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# Fitness component assessments of wild-type and growth hormone transgenic coho salmon reared in seawater mesocosms



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#### ABSTRACT

Growth hormone (GH) transgenic fish have been proposed for use in aquaculture to enhance production efficiency. As part of a risk analysis for use of such fish, the influence of GH transgenesis on the potential to persist and succeed in natural ecosystems is being examined in confined laboratory conditions. GH transgenesis can greatly accelerate growth and, in culture conditions, is associated with secondary effects such as poor swimming capacity and spawning success. However, standard culture has also been shown to negatively affect fitness components of wild-type fish, raising the question of whether culture conditions influence fitness components of transgenic fish in a similar way. To examine factors influencing the phenotype of marine-stage GH transgenic salmon (T), and to determine if genotype-by-environment interactions exist at this life stage, we grew T and wild-type (NT) coho salmon (Oncorhynchus kisutch) over six cohort years in 350,000 L seawater tanks (termed mesocosms) designed to minimize effects of standard culture conditions. Mesocosm rearing partially facilitated development of normal size and morphology of NT fish relative to nature-reared counterparts, but altered overall body shape, indicating mesocosm conditions do not fully mimic natural environmental effects on coho salmon phenotype. T fish reared in mesocosms had larger mass at maturity than mesocosm- or nature-reared NT fish, indicating GH transgenesis can alter maximum obtainable mass in salmon. Unlike NT, T fish obtained maximum size at maturity across environments, suggesting marine environmental conditions may affect T growth less than NT growth. Screening parents for a common disease agent (Renibacterium salmoninarum) improved seawater survival, and T fish had lower survival than NT fish when from unscreened parents and inconsistent relative survival when from screened parents, indicating GH transgenesis may constitute an advantage or disadvantage in terms of survival. Transgenic salmon had lower swimming capacity and aerobic scope, but similar routine metabolic rate and thermal tolerance, demonstrating transgenesis can have different influences depending on what phenotype is examined. Using an alternate strain of T fish in phenotypic comparisons did not greatly influence most fitness components, although had a strong effect on female fecundity. The inconsistent influence of GH transgenesis on different fitness components, and existence of genotype-by-environment interactions during the marine life stage, complicates extrapolation of laboratory data for transgenic fish to natural environments. However, current and previous data do not provide evidence that overall increased performance of GH transgenic salmon over wild-type fish would arise in the marine environment.

*Statement of relevance:* Rearing in seawater mesocosms demonstrate that growth hormone transgenesis has inconsistent effects on marine fitness components in coho salmon.

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*Abbreviations*: bl, body length; CAER, Centre for Aquaculture and Environmental Research; CF, condition factor; COT, cost of transport; COTnet, net cost of transport; DFO, Fisheries and Oceans Canada; EPOC, excess post-exercise oxygen consumption; GH, growth hormone; LOE, loss of equilibrium; LSM, least square means; MO<sub>2</sub>, oxygen uptake; MO<sub>2-max</sub>, maximum obtainable oxygen uptake; MO<sub>2-R</sub>, routine oxygen uptake; NT, non-transgenic (wild-type) coho salmon; OnH3GH1, sockeye salmon histone-3 promoter driving expression of the growth hormone 1 gene from the same species; OnMTGH1, sockeye salmon metallothionein-B promoter driving expression of the growth hormone 1 gene from the same species; PIT, passive integrated transponder; Rs, *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease; SGR, standard growth rate (mass); T, growth hormone transgenic coho salmon containing the OnH3GH1 transgene;  $T_{MT}$ , growth hormone transgenic coho salmon containing the OnH3GH1 transgene;  $U_{crito}$ , maximum sustainable swim speed.

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#### 1. Introduction

Growth hormone (GH) transgenesis is known to greatly accelerate growth rates in many fish species, and the use of this technology for aquaculture production is now approved in the USA and Canada for an Atlantic salmon (*Salmo salar*) model. While the risks of accidental escapes that could allow a transgene to introgress into natural populations is minimized in highly secure land-based facilities coupled with biological containment strategies, other scenarios where transgenic fish might more readily enter nature (e.g. open net pens, pond culture, etc.) dictate an urgent need to assess potential ecological impacts. The purpose of the present study was to contribute to such assessments for transgenic coho salmon (*Oncorhynchus kisutch*) maintained in marine land-based culture conditions where abiotic and biotic factors (e.g. water supply, lighting, rearing density, human interactions) more closely mimicked natural conditions than in previous studies.

The potential for GH transgenic fish to impact natural ecosystems is determined in part by their ability to survive, reproduce and persist within these ecosystems, i.e., fitness. GH transgenic fish are known to have altered factors than can influence fitness (hereafter termed "fitness components") compared to non-transgenic fish. For example, GH transgenic fish reared in standard culture when compared with nontransgenic counterparts reared in nature or standard culture have lower reproductive success, swimming ability, survival, and disease resistance (Bessey et al. 2004; Devlin et al. 2004a; Figueiredo et al. 2013; Jhingan et al. 2003; Kim et al. 2013; Lee et al. 2003a; Li et al. 2007; Moreau et al. 2011) while having a faster growth rate and being more aggressive (Devlin et al. 1999; Devlin et al. 1994; Du et al. 1992; Duan et al. 2011; Rahman et al. 1998, see Devlin et al. 2015). However, a strong effect of culture conditions is known to affect many fitness components in non-transgenic salmon (e.g. reproduction, body size, Berejikian et al. 2001b; Berejikian et al. 2001a; Berejikian et al. 1997; Bessey et al. 2004), and genotype-by-environment interactions have been identified when comparing GH transgenic and non-transgenic fish (e.g. juvenile growth rates and behaviour, Devlin et al. 2004b; Sundström et al. 2007, adult spawning behaviour and success, Leggatt et al. 2014). Consequently, it is very difficult to predict the fitness of GH transgenesis in nature, as well as resulting ecological effects, without better mimicking natural abiotic and biotic factors. Indeed, indications that laboratory culture conditions may be poor indicators of how fish respond to natural environments come from observations that altered laboratory rearing conditions can greatly limit size at maturity and spawning success of non-transgenic coho salmon (Bessey et al. 2004; Devlin et al. 2004a), and potentially limit growth in GH transgenic tilapia (Martínez et al. 2000).

While rearing of juvenile GH transgenic salmonids in semi-natural contained stream systems has already provided insight into how GH transgenic fry may respond to natural conditions as well as interact with ecosystem components (e.g., Sundström et al. 2007, see Devlin et al. 2015), creating contained environmental conditions that partially or fully mimic natural marine conditions of salmon (i.e., the ocean) has proven more of a challenge. To fill this void and directly assess whether or not marine rearing conditions impact fitness of GH transgenic salmon, we raised GH transgenic and non-transgenic coho salmon from the smolt stage to maturation in extremely large 350,000 L seawater tanks (termed mesocosms) where abiotic and biotic factors (e.g. water supply, lighting, rearing density, human interactions) were maintained close to natural conditions. We compared growth and survival of transgenic and non-transgenic salmon over multiple cohort years to gain an understanding of overall fitness effects of GH transgenesis in the marine environment, as well as to assess the influence of year-toyear variation on relative growth and survival of GH transgenic compared to non-transgenic coho salmon. We further examined the effect of GH transgenesis on other marine fitness components (swimming ability, swimming efficiency and thermal tolerance) that are known to influence the ability of near-mature fish to migrate to natural rivers to spawn (e.g., Eliason et al. 2011). We also studied whether fitness components were consistent between two strains of GH transgenic coho salmon to determine if fitness estimates can be inferred across GH transgenic strains within a species.

#### 2. Materials and methods

#### 2.1. Fish

Experiments were conducted at the Fisheries and Oceans Canada (DFO) Centre for Aquaculture and Environmental Research (CAER), West Vancouver, BC, Canada under an institutional animal care permit meeting guidelines established by the Canadian Council for Animal Care. The facility was specifically designed to prevent escape of GH transgenic fish to natural ecosystems. All coho salmon used in this study possessed a Chehalis River, BC, hatchery genetic background and transgenic lines were originally propagated within this hatchery strain. The hatchery strain is propagated at each generation using wild fish collected from nature and hatchery returns both of which are phenotypically highly similar (Chittenden et al. 2010). Unless otherwise stated, GH transgenic salmon were M77-strain fish (termed T or  $T_{MT}$ ) produced by insertion of the OnMTGH1 gene construct containing GH1 driven by the metallothionein-B promoter both from sockeye salmon (O. nerka; Devlin et al. 2004b; Devlin et al. 1994). Some experiments also used a second line of transgenic fish  $(T_{H3})$  that contained the same GH1 construct structure as OnMTGH1, but was coupled to a histone-3 promoter from sockeye salmon (OnH3GH1, H3-3339 line, see Leggatt et al. 2012). Transgenic fish used in this experiment were produced by crossing T parents with wild-caught Chehalis River hatchery salmon, and wild-type non-transgenic smolts (NT) were produced from wild-caught Chehalis River hatchery salmon. Fish were produced by pair or batch crosses using a minimum 7 females and 5 males. Genetic diversity and hatchery-strain background of transgenic fish lines was maintained by back-crossing to wild-caught Chehalis River hatchery salmon at each generation, in order to assess the potential impacts of the transgene in a wild population.

#### 2.2. Rearing conditions

Post-smolt rearing of NT and T coho salmon was conducted in three replicate mesocosms designed to minimize the effects of culture (i.e., 350,000 L seawater tanks as described by Leggatt et al. 2014, see Fig. 1). In brief, mesocosm tanks were supplied with ambient temperature, flow-through, sand-filtered seawater with unidirectional inflow (approximately 460 L/min) to stimulate continuous swimming of fish at low density ( $<2 \text{ kg/m}^3$ ) and with natural lighting. The marine organisms that colonized these mesocosms, presumably entering as larvae through the natural water supply, included chitons (Class Polyplacophora), sea anemones (Subclass Hexacorallia), and nudibranchs (Order Nudibranchia). The mesocosm upper edges were fitted with fine mesh screens that reached 2 m above the floor, which had a primary purpose of minimizing fish disturbance resulting from human presence. Fish were implanted with Passive Integrated Transponder (PIT) tags (BioMark, Idaho USA) prior to introduction to the mesocosm. During mesocosm rearing fish were hand fed 2 times per day to satiation with size-appropriate commercial salmonid diet (Skretting Canada Ltd., Vancouver, BC, Canada). Approximately every 3 months, all fish were seine-netted out of the mesocosms and lightly anaesthetized with 50 mg/L tricaine methanesulfonate for enumeration and measurement of mass and length.

#### 2.3. Effect of transgenic and promoter type on growth and survival

Overall size at maturity and seawater survival of T and NT fish were compared over six year classes (2007–2013). The six year classes were designated by the year that fish entered seawater and termed smolt-



**Fig. 1.** A) Picture of mesocosms (white arrow shows person for scale) and spawning channel (on the left), and B) mesocosm diagram with dimensions and 1 m high jump screen.

years (see Table 1 for complete details of each smolt-year). Due to accelerated juvenile growth under satiation conditions, T coho salmon smolt at a different season (late summer) and age (approximately 8 months) than the normal timing for NT coho salmon (spring, at approximately 18 months of age). To make meaningful comparisons between NT and T fish, three different strategies were used in different smolt-years to produce NT and T that entered seawater at the same time and approximate size: 1) by delaying NT smolt season until the fall (at approximately age 20 months) to match that of T fish, 2) by restricting T juvenile growth by feeding the same ration as separately-reared NT fish so that T fish reached a smolt size at the NT age (18 months), or 3) accelerating T juvenile growth by rearing at warm temperatures (15 °C) so that they reached the smolt stage in their first spring (6 months of age) to match NT smolt timing in their second spring (see Table 1). NT and T fish were reared together, starting in a single mesocosm and later split between two mesocosms and culled where appropriate to keep a low fish density (i.e. more like nature that typical culture conditions). One exception to this was for the 2007 smolt year where two mesocosms had mixed T and NT fish, and one mesocosm had just NT fish to determine if T cohabitation influenced NT growth. As the experiments progressed, several strategies were employed to improve overall survival of the fish. Renibacterium salmoninarum (Rs), the causative agent of bacterial kidney disease, is prevalent in Pacific Northwest coho salmon (e.g. Kent et al. 1998), and can be vertically transmitted to offspring from the maternal but not the paternal parent (Evelyn et al. 1986). After the initial smolt-years where survival was low, maternal parents in laboratoryreared fish were screened for presence of Rs antigens by enzyme-linked immunosorbent assay (ELISA) assays on fresh kidney samples (performed by Pfizer Canada Inc./Microtek International Inc., Saanichton, BC, Canada), and only eggs from Rs-negative mothers were used for experimental fish production. In 2012 and 2013 smolt-years, fish received prophylactic antibiotic treatments (intraperitoneal injection of 20 mg/ kg oxytetracycline) at sample periods (approximately every 3-4 months). In the 2012 smolt year, these were administered to one half of each fish group only. In some smolt-years (2007, 2008, 2011), sufficient numbers of laboratory-reared NT fish were not available, so numbers were supplemented with NT fish of the same strain that were reared as juveniles under culture conditions at the Chehalis River Hatchery facility, Agassiz, BC, Canada. Otherwise, all fish were

#### Table 1

Rearing conditions in different smolt year-classes of non-transgenic (NT) and GH transgenic (T) coho salmon, including whether smolted in the fall (normal season for T fish) or spring (normal season for NT fish), whether from *R. salmoninarum* (Rs) unscreened (no) or negative (-ve) mothers, and whether given prophylactic antibiotic injections (Ab). Transgenic fish are MT strain unless otherwise indicated, and NT and T fish were reared together unless otherwise indicated. Mass, fork length, condition factor (CF) at maturity, and marine survival are given for each fish group and smolt year, and letters (*a*, *b*, *c*; *w*, *x*, *y*, *z*) indicate significant differences in variable among fish groups within smolt year (P < 0.05). Data are presented as mean  $\pm$  SEM.

| Year              | Group               | Smolt season | Rs  | Ab              | Initial # fish <sup>a</sup> | Mass (kg)           | Length (cm)        | CF                   | Survival           |
|-------------------|---------------------|--------------|-----|-----------------|-----------------------------|---------------------|--------------------|----------------------|--------------------|
| 2007              | NT <sup>b</sup>     | Fall         | no  | no              | 400                         | $1.37 \pm 0.08^{x}$ | $46.5 \pm 0.9^{x}$ | $1.28 \pm 0.02^{x}$  | 11.4% <sup>x</sup> |
| 2007              | NT                  | Fall         | no  | no              | 400                         | $1.53 \pm 0.09^{x}$ | $48.5 \pm 1.0^{x}$ | $1.28 \pm 0.03^{x}$  | 12.1% <sup>x</sup> |
| 2007              | Т                   | Fall         | no  | no              | 400                         | $4.12 \pm 0.50^{y}$ | $60.8 \pm 2.9^{y}$ | $1.72 \pm 0.05^{y}$  | 2.8% <sup>y</sup>  |
| 2008 <sup>c</sup> | NT                  | Fall         | no  | no              | 425                         | $1.38 \pm 0.11^{a}$ | $45.2 \pm 1.2^{a}$ | $1.37\pm0.02^a$      | 9.3% <sup>a</sup>  |
| 2008 <sup>c</sup> | Т                   | Fall         | no  | no              | 200                         | $4.87\pm0.78^b$     | $63.1 \pm 4.0^{b}$ | $1.69\pm0.10^b$      | $6.0\%^{b}$        |
| 2010              | NT                  | Spring       | -ve | no <sup>g</sup> | 600                         | $1.61 \pm 0.05^{x}$ | $48.7 \pm 0.6^{x}$ | $1.32 \pm 0.01^{x}$  | 20.0% <sup>x</sup> |
| 2010              | T <sup>d</sup>      | Spring       | -ve | no <sup>g</sup> | 600                         | $3.42 \pm 0.27^{y}$ | $59.2 \pm 1.7^{y}$ | $1.52 \pm 0.03^{y}$  | 25.8% <sup>y</sup> |
| 2011              | NT                  | Fall         | no  | no              | 275                         | $1.27\pm0.04^a$     | $44.5 \pm 0.5^{a}$ | $1.34 \pm 0.02^{a}$  | 11.3% <sup>a</sup> |
| 2011              | NT                  | Fall         | -ve | no              | 190                         | $0.79\pm0.06^b$     | $37.4 \pm 1.0^{b}$ | $1.40\pm0.05^a$      | $29.9\%^{b}$       |
| 2011              | Т                   | Fall         | -ve | no              | 600                         | $2.68 \pm 0.14^{c}$ | $54.0\pm0.9^{c}$   | $1.65\pm0.04^b$      | 42.5% <sup>c</sup> |
| 2012              | NT                  | Spring       | -ve | no              | 230                         | $0.97 \pm 0.0^{x}$  | $42.4 \pm 0.8^{x}$ | $1.19 \pm 0.04^{x}$  | 40.3% <sup>w</sup> |
|                   |                     |              |     | yes             | 230                         | $1.10 \pm 0.03^{x}$ | $43.5\pm0.4^{x}$   | $1.23 \pm 0.01^{x}$  | 54.0% <sup>x</sup> |
| 2012              | Т                   | Fall         | -ve | no              | 190                         | $2.15 \pm 0.18^{z}$ | $50.2 \pm 1.4^{z}$ | $1.58 \pm 0.06^{y}$  | 34.1% <sup>y</sup> |
|                   |                     |              |     | yes             | 190                         | $2.10 \pm 0.15^{z}$ | $50.7 \pm 1.2^{z}$ | $1.42 \pm 0.03^{yz}$ | 49.2% <sup>z</sup> |
| 2012              | T <sub>H3</sub>     | Fall         | -ve | no              | 325                         | $1.75 \pm 0.18^{z}$ | $49.8 \pm 1.8^{z}$ | $1.33 \pm 0.07^{z}$  | 24.7% <sup>y</sup> |
|                   |                     |              |     | yes             | 325                         | $2.24 \pm 0.22^{z}$ | $52.7 \pm 1.7^{z}$ | $1.31 \pm 0.04^{z}$  | 42.8% <sup>z</sup> |
| 2013              | NT                  | Spring       | -ve | yes             | 200                         | $2.23 \pm 0.13^{a}$ | $55.7 \pm 1.1^{a}$ | $1.23\pm0.03^a$      | 32.5% <sup>a</sup> |
| 2013              | T <sup>d</sup>      | Spring       | -ve | yes             | 210                         | $3.79 \pm 0.36^{b}$ | $61.0 \pm 1.7^{b}$ | $1.51 \pm 0.03^{b}$  | $22.0\%^{b}$       |
| 2013              | T <sup>e</sup>      | Spring       | -ve | yes             | 360                         | $2.95\pm0.25^a$     | $55.6 \pm 1.7^{a}$ | $1.54\pm0.04^b$      | 27.3% <sup>c</sup> |
| 2013              | T <sub>H3</sub> e,f | Spring       | -ve | yes             | 30                          | n/a                 | n/a                | n/a                  | n/a                |

Mass, length, and CF at maturity on subset of fish from years 2007, 2008, 2010, and 2011 were presented in Leggatt et al. (2014).

<sup>a</sup> With the exception of the 2007 smolt year, all fish started in a single mesocosm and were split into two mesocosms and culled as appropriate at sample times to keep density <2 kg/m. <sup>b</sup> NT fish reared separately from T fish.

<sup>c</sup> Fish were reared in 12,500 L seawater tanks for 6 months prior to mesocosm entry.

<sup>d</sup> T fish given restricted ration as juveniles to grow and smolt at a NT rate (i.e. smolt at approximately 18 months in the second spring).

<sup>e</sup> T fish reared at 15 °C as juveniles to match smolt time, but not age, of NT fish (i.e. in the spring at approximately 6 months).

<sup>f</sup> Fish used in swimming, temperature trials only and not reared to maturity.

<sup>g</sup> Half of fish received a single antibiotic treatment. This treatment did not significantly influence survival or growth in either genotype and was excluded from analyses.

reared as juveniles at the CAER laboratory. Juvenile fish at the CAER laboratory were reared in freshwater drawn from a well that maintained water temperature of  $10 \pm 0.5$  °C year round, unless otherwise noted, and with simulated natural lighting and photoperiod. NT fish reared at the Chehalis hatchery as juveniles were reared on well water (temperature approximately 9 °C) until 1.5–2 g in size, then transitioned to freshwater from a river with seasonal fluctuations in water temperature (range approximately 0.3-19 °C).

## 2.4. Effect of transgenesis and promoter type on swimming success and metabolic rate

To determine if GH transgenesis and/or GH construct promoter type influences swimming performance and metabolic rate, a subset of late marine-stage coho salmon (n = 8 for  $T_{MT}$  and  $T_{H3}$  fish, n = 7 for NT fish) from the 2013 smolt-year were tested approximately four months prior to maturation (late summer), at an average water temperature of 11.3 °C. To initiate the swimming trials, fish were starved for 24 h, lightly anaesthetized, measured, and placed in one of two seawater-fed Brett-type mobile respirometer swim tunnels (220 and 425 L in size) as described by Farrell et al. (2003), with approximately equal proportions of each fish group being swum in each of the tunnels. Fish were allowed to recover overnight at a resting water velocity of approximately 0.3 body lengths (bl)/s. The following morning routine oxygen uptake  $(MO_{2-R})$  was recorded at 0.3 bl/s, then water speed increased every 5 min to half of expected critical swimming speed – U<sub>crit</sub> (approximately 0.8 bl/s). Water speed was then increased by approximately 0.2 bl/s every 20 min, with oxygen uptake measured in the final 10 min, until fish could no longer swim continuously but rested on the back grid for >5 s. At this point the water speed was returned to 0.3 bl/s and fatigued oxygen uptake recorded (MO<sub>2-max</sub>, defined as fatigued oxygen uptake or oxygen uptake at highest swim speed, whichever was highest), followed by recovery oxygen uptake over 80 min. Background oxygen uptake of the swim tunnels was measured daily and oxygen uptake of fish adjusted accordingly. Aerobic scope was calculated as (MO2- $_{max} - MO_{2-R}$ ). U<sub>crit</sub> was calculated with corrections for blocking effect as per Bell and Terhune 1970 (see Lee et al. 2003a for details). Swimming economy was calculated as (MO<sub>2-max</sub> / U<sub>crit</sub>). Cost of transport (COT) was calculated as MO<sub>2</sub>/U, where U is swim speed in bl/min, and COT is presented as average over all U for each fish. Net cost of transport (COTnet) was calculated as (aerobic scope / U<sub>crit</sub> / 60). Excess post-exercise oxygen consumption (EPOC) was calculated for the first 30 min of recovery post-U<sub>crit</sub> as the area under the recovery chart bounded by MO<sub>2-R</sub> and recovery MO<sub>2</sub> (Lee et al. 2003b).

#### 2.5. Effect of transgenesis and promoter type on tolerance to heat stressors

To determine if GH transgenesis and/or GH construct promoter type influences the ability to withstand heat stress in late-marine stage coho salmon (2 months prior to maturation),  $T_{MT}$  (n = 8),  $T_{H3}$  (n = 8), and NT fish (n = 7) from the 2013 smolt-year were exposed to serial high temperature challenges. High-temperature challenges were performed in static water baths heated to constant temperature with submersible heaters, where oxygen content was continually measured and supplemental oxygen supplied as needed to maintain levels above 6.0 mg/L. To initiate heat challenge, all fish were netted out of holding tanks (temperature 10.2  $\pm$  0.1 °C) and placed together into a 1000 L heated water bath. Behaviour of fish was continually monitored, and once a fish lost equilibrium the fish was removed and placed in a recovery tank with aerated water at ambient temperature. Once equilibrium was regained, fish were returned to holding tanks. Any fish that did not lose equilibrium in the allotted challenge time was returned to holding tanks. Time to loss of equilibrium (resistance time) and time to recovery were recorded for each fish. Fish were not fed for 24 h prior to temperature challenges. Three different temperature challenges were used: 22 °C for 2 h, 24 °C for 2 h, and 26 °C for 1 h. Fish were monitored in the holding tanks between challenges, and the challenges were staggered by 48–72 h, to ensure recovery.

#### 2.6. Effect of promoter on male reproductive success and female fecundity

To determine if strain or transgene construct influences reproductive success in T fish, the spawning ability and success of  $T_{H3}$  and  $T_{MT}$ males (from the 2012 smolt year class) in competition for NT naturereared females was conducted as described in detail in Leggatt et al. (2014). This particular trial was conducted as male spawning success in salmon can be more affected by culture and transgenesis than female spawning success (Fleming and Gross 1993; Leggatt et al. 2014). Mesocosm-reared T<sub>MT</sub> males in these experimental conditions have previously been demonstrated to have greatly inferior spawning success to mesocosm- or nature-reared NT males (Leggatt et al. 2014), and consequently NT males were specifically excluded from the current trial as their superior reproductive success could potentially mask differences between  $T_{MT}$  and  $T_{H3}$  males which was the primary objective. The following groups of fish were put into each of six  $3.7 \times 2.1$  m spawning arenas: four nature-reared NT females: three T<sub>MT</sub> males: three T<sub>H3</sub> males. Daily behavioural observations were recorded for 5 min per arena at 9 am, 11 am, 1 pm and 3 pm until all females within an arena had spawned and died, and behaviour of each fish was averaged over all intervals the fish was alive for. Parental analysis by microsatellite analysis was performed on subset of emerged offspring as per Leggatt et al. (2014). In addition, approximate individual egg mass, gonadosomatic index (GSI = total egg mass / body mass), and estimated fecundity (number of eggs / 1000 per female) were measured on available mesocosm-reared NT (spring smolted), T<sub>MT</sub>, and T<sub>H3</sub> (fall smolted) females from the 2012 smolt year class at maturity.

#### 2.7. Data analyses

All statistical analyses were performed using R 3.1.2 (R R Core Team 2015) or SigmaPlot (Systat Software Inc., San Jose, CA). Within smoltyear differences among fish groups in size, swimming and metabolic performances, resistance and recovery time during temperature challenges, and female fecundity, were performed with ANOVA. Comparison of survival curves of fish groups within years, as well as loss of equilibrium curves when exposed to high temperature challenges, were compared using the Kaplan–Meier LogRank method followed by the Holm–Sidak multiple-comparison test. Proportion of fish that lost equilibrium when exposed to high temperatures were compared with Chi-squared analysis. Oxygen uptake among fish groups during swimming and recovery were compared with linear regression.

Across smolt year T and NT size at maturity (mass was In transformed to adjust for non-equal variance) and seawater survival, including identification of factors that influenced these differences, were compared by generalized linear mixed models using the lme4 package (Bates et al. 2015). Variables were analyzed with genotype and rearing conditions (i.e. smolt season, Rs screening, antibiotic injections, see Table 1) as fixed interacting factors, and genotype was crossed with the random factor Smolt Year. Fish size was modelled using the normal distribution and identity link, and survival was modelled using a binomial distribution, log-link function, and optimizer control bobyqa to ensure model convergence. The full survival model failed the test for singularity, and consequently survival was modelled simply (i.e. Genotype  $\times$  one rearing factor at a time), and only those factors with significant effects returned to the full model. Significance of the model outputs were evaluated using the Anova() function in the car package (Fox and Weisberg 2011) with test type 3, and post-hoc comparisons were performed by Tukey adjusted pairwise comparisons of least square means (LSM) using the lsmeans (Lenth 2016) and multcomp (Hothorn et al. 2008) packages. Data are presented as mean (for individual smolt years) or LSM (across smolt years)  $\pm$  standard error of the mean, and differences were considered significant if P < 0.05. Condition

factor (CF) was calculated as  $(mass \times length^{-3} \times 100)$ , and standard growth rate was calculated as (SGR = [ln mass 2 - ln mass 1] / [time 2 - time 1]. Male reproductive comparisons among fish groups in the six arenas were compared by general linear mixed models, with genotype as a fixed factor and genotype crossed with the random factor Arena, followed post-hoc comparisons on LSM as above.

#### 3. Results

#### 3.1. T vs. NT size at maturity and seawater survival

Transgenic fish had overall greater mass and length at maturity (see Fig. 2A and B, and Table 2 for all among smolt year P-values) than NT fish after rearing in seawater mesocosms, and had greater mass and length at maturity than NT fish in all individual smolt years despite varying conditions among smolt years (see Table 1). However, there was a significant interaction between genotype (T vs. NT) and smolt season (Spring vs. Fall) when either mass or length was compared across years. Smolt season did not significantly impact the mass at maturity of T fish (P = 0.972), but NT fish entering seawater at their normal spring time had larger size at maturity than those that smolted in the fall (P < 0.001). In addition, overall T fish were only significantly larger than NT fish that had smolted in the fall, not the spring. There was also a significant interaction between genotype and antibiotic injection (no vs. yes) on mass but not length. However, T fish were greater in mass than NT fish whether given antibiotic injections or not, and antibiotic injections did not significantly influence mass of either T or NT fish. Rs screening impacted overall size at maturity. Those from unscreened mothers were significantly larger (LSM 3.44  $\pm$  0.29 and 2.19  $\pm$ 0.19 kg, respectively) and longer (LSM 57.8  $\pm$  2.5 and 50.5  $\pm$  1.9 cm, respectively) at maturity than those from Rs-negative mothers. T fish had greater overall CF than NT fish and in each individual smolt year. While there was a significant interaction between genotype and antibiotic treatment on CF, CF was unaffected by antibiotic treatment in both T and NT fish, and T fish maintained greater CF than NT fish regardless of antibiotic treatment (Fig. 2C). Linear regression of mature length versus CF demonstrated high CF in transgenic fish was due in part to genotype, and not due entirely to differences in mature length between groups of fish (data not shown).

The relative seawater survival of T vs. NT varied depending on whether the maternal parents were Rs-unscreened or Rs-negative. NT fish had greater seawater survival than T fish when from Rs-unscreened mothers (P = 0.007, Fig. 3), while the two groups did not significantly differ in overall survival when from Rs-negative mothers. Use of Rs-negative mothers improved survival of all groups above those from Rs-unscreened mothers (P < 0.001 for all), and this was also observed within smolt year, where NT fish from Rs-unscreened mothers (2011 smolt year, see Fig. 4).

The overall trends listed above were mirrored in relevant individual smolt years (see Table 1, Supplemental Tables 1-6), with the following exceptions. T fish were heavier and longer at maturity than NT fish regardless of smolt season in all individual years except the 2013 smolt year. In the 2013 smolt year all fish were introduced in the spring, and T fish were either reared at 15 °C as juveniles to advance their smolt season to 6 months post-fertilization, or ration restricted as juveniles to delay their smolt season to 18 months post-fertilization. NT fish were equal in size to T fish reared at 15 °C as juveniles, but smaller in length (P = 0.021) and mass (P < 0.001) than T fish fed a restricted ration as juveniles. In years where both NT and T fish were from Rs-unscreened mothers (i.e. 2007, 2008), survival followed the above trend of NT fish having greater seawater survival than T fish. However, in individual smolt years where both genotypes were from Rs-negative mothers survival was not equal between genotypes despite the overall trend for equal survival. For fish from Rs-negative mothers, T fish had greater survival than NT in smolt years 2010 and 2011, while NT fish had greater survival in smolt years 2012 and 2013 (P < 0.001 for all). Overall,



**Fig. 2.** Size and shape at maturity of growth hormone transgenic (T) and non-transgenic (NT) coho salmon reared in seawater mesocosms from 6 smolt years. A) Mass and B) length at maturity summed over season at which fish entered mesocosm (Spring or Fall); and C) condition factor at maturity summed over whether fish were give prophylactic antibiotic treatments or not (Ab + or Ab –). In addition, variables are given for NT and T fish reared in standard culture, and NT fish reared in nature (from Bessey et al. 2004; Devlin et al. 2004a; Fitzpatrick et al. 2011; Leggatt et al. 2014, and unpublished data from the authors). Letters indicate significant differences among fish groups within the mesocosm (P < 0.05), and data are presented as least square means  $\pm$  SEM.

there was no significant factor effect of antibiotic treatment on seawater survival (see above), but in the 2012 smolt year all genotypes had higher survival when treated with antibiotics than without (P = 0.026). For all fish groups and smolt years, we examined whether initial body mass had an influence on survival time of fish groups within each smolt year and the slope of the relationship between initial body mass and survival time was never significantly different from zero (P > 0.05).

In the 2007 smolt year, NT fish were reared with or without T fish to determine if there was a significant influence of T presence on NT growth, survival, and size at maturity. Non-transgenic fish grown with

#### Table 2

*P*-values for factor and interaction effects on size (ln-mass, length), condition factor (CF), and overall seawater survival comparing NT and T coho salmon raised in seawater mesocosms over six smolt years. Fixed factors are Genotype (NT or T), smolt Season (spring or fall), Rs maternal screening (none or negative), and use of Antibiotic injections (no or yes). Where interactions are significant (P < 0.05) factor effects should be examined within other factor effects.

| Factor or interaction        | Ln-Mass | Length | CF     | Survival |
|------------------------------|---------|--------|--------|----------|
| Genotype $\times$ season     | <0.001  | 0.029  | 0.891  | 0.455    |
| Genotype $\times$ Rs         | 0.153   | 0.843  | 0.624  | 0.012    |
| Genotype $\times$ antibiotic | 0.038   | 0.487  | 0.034  | 0.414    |
| Genotype                     | <0.001  | <0.001 | <0.001 | 0.005    |
| Season                       | <0.001  | <0.001 | 0.212  | 0.831    |
| Rs                           | <0.001  | 0.029  | 0.630  | <0.001   |
| Antibiotic                   | 0.089   | 0.278  | 0.686  | 0.504    |

T fish were transiently larger in mass (1.2-fold) and length (1.04-fold) than those grown without T fish (P < 0.001, see Fig. 5, Supplementary Table 1), but were equal in size at maturity. Condition factor (Supplemental Table 1) and standard growth rate (SGR, Fig. 5) of NT fish grown with T fish tended to follow the pattern of T fish, while SGR of NT fish grown without T tended to vary more with season. Non-transgenic fish raised with or without T fish had different-shaped survival curves (P < 0.001), with a faster initial mortality and slower later mortality when T fish were not present (Supplemental Table 1), although the final survival rate of NT fish in this year was not affected by presence of T fish (P = 0.790, see Table 1).

In the 2012 smolt year, an additional T strain was included which contained the H3 promoter  $(T_{H3})$  rather than the MT promoter  $(T_{MT})$  of the main T fish groups, to determine whether size at maturity and seawater survival was consistent between GH strains. By maturity,  $T_{H3}$  and  $T_{MT}$  fish did not differ in either size or overall survival, although  $T_{H3}$  fish had a lower CF than  $T_{MT}$  fish and there were minor transient differences in size and growth between  $T_{H3}$  and  $T_{MT}$  fish during seawater rearing (Supplemental Table 5).

#### 3.2. Swimming and metabolic effects

Late marine-stage fish groups used in swimming trials did not differ significantly in mass (average 2.33  $\pm$  0.13 kg, P = 0.289), or length (53.0  $\pm$  0.9 cm, P = 0.921). However, CF for T<sub>MT</sub> and T<sub>H3</sub> fish was 20% and 10% greater, respectively, than NT fish (1.69  $\pm$  0.07, 1.57  $\pm$  0.03, and 1.39  $\pm$  0.04 respectively, P = 0.001). Fin condition was generally good in all groups of fish, although T<sub>MT</sub> and T<sub>H3</sub> fish tended to have smaller, more eroded tails than NT fish (see Fig. 6). Transgenesis



**Fig. 3.** Overall survival from smolt to maturity of growth hormone transgenic (T) and nontransgenic (NT) coho salmon reared in seawater mesocosms from 6 smolt years, summed over whether from Rs (*Renibacterium salmoninarum*) unscreened or negative mothers (Rs no or Rs –ve, respectively). Letters indicate significant differences among fish groups (P < 0.05), and data are presented as least square means  $\pm$  standard error of the mean.



**Fig. 4.** Survival of growth hormone transgenic (T) and non-transgenic (NT) coho salmon reared from smolt in seawater mesocosms, 2011 smolt year.  $NT_{Lab}$  and T fish were reared as juveniles in laboratory conditions and produced from Rs-negative maternal parents, and  $NT_{Hat}$  fish were reared as juveniles at the Chehalis River hatchery and produced from Rs-unscreened maternal parents.

decreased swimming performance, as U<sub>crit</sub> for similarly sized NT fish was 27% faster than  $T_{MT}$  and  $T_{H3}$  fish (Table 3).  $T_{MT}$  and  $T_{H3}$  fish had a similar U<sub>crit</sub>, suggesting that a different transcript did not affect U<sub>crit</sub>. Transgenesis decreased metabolic efficiency with swimming as both T groups had 17% higher average COT at a given speed than NT fish (Fig. 6A, Table 3). NT fish had higher aerobic and anaerobic capacity as they had 16% higher MO<sub>2-max</sub>, 24% higher aerobic scope, and 56% higher EPOC than T fish when the two T genotypes were pooled. However, transgenesis did not significantly affect MO<sub>2-R</sub> or COTnet, while transgenic strain (T<sub>MT</sub> versus T<sub>H3</sub>) did not significantly affect any measured metabolic variable. During recovery from exhaustion at U<sub>crit</sub>, NT fish had a greater peak oxygen uptake (P = 0.018) and faster rate of recovery for the first 10 min (P = 0.018, Fig. 6B) than either T<sub>MT</sub> or T<sub>H3</sub> fish. After 30 min of recovery, oxygen uptake temporarily plateaued at a significantly elevated level when compared with the MO<sub>2-R</sub> measured at the start of the swimming trial in all three fish groups (1.62-fold greater in NT and  $T_{H3}$  fish, 1.25-fold greater in  $T_{MT}$  fish, P < 0.001, Fig. 6B) and oxygen uptake was lower for  $T_{MT}$  fish than either NT or  $T_{H3}$  fish from 30 min to 80 min after the swim trial (P < 0.001).

#### 3.3. Temperature challenges

Late marine-stage fish groups used in temperature trials were the same fish used in swim trials above, with a 2 month recovery period. T<sub>MT</sub>, T<sub>H3</sub> and NT fish did not differ in percent of fish that lost equilibrium (Table 4), resistance time, or time to recovery, after three staggered high temperature trials. The three fish groups did not significantly differ either in the relationship between percent loss of equilibrium and time for 22 °C (P = 0.957) or 24 °C challenges (P = 0.108, Fig. 7). However, the relationship among fish groups did differ during a 1 h 26 °C heat challenge (P = 0.046, Fig. 7), where T<sub>H3</sub> fish had a steeper slope than NT fish, although both were not significantly different from T<sub>MT</sub> fish. Two NT fish (one at 24 °C and one at 26 °C) lost equilibrium when returned to the holding tank at the end of the trial and did not recover, whereas all T<sub>MT</sub> and T<sub>H3</sub> fish recovered.

#### 3.4. T<sub>MT</sub> vs. T<sub>H3</sub> spawning success

 $T_{MT}$  and  $T_{H3}$  males (from the 2012 smolt year class) competing for nature-reared NT females did not significantly differ in any spawning success or behaviour variable examined (Table 5). Fecundity measurements for mesocosm-reared spring smolted NT (n = 11) and fall smolted  $T_{MT}$  (n = 29) and  $T_{H3}$  (n = 11) revealed a gonadal somatic index order of NT >  $T_{MT}$  >  $T_{H3}$  (0.22  $\pm$  0.01, 0.18  $\pm$  0.01, and 0.14  $\pm$  0.01 respectively, P < 0.001), while estimated fecundity was ordered



**Fig. 5.** Growth of fish in the 2007 smolt year. **a)** Mass and **b)** standard growth rates - mass (SGR) of growth hormone transgenic (T) and non-transgenic (NT) fish reared from smolt in seawater mesocosms, where NT fish were reared with or without T fish. SGR is given at end of time interval used for calculations. Letters indicate significant differences among fish groups within time period (P < 0.05). Data are presented as mean  $\pm$  SEM.

 $T_{MT} > T_{H3} = NT$  (2.59  $\pm$  0.14, 1.76  $\pm$  0.15, and 1.43  $\pm$  0.14 respectively, P < 0.001). The difference in these rank orders reflected estimated individual egg mass being significantly greater in NT than  $T_{H3}$  although neither differed from  $T_{MT}$  (0.17  $\pm$  0.01, 0.12  $\pm$  0.01, 0.14  $\pm$  0.01 respectively, P = 0.012).



**Fig. 6.** Oxygen uptake of late marine stage non-transgenic (NT, white shapes, solid lines) or growth hormone transgenic coho salmon containing either the OnMTGH1 ( $T_{MT}$ , black shapes, dashed lines) or OnH3GH1 ( $T_{H3}$ , gray shapes, dotted lines) gene construct during A) increasing swim speeds to exhaustion and B) recovery from exhaustive swimming trial. Inset shows morphology of representative fish used in the swimming trial.

#### 4. Discussion

4.1. How well did fish reared in mesocosms mimic natural growth rates and reproductive performance?

It is well established that standard culture conditions for NT fish stunts growth, changes morphology and colouration, and reduces fecundity. Here we provide definitive evidence that rearing in seawater mesocosms partially removed effects of culture, as mass and length at maturity (see Fig. 2), and spawning success (Leggatt et al. 2014) were intermediate between those for standard culture conditions and those from nature. Morphology of mesocosm-reared fish was more similar to nature-reared than culture-reared fish, although mesocosm-reared fish had the deepest body shape (see Fig. 2 for CF and Fig. 8 for representative fish from each genotype/environment). Thus, while the mesocosm environment did not fully mimic the phenotype of naturereared NT fish, it represents a clear improvement in phenotype over standard culture and more accurately reflects commercial-scale culture conditions. Factors that may have contributed to the more natural phenotype in the mesocosm could include limited human interactions (Sundström et al. 2016), natural lighting and water supply, and low density, whereas cohabitation with T fish, artificial food supply, and limited spatial scope of the mesocosm could be factors potentially limiting the return of NT phenotype to that of nature-reared fish. Although cohabitation with T fish could influence growth and survival of NT fish through competitive interaction effects, the 2007 smolt-year NT salmon were also grown separately from T without any change to size or body shape at maturity. Note, however, that NT fish grown with T fish tended to have less seasonal fluctuation in growth rate and CF (i.e. similar to T fish) than those grown without T fish (Fig. 5). Feeding behaviour of a few individual rainbow trout is known to initiate the same behaviour in the rest of the population (see Ellis et al. 2002). Thus, it is possible that cohabitation of NT and T fish may likewise have increased feeding behaviour during winter where NT normally have minimal feeding behaviour. Conversely, competition with T fish may suppress NT growth during periods of normally high summer growth. The specific influences of other individual environmental components on phenotype have not been examined. Nevertheless, with a more natural phenotype in mesocosm-reared NT fish we believe that a more accurate prediction of the potential phenotype of T escapees from commercial operations to natural ecosystems is now possible.

#### 4.2. T vs. NT size at maturity

The importance of body size is well established in fish. It can influence dominance hierarchies and foraging competition (e.g. Reinhardt 1999), and size at maturity can influence spawning success in salmonids (Berejikian et al. 2009; Fleming and Gross 1993). As such, the potential

#### Table 3

Swimming performance and oxygen uptake of non-transgenic (NT) or growth hormone transgenic late marine stage coho salmon containing either the OnMTGH1 ( $T_{MT}$ ) or OnH3GH1 ( $T_{H3}$ ) gene construct, as well as both transgenic groups combined (T). Measurements were made during a swim trial to determine critical swimming speed ( $U_{crit}$ ), maximum oxygen uptake ( $MO_{2-max}$ ), routine oxygen uptake at resting swimming speeds ( $MO_{2-R}$ ), aerobic scope ( $MO_{2-max} - MO_{2-R}$ ), swimming economy ( $MO_{2-max} / U_{crit}$ ), average cost of transport (COT =  $MO_2 / swim speed$ ), net cost of transport (COTnet = aerobic scope /  $U_{crit}$ ), and excess post-exercise oxygen consumption over the first 30 min of recovery (EPOC). *P*-values for differences among genotypes are given as: all groups (T groups combined). Letters (a, b) indicate significant differences among fish groups within a variable, \* indicates significant difference between NT and T fish when  $T_{MT}$  and  $T_{H3}$  are combined (P < 0.05). Data are presented as mean  $\pm$  SEM.

| Variable measured                                 | NT                    | T <sub>MT</sub>                | T <sub>H3</sub>       | Т                     | P-value           |
|---|-----------------------|--------------------------------|-----------------------|-----------------------|-------------------|
| U <sub>crit</sub> (body lengths/s)                | $2.25\pm0.06^a$       | $1.69\pm0.12^{b}$              | $1.84\pm0.10^{\rm b}$ | $1.77 \pm 0.08^{*}$   | 0.001 (<0.001)    |
| $MO_{2-max}$ (mg·O <sub>2</sub> /kg/min)          | $9.49 \pm 0.55$       | $8.39 \pm 0.76$                | $8.03 \pm 0.50$       | $8.20 \pm 0.45^{*}$   | 0.107 (0.034)     |
| MO <sub>2-R</sub> (mg O <sub>2</sub> /kg/min)     | $2.34 \pm 0.28$       | $2.40 \pm 0.10$                | $2.46 \pm 0.30$       | $2.43 \pm 0.16$       | 0.982 (0.92)      |
| Aerobic scope (mg $\cdot$ O <sub>2</sub> /kg/min) | $7.15 \pm 0.74$       | $5.99 \pm 0.73$                | $5.57 \pm 0.43$       | $5.77 \pm 0.40^{*}$   | 0.099 (0.031)     |
| Swimming economy $(mg \cdot O_2/kg/m)$            | $0.132 \pm 0.006$     | $0.157 \pm 0.014$              | $0.139 \pm 0.007$     | $0.147 \pm 0.008$     | 0.132 (0.14)      |
| COT (mg·O <sub>2</sub> /kg/m)                     | $0.064 \pm 0.003^{a}$ | $0.077 \pm 0.006^{\mathrm{b}}$ | $0.073 \pm 0.004^{b}$ | $0.075 \pm 0.004^{*}$ | < 0.001 (< 0.001) |
| COTnet (mg $\cdot$ O <sub>2</sub> /kg/m)          | $0.052\pm0.005$       | $0.062 \pm 0.018$              | $0.050 \pm 0.009$     | $0.055 \pm 0.004$     | 0.268 (0.618)     |
| EPOC (mg·O <sub>2</sub> )                         | $83.9 \pm 12.9$       | $46.9\pm8.3$                   | $60.3 \pm 11.5$       | $53.6\pm7.0^*$        | 0.081 (0.037)     |

for a GH transgene to influence body size could have implications to overall fitness in fish. Transgenic fish reared in seawater mesocosms obtained larger mass, length and CF at maturity than NT fish in all years despite fluctuations in experimental design among years. However, when juvenile T growth was manipulated to meet NT smolt season requirements (i.e. smolt in the spring), the differences in mass and length were significant in individual years but not overall. The larger fold differences between NT and T fish when entering seawater in the fall were likely due to detrimental effects of delayed smoltification in NT fish, as T fish were unaffected by smolt season. The largest differences between NT and T growth rates were observed in early seawater rearing, as well as in the growth period immediately prior to maturity (see Fig. 5, Supplemental Tables 1-6). This concurs with greater increase in relative growth in T fish in early versus late life stages in standard culture conditions (Devlin et al. 2004a; Oakes et al. 2007; Zhong et al. 2009). The greater growth rate of T fish near maturation demonstrates T fish maintained food intake along with developing less typical spawning morphology (see Fig. 8 and Leggatt et al. 2014), whereas NT fish followed the more typical development of spawning condition with decreased feeding in preparation for gamete production.

Different environment conditions (culture vs. mesocosm, smolt season) had less effect on mass and length at maturity for T fish compared with NT fish (current and previously published data summarized in Fig. 2). Mesocosm-reared T fish tended to be greater in mass, but not length, than nature-reared NT fish. This suggests that GH transgenesis does not influence the maximum obtainable length of coho salmon, although may influence maximum obtainable mass. Unlike NT fish, T fish were able to obtain this maximum size among different marine environments, and in a shorter period of time, suggesting at the marine stage and in terms of growth T fish are less influenced by environmental conditions than NT fish. This could be due to extreme upregulation of appetite peptides (i.e. AgRP1) increasing feeding behaviour and consequent growth in T fish independently of environmental signals (Kim et al. 2015). The limited influence of different marine environments on T growth contrasts with studies showing juvenile T coho salmon are greatly influenced by freshwater environmental conditions and are more similar to NT fish in growth rate and CF in semi-natural conditions compared to culture conditions (Sundström et al. 2007; Sundström et al. 2009). As such T fish may respond to varying environmental conditions differently at different life stages, although differences between life-stages may also reflect the greater control of environmental variables in simulated juvenile versus marine ecosytems. In particular, use of a natural food supply in juvenile studies versus an artificial food supply in marine studies is hypothesized to influence the greater similarity to NT morphology in T fish in the juvenile versus marine life-stage.

Extrapolation of NT and T characteristics reared in the limited number of marine environments examined here suggest that T fish reared in nature might have larger body mass but similar length as NT fish (i.e. a greater CF and altered body shape relative to nature-reared NT fish). If the plastic responses of salmon to nature-like environments were to arise as predicted, such changes are likely to influence the ability of T salmon to compete effectively with the naturally-selected morphology possessed by wild type fish reared in nature. However, the relatively high CF of NT fish grown in the mesocosm indicates that the mesocosm does not simply represent an intermediate environment between standard culture and natural environments, but rather alters the allometric relationships among tissue types (i.e., skeletal length vs. muscle volume). Consequently, extrapolation of T body mass and shape as would exist in natural conditions from what is known from two different non-natural conditions cannot be done with accuracy at this time. Current data indicate that we do not yet have control over all variables influencing growth and phenotypic development in wild-type and GH transgenic coho salmon.

#### 4.3. T vs. NT survival

Fish in all year-classes were not vaccinated, and those in the first four of the six smolt year-classes were not treated with antibiotics in an effort to determine what the relative seawater survival of NT and T fish might be under conditions of natural pathogen exposure. Fish

Table 4

Response of late marine stage non-transgenic (NT) or growth hormone transgenic coho salmon containing either the OnMTGH1 ( $T_{MT}$ ) or OnH3GH1 ( $T_{H3}$ ) gene construct to 2 h (22 and 24 °C) or 1 h (26 °C) static heat challenges. Measured responses include percent that lost equilibrium (% LOE), average time (min) to LOE for those fish that lost equilibrium (Resistance time), and time (min) to recover from LOE. Time data are presented as mean  $\pm$  SEM, differences are considered significant if P < 0.05.

| Variable        | Temperature | NT                | T <sub>MT</sub>   | T <sub>H3</sub>   | P-value |
|-----------------|-------------|-------------------|-------------------|-------------------|---------|
| % LOE           | 22 °C       | 75.0%             | 62.5%             | 71.4%             | 0.856   |
|                 | 24 °C       | 62.5%             | 87.5%             | 57.1%             | 0.384   |
|                 | 26 °C       | 85.7%             | 100%              | 100%              | 0.325   |
| Resistance time | 22 °C       | $61.31 \pm 12.82$ | $48.08 \pm 10.50$ | $57.52 \pm 14.80$ | 0.534   |
|                 | 24 °C       | $42.84 \pm 15.30$ | $30.13 \pm 4.67$  | $23.24 \pm 5.93$  | 0.214   |
|                 | 26 °C       | $17.04 \pm 3.08$  | $16.84 \pm 2.35$  | $14.11 \pm 2.22$  | 0.109   |
| Recovery time   | 22 °C       | $6.78 \pm 0.82$   | $4.42 \pm 2.12$   | $6.05\pm2.00$     | 0.628   |
|                 | 24 °C       | $6.18 \pm 1.48$   | $5.13 \pm 1.52$   | $3.27 \pm 1.15$   | 0.505   |
|                 | 26 °C       | $9.97 \pm 2.82$   | $7.90 \pm 1.73$   | $9.53 \pm 1.90$   | 0.325   |



**Fig. 7.** Time to loss of equilibrium for late marine stage non-transgenic (NT) or growth hormone transgenic coho salmon containing either the OnMTGH1 ( $T_{MT}$ ) or OnH3GH1 ( $T_{H3}$ ) gene construct, exposed to heat challenges. Fish were exposed to static, oxygenated water baths of 22 °C and 24 °C for up to 120 min, and 26 °C for up to 60 min (ambient water temperature 10.2  $\pm$  0.1 °C). Letters at end of curves indicate significant differences among genotypes at 26 °C (P < 0.05).

groups came from a population known to harbour *R. salmoninarum* (Rs), the causative agent of bacterial kidney disease. When maternal parents were not screened for Rs, survival was very poor overall, and importantly NT fish had greater survival than T fish. This concurs with previous reports of T fish having lower survival to experimental infections relative to NT fish (Jhingan et al. 2003; Kim et al. 2013). When fish were produced from Rs-negative maternal parents, overall survival was similar between NT and T fish, although when individual smolt years were examined T and NT each had greater survival in two of the four years. This concurs with conflicting results for relative survival for T fish models in different environments (Devlin et al. 2004a; Higgs et al. 2009; Muir and Howard 1999; Pennington and Kapuscinski 2011; Rahman et al. 2001; Sundström and Devlin 2011; Sundström et al. 2014). It is clear we do not fully understand the factors influencing relative survival of T vs. NT in mesocosm environments, although T fish do appear to have a disadvantage in the presence of infectious disease agents.

Survival increased several-fold when only Rs-negative mothers were used in crosses. This difference in survival was also observed when fish from Rs-unscreened and Rs-negative maternal parents were raised together (NT fish from the hatchery or laboratory, respectively, 2011 smolt-year, see Fig. 4), indicating a significant influence on survival may arise from vertical rather than horizontal transmission of Rs. Previous studies have shown use of Rs-negative versus unscreened maternal parents can decrease prevalence and intensity of Rs levels in resulting salmonid juveniles, smolts, and returning adults (Elliott et al. 1997; Faisal et al. 2012; Guðmundsdóttir et al. 2000; Munson et al. 2010), as well as increase juvenile survival in hatcheries and rivers (Munson et al. 2010; Pascho et al. 1993; Pascho et al. 1991), and during a 98-day saltwater holding trial (Elliott et al. 1995). However, the present study has further shown that maternal Rs screening can be associated with increased overall seawater survival of offspring. It should be noted that the intention of this study was not to examine the effects of maternal Rs screening on marine survival of coho salmon, and hence the results should be considered anecdotal at this point. Whether seawater survival of nature-reared salmonids may be impacted in a similar manner by maternal Rs load is not clear, particularly as horizontal transmission of Rs (e.g. via faecal-oral route, Balfry et al. 1996) in the mesocosm is expected to be much greater than under natural marine conditions due to limited flushing and spatial scope of the mesocosms. Fish from Rs-unscreened mothers were larger in size than those from Rs-negative mothers. This was likely due to decreased density in mesocosms associated with increased mortality, rather than increased survival in larger fish, as initial body mass was not significantly correlated with survival in any smolt year.

#### 4.4. T vs. NT: other fitness components

For most Pacific salmon species, the ability to successfully reproduce relies in part on the ability to navigate up their natal rivers and streams to spawning grounds through high water velocities and at temperatures greater than those experienced in the marine environment (Eliason et al. 2011). As well, factors such as growth rate, dominance and aggression, and maturation, can influence routine metabolic rate, with resulting downstream effects on energy utilization in fish (see Eliason and Farrell 2016). Therefore, we examined metabolic rates, swimming ability and high temperature tolerance of late marine stage salmon to determine if GH transgenesis can influence these fitness components in the absence of the stunting effects of standard culture.

GH transgenic fish are generally reported to have higher routine oxygen uptake than their non-transgenic counterparts (Cook et al. 2000; Deitch et al. 2006; Guan et al. 2008), although in some species, including coho salmon, higher oxygen uptake may be due to increased feeding level and not a basal cost to metabolism (Guan et al. 2008; Leggatt et al. 2003). In the current study T fish did not differ from NT fish in routine oxygen uptake, further demonstrating a lack of basal cost of GH transgenesis in coho salmon, although Lee et al. (2003a) found mature cultured T fish had higher  $MO_{2-R}$  than ocean-ranched NT fish.

In swimming trials, NT fish swam 40% faster (U<sub>crit</sub>) than T salmon, which required a higher maximum oxygen uptake than T fish. Nevertheless, NT fish had a significantly lower COT, suggesting a more efficient swimming as well. These results are consistent with earlier studies in cultured transgenic coho salmon in both juvenile and mature adult stages (Farrell et al. 1997; Lee et al. 2003a, adult comparison was to nature-reared NT fish), and MO<sub>2-max</sub> of T fish reared in the mesocosm compares well with that reported previously for culture-reared coho salmon (8.2 vs. 8.8 mg O<sub>2</sub>/kg min respectively, Lee et al. 2003a). Thus, poor swimming ability and efficiency is a consistent phenotype of GH transgenesis in coho salmon at multiple life-stages and under multiple culture conditions. Similarly GH transgenesis decreased Ucrit in common carp (Li et al. 2007). In contrast, GH transgenic Atlantic salmon only had poorer COT and a similar U<sub>crit</sub> as NT fish (Stevens et al. 1998) whereas GH transgenesis in tilapia had no effect on either U<sub>crit</sub> or COT (McKenzie et al. 2003). Thus, GH transgenesis can influence swimming ability of different fish species in distinct ways.

Johnston et al. (2014) found juvenile T coho salmon in culture did not differ from wild-type fish in skeletal white muscle fibre number, size, or distribution in culture, and T fish in general have larger hearts and greater mass-specific cardiac output than non-transgenic fish (Chen et al. 2015; Deitch et al. 2006; Pitkänen et al. 2001), suggesting the poor swimming capacity in T coho salmon is likely not due to altered

Table 5

Reproductive success and behaviour of  $T_{MT}$  and  $T_{H3}$  mesocosm-reared male coho salmon in competition for NT nature-reared female fish. Letters indicate significant differences among fish groups (P < 0.05). Data are presented as LSM  $\pm$  SEM.

| Reproductive variable   | T <sub>MT</sub> o <sup>*</sup> | T <sub>H3</sub> ♂    | NT Q                | P-value |
|---|--------------------------------|----------------------|---------------------|---------|
| Number of offspring produced per male $(\times 10^3)$                   | $0.58\pm0.14$                  | $0.30\pm0.13$        | -                   | 0.124   |
| % of male fish that spawned   | $67.2 \pm 4.7$                 | $55.8 \pm 2.1$       | -                   | 0.513   |
| % of available females male fish spawned with                           | $29.9\pm0.6$                   | $26.7 \pm 0.5$       | -                   | 0.691   |
| Number of aggressive actions given (per fish per 5 min interval)        | $0.16\pm0.17^{a}$              | $0.09 \pm 0.019^{a}$ | $1.15 \pm 0.15^{b}$ | < 0.001 |
| Number of aggressive actions received (per fish per 5 min interval)     | $0.66 \pm 0.12^{a}$            | $0.71 \pm 0.12^{a}$  | $0.17 \pm 0.11^{b}$ | < 0.001 |
| Number of attending behaviours given (per male fish per 5 min interval) | $0.12\pm0.06$                  | $0.16\pm0.06$        | -                   | 0.641   |



**Fig. 8.** Spawning morphology of growth hormone transgenic (T) and non-transgenic (NT) coho salmon reared from smolt in standard seawater culture conditions (4000 L tank), seawater mesocosms (350,000 L tank), or natural conditions. Morphology of each group was taken from representative fish from this study (mesocosm-reared fish, 2013 smolt-year), Leggatt et al. 2014 (nature-reared fish), and Devlin et al. 2006 (culture-reared fish). Morphology of additional fish from the mesocosm and nature from multiple smolt years are given in Leggatt et al. (2014).

muscle structure or poor cardiac function. Surprisingly, Hill et al. (2000) found muscles of juvenile T coho had variables consistent with greater potential swimming ability (i.e. higher red muscle content, high phosphofructokinase activity), and suggested factors outside muscle function may be limiting T swimming ability. Rather, reduced swimming capacity may be due to their altered body shape and poor caudal fin condition, both of which have been shown to alter swimming ability in other fishes (Fu et al. 2013; Li et al. 2009; Li et al. 2007; Plaut 2000; Qu et al. 2013).

Total metabolic cost during the swim trials (COTnet) did not differ between NT and T fish, indicating T fish were not limited aerobically, even though they expended more energy at a given swim speed. In contrast, EPOC was 57% greater in NT fish, demonstrating a greater ability or desire to utilize anaerobic metabolism to achieve U<sub>crit</sub> than T fish. T<sub>MT</sub> fish also had lower oxygen uptake during recovery than NT fish. This suggests that this strain of T fish either may have been limited by nonmetabolic factors such as morphology, or had less ability or desire to change swimming gaits and use an anaerobic swimming mode (see Peake and Farrell 2004). This is surprising as enzyme profiles of T juvenile coho reared in culture suggest higher ability for anaerobic metabolism in muscle (i.e. higher LDH activity, Leggatt et al. 2012). As well, Lee et al. (2003a) found the reverse of the above, where adult T coho salmon reared in culture had greater EPOC than ocean-ranched NT fish, indicating rearing conditions may influence relative anaerobic capacity of T and NT coho salmon. Whether swimming capacity of T fish would be reduced if reared in even more natural conditions that minimized alterations to fin condition and body shape is not known. If these effects persisted in Tescapees, current data suggest T fish would have relatively lower success during times of high swimming demand such as evading predators, capturing prey, and migrating up rivers to spawn in part because they would incur greater energy costs associated with a reduced capacity for aerobic and anaerobic swimming when compared with NT salmon.

Although NT and T fish had a similar percent that lost equilibrium during incremental high water temperature challenges,  $T_{H3}$  fish lost equilibrium faster than NT fish at the highest test temperature examined (26 °C). This indicates that impaired thermotolerance may not be a consistent feature of GH transgenesis in coho salmon. Chen et al. (2015) found no difference in critical thermal maxima in juvenile T and NT coho salmon. While abrupt temperature changes are not as sensitive in determining strain differences as designs with slower temperature changes (Galbreath et al. 2004), we suggest that performance T fish during periods of high environmental water temperatures, such as migrating through rivers in summer months, may not significantly differ from NT fish.

#### 4.5. Influence of promoter type on fitness components of GH transgenic coho salmon

The H3 promoter is reported to be a weaker promoter than the MT promoter (Chan and Devlin 1993), and T<sub>H3</sub> strains have slower growth than T<sub>MT</sub> strains in standard culture (Leggatt et al. 2012). However, in mesocosms there were no significant differences between the T<sub>H3</sub> and  $T_{MT}$  strains in size at maturity, male reproductive success, late marinestage swimming performance, and thermal tolerance, suggesting minimal effects of strain or promoter on many marine variables influencing GH transgenic coho salmon fitness. This differs from the juvenile lifestage, where T<sub>H3</sub> strains had lower survival and inconsistently effected growth in semi-natural streams relative to T<sub>MT</sub> fry (Leggatt et al. 2017). A few differences did emerged (e.g., lower CF during marine growth, higher oxygen uptake post swimming-recovery) that could potentially impact success in the marine environment. In particular, T<sub>H3</sub> females had lower fecundity, GSI and egg size than T<sub>MT</sub> fish, indicating they may have lower potential to establish populations in natural environments. However, the importance of genotype-by-environment interactions was demonstrated by  $T_{H3}$  fish growing slower than  $T_{MT}$  fish in standard culture conditions (Leggatt et al. 2012), but not in mesocosm environments. Further data examining additional gene constructs and strains will be required to determine to what extent potential fitness consequences may be reliably extrapolated among transgenic strains of different origins at this life stage.

#### 5. Conclusions

Results from our studies have found that, as with juvenile life stages, wild-type and GH transgenic salmon show plastic responses to marine rearing conditions, although unlike wild type, GH transgenic coho salmon can obtain maximum body size in both cultured and semi-cultured environments and under variable environmental conditions (i.e. different levels of disease screening, antibiotic treatments, smolt season, etc.). Transgenic fish appeared to have a more advantageous phenotype for some fitness components (i.e. greater size), less advantageous phenotype for type for others (i.e. morphology at maturity, swimming ability), and

Table 6

Summary of marine fitness components of non-transgenic (NT) and growth hormone transgenic (T) coho salmon reared in seawater mesocosms, including effect of marine (mesocosm vs. nature) environment on NT fitness components, and whether T vs. NT fitness is influenced by marine rearing conditions (presence of genotype-by-environment interaction: G x E) or GH transgene promoter.

| Fitness component             | Mesocosm: T vs. NT | NT: Mesocosm vs. nature | Presence of $G\timesE$ | Influence of transgene promoter |
|-------------------------------|--------------------|-------------------------|------------------------|---------------------------------|
| Mature size                   | T > NT             | Nature > mesocosm       | Yes                    | No                              |
| Mature shape/morphology       | NT ≠ T             | Nature ≠ mesocosm       | Yes                    | Yes                             |
| Marine survival               | $T \ge \langle NT$ | n/a                     | n/a                    | No                              |
| Spawning success <sup>a</sup> | $NT \ge T$         | Nature > mesocosm       | Yes                    | Yes                             |
| Swimming ability              | NT > T             | n/a                     | n/a                    | No                              |
| Heat tolerance                | $NT \approx T$     | n/a                     | n/a                    | No                              |
| Net marine phenotype          | ?                  | Nature > mesocosm       | Yes                    | (Yes)                           |

<sup>a</sup> From Leggatt et al. (2014).

inconsistent relative phenotype for factors directly influencing fitness (i.e. survival, spawning success), making predictions on overall relative marine net fitness problematic (see Table 6). Further, genotype-by-environment interactions have been observed for size, body shape, and other fitness components of NT and T fish during the marine life stage. Consequently, predicting the success of GH transgenic fish in the wild, should they escape confinement, is difficult to do with a high level certainty with the limited available data from culture and semi-cultured conditions of the scale described here. For survival, the direction of relative success was not consistent among years when from Rs-negative parents indicating for some fitness components, whether GH transgenesis represents an advantage or disadvantage may depend on the influence of other unknown factors. Use of an alternate promoter for the GH transgene did not greatly influence most fitness components, although it had a strong effect on female fecundity measurements, indicating limited strain effect on T coho salmon at this life stage and environmental conditions. Our studies have found that while mesocosm rearing can minimize some effects of culture in coho salmon, creating contained environments that adequately mimic the full complexity and dynamic nature of ocean environments would be a significant undertaking. As well, the existence of genotype-by-environment interactions observed within the few marine rearing conditions explored here make predicting the performance of T coho salmon in natural environments difficult, with significant uncertainty. However, the large physical size of the mesocosm may more accurately predict phenotype of transgenic fish reared in large-scale commercial operations than small-scale research projects could. As well, escaped aquaculture transgenic fish could have a domesticated background, and overall consequences of a GH transgene may differ in different genetic backgrounds (see Devlin et al. 2001). Notwithstanding the above caveats, the current and previous data do not provide evidence that overall increased performance of GH transgenic relative to wild-type coho salmon would arise in the marine environment, although whether transgenic fish would be an a disadvantage relative to wild counterparts is not certain.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.aquaculture.2017.01.022.

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