POTENTIAL FOR ECOLOGICAL EFFECTS AND GENE FLOW RESULTING FROM GROWTH HORMONE TRANSGENIC ATLANTIC SALMON (SALMO SALAR) INTERACTIONS WITH WILD CONSPECIFICS

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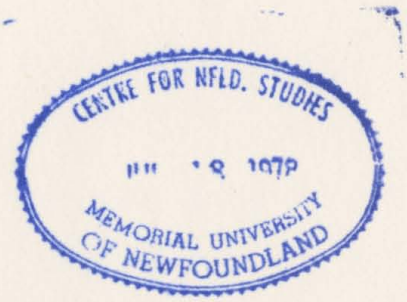
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POTENTIAL FOR ECOLOGICAL EFFECTS AND GENE FLOW RESULTING FROM GROWTH HORMONE TRANSGENIC ATLANTIC SALMON (*SALMO SALAR*) INTERACTIONS WITH WILD CONSPECIFICS

By

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Growth hormone (GH) transgenic Atlantic salmon (*Salmo salar*) exhibit tremendous growth rates under hatchery conditions. This phenotypic response has created interest within the aquaculture industry; however, possible escapee events have raised concerns regarding their potential ecological impacts. This thesis applied an eco-evolutionary approach to empirically assess the potential ecological effects of GH transgenic Atlantic salmon on non-transgenic intraspecific populations. Specifically, my goal was to explore the relative survival and reproductive success of GH transgenic and non-transgenic salmon under near-natural conditions. To accomplish this, key fitness-related traits were compared between GH transgenic and non-transgenic Atlantic salmon over periods of their life cycle when natural selection is typically intense. Specifically, this thesis focused on the young-of-the-year stream and the breeding periods.

Two studies (Chapters 2 and 3) compared fitness-related traits between transgenic and non-transgenic Atlantic salmon during early life history. Chapter two explored the potential differences in developmental rate and respiratory metabolism between transgenic and non-transgenic siblings at three early stages of life; the eyed-embryo, alevin (larval) and first-feeding fry (juvenile) stages. Chapter three explored the foraging behaviour and the growth and survival of transgenic and non-transgenic first-feeding fry reared under low feed, stream-like conditions. Collectively, the results of these chapters suggest that there is an ontogenetic delay in the phenotypic response induced by the transgene, such that biologically significant differences in fitness-related traits between GH transgenic and non-transgenic Atlantic salmon are minimal during this critical early life history period.

The final two studies (Chapters 4 and 5) compared fitness-related traits between transgenic and non-transgenic Atlantic salmon during the reproductive phase of the life cycle. The fourth chapter compared the breeding performance of growth hormone transgenic and non-transgenic Atlantic salmon males of both alternative reproductive phenotypes to test for the potential of the transgene to introgress into wild populations. The fifth chapter used populations of GH transgenic and non-transgenic Atlantic salmon siblings to elucidate the effects of growth on precocious parr maturation. Collectively, these data suggest that transgenic males may experience reduced reproductive success relative to non-transgenic individuals. However, the potential for the transgene to introgress into wild populations was demonstrated. The empirical contributions of this thesis inform decisions regarding the potential ecological impacts associated with GH transgenic Atlantic salmon.

Acknowledgements

This thesis has coincided with my late twenties. As is common with this period of life, I have undergone a great period of personal development and personal understanding. While the next sentence may sound very cliché, it is also very accurate. My years as a graduate student have helped me define who I am and the type of life I would like to lead. The graduate student experience has been an exceptional venue to undertake this phase in the journey of life. There are many people you have helped me either directly or indirectly and I could not possibly do them all justice here. However, there are a few individuals that I would like to mention.

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I am also very thankful to my supervisors, Ian Fleming and Garth Flecther, and my committee member, Kurt Gamperl. It has been a pleasure to work with three researchers that have been so successful within their respective areas of research. My exposure to their often dissimilar perspectives on biological questions has provided me with a well rounded lense with which to design and interpret biological research. This has further been aided by a dynamic group of student colleagues, who, like me, revel in mental exercises that challenge the status quo. Finally, I'd like to thank my family who, despite being supportive, have never quite understood what it is I have been doing out in Newfoundland all this time, or why. Hopefully it will make sense one day.

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List of Abbreviations and Symbols

GH – Growth Hormone

GH-IGF-I - Growth Hormone-Insulin-like Growth Factor I

DNA – Deoxyribonucleic Acid

c. – Circa

RNA – Ribonucleic Acid

mRNA – Messenger Ribonucleic Acid

cDNA – Complementary Deoxyribonucleic Acid

N – North

W – West

spp. – Species (plural)

L – Light

D – Dark

v. – Versus

n – Sample Size

M – Mass

unpubl. - Unpublished

e.g. – Example

i.e. - id est (in other words)

m – Meters

cm - Centimetres

mm - Millimetres

L_F – Fork Length

L - Length

G – Instantaneous Growth Rate

GLM – General Linear Model

Tukey HSD – Tukey Honest Significant Difference

P (-value) – Probability Value

LR - Logistic Regression

χ^2 – Chi-square

= - Equal Sign

× - Multiplication Symbol

S.E. – Standard Error

% - Percent

> - Greater Than

< - Less Than

g – Grams

± - Plus or Minus

et al. - et alii (and others)

USDA –United States Department of Agriculture

OSC – Ocean Sciences Centre

s – Seconds

HD – High Density

LD – Low Density

Ln - Natural Logarithm

Log – Logarithm

PCR - Polymerase Chain Reaction

°C – Degrees Celsius

min – Minutes

mg – Milligrams

O_2 – Oxygen

hr – Hour

ANOVA – Analysis of Variance

AIC - Akaike Information Criterion

$\Delta_i AIC_c$ - Akaike Information Criterion (corrected for small sample size)

Δ_i - Relative Performance (statistical model)

w_i - Akaike Weights

k - Represents the number of predictors in each model

MO_2 – Oxygen Consumption

CFI – Canadian Foundation for Innovation

NSERC – National Science and Engineering Research Council

dd – Degree Days

A – Alevin Characteristics

MS-222 - Tricaine Methane Sulphonate

PVC - Polyvinyl Chloride

PIT - Passive Integrated Transponder

HDD/DVD – Hard Disk Drive/Digital Video Disc

V – Volt or Test Statistic for Wilcoxon Signed Rank Test

µl – Microlitre

ng - Nanograms

mM – Millimolar

dNTP – Deoxynucleotide Triphosphate

µM – Micromolar

KCl – Potassium Chloride

Tris-HCl – Tris(hydroxymethyl)aminomethane-Hydrogen Chloride

$MgCl_2$ – Magnesium Chloride

U – Enzyme Unit

t – Test Statistic for Paired t-test

GnRH - Gonadotropin Releasing Hormone

T – Transgenic

NT – Non-transgenic

F – Test Statistic for models using the F distribution

Co-authorship Statement

The work described in this thesis was not an individual contribution. Co-author contribution for each chapter is described below:

Chapter1: Most of this introductory chapter will also serve as a book chapter in an upcoming Wiley-Blackwell publication. Darek Moreau was responsible for the background research and writing. Ian Fleming (co-author) and Garth Fletcher (book editor) provided editorial assistance and intellectual input.

Moreau, D.T.R., and I.A. Fleming. 2011. The potential ecological and genetic impacts of aquaculture biotechnologies: Eco-evolutionary considerations for managing the blue revolution. In. Aquaculture Biotechnology (ed. Fletcher, G.L. and Rise, M.L.). Wiley-Blackwell, Hoboken, NJ, USA. P. XX-XX.

Chapter 2: This chapter is published in the Journal of Fish Biology (Wiley-Blackwell). Darek Moreau was responsible for the concept development, experimental design, construction, data collection and analysis, and manuscript preparation. Ian Fleming (co-author) assisted with concept development and experimental design, and editorial and intellectual input. Garth Fletcher (co-author) provided editorial assistance and intellectual input. Joe Brown (co-author) assisted with concept development and provided intellectual input *a priori*.

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Chapter 3: Darek Moreau was responsible for the concept development, experimental design, construction, data collection and analysis, and manuscript preparation. Ian Fleming (co-author) assisted with concept development and experimental design, and provided editorial and intellectual input. Kurt Gamperl (co-author) assisted with

experimental design, editing and intellectual input. Garth Fletcher (co-author) provided editorial assistance and intellectual input.

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Chapter 1

General Introduction

1.1: Introduction

Human history has been inextricably shaped by the exploitation of captive animals. In conjunction with plant agriculture, captive-reared animals provided ancestral humans with an abundant and relatively stable food surplus, resulting in increased population densities and stationary communities. The advent of agriculture permitted the diversification of labour leading to rapid advances in technology and more sophisticated, hierarchical political systems that gradually came to dominate traditional hunter-gatherer societies. Diamond (2002) has suggested that increased human population sizes, a reduction (overexploitation) in large mammal populations, and technological advances leading to food storage were primary reasons for the initial transition to agriculture. A similar, contemporary transition appears to be accompanying the global decline in aquatic food resources, which has been brought about largely by increased human exploitation and damage to aquatic ecosystems. In parallel with our Holocene ancestors, we have escalated the development of new technologies for the exploitation of captive aquatic animals (i.e. aquaculture) to supplement capture-based fisheries.

Aquaculture, the cultivation of aquatic organisms, is a primary tool used to supplement depressed global fisheries and, in the future, may help conserve heavily exploited populations. Over the last fifty years, the so called "Blue Revolution" has transformed aquaculture from a localized agricultural activity producing less than one million tonnes of food annually to a global industry, producing nearly sixty million tonnes of food annually (FAO, 2010). Commercial aquaculture accounts for nearly half of all food fish production worldwide and that proportion is expected to continue to grow.

The rapid growth of aquaculture has been aided tremendously by the application of science. Indeed, biotechnology is partly responsible for the unprecedented growth in the volume and diversity of species involved in modern aquaculture production (Duarte et al. 2007). Advancing beyond breeding only the largest and healthiest fish, humans can now select individual fish with the aid of observations at the gene level (e.g. gene expression profiles, quantitative trait loci) or manipulate the genome directly (e.g. transgenesis). It is expected that biotechnologies will continue to aid the development of traits advantageous for production.

While aquaculture is developing in the biotechnology era, it is also developing in an era of environmental consciousness. There is now considerable pressure to minimize anthropogenic impacts on the environment and biodiversity, which is largely in response to the unprecedented environmental changes we are seeing globally. Commercial aquaculture has not escaped attention and associated environmental concerns have been a centre of debate (Costa-Pierce 2002; FAO 2010). From an eco-evolutionary perspective, perhaps the most complex concern involves the potential ecological and genetic impacts of aquaculture escapees on surrounding ecosystems.

There are numerous studies on the ecological and genetic effects of aquaculture escapees (reviewed in Utter and Epifanio 2002; Naylor et al. 2005; Thorstad et al. 2008). While the fundamental questions remain similar, it is widely recognized that modern biotechnologies, such as transgenesis, introduce new challenges to this already complex issue. This dissertation provides a thorough empirical assessment of the potential environmental effects of growth hormone (GH) transgenic Atlantic salmon (*Salmo salar*) entry into the wild; a leading candidate for commercialization. Prior to presenting the

empirical core of this thesis, I review the eco-evolutionary context and existing literature on the potential impacts of aquaculture escapees. Specifically, the current chapter focuses on the similarities and differences between technologies applied in aid of intentional selection (i.e. marker-assisted broodstock development) and those involving direct genetic change (i.e. transgenesis). I shall provide evidence suggesting that the ecological and genetic impacts of transgenic animals may be more difficult to predict than that of animals produced through marker-assisted broodstock development and introduce the empirical work contained in this dissertation.

1.2: Genetic background

Aquaculture biotechnologies, such as marker-assisted broodstock development, that do not involve direct genetic manipulation remain similar to traditional intentional selection (i.e. artificial selection based strictly on the phenotypic expression of traits). This is because the only process that differs is that by which parents are selected. In terms of evolutionary processes, they all involve multiple generations of selection that result in concurrent genomic changes and novel phenotypes under polygenic control (Mignon-Grasteau 2005; Jensen 2006). Thus, I will not distinguish between animals produced using such biotechnologies and those produced using traditional artificial selection and will refer to both as farmed strains.

Farmed species tend to have low genetic diversity within and among populations relative to that of wild populations. Genetic similarities appear to be the result of: 1) the low number of initial broodstock source populations and 2) the selection of similar traits over multiple generations. For example, Atlantic salmon (*Salmo salar*) breeding

programs have developed with local populations in several places, including Eastern Canada, Norway, and Scotland (Ferguson et al. 2007). For each region, however, programs have either begun by, or ended up concentrating on no more than a few strains, which are not likely representative of local population structure (Gjedrem et al. 1991; Gjøen and Bentsen 1997; Glebe 1998). Moreover, some cultured stocks are transplanted or hybridized with local cultured strains, further homogenizing strains used in the industry (Ferguson et al. 2007). Thus, the relatively low number of strains involved in broodstock development contributes to genetic similarities among artificially selected populations and genetic dissimilarities relative to wild populations.

The selective pressures on farmed populations are often very similar; that is selection for high growth and survival under like culture conditions. From an evolutionary perspective, parallel evolution among domesticated strains might be expected, whereby different lineages share genotypic similarities due to similar evolutionary pressures (Foster and Baker 2004; Schluter et al. 2004). Indeed, Roberge et al. (2006, 2008) demonstrated parallels between the transcription profiles of two leading Atlantic salmon aquaculture strains from Eastern Canada and one from Norway. Analogous patterns have also been observed in the transcriptome of closely related cotton congeners (*Gossypium barbadense*, *G. hirsutum*) in response to artificial selection (Chaudhary et al. 2008; Hovav et al. 2008). Evidence for convergent evolution also exists among *Drosophila subobscura* populations when exposed to laboratory environments over multiple generations (Matos et al. 2000; 2002). These findings suggest that both additive and non-additive genetic variation may converge among distinct populations experiencing similar pressures from artificial selection.

The phenotypic enhancement strategy of transgenesis is very different from intentional selection. Specific phenotypes are targeted by gene insertion and thus the traditional, polygenic, process of artificial selection can be bypassed. The most common method of creating transgenic fish is currently cytoplasmic microinjection, where multiple copies of the transgene are inserted into the cytoplasm of a recently fertilized egg and the transgene(s) are incorporated into the developing zygote's genome haphazardly (Du et al. 1992; Iyengar et al. 1996; Twyman 2005). Individuals expressing the desired phenotypic trait are crossed with wild-type fish. The transgenic offspring are then crossed with non-transgenic conspecifics over multiple generations, forming a stable transgenic line. Successful transgenesis can avoid the time and resources involved in an artificial selection program. Moreover, transgenesis may allow for the development of traits not attainable through selective breeding by adding genes that code for proteins not present within the host genome (e.g. carbohydrate metabolism or freeze resistance; Fletcher et al. 1994). As a result, a transgenic broodstock can be developed in the absence of intentional selection. Therefore, transgenic broodstocks may be more similar genetically (i.e. with exception of the transgene(s)) to their wild source populations than are farmed broodstocks, that have been selected for production traits for generations. This may also reduce the negative fitness consequences for transgenic animals in the wild. The fitness consequences, however, will depend on how the transgene(s) interacts with the organism's existing genetic architecture, with which it has not coevolved (discussed below).

1.3: Phenotypic expression

Phenotypic expression among transgenic organisms has been shown to vary by integration position, copy number, construct, strain, and species (Twyman 2005; Gong et al. 2007; Nam et al. 2007). Researchers developing transgenic organisms have noted substantial phenotypic differences between individuals successfully integrating the transgene within the same population. These latter differences are due to epistasis resulting from molecular level variation during the integration process. The genomic location of transgene integration during initial insertion is the major cause of this variation; known as position/integration effects (Iyengar et al. 1996; Twyman 2005). Essentially, epistatic interactions between the transgene and neighbouring genes affect the activity of the local molecular region, which may influence the phenotype. Another cause of molecular level variation is the number of transgene copies that integrate into the host genome; known as dosage effects (Twyman 2005). Copy number may affect the amount of protein produced by the transgene loci and, consequently, the overall phenotype. These sources of phenotypic variation are difficult to predict *a priori* and may have fitness consequences. Continued efforts to develop or adapt more predictable, efficient and practical gene transfer methods for fish and shellfish species could be an asset for the aquaculture industry and the risk assessment process (Nam et al. 2007).

Genetic recombination may rearrange transgenes or their location, as with any endogenous gene sequence over time. In some countries, federal legislation requires the demonstration of genetic stability in a transgenic strain for several generations (CEPA 1999; USFDA 2009). Therefore, genetic stability of the transgenic loci and the targeted phenotype will need to be maintained for several generations to commercialize

aquaculture biotechnologies (Yaskowiak et al. 2006). However, a few stable generations does not preclude recombination at the transgene loci in future generations. The effect of recombination on the location and structure of the transgene loci cannot be predicted nor can the resulting phenotype or effect on fitness. Furthermore, transgenes are invariably designed to behave as genes of major effect; genes that influence the phenotype more so than most genes. Therefore, recombination at the transgene loci may result in a greater influence on fitness-related traits than recombination at most other loci. There is some evidence of transgene instability among some populations of mud loach (*Misgurnus mizolepis*) and carp (*Labeo rohita*; Nam et al. 1999; Kim et al. 2004; Venugopal et al. 2004). Thus, position effects caused by genetic recombination may change the phenotype of a transgenic line between generations. This is complicated further when we consider the effect of the background genotype on the phenotypic effects induced by the transgene.

The phenotypic response to a stable transgene construct can vary considerably within and between populations and species. Aside from differences caused by construct design, pleiotropy induced by a particular transgene is affected by the composition of, and interaction among the genes of the receiving animal. The differential response among species to a specified transgene construct is well documented (Nam et al. 2008). There are also examples of this phenomenon among different populations within a species. For example, Devlin et al. (2001) found that the growth response of wild and farmed rainbow trout (*Oncorhynchus mykiss*) populations to transgene (OnMTGH1) introduction differed. The growth response of the wild strain was far greater than that of the farmed strain, which had been selected for rapid growth over several generations. The non-transgenic farm strain, however, outgrew the transgenic wild strain. These results might have been

influenced by position or dosage effects. However, a recent study by Neregård et al. (2008a) using GH implants to compare the growth responses of two wild and one farmed strain of Atlantic salmon (*Salmo salar*) found similar results (Table 1.2). This confirmed that genetic background can be a key factor in the degree of response to supplementation, whether through transgenesis or implantation.

In summary, intentional selection in aquaculture broodstocks leads to genetic homogeneity brought about by the use of few strains and subsequent parallel evolution. Moreover, intentional selection allows genes, and consequently, phenotypic traits to co-evolve over time. Conversely, phenotypes of different transgenic lines can vary due to transgene position and/or dosage effects or differences in the genetic background of the parent strains. This potential for a higher degree of dissimilarity suggests that the evolutionary pressure on wild populations from interbreeding with transgenic animals may induce a greater array of pleiotropic effects than that observed from interbreeding with farmed animals. However, over time, transgenic strains may experience intentional selection such that fitness reductions are similar to farmed strains. Thus, at least in the absence of intentional selection, the ecological and genetic impacts of transgenic animals may be more difficult to predict than those caused by animals produced through traditional intentional selection.

To illustrate the above, I shall compare what is known about potential ecological and genetic effects caused by aquaculture escapees originating from traditional breeding programs with those originating from transgenic manipulation. Despite the diversity of species used in aquaculture, the focus will be on salmonid fishes because of an unfortunate paucity of data addressing ecological and genetic effects of other species.

Moreover, salmonids are one of few taxon where fitness-related consequences of transgenesis have been investigated and are also the focus of this dissertation.

1.4: Domestication selection and divergence

Aquaculture strains are genetically and phenotypically distinct from their source wild populations due to the process of domestication selection (e.g., Utter and Epifanio 2002; Ferguson et al. 2007). Domestication selection refers to the different forces affecting genetic change in captive-reared versus wild populations. This genetic change may occur for a number of direct and indirect reasons. Direct genetic change or intentional domestication selection, refers to selective breeding for desired traits, such as those targeted in traditional aquaculture practices. Gene transfer biotechnologies are also a direct method of genetic change; however, as previously described, they may differ in fundamental ways.

Domestication selection can also influence both farmed and transgenic animals through indirect genetic change. That is, rearing a population in captivity with no guided selection can lead to divergence from the source population. Unintentional selection may manifest itself through two, most often concurrent, routes. First, changes may result from genetic drift due to an inadvertent sampling bias in the wild founder population of a captive-bred line; known as a founder effect (Frankham et al. 2002; Allendorf and Luikart 2007). Second, changes may result from abiotic and biotic differences between wild and captive rearing environments (Price 1999; Einum and Fleming 2001; Huntingford 2004). The result is the potential for indirect selection of traits that increase fitness in the captive environment (Encomio et al. 2005; Shoemaker et al. 2006) or, conversely, relaxed

selection on traits that decrease fitness in the wild (Fleming and Gross 1989; 1993; Waples 1999). Unintentional selection is hypothesized to correlate with the number of generations in captivity (Araki et al. 2007; Caroffino et al. 2008). So long as the environment is held constant, farmed and transgenic populations should experience similar unintentional selective pressures; unless there is some sort of unique interaction between the transgene and the environment.

The effects of domestication selection on the genetic and phenotypic characteristics of aquaculture animals lead to various potential environmental impacts upon release into nature. Figure 1.1 summarizes the mechanisms responsible for such impacts within four categories; direct ecological effects, indirect ecological effects, direct genetic effects and indirect genetic effects. Most of these potential impacts depend on whether the cultured animals are entering habitat occupied by populations with which they can interbreed. As such, I shall discuss the effects in non-native (exotic) and native habitats separately.

1.5: Effects in Non-native Habitats

The potential genetic effects of farmed and transgenic animals are unlikely to differ fundamentally when invading non-native habitats that lack genetically compatible heterospecific populations. This is because the complex genetic effects associated with species interbreeding and genes introgressing are absent. Interbreeding and introgression are not synonymous. Interbreeding refers only to the act of sexual reproduction between two discrete populations, while introgression refers to the successful transfer of genes from one gene pool to another by interbreeding (Frankham et al. 2002; Allendorf and

Luikart 2007). We recognize that exotic habitats may contain closely related species with which an invading population can introgress (reviewed in Allendorf and Leary 2001; Allendorf and Luikart 2007); however hybridization usually occurs at low rates and results in infertile offspring or offspring with greatly reduced reproductive success. Should hybridization result in the introgression of a transgene into a native species, then the effects are likely to become similar to those that occur in native habitats (see below). Here, however, I focus on effects in the absence of introgression.

The effects on ecology and biodiversity resulting from species invasions have been reported for decades (e.g. Levine and D'Antonio 1999; Simberloff and Von Holle 1999; De Silva et al. 2006). The establishment of an exotic species depends on the frequency and magnitude of the invasion, the relative fitness of the colonizer and the vulnerability of the ecosystem (Ruesink 2005; Olden et al. 2006). An aquatic species that is farmed extensively is likely to have ample opportunity and sufficient numbers to invade host ecosystems due to the potential for recurring escape events and the scale of farming (e.g. Fleming et al. 2000, Bekkevold et al. 2006; Thorstad et al. 2008). Similarly, most aquacultured species are reared in environments where their ability to tolerate local abiotic factors is well understood. Casal (2006) reported a list of the top eighteen invasive finfish species reported to have negative effects on local ecosystems, of which, thirteen (72%) have been used in aquaculture.

Many of the ecological effects of escaped aquaculture animals are common to both non-native and native habitats (see below). However, there are also many ecosystem-level abiotic and biotic indirect effects that are more likely to occur in non-native habitats. Indirect effects refer to changes mediated through a third component

(abiotic or biotic) of the ecosystem. Such indirect mechanisms include habitat modification, apparent facilitation, apparent competition and the trophic cascade effect (Goldschmidt et al. 1993; Shurin et al. 2002; White et al. 2006). Indirect effects are more likely in exotic habitats due to the potential for exotic species to interact with the environment in a novel manner and, thus, influence community structure and function.

The genetic impacts of aquaculture animals entering non-native habitats can be mediated indirectly through ecological interactions. Indirect genetic effects refer to changes in local heterospecific populations. Such interactions may change the selective pressures experienced by wild populations, resulting in genotypic and phenotypic adaptations (Waples 1991). In contrast, such changes may manifest themselves through negative interactions resulting in a reduction in population size and genetic diversity.

1.6: Effects within Native Habitats

1.6.1: Competition

In terms of risk assessment, the effects of farmed and transgenic animals interacting with wild conspecific or heterospecific populations with which they can interbreed is likely the most critical scenario. This is because the consequences of direct genetic mechanisms, such as an invasion of farmed fish gene complexes or transgenes, are exceptionally difficult to predict (Devlin et al. 2006, 2007; Hindar et al. 2006; Kapuscinski et al. 2007).

Ecological effects can be caused by direct mechanisms, such as disease, predation, and interference competition, or indirect mechanisms, such as exploitative competition (Weir and Grant 2005). In salmonids, competition between farmed and wild conspecifics

is well described. Salmonids are interference competitors both as juveniles, when competing for foraging territories, and as adults, when competing for breeding opportunities. Lab-based studies generally report increased juvenile aggression and poor reproductive behaviours in farmed individuals (Einum and Fleming 2001; Fleming and Petersson 2001; Weir and Grant 2005). These distinct patterns of competition indicate substantial resource overlap. Therefore, ecological effects may depend on their respective densities, their relative competitive abilities and the carrying capacity of the ecosystem (Weber and Fausch 2003). However, it should be noted that gene transfer modifications, such as, an addition of a gene supporting carbohydrate metabolism (Pitkänen et al. 1999) could affect prey choice and large scale foraging patterns. In fact, there is some evidence of small scale differences between GH transgenic and non-transgenic coho salmon foraging patterns (Sundström et al. 2007).

1.6.2: Interbreeding and introgression

The differences observed in the competitive ability of farmed and wild salmonids suggest differences in survival and reproductive success. Despite lower survival of farmed juveniles, competitive displacement of wild individuals has been observed in Atlantic salmon, indicating potentially negative ecological effects (Fleming et al. 2000; McGinnity 1997; McGinnity et al. 2003). However, adult farmed strains show poor rates of return to the spawning grounds (McGinnity et al. 2003) and differences in spawning behaviour that correlate expectedly with reduced reproductive success (Fleming et al. 1996, 2000; Weir et al. 2004). Reduced reproductive success, however, appears not to carry over to males that mature precociously in fresh water as parr, having never been to

the ocean. Males of this alternative life history tactic, which are a fraction of the size of the anadromous males (i.e. males that have been to sea), may breed successfully by 'sneaking' fertilizations (reviewed by Fleming and Reynolds 2004). Farmed males expressing this alternative reproductive phenotype can compete successfully with their wild counterparts for breeding opportunities, leading to equal or even superior reproductive success (Garant et al. 2003; Weir et al. 2005). Thus, the available evidence from salmonid fishes demonstrates that competition can result in the reduced fitness of wild strains. Moreover, farmed individuals have poor lifetime reproductive success in the wild, but can contribute to subsequent generations and may therefore influence the fitness of wild populations (Fleming et al. 2000; McGinnity et al. 2003; Hindar et al. 2006).

The interbreeding of farmed and wild populations gives rise to concerns about the potential negative effects of altering wild gene pools via introgression. Such concerns are based on the evidence suggesting that genetic and phenotypic differentiation between salmonid populations has adaptive significance (Garcia de Leaniz et al. 2007; Carlson and Seamons 2008) and that this would be threatened by introgression.

Captively reared populations usually have lower genetic diversity because they are often closed and have reduced effective population sizes relative to those in the wild (Frankham 2008). This pattern has been observed repeatedly in aquaculture broodstocks (Exadactylos et al. 1999; Skaala et al. 2005 Frost et al. 2006). Significant one-way gene flow due to escapees could shift the genetic composition of the wild populations towards that of the cultured broodstock (Fleming et al. 2000; McGinnity et al. 2003; Hindar et al. 2006). Subsequent reductions in the genetic diversity of wild populations would make them more vulnerable to environmental change and, in extreme cases, could lead to

extinction (Frankham et al. 2002; Allendorf and Luikart 2007; Carlson and Seamons 2008).

Species where there is low gene flow between populations and a high degree of local adaptation, such as salmonids, are particularly vulnerable to outbreeding depression. Outbreeding depression refers to combining alleles from different populations adapted to different environmental conditions, resulting in the reduced fitness of the hybrid population (Wolf et al. 2000; Frankham et al. 2002; Allendorf and Luikart 2007). Further harmful effects may occur if the interbreeding populations disrupt co-adapted gene complexes upon recombination in subsequent generations. Co-adapted gene complexes are sets of loci that undergo fitness-related epistatic interactions (Wolf et al. 2000; Frankham et al. 2002; Allendorf and Luikart 2007). Consequently, interbreeding between a wild and a captive-reared population may result in outbreeding depression in the hybrid progeny and the breakdown of co-adapted gene complexes in subsequent generations. There is fairly consistent empirical evidence of outbreeding depression caused from the interbreeding of wild and farmed or wild and non-local salmonid populations (reviewed in Ferguson et al. 2007; Garcia de Leaniz et al. 2007; Fraser 2008).

1.7: Case study of salmonid growth enhancement

The goal of this thesis is to provide empirical information on the potential environmental and genetic effects of growth hormone (GH) transgenic Atlantic salmon (*Salmo salar*) entry into the wild. The preceding discussion set the eco-evolutionary context within which the issue of aquaculture biotechnologies may be assessed. The

following discussion provides information on the state of current empirical knowledge and a commentary on the literature related directly to the focus of this dissertation.

The salmonid growth-enhancement literature consists of studies investigating the fitness-related traits of growth-selected aquaculture (farmed) fish, growth hormone (GH) transgenic fish and fish administered exogenous GH. The latter group has been utilized extensively in the last 15 years as a proxy for transgenic individuals. Typically, this method relies on a continuous, slow-release bovine GH formulation (Posilac®; Monsanto Company; St. Louis, USA) that is implanted into the peritoneal cavity (McLean et al. 1997). The appeal of this substitute is that it allows field comparisons of treated and untreated wild fish; an option not available for transgenic animals. Field experiments allow for the complexity of nature, which cannot be mimicked fully in laboratory environments. Furthermore, the use of wild fish eliminates the potentially confounding effects of the captive rearing environment on phenotypic development. Molecular-level complications associated with the development of transgenic lines, such as position or dosage effects, also need not be a concern.

The obvious limitation of exogenous GH administration is that it is not a complete physiological equivalent of the endogenous GH production induced by a transgene. Scientific understanding of the effects of the growth hormone-insulin-like growth factor I system (GH-IGF-I) on several aspects of salmonid physiology remains uncertain (Björnsson 1997; Björnsson et al. 2002). Moreover, the endocrinological effects of a GH implant compared to a transgene are not known. This may be particularly significant when we consider the inherent complexity of biological cycles. Specifically, the effects of seasonality and age on GH-IGF-I induced phenotypic variation. With this caveat in

mind, the effects of exogenous GH administration appear to stimulate similar phenotypic changes as that seen with transgenesis (Tables 1.1 and 1.2). This makes it a useful tool when studying life history periods where we expect animals are under the influence of sustained GH production, such as that of juvenile salmonids in the spring and summer (Björnsson 1997; Fleming et al. 2002). Therefore, I shall consider exogenous GH administration as analogous to GH transgenesis for the purposes of this chapter and refer to them collectively as GH-enhanced fishes.

1.7.1: Comparing growth-selected and GH-enhanced phenotypes

A range of similar experiments have been performed on growth selected (farmed) and GH-enhanced (GH transgenic and GH treated) salmonid fishes. Overall, the differences observed for fitness-related traits of farmed and GH-enhanced fishes relative to wild-type individuals are quite similar in hatchery-type environments (Table 1.1). These data generally indicate increased growth potential and feeding motivation, reductions in antipredator behaviour and differences in various physiological correlates relative to wild salmonids.

These similar patterns may correspond to equivalent processes influencing farmed and GH-enhanced animals. A principal phenotypic change resulting from GH transgenesis or exogenous treatment is, unequivocally, increased GH production. The principal phenotype targeted in salmonid aquaculture is growth and farmed salmonids have been associated with an increase in circulating growth hormone during the juvenile growth phase of their life history (Fleming et al. 2002; Devlin et al. 2009). Thus, similar

pluripotent effects on fitness-related traits may be intimately associated with the changes in endocrine growth regulation in both farmed and GH-enhanced salmonids.

Upon comparison of farmed and GH-enhanced individuals relative to wild individuals in more complex environments, variability begins to emerge (Table 1.2). In natural and near-natural experimental streams, evidence of increased growth and decreased antipredator behaviour is consistent. However, the direction of fitness-related traits such as survival, reproductive success, energy use and competitive behaviour is inconsistent. Trait divergence between farmed and GH-enhanced individuals relative to wild individuals may reflect genetic and/or environmental differences. For comparison among studies, it is important to identify and, where applicable, control for such sources of trait differentiation. Otherwise it is difficult to infer whether a genetic predisposition is responsible for trait differences or, alternatively, if differences reflect a plastic response to unique environments. This is not always easy to accomplish because, for example, research cannot be conducted with transgenic organisms in the wild. Therefore, unlike farmed strains, comparing fitness-related traits between wild-reared transgenic and non-transgenic strains may not be possible. In one study, Bessey et al. (2004) showed similar patterns of reproductive trait divergence between GH transgenic coho salmon and wild individuals as has been observed between farmed and wild individuals; suggesting that for these traits, rearing history may be a more critical factor than transgenesis (Table 1.2).

When GH-enhanced individuals are compared to wild-type individuals with the same background genotype and rearing history, under natural or near-natural laboratory conditions, many fitness-related traits appear unaffected by treatment (Table 1.2). For example, natural breeding experiments show no differences in courtship behaviour and

reproductive success between GH-enhanced and -unenhanced individuals when they share the same background genotype and rearing history (Bessey et al. 2004; Sundt-Hansen 2008). Similarly, studies measuring traits in juvenile parr (> 2g) under the threat of predation consistently find increases in growth, but no differences in survival between GH-enhanced and wild individuals (Johnsson et al. 1999; Johnsson et al. 2000; Johnsson and Björnsson 2001; Sundström et al. 2007; Sundt-Hansen 2008, Sundt-Hansen et al. 2009).

In terms of fitness, many studies on juvenile fish fail to capture a significant period of early life history; the onset of exogenous feeding. The transition to exogenous food resources is a critical period of survival for young fry, where individuals confront an environment saturated with competitors and predators; resulting in high rates of mortality (Elliot 1994; Einum and Fleming 2000; Kennedy et al. 2008). Few studies have measured fitness-related traits in farmed and GH-enhanced fry (< 2 g). In near-natural laboratory conditions, Sundström et al. (2004; 2005) showed that GH transgenic coho fry experience increased predator-induced mortality, reduced dispersal and equal growth under moderate to high feed levels relative to wild-type fry. However, decreased growth was observed under low feed levels. Conversely, a recent mark-recapture study, where GH-implanted Atlantic salmon fry were released into a natural stream found no differences in survival or dispersal, and reduced growth of GH-implanted relative to control fry (Sundt-Hansen et al. 2008). It is unclear whether these inconsistencies reflect phenotypic differences resulting from intrinsic variation between species, enhancement methods (i.e. transgenesis versus implantation) or experimental environments (e.g. relative levels of predation and competition). It is, however, likely a combination of such

factors. Nonetheless, when genetic background and rearing environment are controlled, differences in fitness-related traits appear less likely between GH-enhanced and unenhanced individuals compared under near-natural conditions. Furthermore, there is some evidence to suggest that high levels of predation and foraging competition can reduce survival and growth in GH-enhanced individuals at the critical, first-feeding stage.

The above discussion highlights a principal reason why transgenic animals may have a lower element of predictability than farmed animals with respect to risk assessment. Farmed individuals have experienced generations of intentional selection for production-relevant traits that are likely controlled by many genes of small effect. This results in genetic divergence from wild populations. Evidence of consistent, negative fitness consequences have been demonstrated between farmed and wild salmon populations. Due to low strain variation and similar selective pressures among aquaculture broodstocks, variability in the consequences of recombination is principally caused by the response of the receiving population to the breakdown of co-adapted polygenic complexes.

In contrast to aquaculture strains, transgenesis can induce growth in fishes of unselected and diverse genetic backgrounds under controlled environments. As genes of major effect, transgenes can have a greater influence on phenotypic expression than most other genes. When growth-enhanced fish are compared to wild-type individuals of similar genetic background, trait differences become evident under laboratory conditions. However, under natural conditions, fitness-related trait differences are variable, weak or nonexistent; indicative of gene by environment interactions (Devlin et al. 2004; 2006; 2007; Sundström et al. 2007b). Evidently, it appears the background genome can

moderate the influence of a transgene on the phenotype in response to the environment. This suggests that invading transgenic individuals can experience greater fitness relative to that of invading farmed strains because they have not undergone intentional selection on the whole genome and, therefore, may have a greater capacity to exhibit a plastic response suitable to wild environments. However, transgenic strains that have undergone intentional selection may experience reduced fitness similar to, or greater than, that of farmed strains. The evidence supports this contention, with transgenic strains showing less predictable phenotypic responses to naturalized environments. Consequently, this may also lead to more variable rates of transgene integration into receiving populations. Additionally, the phenotypic effects of transgene position/dosage differences among transgenic lines complicate the ability to accurately conduct risk assessments.

1.8: Thesis Outline

Growth hormone transgenic Atlantic salmon (gene construct: opAFP-GHc2; transgene: EO-1α; Yaskowiak et al. 2006) are the first genetically modified animal to undergo extensive regulatory assessment and, thus, stand to be the first approved for commercial food production. However, there has been little empirical work addressing the potential ecological and genetic effects of this transgenic line. As the above discussion attests, ecological risk assessments require empirical data collected on the specific genetically modified organism under review. The goal of this thesis was to provide such information by comparing key fitness-related traits between GH transgenic and non-transgenic Atlantic salmon.

The life cycle of Atlantic salmon is complex, thus I have focused my efforts on periods of strong selection; specifically, the young-of-the-year stream period (Chapters 2 and 3) and the breeding period (Chapters 4 and 5). First-feeding stream salmonid fry experience intense competition for food and space that can contribute to low (< 20%) rates of survival during this period (Elliott 1994; Einum and Fleming 2000; Kennedy et al. 2008). Thus, any pleiotropic effects on physiological and developmental processes or foraging and anti-predator behaviour during early ontogeny could severely impact the viability of the transgene in nature. Chapters 2 and 3 aim to elucidate such potential pleiotropic effects. The second chapter compared the developmental rate and respiratory metabolism of GH transgenic and non-transgenic full sibling Atlantic salmon during early ontogeny. Specifically, the routine metabolism of GH transgenic and non-transgenic siblings was quantified at three early stages of ontogeny; eyed-eggs, alevins (larvae) and first-feeding fry. Furthermore, this chapter tested for differences in hatch time and, near exogenous feeding, alevin mass, length and the amount of yolk remaining within (transgenic versus non-transgenic) and among families. The third chapter examined the relative competitive ability and performance of first-feeding GH transgenic and non-transgenic Atlantic salmon fry under low food conditions. The first part of chapter three consisted of pair-wise behavioural trials that investigated the relationships of dominance and aggression between transgenic and non-transgenic first-feeding fry competing for food. The second part of Chapter 3 compared the effect of density and low food conditions on the survival and growth of transgenic and non-transgenic fry in semi-natural stream microcosms.

The genetic effects associated with interbreeding and introgression are among the greatest concerns associated with the introduction of transgenic organisms into nature (Muir and Howard 2002; Howard et al. 2004; Devlin et al. 2006). Moreover, age at sexual maturity is considered a key fitness-related trait influencing the invasion of foreign genes into wild populations because early maturation reduces generation time and increases the probability of survival to reproduction (Muir and Howard 2001; Garant et al. 2008). The fourth chapter used mixed populations of GH transgenic and non-transgenic Atlantic salmon siblings to elucidate the effects of growth and energy accumulation on precocious parr maturation. Specifically, mixed tanks of yearling (0+) parr were exposed to two different feed levels, and, subsequently, in their second year (1+) exposed to a single feed level, to test the effect of growth and energy accumulation on the precocious maturation of transgenic and non-transgenic parr. The fifth chapter compared the breeding performance of growth hormone transgenic and wild-type Atlantic salmon males of both alternative reproductive phenotypes to test for the potential of the transgene to introgress into wild populations. We conducted two separate experiments in a naturalised stream mesocosm. The first experiment assessed the ability of farmed, first-generation transgenic males to contribute reproductively by quantifying the breeding behaviour and participation of captively-reared, anadromous transgenic and wild males. The second experiment assessed the ability of transgenic fish to contribute reproductively as precocial parr by quantifying the breeding behaviour, performance and reproductive success of captively-reared, transgenic and wild-type precocial parr. The results of these empirical chapters are discussed within the broader context of the ecological risk assessment of transgenic organisms.

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Table 1.1. General patterns of fitness-related trait divergence between wild and growth selected (farmed), growth hormone (GH) transgenic or GH treated salmonid populations compared in artificial laboratory tanks or aquaria. Trait direction reflects a comparison of the growth enhanced relative to wild-type fishes. References provided are not exhaustive and are intended merely as examples.

Phenotypic trait	**Direction**	**References**		
		Growth selected	GH transgenic	GH treated
Behavioural				
Dominance/Aggression*	Increased	Einum and Fleming 1997; Fleming and Einum 1997; Metcalfe et al. 2003	Devlin et al. 1999; Devlin et al. 2004b	Johnsson and Björnsson 1994; Jönsson et al. 1998; Neregård et al. 2008b
Antipredator behaviour	Decreased	Fleming and Einum 1997; Johnsson et al. 2001; Reinhardt 2001; Yamamoto and Reinhardt 2003; Tymchuk et al. 2006	Abrahams and Sutterlin 1999; Sundström et al. 2003; Sundström et al. 2007a	Jönsson et al. 1996
Spatial movement	Increased	n/a	Sundström et al. 2007a	Herbert et al. 2001; Jönsson et al. 2003; Johansson et al. 2004; Johansson et al. 2005;
Feeding Motivation/Appetite	Increased	Thodesen et al. 1999; Reinhardt 2001;Sanchez et al. 2001; Yamamoto and Reinhardt 2003; Biro et al. 2006	Devlin et al. 1999; Sundström et al. 2003; Sundström et al. 2004a Tymchuk et al. 2005; Lõhmus et al. 2008	Johnsson and Björnsson 1994; Jönsson et al. 1996; Johansson et al. 2005;

Table 1.1. Continued.

Phenotypic trait	Direction	References		
		Growth selected	GH transgenic	GH treated
Physiological				
Growth rate	Increased	Gjøen and Bentsen 1997; Glebe 1998; Thodesen et al. 1999 Gjedrem 2000; Fleming et al. 2002; Handeland et al. 2003a,b; Biro et al. 2006; Tymchuk et al. 2006	Du et al. 1992; Cook et al. 2000a,b; Devlin et al. 2001; Leggatt et al. 2003; Sundström et al. 2007b; Lõhmus et al. 2010a,b	Johnsson and Björnsson 1994; Neregård et al. 2008a; Sundt-Hansen et al. 2008
Stress response [Ψ]	Decreased	Lepage et al. 2000; Johnsson et al. 2001;	n/a	n/a
Metabolic rate	Increased	n/a	Stevens et al. 1998; Cook et al. 2000b,c; Lee et al. 2003; Leggatt et al. 2003; Deitch et al. 2006	Seddiki et al. 1995,1996
Swimming performance	Decreased	Enders et al. 2004	Farrell et al. 1997; Lee et al. 2003; Deitch et al. 2006	n/a
Hypoxia tolerance	Decreased	n/a	Sundt-Hansen et al. 2007	n/a

Table 1.1. Continued.

Phenotypic trait	Direction	References		
		Growth selected	GH transgenic	GH treated
Physiological				
Developmental rate	Increased	n/a	Devlin et al. 2004a; Lõhmus et al. 2010b	n/a

* Aggression and dominance are not always increased (Mork et al. 1999; Yamamoto and Reinhardt 2003). See Huntingford (2004) for hypotheses as to why this inconsistency is observed.

Ψ Jhingan et al. 2003 found similar stress responses to heat shock in GH transgenic and non-transgenic coho salmon.

Table 1.2. General patterns of fitness-related trait divergence between wild and growth selected (farmed), growth hormone (GH) transgenic or GH treated salmonid populations compared in natural or near-natural laboratory environments. Trait direction reflects a comparison of the growth enhanced relative to wild-type fishes. Each numerical value in the direction column represents one measurement from a single treatment within a given study. Thus, some studies contribute multiple values to the direction column. References provided are not exhaustive and are intended merely as examples.

Modification	Trait	Direction			References	Comments
		>	=	<		
GH selected	Survival	3	5	8	Fleming et al. 1996; Einum and Fleming 1997; Fleming and Einum 1997; McGinnity et al. 1997; Fleming et al. 2000; McGinnity et al. 2003; Biro et al. 2004 Weir et al. 2004; Weir et al. 2005; Biro et al. 2006	Biro et al. 2004, 2006: Observed increased survival when predation was absent or low. Only studies to observe increased survival. Three studies show decreased survival during breeding period.
GH treated	Survival	0	4	1	Johnsson et al. 1999; Johnsson et al. 2000; Johnsson and Björnsson 2001; Sundt-Hansen et al. 2008	Sundt-Hansen et al. 2008: Decreased survival in mature Atlantic salmon parr during breeding season.
GH transgenic	Survival	0	3	5	Sundström et al. 2004b; Sundström et al. 2005	Both studies with first-feeding fry. Demonstrate high susceptibility to predation and low food levels.
GH selected	Growth	8	1	1	Einum and Fleming 1997; Fleming and Einum 1997; McGinnity et al. 1997; Fleming et al. 2000; McGinnity et al. 2003; Biro et al. 2004; Biro et al. 2006; Tymchuk et al. 2006	
GH treated	Growth	7	0	1	Johnsson et al. 1996; Johnsson et al. 1999; Johnsson et al. 2000; Johnsson and Björnsson 2001; Martin-Smith et al. 2004; Sundt-Hansen et al. 2008; Sundt-Hansen et al. 2009	Martin-Smith et al. 2004: Increased only in summer. Sundt-Hansen et al. 2008: Decrease observed is the only study on first feeding fry.

Table 1.2. Continued.

Modification	Trait	Direction			References	Comments
		>	=	<		
GH transgenic	Growth	6	8	4	Sundström et al. 2004b; Sundström et al. 2005; Sundström et al. 2007b; Sundström et al. 2009	Sundström et al. 2004b, 2005: Studies with first-feeding fry. Results varied by level of predation, food availability and rearing environment.
GH selected	Antipredator behaviour	0	0	2	Biro et al. 2004; Biro et al. 2006	Inferred.
GH treated	Antipredator behaviour	0	0	1	Johnsson et al. 1996	
GH transgenic	Antipredator behaviour	0	0	2	Sundström et al. 2004b; Sundström et al. 2005	Inferred.
GH transgenic	Predation rate	3	2	0	Sundström et al. 2007b; Sundström et al. 2009	
GH treated	Appetite	0	1	0	Johnsson et al. 1996	
GH selected	Competitive behaviour	0	2	4	Fleming and Einum 1997; Fleming et al. 1996; Fleming et al. 2000; Weir et al. 2004	Consistent disadvantage for adult breeding males but not females or mature male parr.
GH treated	Competitive behaviour	0	2	0	Johnsson et al. 1996; Martin-Smith et al. 2004;	
GH transgenic	Competitive behaviour	0	0	1	Bessey et al. 2004	Groups experienced different rearing environments.

Table 1.2. Continued.

Modification	Trait	Direction			References	Comments
		>	=	<		
GH transgenic	Time to redd emergence	0	0	1	Sundström et al. 2005	
GH selected	Dispersal/ spatial movement	0	0	5	Nagata et al. 1994; McGinnity et al. 1997; Fleming et al. 2000; McGinnity et al. 2003	Reduced dispersal inferred to be example of competitive displacement.
GH treated	Dispersal/ spatial movement	2	3	0	Martin-Smith et al. 2004; Sundt-Hansen et al. 2009	Increased spatial movement but no effect on dispersal or diel movement patterns.
GH transgenic	Dispersal/ spatial movement	0	0	2	Sundström et al. 2005; Sundström et al. 2007a	
GH treated	Energy reserves	0	3	2	Johnsson et al. 1999; Johnsson et al. 2000; Neregård et al. 2008a; Sundt-Hansen et al. 2008; Sundt-Hansen et al. 2009	Observed differences most evident in summer and early fall.
GH selected	Reproductive/courtship behaviour	1	1	5	Fleming et al. 1996; Fleming et al. 2000; Garant et al. 2003; Weir et al. 2004; Weir et al. 2005	Weir et al. 2004: Farmed adult Atlantic salmon males make more spawning attempts but often fail to release sperm.
GH treated	Reproductive/courtship behaviour	0	1	0	Sundt-Hansen 2008	Mature Atlantic salmon male parr.
GH transgenic	Reproductive/courtship behaviour	0	1	1	Bessey et al. 2004	Divergence found when groups had different rearing environments.

Table 1.2. Continued.

Modification	Trait	Direction			References	Comments
		>	=	<		
GH selected	Reproductive success	1	1	4	Fleming et al. 1996; Fleming et al. 2000; Garant et al. 2003; Weir et al. 2005	Increased and equal reproductive success observed with mature male Atlantic salmon parr.
GH treated	Reproductive success	0	1	0	Sundt-Hansen 2008	Mature Atlantic salmon male parr.
GH transgenic	Reproductive success	0	1	1	Bessey et al. 2004	Divergence found when groups had different rearing environments.

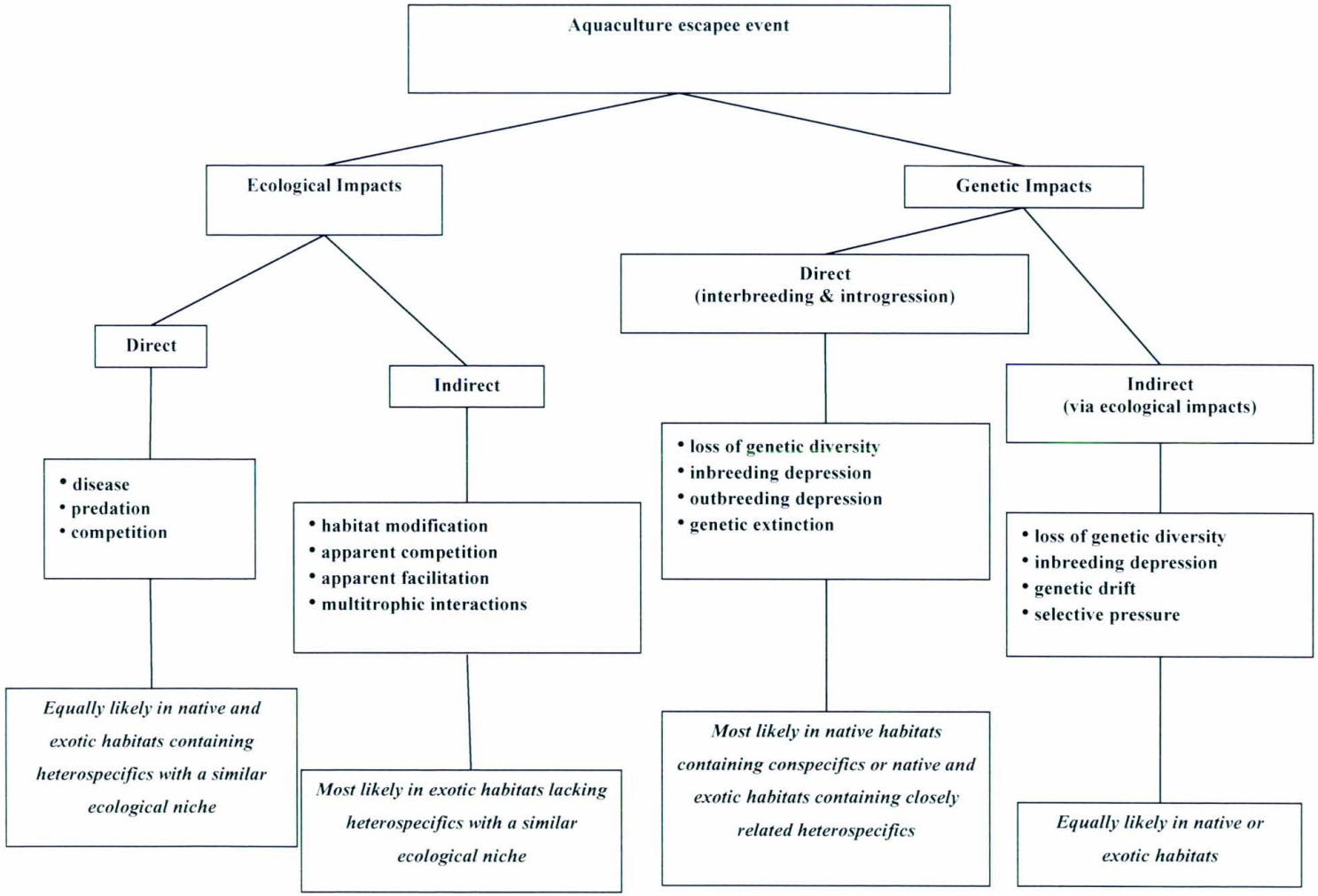

Figure 1.1. Flow chart summarizing the potential ecological and genetic impacts of aquaculture escapees in native and non native habitats.

Chapter 2

Growth hormone transgenesis does not influence territorial dominance or growth and survival of first-feeding Atlantic salmon *Salmo salar* in food-limited stream microcosms

Abstract

This study explored the relative competitive ability and performance of first-feeding growth hormone (GH) transgenic and non-transgenic Atlantic salmon *Salmo salar* fry under low food conditions. Pair-wise dominance trials indicated a strong competitive advantage for residents of a contested foraging territory. Transgenic and non-transgenic individuals, however, were equally likely to be dominant. Similarly, in stream environments with limited food, the transgene did not influence growth in mass or survival at high or low fry densities. Fry in low density treatments, however, performed better than fry in high density treatments. These results indicate that, under the environment examined, the growth performance of GH transgenic and non-transgenic *S. salar* may be similar during first-feeding, an intense period of selection in their life history. Similarities in competitive ability and growth performance with wild-type fish suggest the capacity of transgenic *S. salar* to establish in natural streams may not be inhibited during early life history.

2.1: Introduction

There is increasing interest in the development of biotechnologies in support of the burgeoning aquaculture industry worldwide. Transgenesis, a method of genetic modification involving the insertion of novel DNA into the genome, is one such application that has received considerable attention. In particular, growth hormone transgenesis has been applied to several popular finfish aquaculture species, including tilapia (*Oreochromis niloticus* (L.), Rahman and Maclean, 1999; *Oreochromis* spp., Martinez et al., 2000; *O. niloticus*, Maclean et al., 2002), carp (*Cyprinus carpio* L., Fu et al., 2005), and various salmonids: Atlantic salmon *Salmo salar* L. (Du et al., 1992), coho salmon *Oncorhynchus kisutch* (Walbaum), rainbow trout *Oncorhynchus mykiss* (Walbaum), cutthroat trout *Oncorhynchus clarki* (Richardson), Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) (Devlin et al., 1995) and Arctic charr *Salvelinus alpinus* (L.) (Pitkanen et al., 1999). The popularity of growth hormone transgenesis as a potential aquaculture biotechnology is due largely to the success of achieving substantially increased growth rates, the desired phenotypic trait.

There are concerns regarding the potential environmental risks associated with the commercial production of growth hormone (GH) transgenic *S salar*. *Salmo salar* aquaculture is largely undertaken through coastal sea cage operations that are subject to escape events. The occurrence of farmed *S. salar* escapees entering the surrounding environment and interacting with local intra and interspecific populations is a well documented ecological concern (Ferguson et al. 2007; Morris et al. 2008; Thorstad et al. 2008). Research on the genetic and ecological interactions between domestic escapees and wild *S. salar* have suggested the depression of locally adapted traits through

interbreeding and introgression, and competitive asymmetries that may have fitness consequences in nature (Fleming et al., 2000; McGinnity et al., 2003; Hindar et al., 2006). The potential fate of the transgene and its fitness effects on wild populations following such escapee events are uncertain (Devlin et al., 2006).

GH transgenic *S. salar* possess distinct phenotypic traits that may influence their survival and reproductive success, including increased rates of growth and respiratory metabolism (Stevens et al., 1998; Cook et al., 2000; Deitch et al., 2006) and decreased anti-predator behaviour (Abrahams and Sutterlin, 1999). Similar observations have been made with fast growing domestic salmonid strains that have displayed more foraging motivation, less anti-predator behaviour and, consequently, experience increased predator-induced mortality (*S. salar*, Einum and Fleming, 1997; *S. salar*, Fleming and Einum, 1997; *O. mykiss*, Biro et al., 2004, 2006). Evidence of such pleiotropic responses, however, has not been investigated in GH transgenic *S. salar* at first-feeding, a critical life history period for survival, when juvenile *S. salar* (fry) emerge from the gravel to begin exogenous feeding. Upon emergence, young fry experience intense territorial competition for food and space resources, where <5% are estimated to survive their first few months of life (brown trout *Salmo trutta* L., Elliott, 1994; *S. salar*, Einum and Fleming, 2000; Nislow et al., 2004). Thus, any pleiotropic effects on developmental processes or foraging and anti-predator behaviour during early ontogeny could severely impact the viability of the transgene in nature.

Studies with GH transgenic *O. kisutch* during early life history have found advanced development both at the time of hatch (2-3 days; Devlin et al., 2004a) and emergence from the gravel (*c.* 14 days; Sundstrom et al., 2005). Moreover, first-feeding

O. kisutch fry have also shown an increased susceptibility to predation and food shortages, suggesting greater food requirements and foraging motivation (Devlin et al., 2004b; Sundstrom et al., 2004). Reverse transcription-polymerase chain reaction data indicate messenger RNA (mRNA) expression of the transgene during these early stages in *S. salar* (M. King and G. L. Fletcher, unpub. data), suggesting that the advanced development and behavioural modifications observed in GH transgenic *O. kisutch* fry may be paralleled in GH transgenic *S. salar*.

If the physiological and/or behavioural differences observed in older GH transgenic *S. salar* exist during early ontogeny, time to emergence may be shorter due to increased energy requirements and behavioural motivations. Early emergence allows first access to foraging territories and as a result, a prior resident advantage. The prior resident effect is the competitive advantage held by existing occupants over a contested space and has been observed in numerous animal taxa, including salmonids (Alford and Wilbur, 1985; Snell-Rood and Cristol, 2005; Geange and Stier, 2009). Resident salmonids have repeatedly demonstrated a tendency to dominate dyadic conflicts with intruders of similar size (*S. salar*, Cutts et al., 1999a; Metcalfe et al., 2003; *S. trutta*, Johnsson *et al.*, 1999). Therefore, first access to feeding territories may provide a competitive advantage for transgenic fry. Moreover, greater foraging motivation may allow transgenic fry greater success at supplanting resident non-transgenic fry (Leimar and Enquist, 1984; Elwood et al., 1998). In contrast, increased susceptibility to food shortages may represent a higher metabolic demand for energy consumption and a disadvantage under conditions where food is scarce or predation pressure is high.

If GH transgenesis affects metabolic rates and foraging motivation in fry as it does in older juveniles, then transgenic fry can be expected to dominate non-transgenic fry for prime foraging territories. Under highly competitive, low-food environments, however, their increased metabolic demand may reduce survival and growth. To investigate these ideas, as well as test whether patterns of phenotypic change resulting from GH transgenesis are similar to that observed in *O. kisutch* with a differing transgene construct, the relative competitive ability and performance of first-feeding GH transgenic and non-transgenic *S. salar* fry under low food conditions was tested. Specifically, this study aimed to (1) quantify territorial competition for food and space between first-feeding fry to test for the influence of the transgene on pair-wise dominance relationships, with and without prior residency, and (2) quantify the effects of density on the growth and survival of transgenic and non-transgenic fry during competition under low food conditions in stream microcosms to test how the transgene will affect performance in highly competitive, food-limited environments.

2.2: Methods

2.2.1: Experimental Animals

In 1989, a gene construct (opAFP-GHc2) consisting of growth hormone complementary DNA (cDNA) from *O. tshawytscha*, and driven by an ocean pout, *Zoarces americanus* (Bloch and Schneider), antifreeze protein gene promoter, was introduced into the genome of wild *S. salar* collected from the Exploits and Colinet Rivers, Newfoundland, Canada (Du et al., 1992). A stable transgenic line was created (EO-1α transgene; Yaskowiak et al., 2006) and has been maintained in captivity at the

Ocean Sciences Centre since its inception. During August 2005, wild adult *S. salar* were collected from the Exploits River (48° 55' N, 55° 40' W), Newfoundland, Canada, and transferred to the Ocean Science Centre, Memorial University of Newfoundland. The *S. salar* population of the Exploits River represents one of the largest in Newfoundland; primarily consisting of one year sea-winter spawning fish (grilse; O'Connell et al., 2003).

To control for maternal effects, eggs from five wild females were divided evenly and crossed individually with milt from five wild males and five homozygous transgenic males on 17 November 2005. Consistent with the basic principles of Mendelian inheritance for dominant genes on a single chromosome, a homozygous transgenic out-cross will produce 100% transgenic offspring that are hemizygous carriers of the transgene. Following water hardening, all transgenic and wild crosses were pooled separately and reared in separate Heath incubation trays.

As the yolk-sac fry neared complete yolk absorption (i.e. the start of exogenous feeding), the transgenic and non-transgenic crosses were transferred into holding tanks (1 m × 1 m) and fed *ad libitum*, with a combination of *Artemia* spp. and a salmonid starter dry feed (Corey Feed Mills Ltd., Fredericton, Canada; www.corey.ca). Photoperiod was maintained at a 12L:12D schedule during holding and experimentation. All animals were treated in accordance with the guidelines provided by the Canadian Council on Animal Care during holding and experimentation, and approval was granted by Memorial University's Institutional Animal Care Committee (AUP 05-03-IF).

2.2.2: Dominance Trials

To compare the relative competitive ability of transgenic and non-transgenic fry, pair-wise dominance trials were conducted in 40 replicate stream-like contest arenas. Each arena approximated the territory size used by an emerging fry (Grant and Kramer, 1990; Figure 2.1). Three sets of pair-wise comparisons were made: (1) transgenic *v.* non-transgenic cohabitants (no prior-residency advantage; $n = 37$), (2) transgenic intruders *v.* non-transgenic residents ($n = 41$), and (3) transgenic residents *v.* non-transgenic intruders ($n = 47$). For cohabitant contests, individuals were introduced simultaneously and allowed a 48 h acclimation period prior to observation. In prior residence trials, the 48 h acclimation period occurred in the separate enclosures, after which the intruder was introduced by temporarily lifting the partition between contest arenas and guiding the intruder into the resident arena. Observations commenced 24 h following the introduction of the intruder. Preliminary trials conducted with non-transgenic individuals the year previous confirmed that the contest arenas and protocol were suitable for detecting dominance behaviour.

Fish were anesthetized (MS-222, Western Chemical Inc.; www.wchemical.com), and size matched to within a mass (M) range of 5% for each trial (mean ± S.E. $M_{\text{Transgenic}} = 0.209 \pm 0.004$ g; $M_{\text{Non-transgenic}} = 0.208 \pm 0.004$ g; the transgenic and non-transgenic populations do not diverge in size until later in life; unpubl. data) and were marked by applying Alcian blue dye (Sigma-Aldrich; www.sigmaaldrich.com) with a fine needle to opposing pectoral fins to distinguish individuals within pairs. After measurements and marking, the fish were transferred into the test aquarium. Trials began *c.* 2 weeks following emergence to ensure all individuals had adjusted to exogenous feeding, and

occurred between 16 May and 19 June 2006, during which the ambient temperature ranged between 8 and 13°C. There were no obvious developmental differences (*e.g.* size, yolk absorption) between the original populations of transgenic and non-transgenic fish prior to experimentation.

Dominance was assessed with a point system accounting for feeding attempts and spatial position (Metcalfe et al., 1992; Johnsson and Bjornsson, 1994; Metcalfe et al., 1995; Cutts et al., 1999b). Six observational trials were performed for each pair. Each observation commenced with the introduction of a consistent quantity of live *Artemia* spp. (*n c.* 10) through a feeding tube centred at the upstream end of the contest arena. Overt feeding behaviour was defined as a distinct biting or lunging motion following the *Artemia* spp. injection. Feeding attempts earned individuals a single point. In cases where both fish displayed feeding behaviour, the first to make a feeding attempt received two points. Spatial position was scored from one to minus one, with a single point awarded to fish positioned directly downstream of the feeding tube, zero points for fish positioned along the margins next to the feeding tube and minus one for fish in the corners, such that their view of the feeding tube was likely hindered. Scores for each fry were tallied and winners were defined by an advantage of greater than three points. Trials in which neither individual exhibited feeding behaviours ($n = 6$) were excluded from the analysis.

2.2.3: Stream Mirocosm

To compare the effects of density on the growth and survival of transgenic and non-transgenic first-feeding fry during competition, eight stream microcosms were

established, four at high (n = 40) and four at low density (n = 10). This was accomplished by partitioning four fibreglass troughs length-wise to create eight semi-natural stream microcosms (2.6 m × 0.25 m × 0.10 m; Figure 2.2). Each trough contained two stream channels separated with window screening. A current was generated within each stream channel using an inflow spray bar positioned behind window screening at the upstream end of each channel. Each trough consisted of one channel with a spray bar attached directly to a facility freshwater supply and the other attached to a pump, creating a partially recirculating, unidirectional flow.

Prior to the experiments, fish were selected haphazardly, anaesthetized with MS-222, measured for M and fork length (L_F), and tagged with visible implant elastomer (Northwest Marine Technologies Inc.; www.nmt-inc.com) and/or Alcian blue dye (Sigma-Aldrich). An elastomer tag in the dorsal musculature was used to differentiate between transgenic and non-transgenic individuals. A second tag made either on the jaw or a fin (caudal, pectoral or anal) was used to uniquely identify individuals. This was done for all fry in the low density treatment (n = 10) and for half of the fry in the high density treatment (n = 10 for each type), because of limited numbers of unique tag combinations. Equal numbers of transgenic and non-transgenic individuals were placed in the low (five of each type) and high density (20 of each type) treatments.

Similar quantities of *Artemia* spp. were delivered to each stream one to four times daily to reflect the fluctuating availability of food in nature. The high and low density channels received mean food levels equivalent to 2.30% (range: 0.65 - 12.20%) and 9.30 % (range: 2.63 - 49.30%) of fish biomass per channel, per day, respectively. The *Artemia* spp. were supplied through tubes hidden behind a blind to prevent disturbance. Feeding

tubes were positioned just above the water surface at 100-140 mm below the upstream screen and again half way down the microcosm to ensure feed would be accessible the full length of each channel. Estimates of invertebrate drift in natural streams coupled with the pulsated feed delivery method suggested that this food level would be representative of a strongly food-limited environment (Wilzbach et al., 1986; Keeley and Grant, 1995). Experimentation occurred *c.* 2 weeks following emergence, between 15 May and 20 June 2006 (37 days), during which the ambient temperature range was 8-13°C. There were no obvious developmental differences (e.g. size, yolk absorption) between the original populations of transgenic and non-transgenic fish prior to experimentation.

2.2.4: Statistical Analyses

To assess dominance, logistic regressions with binomial error (LR) were used to evaluate the number of wins *v.* non-wins (losses and draws) with respect to genotype (transgenic or non-transgenic), prior residency and M, where M was treated as a categorical variable indicating the large and small fish from each trial. In the stream study, L_F (mm) and M (g) measurements of all fry were taken before and after the experiment and used to calculate instantaneous growth rate (G; $G = (\ln X_2 - \ln X_1)\ t^{-1}$). Initial size and G were compared using general linear models (GLM) with genotype and density as factors of fixed effects. To examine body condition, initial and final M - L_F relationships were assessed with a similar GLM that included M as the response variable and L_F as a covariate (García-Berthou, 2001). Where applicable, Tukey HSD *post hoc* comparisons were performed to compare means among transgenic and non-transgenic fry

from both density treatments, using *P*-values adjusted for single-step multiple comparison procedures. For Tukey HSD *post hoc* comparisons of body condition, residuals were produced from a regression analysis of the corresponding M (y-axis) and L_F (x-axis) measurements and used as the response variable. A logistic regression with binomial error was also used to assess stream study mortality with respect to genotype and density. All statistical analyses followed a model simplification approach using the computing program SAS® 9.13 (SAS Institute Inc.; www.sas.com) and significance was measured at a 5% alpha level of type I error.

2.3: Results

2.3.1: Dominance Trials

Transgenic and non-transgenic fry cohabitants (*i.e.* no prior residency) did not differ significantly in their ability to dominate a single foraging territory (LR, $n = 37$, $\chi^2 = 0.02$; $P > 0.05$, Figure 2.3). Similarly, the transgene did not influence dominance in prior residence trials (LR, $n = 88$, $\chi^2 = 2.50$; $P > 0.05$). Residents, however, won significantly more contests than intruders (LR, $n = 88$, $\chi^2 = 12.73$; $P < 0.01$). M did not influence dominance in either cohabitant (LR, $n = 37$, $\chi^2 = 0.02$; $P > 0.05$) or prior residence trials (LR, $n = 88$, $\chi^2 = 2.16$; $P > 0.05$).

2.3.2: Stream Microcosm

Initial L_F (mm) was similar between transgenic and non-transgenic fry (GLM; $F_{1, 199} = 0.01$, $P > 0.05$) and between density treatments (Table 2.1; GLM, $F_{1, 199} = 0.90$, $P > 0.05$). A GLM for initial M (g) indicated a significant interaction between genotype and

density (GLM, $F_{1, 199} = 5.10$, $P < 0.05$). Tukey HSD *post hoc* comparisons indicated that non-transgenic fry in the low density treatment had lower initial M than all other fish groups, including both transgenic and non-transgenic fish in the high density treatment and the transgenic fry of the low density treatment. To examine body condition, a GLM representing the initial M and L_F relationship indicated a significant difference between density treatments ($F_{1, 199} = 7.00$, $P < 0.01$); however, transgenesis had no influence (GLM, $F_{1, 199} = 0.96$, $P > 0.05$). Tukey HSD *post hoc* comparisons indicated that the low density non-transgenics had less M for a given L_F than both the high density transgenic and non-transgenic groups.

Negative mean instantaneous growth rates (G_{length}: mm day^{-1}; G_{mass}: g day^{-1}) were observed for transgenic and non-transgenic fry in both high and low density treatments (Figure 2.4). Transgenic fish maintained significantly more G_{length} than non-transgenic fish (GLM, $F_{1, 90} = 4.93$, $P < 0.05$). The transgene, however, did not influence G_{mass} across density treatments (GLM, $F_{1, 90} = 0.02$, $P > 0.05$). Individuals in the low density treatment lost less size than high density individuals (GLM, G_{length}: $F_{1, 90} = 7.20$, $P < 0.05$; G_{mass}: $F_{1, 90} = 37.17$, $P < 0.01$; Figure 2.5). For body condition, a GLM representing the final M and L_F relationship indicated a significant difference between density ($F_{1, 90} = 11.61$, $P < 0.01$); however, transgenesis provided no influence ($F_{1, 90} = 2.50$, $P > 0.05$). Tukey HSD *post hoc* comparisons indicated that the low density transgenic fish maintained more M for a given L_F than the high density transgenic group.

The mean proportion of survivors in the high (mean ± S.E. = 0.83 ± 0.05) and low density (mean ± S.E. = 0.78 ± 0.14) treatments did not differ (LR, $n = 164$, $\chi^2 = 0.66$; $P > 0.05$). Likewise, there was no significant difference in survivorship between transgenic

(mean ± S.E. = 0.81 ± 0.10) and non-transgenic individuals (mean ± S.E. = 0.79 ± 0.11; LR, n = 164, $\chi^2 = 0.05$; $P > 0.05$).

2.4: Discussion

No differences were found between GH transgenic and non-transgenic *S. salar* fry in any of the fitness-related phenotypic traits measured. During pair-wise dominance trials, prior residency provided a clear advantage. Transgenic fry, however, were equally likely to win territorial dominance contests as were non-transgenic fry. Consistent with the dominance trials, the survival of GH transgenic first-feeding fry in stream microcosms under low food availability did not differ from that of non-transgenic individuals. Moreover, both groups experienced negative growth, though the pattern differed somewhat, with transgenic individuals maintaining greater L_F for a given M than non-transgenic individuals. This result is consistent with previous findings suggesting preferential investment in skeletal growth in GH transgenic *O. kisutch* (Devlin et al., 1995). Having controlled for maternal effects in the experimental design, our results suggest that competition for a limited resource and vulnerability to conditions of low food were not influenced by the transgene. Moreover, the similarity in competitive behaviour, growth and survival of transgenic and non-transgenic individuals, indicates that, in the absence of predation, the EO-1α transgene may not influence the fitness of *S. salar* strongly at the onset of exogenous feeding. While the fry had been fed as they underwent the transition from endogenous to exogenous resources for two weeks prior to the experiments (i.e. to ensure they had switched to exogenous food), both transgenic and

non-transgenic individuals were treated similarly and thus the patterns observed are likely reflective of competition at this life stage.

The adjustment to exogenous feeding is a period of strong selection in stream salmonids, during which individuals establish foraging territories in response to heterogeneous habitat quality (*S. salar* and *S. trutta*, Kalleberg, 1958; *S. salar*, Keenleyside and Yamamoto, 1962; *S. trutta*, Elliott, 1994). The establishment of an economically defendable feeding territory has been suggested to provide an energetic advantage to territorial over non-territorial individuals [*O. kisutch*, Puckett and Dill, 1985; *S. salar*, *S. trutta*, *O. mykiss*, *O. kisutch*, brook charr *Salvelinus fontinalis* (Mitchill), Grant and Kramer, 1990], thus improving the odds of survival. In salmonids, success in territorial contests has been linked to aggression (*S. trutta*, Deverill et al., 1999), body size (*S. trutta*, Johnsson et al., 1999), experience (*O. kisutch*, Rhodes and Quinn, 1998) and prior residency (*S. salar*, Metcalfe et al., 2003). Moreover, GH-enhanced salmonids, including GH transgenic, GH implanted and growth selected fishes, have shown traits associated with territorial dominance, such as increased size and aggression (*O. mykiss*, Johnsson and Bjornsson, 1994; *O. kisutch*, Devlin et al., 1999; *S. salar*, Metcalfe et al., 2003). The correlation between aggression and dominance, however, has not been observed consistently (*O. mykiss*; Jonsson et al., 1998; *O. kisutch*, Sundstrom et al., 2003; *S. trutta*, Neregard et al., 2008). For example, in a study investigating territorial relationships between GH implanted *S. trutta* and wild-type parr, Neregard et al. (2008) observed an increase in aggression among intruding GH treated parr relative to wild-type individuals. Nevertheless, this increased aggressive behaviour did not influence the outcome of territorial conflicts, suggesting motivational changes may not always equate

to foraging success. Similarly, in the current study, no differences in territorial dominance due to the transgene in first-feeding *S. salar* fry were observed. The similarity in ability of the transgenic fry to withstand low feed stream environments relative to non-transgenic individuals, however, suggests the results may reflect a delay in the phenotypic response to the transgene rather than the ineffectiveness of a change in foraging motivation on territoriality.

Juvenile GH transgenic salmonids have been shown to exhibit greater rates of metabolism (*S. salar*, Stevens et al., 1998; Cook et al., 2000; Levesque et al., 2008), aggression and feeding motivation relative to non-transgenic individuals (*O. kisutch*, Devlin et al., 1999; Sundstrom et al., 2003, 2004). Much of these data reflect older juveniles (parr) that have long since undergone the ontogenetic shift to exogenous feeding. Work with GH transgenic *O. kisutch* first-feeding fry, carrying the OnMTGHI gene construct, however, supports previous observations on older juveniles, suggesting metabolism may be driving differences in risk taking and foraging-induced aggressive behaviour. For example, in *O. kisutch* the survival of first-feeding fry in low feed rearing tanks has been shown to dramatically decrease due to the presence of GH transgenic individuals (Devlin et al., 2004b). Specifically, low food abundance caused greater mortality in transgenic-containing tanks, brought upon by the aggressive behaviour of dominant transgenic fry. Similarly, GH transgenic *O. kisutch* have demonstrated increased mortality due to predation and decreased growth rates at first-feeding in low food stream environments relative to non-transgenic fry (Sundstrom et al., 2004). Further support for the suggestion that metabolism may be driving differences is provided by observations of enhanced egg and alevin developmental rates and reduced egg survival

under low oxygen conditions in GH transgenic *O. kisutch* (Devlin et al., 2004a; Sundstrom et al., 2005; Sundt-Hansen et al., 2007). Metabolism, however, has not specifically been measured during early ontogeny.

This study represents the first attempt to quantify phenotypic differences between first-feeding growth hormone transgenic and non-transgenic *S. salar* fry. These data suggest important phenotypic differences between GH transgenic *S. salar* and, previously studied, *O. kisutch* populations during this critical early life history period. The observed similarities in behaviour, growth and survival suggest that there is a delayed ontogenetic response to the presence of a growth hormone transgene in *S. salar*, such that the critical period of survival associated with emergence may not influence the fitness of the transgenic fry strongly. This may allow a greater proportion of transgenic individuals to survive past first-feeding, and as a consequence interact ecologically and genetically with wild fish at later life stages, than that expected based on observations of older juveniles or other GH transgenic strains. However, it is important to acknowledge that this study represents one of many potential ecological scenarios where empirical investigation is recommended prior to any future risk assessment efforts.

The phenotypic response to transgenesis in fishes may vary considerably in response to construct design, the genome of the receiving organism and the dominance and epistatic interactions between the transgene and the background genome (Twyman, 2005; Gong et al., 2007; Nam et al., 2007). GH transgenic *S. salar* and *O. kisutch* have been derived from populations of two different species, with two different gene constructs. While both transgenic strains display many similar phenotypic changes later in ontogeny, they also display differences in response to transgenesis during an important

life history period, the onset of exogenous feeding. Such a difference emphasizes the importance of assessing the environmental risk of transgenic organisms on a case-by-case basis because the phenotypic effects of transgenesis may vary between species and constructs designed for the same purpose.

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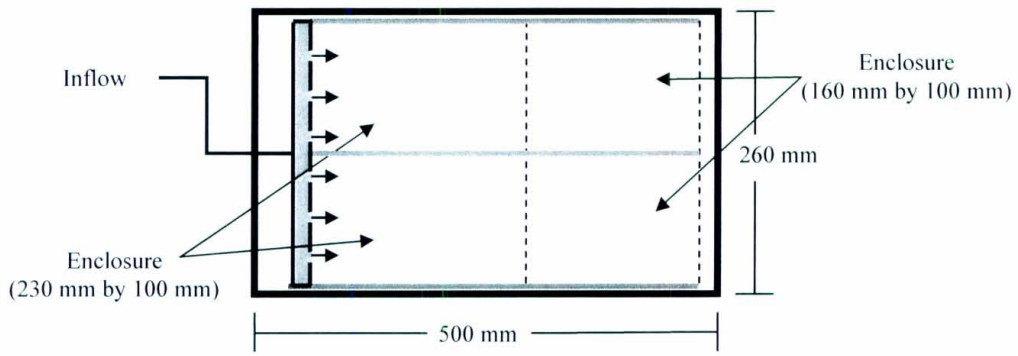

Figure 2.1. The experimental apparatus used during pair-wise dominance trials with *Salmo salar* fry (n = 10). Located immediately downstream of each other were contest enclosures. Each enclosure consisted of mesh ends and PVC side partitions. The mesh partition separating each enclosure could be raised, allowing intruder entry during prior residence trials. Freshwater flowed through a spray bar allowing a current across the width of the apparatus at approximately 30-50 mm sec^{-1}. A water depth of 70-100 mm was maintained by the addition of a modified, grey plastic bottom covered with a thin layer of a natural gravel substrate. Size differences between the upstream and downstream contest areas were accounted for in the experimental design. Specifically, all prior residence trials were conducted in the downstream contest enclosures and an equal number of cohabitant trials were conducted in upstream and downstream enclosures.

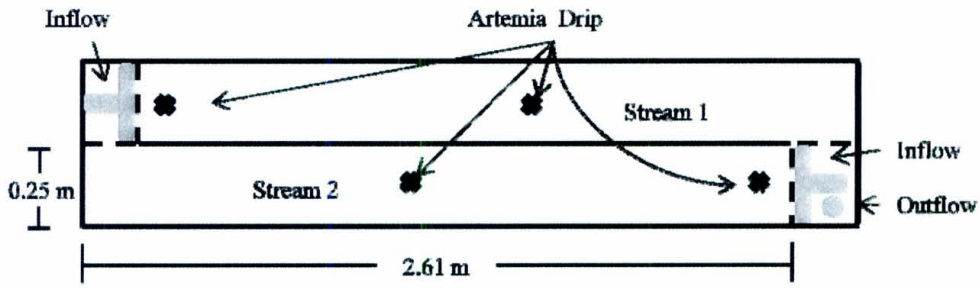

Figure 2.2. Experimental stream microcosms (n = 8) used to compare the effects of density on the growth and survival of transgenic and non-transgenic *Salmo salar* fry. Inflow spray bars were positioned behind a screen partition at the upstream end of each stream, creating a unidirectional, clockwise flow within each trough. *Artemia* spp. drip food delivery tubes were positioned just above the water surface at 100-140 mm below the upstream screen and again half way down the microcosm to ensure feed would be accessible the full length of each stream. The current speed within each stream ranged from 120-180 mm s^{-1} upstream to 30-80 mm s^{-1} downstream. The bottom of each channel was covered with 5-15 mm gravel and 50-150 mm rocks to create habitat heterogeneity.

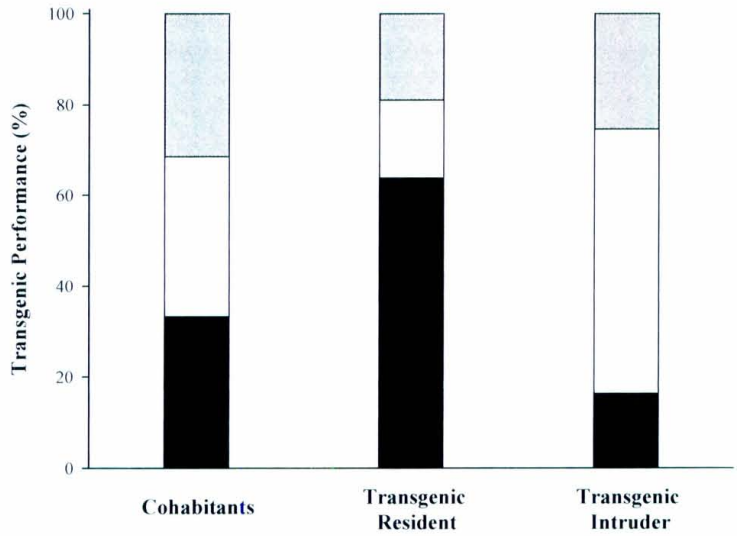

Figure 2.3. The performance, displayed in percentages, of growth hormone transgenic *Salmo salar* fry during pair-wise dominance contests with non-transgenic fry under three scenarios of competition (cohabitant, resident and intruder). Performance was measured by wins (black), losses (white) and draws (grey).

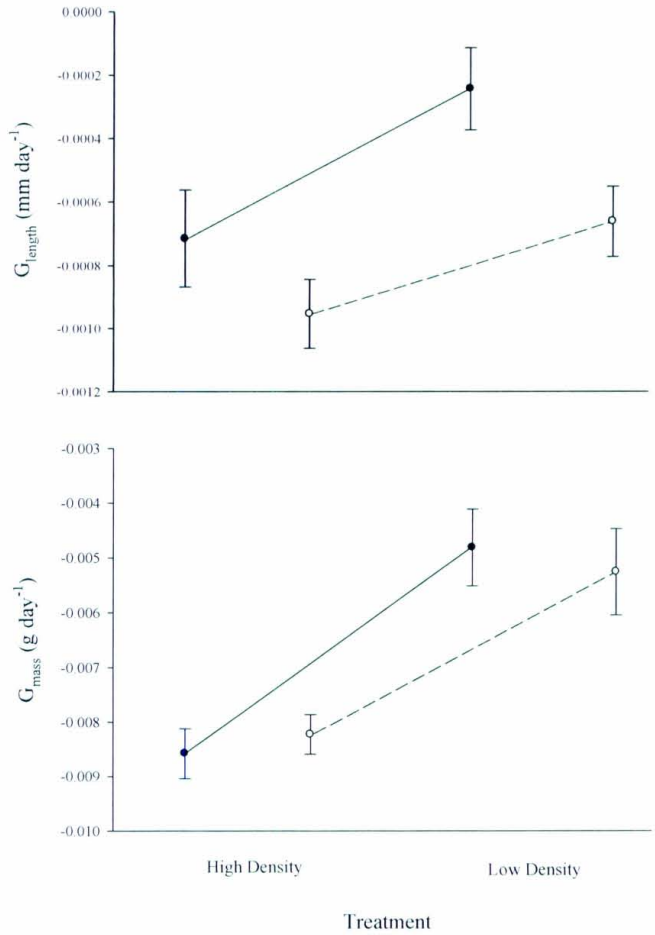

Figure 2.4. The mean ± S.E. instantaneous growth rates (G) for fork length (G_{length}: mm day^{-1}) and mass (G_{mass}: g day^{-1}) of growth hormone transgenic (filled circles) and non-transgenic (open circles) *Salmo salar* fry reared at high and low densities in near-natural stream microcosms under low feed conditions.

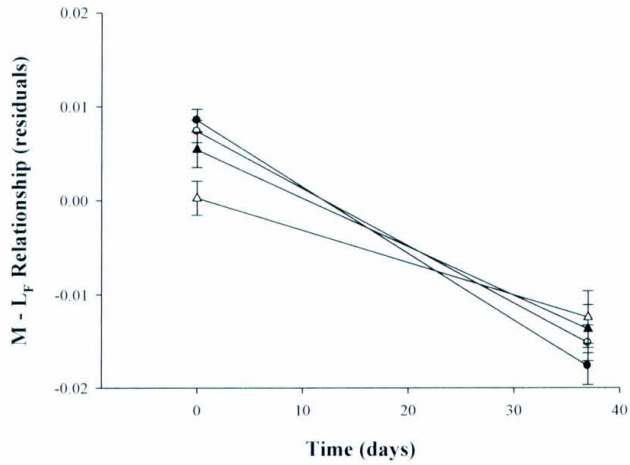

Figure 2.5. The body condition [mass (M) - fork length (L_F) relationship] of growth hormone transgenic and non-transgenic *Salmo salar* fry reared at high (HD) and low (LD) densities in near-natural stream microcosms under low feed conditions. The fry are categorized as follows: HD transgenic (filled circle), HD non-transgenic (open circle), LD transgenic (filled triangle) and LD non-transgenic (open triangle). The M and L_F relationship is represented by mean ± S.E. residuals produced with a linear regression of initial and final natural log-transformed M (g) and L_F (mm) variables.

Table 2.1. The mean ± S.E. initial and final mass (M) and fork length (L_F) measurements of first-feeding growth hormone transgenic and non-transgenic *Salmo salar* fry. Transgenic and non-transgenic fry were reared together at high and low densities in near-natural stream microcosms under low feed conditions.

Genotype	Density Treatment	Initial n	Final n	Initial M (g)	Final M (g)	Initial L_F (mm)	Final L_F (mm)
Transgenic	High	80	66	0.15 ± 0.00	0.11 ± 0.00	28.53 ± 0.08	27.75 ± 0.15
Transgenic	Low	20	16	0.16 ± 0.00	0.13 ± 0.00	28.80 ± 0.16	28.69 ± 0.17
Non-transgenic	High	80	67	0.16 ± 0.00	0.12 ± 0.00	28.70 ± 0.07	27.91 ± 0.15
Non-transgenic	Low	20	15	0.15 ± 0.00	0.12 ± 0.01	28.65 ± 0.17	27.99 ± 0.20

Chapter 3

Delayed phenotypic expression of growth hormone transgenesis during early ontogeny in Atlantic salmon (*Salmo salar*)

Abstract

There is considerable uncertainty regarding the potential ecological and genetic impacts that the escape of growth hormone (GH) transgenic Atlantic salmon might have on wild populations. This study compared the developmental rate and respiratory metabolism of GH transgenic and non-transgenic full sibling Atlantic salmon during early ontogeny; a life history period of intense selection that may provide critical insight into the fitness consequences of transgenesis. Transgenesis did not affect the routine oxygen consumption of eyed embryos, newly hatched larvae (alevins) or first-feeding juveniles (fry). Moreover, the timing of early life history events was similar, with transgenic fish hatching less than one day earlier, on average, than their non-transgenic siblings. By the time emergence neared, however, transgenic fish were somewhat developmentally behind, having more unused yolk and being slightly smaller than their non-transgenic siblings. Although such differences were found between transgenic and non-transgenic siblings, family differences were considerably more important in explaining phenotypic variation. Overall, this research suggests that biologically significant differences in fitness-related traits between GH transgenic and non-transgenic Atlantic salmon are minimal during the critical early life history period.

3.1: Introduction

There is considerable interest in growth hormone (GH) transgenic Atlantic salmon (*Salmo salar* L.) as a candidate biotechnology for aquaculture. Similar to those associated with domesticated aquaculture strains (Ferguson et al. 2007; Morris et al. 2008; Thorstad et al. 2008), there are concerns regarding the potential impacts of ecological and genetic interactions between transgenic and wild salmon in nature (Kapuscinski and Hallerman 1991; Muir and Howard 2002; Devlin et al. 2006). Currently, however, there is little empirical data with which to assess the possible environmental risks of this transgenic fish strain; a leading candidate for commercialization.

Early ontogeny represents a highly selective life history period for many stream salmonids, and thus, may provide valuable information regarding the fitness of transgenic salmon strains relative to wild-type individuals. At fertilization, eggs are buried in gravel nests and remain immobile until hatch. During this stage, eggs can experience lethally low levels of dissolved oxygen, resulting in high mortality (Lacroix 1985; Chapman 1988; Peterson and Quinn 1996). Upon hatch, alevins (larval phase) remain underneath the gravel until their endogenous yolk reserves are near fully consumed. At this point, individuals emerge and commence exogenous feeding. First-feeding is a critical period of survival and performance for the fry of many salmonid species (early stage juveniles), where individuals must learn to attain food, compete for and/or migrate to foraging territories and avoid predation (Chandler and Bjornn 1988; Brannas 1995; Einum and Nislow 2005). Mortality during the first few weeks of life can be greater than 80% (Elliott 1994; Einum and Fleming 2000; Nislow et al. 2004). Thus, any transgene-induced effects on physiological and behavioural traits during early ontogeny may impact survival and thus, the viability of the transgene in nature.

Beyond its affects on growth (Du et al. 1992; Devlin et al. 1994), GH transgensis is known to have pleiotropic effects on other phenotypic traits in salmon, including elevated metabolic rates, increased foraging motivation and reduced anti-predator behaviour (Abrahams and Sutterlin 1999; Cook et al. 2000; Leggatt et al. 2003; Sundstrom et al. 2003; Tymchuk et al. 2005). Many of these studies have concentrated on juveniles ca. 8 months or older, bypassing the intense selection experienced during early ontogeny. However, research with GH transgenic coho salmon, *Oncorhynchus kisutch* (Walbaum), has shown phenotypic effects during early life history. These include GH transgenic coho displaying reduced survival as eyed-eggs during hypoxic (low oxygen) conditions (Sundt-Hansen et al. 2007), advanced embryo and larval development (Devlin et al. 2004a; Sundstrom et al. 2005; Lohmus et al. 2010) and greater susceptibility of first-feeding juveniles (fry) to predation and starvation than non-transgenic coho (Devlin et al. 2004b; Sundstrom et al. 2004). Collectively, these studies suggest that the relative fitness of transgenic and non-transgenic coho salmon during early life history may differ considerably in nature.

As part of a continuum of correlated traits, resting metabolism has been linked to variation in behaviour, performance, and life history strategies among individuals at both inter- and intra-specific levels (Symonds 1999; Sih et al. 2004; Biro and Stamps 2008, Careau et al. 2008). In intra-specific laboratory studies with salmonids, high resting metabolic rates correlate with fast growth (Metcalfe et al. 1995; Yamamoto et al. 1998), foraging-induced aggression and dominance (Cutts et al. 1998; Cutts et al. 2001; McCarthy 2001; Lahti et al. 2002); all of which have been observed for GH transgenic salmon parr. Resting metabolism is the minimum energy requirement of an individual within a specific environment, and represents an internal constraint on energy allocation that has significant implications for an animal's survival (Brown et al. 2004). For example, fish with elevated resting metabolic rates require more energy and,

consequently, more oxygen to maintain normal body function. Thus, the transgene-induced differences in resting metabolic rate during early life history may explain observations of increased sensitivity to hypoxia, advanced development, higher foraging-induced aggression and decreased anti-predator behaviour in GH transgenic salmon. However, to our knowledge, respiratory metabolism has not been compared between GH transgenic and non-transgenic salmon during early ontogeny. Moreover, previous work with GH transgenic coho salmon, a Pacific species that carries a distinct transgene construct, may not represent the early phenotypic responses of all growth hormone transgenic salmonid strains (Nam et al. 2007).

If the GH transgene elevates metabolic rates during early ontogeny, as observed for older juveniles (aged > 8 months; Stevens et al. 1998), then a similarly advanced development to that of coho salmon may also result in Atlantic salmon. Such phenotypic shifts could influence the relative survival of transgenic and non-transgenic salmon during this critical life history period. To test for these potential phenotypic effects and compare how they may differ from other manifestations of GH salmon transgenesis, this study compared the respiratory metabolism and development of GH transgenic and non-transgenic Atlantic salmon siblings during early ontogeny. Using multiple family replicates, we quantified the routine metabolism of GH transgenic and non-transgenic siblings at three early stages of ontogeny; eyed-eggs, alevins (larvae) and first-feeding fry. Furthermore, we tested for differences in hatch time and, near exogenous feeding, alevin mass, length and the amount of yolk remaining within (transgenic versus non-transgenic) and among families.

3.2: Methods

3.2.1: Experimental Animals

A gene construct (opAFP-GHc2) consisting of growth hormone cDNA from Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and an antifreeze protein gene promoter from ocean pout, *Macrozoarces americanus* L., was introduced into the genome of wild Atlantic salmon collected from the Exploits and Colinet Rivers, Newfoundland, Canada in 1989 (Du et al. 1992). A stable transgenic line (EO-1α transgene) resulting from these gene insertion experiments was produced at the Ocean Sciences Centre, Memorial University of Newfoundland (Yaskowiak et al. 2006). During August 2005, wild adult Atlantic salmon were also collected from the Exploits River (48°55'N, 55°40'W), Newfoundland, Canada, and transferred to the Ocean Science Centre. The Exploits River salmon population is one of the largest in Newfoundland. A characteristic of anadromy in this system is that fish typically return to the river following a single year at sea (O'Connell et al. 2003).

Eleven single family crosses were produced between wild, non-transgenic females and captive-reared, transgenic males that were hemizygous for the GH transgene between the 3rd and 22nd of November, 2005. True to Mendelian inheritance patterns, this cross results in approximately half of the offspring inheriting the GH transgene (Shears et al., 1992). This enabled the comparison of full siblings differing primarily by the presence or absence of the transgene (i.e. other genetic differences tending to be randomized), allowing for the control of maternal effects and general genetic background.

All families were reared separately in Heath incubation trays. Shortly following fertilization, 10 eggs from each family were collected and both wet and dry mass (g) were determined. At first-feeding, families were pooled into two separate family groups, reared in 1m by 1m holding tanks, and fed *ad libitum* with a combination of *Artemia spp.* and a salmonid

starter dry feed (Corey Feed Mills, Fredericton, NB). During holding and experimentation, both temperature and photoperiod were kept at ambient conditions, except in association with the respirometry trials (see below). Following all experiments, a tissue sample of each individual was screened for the transgene using the polymerase chain reaction (PCR) protocol described in Deitch et al. (2006). All animals were treated in accordance with the guidelines provided by the Canadian Council on Animal Care and with the approval of Memorial University's Institutional Animal Care Committee.

3.2.2: Respirometery Systems

To estimate the metabolic rate of individual embryos and fish, we measured their routine oxygen consumption (Jobling 1994) in one of two respirometery systems. Jobling (1994) defined routine metabolic rate as a measure of oxygen consumption (mg O_2 g^{-1} hr^{-1}) for fasted, unstressed animals experiencing minimal movement. In the case of endogenously feeding eyed embryos and alevins (larvae), we consider our measurements representative of routine metabolism.

The first respirometer was a custom glass design, used to measure the oxygen consumption of individual salmon eyed-eggs. It consisted of an inner experimental chamber, where the animal was located, and an outer chamber connected to an external water bath (model 1150S, VWR International, Mississauga, ON, Canada) that maintained the inner chamber at 3 °C. Freshwater was pumped into the bottom of the 6.75 ml inner chamber from an oxygenated glass reservoir (situated in the chiller basin) and returned through an exit port at the top of the chamber with the aid of a peristaltic pump (Masterflex L/S model 77200-12, Cole-Palmer Inc., Barrington, USA) and low gas permeability tubing (Tygon® Food and LFL, Cole Palmer Inc., Barrington, USA). Individual eyed-eggs were elevated above the bottom of the inner chamber on the mesh surface of a perforated, circular glass tube. The entire respirometer was suspended over a magnetic

stirrer such that the stir bar, located within the inner chamber, ensured water was mixing slowly and no oxygen gradients were present. Immediately prior to oxygen consumption measurements, the peristaltic pump was turned off and the inner chamber was closed with stop-cocks. The drop in oxygen concentration was then measured using a computer controlled fiber-optic minisensor system (Fibox 3®, PreSens GmbH, Regensburg, Germany) and an oxygen sensitive spot attached to the inner surface of the inner chamber. The fiber-optic oxygen system was calibrated regularly using oxygen-saturated water and water from which all oxygen had been removed by the addition of sodium sulphite (0.25g per 10 ml).

The second respirometer was a custom-built, glass, Blazka-type respirometer (Brett 1964) that had an 82 ml inner chamber volume. This device was used to measure the routine metabolism of individual alevins and first-feeding fry. The design and operation of this respirometer was similar to that previously described in Killen et al. (2007), with one exception. As with the respirometer used to measure embryo metabolism, water temperature was controlled using an outer water jacket that was connected to an external water bath. The water temperature was maintained at 4.5 °C and 8.5 °C for alevins and first-feeding fry, respectively. A weak current was induced within the resirometer to ensure proper mixing and prevent the formation of oxygen gradients. The current, however, was weak and no swimming activity was required by the animals. A black cloth was draped over the respirometer to prevent disturbance and a mirror was used to monitor the activity of the fish during the oxygen measurement period.

3.2.3: Respirometry Protocol

Fish used in the respirometry experiments were maintained at the experimental temperatures for a minimum of two weeks prior to measurement. These temperatures corresponded to the ambient conditions at the initiation of experimentation. For eyed-eggs, the

oxygen consumption of 6-7 eggs from six families (n = 39; 0.10-0.16g) was measured at ages ranging from 385-415 degree days (a developmental index representing the sum of daily mean temperatures). Individual eyed-eggs were acclimated to the respirometer for 90 minutes prior to oxygen consumption measurements. Two successive, 30-min measurements, separated by 15 min, were taken on each eyed-egg and averaged. All eyed-eggs within each family were measured within an 18-hour period to limit potential developmental effects on metabolic rate. To imitate both the rearing and natural environments, all measurements were performed in total darkness.

For alevins, the oxygen consumption of 9-10 individuals from four families (n = 39; 0.12-0.19g) was measured at ages ranging from 668-725 degree days. Individuals were acclimated to the respirometer for 90 min prior to a 60-min oxygen consumption measurement. All individuals within each family were measured over 3 days to limit potential developmental effects on metabolic rate. As with the eyed-eggs, all measurements were performed in total darkness.

For first-feeding fry, the oxygen consumption was measured on individuals (n = 32; 0.13-0.27g) that had been fasted for 48 hours. These animals were haphazardly selected from two tanks, each containing 5 and 6 families, respectively. Individuals were acclimated to the respirometer for 150 min prior to a 30 min measurement of oxygen consumption. All measurements were performed under low light conditions.

Acclimation time periods were based on preliminary experiments, and ensured that the fish/eyed-eggs were in a steady state of constant low oxygen consumption (i.e. they had recovered from any stress associated with handling). Rates of oxygen consumption (mg O_2 g^{-1} hr^{-1}) for each trial were calculated using the slope of a linear regression between water oxygen level and time, then multiplied by the chamber volume and divided by the animal's mass. At the end of each day, blank measurements were made to ensure minimal background oxygen

consumption, and the respirometers were cleaned with 100% ethanol. Any observed background oxygen consumption rate was subtracted from the experimental values. Trials where background oxygen consumption rates were greater than 5% of animal oxygen consumption were not included in the data set.

3.2.4: Development

Hatch time, alevin yolk surface area (mm^2), mass (g) and fork length (mm) near emergence were used as indices for examining the effect of the transgene on developmental rate. For each of 8 families, ca. 100 eyed-eggs were haphazardly sub-sampled from family-specific Heath incubation trays and placed into plastic canvas mesh baskets housed within separate trays. During incubation, the ambient temperature ranged between 2-8 °C, with a temperature of 4 °C at hatch. Baskets were checked once daily for hatched individuals. At hatch, individuals were preserved in 95% ethanol for subsequent PCR analysis. For the same 8 families, 40 late stage alevins (ca. 774 degree days), near emergence, were haphazardly sub-sampled from the family-specific incubation trays and photographed using the Pixera Viewfinder 2.6 software application (Pixera Corp., Los Gatos, USA). Fork length (cm), total body surface area (mm^2) and yolk surface area (mm^2) were recorded using ImageJ 1.37v processing and analysis software (ImageJ, http://rsbweb.nih.gov/ij/index.html). Following measurements, the animals were placed into individual microcentrifuge tubes containing 98% ethanol for subsequent PCR analysis.

3.2.5: Data Analyses

Mixed model, nested, two-way ANOVA's were performed to test for the effects of family origin and genotype (transgenic or non-transgenic) on the response variables of mass (mg) and oxygen consumption (mg O_2 g^{-1} hr^{-1}), where genotype was nested within family. Family and

genotype were treated as random and fixed factors, respectively. For first-feeding respirometry, families were split into two groups and placed in separate tanks; therefore tank was included as a fixed factor.

To test for differences in hatch time between families and genotypes, a mixed effects binomial logistic regression was fit, where the response variable represented the proportion of all individuals carrying the transgene. Explanatory variables including hatch day (represented by degree days) and family where treated as fixed and random factors, respectively. To test for the effects of family and genotype on yolk surface area (mm^2), mass (g) and fork length (mm) of alevins near emergence, mixed model, two-way ANOVAs were used with genotype nested into family.

To explore associations between metabolic rate, size and development during early ontogeny, a series of Pearson's product-moment correlations were performed with family-level means. The first set explored the relationship between initial dry egg mass and degree days at 50% hatch, mass-independent egg oxygen consumption (mg O_2 hr^{-1}), alevin yolk area, mass and length near emergence. The second set investigated family-level mean correlations between egg O_2 consumption (mg O_2 g^{-1} hr^{-1}) and degree days at 50% hatch, alevin O_2 consumption (mg O_2 g^{-1} hr^{-1}) and alevin yolk area near emergence, and degree days at 50% hatch and alevin yolk area near emergence. All data were analyzed using the R statistical software application (version: R-2.9.2.; http://www.r-project.org/).

For all non-correlative data, statistical inference followed a model comparison approach using the Akaike information criterion (AIC; Burnham and Anderson 2004; Hobbs and Hilborn 2006). Each model can be viewed as a competing hypothesis where the relative weight of each hypothesis is compared using model selection measures of relative performance (Δ_i) and Akaike weights (w_i). Δ_iAIC ($\Delta_i AIC_c$: corrected for small sample size) refers to the change in AIC

between the focal model and the model with lowest AIC score. Δ_iAIC interpretation adhered to the following guidelines. Relative to other candidate models, strong evidence or support was considered for models with $\Delta_i \leq 2$, little evidence for models with $4 < \Delta_i < 7$ and no support for models with $\Delta_i > 10$. Akaike weights represent the probability that the focal model provides the best representation of the data relative to the other candidate models following repeated analyses. For all response variables mentioned above, two or three models were compared to assess the relative influence of family and genotype (transgenic vs. non-transgenic). Model selection criterion between fixed and mixed models is not appropriate (Bolker 2008), therefore, a separate set of fixed and mixed models were produced. Interpretation is based predominately on the mixed models as they capture the experimental design most appropriately. However, the fixed effects models were used to support inferences on the influence of the fixed effect (genotype) variable.

3.3: Results

3.3.1: Respirometry

At both the eyed-egg and alevin stages, oxygen consumption (MO_2) and mass were strongly influenced by family, with little influence caused by the transgene (Figure 3.1). Candidate models for oxygen consumption (MO_2; Table 3.1) and mass (Table 3.2) provided strong evidence that family origin was the most influential predictor for both eyed-egg and alevin stages. The overall mean oxygen consumption of transgenics was slightly higher than non-transgenics during the eyed-egg stage, with the trend reversing at the alevin stage. However, the presence or absence of the transgene had effectively no predictive value at the eyed-egg and alevin stages. Similarly, at the first-feeding stage, the transgene had no influence on oxygen consumption (mean ± S.E.; Transgenic: 0.170 ± 0.004 mg O_2 g^{-1} hr^{-1}; Non-transgenic: 0.164 ±

0.007 mg O_2 g^{-1} hr^{-1}) or mass (Transgenic: 0.187 ± 0.007g; Non-transgenic: 0.172 ± 0.01g). All candidate models for the oxygen consumption of first-feeding fry provided strong support for the data, however, neither model had substantial Akaike weight (Tables 3.1 and 3.2). This reflects the little to no relationship observed between the explanatory and response variables. Candidate models for the mass of first-feeding fry provided strong evidence that holding tank was a strong predictor. This likely reflects a family effect; specifically a bias for larger families in one holding tank over the other.

3.3.2: Development

The majority of individuals (> 60 %) within each family hatched over a three to four day period (Figure 3.2). The proportion of transgenics that hatched was strongly influenced by both family and hatch time (Table 3.3), such that transgenic individuals tended to hatch less than one day (i.e. 4 degree days) earlier (mean ± S.E.; Transgenic: 493.8 ± 8.2 degree days; Non-transgenic: 497.2 ± 8.1 degree days). The model representing family had strong support; however, the Akaike weight indicated this was unlikely to be the best candidate model, whereas hatch time had virtually no support. Thus, to some extent, the effect of transgenesis on hatch time was dependant on family. Comparing the time to hatch of transgenic and non-transgenic individuals within families indicated that the tendency for transgenics to hatch earlier was strong in some families (e.g. E, F; Figure 3.2) and weak in others (e.g. A, G).

Near emergence (i.e. the start of exogenous feeding), transgenic alevins had a slightly greater amount of yolk remaining than non-transgenics (mean ± S.E.; Transgenic: 13.38 ± 0.27 mm^2; Non-transgenic: 12.99 ± 0.26 mm^2). The amount of yolk-sac remaining was represented best by a model containing both family of origin and genotype (Table 3.4; Figure 3.3). With respect to both mass (Transgenic: 0.148 ± 0.001 g; Non-transgenic: 0.151 ± 0.002 g) and length

(Transgenic: 25.08 ± 0.09 mm; Non-transgenic: 25.26 ± 0.12 mm), non-transgenic alevins were slightly larger than the transgenics. Mass and length were also represented best by models containing both family of origin and genotype (Table 3.4; Figure 3.3). Similar to hatch time, there was considerable variation between families such that not all followed these overall trends.

Oxygen consumption increased with size during early ontogeny. Initial egg dry mass correlated strongly with all but one of the measured variables (Table 3.5). Positive associations with mass-independent egg oxygen consumption, alevin yolk area, mass and length near emergence were found. In contrast, there was no correlation between initial egg dry mass and degree days at 50% hatch. Nor were there correlations between egg O_2 consumption (mg O_2 g^{-1} hr^{-1}) and degree days of 50% hatch, alevin O_2 consumption (mg O_2 g^{-1} hr^{-1}) and alevin yolk area or degree days of 50% hatch and alevin yolk area.

3.4: Discussion

Family differences had a stronger influence on the routine metabolism and developmental rate of Atlantic salmon during early ontogeny than did GH transgenesis (Figures 3.1, 3.3 and 3.4). Transgenesis did not affect the oxygen consumption of individuals at the eyed-embryo, alevin (larval) or first-feeding fry (juvenile) stages. Hatching followed the characteristic pattern observed for salmonids, where the majority of individuals (> 60%) within each family hatched over a three or four day period (Gustafson-Marjanen and Dowse 1983) and the effect of transgenesis was weak. Transgenic fish hatched less than one day earlier than their non-transgenic siblings. Conversely, near emergence, transgenic individuals contained more yolk and were smaller in terms of both mass and length. However, the influence of genotype on all these measures was less than that of family, suggesting that family of origin contributes more to the variation of these traits than the GH transgene.

The vulnerability of salmonid eggs to low oxygen conditions has been demonstrated previously (Peterson and Quinn 1996; Rubin and Glimsater 1996; Einum et al. 2002). If GH transgenesis were to affect the basal metabolic rate of Atlantic salmon eggs, there could be survival differences during egg incubation relative to non-transgenic individuals (Alderdice et al. 1958; Sundt-Hansen et al. 2007). However, both metabolic and developmental measurements were similar between transgenic and non-transgenic eggs of Atlantic salmon, suggesting that the threat of exposure to periods of hypoxia in the gravel beds would be similar.

The transition from endogenous to exogenous feeding at emergence is considered a critical period of survival for stream salmonids (Elliott 1994; Einum and Fleming 2000; Nislow et al. 2004). Suitable spawning habitat can contain dense aggregations of nests, a situation that results in density-dependant competition among emerging fry for foraging territories (Einum and Nislow 2005; Einum et al. 2008). Body size at emergence and timing of emergence are thought to be important determinants of survival during this period. Larger fish tend to win laboratory-based contests against smaller fish, and this has been shown to carry over in the performance of individuals in wild release experiments (Johnsson 1993; Rhodes and Quinn 1998; Einum and Fleming 2000). However, the advantages and disadvantages of emerging early or late, relative to the rest of the population, are likely dependant on local environmental conditions. Early emergence may provide the beneficial opportunity to establish prime foraging territories (prior residency), and perhaps, an additional chance to grow (Cutts et al. 1999; O'Connor et al. 2000; Johnsson and Forser 2002). Conversely, environmental stressors such as temporal variation in predation pressure, food resources and suitable habitat characteristics provide possible selective pressures against early emergence (Brannas 1995; Jensen and Johnsen 1999; Nislow et al. 2000). Thus, transgene-induced changes in body size at emergence and/or the timing of emergence, in either direction, have the potential to influence the fitness of the transgene in nature.

While consistent differences were observed in the yolk area, mass and length measures between transgenic and non-transgenic alevins close to emergence, such small differences may not have large effects on relative fitness at emergence. More importantly, family of origin was responsible for more variation in alevin characteristics than was the transgene. From a population perspective, the most dominant trait influencing emergence time may be spawning time (Brannon 1987; Heggberget 1988; Fleming 1996). If we are to assume the transgene does not influence female spawn time then the key traits influencing fitness at emergence are the rate of development (emergence time) and size at emergence. In the current study, non-transgenic alevins contained less yolk reserves and were slightly larger near emergence, suggesting that transgenic Atlantic salmon may be competitively disadvantaged at the onset of first-feeding. However, the differences in the mean value of these three measurements between transgenic and non-transgenic individuals were less than 5%. Stream-dwelling salmonid fry have demonstrated considerable variation in the amount of yolk remaining at emergence (De Leaniz et al. 2000; Skoglund and Barlaup 2006). Thus, the small differences observed in yolk reserves between transgenics and non-transgenics suggest that emergence time would be similar, unless the transgene affects the emergence behaviour of yolk-sac fry. Previous studies assessing the effect of emergence time on performance in the wild have found that early emergence provides a competitive advantage. Such studies, however, have either compared individuals with substantial differences in emergence time (5.6 days; Einum and Fleming 2000) or the early emerging group had a confounding, albeit natural, size advantage (Nislow et al. 2004). The body size differences detected in the current study, using photo imaging software capable of measuring small differences, may be so small as to not influence contests for foraging territories in stream salmonids as suggested by previous behavioural experiments (Johnsson et al. 1999; Metcalfe et al. 2003; Moreau et al. 2011). Thus, the high levels of family variation combined with the small

transgene–induced differences in characteristics of emerging alevin siblings suggest that transgenesis may not have a considerable influence on fitness at first-feeding.

The similarity in metabolic and developmental rate measures of GH transgenic and non-transgenic Atlantic salmon siblings' contrast with observations made with GH transgenic coho salmon during early ontogeny. GH transgenic coho salmon have been shown to experience increased mortality under hypoxic conditions (Sundt-Hansen et al. 2007), hatch 2-3 days earlier (Devlin et al. 2004a; Lohmus et al. 2010) and emerge from the gravel 1-2 weeks earlier (Sundstrom et al. 2005; Lohmus et al. 2010). An increased sensitivity to hypoxic conditions suggests higher basal metabolic rates (Metcalfe et al. 1995), as observed in older GH transgenic salmonids (Cook et al. 2000; Deitch et al. 2006). A higher metabolic rate during early ontogeny may speed the mobilisation of yolk-sac reserves to body tissues and/or for maintenance processes (Metcalfe et al. 1995) and is thus a plausible explanation for observations of advanced development to first-feeding and greater susceptibility of eyed eggs to low oxygen conditions in GH transgenic coho salmon carrying the OnMTGHI gene construct. However, the current study has shown the opAFP-GHc2 gene construct (EO-1α line) has little to no phenotypic effect on pre-emergent Atlantic salmon. This suggests there are ecologically important phenotypic differences between two GH transgenic lines during a critical period of survival.

Elevated respiratory metabolism has been shown to correlate with fast growth (Metcalfe et al. 1995; Yamamoto et al. 1998), foraging-induced aggression and dominance (Cutts et al. 1998; Cutts et al. 2001; McCarthy 2001; Lahti et al. 2002) in juvenile salmon. In addition, it is hypothesized that higher basal metabolic rates concomitantly increase energy requirements that are addressed by a suite of compensatory behavioural changes toward greater foraging motivation and risk taking actions (Cutts et al. 2002; Biro et al. 2006; Careau et al. 2008). GH transgenic coho salmon juveniles, from as young as first-feeding have shown changes in behaviour and

performance that are consistent with this hypothesis (Devlin et al. 2004b; Sundstrom et al. 2004). The current study is the first to measure the respiratory metabolism of GH transgenic salmon fry at first-feeding and we find no effect of transgenesis on the metabolic rate of fry up to one month following emergence. Our results support the findings of a study conducted concurrently, where Moreau et al. (2011) observed no differences in the competitive ability or survival of first-feeding GH transgenic and non-transgenic Atlantic salmon fry reared in low feed, near-natural stream environments. Collectively, our work with GH transgenic Atlantic salmon indicates a delay in the phenotypic expression of the transgene, suggesting that fitness may not be affected during this critical period of early ontogeny. Previous measurements on GH transgenic Atlantic salmon parr (> 2 months post-emergence) have demonstrated elevated routine metabolic rates that are consistent with shifts in behaviour and performance relative to the non-transgenics (Stevens et al. 1998; Abrahams and Sutterlin 1999). We have observed changes in growth prior to this time (personal observations). The absence of an effect during this most critical period of survival, however, suggests that the early recruitment of transgenic parr may be similar to that of non-transgenic individuals in the wild.

The relationships between metabolic rate, size and development, while based on a small sample of families, are consistent with the patterns observed in previous studies. Oxygen consumption increased with size in eyed-eggs; however, did not correlate with any measures of development (hatch time and alevin yolk area). Moreover, hatch time was unrelated to egg mass and alevin yolk area near emergence. These relationships, or lack thereof, are consistent with previous studies (Einum and Fleming 1999; Einum et al. 2002; Valdimarsson et al. 2002; Pakkasmaa et al. 2006).

In the current study, we controlled for genetic background by comparing transgenic and non-transgenic full siblings such that we accounted for intra-population variation in the traits

measured. We found that family of origin explained considerably more trait variation than did transgenesis. Pakkasmaa et al. (2006) found a similarly strong family effect on the metabolic rate of Arctic charr (*Salvelinus alpinus*) eyed-eggs. This suggests that selection acting upon the GH transgene during early life history may be overshadowed by selection acting at the family level. This finding is relevant to understanding the potential implications of the offspring of GH transgenic Atlantic salmon escapees. Firstly, it suggests that there may not be a set of environmental conditions that differentially affect the fitness of the transgene during early life history, because any fitness effect would be less than that acting on trait variation due to other parental effects. Secondly, as transgenic invasions would most often occur in systems with different background strains, any fitness differences during early life history may represent differences in background genotype more so than differences due to transgenesis. Thus, the strong effect of family contributes to the uncertainty in predicting the fate of the transgene in nature.

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Table 3.1. Candidate models (ANOVA) describing the effects of family origin and growth hormone transgenesis on the routine oxygen consumption (mg O_2 g^{-1} hr^{-1}; response variable) of Atlantic salmon (*Salmo salar*) at three stages of early life history. k represents the number of predictors in each model. Model fit is represented by comparing $\Delta_i AIC_c$ and Akaike weights (w_i). $\Delta_i AIC_c$ refers to the change in AIC_c between one model and the model with lowest AIC_c score and w_i refers to the probability that the focal model provides the best representation of the data relative to the other candidate models following repeated analyses (w_i sum to 1.0). Both fixed and mixed models are presented because a single selection criterion was inappropriate. Interpretation is based predominately on the mixed models, with fixed effects models used for inferences about the fixed effect variable (genotype).

Model Type	**Explanatory Variables**	k	AIC_c	$\Delta_i AIC_c$	w_i
Egg Respirometry					
Mixed effects	Genotype nested within Family	3	-381.25	0	0.67
Mixed effects	Family	2	-379.81	1.44	0.33
Fixed effects	Genotype nested within Family	3	-387.16	0	0.70
Fixed effects	Family	2	-384.99	2.16	0.24
Fixed effects	Genotype	2	-382.25	4.90	0.06
Alevin Respirometry					
Mixed effects	Genotype nested within Family	3	-208.60	0.72	0.41
Mixed effects	Family	2	-209.30	0	0.59
Fixed effects	Genotype nested within Family	3	-211.44	3.55	0.14
Fixed effects	Family	2	-215.00	0	0.84
Fixed effects	Genotype	2	-207.00	8.01	0.02
Fry Respirometry					
Fixed effects	Tank and Genotype	3	-155.47	0.03	0.35
Fixed effects	Tank	2	-155.50	0	0.36
Fixed effects	Genotype	2	-155.41	0.39	0.29

Table 3.2. Candidate models (ANOVA) describing the effects of family origin and growth hormone transgenesis on the mass (mg; response variable) of Atlantic salmon (*Salmo salar*) at three stages of early life history. k represents the number of predictors in each model. Model fit is represented by comparing $\Delta_i AIC_c$ and Akaike weights (w_i). $\Delta_i AIC_c$ refers to the change in AIC_c between one model and the model with lowest AIC_c score and w_i refers to the probability that the focal model provides the best representation of the data relative to the other candidate models following repeated analyses (w_i sum to 1.0). Both fixed and mixed models are presented because a single selection criterion was inappropriate. Interpretation is based predominately on the mixed models, with fixed effects models used for inferences about the fixed effect variable (genotype).

Model Type	**Explanatory Variables**	k	AIC_c	$\Delta_i AIC_c$	w_i
Egg Respirometry					
Mixed effects	Genotype nested within Family	3	-260.60	2.45	0.23
Mixed effects	Family	2	-263.10	0	0.77
Fixed effects	Genotype nested within Family	3	-283.90	1.53	0.32
Fixed effects	Family	2	-285.40	0	0.68
Fixed effects	Genotype	2	-202.70	82.72	0
Alevin Respirometry					
Mixed effects	Genotype nested within Family	3	-252.40	3.13	0.17
Mixed effects	Family	2	-255.60	0	0.83
Fixed effects	Genotype nested within Family	3	-264.06	5.36	0.06
Fixed effects	Family	2	-269.43	0	0.94
Fixed effects	Genotype	2	-216.13	53.30	0
Fry Respirometry					
Fixed effects	Tank and Genotype	3	-130.75	0.52	0.44
Fixed effects	Tank	2	-131.27	0	0.56
Fixed effects	Genotype	2	-117.79	13.48	0

Table 3.3. Candidate models (binomial logistic regression) describing the effects of family origin and growth hormone transgenesis on the hatch time of Atlantic salmon (*Salmo salar*). The response variable represents the proportion of hatched individuals carrying the transgene. k represents the number of predictors in each model. Model fit is represented by comparing Δ_iAIC and Akaike weights (w_i). Δ_iAIC refers to the change in AIC between one model and the model with lowest AIC score and w_i refers to the probability that the focal model provides the best representation of the data relative to the other candidate models following repeated analyses (w_i sum to 1.0). Both fixed and mixed models are presented because a single selection criterion was inappropriate. Interpretation is based predominately on the mixed models, with fixed effects models used for inferences about the fixed effect variable (genotype).

Model Type	**Explanatory Variables**	***k***	**AIC**	**Δ_iAIC**	**w_i**
Hatch Time					
Mixed effects	Degree days and Family	3	124.11	0	0.82
Mixed effects	Family	2	127.15	3.05	0.18
Fixed effects	Degree days and Family	3	256.93	0	0.96
Fixed effects	Family	2	263.41	6.48	0.04
Fixed effects	Degree Days	2	267.93	11.00	0

Table 3.4. Candidate models (ANOVA) describing the effects of family origin and growth hormone transgenesis on alevin physical characteristics [response variables: yolk-sac area (mm^2), mass (g), and length (mm)] of Atlantic salmon (*Salmo salar*). k represents the number of predictors in each model. Model fit is represented by comparing Δ_iAIC and Akaike weights (w_i). Δ_iAIC refers to the change in AIC between one model and the model with lowest AIC score and w_i refers to the probability that the focal model provides the best representation of the data relative to the other candidate models following repeated analyses (w_i sum to 1.0). Both fixed and mixed models are presented because a single selection criterion was inappropriate. Interpretation is based predominately on the mixed models, with fixed effects models used for inferences about the fixed effect variable (genotype).

Model Type	**Explanatory Variables**	k	**AIC**	Δ_i**AIC**	w_i
Yolk-sac Area					
Mixed effects	Genotype nested within Family	3	1056.42	0	0.90
Mixed effects	Family	2	1060.83	4.41	0.10
Fixed effects	Genotype nested within Family	3	1024.75	0	0.74
Fixed effects	Family	2	1026.82	2.08	0.26
Fixed effects	Genotype	2	1439.45	414.71	0
Mass					
Mixed effects	Genotype nested within Family	3	-2212.50	0	0.99
Mixed effects	Family	2	-2203.70	8.80	0.01
Fixed effect	Genotype nested within Family	3	-2253.73	0	0.99
Fixed effects	Family	2	-2244.62	9.11	0.01
Fixed effects	Genotype	2	-1605.21	648.52	0
Length					
Mixed effects	Genotype nested within Family	3	578.20	0	0.88
Mixed effects	Family	2	582.17	3.97	0.12
Fixed effects	Genotype nested within Family	3	557.37	2.30	0.24
Fixed effects	Family	2	555.07	0	0.76
Fixed effects	Genotype	2	794.06	238.99	0

Table 3.5. Pearson's product-moment correlations performed using family-level means to explore associations between egg characteristics of transgenic and non-transgenic Atlantic salmon (*Salmo salar*). Egg characteristics included initial dry mass (M_{egg}), degree days at 50% hatch ($dd_{50\%}$) and mass-independent oxygen consumption ($eggMO_{2\ m\text{-}i}$; mg O_2 hr^{-1}), and alevin characteristics near emergence, including yolk area (A_{yolk}), wet mass (M_{alevin}) and length (L_{alevin}).

Variables	Genotype	n	*r*	95% C.I.	P-value
M_{egg} vs. $dd_{50\%}$	Transgenic	8	0.17	$-0.51 \leq r \leq 1$	0.340
	Non-transgenic	8	0.16	$-0.52 \leq r \leq 1$	0.350
M_{egg} vs. $eggMO_{2\ m\text{-}i}$	Transgenic	6	0.82	$-0.02 \leq r \leq 0.98$	0.054
	Non-transgenic	6	0.90	$0.31 \leq r \leq 0.99$	0.016
M_{egg} vs. A_{yolk}	Transgenic	8	0.90	$0.63 \leq r \leq 1$	0.001
	Non-transgenic	8	0.85	$0.48 \leq r \leq 1$	0.004
M_{egg} vs. M_{alevin}	Transgenic	8	0.88	$0.57 \leq r \leq 1$	0.002
	Non-transgenic	8	0.88	$0.57 \leq r \leq 1$	0.002
M_{egg} vs. L_{alevin}	Transgenic	8	0.88	$0.57 \leq r \leq 1$	0.002
	Non-transgenic	8	0.94	$0.76 \leq r \leq 1$	0.000
$eggMO_2$ vs. $dd_{50\%}$	Transgenic	6	0.41	$-1 \leq r \leq 0.88$	0.79
	Non-transgenic	6	0.49	$-1 \leq r \leq 0.90$	0.84
$alevinMO_2$ vs. A_{yolk}	Transgenic	3	-0.97	n/a*	0.06
	Non-transgenic	3	-0.66	n/a*	0.27
$dd_{50\%}$ vs. A_{yolk}	Transgenic	8	0.16	$-0.52 \leq r \leq 1$	0.35
	Non-transgenic	8	0.23	$-0.46 \leq r \leq 1$	0.29

* Sample size was not large enough to generate confidence intervals

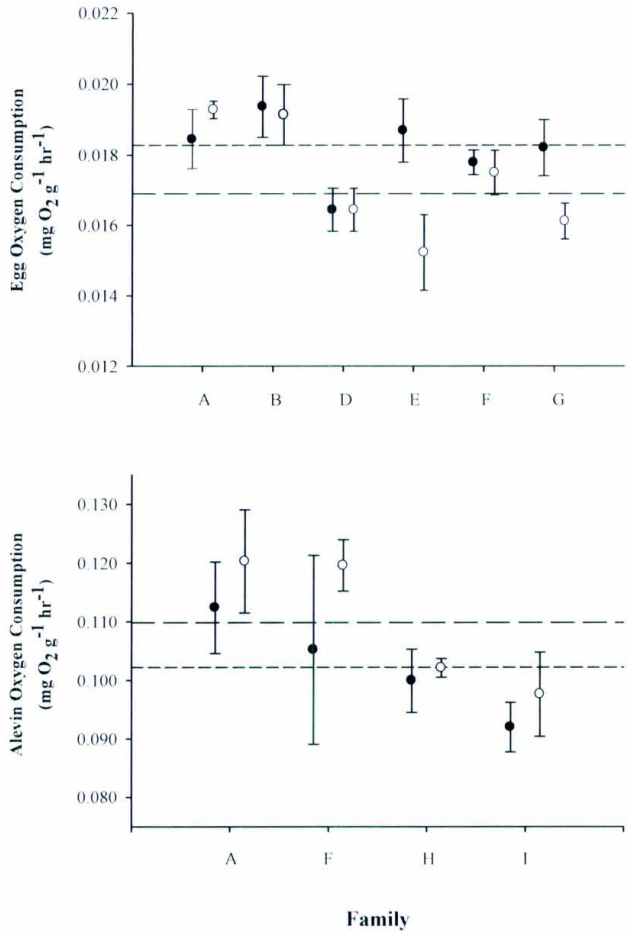

Figure 3.1. The mean (±S.E.) routine oxygen consumption (mg O_2 g^{-1} hr^{-1}) of transgenic and non-transgenic Atlantic salmon (*Salmo salar*) eyed-egg and alevin full siblings. Transgenic and non-transgenic mean values within families are represented by black and white circles, respectively. The short and long dashed lines represent the overall transgenic and non-transgenic means, respectively.

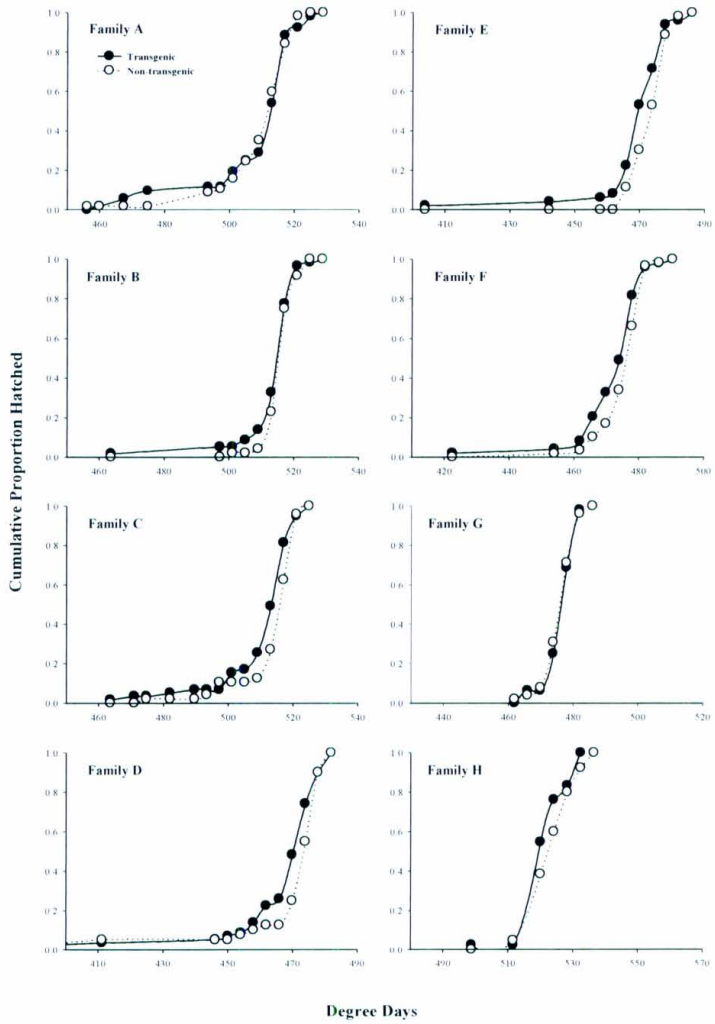

Figure 3.2. The time of hatch (degree days) of full-sibling transgenic and non-transgenic Atlantic salmon (*Salmo salar*) from eight families. These data are represented as cumulative proportions of approximately 100 individuals per family.

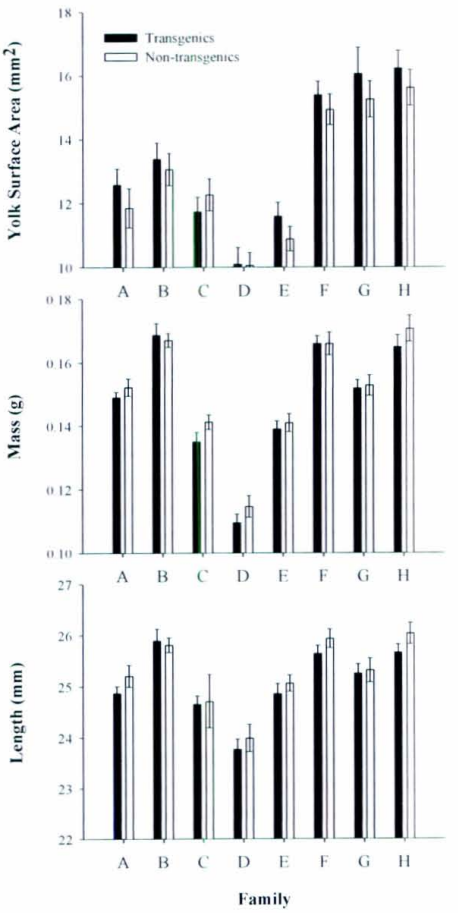

Figure 3.3. Mean (±S.E.) yolk surface area (mm^2), mass (g), and fork length (mm) of transgenic and non-transgenic Atlantic salmon (*Salmo salar*) alevins near emergence.

Chapter 4

Reproductive performance of alternative male phenotypes of growth hormone transgenic Atlantic salmon (*Salmo salar*)

Abstract

Growth hormone (GH) transgenic Atlantic salmon (*Salmo salar*) are one of the first transgenic animals being considered for commercial farming, yet ecological and genetic concerns remain should they enter the wild and interact reproductively with wild fish. Here we provide the first empirical data reporting on the breeding performance of GH transgenic Atlantic salmon males, including that of an alternative male reproductive phenotype (i.e. small, precocially mature parr), in pair-wise competitive trials within a naturalised stream mesocosm. Wild anadromous (i.e. large, migratory) males outperformed captively-reared transgenic counterparts in terms of nest fidelity, quivering frequency and spawn participation. Similarly, despite displaying less aggression, captively-reared non-transgenic mature parr were superior competitors to transgenic counterparts in terms of nest fidelity and spawn participation. Moreover, non-transgenic parr had higher overall fertilisation success than transgenic parr and their offspring were represented in more spawning trials. Although transgenic males displayed reduced breeding performance relative to non-transgenics, both male reproductive phenotypes demonstrated the ability to participate in natural spawning events and thus, the potential to contribute genes to subsequent generations.

4.1: Introduction

Growth-enhancing transgenic biotechnologies have attracted considerable interest from the global aquaculture industry, particularly with regards to Atlantic salmon. However, similar to domesticated strains, concerns have been raised regarding the ecological and genetic effects that may arise if these organisms were to enter the wild (Kapuscinski and Hallerman 1991; Devlin et al. 2006; Kapuscinski et al. 2007). A principal concern involves the potential genetic impacts of fertile transgenic organisms interbreeding with wild populations into which their genes may introgress. For example, risk models indicate that Trojan gene effects may occur, whereby the transgene spreads by enhanced mating advantage but the resulting offspring have reduced viability, which leads to the eventual extinction of populations (Muir and Howard 1999, 2002; Howard et al. 2004). However, there has yet to be any empirical research documenting the ability of growth hormone (GH) transgenic Atlantic salmon to breed naturally and introgress with wild populations. Moreover, there is little understanding of the role that alternative reproductive phenotypes may play in such introgression.

The breeding system of Atlantic salmon exhibits two alternative male reproductive phenotypes, large anadromous adults that have migrated to sea and returned to their natal streams, and small precocial parr that have matured in freshwater, having never been to sea. Anadromous males develop specialized secondary sexual characters to fight other males and court for access to ovipositing females, while precocial parr mature, at a fraction of the size of the anadromous phenotype, use their small size and cryptic colouration to sneak fertilisations (reviewed in Fleming 1996). Both male reproductive phenotypes may form dominance hierarchies among themselves for access to spawning females through aggressive behavioural interactions. While the fertilisation success of anadromous males is typically greater than that of mature parr, reports of precocial parr fertilization rates have ranged from 11-65% of the available

eggs (reviewed in Fleming and Reynolds 2004). Thus, both male reproductive phenotypes can contribute substantially to the next generation and represent potential routes for the introduction of transgenes into wild populations.

The extent of transgene introgression into wild populations would depend on the fitness of transgenic individuals in the receiving environment, which may vary along a continuum featuring high fitness, leading to the fixation of the transgene, at one end and low fitness, leading to its elimination within a few generations, at the other (Muir and Howard 1999, 2002). Perhaps more commonly, however, the fitness of transgenic organisms would lie between these poles and create, for example, an outbreeding depression scenario where transgene-induced maladaptive traits pose a threat to the viability of the entire receiving population (Hedrick 2001).

This outbreeding depression scenario is representative of the concerns associated with wild salmonid populations exposed to strains that have experienced domestication selection (McGinnity et al. 2003; Tymchuk et al. 2007; Fraser et al. 2008). In Atlantic salmon, anadromous adults from aquaculture strains (farmed) exhibit atypical spawning behaviour, including reductions in aggressive displays toward other males, quivering and nest fidelity, which may contribute to observations of reduced reproductive success (Fleming 1996; Fleming et al. 2000; Weir et al. 2004). In contrast, studies exploring the relative reproductive behaviour and success of mature farmed and wild parr have found that farmed parr perform similarly to or better than wild parr (Garant et al. 2003; Weir et al. 2005). Regardless of the relative spawning success of farmed and wild males, both reproductive phenotypes have demonstrated the potential for the introgression of farmed genes into wild populations and the disruption of locally adapted phenotypic traits (Hindar et al. 2006; Garcia de Leaniz et al. 2007; Fraser et al. 2010).

Comparisons of reproductive performance between growth hormone (GH) transgenic and non-transgenic salmonids are limited. Similar to observations with farmed adults, previous

efforts have reported reduced reproductive performance in hatchery-reared transgenic relative to wild coho salmon (*Oncorhynchus kisutch*; Bessey et al. 2004; Fitzpatrick et al. 2011). While these results represent the expectations of a first generation transgenic escapee scenario, GH transgenic Atlantic and coho salmon represent two species carrying two unique transgene constructs with two distinct life histories (e.g. rarely do coho salmon mature precocially as parr; Fleming 1998). For example, previous work has demonstrated differences in the onset of transgene-induced phenotypic expression between the two species, which may have important implications for early survival (Sundstrom et al. 2004, 2005; Lohmus et al. 2010; Moreau et al. 2011). Potentially more important are the distinct differences in reproductive phenotypes that may have important implications for introgression (Valosaari et al. 2008), as seen in the reproductive performance differences between anadromous and mature parr Atlantic salmon males of farmed origin (Fleming 1996; Fleming et al. 2000; Garant et al. 2003; Weir et al. 2005).

The aim of this study was to compare the breeding performance of growth hormone transgenic and non-transgenic Atlantic salmon males of both alternative reproductive phenotypes to test for the potential of the transgene to introgress into wild populations. We conducted two separate experiments in a naturalised stream mesocosm. First, to assess the ability of first-generation, farmed transgenic males to contribute reproductively, the breeding behaviour and participation of captively-reared, anadromous transgenic males (approximating farmed fish) were observed in pair-wise competitive trials with wild males, as well as alone with wild females. Second, to assess the ability of transgenic fish to contribute reproductively as precocial parr, the breeding behaviour, performance and reproductive success of captively-reared, transgenic and non-transgenic precocial parr were compared in pair-wise competitive trials.

4.2: Methods

4.2.1: Experimental Fish

In 1989, a transgene construct consisting of growth hormone cDNA from Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), and an ocean pout, *Macrozoarces americanus* L., antifreeze protein gene promoter (opAFP-GHc2) was inserted into the genome of wild Atlantic salmon collected from the Exploits and Colinet Rivers, Newfoundland, Canada (Du et al. 1992). A stable transgenic line was created (EO-1α; Yaskowiak et al. 2006) and has since been cultured at the Ocean Sciences Centre (OSC), Memorial University of Newfoundland. The competitive breeding trials between transgenic and wild anadromous salmon were conducted in 2006 and involved fifth and sixth generation anadromous males from this captive transgenic line. Wild anadromous males and females for these trials were collected from the Exploits River (48°55'N, 55°40'W), Newfoundland, Canada, in September of that year and transferred to the OSC. Parr, both mature and immature individuals, were also included in the 2006 trials to simulate the natural structure of the breeding system. They were derived from eight single pair crosses produced in the fall of 2004 that involved wild, Exploits River salmon, with the subsequent offspring captively-reared to the parr stage at the OSC.

The competitive breeding trials to assess the ability of transgenic relative to non-transgenic fish to contribute reproductively as precocial parr were undertaken in 2007. The mature transgenic parr were age 0+, having been produced in the fall of 2006 by eight single pair crosses between St. John River (aquaculture strain) males, hemizygous for the EO-1α transgene, and wild Exploits River females. True to Mendelian inheritance patterns, crosses of hemizygous to wild-type individuals result in ca. half the offspring inheriting the GH transgene (Shears et al. 1992). Due to the tremendous growth induced by transgenesis, it is difficult to compare size and age matched transgenic and non-transgenic individuals. Therefore, to reduce these potential

sources of variation, half of the mature non-transgenic parr used in the trials were 0+ offspring from the above 2006 crosses and the other half were 1+ offspring from 5 single pair crosses in 2005 of wild, Exploits River parents. To facilitate natural breeding and competitive interactions, anadromous females and males, collected from the Exploits River during September 2007 were transferred to the OSC and used in the trials.

Prior to both the anadromous and parr competition experiments, all animals were housed in fibreglass tanks under a natural photoperiod and fed a standard salmonid dry feed (Corey Feed Mills, Fredericton, NB, Canada) *ad libitum*, 3-5 times weekly. Feeding of the anadromous transgenic fish ceased in early fall, preceding the breeding season (wild anadromous fish captured in early fall were not fed). Parr continued to be fed until they were introduced into the breeding trials. Prior to experimentation, all potential transgenic individuals were screened using the polymerase chain reaction (PCR) amplification protocol described in Deitch et al. (2006). To facilitate night behavioural observations in the breeding trails, the fish were exposed to a low-light regime with standard facility light installations. All measurement and tagging procedures were performed under mild anaesthesia (MS-222, Western Chemical Inc., Ferndale, USA) and fish were treated in accordance with the guidelines provided by the Canadian Council on Animal Care and with the approval of Memorial University's Institutional Animal Care Committee.

4.2.2: Experimental Design

A fully contained stream mesocosm was constructed out of a large, indoor concrete raceway and used for the competitive breeding trials (Figure 4.1). To divide the mesocosm into two replicate breeding channels (1.25 m × 7.8 m × 0.25 m), a fibreglass partition was placed along the centre of the mesocosm and screens of plastic mesh fencing, framed with PVC pipe, were installed at each end. Two external pumps (1.5 hp, Dynamo®, Pentair Water Pool and Spa,

Inc., Sanford, NC, USA) were placed at opposite ends of the mesocosm to generate a unidirectional, circulating current (mean ± SE: 22.3 ± 0.24 cm/s). The bottom of the mesocosm was covered with cobble (~5-10 cm diameter; ~40 cm deep) and large rocks (20-30 cm diameter) to naturalise the breeding channels and provide the salmon with nest substrate.

Anadromous Male Experiments

The behaviour of anadromous transgenic and non-transgenic males was compared during pair-wise competitive breeding trials between 18 November – 16 December 2006. Each trial consisted of a single female, a focal pair of anadromous males and a complement of parr (5 mature males and 10 immature) to naturalise the mesocosm with respect to the Atlantic salmon breeding system. Six weeks prior to the onset of experimentation, fork length (cm) and mass (g) measurements were recorded for all anadromous males and females. It was not possible to size-match competing anadromous males due to substantial size differences between the transgenic and non-transgenic fish (Table 4.1). To allow for individual identification, anadromous fish were marked with uniquely coded Petersen disc tags (3.4 cm diameter; Floy Tag and Manufacturing Inc., Seattle, WA, USA) just below the dorsal fin.

Each breeding trial (n=11) consisted of two phases; the competitive and non-competitive phases. The competitive phase included both the anadromous transgenic (n=11) and non-transgenic (n=11) males competing directly for breeding opportunities with the female. To separate the effects of courting and mate choice from intersexual competition on breeding performance, the non-competitive phase involved providing each of the transgenic (n=8) and non-transgenic (n=6) males sole access to the female. The order by which each of the two males had sole access to the female was alternated among trials. Each trial phase consisted of 1-5 spawning events (a female will spawn 3-8 times typically, depending on her size; Fleming 1996).

However, to standardize among trials, a maximum of two spawns per phase were included in the behavioural analyses. The duration of each phase was dependent on the spawning behaviour of the individuals, with a phase being terminated following two confirmed spawning events. In cases where no spawning occurred (n=4; all transgenic males in the absence of competition), a maximum duration of 36 hours was applied to each phase.

Precocious Male Parr Experiments

The behaviour of transgenic and non-transgenic precocious male parr were compared in pair-wise competitive breeding trials (n=11) between 15 November – 22 December 2007. Each trial consisted of an anadromous male and female pair, a focal pair of mature male parr and 4 immature parr (2 transgenic and 2 non-transgenic). In most cases, it was not possible to size-match competing mature parr due to substantial size differences between transgenic and non-transgenic parr (Table 4.1). Each breeding trial consisted of 1-4 spawning events, however, a maximum of the first two spawns per trial (referred to subsequently as spawn A or spawn B) were included in the behavioural analyses. Similar to the 2006 experiments, the anadromous fish were measured for fork length (cm) and mass (g), and tagged with uniquely coded Petersen disc tags, all of which was completed three weeks prior to the experiments. The parr to be used in the experiments were either tagged with a passive integrated transponder (PIT; model RI-TRP-WRHP; Texas Instruments Inc., Dallas, TX, USA; 23.1 x 3.9 mm and 0.6 g) or marked using visible implant elastomer (Northwest Marine Technology Inc., Shaw Island, WA, USA) six weeks prior to the experiment. PIT tags were inserted into the body cavity through a small, ventral incision made anterior to the pelvic girdle, which was closed with a single suture using surgical thread. For parr deemed too small for a PIT tag (i.e. < 10 cm), elastomer was injected ventrally, just under the skin with a fine needle to provide a small, unique mark. Just prior to the

beginning of each trial, the fork length (cm) and mass (g) of the experimental parr were measured. Adipose fin clips were collected following the trials for all fish involved and placed in 1.5 ml microcentrifuge tubes containing 99% ethanol and stored in a -20°C freezer.

Between 1-3 h following each spawning event, the trial was temporarily disrupted to collect the eggs laid for subsequent parentage analyses. Eggs were excavated from the gravel with the aid of a suction system based on the venturi effect and then counted, transferred into spawn-specific plastic mesh baskets and reared in Heath incubation trays. The effect of these disruptions appeared to be limited to the latency of breeding behaviour resumption, which ranged from 15 minutes to 3 hours.

4.2.3: Behavioural Observations

For the anadromous male experiments, breeding behaviour was monitored 24 h per day using a combination of live and recorded video observations. The video monitoring system included two overhead surveillance cameras, equipped with remote pan, tilt and zoom capabilities that recorded directly to a computer, and underwater cameras (SEA-CAM; Borel Manufacturing Inc., Alameda, CA, USA) positioned near female nest sites that recorded directly to individual HDD/DVD recorders. Each spawn was monitored with one overhead and 2-3 underwater cameras, simultaneously.

During the anadromous male experiments, behavioural data were collected for 60 min before (pre-spawn) and 30 min after (post-spawn) each spawning event. For trial phases where no spawning event occurred, observations were conducted for 5 min intervals every 30 min for the duration of the phase (i.e. a total of 360 min of observation time). The behaviours recorded included, nest fidelity, anadromous male-male aggression, quivering and spawn participation (Table 4.2).

For the precocious male parr experiments, breeding behaviour was also monitored 24 h per day. A PIT tag detection system was used in addition to live and recorded video observations from 3-4 underwater cameras stationed around the nest site. The PIT tag detection system monitored presence/absence and time data on parr around the nest site (Armstrong et al. 2001) and was designed in a manner similar to that detailed in Roussel et al. (2000). Each unit (n=2) consisted of a double gate loop antenna (100cm diameter) that was positioned so as to encircle an individual nest site of a spawning female. The antennae was connected to a PIT tag reader (model Series 2000; RI-CTL-MB2A; Texas Instruments Inc, Dallas, TX, USA) powered by a 12V battery. Data were input into a palmtop computer (Dell™ Axim™ X51, Round Rock, TX, USA) with a custom-designed software program (Roussel et al. 2000). Both cameras and PIT tag systems were positioned at nest sites shortly following female nest site selection (as indicated by the female's consistent digging at a focal site).

Based on observations conducted during the anadromous male experiments, parr behavioural data collection and analyses were adjusted to capture perceived differences between the two reproductive phenotypes. As such, behavioural data were collected over a continuous 75 minute period, 52.5 minutes before and 22.5 minutes after each spawning event. For analysis, these data were segregated into three time periods including the spawn period (12.5 minutes before and after the spawning event), the pre-spawn period (40 minutes prior to the spawn period) and the post-spawn period (10 minutes immediately after the spawn period). Behaviours recorded included, nest fidelity, parr-parr aggression, and spawn participation (Table 4.2).

4.2.4: Parentage Analyses

Parentage analyses were conducted exclusively for the mature parr experiments because the behavioural results from the anadromous male experiments made it unnecessary to assess

breeding success at the genetic level (see results, page 138). Shortly following hatching, a subsample of offspring from each spawn was placed in 1.5 ml microcentrifuge tubes with 99% ethanol and stored in a -20°C freezer. A total of 32 alevins were sampled from each spawn, unless fewer had survived. Parentage analyses were conducted on individuals from all 11 trials, with representation ranging from 1-4 spawns per trial, 27-119 eggs per trial and 13-32 eggs per spawn for a total of 715 eggs.

Microsatellite analyses were conducted at 3 highly polymorphic, tetranucleotide loci using primer sequences developed specifically for Atlantic salmon (*Ssa*202, O'Reilly et al. 1996; *SSsp*2215, *SSsp*2216, Paterson et al. 2004). The DNA of potential parents and offspring were extracted and purified using the Wizard® SV 96 Genomic DNA Purification System (Promega Corp. Madison, WI, USA), following the protocol provided by the manufacturer. PCR amplifications were performed in 10 µl solutions, containing 2-10 ng of sample DNA template, 0.2 mM of each dNTP, 0.5 µM of each of the labelled and unlabelled primers, 1* KCl buffer (10mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase. Thermal cyclers (model 2720, Applied Biosystems™, Foster City, CA, USA) were programmed under the following regime: (94°C for 2 min)*1, (94°C for 45 sec, 58°C for 45 sec, 72°C for 1 min)*35, (72°C for 15 min)*1, and finished with a 4°C hold. Subsequent to DNA amplification, the PCR products representing different primer sets from like samples were combined and purified using the MiniElute® 96 UF PCR Purification method (Qiagen Inc., Hilden, NRW, Germany), following the manufacturer's protocol. Microsatellite fragments were then separated and visualised with an Applied Biosystems™ 3130 Genetic Analyzer and the accompanying GeneMapper® 4.0 software (Applied Biosystems™, Foster City, CA, USA). Two known reference samples were used as standards and run on each plate to monitor for allele size shifts and function as an internal plate indicator.

Given that each spawn involved a single female and 3 potential males, we used an allele exclusion-based approach to assign parentage, where potential parents are eliminated on the basis of Mendelian inheritance patterns at primer loci (O'Reilly et al. 1998). Specifically, offspring genotypes were compared to all potential parental genotype combinations from all breeding trials, using a custom-designed Microsoft® Excel exclusion macro. In cases where multiple parental crosses shared the most complete genotypic match (allelic match at two or three loci) to an offspring, assignment was assumed to the parental cross representing the particular trial and spawn corresponding to that offspring. In no circumstance did two parental crosses from the same trial and spawn share the most complete genotypic match. Moreover, all offspring were successfully assigned to a parental cross corresponding to the trial and spawn from which they were collected. All exclusion–based assignments were corroborated with the likelihood-based assignments produced using Cervus 3.0.3 (Field Genetics Ltd., London, UK).

4.2.5: Statistical Analyses

For the anadromous male experiment, nest fidelity was modelled as a binomial logistic regression (LR_b) with trial and genotype (transgenic or non-transgenic) as explanatory variables. Pre-spawn and spawn periods were analysed separately for the competitive phase, however, all periods were summed during the non-competitive phase to allow for the comparison of the two genotypes because half of the transgenic males failed to spawn. Spawn participation was also modelled as a binomial logistic regression with explanatory variables that included genotype and phase. Quivering count data from pre-spawn and spawn periods were summed, as there were no differences between the periods, and a logistic regression with poisson error (LR_p) was fit, where genotype, phase and trial served as explanatory variables. For similar reasons, overt aggression count data were summed across spawn periods and phases and analysed with the Wilcoxon

signed rank test with continuity correction. In cases where data were available for multiple spawns within a phase, the mean value of the behavioural measure was used for analyses. All observations were standardised with respect to observation time.

Similar statistical models to those used for the competitive phase of the anadromous male experiments were used for analogous behavioural data in the mature parr experiments. Spawn identity (spawn A or B) was used in an analogous fashion to experimental phase in the anadromous male experiments. For analysis of male fertilisation success in the parr experiments, the number of eggs fathered by either the anadromous male, transgenic parr or non-transgenic parr from each trial was summed across spawns and tested using two approaches. First, a series of Wilcoxon signed rank tests were used to compare the relative fertilisation success between all three male types. Second, the overall proportions of offspring fertilized by transgenic and non-transgenic parr across all trials was compared by a two sample test of binomial proportions.

Any over-dispersed data were accounted for by applying an empirical scale parameter by specifying either quasi-likelihood binomial or poisson errors in the model. All data were analyzed using the R statistical software application (version: R-2.10.1.; http://www.r-project.org) following a hypothesis testing approach. Statistical significance was measured at a 5% alpha level of type I error.

4.3: Results

4.3.1: Anadromous Males

The captive-reared, transgenic males were significantly larger than the wild, non-transgenic males in terms of both mass (Table 4.1; paired t-test; $t_{1, 10} = 6.03$, $P < 0.001$) and length (paired t-test; $t_{1, 10} = 5.14$, $P < 0.001$). Despite a clear size advantage for transgenic males, there were no differences in the frequency of overt aggressive behaviours relative to non-

transgenic males (Figure 4.2A; Wilcoxon signed rank test: $V_{1, 10} = 34.20$, $P = 0.057$). However, non-transgenic males demonstrated a competitive advantage over transgenic males in all other breeding behaviours measured. In the presence of competition, non-transgenic males spent significantly more time at the nest with the females (nest fidelity) than did transgenic males during both the pre-spawn and post-spawn periods (Table 4.3). Non-transgenic males also had higher nest fidelity than transgenic males in the absence of competition; although the difference was much less. Moreover, unlike both the pre-spawn (LR_b; $\chi^2 = 0.40$, $P = 1$) and post-spawn (LR_b; $\chi^2 = 6.79$, $P = 0.731$) periods of direct competition, there was a significant trial effect on nest fidelity (LR_b; $\chi^2 = 20.56$, $P < 0.001$) in the absence of competition, indicative of the high variation in behaviour observed. The quivering frequency of non-transgenic males was greater than that of transgenic males (LR; $\chi^2 = 41.45$, $P < 0.001$); with no effect of competition (Figure 4.2; LR_p; $\chi^2 = 1.00$, $P = 0.606$) or trial (LR_p; $\chi^2 = 15.63$, $P = 0.111$). Furthermore, non-transgenic males participated in more spawning events than transgenic males regardless of the presence or absence of competition (Figure 4.3; LR_p; $\chi^2 = 22.60$, $P < 0.001$).

4.3.2: Precocious Male Parr

In trials involving 1+ non-transgenic and 0+ transgenic parr, there were no significant differences in mass (paired t-test; $t_{1, 5} = -1.37$, $P = 0.231$) and length (paired t-test; $t_{1, 5} = -1.63$, $P = 0.163$) between the two groups (Table 4.1). However, in trials where both parr types were age 0+, the transgenic parr were significantly larger than the non-transgenic parr in terms of both mass (paired t-test; $t_{1, 4} = -5.325$, $P = 0.006$) and length (paired t-test; $t_{1, 4} = -3.47$, $P = 0.026$). Similarly, when age is ignored and the above data are analysed collectively, the transgenic parr were significantly larger than the non-transgenic parr in terms of both mass (paired t-test; $t_{1, 10} = -3.42$, $P < 0.001$) and length (paired t-test; $t_{1, 10} = -3.26$, $P < 0.001$). There were no significant

differences in behaviour between trials involving 0+ and 1+ non-transgenic parr, thus these were combined for subsequent analyses. Transgenic parr performed more overt aggressive behaviours than non-transgenic parr (Figure 4.2; Wilcoxon signed rank test; $V_{1,\ 10} = 26.5$, $P = 0.042$). However, non-transgenic parr demonstrated greater nest fidelity than transgenic parr during all the comparisons save one; nest fidelity was similar during the post-spawn period of spawn A (Table 4.4). There were no trial effects observed on nest fidelity. Greater nest fidelity was accompanied by greater spawn participation by non-transgenic relative to transgenic parr (Figure 4.3; LR_b; $\chi^2 = 11.20$, $P < 0.001$) and the levels of participation were similar across spawns (LR_p; $\chi^2 = 0.13$, $P = 0.72$).

The fertilisation success of both transgenic and non-transgenic parr was low (Table 4.5). Wilcoxon signed ranked tests confirmed that anadromous males dominated both transgenic ($V_{1,\ 10} = 66.0$, $P < 0.001$) and non-transgenic ($V_{1,\ 10} = 66.0$, $P < 0.001$) parr in fertilization success across breeding trials. Furthermore, transgenic and non-transgenic parr fertilization success did not differ significantly across trials (Wilcoxon signed rank test; $V_{1,\ 10} = 16.0$, $P = 0.295$). The overall (trial ignored) fertilisation success of non-transgenic parr, however, was significantly higher than that of transgenic parr (binomial test; $\chi^2 = 15.98$, $P < 0.001$) and offspring fathered by non-transgenic parr were represented in more trials.

4.4: Discussion

This study provides the first empirical observation on the breeding of, and potential for transgene introgression by GH transgenic male Atlantic salmon, including that of alternative reproductive phenotypes. Transgenic anadromous males (i.e. large, fighter males), reared to maturity in captivity, were behaviourally outcompeted by their wild counterparts in terms of nest fidelity, quivering frequency and spawn participation. Similarly, despite having similar rearing

histories and displaying more aggression, transgenic male parr (i.e. precocially mature, sneaker males) were inferior competitors to wild-type parr in terms of nest fidelity and spawn participation. Moreover, wild-type parr had higher overall fertilisation success than transgenic parr and their offspring were represented in more spawning trials. Although transgenic males displayed reduced breeding performance relative to non-transgenics, both male reproductive phenotypes demonstrated the ability to participate in natural spawning events and, thus, the potential to contribute genes to subsequent generations.

The reduced reproductive performance of captively-reared, anadromous transgenic males relative to wild males parallels the results of similar studies comparing captively-reared salmon to wild salmon. Varying degrees of exposure to captive environments and domestication selection have been shown to affect the breeding behaviour and success of adult salmonids negatively (Fleming and Gross 1993; Fleming et al. 1997; Berejikian et al. 2001a; Weir et al. 2004). Moreover, Bessey et al. (2004) observed that wild-exposed coho salmon males out-competed captively-reared transgenic males in terms of spawn participation, courtship and aggressive behaviours. However, Bessey et al. (2004) also observed that when transgenic and non-transgenic males were both reared in the laboratory, performance was poor irrespective of transgenesis (see also Fitzpatrick et al. 2011). Thus, the captive rearing environment appears to diminish the competitive and reproductive performance of the anadromous salmonid phenotype, irrespective of genetic background (Berejikian et al. 1997, 2001a, 2001b). The current study can, therefore, not eliminate the possibility that the poor performance of the anadromous transgenic males has more to do with rearing environment than transgenesis because these variables were confounded. Nevertheless, comparisons of captively-reared transgenic and wild andromous males mimic the environmental differences that represent an initial transgenic escapee invasion

scenario and are thus valuable for predicting the probability of first generation intraspecific hybridisation.

Reproductively isolated populations are predicted to genetically diverge due to adaptive and/or non-adaptive evolutionary pressures, such as selection to environmental variation, genetic drift, gene flow and chance mutations (Frankham et al. 2002; Allendorf and Luikart 2007; Garcia de Leaniz et al. 2007; Carlson and Seamons 2008). This evolutionary theory provides some perspective on two elements of the current study. First, the captively-reared, anadromous transgenic males did not have an identical genetic background to the wild anadromous males with which they were compared. Specifically, the genetic background of the transgenic males consisted of two wild populations, one of which was the same as that of the wild males. Thus, in addition to captive rearing, intraspecific population differences may also have contributed to observations of reduced reproductive performance in transgenic relative to non-transgenic males. Second, evolutionary divergence among wild Atlantic salmon populations can potentially influence their relative reproductive performance when competing against transgenic invaders (Devlin et al. 2006; Kapuscinski et al. 2007; Hutchings and Fraser 2008). This study correctly mimics a likely invasion scenario, where the genetic background of the transgenic population differs from that of the wild population. However, contextualising these results with the general concerns of GH transgene introgression into wild populations must be done so with caution. It remains uncertain how the reproductive performance of this GH transgenic population would compare with other wild populations. Similarly, it is uncertain how the reproductive performance of this wild population would compare with other GH transgenic populations.

Previous studies comparing the reproductive behaviour and success of farmed and wild-type mature male parr have suggested that this alternative male reproductive phenotype may facilitate the interbreeding and introgression of farmed genes into wild populations (Garant et al.

2003; Weir et al. 2005). This rationale is based on observations of equal or greater breeding performance among farmed parr relative to wild-type parr coupled with the fact that maturation prior to anadromy increases the probability of survival to maturity and reduces generation time. In the current study, we found that the breeding behaviour and success of transgenic parr was inferior to that of wild-type parr, despite transgenic parr displaying more aggression. Moreover, transgenic parr sired fewer eggs than non-transgenic parr. When the data were paired by trial, however, no differences were observed in fertilisation success between the two groups, which may be due largely to a number of spawns where there was no parr contribution and the associated low statistical power. Interpretations based on the entirety of the behavioural and fertilization findings suggest that the non-transgenic parr marginally outcompeted transgenic parr during spawning. Nevertheless, transgenic parr demonstrated a behavioural interest in spawning and contributed gametes to the next generation. Thus, the alternative male reproductive phenotype of early maturation in Atlantic salmon may facilitate the introgression of transgenes into wild populations in a similar manner to that observed with farmed strains.

In an effort to limit size differences between transgenic and non-transgenic parr during the paired behavioural trials, age differences existed between competing parr in some of the trials. There was no significant difference in transgenic performance, whether competing with 0+ (n=5) or 1+ (n=6) non-transgenic parr, although we acknowledge the statistical limitations associated with the low sample sizes. Moreover, despite holding a significant body size advantage, irrespective of non-transgenic parr age, and exhibiting increased overt aggressive behaviour, the reproductive success of transgenic parr was less than that of non-transgenic parr. While there is evidence both for (Thomaz et al. 1997; Koseki and Maekawa 2000) and against (Jones and Hutchings 2001, 2002) parr body size influencing spawning success, it has been suggested that large body size may be a stronger predictor of dominance under scenarios with few competing

parr (Hutchings and Myers 1994; Jones and Hutchings 2001). However, in the present study, the breeding performance of transgenic parr appears to be inferior independent of size.

The reduced breeding performance of transgenic parr may be due, in part, to behavioural changes associated with GH transgenesis. Juvenile salmonids have shown distinct shifts in behavioural phenotypes in response to GH transgenesis, including increased foraging-induced aggression and reduced anti-predator behaviour (Abrahams and Sutterlin 1999; Sundstrom et al. 2003, 2004). The reduced nest fidelity and spawn participation by mature transgenic parr relative to non-transgenic parr may be driven by transgene-induced hormonal changes. Gonadotropin releasing hormone (GnRH) is thought to increase the expression of reproductive behaviours in many species (Maney et al. 1997; Yamamoto et al. 1997; Johnson et al. 2007; Munakata and Kobayashi 2010), including salmonids (Berejikian et al. 2003). For example, studies with the dwarf gourami (*Colisa lalia*) have indicated that male nest building behaviour is reduced when GnRH function is disrupted (Yamamoto et al. 1997; Munakata and Kobayashi 2010). Moreover, there is an existent, but poorly understood, association between the GH-IGF-I axis and the GnRH-gonadotropin-sex steroid axis (Holloway and Leatherland 1997a, 1997b; Mercure et al. 2001; Bjornsson et al. 2002). Thus, GH transgenesis may influence the interactions between these two hormonal axes such that the breeding behaviour of mature male parr is negatively affected. However, empirical investigations are required to explore the effects of GH on reproductive hormones and behaviour.

A common method for conducting environmental risk assessments involves the use of quantitative models that estimate a defined measure of risk. For genetically modified organisms, the prospect of gene flow from transgenic escapees into wild populations is a key issue due to the potential influences the transgene may have on fitness. In response, models have been developed to estimate the fitness outcome of transgene introgression into wild populations (Muir and

Howard 1999, 2001; Aikio et al. 2008; Valosaari et al. 2008; Ahrens and Devlin 2010). Frequently, the model parameters consist of empirical measurements of fitness-related life history traits such as growth, survival and reproductive probabilities, age at sexual maturity, female fecundity and male fertility (Muir and Howard 2002). The current study provides data on the relative breeding success of male salmon that are applicable to such predictive quantitative models. Specifically, we contribute to observations indicating captive-reared GH transgenic and farmed adult male salmon have a mating disadvantage relative to wild individuals; a gene flow scenario indicative of an initial invasion. Moreover, captive-reared non-transgenic precocial male parr demonstrated a modest mating advantage over transgenic individuals; a gene flow scenario comparable to subsequent generations following an invasion. Similar to the Japanese medaka (*Oryzias latipes*) work of Pennington et al. (2010), these findings are inconsistent with the assumption of a transgenic male mating advantage used in previous quantitative models (Hedrick 2001; Aikio et al. 2008; Valosaari et al. 2008), but see Howard et al. (2004) and emphasise the importance of basing parameter values on empirical data.

The present study, however, only provides an estimate of breeding success under a single set of physical and demographic environmental conditions consisting of paired males competing for single females. In the wild, male salmon will typically have access to multiple females simultaneously and have to contend with multiple competitors (Fleming 1996; Fleming and Reynolds 2004). Moreover, should transgenic animals get exposure to the wild environment prior to breeding (i.e. escape prior maturation), this may well alter their reproductive performance in a similar way, but opposite, to the effects captive rearing has on wild fish (e.g. Berejikian et al. 1997, 2001a; Bessey et al. 2004). As pointed out by Devlin et al. (2006), there are limitations and difficulties associated with collecting the breadth of empirical data required to accurately represent the full range of genotype by environment interactions affecting fitness-related life

history traits in the wild. The findings of this study are valuable with respect to a first generation invasion scenario; but beyond that, reproductive performance is difficult to predict and is, therefore, an unavoidable source of epistemic uncertainty for both quantitative and qualitative invasion models. Further work is thus required to compare the breeding performance of transgenic and non-transgenic salmon in a range of ecologically relevant scenarios.

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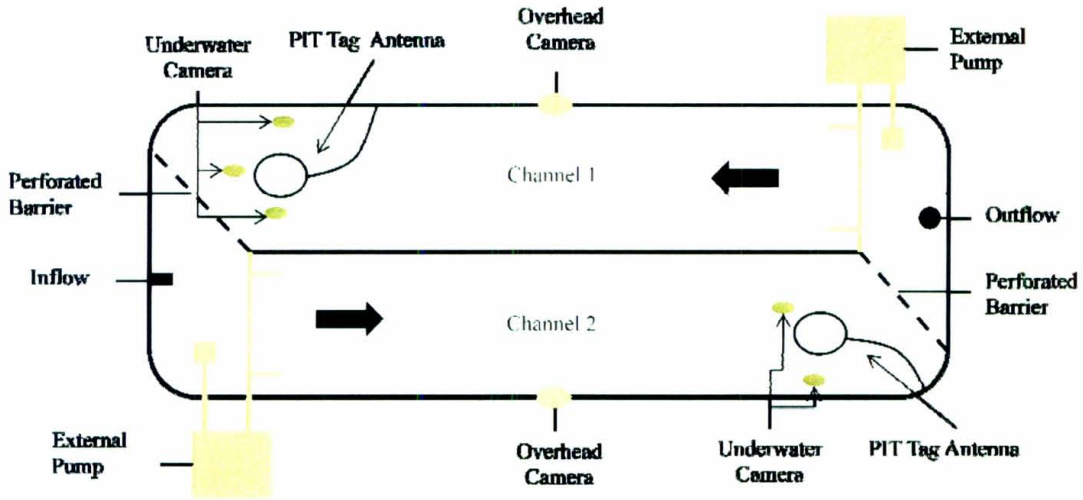

Figure 4.1. An illustration of the naturalised stream mesocosm (1.25 m × 7.8 m × 0.25 m per channel), which was divided into two channels and used to compare the reproductive performance of growth hormone transgenic and non-transgenic Atlantic salmon (*Salmo salar*) males, both as anadromous fish and precocial parr. Behavioual data were collected using a combination of video observation and PIT tag detection, with the respective underwater cameras and antenna moved in response to the location of female nesting activity. Thick arrows indicate the direction of water flow.

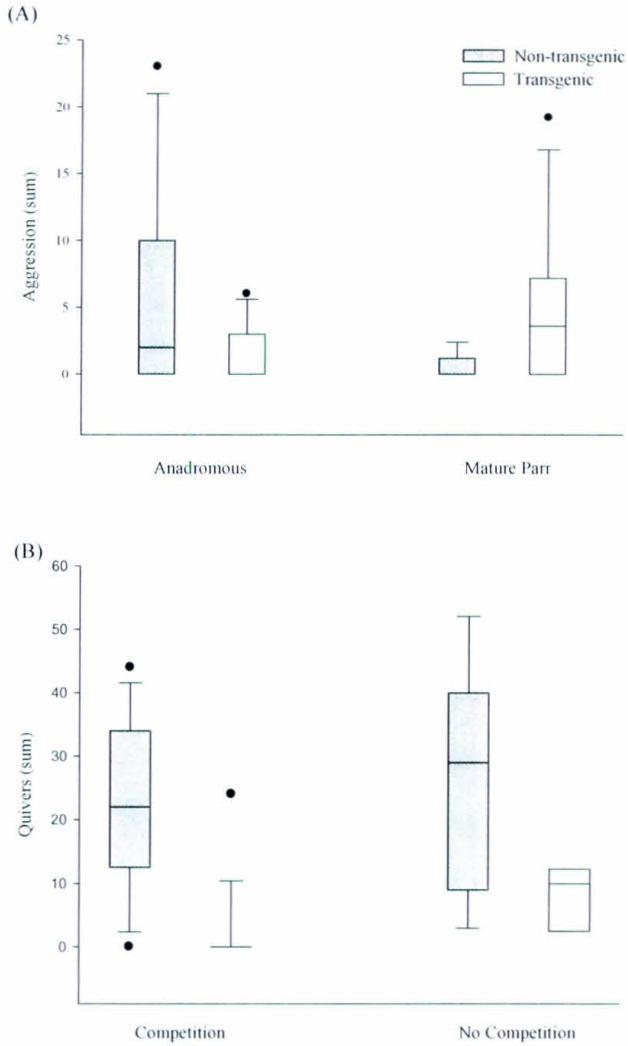

Figure 4.2. Standard box plot frequencies of (A) overt aggressive behaviours by transgenic and non-transgenic anadromous and parr males during paired competitive breeding trials and (B) quivering by transgenic and non-transgenic anadromous males during the competitive and non-competitive phases. For graphical purposes, these data were standardised to a 90 minute observation period. The top and bottom of each box represents the upper (75%) and lower (25%) quantiles, respectively. The horizontal line within each box indicates the median. The vertical lines (whiskers) extending from the upper and lower quantiles represent the maximum and minimum values of the distribution, excluding the outliers. The outliers are represented by the dots located beyond the maximum and minimum whiskers.

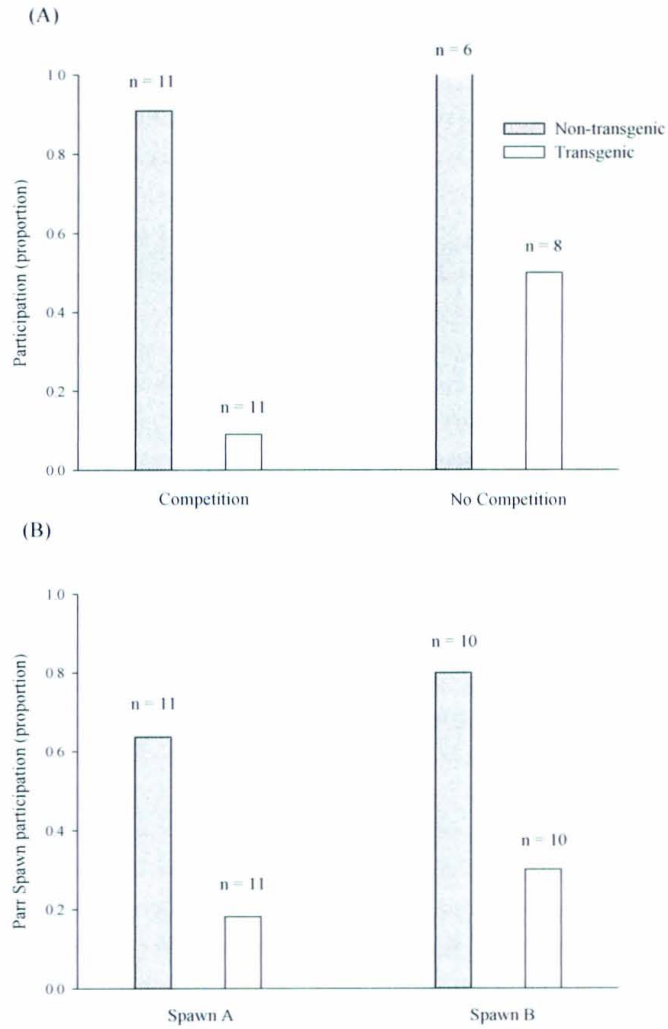

Figure 4.3. The spawn participation (presence/absence during a spawning event) of growth hormone transgenic and non-transgenic Atlantic salmon (*Salmo salar*) males during paired competitive breeding trials. Spawning behaviour and success was measured between transgenic and non-transgenic males of both the anadromous (A) and parr (B) reproductive phenotypes.

Table 4.1. Mean fork length (cm; ±S.E.) and mass (g; ±S.E.) of the mature Atlantic salmon used in competitive breeding experiments comparing GH transgenic and non-transgenic alternative reproductive phenotypes. In 2007, six trials compared age 0+ transgenic (T) versus 1+ non-transgenic (NT) parr and five trials compared 0+ transgenic versus 0+ non-transgenic parr; the size of parr involved are reported separately for each age comparison below. The N for each fish type is provided in parentheses.

Year	Fish Type	Length (cm)		Mass (g)	
		Transgenic	Non-transgenic	Transgenic	Non-transgenic
2006	Anadromous female	---	54.26±1.20 (11)	---	1620.3±150.8 (11)
	Anadromous male[a]	65.45±0.83 (11)	55.74±1.61 (11)	2862.2±134.2 (11)	1604.6±180.1 (11)
	Mature parr	---	14.39±1.14 (30)	---	37.99±3.18 (30)
2007	Anadromous female	---	59.36±1.83 (9)	---	1925.6±140.4 (9)
	Anadromous male	---	62.13±1.49 (7)	---	2202.3±287.5 (7)
	Mature parr (T 0+ vs. NT 1+)	15.22±0.56 (6)	14.14±0.28 (6)	36.10±3.52 (6)	30.9±1.91 (6)
	Mature parr[a] (T 0+ vs. NT 0+)	9.33±0.70 (5)	7.84±0.39 (5)	20.6±2.45 (5)	12.85±1.40 (5)

[a] indicates instances where transgenic males were larger than non-transgenic males in length and mass

Table 4.2. An ethogram describing the spawning behaviours measured during paired competitive trials between transgenic and non-transgenic Atlantic salmon males of both the anadromous and parr reproductive phenotypes.

Behaviour	Description	Unit of Measure
Nest Fidelity	The time the focal male spends with a nesting female.	Proportion of time the focal male attends the nest with the female present.
Overt Aggression	Male-male overt aggressive actions including chasing, charging, biting and fighting (Fleming 1996).	Frequency of all overt aggressive behaviours performed by the focal male.
Quivering	A courting behaviour, where the focal male vibrates its body while aligned in parallel with the female.	Frequency of all quivers performed by the focal male.
Spawn Participation	The active participation of the focal male during a spawning event.	Presence or absence of active participation during a spawning event.

Table 4.3. Nest fidelity (proportion of time spent with nesting female) of anadromous growth hormone transgenic and non-transgenic Atlantic salmon males during paired competitive breeding trials. Each breeding trial included phases of competition and no competition. During competition, both the transgenic and non-transgenic males competed directly for breeding opportunities with the female. During no competition, males had sole access to a spawning female. Data from each trial were analysed 60 min before (pre-spawn) and 30 min after (post-spawn) each spawning event. In trials with no spawning (n=4; all transgenic males in the absence of competition), analyses were based on observations conducted for 5 min intervals every 30 min for the duration of the phase (i.e. a total of 360 min of observation time). For statistical analyses, nest fidelity during the no competition phase was not segregated into periods.

Phase	Period	Genotype	N	Median	.25 Quantile	.75 Quantile	Range	Statistics
Competition	*Pre-spawn*	Transgenic	11	0	0	0.06	0-0.98	$\chi^2 = 19.33$, $P < 0.001$
		Non-transgenic[a]	11	1	0.89	1	0-1	
	Post-spawn	Transgenic	11	0.07	0	0.52	0-0.88	$\chi^2 = 14.85$, $P < 0.001$
		Non-transgenic[a]	11	0.83	0.58	1	0-1	
No Competition	---	Transgenic	8	0.91	0.64	0.97	0.02-1	$\chi^2 = 7.09$, $P < 0.01$
		Non-transgenic[a]	6	0.96	0.94	0.98	0.89-1	

[a]indicates the genotype with greater nest fidelity for each comparison

Table 4.4. Nest fidelity (proportion of time spent with nesting female) during paired competitive breeding trials of mature male parr that were growth hormone transgenic and non-transgenic. The first (A) and second (B) spawns from each trial were analysed for the period 52.5-12.5 min before the spawn (*pre-spawn*), 12.5 min on either side of the spawn (*spawn*), and 12.5-22.5 after the spawn (*post-spawn*).

Spawn	Period	Genotype	N	Median	.25 Quantile	.75 Quantile	Range	Statistics
A	*Pre-spawn*	Transgenic	11	0	0	0.04	0-0.88	$\chi^2 = 5.27$
		Non-transgenic[a]	11	0.87	0.09	1	0-1	P = 0.022
	Spawn	Transgenic	11	0	0	0.04	0-1	$\chi^2 = 4.70$
		Non-transgenic[a]	11	0.98	0.64	1	0.01-1	P = 0.030
	Post-spawn	Transgenic	11	0	0	0.38	0-1	$\chi^2 = 1.58$
		Non-transgenic	11	0.77	0.11	1	0-1	P = 0.209
B	*Pre-spawn*	Transgenic	10	0.02	0	0.72	0-1	$\chi^2 = 4.51$
		Non-transgenic[a]	10	0.98	0.52	1	0-1	P = 0.034
	Spawn	Transgenic	10	0.02	0	0.40	0-1	$\chi^2 = 5.19$
		Non-transgenic[a]	10	1	0.55	1	0.05-1	P = 0.023
	Post-spawn	Transgenic	10	1	0	0.39	0-1	$\chi^2 = 5.89$
		Non-transgenic[a]	10	1	1	1	0-1	P = 0.015

[a] indicates the genotype with greater nest fidelity

Table 4.5. The fertilisation success (proportion of eggs fertilized) of wild anadromous males and growth hormone transgenic and non-transgenic mature male parr during 11 pair-wise competitive breeding trials. Representation indicates the number of trials where successful fertilisation was observed by a male type.

Male Type	Median	.25 Quantile	.75 Quantile	Range	Representation
Anadromous male[ab]	0.98	0.92	1	0.59-1	11
Transgenic parr	0	0	0.06	0-0.22	1
Non-transgenic parr	0	0	0	0-0.41	5

[ab] indicates the anadromous males fertilised significantly more offspring than either parr genotype

Chapter 5

Enhanced growth reduces precocial male maturation in Atlantic salmon (*Salmo salar*)

Abstract

Understanding the proximate and ultimate mechanisms shaping the expression of alternative reproductive phenotypes in fishes is a fundamental question in life history evolution. Precocial maturation, one such alternative phenotype, has been thought to reflect rapid growth and/or energy accumulation; however, mechanistically linking these specific traits to discrete life history patterns is complex and poorly understood. Here we use mixed populations of growth hormone transgenic and non-transgenic Atlantic salmon (*Salmo salar*) siblings to elucidate the effects of intrinsically fast growth on precocious maturation at the freshwater parr stage. Despite facilitating growth to sizes typical of one-year-old mature wild-type parr, transgenesis did not influence maturation in the first year of life. In the second year, the number of maturing transgenic parr was 46% less than that of non-transgenic individuals. By manipulating intrinsic growth and controlling for both environment and genetic background (i.e. beyond the transgene), this study provides direct empirical evidence suggesting that the physiological mechanisms promoting growth do not necessarily play a causative role in precocial male maturation in fish, such as Atlantic salmon. The significance of these data is discussed in light of the ecological risk assessment of genetically modified organisms.

5.1: Introduction

Phenotypic variation within populations can be expressed in discrete life history forms that evolve through disruptive selection. A well documented category of discrete, alternative phenotypes are those associated with reproductive competition, where intraspecific (usually male) individuals adopt discrete traits or tactics that are believed to be part of a single conditional strategy maintained by both frequency- and condition-dependent selection (Hutchings and Myers 1994; Gross 1996; Calsbeek et al. 2002). Many of these alternative phenotypes are controlled by threshold switches, which are genetically-determined, internal triggers that direct the expression of one phenotype over another and are responsive to the environmental conditions experienced during ontogeny (Shuster and Wade 2003; Oliveira et al. 2008). Threshold switches are thought to initiate distinct internal resource allocation pathways leading to alternative phenotypes; however, the underlying proximate mechanisms are not well documented.

Atlantic salmon, *Salmo salar*, provide a model system with which to study the underlying mechanisms influencing alternative reproductive phenotypes. The breeding system includes anadromous (ocean migratory) and precocially maturing male (i.e. non-migratory parr) phenotypes that compete for fertilizations with spawning females. Anadromous males, which are large and display specialized secondary sexual characters, fight for access to breeding females, while mature male parr adopt a breeding tactic reliant on small size (often weighing two orders of magnitude less than anadromous males) and crypsis to sneak fertilization attempts (Fleming and Reynolds 2004).

An intriguing feature of this life history dichotomy involves the proximate and ultimate mechanisms that determine whether stream-dwelling male parr follow a

developmental trajectory toward precocial maturation or anadromy. Current evidence suggests that precocious maturation is initiated by parr reaching a polygenic performance threshold that, within populations, correlates with fast growth/size at age (Aubin-Horth and Dodson 2004; Baum et al. 2004; Hutchings and Myers 1994). Proximate causation is thought to be associated with the available energy (lipid) reserves, and the rate of energy reserve allocation, an individual has attained prior to the onset of environmental triggers initiating sexual development (Thorpe et al. 1998; Jonsson and Jonsson 2003; Mangel and Satterthwaite 2008). A similar proximate causation, however, is thought to underlie an alternative life history trajectory that involves phenotypic transformation for migration to sea (i.e. smoltification), whereby the largest fish from within a cohort are those most likely to exceed the performance threshold to become sea-migrating smolts. Thus, a developmental conflict may exist between precocial maturation and smoltification.

Proximate models suggest that parr maturation occurs if an energetic threshold is reached and maintained for a year prior to the fall breeding season, while smoltification occurs if a growth rate/size threshold is surpassed seven months prior to the spring seaward migration (Metcalfe 1998; Thorpe et al. 1998; Mangel and Satterthwaite 2008). Under these models, smolt transformation is viewed as an alternative developmental pathway for individuals that have not obtained enough energy reserves to produce sufficient gametic tissue by the breeding season. However, the allocation of energy between storage tissues, which may support maturation, and structural tissues (growth), which may promote smoltification, remains poorly understood.

Growth hormone (GH) transgenesis allows a unique opportunity to empirically test this proximate life history framework by permitting the manipulation of intrinsic

growth (Du et al. 1992; Cook et al. 2000a), while controlling for other genetic effects not associated directly with the transgene. Based on observed covariation between size at age, fast growth, and lipid investment with parr maturation, GH transgenesis may allow precocial maturation thresholds to be reached faster and in greater proportion relative to non-transgenic individuals, as previously proposed (Valosaari et al. 2008). Fast-growing transgenic parr, however, have greater metabolic requirements and preferentially invest energy into structural rather than storage tissues relative to non-transgenic parr (Stevens et al. 1998; Cook et al. 2000a, b). Such physiological differences may alter the internal triggers and conditional requirements necessary to reach maturation thresholds; potentially toward life history trajectories favouring smolt transformation (Metcalfe 1998; Saunders et al. 1998; Thorpe and Metcalfe 1998). Thus, GH transgenesis may provide empirical insight, allowing the separation of proximate mechanisms responsible for parr maturation from characteristics that covary.

Using mixed populations of GH transgenic and non-transgenic Atlantic salmon siblings we test the effect of growth on precocious maturation. If growth has a causative role in parr maturation, then GH transgenesis should increase the likelihood of early male maturation over the first and second years of life. However, if energy accumulation causes maturation, then GH transgenesis may reduce the incidence of parr maturation due to higher routine metabolic rates and preferential investment in structural tissues.

5.2: Methods

During September 2006, wild adult Atlantic salmon females were collected from the Exploits River, Newfoundland, Canada and transferred to the Ocean Science Centre,

Memorial University of Newfoundland. Upon egg ripening (21 November – 12 December), male gametes of hemizygous growth hormone transgenic Atlantic salmon (Gene construct: opAFP-GHc2; transgene: EO-1α [Yaskowiak et al. 2006]) were crossed with the wild females to produce eight single family crosses. The background genome of this transgenic strain is derived largely from Saint John River (NB, Canada) salmon. True to Mendelian inheritance patterns, such hemizygous crosses result in ca. half the offspring in each family inheriting the GH transgene (Shears et al. 1992). This allows for the comparison of full siblings, facilitating the control of general genetic background and maternal effects.

During early ontogeny, all families were reared separately in Heath incubation trays. At first-feeding (May 28 2007), families were reared separately in randomly-assigned, individual compartments of two rearing troughs (261 cm × 24.5 cm × 10 cm) and fed 4-8 times daily with a combination of *Artemia spp.* and a salmonid starter dry feed (Corey Feed Mills, Fredericton, NB). Temperature and photoperiod were kept at ambient conditions throughout the lives of these animals.

On July 30, 2007, 32 fry from each of eight families were haphazardly assigned to one of six $1m^2$ circular rearing tanks (n=256 per tank) and subsequently fed dry feed from automated feeders every 30 min. throughout each day. Initially, the tank replicates were split into high (8% tank biomass per day) and low feed (2%) treatments. In October 2007, both high and low feed treatments were decreased to accommodate reduced feeding levels (i.e. to 4% and 1% tank biomass per day, respectively). In January 2008, high and low feed treatments were discontinued and maintenance feed levels were delivered to all six tanks with hand feedings 1-3 times daily. Previous observations of salmon from the

same source wild population indicate that such conditions produce high male maturation rates (ca. 50%) in the second year of life (1+; unpubl. data).

In February 2008, the number of individuals in each tank was reduced (n=100) to accommodate expected biomass increases in the following spring/summer growing season. To prevent sampling bias in both family and transgenic composition, a mass frequency-based selection process (5 g intervals) was used whereby individuals were haphazardly removed in a manner consistent with maintaining the mass distribution among fish within each tank. To assess the ratio of transgenic to non-transgenic fish, a representative sample (n=40 per tank) of individual adipose fin clips were placed in microcentrifuge tubes containing 99% ethanol and subsequently screened for the transgene using a previously described polymerase chain reaction (PCR; Deitch et al. 2006).

In 2007 and 2008, male maturation was assessed on all fish once weekly between mid-October and the end of December, by gently squeezing the belly along the length of the body and looking for the presence of sperm at the genital papilla. The mass (g) and fork length (mm) of each mature parr was documented upon assessment. Subsequently, a tissue sample of each mature parr was collected for transgenic identification and the animals were euthanized with MS-222 (Western Chemical Inc., Ferndale, USA) prior to being frozen whole (-20°C). Frozen gonadal and whole body mass (g) was later collected to determine the gonadal-somatic investments of transgenic and non-transgenic mature parr.

5.2.1: Data Analyses

Logistic regressions with binomial error (LR) were used to test for tank effects on proportions of transgenic parr and to evaluate the proportion of mature parr with respect to feed level during the early growth phase (July – December 2007) and genotype (transgenic or non-transgenic). General linear models (GLM) were used to compare fork length, body mass, gonad mass and body condition (mass as the response variable and length as a covariate (García-Berthou 2001) with respect to genotype and early feed level. Data fit with GLM's that did not meet the requirements of normality were either fit to a gamma distribution (link: inverse) or natural log transformed prior to insertion into a linear model. All data were analyzed using the R statistical software application (version: R-2.10.1.; http://www.r-project.org).

5.3: Results

In 2007, the first year of life (0+), 1.3% of the total population consisted of mature male parr (Figure 5.1). While more transgenic (n=11) than non-transgenic (n=8) parr matured, there was no significant difference in the rates of maturity (LR; n = 19; χ^2 = 0.90; P = 0.35). Similarly, feed level did not influence the rates of early maturity (LR; n = 19; χ^2 = 0.18; P = 0.67). Among 0+ mature parr, mass and fork length were greater in high than low feed tanks (GLM; n = 19; mass: χ^2 = 14.26, P < 0.01; length: χ^2 = 13.10, P < 0.01) and transgenic fish were larger than non-transgenics (GLM; n = 19; mass: χ^2 = 102.05, P < 0.01; length: χ^2 = 48.82, P < 0.01; Figure 5.2A, B). Testing for differences in body condition (length-adjusted mass) between 0+ transgenic and non-transgenic mature parr indicated a three-way interaction between length, feed level and genotype (n = 19; P

< 0.001; Figure 5.3A). Qualitatively, the transgenic parr tended to have a higher mass for a given length than the non-transgenic parr.

Among the immature fish, the proportion of transgenic (n = 119) to non-transgenic (n = 120) parr did not differ (Exact Binomial test; $\chi^2 = 0.12$, P = 0.73); moreover, this pattern was consistent across tanks (LR; n = 239; $\chi^2 = 0.44$, P = 0.51). There was a strong interaction between feed level and genotype on fish mass ($P < 0.01$), indicating that transgenic and non-transgenic parr responded differently to the feed treatments. Transgenics outgrew non-transgenics, being heavier in both high (mean ± SE transgenics: 31.85 ± 1.26 g; non-transgenics 6.39 ± 0.25 g; GLM; n = 119; $\chi^2 = 783.37$, P < 0.01) and low feed treatments (transgenics: 18.45 ± 0.71g; non-transgenics 6.41 ± 0.54 g; GLM; n = 120; $\chi^2 = 127.43$, $P < 0.01$). However, the size of non-transgenics did not differ between feed levels (GLM; n = 120; $\chi^2 = 0.001$, P = 0.98), while that of transgenics did (GLM; n = 119; $\chi^2 = 97.62$, $P < 0.01$).

In 2008, the second year of life (1+), 35% of the total population consisted of mature male parr (Figure 5.1), of which non-transgenics (n=129) were 1.8 times more likely to mature than transgenics (n = 70; LR; n = 199; $\chi^2 = 14.12$; $P < 0.01$). Maturation was not influenced by the feed level in the first year (LR; n = 199; $\chi^2 = 1.55$; P = 0.21). Similar to the immature parr, mature parr showed strong interactions between feed level and genotype for all size measures ($P < 0.05$). Transgenics outgrew non-transgenics, being larger in both high (GLM; n = 106; mass: $\chi^2 = 563.22$, $P < 0.01$; length: $\chi^2 = 542.74$, $P < 0.01$) and low feed treatments (GLM; n = 91; mass: $\chi^2 = 69.52$, $P < 0.01$; length: $\chi^2 = 68.76$, $P < 0.01$; Figure 5.2A, B). Under low feed, body condition (length-adjusted mass) did not differ between transgenic and non-transgenic parr (GLM; n = 91; $\chi^2 = 2.21$, P =

0.130; Figure 5.3B). Under high feed, a strong interaction occurred between length and genotype ($n = 106$; $P < 0.001$). These data qualitatively suggest that non-transgenic parr had a greater length-adjusted mass than transgenic parr at the larger end of the size distribution, with the opposite pattern occurring among smaller fish. However, the small overlap in size distribution between transgenic and non-transgenic parr makes interpretation difficult. The growth effects of differing feed levels during the first year persisted among transgenic mature parr into the following year, with those from high feed being larger than those from low feed at age 1+ (GLM; mass: $n = 70$; $\chi^2 = 10.30$, $P < 0.01$; length: $n = 70$; $\chi^2 = 11.43$, $P < 0.01$; Figure 5.2A, B). However, the opposite occurred among non-transgenic parr, with those experiencing low feed during the first year being larger than those experiencing high feed (GLM; mass: $n = 129$; $\chi^2 = 14.05$, $P < 0.01$; length: $n = 129$; $\chi^2 = 7.02$ $P < 0.01$).

Comparing size across years, mature 0+ transgenic parr (29.6 ± 3.5 g; mean ± S.E.) did not differ in mass from that of 1+ non-transgenic parr (27.2 ± 1.4 g; GLM; $n = 140$; $\chi^2 = 0.22$, $P = 0.64$; Figure 5.2A). However, 1+ non-transgenic parr (132.4 ± 1.7 mm; mean ± S.E.) were significantly smaller in length (113.6 ± 9.9 mm; GLM; $n = 140$; $\chi^2 = 8.58$, $P < 0.01$; Figure 5.2B). Thus, mature 0+ transgenic parr were heavier for their length relative to 1+ non-transgenic parr (GLM; $n = 140$; $\chi^2 = 15.50$, $P < 0.01$).

At age 1+, the absolute gonadal mass of mature transgenic parr was greater than that of non-transgenic parr (GLM; $n = 163$; $F = 15.69$, $P < 0.01$), however, this was mainly due to their larger body size (Figure 5.4). For a given body mass, non-transgenic parr actually invested proportionately more in gonadal mass than transgenic parr (GLM, $n = 163$, slope: $F = 0.61$, $P = 0.55$; intercept: $F = 224.20$, $P < 0.01$). Unlike the non-

transgenic parr, many of the immature transgenic parr, both as 0+ and as 1+, exhibited secondary smolt characteristics, including long, silver bodies with darkened fins.

5.4: Discussion

During the first year of life (O+), growth hormone transgenic parr showed accelerated growth, reaching sizes typical of two (1+) or three (2+) year old non-transgenic parr (Hutchings and Jones 1998). Moreover, while transgenics exposed to high feed outgrew those exposed to low feed, the size of non-transgenics in both feed treatments was equal, suggesting that transgenic individuals were limiting the energy consumption of non-transgenics through direct competition. Notwithstanding this fast growth and domination of food resources, precocious maturation was low (1.3%) and not influenced by transgenesis or feed level. With the feed treatment eliminated for the second year of life, the incidence of 1+ non-transgenic mature parr was nearly twice that of the much larger transgenic parr. Moreover, while the absolute gonadal mass of mature 1+ transgenic parr was greater than that of non-transgenic parr, the relative investment for a given somatic mass was less. These results suggest that growth rate and/or size at age are not proximate mechanisms responsible for precocial male maturation in Atlantic salmon and support the idea that energy accumulation thresholds dictate the proximate basis of this life history decision, a pattern that may be common to organisms with similar alternative life histories.

Prior to the current study, it had been difficult to identify the direct effects of intrinsic growth and the potential implications of energy accumulation on precocious maturation. Contemporary thought suggests that, in late summer, large Atlantic salmon

parr that have exceeded a threshold level of energy reserves will mature that fall; whilst, those large parr lacking such energy reserves may undergo smolt transformation (Metcalfe 1998; Thorpe et al. 1998; Thorpe and Metcalfe 1998). Supporting evidence includes observations that precocious maturation is more common in resource-rich environments (Berglund 1995; Letcher and Terrick 1998; Rowe and Thorpe 1990; Saunders et al. 1982). Moreover, within populations, both parr maturation and smoltification correlate with high growth rates (Metcalfe et al. 1988; Saunders et al. 1994; Kadri et al. 1996). By manipulating intrinsic growth and controlling for both environment and genetic background (transgene excluded), this study provides direct empirical evidence suggesting that the physiological mechanisms promoting growth do not play a causative role in the early maturation of male parr, and may even hinder it.

Growth hormone contributes to a wide array of biological processes. The most well documented effect of GH is growth stimulation, which occurs in part by promoting lipolysis and protein synthesis (Björnsson 1997; Björnsson et al. 2002; see also Raven et al. 2006). High levels of circulating growth hormone, such as that experienced by transgenic fish, have been shown to reduce energy reserves in stream salmonids, which likely reflects the metabolic effects mentioned above (Johnsson et al. 1999; Neregård et al. 2008). In addition to stimulating growth, GH is known to be involved in smolt transformation; both directly and as a regulatory factor (McCormick 1996; Pelis and McCormick 2001; Bjornsson et al. 2002, 2011). Growth hormone is also implicated in the maturation of salmonids (Björnsson et al. 1994; Benedet et al. 2010); however, the exact role remains uncertain (Björnsson 1997; Björnsson et al. 2002). While the extent of physiological changes associated with the GH transgene may not be fully understood, a

suite of pleiotropic effects have been observed including higher metabolic demands, increased activity and preferential investment in somatic tissue over energy reserves (Stevens et al. 1998; Cook et al. 2000b, 2000c). These physiological differences, coupled with the reduced maturation rates and secondary smolt characteristics observed here, suggest that the transgene may induce physiological pathways toward smoltification preferentially; an observation that is consistent with previous research on GH transgenic salmonids (Saunders et al. 1998; Devlin et al. 1994, 2000, 2004a). Collectively, these results suggest that the proximate mechanisms underlying intrinsically fast growth promote life history shifts toward smolt transformation as opposed to precocial maturation.

Presumably, the investment of energy into structural tissues leads to high growth rates at the expense of investment into storage tissues for other purposes. Thus, from an ultimate perspective, it is unclear why fast growth consistently correlates with precocial maturation, in natural populations, if it may reduce available resources for other functions, such as maturation. This may be explained in part by the importance of body size in the breeding success of precocial males, with selection likely stabilizing. Large size affords larger gonads (Fleming 1998) and an ability to behaviourally dominate smaller parr during competition for access to breeding females (Thomaz et al. 1997; Koseki and Maekawa 2000); although this advantage appears to decline at high parr densities (Jones and Hutchings 2001, 2002). By contrast, small size may afford crypsis during sneak mating and reduce the likelihood of targeted aggression by anadromous adults. Thus, the present study suggests that patterns of fast growth/large size at age with precocious maturation are correlations reflecting limits to plasticity in the partitioning of

energy between structural and storage tissues. Neither growth rate nor size at age are proximate mechanisms responsible for precocial male maturation in Atlantic salmon. Rather, energy accumulation thresholds may be dictating the proximate basis of this alternative life history decision.

The results of this study suggest that growth rate and/or size at age are not proximate mechanisms responsible for precocial male maturation in Atlantic salmon. Further, it is proposed that these data support the hypothesis that energy accumulation thresholds dictate the proximate basis of this alternative life history decision. However, this study did not quantify energy accumulation in mature or immature parr and, therefore cannot rule out alternative hypotheses. The physiological effects of GH transgenesis on Atlantic salmon maturation are not known, while the effects of GH on internal biological processes are broad, and complex (Björnsson 1997; Björnsson et al. 2002). Thus, the reduced maturation rates induced by GH transgenesis may be the result physiological interactions unrelated to energy accumulation.

5.4.1: Implications

The genetic effects associated with interbreeding and introgression are among the greatest concerns associated with the potential entry of transgenic organisms into nature (Muir and Howard 2002; Howard et al. 2004; Devlin et al. 2006). Moreover, age at sexual maturity is considered a key fitness-related trait influencing the invasion of foreign genes into wild populations because early maturation reduces generation time and increases the probability of survival to reproduction (Muir and Howard 2001; Garant et al. 2003). This study provides the first empirical data on the relative incidence of

precocial male maturation in GH transgenic and non-transgenic Atlantic salmon and, therefore, provides valuable information for the ecological and genetic risk assessment process.

Among farmed Atlantic salmon strains, mature male parr have been identified as a potential means of increasing the pace of farmed gene introgression into wild populations (Garant et al. 2003; Weir et al. 2005). From a demographic perspective, the reduced expression of precocial male maturation among GH transgenic parr relative to non-transgenic parr suggests that the rate of trangene introgression may be limited by the number of maturing parr. However, mature male parr compete with one and other for proximity to nesting females (Fleming 1996). Thus, differences in competitive ability could either enhance or reduce the influence of proportional differences between mature male transgenic and non-transgenic parr. While our observations are valuable, caution is required when inferring risk scenarios from these data because the relative incidence and size of mature male transgenic and non-transgenic parr under natural conditions is not known. Previous efforts have shown that there are strong genotype by environment interactions on juvenile growth in transgenic salmon (Devlin et al. 2004b; Sundstrom et al. 2007; Moreau et al. 2011). Therefore, when used contextually, these data may provide valuable information for decision makers assessing the risks of GH transgenic salmonid biotechnologies.

5.5: Acknowledgments

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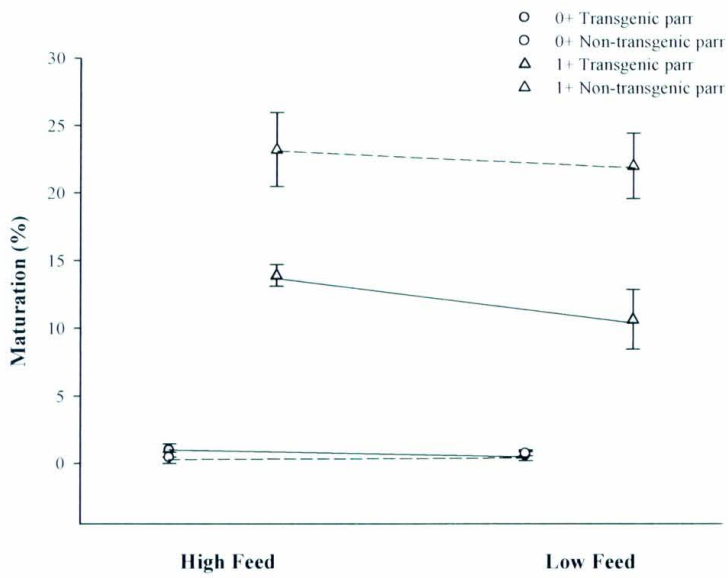

Figure 5.1. The incidence (%) mature male transgenic and non-transgenic Atlantic salmon parr (*Salmo salar*) during the first (0+) and second (1+) years of life. High and low feed levels were applied only during the first year of life. Thereafter maintenance levels were used. The error bars represent the 95% confidence intervals around the mean.

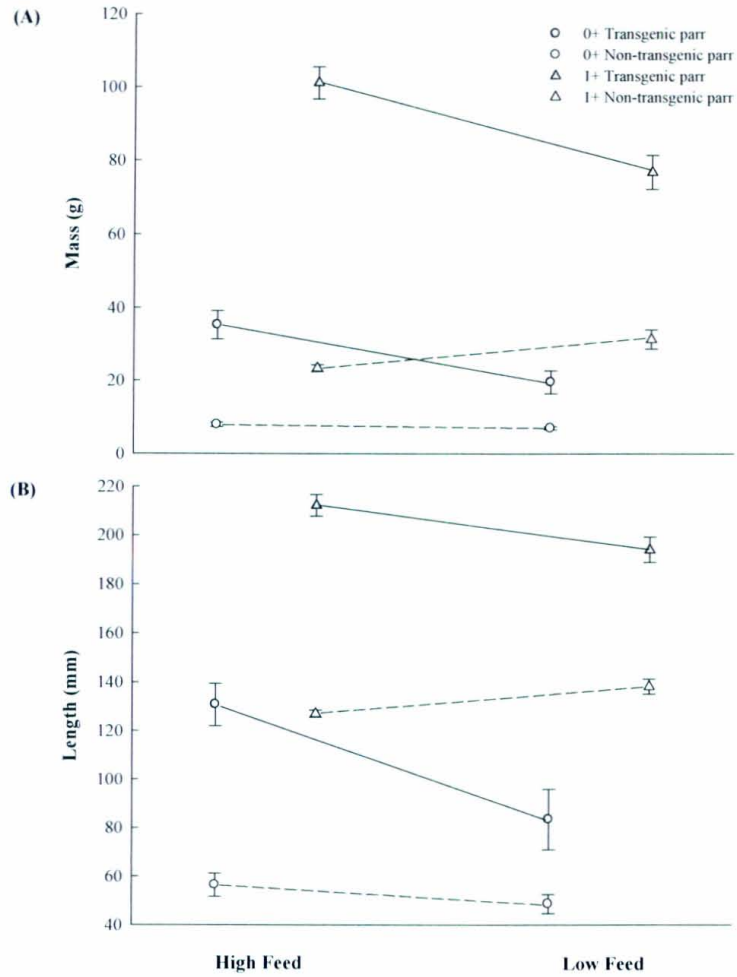

Figure 5.2. The mean (A) wet mass (g) and (B) fork length (mm) of transgenic and non-transgenic precocious male Atlantic salmon (*Salmo salar*) during the first (0+) and second (1+) years of life. High and low feed levels were applied only during the first year of life. Thereafter maintenance levels were used. The error bars represent the 95% confidence intervals around the mean.

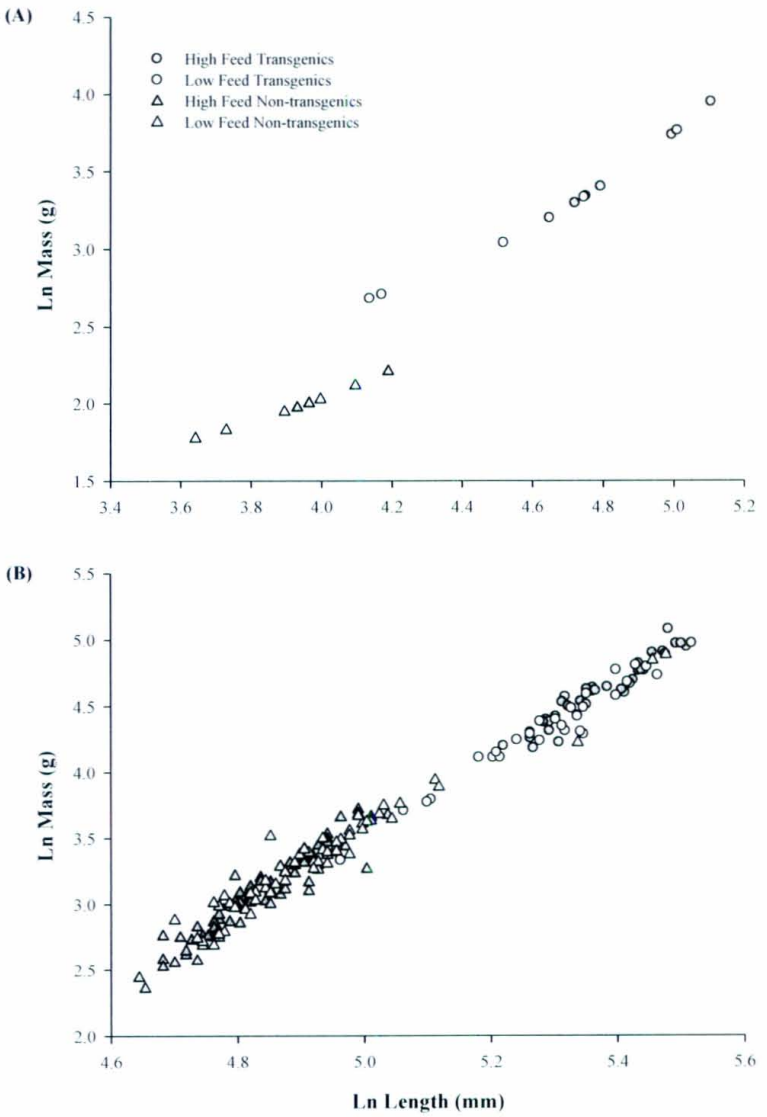

Figure 5.3. The length-mass relationship for transgenic and non-transgenic precocious male Atlantic salmon (*Salmo salar*) during the first (A) and second (B) years of life. High and low feed levels were applied only during the first year of life.

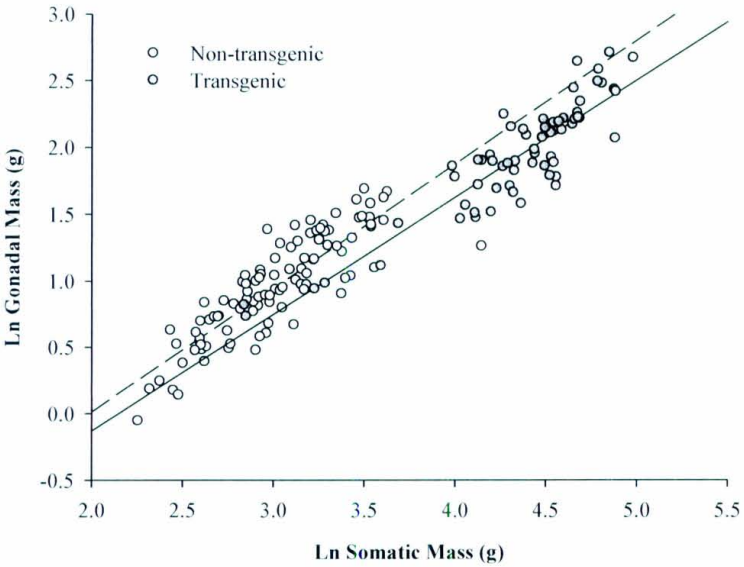

Figure 5.4. Natural log transformed gonadal and somatic mass (g) of 1+ mature male transgenic and non-transgenic Atlantic salmon (*Salmo salar*) parr. The dashed and solid lines of best fit represent the non-transgenic and transgenic parr, respectively.

Chapter 6

General Discussion

This thesis applied an eco-evolutionary approach to empirically assess the potential environmental effects of growth hormone (GH) transgenic Atlantic salmon (*Salmo salar*) entry into the wild. Specifically, my goal was to explore the relative survival and reproductive success of GH transgenic and non-transgenic salmon under near-natural conditions. To accomplish this, key fitness-related traits were compared between GH transgenic and non-transgenic Atlantic salmon over highly selective periods of their life cycle. Specifically, this thesis focused on the young-of-the-year stream and the breeding periods.

Two studies (Chapters 2 and 3) compared fitness-related traits between transgenic and non-transgenic Atlantic salmon during early life history. Chapter 2 explored the potential differences in developmental rate and respiratory metabolism between transgenic and non-transgenic siblings at three early stages of life; the eyed-embryo, alevin (larval) and first-feeding fry (juvenile) stages. Overall, the effect of GH transgenesis was weak to non-existent, with family differences having a much stronger influence on both routine metabolism and developmental rate. In Chapter 3, the foraging behaviour and the growth and survival of transgenic and non-transgenic first-feeding fry reared under low feed, stream-like conditions were explored. Similar to Chapter 2, the transgene did not influence any of the fitness-related phenotypic traits measured. During pair-wise dominance trials, transgenic fry were equally likely to win territorial dominance contests as were non-transgenic fry. Consistent with the dominance trials, the survival of GH transgenic first-feeding fry in stream microcosms under low food availability did not differ from that of non-transgenic individuals. Moreover, both groups experienced negative growth, though the pattern differed somewhat, with transgenic individuals

maintaining greater length for a given mass than non-transgenic individuals. Collectively, these chapters suggest that there is an ontogenetic delay in the phenotypic response induced by the transgene, such that biologically significant differences in fitness-related traits between GH transgenic and non-transgenic Atlantic salmon are minimal during this critical early life history period.

The final two studies (Chapters 4 and 5) compared fitness-related traits between transgenic and non-transgenic Atlantic salmon during the reproductive phase of the life cycle. The fourth chapter compared the breeding performance of growth hormone transgenic and wild-type Atlantic salmon males of both alternative reproductive phenotypes to test for the potential of the transgene to introgress into wild populations. Although transgenic males displayed reduced breeding performance relative to non-transgenics, both male reproductive phenotypes demonstrated the ability to participate in natural spawning events and, thus, the potential to contribute genes to subsequent generations. The fifth chapter used mixed populations of GH transgenic and non-transgenic Atlantic salmon siblings to elucidate the effects of growth on precocious parr maturation. Precocious maturation was low (1.3%) during the first year of life and was not influenced by transgenesis or feed level. With the feed treatment eliminated for the second year of life, the incidence of 1+ non-transgenic mature parr was nearly twice that of the much larger transgenic parr. This reduction in transgenic maturation rates relative to non-transgenics suggests that the ability to reach threshold levels of energy required to accommodate maturation is negatively affected by transgenesis.

Collectively, the empirical studies contained in this thesis provide insight into the potential ecological and genetic effects that GH transgenic Atlantic salmon may have on

natural conspecific populations. Specifically, transgenesis did not influence any fitness-related traits measured during the critical egg through first-feeding stages, suggesting that selective pressures would affect transgenic and non-transgenic individuals in a similar manner. Additionally, data collected during the reproductive phase suggest that transgenic males may experience reduced reproductive success relative to non-transgenic individuals. The potential for the transgene to introgress into wild populations, however, was demonstrated.

Ecological risk assessments frequently make use of quantitative models that estimate a defined measure of risk. Consistent with the data produced by this thesis, model parameters often consist of empirical measurements of fitness-related life history traits such as growth, survival and reproductive probabilities, age at sexual maturity, female fecundity and male fertility (Muir and Howard 2002). While it is imperative to use empirical data to derive parameter values, it is also essential to consider the limitations of such models when incorporating their results into an overall risk assessment framework. These limitations address the ecological complexities associated with empirical data collection and can be categorised as either: (1) genotype by environment interactions and (2) strain specificity.

For quantitative models to accurately predict the outcomes of real transgene invasion scenarios, representative parameter values for a given phenotypic trait are required for the broad range of environments that may be experienced. As detailed in Chapter 1, previous work has demonstrated that phenotypes of transgenic salmon display highly plastic responses to varied environmental pressures (Devlin et al. 2004, 2006, 2007; Kapuscinski et al. 2007; Lõhmus et al. 2010a, b). This suggests that the

background genome can moderate the effect of a transgene on the phenotype in response to the environment. Given the complexity and dynamism of natural environments and the time and monetary expense of empirical data collection, predicting the fitness of transgenic organisms is a difficult and uncertain task. Thus, from a risk assessment perspective, quantitative models are limited by the narrow environmental scenarios represented by the empirical data representing parameter values.

The phenotypic expression of transgenic organisms has been shown to vary considerably by transgene construct and background genome; both within and between populations and species (Devlin et al. 2001; Nam et al. 2008). The current thesis provides further evidence of this phenomenon. Specifically, there appear to be ecologically important phenotypic differences between existing strains of GH transgenic Atlantic and coho salmon populations, which carry unique GH transgene constructs. During early life history the phenotypic effects of transgenesis in Atlantic salmon appear to be delayed or reduced relative to that observed for coho salmon (Chapter 1, 2, 3; Sundstrom et al. 2004, 2005; Lohmus et al. 2010b). Such observations suggest that the fitness of young-of-the-year transgenic Atlantic and coho salmon may differ considerably relative to wild-type conspecifics. From a risk assessment perspective, the uncertainty associated with predicting such responses and how they may influence fitness accentuates the importance of strain-specific empirical data requirements.

Acknowledging the limitations discussed above, ecological risk assessment protocols will require a more qualitative approach that recognises and accounts for the unavoidable limitations and uncertainties inherent with empirical data in support of quantitative risk assessment models. A precautionary regulatory policy must not exclude

the use of quantitative models. The parameterised data, however, must be contextualised to the ecological scenario it represents and the model assumptions need to accurately reflect the ecology of the organism. The data provided here represent an initial step toward understanding the potential ecological and genetic effects of GH transgenic Atlantic salmon on wild conspecific populations.

6.2: Conclusion

Our ability to understand and predict the environmental consequences of transgenic organisms is lagging behind its technological development. We can engineer gene transfer biotechnologies with viable, attractive production traits for aquaculture. However, it is difficult to predict the effects of genetic and environmental variation on complex phenotypes, like that resulting from the interbreeding of fish populations in nature. Transgenesis adds another layer of complexity to the challenge of estimating risks associated with aquaculture escapees. In many countries, regulatory legislation will respond to this scientific uncertainty by administering detailed environmental risk assessments and potential restrictions on the commercialization of gene transfer biotechnologies deemed unsafe.

A long term solution to dealing with the uncertainty and risk associated with aquaculture biotechnologies is the development of biological containment methods. There is little doubt that aquaculture biotechnologies will have an increasingly important role in the global food supply of the 21st century. The challenge is to ensure that aquaculture development does not contribute to further declines in the health of aquatic ecosystems. Aquaculture biotechnology can play a major role in the sustainable

development of the industry by developing practical biological containment methods. The challenge of the biotechnology community is to develop ecologically neutral technologies; that is, animals that cannot contribute genetically to wild populations and have little or no impact on the ecosystem. To accomplish this, public and private institutions will need to prioritize research into chromosome set manipulations, sterility transgenes, sex-control and other such technologies to complement favourable production traits, while recognizing there may also be inherent dangers with these applications as well.

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