0±0.1	0.91	0.04*

Values are means ± 1 standard error (N=8).
*Significant difference between groups (P<0.05).

No significant differences were found in the number of filaments per arch, total filament length, lamellar density, lamellar area, or total gill area between groups (Table 2). Interestingly, however, a significant relationship existed between routine $\dot{M}_{O_2}$ and total gill surface area in the transgenics (R^2=0.63) (Fig. 4), but not controls.

Resting cortisol levels (~12 ng ml^{-1}) were not significantly different between control and transgenic salmon (Table 3). Both the transgenic and control salmon showed significant stress-related increases in plasma cortisol (by 35% and 51%, respectively). However, post-stress cortisol levels in the control salmon were significantly higher (by 28%). Resting EPI and NE levels ranged from 2 to 6 nmol l^{-1}, and catecholamine concentrations (EPI, NE, and total) increased by 2.1–3.7× following the 45-s net stress. With one exception (resting EPI, P=0.17), plasma catecholamine levels were significantly higher in the transgenic salmon as compared with the controls (Table 3).

Erythrocyte perimeter and compactness were significantly greater (by 3% and 8%, respectively) in control salmon than in the transgenics (Table 4). These differences, however, may have limited biological significance because there was no difference in erythrocyte optical surface area (P=0.10). The 45-s net stress did not lead to a significant change in blood haematocrit, haemoglobin concentration or MCHC in either group (Table 4). Further, there was only one difference in these parameters between control and transgenic fish: post-stress haemoglobin levels were 14% higher in transgenic salmon (7.3±0.3 *vs* 6.4±0.2).

In both groups, protein content was greatest in the white muscle, followed by the red muscle, and then the myocardium (ranging from 147.8 to 118.1 mg g wet $mass^{-1}$) (Table 5). No

Table 2. *Gill morphometric parameters for transgenic and control Atlantic salmon*

	Control	Transgenic	Trans/Con ratio	*P* value
Number of filaments	263±21	260±13	0.99	0.72
Total filament length (mm)	19530±832	20432±494	1.08	0.35
Lamellar density (mm^{-1})	26.6±3.5	22.8±3.2	0.86	0.25
Lamellar area (mm^2)	0.098±0.002	0.112±0.001	1.15	0.42
Total gill area (mm^2 g^{-1})	143.37±10	134.8±6.0	0.94	0.49

Number of filaments is the average number of filaments on one side of each gill arch; total filament length is the total length of all filament on the entire fish; lamellar density is the average number of lamellae per mm of filament; lamellar area is the average area of all lamellae; and total gill area represents the gill area for the entire fish.

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 comparison-wise P value (P=0.0032, see Materials and methods).

Values are means ± 1 standard error (N=8).

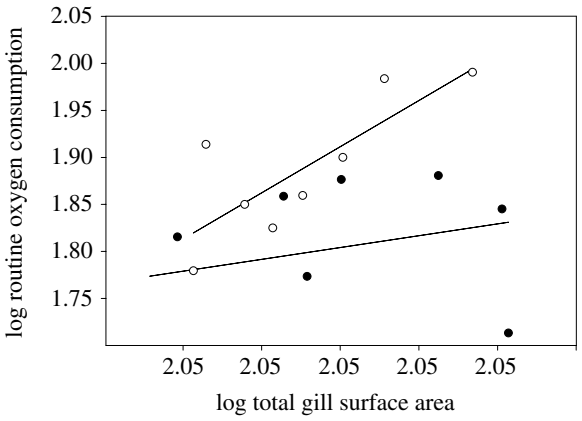

Fig. 4. Relationships between routine oxygen consumption and total gill surface area for transgenic (open circles, y=–0.02+0.98x, R^2=0.63, P=0.02) and control (closed circles, y=1.26+0.25x, R^2=0.10, P=0.50) Atlantic salmon (N=8).

difference was found in protein content of the heart or the red muscle when fish groups were compared. However, white muscle of the transgenics had a significantly greater protein concentration (by 4%) than the controls. CS and CCO activity were greatest in the heart (~0.12 and 0.190 units mg^{-1} protein, respectively), whereas white muscle had the lowest activity (~0.010 and 0.018 units mg^{-1} protein, respectively), and the red muscle was intermediate (~0.07 and 0.15 units mg^{-1} protein, respectively). The hearts of transgenic fish had a significantly greater CS activity, both in terms of activity per wet mass, and mg protein (both P<0.001). Although, no difference in CS activity was observed in the white or red muscle, CCO activity was significantly higher in the red muscle of the transgenics, on both a per gram wet mass and per mg protein basis (P=0.018 and P=0.007, respectively).

Relative ventricular mass (RVM) was 29% greater in the transgenic fish than in the controls (Table 6). In contrast, atrial and bulbar masses were not significantly different. The relative amount of compact myocardium (approx. 45%) 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 ventricle:atrium and ventricle:bulbus mass ratios were compared between groups, no significant differences were found. However, the transgenic salmon tended to have higher ratios in terms of both the atrium and the bulbus (P=0.1 and 0.054, respectively) (Table 6).

Resting *in situ* input pressures were subambient, and although there was a 36% difference in resting input pressure between groups, a high degree of variation made this difference non-significant (Table 7). Heart rates were significantly greater in the transgenic salmon than in the controls, with resting heart rate and heart rate at maximum cardiac output being 14% (or approximately 11 beats min^{-1}) and 7% (or approximately 5 beats min^{-1}) higher, respectively. The transgenic fish were able to develop a significantly greater maximum cardiac output (by 18%) when measured in ml min^{-1} kg^{-1}, however, this difference was not evident when $\dot{Q}$ was expressed per gram of ventricular mass. Although maximum stroke volume, measured as per gram body mass or per gram ventricle, was not significantly different between the transgenic and control fish, the transgenic salmon tended to have lower ventricle-specific stroke volumes because of their larger ventricles and higher heart rates (Fig. 5B). Maximum power output was not different between groups (control fish, 9.69 mW g^{-1} ventricle; transgenic fish, 9.67 mW g^{-1} ventricle; Table 7). Further, maximum power was achieved at similar output pressures in both groups (7.3 kPa), and the relationships between power output and output pressure were nearly identical (Fig. 6).

Discussion

Study overview

This study showed that GH transgenic Atlantic salmon had elevated routine and standard metabolic rates (by 21 and 25%, respectively), and that many aspects of their cardiorespiratory system were upregulated. For instance, their 29% larger heart

Table 3. *Resting and post-stress plasma cortisol and catecholamine levels in GH transgenic and control Atlantic salmon*

		Control	Transgenic	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 (nmol l^{-1})	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 (nmol l^{-1})	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 (nmol l^{-1})	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*

Resting measurements were taken 48 h after cannulation and black box confinement. Post-stress catecholamine levels were measured immediately after a 45 s net stress, whereas post-stress cortisol levels were assessed 30 min later.

Values are means ± 1 standard error (N=8).

*Significant difference (P<0.05) between transgenic and control salmon; †significant difference (P<0.05) between resting and stressed fish.

Table 4. *Erythrocyte morphometrics and haematological parameters for GH transgenic and control Atlantic salmon*

		Control	Transgenic	Trans/Con ratio	*P* value
Morphometric parameters					
Optical surface area (μm^2)		129.7±2.0	125.5±2.0	0.97	0.10
Perimeter (μm)		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 parameters					
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 (g dl^{-1})	Resting	6.2±0.2	6.6±0.2	1.06	0.24
	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

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 are means ± 1 standard error (control, *N*=7; transgenic, *N*=8).

*Significant difference (*P*<0.05) between transgenic and control salmon.

with an 18% better *in situ* maximum pumping capacity, when combined with a 14% higher post-stress haemoglobin content, would allow for a 35% greater maximum O_2 transport capacity. The increased CCO (by 5%) and CS (by 10%) activities in the red and heart muscle, respectively, would enhance the capacity of these tissues to generate energy through oxidative metabolism. Finally, their circulating epinephrine and norepinephrine levels were significantly elevated, a modification that would provide additional stimulation to the cardiorespiratory system [improving gill perfusion, haemoglobin–oxygen binding, cardiac function etc (Randall and Perry, 1992)] during stressful periods or peaks in oxygen

Table 5. *Protein content and enzyme activities in heart, red and white muscle tissue from GH transgenic and control Atlantic salmon*

	Control	Transgenic	Trans/Con ratio	*P* value
Protein (mg g^{-1} wet tissue)				
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 (units g^{-1} wet mass)				
Heart	13.9±0.23	15.3±0.24	1.10	<0.001*
Red	9.58±0.23	9.22±0.15	0.96	0.19
White	1.63±0.135	1.42±0.134	0.87	0.28
CS activity (units mg^{-1} protein)				
Heart	0.124±0.002	0.129±0.002	1.10	<0.001*
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 (units mg^{-1} wet mass)				
Heart	22.4±0.29	22.7±0.32	1.01	0.64
Red	19.5±0.30	20.3±0.15	1.04	0.018*
White	2.46±0.12	2.77±0.10	1.12	0.059
CCO activity (units mg^{-1} protein)				
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

CS, citrate synthase; CCO, cytochrome *c* oxidase.

Values are means ± 1 standard error (*N*=8).

*Significant difference (*P*<0.05) between transgenic and control salmon.

Table 6. *Heart chamber morphometrics of transgenic and control Atlantic salmon*

	Control	Transgenic	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	4.4±0.3	5.2±0.3	1.17	0.10
Ventricle:bulbus	3.6±0.4	4.8±0.5	1.32	0.06
% Compact myocardium	44.8±1.0	46.5±1.1	1.04	0.45

Values are means ± 1 standard error (*N*=8).
*Significant difference (*P*<0.05) between groups.

demand. However, the cardiorespiratory system of the GH transgenic salmon was unable to cope with the high functional demands placed on it during the U_{crit} test (as evidenced by their unchanged $\dot{M}_{O2,max}$, and subsequently, reduced values for U_{crit} and metabolic scope), and thus has not adapted in a manner suggested by the theory of symmorphosis. Of the parameters measured, only gill surface area failed to increase, and, below, we discuss whether limited gill O_2 diffusive capacity might explain the inability of the GH transgenic salmon to elevate metabolic capacity.

Metabolism and swimming performance

Our post-smolt (adult) transgenic salmon had a significantly greater routine metabolic rate (1.2×) than the controls. This is in agreement with Lee et al. (Lee et al., 2003) who found an elevated routine $\dot{M}_{O_2}$ of 1.21× in adult transgenic coho salmon 4 days post-feeding. However, it is much lower than the ~1.7× increase found by Stevens et al. (Stevens et al., 1998) and Cook et al. (Cook et al., 2000b). The larger difference reported by Stevens et al. (Stevens et al., 1998) and Cook et al. (Cook et al., 2000b) was probably the result of their fish being smaller, and the fact that respirometry was performed on groups of fish, and the more active and aggressive nature of the transgenics (Abrahams and Sutterlin, 1999; Herbert et al., 2001; Leggatt et al., 2003) would lead to higher oxygen consumption rates than true routine levels. Although Leggatt et al. (Leggatt et al., 2003) suggest that differences in O_2 consumption between transgenic and non-transgenic salmon are due to the effects of feeding, activity level and acclimation conditions, our study does not support this conclusion. In this study, we controlled for differences in activity and SDA between groups by taking routine $\dot{M}_{O_2}$ measurements on individual food-deprived fish (fasted for approx. 2.5 days prior to testing) while they were stationary and made no large, energetically costly, movements. Further, we reared the control and transgenic salmon together for 9 months prior to experimentation to remove the potentially confounding effects of environmental/rearing history on metabolic differences. Clearly, at least in this line of Atlantic salmon, there are significant metabolic costs directly associated with GH transgenesis.

Table 7. *Resting heart rate and input pressure, and maximum cardiac performance of GH transgenic and control Atlantic salmon in situ hearts*

	Control	Transgenic	Trans/Con ratio	*P* value
Resting				
P_{in} (kPa)	–0.15±0.060	–0.001±0.025	0.64	0.35
Heart rate (beats min^{-1})	73±2	84±3	1.14	0.007*
Parameters at maximum cardiac output				
$\dot{Q}$ (ml min^{-1} kg^{-1})	63.8±1.9	75.5±2.8	1.18	0.005*
V_S (ml kg^{-1})	0.93±0.03	1.03±0.05	1.11	0.16
Heart rate (beats min^{-1})	69±1	74±2	1.07	0.04*
$\dot{Q}$ (ml min^{-1} g^{-1} ventricle)	96.0±4.4	95.7±1.9	0.99	0.95
V_S (ml g vent^{-1})	1.4±0.09	1.3±0.04	0.94	0.28
Power (mW g^{-1} ventricle)	9.69±0.41	9.67±0.5	0.99	0.98

Resting P_{in} was set to achieve a physiological resting cardiac output of approximately 16 ml min^{-1} kg^{-1}.
Values are means ± 1 standard error (control, *N*=8; transgenic, *N*=7).
*Significant difference (*P*<0.05) between groups.

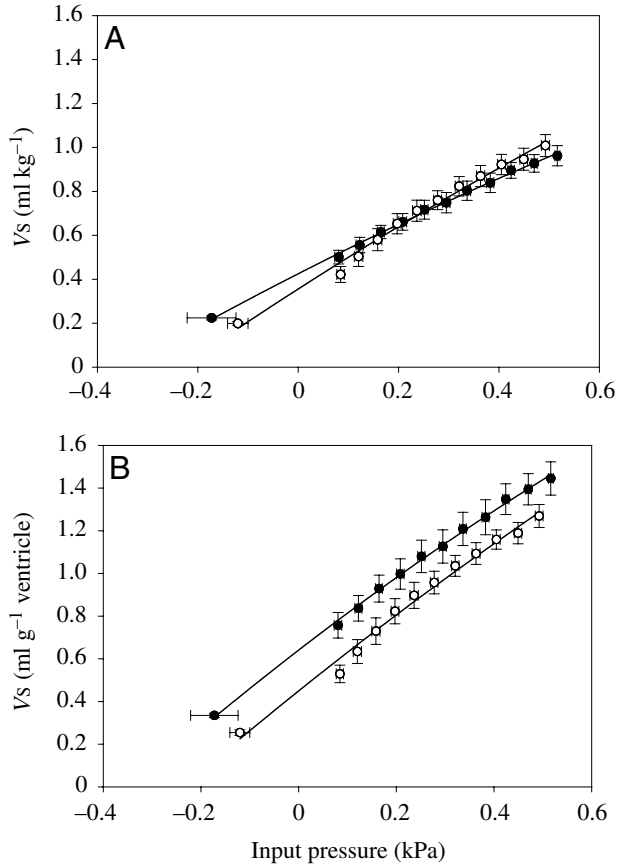

Fig. 5. The effect of increased input pressure (kPa) on maximum *in situ* stroke volume (A, ml kg^{-1}) (B, ml g ventricle^{-1}) of transgenic and control Atlantic salmon hearts. (A) Transgenic salmon (N=7) are represented by open circles (y=0.36+0.14x–0.002x^2, R^2=0.98) and controls (N=8) are represented 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 using second order regressions. Values are means ± 1 standard error.

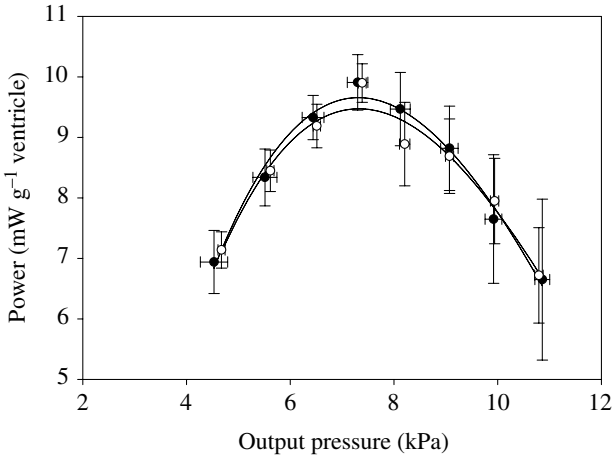

Fig. 6. The effect of increased output pressure (kPa) on myocardial power output (mW g^{-1} ventricle) of *in situ* transgenic (open circles, y=–13.2+0.71x–6.91x^2+1.83x^3, R^2=0.95) and control (closed circles y=–14.2+0.75x–7.2x^2+1.83x^3, R^2=0.98) Atlantic salmon hearts. Hearts were left at the P_{in} at which $\dot{Q}_{max}$ was obtained while P_{out} was being manipulated. Curves were fitted using a second order regression. Values are means ± 1 standard error (N=7 for transgenic salmon; N=8 for controls).

Several physiological changes have been noted in GH transgenic fish that may explain their inherently greater routine and standard $\dot{M}_{O_2}$. These include substantially elevated growth rates, a greater white muscle protein content (Blier et al., 2002) (this study), a 2.2× greater intestinal surface area (Stevens and Devlin, 1999), and an increased red muscle mass (Hill et al., 2000). Faster growth has been linked with a greater $\dot{M}_{O_2}$ in a number of fish species (Pauly, 1998; Galarowicz and Wahl, 2003), as growth-associated increases in protein synthesis require an elevated aerobic capacity (Mathers et al., 1992; Couture et al., 1998). Thus, the 3.6× greater growth rate in our transgenic Atlantic salmon probably explains the majority of the increase in routine and standard metabolism. However, it has also been shown that tissue protein turnover rate and oxygen consumption are linked (Waterlow and Millward, 1989; Houlihan, 1991). Therefore, it is possible that the more proteinaceous white muscle (which represents ~70% of body mass) and increased intestinal surface areas are more expensive to maintain, and this further elevated metabolic demands.

The higher routine $\dot{M}_{O_2}$ in our GH transgenic salmon was not compensated for by an increase in $\dot{M}_{O_2,max}$. This agrees with the findings of McKenzie et al. (McKenzie et al., 2003) who found no significant difference in $\dot{M}_{O_2,max}$ in transgenic tilapia when compared to non-transgenic controls, but contrasts with Lee et al. (Lee et al., 2003) and Stevens et al. (Stevens et al., 1998) who reported 11% lower and 60% higher $\dot{M}_{O_2,max}$ values in transgenic adult coho and juvenile Atlantic salmon, respectively. The lower $\dot{M}_{O_2,max}$ reported for the tank-reared transgenics of Lee et al. (Lee et al., 2003) was not surprising, as the 'control' fish were wild salmon captured during their spawning migration. The relatively sedentary lifestyle of the tank-reared transgenics would not have necessitated a high $\dot{M}_{O_2,max}$, as compared with the ocean-ranched controls that were required to perform metabolically demanding activities (migration, prey capture, predator avoidance, etc.). The difference between this study and Stevens et al. (Stevens et al., 1998) is more difficult to explain, but may be related to fish size. Small fish eat more per gram body mass [two- to fivefold between 30 and 650 g (Silverstein and Freeman, 2001; Damsgard et al., 1999)], and this combined with the enhanced ability of the transgenics to process food (Cook et al., 2000a; Leggatt et al., 2003) may have caused their $\dot{M}_{O_2,max}$ to increase to accommodate higher digestive costs (SDA). Further, fish feeding at high rates 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 $\dot{M}_{O_2,max}$.

The higher routine $\dot{M}_{O_2}$, and unchanged $\dot{M}_{O_2,max}$, resulted in the transgenic salmon having a 29% lower scope for activity

as compared with controls. Fish must be able to multitask and partition the oxygen available between competing processes (Korsmeyer et al., 1996), and Pauly (Pauly, 1998) concluded that fish have to 'choose' to allocate the limited oxygen they have to fuel either a higher growth rate or a greater performance. Thus, given their higher standard and routine $\dot{M}_{O_2}$, our adult GH transgenic salmon appear to be favouring growth, at the expense of maximum performance (as evidenced by their 9% lower U_{crit}). A lower U_{crit} was also shown by Farrell et al. (Farrell et al., 1997) and Lee et al. (Lee et al., 2003) for GH transgenic coho salmon (by 37% and 22%, respectively), and by Stevens et al. (Stevens et al., 1998) for GH transgenic Atlantic salmon (15%, P=0.09). Based on this data, it appears that a lower maximum swimming speed is characteristic of GH transgenic salmonids.

Gill surface area and limitations on performance?

In contrast to our findings for post-smolt (adult) GH transgenic Atlantic salmon, Stevens and Sutterlin (Stevens and Sutterlin, 1999) showed that transgenic pre-smolts had a 1.25× greater gill surface area than non-transgenic controls. This discrepancy was not due to differences in growth rate as fish in both studies were growing at approx. 2–3× that of controls, but may be related to the higher mass-specific oxygen requirements of the freshwater pre-smolts. The transgenic pre-smolts (when measured in groups) had a metabolic rate 1.6× that of controls, and would have higher mass specific-oxygen requirements because of the allometric relationship between body mass and $\dot{M}_{O_2}$ (e.g. Rodnick et al., 2004), and greater foraging and digestive costs (see above).

In this study, we report that U_{crit} and metabolic scope were reduced significantly in the transgenic salmon, despite increases in tissue aerobic enzyme activities, maximum cardiac function, resting and post-stress plasma catecholamine concentrations, and post-stress haemoglobin levels. This data suggests that the inability of adult GH transgenic Atlantic salmon to elevate maximum metabolic rate (maintain metabolic scope) and swimming performance was related to limitations in O_2 diffusion, not perfusion/oxygen delivery. Clearly, the lack of an increase in gill surface area in the transgenic salmon, and the positive relationship between routine $\dot{M}_{O_2}$ and gill surface area in this group (Fig. 4), suggest that the gills may be the site of this diffusion limitation. This hypothesis is supported by a number of pieces of evidence. For example, Piiper et al. (Piiper et al., 1977) provided evidence of a diffusion limitation to branchial O_2 transfer in dogfish during strenuous exercise, Gallaugher et al. (Gallaugher et al., 1992; Gallaugher et al., 1995; Gallaugher et al., 2001) reported arterial hypoxemia in rainbow trout, and Gallaugher et al. (Gallaugher et al., 1995) show that the slope of relationship between CaO_2 and [Hb] at U_{crit} is less than 1.

Aerobic enzyme activity

Mitochondrial enzymes provide the energy needed to fuel growth and metabolism, and it was hypothesized that the presence of the transgene would provide enough selective pressure to upregulate aerobic enzyme activity. This appears to be the case, as the GH transgenic salmon had higher red muscle CCO and myocardial CS activities (measured as units mg^{-1} protein and units g^{-1} tissue). By contrast, however, there were no changes in white muscle enzyme activity, a finding that agrees with Blier et al. (Blier et al., 2002) who studied GH transgenic coho salmon. The lack of a change in white muscle aerobic capacity suggests that the increased enzyme activities in the heart and red muscle were not growth related, but a consequence of the higher physical activity of the transgenic fish (Abrahams and Sutterlin, 1999; Herbert et al., 2001; Leggatt et al., 2003). Both the heart and red muscle are aerobic tissues, the use of which would increase with even modest rises in activity level, and there are several reports (e.g. Johnston and Moon, 1980; Farrell et al., 1989; Farrell et al., 1991) of elevated aerobic enzyme capacity in these tissues following training at moderate speeds. Although Farrell et al. (Farrell et al., 1991) have shown that exercise training increases white muscle enzyme activity, it is only recruited at swimming speeds greater than 80% of U_{crit} (Jones, 1982; Burgetz et al., 1998). This is a level higher than our tank-reared fish were likely to routinely swim.

Haematology and stress hormone levels

At rest, there were no significant differences in haematocrit, haemoglobin levels or MCHC between the two groups (Table 4). In contrast, post-stress haemoglobin levels were 14% higher in the transgenic salmon. This finding is in agreement with Cogswell et al. (Cogswell et al., 2002) who showed that GH transgenic Atlantic salmon sampled by caudal puncture have 20% higher haemoglobin levels (pg $cell^{-1}$), and indicates that these fish have an elevated capacity to transport oxygen during stressful situations as compared with non-transgenic conspecifics.

Resting cortisol levels 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., 1994a; Ackerman et al., 2000). The lack of a significant difference between the groups is, however, in contrast to the work of Jhingan et al. (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 Jhingan et al. (Jhingan et al., 2003), but it is most probably the result of differences in species/strain, the position or type of transgene used (i.e. pleiotropic effects), or that we used cannulated fish, held in black boxes. This latter procedure has been shown to elevate cortisol levels (Gamperl et al., 2004b).

The cortisol stress response of our fish (approx. twofold increase) was low compared to other studies on fish, in which 10- to 100-fold post-stress increases are normally observed (reviewed by Barton and Iwama, 1991; Gamperl et al., 1994a). Our control salmon did, however, have a significantly greater (by 28%) post-stress cortisol response as compared with the transgenics, suggesting that GH transgenic Atlantic salmon

have a blunted stress response. Although there are no comparable post-stress data for GH transgenic fishes, this result is opposite to what would be expected based on studies that have examined the relationship between GH and cortisol levels in fishes. For instance, Nielsen et al. (Nielsen et al., 1994) showed concurrent increases in GH and cortisol in exercised rainbow trout, and GH injection studies suggest that GH increases interrenal function in coho salmon, resulting in elevated plasma cortisol levels (Higgs et al., 1977; Young, 1988).

Although resting plasma epinephrine values were not significantly different between groups, resting norepinephrine and total catecholamine values were significantly greater in the transgenic salmon (by 22% and 30%, respectively). Furthermore, the post-stress epinephrine, norepinephrine and total catecholamine levels were significantly higher. Although the cause of the elevated resting and post-stress catecholamine levels is not known, it is clear that the increased concentrations of these hormones would benefit numerous physiological functions (Randall and Perry, 1992; Wendelaar Bonga, 1997; Perry and Bernier, 1999). As with cortisol, post-stress increases in plasma catecholamines (approx. fourfold) were significantly less than observed in other studies (Gamperl et al., 1994a). The lack of a robust stress response in these salmon is an interesting finding that deserves further study, and suggests that this strain is a good candidate for commercial aquaculture.

Cardiac morphometry and performance

This is the first study to investigate whether GH transgenesis affects cardiac function in salmonids. The *in situ* hearts of the transgenic salmon exhibited marked increases in maximum cardiac output (18%) and RVM (29%) as compared with the controls. The greater RVM agrees with Pitkanen et al. (Pitkanen et al., 2001) who found that RVM was 38% larger in GH transgenic Arctic char (*Salvelinus alpinus*), and with the 20% higher (but not significant, N=6) RVM reported by McKenzie et al. (McKenzie et al., 2003) for GH transgenic tilapia. 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) tissues. How the increase in heart size influenced cardiac output is not directly obvious, as Vs (in ml kg^{-1} or ml g^{-1} ventricle) was not significantly 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 limitations on ventricular filling (Farrell et al., 1996)] at their 7–14% higher heart rates; ultimately enabling them to achieve a significantly greater cardiac output. This cardiovascular plasticity would allow GH transgenic Atlantic salmon to accommodate alterations in their physiology (increased activity, feeding, growth rate) due to the transgene, and is consistent with studies showing that heart size in salmonids is influenced by training (Farrell et al., 1988; Farrell et al., 1991), and that the increased metabolic demands associated with digestion (SDA) and activity (swimming) are tightly coupled with alterations in cardiac function (Thorarensen et al., 1996; Axelsson et al., 2000). We did not measure plasma GH levels in this study, and thus cannot exclude the possibility that the increase in heart size was related to differences in circulating GH concentrations. However, Fleming et al. (Fleming et al., 1996) found that domestic Atlantic salmon had smaller hearts than their wild counterparts, even though they possessed higher plasma GH levels (Fleming et al., 2002). These results suggest that the increase in heart size/function observed in this study was a direct result of an increase in metabolic demands, and not due to GH stimulated cardiac growth.

A particularly interesting finding was the elevated intrinsic *in situ* heart rate of the transgenic fish. The higher heart rate (14% resting, 7% at $\dot{Q}_{max}$) of the transgenic salmon could be the result of several factors. First, epinephrine has a positive chronotropic effect (Farrell et al., 1991), and alterations in myocardial *B*-adrenergic receptor density/affinity could have elevated the sensitivity of the transgenic salmon to the 10 nmol l^{-1} epinephrine that was used to maintain the viability of the *in situ* hearts. Although the myocardial B-adrenergic/signal transduction system has not been studied in GH transgenic fishes, rat studies have shown that B-receptor affinity can be heightened by GH injection (Iwasaki et al., 1982; Popova et al., 1990). Second, the greater food intake of the transgenics could have affected heart rate, as both food-deprived cod (A. G. Genge, K. J. Rodnick and A.K.G., unpublished data) and sturgeon (Agnisola et al., 1999) fed lipid-reduced diets exhibited 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, and thus their excitability/rate of firing. However, it is unclear whether the results of these *in situ* experiments translate to the *in vivo* situation, where cholinergic and adrenergic nervous control may compensate for alterations in pacemaker physiology.

Conclusions/implications of this study

Although this study provides a wealth of information on the physiology of GH transgenic Atlantic salmon, it creates numerous questions that require further experimentation. The first of these is a basic one: was increased growth rate or activity responsible for the upregulation of many aspects of the cardiorespiratory system? Irrespective of the ultimate cause, we report that GH transgenic Atlantic salmon had a greater requirement for oxygen at rest, and that they showed enhancements in heart morphology and performance, blood oxygen carrying capacity ([Hb]), tissue enzyme activities, and the stress response. Given the above modifications in the cardiorespiratory system, one might conclude that these salmon are compensating for increased metabolic demands using a 'whole system' approach. Interestingly, however, our results do not support the theory of symmorphosis. There was no increase

in gill surface area, and it appears from our data (Fig. 4) and the work of Gallaugher et al. (Gallaugher et al., 1992; Gallaugher et al., 1995; Gallaugher et al., 2001) that this lack of respiratory diffusive capacity was limiting oxygen uptake and metabolic capacity. The finding that gill surface area did not change in accordance with the theory of symmorphosis was not entirely unexpected since Weibel et al. (Weibel et al., 1991) concluded that the hypothesis of symmorphosis must be refuted for the mammalian lung. However, Weibel et al. (Weibel et al., 1991) report that pulmonary gas exchange does not fit the theory of symmorphosis because it has a considerable excess diffusing capacity, a result which argues against gill surface area limiting metabolic capacity in our transgenic salmon. This latter conclusion fits with the notion that enhanced oxygen extraction ($E_{O_2,max}$) is a primary determinant of intra-specific improvements in metabolic capacity (aerobicity) (Gallaugher et al., 2001), but not with data on juvenile (pre-smolt) Atlantic salmon where a 1.21-fold increase in gill surface area was concomitant with a 1.7-fold increase in $\dot{M}_{O_2,max}$ (Stevens et al., 1998; Stevens and Sutterlin, 1999). Clearly, more research is needed to better understand how ontogenetic stage influences gill structural and physiological plasticity, and the extent that intra-specific differences/alterations in $\dot{M}_{O_2,max}$ are dependent upon diffusion-limited *versus* perfusion (i.e. $\dot{Q}_{max}$ and CaO_2)-limited processes.

List of symbols and abbreviations

CCO	cytochrome *c* oxidase
CS	citrate synthase
DHBA	(3,4-dihydroxybenzylamine)
DTNB	5,5′-dithiobis-(2-nitrobenzoic acid)
EPI	epinephrine
F_H	heart rate
FL	fork length
GH	growth hormone
$\dot{M}_{O_2}$	rate of oxygen consumption
NE	norepinephrine
P_{in}	input pressure
P_{out}	output pressure
$\dot{Q}$	cardiac output
RIA	radioimmunoassay
RVM	Relative ventricular mass
*V*s	stroke volume

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