Environmental Assessment for *AquAdvantage*® Salmon

An Atlantic salmon (*Salmo salar* L.) bearing a single copy of the stably integrated α-form of the opAFP-GHc2 gene construct at the α-locus in the EO-1α line

Aqua Bounty Technologies, Inc.

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Center for Veterinary Medicine
US Food and Drug Administration

For Public Display

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List of Acronyms & Abbreviations

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~	approximately
AAS	*AquAdvantage* Salmon
ABRAC	Agricultural Biotechnology Research Advisory Committee
ABT	Aqua Bounty Technologies
AFP	antifreeze protein
amp^r	ampicillin resistance
BOD	biochemical oxygen demand
bf	base-fragment
bla	β-lactamase
bp	base-pair
CEQ	Council on Environmental Quality
CFR	Code of Federal Regulations
COD	chemical oxygen demand
CVM	Center for Veterinary Medicine
DHHS	Department of Health and Human Services
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
DO	dissolved oxygen (content)
EA	environmental assessment
EIS	environmental impact statement
EC	Environment Canada
EO-1	The female founder of the *AquAdvantage* Salmon line
EO-1α	The integrated form of the *AquAdvantage* transgene
EPA	US Environmental Protection Agency
ERA	early-rearing area
ESA	Ecological Society of America
EU	European Union
FAO	Food and Agricultural Organization (of the United Nations)
FCR	feed conversion ratio
FDA	US Food and Drug Administration
FFDCA	Federal Food, Drug, and Cosmetic Act
FL	Fork length; length of a fish from nose to tail-fork
FONSI	finding of no significant impact
FWS	US Fish and Wildlife Service
GOA	grow-out area
GE	genetically engineered
GH	growth hormone
GXE	genotype-by-environment

List of Acronyms & Abbreviations

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INAD	Investigational New Animal Drug (exemption)
LHO	Low head oxygenator
MM	million
MT	metric ton
MUNLV	German [Ministry of Environment, Conservation, Agriculture & Consumer Protection]
NADA	New Animal Drug Application
NASCO	North Atlantic Salmon Conservation Organization
NEPA	National Environmental Policy Act
NRC	National Research Council
NTU	nephelometric turbidity units
ONADE	Office of New Animal Drug Evaluation
opAFP-GHc2	The plasmid form of the *AquAdvantage* transgene
OSTP	Office of Science and Technology Policy
PB	polar body
PCR	polymerase chain reaction
PEI	Prince Edward Island, Canada
PIT	passive integrated transponder
PVC	polyvinyl chloride
rDNA	recombinant deoxyribonucleic acid
SW	Sea winter
US	United States
UV	ultraviolet

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List of Definitions

AquAdvantage gene construct	The plasmid form of a transgene used to modify phenotype.
AquAdvantage Salmon	Atlantic salmon modified with the gene construct, opAFP-GHc2.
°C-day [min]	Compound unit of "time" (°C x days [min]) for relative determination of growth rate that accounts for effect of water temperature.
Diploid	Having two complete sets of chromosomes.
EO-1	The mosaic, female founder of the *AquAdvantage* Salmon line created by microinjection of the opAFP-GHc2 transgene into a fertilized egg.
EO-1α	Functional, stably integrated form of opAFP-GHc2 in the *AquAdvantage* Salmon genome.
Flow cytometry	Method used to confirm ploidy by determination of DNA content in a dye-labeled cell population via relative fluorescence intensity.
Genotype	An organism's full hereditary information, even if not expressed.
Hemizygous	Having one copy of a given (trans)gene.
Homozygous	Having matching, homologous copies of a particular (trans)gene on each of two paired chromosomes in a diploid genotype.
Neomale	A female fish converted to a phenotypic male by hormone treatment.
opAFP-GHc2	*AquAdvantage* rDNA construct comprising regulatory sequence from an ocean pout AFP gene and GH-coding sequence from chinook salmon.
PCR	Polymerase chain reaction; method used to confirm genotype by primer-extension and identification of select sequences unique to EO-1α.
Phenotype	An organism's actual observed properties, such as morphology, development, or behavior, which derive from its genotype.
PIT	Passive integrated transponder; implantable radio-beacon for fish identification.
Plasmid	A class of episomal bacterial DNAs employed in the molecular cloning of small to mid-size DNA fragments.
Ploidy	Either diploid or triploid.
Protein-coding sequence	The DNA sequence of a gene that is transcribed into mRNA and subsequently translated into protein.
Regulatory sequence	Non-protein coding DNA sequence of a gene controlling its expression.
SW	Sea winter: Number of winters spent at sea (e.g., 1-SW, 2-SW).
Transgene	Synthetic gene comprising regulatory and coding sequences constructed *in vitro*, incorporated into genome of an organism to modify phenotype.
Triploid	Having three complete sets of chromosomes.

Summary

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AquAdvantage Salmon is a genetically-engineered Atlantic salmon with a rapid-growth phenotype that has been developed over the past 15 years. The genetic modification comprises one copy of a salmon growth hormone transgene that is stably integrated at a specific site in a specific line. Triploid eyed-eggs for *AquAdvantage* Salmon are produced in a manner that results in the culture of an all-female population of triploid fish that are otherwise substantially equivalent to farmed Atlantic salmon. The monosex nature of the population derives from the use of a breeding strategy that is 100% effective; and, the induction of triploidy, which renders the animal reproductively incapable, is achieved using a validated method with an average effectiveness of more than 99% at commercial scale.

The product is intended for the land-based culture of Atlantic salmon for commercial sale and human consumption. This Environmental Assessment describes the potential environmental risks associated with *AquAdvantage* Salmon under the following specific conditions: production of eyed-eggs in Canada; shipment of eyed-eggs to Panama; grow-out and processing of fish in Panama; and, shipment of table-ready, processed fish to the United States for retail sale.

Assessment of the potential risks to the environment from *AquAdvantage* Salmon involves consideration of the likelihood and consequences of the fish escaping, becoming established in the environment, and spreading to other areas. If the likelihood of these events, which are analogous to *exposure* in the traditional risk-assessment paradigm, is zero or close to zero, it is reasonable to conclude that the consequences of these events, which are analogous to the *effects*, are not of concern. In other words, if there is no exposure, there is no risk.

The likelihood of escape, establishment, and spread of *AquAdvantage* Salmon is extremely small due to redundant containment measures, including physical, physico-chemical, geographic/geophysical, and biological measures, that are being implemented at the sites of egg production, grow-out, and disposal. The combination of these various methods results in a very high degree of effective control. Physical measures include multiple mechanical means to prevent escape (e.g., screens, filters, etc.), while physico-chemical measures include the use of chlorine to kill any potential escapees. A strong management operations plan ensures that these containment measures are reliably implemented. Geographical and geophysical containment is provided by the location of the egg production and grow-out sites: the environment surrounding the egg-production site in Canada is inhospitable to early-life stages of Atlantic salmon due to high salinity; and, the environment downstream of the grow-out site in Panama is inhospitable to all life stages of Atlantic salmon due to high water temperatures, poor habitat, and physical barriers (e.g., several hydro-electric facilities). Biological containment is accomplished through the production of all-female triploid fish, which reduces the chance of breeding with native species, and significantly reduces the risk of transgene propagation in the environment.

Summary

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In summary, production and rearing of *AquAdvantage* Salmon will involve simultaneous, multiple, and redundant containment measures of various types that serve to mitigate the environmental risk quite adequately. These measures consist of producing triploid, all-female salmon that will be reared in land-based aquaculture systems possessing redundant physical containment measures engineered and managed to confine the fish to the culture systems and minimize the potential for escape. Furthermore, the facilities are located in geographical areas that are highly unfavorable to the survival, establishment and spread of *AquAdvantage* Salmon, should there be an escape.

Consequently, the production, grow-out, and disposal of *AquAdvantage* Salmon under the conditions described in this Environmental Assessment are highly unlikely to cause any significant effects on the environment, inclusive of the global commons, foreign nations not a party to this action, and stocks of wild Atlantic salmon.

####

1.0 INTRODUCTION

AquAdvantage Salmon (***AAS***) is a genetically-engineered (***GE***) Atlantic salmon (*Salmo salar*) with a rapid-growth phenotype for use in commercial aquaculture. The development of *AAS* has been ongoing for approximately (**~**) 15 years, and the Sponsor, Aqua Bounty Technologies, Inc. (***ABT***), is seeking regulatory approval from the Center for Veterinary Medicine (***CVM***) of the United States (***US***) Food and Drug Administration (***FDA***) through a New Animal Drug Application (***NADA***). This document constitutes the Environmental Assessment (***EA***) that addresses the potential environmental risk associated with *AquAdvantage* Salmon, and the mitigation thereof, should that NADA be approved.

1.1 Background and Overview

In 2006, the world consumed ~110.4 million (***MM***) metric tons (***MT***) of fish, with almost half of that (~51.7MM MT) coming from commercial aquaculture. To meet increasing demand for this valuable source of protein in light of declining stocks and diminishing capture of wild fish, commercial aquaculture will need to expand significantly. The Food and Agricultural Organization (***FAO***) of the United Nations has estimated that by 2030, annual commercial production will need to increase by an additional ~28.8MM MT (i.e., ~80.5MM MT total) in order to maintain per capita fish consumption at current levels (FAO, 2008a).

The demand for farmed salmon has followed a trend similar to that of other fish species, increasing steadily year-by-year with new markets opening up worldwide (FAO, 2009). Commercial aquaculture was the source of ~69% of worldwide salmon production in 2006 (FAO, 2008b). During the years 2000-2004, Americans consumed an average of ~284,000 MT of salmon annually, of which two-thirds were farmed rather than wild caught (Knapp *et al*., 2007). This is especially true for Atlantic salmon, since the last wild fishery for this species in the US was closed in the 1980s: 99% of the Atlantic salmon consumed in the US from 2000-2004 was farmed (Knapp *et al*., 2007), almost all of that being supplied by aquaculture operations in Canada, Chile, Norway, and Scotland.

The intentional selection imposed on salmon during commercial domestication has improved phenotype to the benefit of both productivity and marketability; however, the inherent biological limitations and slow pace of the traditional approach can only be overcome through the use of biotechnology. In response, Aqua Bounty Technologies has developed *AquAdvantage* Salmon, a GE Atlantic salmon with a rapid-growth phenotype that is intended to benefit commercial farming by significantly reducing time-to-market and improving the economics of land-based production.

The *AquAdvantage* founder animal was developed by inserting the coding sequence from a chinook salmon (*Oncorhynchus tshawytscha*) growth hormone (***GH***) gene under the control of regulatory sequences from an ocean pout (*Macrozoarces americanus*) antifreeze protein (***AFP***) gene into wild Atlantic salmon. The marketed product is a population of

fish that are triploid females,[1] which serves to prevent spread of the genetic modification in the environment in the event of their escape. The limitations on use of the product and the measures designed to mitigate potential environmental impacts are discussed herein.

1.2 Description of Product

The *AquAdvantage* Salmon to be sold into commerce is a triploid female Atlantic salmon bearing a single copy of the stably integrated α-form of opAFP-GHc2 at the α-locus in the EO-1α line. For the use covered in this EA, the product subject to regulatory approval is an eyed-egg produced at a specific site on Prince Edward Island, Canada (***PEI***), and delivered to a specific site in Panama for grow-out (i.e., culture to market size) and processing pursuant to retail sale in the United States.

opAFP-GHc2 is a recombinant deoxyribonucleic acid (***rDNA***) construct comprising regulatory sequences from an ocean pout AFP gene and protein-coding sequence from a chinook salmon GH gene. The founder animal from which the *AquAdvantage* line derives was a mosaic, transgenic female (***EO-1***) generated by injecting the construct into the fertilized eggs of wild Atlantic salmon. Two rapidly-growing, transgenic F_1-progeny of EO-1 were selected for further development and found to harbor two independently segregating integrants: a functional α-form and a non-functional β-form. The breeding of six subsequent generations has led to the establishment of an *AquAdvantage* Salmon line (***EO-1α***) which bears a single copy of the α-form of the integrated transgene. This is described more fully in §2.2.

The broodstock used in spawning of *AquAdvantage* Salmon are homozygous females (i.e., having two copies of the transgene) that have been phenotypically sex-reversed for breeding purposes. These so-called neomales are crossed with non-transgenic female Atlantic salmon to produce eggs containing a single-copy of the transgene that are pressure-shocked to induce triploidy, which renders the fish sterile. Therefore, the salmon deriving from these eggs are females incapable of reproduction; the significance of this will be discussed in subsequent sections of this EA. The fish that develop from these eggs have an enhanced growth rate compared to non-transgenic Atlantic salmon.

1.3 Description of the Proposed Action: an NADA Approval for *AAS*

1.3.1 The regulatory mandate

As described in Guidance Document 187 (CVM, 2009), GE animals are regulated under the new animal drug provisions of the Federal Food, Drug, and Cosmetic Act (***FFDCA***), and the CVM has established a risk-based hierarchical approach to demonstration of safety and effectiveness that is consistent with FFDCA (21 USC 321 *et seq*.) and its enabling regulations (21 CFR 511 & 514).

[1] ***Triploidy*** is induced in fin-fish to inhibit their sexual development and render them sterile; and, pressure shock has exhibited an average efficiency exceeding 99% in inducing triploidy in *AAS* eggs at commercial scale. While the vast majority of *AAS* being cultured for retail sale will have no reproductive capacity, triploidy is not necessarily 100% effective in producing infertility (*see*, §6.1.2.2), and reference to "sterile" *AAS* in this document should be interpreted in that context. The production of ***monosex*** (i.e., all-female) populations of *AAS*, which is accomplished through a biological process that is 100% efficient, is being used to further diminish the possibility that *AAS* could become established in the wild in the event of their escape from physical containment.

This approach, which is illustrated in Figure 1, begins with a product definition, and proceeds through a step-wise series of investigations to characterize the potential hazards associated with the rDNA construct, the lineage of the GE animal, and the durability of its genotype and phenotype. This information enables the CVM to determine the likelihood and potential severity of impacts on animal or human health and the environment.

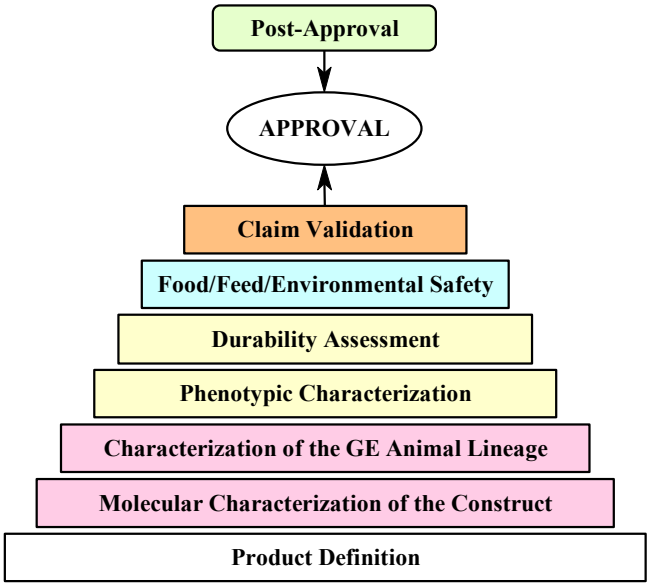


Figure 1. Regulatory Review Process for GE Animals

Major agency actions such as an NADA approval trigger the requirement for preparation of an EA addressing the potential environmental impact of that action under the National Environmental Policy Act (***NEPA***). This document constitutes an assessment of potential environmental risk that: satisfies Sponsor obligations under the Food/Feed/Environmental Safety step of the review process; addresses NEPA-related responsibilities of the Sponsor and FDA described in 21 CFR Part 25; and, provides material assistance to the FDA for making a decision whether to prepare a finding of no significant impact (***FONSI***) or an environmental impact statement (***EIS***). Of principal importance in the consideration of this EA is the proposed production of eyed-eggs in PEI and grow-out of *AquAdvantage* Salmon in Panama using a land-based culture system: since these activities involve sites outside the US, concern is directed toward environmental impact on the global commons under Executive Order 12114 (21 CFR 25.60), and those foreign countries not participating in the action who may nevertheless be affected by it.

1.3.2 Purpose of and need for the proposed action

ABT is in the process of requesting an FDA approval for *AquAdvantage* Salmon on the basis of numerous studies that have been submitted to the CVM in satisfaction of the established paradigm for regulatory review of GE animals. In doing so, ABT intends to address an industry need for more rapidly-growing Atlantic salmon broodstock that will significantly decrease time-to-market and increase the productivity of farming operations.

Provided that the Sponsor has satisfactorily addressed the technical considerations required under the established review process for *AquAdvantage* Salmon, and can reasonably assert the absence, or effective mitigation, of any significant environmental risk associated with manufacture and use of *AquAdvantage* Salmon, the FDA may take affirmative action with regard to the Sponsor's anticipated request for an NADA approval.

1.3.3 Alternatives to the proposed action

If the FDA does not find *AquAdvantage* Salmon to be safe and effective for its intended use based upon sound and objective consideration of the aggregate information provided as the basis for its decision, it may also choose to not approve *AquAdvantage* Salmon.

1.4 Regulatory Background

ABT requested an Investigational New Animal Drug (***INAD***) exemption in 1995 from CVM to pursue the development of *AquAdvantage* Salmon. The development of *AAS* has continued with oversight by the Office of New Animal Drug Evaluation (***ONADE***) subject to the review paradigm for GE animals, which comprises a risk-based, case-by-case assessment that includes the technical objectives of the traditional drug paradigm, but does so in a series of obligate, step-wise reviews incorporating a weight-of-evidence approach.

Under the FFDCA (21 USC 321 *et seq.*), the FDA has the authority to regulate new animal drugs. The definition of a drug includes the rDNA construct in a GE animal that is intended to affect the structure or function of the body of the GE animal. All GE animals derived from the same transformational (i.e., genetic) event are considered to contain the same test article and are subject to evaluation under a single NADA. Because GE animals that are being used in commerce are descendents of the parent GE animal itself, assurance of the stability of the genotype and phenotype is of primary importance.

FDA review of an NADA involves compliance with NEPA requirements, which mandate consideration of potential environmental effects. According to 21 CFR 514.1(b)(14), the NADA must include either a claim for categorical exclusion or an EA. As was previously noted, the EA is a public document that provides sufficient evidence and analysis to allow the FDA to make a determination whether to prepare a FONSI or an EIS.

This document constitutes the EA for *AquAdvantage* Salmon to be produced and grown under the conditions described herein.

1.5 Approach Taken in This Assessment

This EA describes the potential environmental risks of *AquAdvantage* Salmon under the following specific conditions: production of eyed-eggs at a specific site on PEI; shipment of eyed-eggs to a specific site in Panama; grow-out of fish at that site in Panama; and, processing and shipment of table-ready fish from Panama to the US. This EA does not consider risks under other production or grow-out conditions, although portions of this document are likely to be useful in addressing the environmental risks that may be presented by other scenarios.

This EA uses principles of ecological risk assessment employed by the Environmental Protection Agency (***EPA***, 1998) as modified to address GE organisms by the National Research Council (***NRC***, 2002). Risk is the joint probability of exposure and the conditional probability of harm given that exposure has occurred. In this context, the steps in the risk analysis, as outlined by NRC (2002), are as follows: (1) to identify the potential harms regardless of likelihood; (2) to identify the potential hazards that might produce these harms; (3) to define what exposure means for a GE organism and the likelihood of exposure; (4) to quantify the likelihood of harm given that exposure has occurred; and, (5) to combine the resulting probabilities to characterize risk.

2.0 PRODUCT

2.1 Product Definition

For the purposes of US regulatory approval, the Product Definition of *AquAdvantage* Salmon is as follows:

- ***Product Identity:*** Triploid hemizygous, all-female Atlantic salmon (*Salmo salar*) bearing a single copy of the α-form of the opAFP-GHc2 rDNA construct at the α-locus in the EO-1α lineage.
- ***Claim:*** Significantly more of these Atlantic salmon grow to at least 100 g within 2700°C-day than their comparators.
- ***Limitations for Use:*** These Atlantic salmon are produced as eyed-eggs for grow-out only in the FDA-approved, physically-contained freshwater culture facility.

This EA is limited to specific facilities for the production of eyed-eggs on PEI and grow-out to market size in Panama. The production plan is described below.

The ABT product is a population of triploid, eyed-eggs from a proprietary line of Atlantic salmon that has been genetically engineered to increase growth rate and reduce overall time-to-market. The genetic modification comprises one (1) copy of a salmon GH transgene (opAFP-GHc2) that is stably integrated at a specific site (the α-locus) in a specific line (EO-1α). The eggs are produced in a manner that provides an all-female population of triploid fish that are otherwise substantially equivalent to farmed salmon.

2.2 Characterization of the rDNA Construct

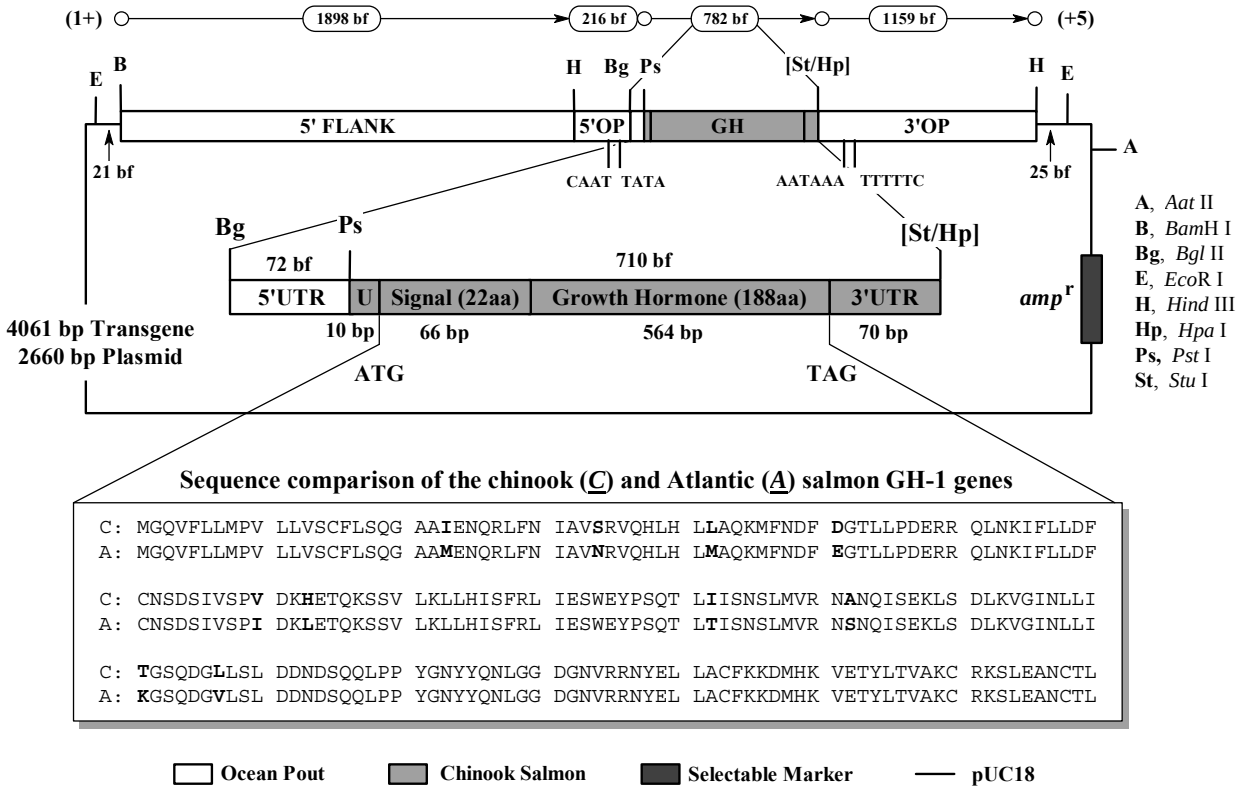
ABT has submitted several studies to CVM detailing the development and molecular-genetic characterization of the *AquAdvantage* rDNA construct (opAFP-GHc2), the development of the *AquAdvantage* Salmon line from founder animal through the F_7-generation, and the molecular-genetic characterization and stability of the integrated transgene (EO-1α) therein. CVM has reviewed these submissions and determined that they are satisfactory in addressing the characterizations required under the first two steps of the GE review process (*see*, Figure 1). The following section summarizes this information.

2.2.1 Characterization of the plasmid form, opAFP-GHc2

The plasmid form of the *AquAdvantage* rDNA construct, opAFP-GHc2, comprises 5′- and 3′-regulatory sequences from an ocean pout AFP gene and the complementary deoxyribonucleic acid (***cDNA***) sequence of a chinook salmon GH gene as an integrated transcriptional unit, which has been shown to retain the molecular-genetic integrity required for GH expression in salmonid cells (Sponsor submissions to CVM).

As illustrated in Figure 2, opAFP-GHc2 is a 6721 base-pair (***bp***) recombinant plasmid comprising 4061 bp of fish DNA and 2660 bp of vector DNA derived primarily from pUC18. As noted above, the characterization of opAFP-GHc2 has been the subject of Sponsor submissions providing a thoroughly detailed account of the following: source of fish DNA sequences used in construct development; molecular-genetic methods used to prepare the construct; *in vitro* expression studies confirming transcriptional capacity of the construct in fish cells; and, consensus nucleotide sequence of the transgene, including a comparison of that sequence to the published sequences of the constituent fish DNAs. CVM has found these submissions to be acceptable for characterization of the plasmid construct.

Figure 2. Physical Description of the *AquAdvantage* Construct, opAFP-GHc2 *



* Base-pair (***bp***) length is used in the narrative and figures in reference to the physical size of a DNA in fully-duplexed form; base fragment (***bf***) length is used in reference to the number of bases between, and inclusive of, the 5′- and 3′-nucleotides comprising the restricted recognition sequences on the boundaries of the + strand. ***amp***^{r}, *bla* gene providing ampicillin resistance.

In evaluating potential environmental risk associated with the construct itself, three specific elements of design and deployment should be taken into consideration: the selection of suitable promoters; the retention of antibiotic resistance genes after transgenesis; and, the use (or not) of viral vectors and transposons for improving integration efficiency.

The *AquAdvantage* construct employs a fish-derived promoter, opAFP, thereby avoiding the introduction of a promoter from another type of organism. The use of the opAFP promoter to develop an all-fish gene cassette suitable for gene transfer in aquaculture has been described in the published literature (Du *et al*., 1992a). Gene expression driven by the opAFP promoter in *AquAdvantage* Salmon has been well characterized in seven generations of fish, as described in §2.3.

The vector used to prepare the *AquAdvantage* construct is a bacterial (*E. coli*) plasmid called pUC18, which contains the gene for beta-lactamase (*bla*), an enzyme that confers resistance to ampicillin (***amp*^{r}**) and is used as a selectable marker in plasmid-cloning operations. Less than 50 bp of this plasmid DNA has been introduced into the GE fish genome, none of which encodes *bla* or any other gene of bacterial origin.

Viral vectors and transposons were not used in the *AquAdvantage* construct to improve transgene integration efficiency. Not using viral vectors and transposons eliminates a major mechanism for unexpected movement of genetic material within the genome of the GE fish or transfer to other unrelated species.

Thus, the *AquAdvantage* construct contains no intrinsic hazards to the environment.

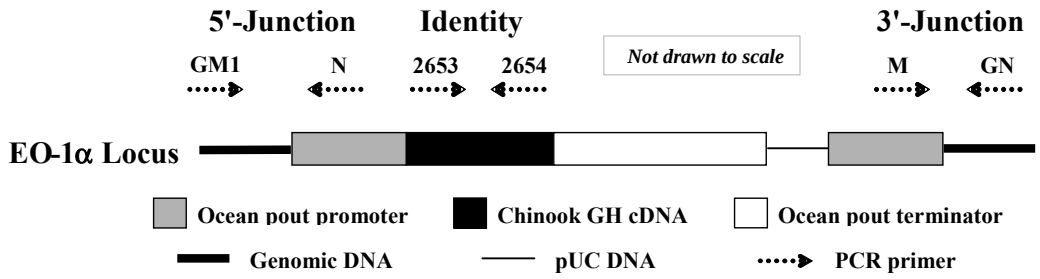
2.2.2 Characterization of the integrated form, EO-1α

The founder animal from which the *AquAdvantage* Salmon line derives was a mosaic, transgenic female (EO-1) generated in 1989 by micro-injecting a linearized form of opAFP-GHc2 into the fertilized eggs of wild Atlantic salmon. Two rapidly-growing, transgenic F_1-progeny of EO-1 were selected for further development and found to harbor two independently segregating integrants: a functional α-form and a non-functional β-form. During the breeding of six subsequent generations (i.e., F_2-F_7), an *AquAdvantage* Salmon line (EO-1α) was established that bears a single copy of the α-integrant, which has been the subject of several submissions to CVM providing a thorough account of the following: the development of the EO-1α line; diagnostic methods able to discriminate the α- and β-integrants; functional and molecular-genetic characterization of the EO-1α locus; multi-generational heritability and stability of the EO-1α locus; and, the consensus nucleotide sequence of the α-integrant in F_2- and F_4-generation *AquAdvantage* Salmon, including its comparison to the input-construct sequence (Sponsor submissions to CVM).

As shown in Figure 3, the α-form was subject to partial 5′→3′ rearrangement during its integration into the genome of EO-1. This particular integration event, the location thereof, and the molecular-genetic form of the transgene therein (collectively, the EO-1α locus) compose the defining characteristic of the *AquAdvantage* Salmon for which FDA approval is being sought. The molecular-genetic tools that were developed during investigation of

this integrated form have provided diagnostic means of determining its presence and stability, which has been done across numerous families of *AquAdvantage* Salmon through the F_6-generation, and which will continue to be done during commercial production as a matter of post-approval surveillance of product integrity and durability. The submissions made regarding the molecular-genetic characterization of the integrated form of the construct in *AquAdvantage* Salmon, and the heritability and stability of the transgene across multiple generations, have been accepted by CVM as satisfactory.

Figure 3. Physical Description of the Integrated *AquAdvantage* Transgene & Means of Diagnostic Assessment *



Diagnostic PCR Assessments		
Objective	**Transgene Target**	**Primers**
Identity	GHc cDNA	2653-2654
Stability	5′-junction	GM1-N
	3′-junction	GN-M

Differential Signals	
EO-1α	**Endogenous GH**
207 bp	798 & 1150 bp
572 bp	*None*
350 bp	

* Abbreviations: ***bp***, base-pair; ***cDNA***, complementary DNA; ***Endogenous***, native GH genes in the Atlantic salmon genome (i.e., GH-1 & GH-2); ***GH***, growth hormone; ***GHc***, chinook salmon GH; ***PCR***, polymerase chain reaction.

NB: The pUC DNA sequence residing between the ocean pout terminator and downstream portion of the ocean pout promoter subject to 5′→3′ rearrangement during transgene integration comprises 45 bp derived from the polycloning sites of the parent pUC vectors used in transgene construction. These sequences are non-coding and beyond the open-reading frame of EO-1α.

In evaluating potential environmental risk associated with the integrated form of the construct as a transgene in the *AquAdvantage* line, four specific elements of the structure and means of integration of this rDNA should be taken into consideration: foreign DNA could interrupt endogenous (i.e., host) genes and cause alteration or loss of function; foreign DNA could affect adjacent host genes and either decrease or increase their expression; the transgene could be expressed in an unexpected manner under promoters of adjacent host genes, or conversely, host genes could be expressed unexpectedly by the transgene promoter; and, transgene rearrangements during integration could create spurious open reading frames, which could result in the expression of *de novo* protein sequences.

These concerns have been evaluated through the functional and molecular-genetic characterization of the EO-1α locus with respect to its multi-generational heritability and stability, and detailed nucleotide sequence in F_2- and F_4-generation *AquAdvantage* Salmon.

No evidence of interruption of endogenous genes has been identified, lowering the risk of unanticipated phenotypic effects in the GE fish. No such effects have been observed in seven generations cultured over the last 15 years, as discussed in §2.4.

The results of these evaluations indicate that the structure of the transgene locus poses no risks to the environment.

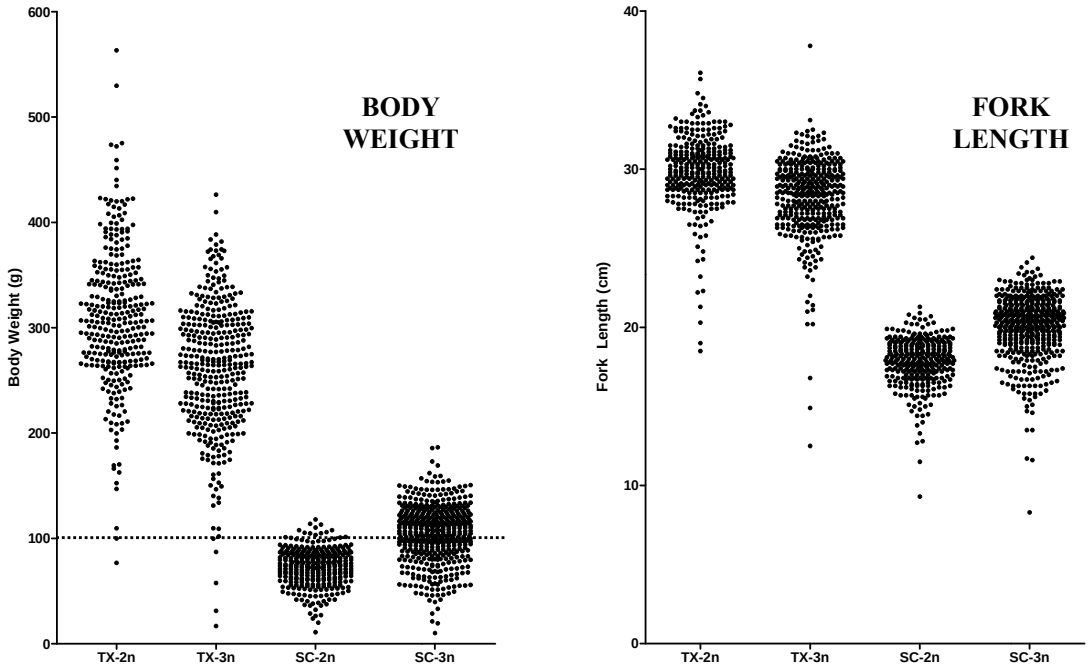
2.3 Durability of the Associated Genotype and Phenotype

The *AquAdvantage* phenotype, which has been linked to inheritance of the EO-1α locus, is characterized by an increase in rate of growth that is intended to improve the economics of salmon aquaculture by reducing time-to-market (Sponsor submissions to CVM). As shown in Figure 4, the mean body weight of an *AquAdvantage* Salmon population (261.00 ± 60.93 g) was significantly greater ($P < 0.0001$) than that of a population of non-transgenic, diploid siblings (72.63 ± 17.80 g) grown under the same culture conditions for ~2700°C-day after first-feeding; moreover, the percentage of *AquAdvantage* Salmon having a body weight greater than 100 g (98.64%) was significantly greater ($P < 0.0001$) than that of the non-transgenic, diploid comparators (4.90%).

Figure 4. Early-Life Growth Performance of *AquAdvantage* Salmon *

BW (g)	TX-2n	TX-3n	SC-2n	SC-3n
Sample (n)	309	369	306	464
Mean	309.90	261.00	72.63	104.21
SD	65.63	60.93	17.80	27.82
Max	563.3	426.3	118.0	186.5
Min	76.8	16.9	11.0	10.2
n > 100g	307	364	15	276
% > 100g	99.35%	98.64%	4.90%	59.48%

FL (cm)	TX-2n	TX-3n	SC-2n	SC-3n
Sample (n)	309	368	306	464
Mean	29.85	28.22	17.81	20.03
SD	2.30	2.53	1.55	1.99
Max	36.1	37.8	21.3	24.4
Min	18.5	12.5	9.3	8.3



* Diploid (TX-2n) and triploid (TX-3n) *AquAdvantage* Salmon and non-transgenic, diploid (SC-2n) and triploid (SC-3n) comparators grown for ~2700°C-day after first-feeding.

Supportive health and welfare data for *AquAdvantage* Salmon and their non-transgenic siblings captured in a 10-year historical record of the breeding program provide no indication of significant animal safety issues (Sponsor submissions to CVM). Early-generation *AquAdvantage* Salmon were found to be more subject to an increased incidence of certain morphologic irregularities (e.g., of the jaw & operculum), which are common in farm-raised salmon that have been selected for their rapid growth rates. The cause of these morphologic irregularities is a complex, poorly understood, multi-factorial phenomenon that does not appear to be a pathobiologic response specific to *AquAdvantage* transgenesis. The incidence of such morphologic irregularities decreased in later-generation fish, and was found to be comparable to the incidence observed in non-transgenic salmon grown under the same culture conditions in a blinded, comparator-controlled animal safety study comprising assessments of gross anatomy, histopathology and clinical chemistry, which is discussed further in §2.4.3.

ABT has examined the durability of the *AquAdvantage* genotype and phenotype in multiple families over seven generations. This experience has presented no indication of change or instability; and, as shown in Figure 5, the molecular-genetic stability of the integrated transgene has been examined specifically in representative individuals from the multiple families representing the source of animal subjects for regulatory investigations that have been submitted in support of NADA approval.

In addition, the molecular-genetic integrity of the integrated transgene will be monitored during the production cycle. This will be accomplished by analysis of a blood sample from each broodstock individual using a multiplex, polymerase chain reaction (***PCR***) assay. Animals failing the acceptance criteria will be re-tested and eliminated from prospective use in spawning upon confirmed testing failure.

The submissions made regarding the durability of the *AquAdvantage* Salmon phenotype, and the means of continuing surveillance and reporting thereof by the Sponsor following NADA approval, have been accepted by CVM as satisfactory.

2.4 Phenotypic Characterization of *AAS vs.* Wild and Domestic Salmon

This section provides a discussion of the *AquAdvantage* Salmon phenotype relative to non-transgenic Atlantic salmon, since phenotypic differences may also affect the ability of *AquAdvantage* Salmon to pose an environmental hazard, as discussed in subsequent sections. In general, Atlantic salmon display a high degree of phenotypic plasticity and complex life history that enable them to adapt to variable conditions and rigorous environments. In addition, genotype-by-environment (***GXE***) interactions will produce different phenotypes when animals with the same genetic background are exposed to different environmental conditions.

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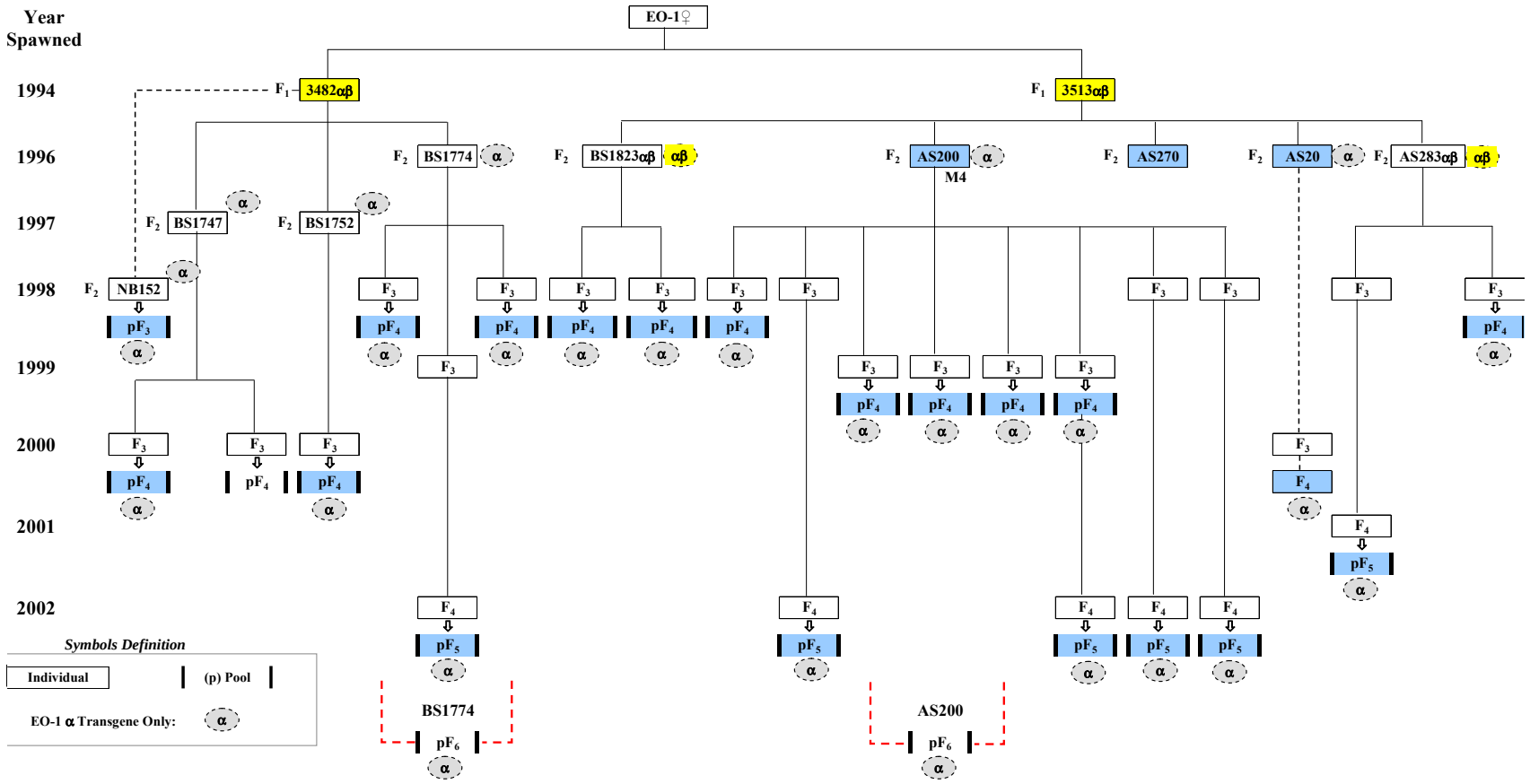
Figure 5. Summary Genealogy of *AquAdvantage* Salmon Sourced for NADA Studies & Durability Assessments *



* All individual fish and pools of fish sourced for enrollment in NADA investigations are colored blue. These individuals and pools were subjected to molecular-genetic confirmation of transgenotype using the PCR-based methods described in Figure 3, and were found to have the same EO-1α locus.

2.4.1 Biology of Atlantic salmon

Information on the biology of Atlantic salmon (*Salmo salar* L.) was obtained largely from reviews by Klemetsen *et al*. (2003) and Teufel *et al*. (2002).

2.4.1.1 Home range

Atlantic salmon inhabit both the east and west coasts of the North Atlantic Ocean, from the Connecticut River to Ungava Bay (Canada) in the northwest, and northern Portugal to the tributaries of the Barents Sea and White Sea (Russia) in the northeast.

2.4.1.2 Life history

Atlantic salmon populations exhibit diverse physiological, anatomical and behavioral characteristics that derive in part from local genetic adaptation. In populations for which seaward migration is not prevented by physical barriers, females are usually anadromous (i.e., living in salt water & spawning in fresh water); however, males often reproduce after living 1-4 years in fresh water, after which they may or may not migrate to sea. Anadromous populations also exhibit considerable variation in the type of freshwater habitat chosen for rearing (estuarine or lacustrine), the total duration of their seawater habitation (20-50% of lifetime), and the timing of spawning migration (spring or fall). Some Atlantic salmon complete their entire life cycle in fresh water, such populations being common throughout the North American range, but more limited to large lakes in the European distribution. The developmental phases of Atlantic salmon include the following:

- *Alevin:* A newly-hatched fish in the larval stage that has not yet emerged from the nesting area and is dependent upon a yolk sac for its nutritional requirements;
- *Fry:* An alevin that has fully absorbed its yolk sac and can now hunt for and consume live food;
- *Parr:* A young salmon in fresh water that has developed a characteristic skin coloration known as "parr marks;"
- *Smolt:* A young salmon that has undergone the physiologic adaptation necessary for transition to salt water;
- *Grilse:* A salmon returning to fresh water one year after migrating to the sea;
- *Kelt:* A salmon after spawning.

The Atlantic salmon is iteroparous, meaning it may spawn repeatedly. Typically, Atlantic salmon spawn during October to February, with the peak of spawning usually occurring in late October and November. The nesting site, or redd, is chosen by the female, and is usually a gravel-bottom riffle upstream from a pool (Bigelow, 1963; Scott & Crossman, 1973). The ecomorphological demands of the spawning grounds are as follows: *water descent* of 0.2-3%; *water depth* of 50 to 90 cm; *running speed* of 0.3 to 0.7 m/s; *gravel size* of 3 to 5 cm; and, *nest size* of 1 to 2 m (MUNLV, 2001).

The eggs are buried in gravel at a depth of about 12-25 cm (Bigelow, 1963; Scott & Crossman, 1973). The female rests after spawning and then repeats the operation, creating a new redd, depositing more eggs, and resting again until spawning is complete. Thereafter, the adult fish, or kelt, may return to the ocean without delay, move to a pool down-river for a period of rest, or over-winter in the nursery river and return to sea in the spring.

Egg hatching usually occurs in April, but the alevin remain in the gravel until the yolk sac is absorbed; the young fry emerge to feed on their own in May or June, and remain in rapid water where they continue development to the parr-stage (~6.5 cm fork length [***FL***]). After one winter, only the most rapidly growing parr (~10-15 cm FL) start their seaward migration and become smolt, which have undergone the physiologic adaptation necessary for survival in salt water.

Atlantic salmon grow rapidly while at sea. Some may return to the river to spawn as grilse after one winter at sea (***1-SW***), but they more typically spend two years at sea (***2-SW***). They school at sea entry and may travel with or be mistaken for herring, mackerel or other pelagic fish, since post-smolts occur as by-catch in these fisheries according to the North Atlantic Salmon Conservation Organization (***NASCO***, 2007). Post-smolts follow ocean currents, feeding as they migrate, and adding fish to their diet of marine invertebrates at about 27 cm FL after a few months at sea. Survival in fresh water from egg to smolt varies from 0.3-2.6%. Survival in the sea from smolt to return as grilse varies from 1.3-17.4% (Hutchings & Jones, 1998). Most Atlantic salmon (70-80%) survive spawning and migrate to sea a second time as kelt; only about 10% of them return to spawn a second time (Fleming, 1998).

The size of the adult fish is more dependent on time spent feeding at sea than on age. Sea-run Atlantic salmon usually attain a larger size than do landlocked salmon (i.e., those living entirely in fresh water). Sea-run salmon range from 2.3 to 9.1 kg and commercially-raised fish average 4.5 to 5.4 kg. (Teufel *et al*., 2002). Many aspects of Atlantic salmon behavior are affected by size. Investigations of growth in parr have shown that they may segregate into two or more groups at the end of the first growth season. Parr in the upper modal group may smoltify at 1+ years vis-à-vis the lower modal groups, which may smoltify later (Metcalfe *et al*., 1988). Within populations, therefore, the onset of the parr-smolt transition is dependent on growth rate. Smolt size can also vary widely among populations (Klemetsen *et al*., 2003). 1-SW salmon spawn usually every year, while older sea-age salmon are primarily biennial spawners; within populations, the proportion of biennial spawners increases with the size of fish at first maturity. The proportion of repeat spawners decreases with size of fish. This may be related to energy expenditure due to spawning: 1-SW salmon may allocate 50% of their energy (Jonsson *et al.*, 1991) for spawning compared to 70% for older salmon (Jonsson *et al.*, 1997).

Fecundity is another trait that varies considerably both within and among salmon stocks. Egg number and egg size increase with body size (Thorpe *et al.*, 1984; Jonsson *et al.*, 1996). Although absolute fecundity varies greatly among individuals, as expected owing to high variability in adult body size, relative fecundity (eggs/kg total egg mass) as a measure

of reproductive effort varies much less. The faster that parr grow in fresh water before smoltification, the smaller their relative egg size becomes when they attain maturity. This phenotypic response has been explained as an adaptation to the potential growth opportunities in their nursery river. Usually, both egg size and fecundity increase with size of fish (Klemetsen *et al.*, 2003).

Atlantic salmon compete for food and space in fresh water (Chapman, 1966) where they may be "keystone species" like Pacific salmon (steelhead, *Oncorhynchus mykiss*), which along with California roach (*Hesperoleucas symmetricus*) were found to influence the entire food web in a Northern California river (Power, 1990). In marine waters, however, even at their highest levels of historical abundance, Atlantic salmon are rare relative to the available space and few in proportion to total biomass of fish populations, and are thus expected to play a more minor role (Hindar, 2001).

2.4.1.3 Habitat

The physical habitat requirements of the Atlantic salmon vary depending upon the life stage. The preferred spawning habitat is a transitional area between pool and riffle with coarse gravel. Shelter (e.g., undercut banks or overhanging vegetation) is also important. Juvenile freshwater habitat includes rivers, lakes and estuarine (i.e., brackish) environments. Highest population densities are typically found in rivers with riffle, run and pool sections, with moderate-size cobble substrates. As parr grow, they prefer deeper and swifter parts of riffles. In general, juvenile salmon occupy shallow fast-flowing water with a moderately coarse substrate and overhead cover provided by surface turbulence. Once in the sea, the distribution of adult salmon appears to reflect environmental factors such as surface temperature, currents, and food availability.

2.4.1.4 Tolerance of physical factors

Temperature plays a major role in influencing salmon behavior. Fish move to sea earlier in southern than in northern rivers; and, in Europe, sea temperature is close to 8°C when smolt enter the ocean whether the river is southern or northern (Klemetsen *et al*., 2003). An optimal surface-seawater temperature range for Atlantic salmon is estimated to be 4-10°C (Reddin, 2006). The upper incipient lethal temperature (i.e., the temperature at which all salmon would exit a habitat if the opportunity were available) is estimated to be ~28°C (Garside, 1973); the lower lethal temperature is below 0°C (Reddin, 2006). Stead and Laird (2002) have cited the upper lethal temperature for salmon as being 23ºC. In a study examining the tolerance and resistance to thermal stress in juvenile Atlantic salmon, Elliot (1991) acclimated the fish for two weeks to various temperatures (5, 10, 15, 20, 25 & 27ºC) then raised or lowered the temperature by 1ºC per hour. The incipient lethal levels defined the tolerance zone within which salmon lived for a considerable time (i.e., survival over seven days). Salmon acclimated to 27ºC initially demonstrated the highest incipient lethal level at 27.8 ± 2ºC; for these fish, the lower mean incipient lethal level was 2.2 ± 4ºC. Temperature limits for feeding increased slightly with acclimation temperature to upper- and lower-mean values of 22.5 ± 0.3ºC and 7.0 ± 0.3ºC, respectively. The fish acclimated to 25ºC and 27ºC did not feed, while fish acclimated to the lower temperatures fed normally at 21.6-22ºC (Elliot, 1991). This indicates that, although fish acclimated to

relatively high temperatures may be able to survive more than seven days at these high temperatures, they do not feed at temperatures above ~23ºC and would eventually starve. Willoughby (1999) presents the feeding and activity range for smaller Atlantic salmon (i.e., < 100 g) in fresh water as favorable up to ~23ºC, with mortality occurring at ~26ºC. For larger Atlantic salmon, the available data for sea water show the feeding and activity range as favorable up to ~20ºC, with mortality occurring at ~22ºC. Elliott (1991) noted that little is known about the upper temperature limits for survival of Atlantic salmon in the field, and reported studies showing tolerances similar to those observed in his laboratory. Other experimental studies summarized by Elliott (1981, 1991) indicate that the optimum temperatures for growth of young Atlantic salmon are in the range 16-19ºC.

The minimum ***pH tolerance*** is between pH 5.0-5.4 depending on other river variables (e.g., aluminum levels), with eggs being the developmental stage least sensitive to acidity, followed by parr, and then smolt and fry, which are the most sensitive (Amiro, 2006).

Salmonids are known for requiring more ***dissolved oxygen*** (***DO***) than "warm-water fish." Shepherd and Bromage (1995) state that the oxygen content of water in a salmonid farm should never drop below 6 mg/L and that carbon dioxide starts to be a problem for salmonids above 15 mg/L. Similarly, Stead and Laird (2002) suggest that DO levels should never fall below 5 mg/L; for good growth, a minimum of 7 mg/L is essential.

Other challenges to survival come from ***obstruction and siltation***. Passage of salmon upstream can be blocked by natural and man-made obstructions. Generally, most vertical obstructions in excess of 3.4 m will block the upstream passage of salmon. As little as 0.02% silt has been shown to decrease egg survival (Amiro, 2006).

Atlantic salmon have the capacity to cope with a wide variety of ***flow conditions***, and juvenile salmon have been known to prefer pools at lower discharges and move from pool to riffle habitats at higher discharges. Their ability to adapt to changes in flow and tolerance of relatively high water temperatures enables juvenile salmon to occupy extensive sections of streams that experience variations in flow outside the range of useful habitat of some competitive sympatric species (Amiro, 2006).

2.4.1.5 Interaction with other organisms

In fresh water, Atlantic salmon compete with other conspecifics, grayling, brown trout, and brook trout. Carps, minnows, darters, perches, and similar fishes compete with Atlantic salmon in pools. It is difficult to determine the extent of competitive interactions in marine waters due to the vast scale of the habitat that is used.

Predators of smolt and juvenile salmon in fresh water include birds, reptiles, mammals, and other fish (including salmon and trout); predators in estuaries, coastal waters, and the sea include birds, fish, and mammals.

In fresh water, juvenile salmon are opportunistic predators of invertebrates, especially those drifting at the surface (including mayflies, stoneflies, caddisflies, midges, and beetles). Larger parr eat fish (including smaller trout and salmon) and their eggs. In marine waters,

post-smolts feed primarily on small fish and crustaceans such as euphausiids (krill), amphipods (scud), copepods, and crab larvae. Large juveniles prey mostly upon fish.

2.4.2 Domesticated and wild salmon

This section provides information about general practices used in salmon aquaculture; specific culturing practices for *AquAdvantage* Salmon are described in §3.0. In addition, information about the interaction of domestic salmon with their wild counterparts is discussed.

2.4.2.1 Salmon farming

Salmon farming industries rely on domesticated breeding lines selected for commercially important phenotypic traits, most importantly for faster growth and delayed sexual maturation (Gjedrem *et al*., 1991). The oldest of these lines, developed in Norway and incorporated into virtually all commercial breeding programs (except those in eastern Canada), achieved a growth rate improvement of about 10% per generation over the first seven generations of development (Gjøen & Bentsen, 1997).

Although Atlantic salmon can complete their entire life cycle in fresh water, most commercial Atlantic salmon farming involves both fresh and saltwater phases. In the freshwater phase, eggs are provided with a continuous flow of oxygenated water until they hatch. Typically, the alevin are transferred to small fiberglass tanks while they absorb the yolk sac prior to first-feeding. Once established on feed, the fry are transferred to larger tanks and grown to the parr stage, when they are sorted by size, segregated by growth rate, and transferred to separate tanks. In some locations, the parr may be transferred to lakes for the final phase of freshwater rearing. When the parr reach 60-120 g and begin to take on the silver coloration of smolt, they are typically transferred to saltwater production units called net pens or sea cages.

Under ambient light and temperature conditions, the freshwater phase takes 14-16 months, but is often shortened to eight months by increasing the early-rearing temperature and introducing a short period of darkness after the summer solstice to trigger smoltification at the next equinox, in the fall rather than spring (McCormick *et al*., 1987). Not all parr respond to such photo-manipulation. Virtually all commercial smolt are vaccinated against pathogens of local concern before transfer to sea water, which reduces the risk of disease, pathogen amplification, and the need for antibiotic treatment. The saltwater grow-out phase begins when the smolt are transferred to sea water and lasts for 12-26 months, depending on ambient sea temperature and the contingencies of harvest-to-order marketing. Feeding usually occurs twice a day, when the feed is generally moved by compressed air through tubes from a central hopper to each individual sea cage, and continues until uneaten feed is detected by an underwater sensor located below the layers of the water column where the fish are observed to feed.

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2.4.2.2 Interactions between domesticated and wild salmon

There are four general areas of potential interaction between natural salmonid populations and escaped, hatchery-reared fish that can pose hazards, which are as follows:

- Transfer of exotic pathogens or amplification of endemic pathogen loads (Saunders, 1991; McVicar, 1997);
- Genetic disturbance caused by transmission of fitness-reducing alleles (Ryman & Utter, 1987; Frankham, 1995), disruption of locally-evolved allelic combinations (Templeton, 1986; Ryman, *et al*. 1995; McGinnity *et al*., 2003), or "swamping" of the native gene pool (Sægrov *et al*., 1997);
- Direct competition for environmental resources, such as habitat, food, or mating opportunities (McGinnity *et al*., 1997; Fleming *et al*., 2000); and,
- Ecological disturbance through interference competition or disruption of local equilibria in complex systems, such as food webs, predator-prey relationships, or migration patterns (Lacroix & Fleming, 1998).

Pathogen transfer: Documented examples of pathogen transmission between artificially-propagated and wild fish are not common, but have been known to occur through stock enhancement programs (Brackett, 1991). Although there is no direct evidence of disease transmission from commercial to wild salmon, several incidents in the late 1980s suggest circumstantial involvement of farmed salmon in the movement of an endemic bacterium, *Aeromonas salmonicida*, from Scotland to Norway (Johnsen & Jensen, 1994; Inglis *et al*., 1991). The transmission of parasites by cultured fish on the other hand is less subject to debate (McVicar *et al*., 2007). The introduction of *Gyrodactylus salaris* to Norwegian waters in 1975 has been clearly linked to resource management activities (Johnsen & Jensen, 1991), but the role of farmed salmon in the subsequent epidemiology remains under investigation (Bakke & Harris, 1998). While *Gyrodactylus* was confined to Baltic waters before the Norwegian introduction, salmon lice, *Lepeophtheirus salmonis*, are endemic throughout the native range of Atlantic salmon, making a direct link to salmon aquaculture difficult to establish. Natural populations of parasites may be amplified in areas associated with salmon farming (Bakke & Harris, 1998), but sea lice abundance may be associated with rising marine temperatures as much as with the availability of hosts. At least one epizootic was reported before the advent of commercial salmon farming (White, 1940).

Genetic disturbance: Atlantic salmon have been subject to significant selection pressure, both intentional and inadvertent, as a result of human activity for more than a century. The former include, but are not limited to, size-selective harvesting, stock-enhancement efforts, transplantation across drainages and ecosystems, and increasing importance of commercial and recreational objectives; the latter derive (in part) from hydro-electric dams, acid rain, agricultural (and other) run-off, increased sedimentation and water temperature due to deforestation, and stocking of native (striped bass) and non-native (rainbow & brown trout) salmonid predators. Despite these challenges, evidence of genetically-differentiated population structuring is still evident for salmon at local, regional, and continental scale based on allozyme, mitochondrial, and nuclear DNA analyses (Ståhl, 1987; Bourke *et al*.,

1997; Bermingham, *et al*., 1991; McConnell *et al*., 1995; Taggart *et al*., 1995; King *et al*., 2001). The temporal stability of this structure has been traced over decades through the analysis of genetic material contained in archived scales (Nielsen *et al*., 1997; Tessier & Bernatchez, 1999).

Farmed salmonid strains are typically genetically distinct from local wild populations; for example, many farmed strains used in Ireland and Scotland are of Norwegian origin. Numerous studies have demonstrated that escaped farmed salmon can interbreed with local populations, thereby causing genetic change in them (Teufel *et al*., 2002). "*Inbreeding*" refers to mating between individuals more closely related than those drawn by chance from the general population, which can often result in a decrease in fitness. "*Outbreeding*" refers to mating between individuals from different populations, which can increase (enhance) or decrease (depress) fitness relative to both parental genotypes. Outbreeding depression can be the result of poor adaptation of the hybrid to the environment (e.g., the hybrid inherits a combination of traits that make it less suitable for that environment than either parent) or of the combination of alleles in the hybrid to each other. Outbreeding depression has been observed in an Irish experiment with first- and second-generation offspring of wild and farmed Atlantic salmon (McGinnity *et al*., 2003) and in hybrid offspring produced by the crossing of anadromous and landlocked Atlantic salmon (Sutterlin *et al*., 1987).

The persistence of genetic population structuring, even in the extreme circumstance of low population abundance and significant management intervention, indicates a degree of genetic resilience in locally-adapted wild populations that is critically important (NRC, 2003). Evidence of such persistence in nearly-extirpated Atlantic salmon populations raises doubt about the capacity of cultured salmon (ranched, farmed, or genetically-engineered) to undermine even small populations of wild salmon over time through genetic introgression or parallel colonization, and justifies the effort to rebuild and sustain those indigenous populations.

Direct competition for resources: Although domesticated Atlantic salmon are known to survive and breed successfully in the wild (Lura & Sægrov, 1991; Webb *et al*., 1991), they do so in a small proportion of the numbers that escape from farms (Webb *et al*., 1993; Clifford *et al*., 1998) and at a fraction of the spawning rate of wild salmon (Fleming *et al*., 1996; Clifford *et al*., 1998) for two primary reasons:

- ***Although socially dominant in culture environments, farmed Atlantic salmon are subordinate in nature:*** since salmon form dominance hierarchies around foraging opportunities, farmed salmon establish their social status in confinement; in nature, imposition of dominance is dampened by a resident advantage that deters even the largest fish from evicting territory holders from home ground; and,
- ***Farmed salmon compete poorly for mates and spawning locations:*** males are particularly disadvantaged in both access to mating opportunities and breeding success (Fleming *et al*., 2000); farmed females enter rivers out-of-phase with wild salmon, make fewer, poorly-covered nests, breed for a shorter period of time, and retain more eggs that remain unfertilized (Jonsson *et al*., 1997; Webb *et al*., 1991).

Consequently, even in their home range, the reproductive success of escaped, domesticated Atlantic salmon from spawning to F_1-adult return can range from 2-19% (Clifford, 1998; McGinnity *et al*., 2003; Fleming *et al*., 2000) of that achieved by wild salmon; the additional loss of 68% of eggs in the F_2-generation is a further barrier to successful introgression or establishment of escaped farmed salmon within or co-existent with natural populations (McGinnity *et al*., 2003).

Ecological disturbance: Farmed salmon can enter marine systems in large numbers by escape from containment nets, but can only become established by reproducing in adjacent freshwater ecosystems. Consequently, the fitness and behavior of feral Atlantic salmon is of continuing interest as a matter of risk management in Atlantic salmon aquaculture, specifically with regard to their escape at sea, homing migration, freshwater spawning and survival of offspring. A fundamental risk parameter, the number of animals escaping containment, is difficult to know with certainty due to inconsistencies in reporting, lack of long time-series, decomposition of small fish that die in sea cages, and limited data collection on escapees at sea; however, two million escapees in the North Atlantic has come to be an acceptable estimate (McGinnity *et al*., 2003) that represents an escape rate of about 1%. When applied to operations in the Pacific, this rate of escape would suggest that an additional one million escapees could derive from Chilean production and one-half million from farms in the Pacific Northwest. Escaped farmed salmon feed poorly in fresh and salt water, and may not begin feeding on wild prey for a considerable period after escape owing to their acclimation to pelleted feed; by way of example, only 5-15% of escaped Atlantic salmon recovered from British Columbian and Alaskan waters had fed after their release (Alverson & Ruggerone, 1997). Less than 2% of wild Atlantic salmon currently return to spawn; escaped farmed salmon survive marine conditions and migration at one-third to one-half of the rate for wild Atlantic salmon and return to fresh water at about 1% of the numbers that are estimated to escape (Butler *et al*., 2005). In studies of farm sites worldwide over a period of two decades, Nash (2001) concluded that "*a common denominator is that the potential for environmental impact depends primarily on the site of each individual farm. The most important rule in the management of risk is therefore the careful selection of the site.*"

2.4.3 Genetically-engineered salmon

Studies of *AquAdvantage* Salmon have demonstrated that they are similar in many ways to their non-transgenic counterparts, but very different in other respects; the same may be said of comparisons between GE salmon. Devlin and colleagues have published a significant body of work on GH-transgenesis in salmonids that is of considerable interest, but has limited, direct impact on the objective considerations required under this EA. For example, Devlin *et al*. (2000) micro-injected coho salmon (*Oncorhynchus kisutch*) eggs with an rDNA construct (opAFP-GHc) differing in sequence from opAFP-GHc2 only with respect to the ~70 bp 5'-untranslated region (which derives from chinook salmon rather than ocean pout). The GE coho salmon thus derived had a growth rate that was just ~1.7-2.7 times greater than that of non-transgenic siblings, even though GH levels were significantly elevated (19.3- to 32.1-fold). This finding is dissimilar from the experience with *AquAdvantage* Salmon, which have a much higher growth rate than their non-transgenic

siblings that occurs in the absence of a demonstrable increase in GH levels. Moreover, the experimental efforts of Devlin and colleagues have focused more generally on transgenic coho salmon in which a metallothionein promoter was used in GH-transgenesis. These OnMTGH1 transgenic coho salmon exhibit an ~11-fold higher growth rate and 40-fold elevation of GH levels that appear to be derived from the integration of multiple, tandem-repeats of the rDNA construct (Devlin *et al*., 1994; Uh *et al*., 2006). In view of these considerable differences, no concerted attempt will be made to draw upon the work of Devlin and colleagues herein, considering the difficulty in assessing its true relevance to *AquAdvantage* Salmon.

Given the high degree of phenotypic plasticity of *Salmo salar*, and impact of GXE interactions, it is not surprising that the wide spectrum of traits observed in wild-type Atlantic salmon generally encompasses that of *AquAdvantage* Salmon.

Investigations of the human food safety of *AquAdvantage* Salmon have shown that the nutritional and hormonal composition of their muscle and skin is similar to that of present-day farmed salmon (Sponsor submission to CVM); of particular interest is the fact that no elevation of GH in muscle and skin was detected.

A blinded, comparator-controlled animal safety study (Sponsor submission to CVM) was conducted in which the gross anatomy, histopathology, and clinical chemistry of male and female, diploid and triploid, *AquAdvantage* Salmon and size-matched, non-transgenic comparator salmon were examined. Normal behavior was observed in all groups of fish. Eight physical features were evaluated and the incidence of abnormalities was similar for *AquAdvantage* Salmon and the non-transgenic comparators, with the number of abnormal findings being greater for triploid fish of both treatments, especially with regard to irregularities in gill structure. An examination of nine internal organs or structures, as well as relative organ weights, revealed no differences between transgenic and non-transgenic salmon or between diploid and triploid salmon. The majority of values for hematology and serum chemistry parameters of *AquAdvantage* Salmon were consistent with published values that represent the normal range for Atlantic salmon; and, the statistically significant differences that were observed are believed to be related to the inherent difference in metabolic rates between *AquAdvantage* and comparator salmon, the effect of triploidy on red cell number and size, and unavoidable limitations in study design. The pathology findings associated with *AquAdvantage* transgenesis were limited to an increased presence of minimal-to-mild focal inflammation of unknown cause in some tissues, especially among diploid fish, and a low occurrence of jaw erosions among both male and female diploids. The majority of other findings, which included gill and fin abnormalities, soft tissue mineralization, hepatic vacuolization, and cardiac shape abnormalities, affected the triploids of both groups. In the aggregate, these findings were generally of low magnitude, limited distribution, and non-debilitating nature; they were deemed unlikely to compromise the overall health of *AquAdvantage* Salmon in commercial production.

It should be noted that morphologic irregularities do occur in salmonids, most commonly affecting cartilaginous and boney structures (Brown & Nuñez, 1998), and are often associated with the development of new commercial lines or husbandry techniques and

culture conditions. Developmental malformations of cartilage and bone have been observed quite commonly in association with intensive commercial farming of salmon (*Salmo*) and trout (*Oncorhynchus*) species, including *S. salar* (Bæverfjord *et al*., 1996; Silverstone & Hammell, 2002; Vägsholm & Djupvik, 1998), *S. trutta*, (Poynton, 1987), *O. mykiss* (Mbuthia, 1994; Madsen & Dalsgaard, 1999), and *O. kuta* (Akiyama *et al*., 1986), as well as salmonids in the wild (DeVore & Eaton, 1983). These malformations include irregularities of the head, jaw, and operculum, and twisting or compression of the spine. Although the incidence of these malformations has not been studied systematically, a background incidence of 3-5% is not uncommon in experimental control animals (Ørnsrud *et al*., 2004). Veterinary field studies have identified the periodic occurrence of spinal compression (humpback) in 70% of salmon in Norwegian farming operations (Kvellestad *et al*., 2000) and jaw malformation in 80% of salmon at commercial sites in Chile (Roberts *et al*., 2001). However, aggregate data for the industry have not been reported, and the experience of individual commercial operations remains closely held. Such irregularities are not limited to salmonids, but have also been reported in the culture of other fish species.

Neither intensive selection for growth nor inbreeding depression are deemed responsible for these morphologic irregularities (Bæverfjord *et al*, 1996), which have been linked more commonly to suboptimal culture conditions (e.g., nutrition, water quality & environmental stressors), inclusive of, but not limited to, the following: phosphorus or vitamin deficiency or excess; high or variable temperatures at incubation, early-rearing, or saltwater transfer; and, exposure to therapeutic treatments, pollutants, or parasites. In general, mild-to-moderate malformations of the head, jaw, operculum, or spine have limited impact on morbidity or mortality when other rearing conditions are optimized; however, rearing conditions that are otherwise deficient and present significant environmental stressors can lead to the increased mortality of these fish. Even in the best circumstance, severe malformation can render a fish non-viable, and multiple malformations can be cause to reject an otherwise viable fish for commercial sale.

In the aforementioned comparator-controlled study (Sponsor submission to CVM), no severe malformations were noted among the *AquAdvantage* Salmon enrolled. Irregularities in the fins and gill structure of triploid transgenic salmon as well as triploid non-transgenic salmon were noted, while diploids in both groups had a low incidence of jaw erosion. The observed abnormalities are within the range of frequency and severity commonly noted in cultured salmonids.

The main difference between *AquAdvantage* Salmon and non-transgenic *Salmo salar*, and the basis for the value of the product, is the significant increase in growth rate of the former. Academic studies of early-generation GE salmon deriving from the program that led eventually to identification and development of the EO-1α line provided estimates of growth rate that were 2- to 6-fold greater than non-transgenic comparators during the first year of life (Du *et al*., 1992b). As was previously noted, a comparator-controlled study of growth performance in F_6-generation *AquAdvantage* Salmon (Sponsor submission to CVM) has confirmed their significant growth advantage over a period of ~2700°C-day in both average size (261.0 g *vs*. 72.6 g for diploid controls) and proportion of animals larger than 100 g (98.6% *vs*. 4.9% for diploid controls).

Transgenesis for improved growth rate has been described as an alternative method of domestication through which traits can be targeted and enhanced in less time than would otherwise be required via selective breeding. Domestication, in turn, occupies a point on a trait continuum across phenotypes comprising different wild, hatchery-reared, sea-ranched, farmed, and GE variants (Tymchuk *et al*., 2006). Rapid-growth phenotypes appear to share several key physiological and behavioral attributes regardless of breeding methodology, including the following: the use of a common endocrine pathway to accelerate growth; elevated metabolism, feeding motivation, and efficiency; increased aggression and foraging activity; and, reduced antipredator response (in farmed Atlantic salmon, Fleming *et al*., 2002; in early-generation, *AquAdvantage*-related transgenics, Cook *et al*., 2000a and Abrahams & Sutterlin, 1999). Differences appear to occur in the scale of trait expression rather than in the scope or character of the trait expressed. Major behavioral changes in GH-transgenic fish include significantly enhanced feeding motivation, increased predation mortality, reduced discrimination of prey choice, and reduced schooling tendency (Devlin *et al*., 2006). The complexity of the interactions between these effects and, in turn, their interactions with the environment, makes it difficult to predict the overall fitness of GH-transgenic salmon in the environment relative to their wild counterparts.

The primary feature of GE individuals that affects the questions to be addressed in the EA is characterization of the nature and magnitude of specific phenotypic changes elicited by expression of the transgene (Kapuscinski & Hallerman, 1991). These authors have suggested that the specific phenotypic changes to be examined comprise the following:

- Metabolic rate;
- Range of tolerance values for physical factors;
- Behavior;
- Resource or substrate use; and,
- Resistance to disease, parasites, or predation.

These factors will affect the fitness of the GE individuals, which will in turn affect their interaction with other organisms and their role in ecosystem processes.

2.4.3.1 Metabolic rates

Metabolic rates influence the components of the overall energy budget for an individual; the components of the energy budget in turn influence an individual's impact on nutrient and energy flows, and other organisms. The distinguishing feature of *AquAdvantage* Salmon is rapid growth, which is a composite of many physiological rates. *AquAdvantage* Salmon have metabolic traits that also appear in other fast-growing Atlantic salmon or in fish that have been treated with time-release GH implants (Johnsson & Björnsson, 2001). Selection for faster growth in domesticated Atlantic salmon is generally associated with increases in pituitary and plasma GH levels (Fleming *et al*., 2002); however, such increases are also observed in wild salmon during winter famine, smoltification, and sexual maturation (Björnsson, 1997). The only unique attributes of GE fish appear to be an increase in the magnitude of trait expression associated with the increase in growth rate

when food is available, and the allocation of energy to growth that occurs at the expense of stored reserves (Cook *et al*., 2000b).

The expression of growth hormone changes aggregate metabolic activity in several ways: lipid breakdown and mobilization are increased, and energy is deployed more readily for maintenance or growth; protein synthesis is increased, providing the raw material for additional body mass; mineral uptake is increased, promoting skeletal development and a longer, leaner morphology; and, feeding efficiency (i.e., feed conversion ratio, or ***FCR***) is improved (Björnsson, 1997). The cost to the animal is higher oxygen utilization due to increased digestive demand and protein synthesis. In comparison to non-transgenic controls, early-generation relatives of *AquAdvantage* Salmon (hereinafter "*AquAdvantage* relatives") had lower initial energy reserves, 2.1 to 2.6-fold greater feed consumption, and a propensity to deplete body protein, dry matter, lipids, and energy more quickly during starvation (Cook *et al*., 2000a & 2000b). Routine oxygen uptake in *AquAdvantage* relatives was 1.7 times that of controls (Stevens *et al*., 1998) and oxygen consumption during activity was 1.6-fold greater, further increasing with effort (Stevens & Sutterlin, 1999). Although these *AquAdvantage* relatives have demonstrated an ability to reduce their metabolic rate in response to starvation, their enhanced metabolic profile and lower initial energy reserves greatly reduce the likelihood of their growing rapidly, or even surviving, outside of the highly supportive conditions provided by commercial farming (Hallerman *et al*., 2007).

2.4.3.2 Tolerance of physical factors

Tolerance of physical factors such as temperature, salinity, pH, etc., can be altered in GE organisms. Changes in lethal limits or optimum values can shift preferred habitats, seasonal patterns, or the geographic range.

As discussed above, the increased requirement for oxygen exhibited by *AquAdvantage* relatives (Abrahams & Sutterlin, 1999; Cook *et al*., 2000a; Cook *et al*., 2000b; Deitch *et al*., 2006) would engender a reduced tolerance for diminished oxygen content in general, and a reduced capacity for survival when DO content is critically low, compared to their non-transgenic counterparts in the wild. In experiments with *AquAdvantage* relatives, oxygen uptake was independent of oxygen concentration above 10 mg/L, but started to decrease at about 6 mg/L DO in transgenic fish versus 4 mg/L in control fish (Stevens *et al*., 1998). Under conditions of oxygen saturation, transgenics are not at a disadvantage compared to controls, since oxygen demand is readily satisfied.

GH appears to have a role in osmoregulation in anadromous salmonids (Down *et al*., 1989; Powers, 1989). During migration from fresh water to sea water, levels of GH are elevated, leading to an increase in sodium exclusion at the gills. Migrating transgenic smolt would therefore be likely to avoid predation better than wild smolt upon entering sea water, because they would adjust faster to the saline environment and thereby escape estuarine and coastal predation (Hindar, 1993). However, other factors (discussed in subsequent sections) tend to increase the predation risk for GE fish.

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AquAdvantage Salmon are triploid fish, and triploidy may be another factor apart from transgenesis affecting tolerance limits. Atkins and Benfey (2008) reported that triploids of Atlantic salmon had lower thermal optima than diploids, which could explain prior observations of mortality of other triploid salmonids (brown trout, brook trout & rainbow trout) at chronically elevated, but sub-lethal rearing temperatures. Data exist for a variety of species of fish to indicate that triploidy could be responsible for reduced survival of early-life stages and reduced survival and growth of later-life stages, particularly when environmental conditions are not optimal (Piferrer *et al*., 2009).

2.4.3.3 Behavior

Behaviors associated with swimming, feeding, reproduction, territorial defense, migration, or other developmental events could be affected by transgenesis. The ecological impacts of these changes in behaviors could affect life history patterns, population dynamics, and species interactions (ABRAC, 1995). *AquAdvantage* relatives did not differ from wild counterparts in critical swimming speed (Stevens *et al*., 1998); however, they did demonstrate double the rate of movement of wild-type fish (Abrahams & Sutterlin, 1999). In nature, swimming performance is important in foraging and predator avoidance. Abrahams and Sutterlin (1999) also demonstrated that *AquAdvantage* relatives would spend significantly more time feeding in the presence of a predator than non-transgenic salmon, indicating that they possess a higher tolerance for predation risk.

GH also increases appetite in various species of salmonids (Raven *et al*., 2006; Abrahams & Sutterlin, 1999; Devlin *et al*., 1999), which influences behavioral traits associated with feeding, foraging, and social competition. The availability of food also influences behavior. The difference in scale between GE and other fast-growing Atlantic salmon is less quantifiable for behavioral traits and further confounded by the effects of hatchery culture, particularly in acclimation to high rates of social interaction. Salmon form dominance hierarchies around foraging opportunities, and hatchery fish have more opportunities to reinforce their social status in confinement. In nature, social dominance is dampened by a resident advantage that generally deters other fish from evicting territory holders from home ground. A 25% difference in size is necessary to overcome the resident advantage (Metcalfe *et al*., 2003).

Under laboratory conditions, GH-transgenic coho salmon (*Oncorhynchus kisutch*) bearing the OnMTGH1 construct have been observed to be more competitive (Devlin *et al*., 1999), less discriminate in choosing prey (Sundström *et al*., 2004), more likely to attack novel prey (Sundström *et al*., 2004), and better at using lower quality food (Raven *et al*., 2006) when compared to wild relatives. Although these effects would have the potential to influence wild relatives both directly and indirectly, such observations were demonstrably muted when the GE fish were reared under simulated natural conditions (Sundström *et al*., 2007), indicating the complexity of gene-environment interactions.

[End of Page]

*AquAdvantage S*almon are triploids; thus, information on the performance of triploid Atlantic salmon in the wild is relevant to consider. Ocean migration studies in Ireland revealed that male triploids returned to their natal area in nearly the same proportions as diploids, whereas female triploids mostly did not (Wilkins *et al*., 2001). Similar results were found in another trial in which the return rate of triploid Atlantic salmon was substantially reduced (Cotter *et al*., 2000a).

2.4.3.4 Resource or substrate use

Changes in resource or substrate use might occur through direct or indirect impact of transferred genes. An example of an indirect impact is the potential for fish bearing a GH transgene to alter food webs; their increased size at a given age can lead to increases in size of their selected prey (Kapuscinski & Hallerman, 1990). As mentioned above, GH increases appetite; and, Cook *et al*. (2000c) found that feed conversion efficiency was improved by 10% for *AquAdvantage* relatives.

2.4.3.5 Impact of disease, parasites, and predation

If the GE organism had improved resistance to disease, parasites, or predation, it could out-compete its non-transgenic counterparts. No evidence has been found that *AquAdvantage* Salmon have any improved resistance to disease or parasites. In fact, although not strictly applicable to *AquAdvantage* Salmon, Devlin *et al*. (2006) found that GH-transgenic coho salmon have reduced disease resistance, impaired swimming ability (which would lead to greater predation risk), and increased predation mortality. As observed by Abrahams and Sutterlin (1999), *AquAdvantage* relatives were more likely to ignore potential predators when they were foraging (at least under laboratory conditions), which could make them more susceptible to predation.

2.4.3.6 Morphology and limits to growth maximization

Changes in the morphology of the organism (e.g., size, shape & color) could alter species interactions (ABRAC, 1995); however, it should be noted that accelerated growth is not an assured outcome for GE salmon in nature. The rapid-growth phenotype is expressed only if supported by sufficient food, as has been shown in both transgenic coho salmon (Devlin *et al*., 2004b; Sundström *et al*., 2007) and *AquAdvantage* relatives (Cook *et al*., 2000b). This is a function of both the productivity of the habitat and the density and behavior of competitors for the resource. GH-transgenesis influences the GXE interaction via powerful stimulation of appetite in the presence of food and a larger capacity for food consumption given the opportunity. *AquAdvantage* relatives consumed ~five times more food than same-age controls that were also size-matched by delaying hatch time of the transgenics: this consumption differential appears to derive from the increased feeding motivation of the transgenics, which were 60% more likely than controls to be observed at both safe and risky foraging sites, and the increased willingness of the transgenics to feed in the presence of a predator (Abrahams & Sutterlin, 1999).

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The aforementioned, considerable differences in growth and feeding behavior between non-transgenic, wild-type, or domesticated salmon and GE salmon have been observed in simplified hatchery environments; outcomes in more complex naturalized environments may be much less dramatic: by way of example, hatchery-reared, GH-transgenic coho salmon exhibited greater predation and ~3-fold greater fork-length than age-matched wild type conspecifics; when reared under naturalized stream conditions, they exhibited more modest predation activity and were only 20% longer than controls (Sundström *et al*., 2007).

2.4.3.7 Reproduction

Changes in the age at maturation, fecundity, and sterility could alter population and community dynamics and interfere with the reproduction of related organisms (ABRAC, 1995). Due to their enhanced growth rate, *AquAdvantage* Salmon achieve reproductive status in a shorter time-frame than their non-transgenic siblings; and, many animals, including Atlantic salmon, select mates based upon male body size, so GE males exhibiting larger-than-average body size might be perceived as having an advantage over their wild counterparts. However, significant impacts are unlikely to be observed, since domesticated salmon in general have reduced spawning performance relative to wild fish, and *AquAdvantage* Salmon will be cultured only as sterile females.

2.4.3.8 Life history

Changes in embryonic and larval development, metamorphosis, and life span could alter life-history patterns as well as population and community dynamics (ABRAC, 1995). GH constructs in salmonids have been shown to influence larval developmental rate (in coho salmon, Devlin *et al*., 1995b & 2004a) and smoltification (in Atlantic salmon, Saunders *et al*., 1998; in four species of Pacific salmon, Devlin *et al*., 1995a). Saunders *et al*. (1998) found that *AquAdvantage* relatives reached smolt size sooner than normal and the smoltification process was not inhibited by high temperatures (19ºC) or constant light.

2.4.3.9 Summary characterization vs. non-transgenic salmon

Atlantic salmon display a wide range of characteristics and can adapt to a variety of conditions. *AquAdvantage* Salmon share many of these traits, the notable exception being their increased growth rate and the physiologic sequelae thereof (e.g., increased oxygen consumption).

Table 1 summarizes the observed differences between GH-transgenic salmonids and non-transgenic Atlantic salmon. In many cases, these differences were of greater magnitude under laboratory conditions than in a simulated natural environment. Consequently, not all of these differences may be expressed, or may be expressed to a lesser extent, in the wild.

The submissions made by the Sponsor regarding the phenotype of *AquAdvantage* Salmon have been accepted by CVM as satisfactory.

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Table 1. Differences between GE- and Non-transgenic Salmonids

Trait	Transgenic Relative to Non-transgenic
Metabolic rates	Increased metabolic rates Increased growth when food is available Reduced initial energy reserves Increased oxygen consumption
Tolerance of physical factors	Reduced tolerance to low oxygen availability Reduced thermal optimum range (effect of triploidy not GH)
Behavior (lab conditions)	Increased feeding motivation and reduced prey discrimination Reduced schooling tendency Reduced anti-predator response
Resource or substrate use	Increased utilization of lower quality food (lab conditions) Increased utilization of larger prey (potential)
Resistance to disease, parasites or predation	Reduced disease resistance Reduced anti-predator response, increased predation mortality
Reproduction	Accelerated growth to sexually-mature size Larger males can have a mating advantage
Life history	Accelerated growth to smolt-size Smoltification at higher temperatures and constant light

3.0 PRODUCTION, GROW-OUT, AND DISPOSAL

3.1 Description of *AquAdvantage* Salmon Egg Production

The production of eyed-eggs for *AquAdvantage* Salmon will occur at a specific site on PEI. In the following narrative, the general characteristics of the production process that are not location-specific will be presented, followed by a detailed description of the production facility and local environs.

3.1.1 General production plan

3.1.1.1 Reproductive biology of AquAdvantage broodstock

The production of *AAS* eyed-eggs requires the development of *AquAdvantage* broodstock, which are neomales (i.e., genetic females) homozygous for EO-1α (i.e., having two copies of the transgene), through a process involving two methodologies for the manipulation of salmonid reproductive biology: gynogenesis and sex reversal. Milt from *AquAdvantage* broodstock is used to fertilize eggs from non-transgenic, female Atlantic salmon, and the fertilized eggs are pressure shocked to induce triploidy. The resulting product is a triploid, eyed-egg that will produce a sterile female Atlantic salmon that is hemizygous for EO-1α (i.e., having one copy of the transgene).

In order to produce *AquAdvantage* broodstock, individual *AquAdvantage* females homozygous for EO-1α are subjected to gynogenesis, a reproductive method that generates a monosex population of homozygous females, which are then sex-reversed via treatment

with androgen. The resulting neomales are genotypic females that produce sperm, which can only produce female offspring when crossed with a true female. The original source of homozygous females derives from matings between male (T-, XY) and female (T-, XX) *AquAdvantage* Salmon, and the identification of homozygous animals (TT, XY & TT, XX) that produce 100% *AquAdvantage* Salmon when back-crossed.

The process of gynogenesis involves the destruction of the genetic component in fish sperm, use of those "empty" sperm for egg activation, and restoration of a diploid state in the activated egg by forced retention of the second polar body. All of the offspring from this process are genetic females with a full complement of maternal DNA. The induction of gynogenetic Atlantic salmon is a proven methodology that has most often been accomplished by destruction of sperm DNA via ultraviolet (***UV***)-irradiation, followed by the use of pressure- or heat-shock to prevent loss of the second polar body (Refstie, 1983; Quillet & Gaignon, 1990; Johnstone & Stet, 1995; Slettan *et al*., 1997). To avoid any contribution of genetic material from sperm that may inadvertently escape destruction during irradiation, a different fish species can be used for egg activation. Thus, the sperm that escape destruction will produce either non-viable offspring or hybrid progeny that can be distinguished visually. In the process applicable to *AquAdvantage* Salmon, gynogenesis is done using UV-irradiated milt from Arctic charr (*Salvelinus alpinus*), followed by pressure shock to restore diploidy. Any salmon-charr hybrids that may be produced are easy to distinguish from pure salmon due to a distinct difference in their coloration pattern.

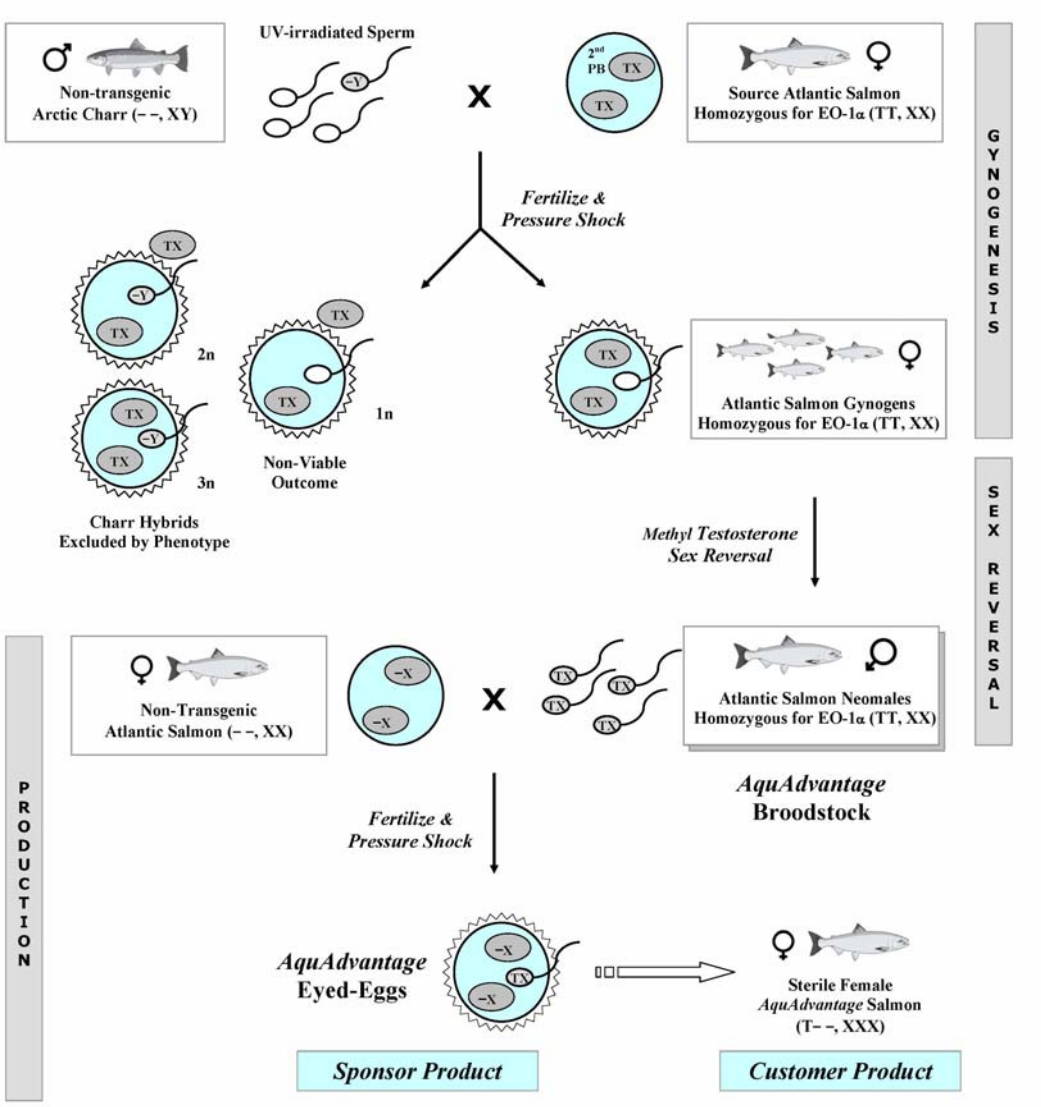
Atlantic salmon have an XY system of sexual determination, such that females are homogametic (XX) and males are heterogametic (XY). Many fish species experience a labile period after hatch when intentional exposure to sufficient levels of androgen or estrogen can influence phenotypic sexual maturity (Pandian & Sheela, 1995). A genetic female can be induced to develop as a phenotypic male, or so-called neomale (XX), the milt from which will produce only genetically female offspring when crossed with a true female (XX). The monosex nature of the progeny derived from neomale-female matings has been demonstrated in several salmonid species, including Atlantic salmon and rainbow trout (Johnstone & Youngson, 1984; Johnstone *et al*., 1978; Johnstone & MacLachlan, 1994; Lee *et al*., 2004). In the *AquAdvantage* production process, 17α-methyl-testosterone (***MT***) administered in the diet is used to produce *AquAdvantage* neomales. In the aforementioned claim validation study conducted by ABT (Sponsor submission to CVM), the animal subjects enrolled were derived from 20 non-transgenic Atlantic salmon females that were crossed with nine hemizygous *AquAdvantage* neomales: the sex of 180 progeny tested for confirmatory purposes was determined to be female.

The reason for generation of an all-female population, which is subsequently sex-reversed, is that it is tedious and time-consuming to distinguish neomales from true males following MT treatment of a mixed-sex population. Consequently, gynogenesis is used to produce an all-female population of salmon homozygous for EO-1α, which will generate *only* the homozygous transgenic neomales required for eyed-egg production when they are treated subsequently with MT.[2]

[2] As noted by Piferrer *et al*. (2009), sex reversal is commonly used in the commercial production of rainbow trout per European Union (***EU***) Directive 96/22/CE (26 April 1996).

The homozygous *AquAdvantage* neomales are mated with non-transgenic females to produce egg populations that are 100% hemizygous *AquAdvantage* females. Triploidy in the eggs is then induced by pressure shock to render the animal sterile. The reproductive biology of broodstock and eyed-egg production is summarized schematically in Figure 6.

Figure 6. Reproductive Biology of *AquAdvantage* Broodstock & Eyed-Egg Production *



* For broodstock development, eggs from a female salmon homozygous for EO-1α are fertilized with UV-irradiated charr sperm, and forced retention of the second polar body (***PB***) is accomplished by pressure shock [**Note:** As shown, the 2nd PB is disproportionately large to allow for indication of genotype]. Salmon-charr hybrids that develop from any sperm that retain viable DNA are identified and removed from the gynogenetic population desired. For production, eggs from non-transgenic Atlantic salmon are fertilized with milt from neomales homozygous for EO-1α and pressure shocked to induce triploidy to ensure that the eyed-eggs sold into commerce can only generate sterile female *AquAdvantage* Salmon. Abbreviations: ***T-***, hemizygous transgenic; ***TT***, homozygous transgenic; **--**, non-transgenic; ***XX***, genetic female; ***XY***, genetic male.

3.1.1.2 Technical details and logistics of commercial production

The activities comprising the technical and logistic details of *AquAdvantage* product manufacture are discussed below and summarized schematically in Figure 7.

Development of AquAdvantage broodstock for post-approval manufacture: Eggs collected from sexually mature, genetic-female salmon homozygous for EO-1α (TT, XX), in which the identity and integrity of the *AquAdvantage* transgene has been confirmed using diagnostic methods, will be fertilized with irradiated milt from Arctic charr, pressure shocked, and incubated until hatch. The fry (TT, XX) will be sex-reversed using 17α-methyl-testosterone, then graded and PIT-tagged at a body weight of ~10-20 g, at which time any salmon-charr hybrids in the population will be identified for disposal. These *AquAdvantage* (neomale) broodstock (TT, XX) will be reared to sexual maturity, when their neomale status will be confirmed by the presence of spermiation.

Maintenance of AquAdvantage broodstock for commercial manufacture: Subsequent generations of *AquAdvantage* broodstock can be derived from existing neomales homozygous for EO-1α by using the milt from those animals to fertilize eggs from true females homozygous for EO-1α (TT, XX); the offspring will be sex-reversed, graded, tagged, and subject to molecular-diagnostic confirmation of genotype prior to their qualification for use in future spawnings.

Production of *AquAdvantage* eyed-eggs for commercial sale: Eggs from non-transgenic female Atlantic salmon (--, XX) will be fertilized with the milt from *AquAdvantage* (neomale) broodstock (TT, XX), and the fertilized eggs (T-, XX) will be pressure shocked to induce triploidy (T--, XXX). The eyed-eggs will be incubated in Heath stack incubators (~10,000 eggs/tray **x** 12-16 trays) or upwelling jars (100-200,000 eggs) for 325-400°C-day, at which time batch-wise sampling will be done to confirm the successful induction of triploidy via flow cytometry prior to release for commercial sale. Confirmation of triploidy is further discussed in §6.1.2.

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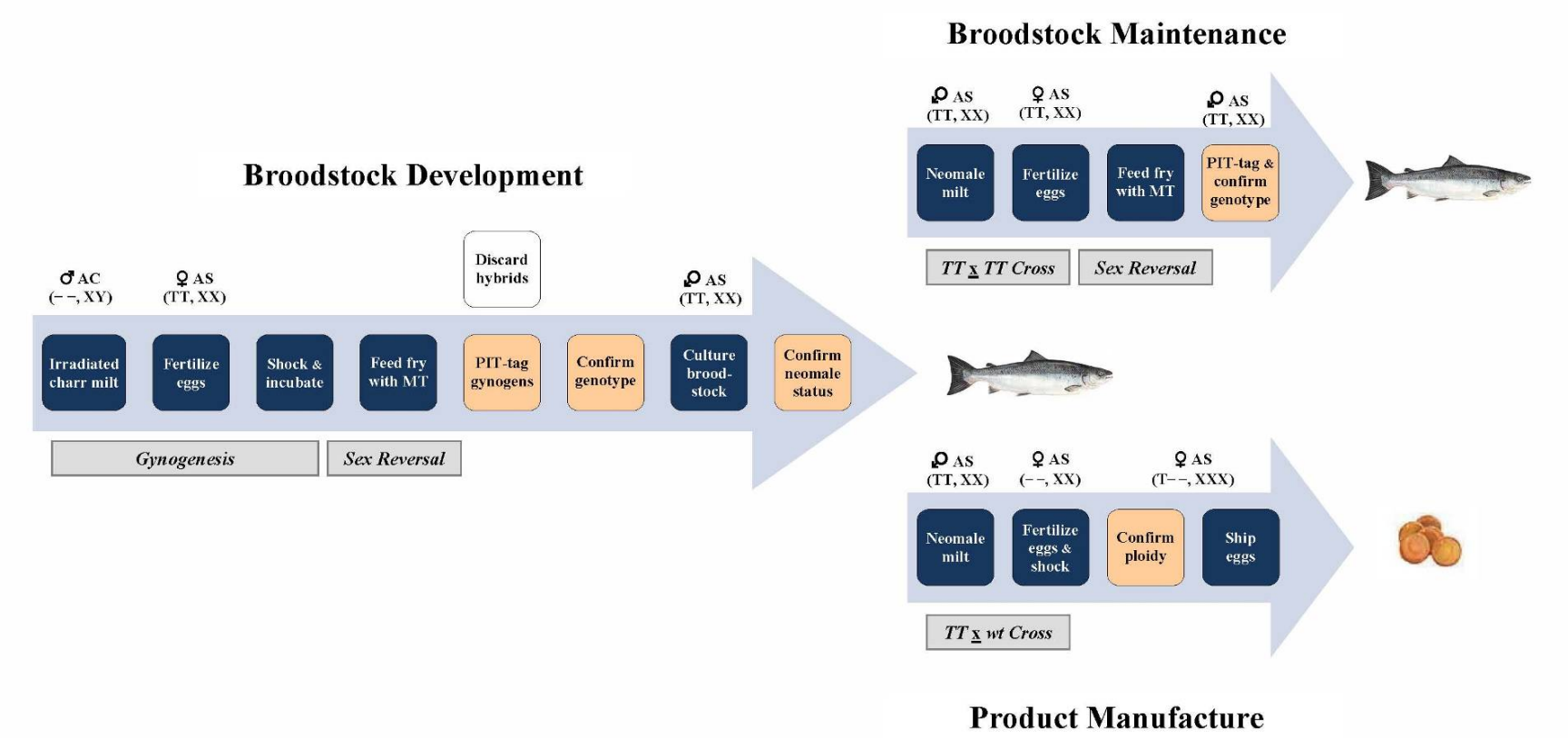
Figure 7. Technical Details & Logistics of Commercial Production *



* Abbreviations for genotypes are defined in the footnote to Figure 6 and the narrative under §3.1.1.2.

3.1.2 Specific production plan

This EA addresses the production of eyed-eggs at only one site, the Sponsor's land-based, freshwater aquaculture facility on PEI, which comprises a main building, storage facility, and ancillary enclosures for operational structures that are secured as follows:

- ***Perimeter security:*** ~1590 linear feet of 2″ x 9 ga x 8′ galvanized chain-link fence of commercial quality surrounds the property, inclusive of freshwater well-heads, back-up generators, liquid oxygen containment, and the storage facility. A service entry adjacent to the storage building remains secured by a double-swing, chain-link gate except when service access to the property is required. A roll-away, chain-link gate spanning the main entry to the property, which is adjacent to the main building, is secured during non-business hours. At night, the entire perimeter remains well-lit.
- ***Outside entries:*** Windows on the lower-level of the main building are barred, and all exterior steel-doors on the main and storage buildings are dead-bolted. Entry into the main building requires a key or intercom-interrogation and remote unlocking by facility staff. Within the main building, access to the first-floor aquaculture facility is further protected by a cipher-locked, interior entry.
- ***Security monitoring:*** Eight motion-activated security cameras are positioned for maximum surveillance of the property immediately surrounding the main building. These cameras are in continuous operation and automatically capture digital images that are stored for later retrieval. Magnetic door-contacts and interior motion-detectors deployed throughout the main building, storage facility, and out-buildings comprise a network of zones that are monitored by a commercial security service.
- ***Water supply & pump-house:*** The primary well and pumping facilities (one primary, two back-ups) that supply the aquaculture facility are securely enclosed in a steel containment structure.
- ***Remote notification of status:*** Environmental alarms indicating emergent change in operational conditions (e.g., water level, DO content), and security alarms indicating suspected intrusion during non-working hours, are conveyed by the security service to senior facility staff via numeric page; in addition, direct telephone contact with the facility manager or other on-call staff is pursued until successfully made, so that clear communication of the event occurs and proper and immediate response is managed.
- ***Additional security:*** The Sponsor may employ professional security personnel to remain on-site during non-business hours. In addition to their direct surveillance of the property, these personnel would have access to the central, security-monitoring system in the main building, but would not have access to the facility at-large, which would remain locked-down and subject to the network of electronic sensors and motion-activated cameras comprising that system. An apartment in the main building provides for additional surveillance by staff living on-site.

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The main building comprises ~9,240 sq ft used for aquaculture operations and ~3,020 sq ft used for laboratory, office, and living space. Inspections for various purposes over the past 10 years have resulted in the facility having been: 1) deemed compliant with containment practice and licensed to conduct research on GE fish under applicable Canadian regulations; and 2) classified as an acceptable manufacturing establishment and judged as having no significant environmental impact by FDA.

Aquaculture operations are conducted in two principal areas comprising four categories of tanks that provide for maintenance of the following: the Early-Rearing Area (***ERA***) for eggs, alevin, and fry; and, the Grow-Out Area (***GOA***) for fry and smolt, as well as longer-term cultivation of juveniles and broodstock.

The ERA comprises 32 (1.8 m D x 0.8 m H) combi-tanks of 1.5 m^3 capacity and 73 (0.59 m W x 0.60 m D) combi tanks of 0.16 m^3 capacity, all of which are fitted with an internal standpipe and mesh-net covering to enforce containment. The GOA comprises 12 (1.8 m D x 0.8 m H) combi-tanks of 1.5 m^3 capacity and 24 (3.6 m D x 1.1 m H) grow-out tanks of 11.2 m^3 capacity that are also outfitted with mesh netting. A variety of other physical barriers and containment practices have been established to ensure that living animals do not escape from the facility into the local environment (*see*, §6.2.1).

A site description, detailed containment diagram, and procedures governing husbandry practice and maintenance have been provided to FDA, which (as noted above) has conducted an on-site inspection that identified no material deficiencies pursuant to use of the facility for product manufacture.

3.2 Description of *AquAdvantage* Salmon Grow-Out

This EA addresses the grow-out of eyed-eggs at only one site, the Sponsor's land-based, freshwater aquaculture facility in Panama.

The facility, which is designed for rearing *AquAdvantage* Salmon from the eyed-egg stage to market-size, comprises a small building that is used for fry-tank housing, quarantine, feed storage and office space, and four outdoor culture tanks. Other components of the facility include water-intake structures, header tanks, low-head oxygenators (***LHO***), containment structures and devices, and four sedimentation ponds. A site description and detailed containment diagram have been provided to FDA, which has conducted an on-site inspection that identified no material deficiencies pursuant to use of the facility for product grow-out. The facilities at this site are secured as follows:

- The site is located in a remote, highland area with very limited access.
- Entry onto the site requires passage via a securely-gated footbridge.
- Culture facilities are enclosed by an 8-foot security fence topped with barbed wire.
- Entrance gates are securely locked and the area is protected by dogs.
- A private residence adjacent to the property provides for additional surveillance by management living on-site.

Eyed-eggs will be received at the site, and acclimated to ambient water temperature and pH. After the eggs hatch, the alevin will be moved to the fry tanks, where they will remain until they are later transferred to the grow-out tanks.

The fry-tank building contains six fiberglass tanks, each with a capacity of 3 m^3. These tanks are assembled with an upper insert containing an interior standpipe that controls the water level. The standpipe is covered by a 1 mm screen when fry are being fed the smallest feed sizes and a 1.5 mm screen when they graduate to larger feed sizes. An exterior screen with a 1.5 mm slot-aperture is placed outside the interior standpipe screen. The lower (primary) tank is equipped with a basket screen (3 or 6 mm) and top screen (3, 6 or 12 mm). In addition, inside the standpipe, affixed by screws to the base of a basket screen, is a permanent metal screen with 5 mm openings that prevent fish of larger diameter from leaving the tank. All water leaving the fry tanks must pass through a 500 µm sock filter that is inspected daily and empties into an excavated earthen drainage canal. Water flow in the fry tanks is regulated at 2-2.5 L/min-kg biomass.

When the fish reach an average size exceeding 25 g, they are transferred to the grow-out tanks. Each grow-out tank is equipped with a rigid, polyvinyl chloride (***PVC***) drain-screen plate having slots of 0.9 cm aperture that is anchored by screws to the one-and-only drain opening of the tank. Fish are transferred from fry tanks to the grow-out tanks when 100% of the animals are more than 1 cm in diameter, so that no animals can pass through the drain-screen plate. The individual grow-out tanks have a maximum capacity of 100 m^3, but are operated at an operational volume of 85 m^3. Densities in the grow-out tanks will be maintained at values below 35 kg/m^3 for optimal water quality and growth conditions, with water flow that supports complete turnover of tank capacity ~once every hour. Water leaving the grow-out tanks flows through the slotted drain-screen and is discharged into a concrete containment sump, from which it flows into an excavated earthen drainage canal.

Drainage from both the fry and grow-out tanks enters the drainage canal and flows through a second concrete containment sump equipped with a 12 mm steel screen-plate, which is anchored in such a way that all water passing through the sump is screened. Distal to the sump, the water flows into a sequential series of four settling ponds, each of which is equipped with a 12 mm rigid-metallic outlet screen on which a secondary, variable-gauge screen is placed to facilitate flow, while maintaining exclusion of fish as they increase in size from fry to market size. From these ponds, the water is recycled into a local river.

The fry tanks and building containing them, as well as the outdoor grow-out tanks, are covered with netting to prevent avian predation and "jumpers" (i.e., fish that escape confinement by jumping out of the tank). In particular, the grow-out tanks are sealed horizontally and vertically inside a cage comprised of netting supported by a rigid structure. Escape from the tanks by jumping, or removal of fish by avian predators, is impossible.

[End of Page]

The primary water supply for the fry tanks derives from a spring located north of the site that is delivered through two 6 in pipes, which converge prior to entering an oxygenation tank. The tank is equipped with a water-level control sensor and alarm, and two small LHOs that are supplied with pure oxygen via hoses from liquid-oxygen cylinders. Oxygen is injected into the spring water, which then flows by gravity to the fry tanks. Water flow to the fry tanks is controlled by means of valves located on the incoming supply line to each tank. In the event of an interruption in spring-water flow, a secondary, emergency water line can be employed. The water intakes are inspected on a daily basis, or more frequently during inclement weather.

The primary water supply for the grow-out tanks derives from an intake canal that diverts water from a local river. Water flows to a basin, which in turn supplies a very large LHO. The water is then gravity-fed to the grow-out tanks through a 16 in pipe. Water flows are adjusted by two valves located on the incoming water supply to each tank. The intake canal is inspected weekly and cleaned when debris accumulates.

When the fish are of sufficient size to be transferred from the fry tanks to the grow-out tanks, they are initially stocked into two of the four grow-out tanks. Subsequently, as they grow and biomass approaches 35 kg/m³, the fish are distributed among all four grow-out tanks, where they will remain until they reach market size (1-3 kg body weight). Fish may be harvested at different weights to test different markets and product presentations. The fish will be harvested by netting, euthanized on-site using ice water, and shipped by truck to a local plant for processing and shipment to local and export markets. The fish will be marketed in different presentations (e.g., whole on ice, whole-dressed on ice, fillet on ice & frozen or smoked fillet). Fish sold into the local market in Panama will be distributed by truck; those exported to the US will be shipped in refrigerated containers by established wholesalers subject to Panamanian law and regulatory authority.

3.3 Description of *AquAdvantage* Salmon Disposal

The disposal of non-viable waste material associated with the production, processing, and consumption of *AquAdvantage* Salmon will not require handling that is different from that used for wild or domesticated non-transgenic fish: the gene construct is not infectious, communicable, or transmissible from waste material. Nevertheless, the Sponsor will handle in-process disposal with greater consideration than may be necessary, given the density and aggregate volume of waste material involved.

3.3.1 Disposal of eggs and fish

At the production site on PEI, triploid eyed-eggs will be sold for commercial grow-out of *AquAdvantage* Salmon following the annual spawn, or destroyed by incineration. Live animals retained in inventory for other research and development activities will be tagged at appropriate size (10-20 g) with a passive integrated transponder (***PIT***) for subsequent identification. The vast majority of transgenic animals requiring disposal are those being culled to maintain acceptable stocking densities or being used in regulated studies requiring lethal termination; morbid or dead animals, and incidental losses (e.g., "jumpers" or "escapees" recovered from containment), are removed during the daily surveillance and

maintenance of the facility. All of these animals requiring disposal are stored frozen within the confines of the aquaculture facility until they are incinerated *en masse*.

The small number of dead eggs and morbid or dead animals removed during daily surveillance and maintenance of the grow-out facility in Panama will be buried on-site. Each burial pit will be excavated to an initial depth of 1.0 m (0.5-0.75 m diameter). As dead fish are deposited, they will be covered with caustic lime, followed by another layer of dead fish and caustic lime, etc., until the burial pit is ~0.5 m deep, at which point it will sealed with plastic and covered with soil. Successive pits will be located at a minimum distance of 0.5-1.0 m from those used previously; the aggregate collection of such pits will be located on high ground that is not within the 100-year flood plain (*see*, §4.2.1). In the event that disposal capacity at the site is inadequate to handle the immediate or aggregate waste volume, alternative means of disposal will be sought.

3.3.2 Disposal of fish wastes

Effluent from the aquaculture facility on PEI is subjected to highly redundant containment measures using both physical (e.g., screens & drum filters) and physico-chemical (e.g., high water temperatures & chlorine exposure) barriers. Water-exchange rates and routine monitoring of water quality preclude excess chemical- (***COD***) or biochemical-oxygen demand (***BOD***) in the effluent from attendant metabolic wastes (e.g., ammonia, nitrate, nitrite). Particulate matter (e.g., feces & uneaten feed) is trapped by screened sumps, then recovered and bagged for incineration. Effluent leaving the facility is released into the local watershed, which flows without diversion into the local marine environment.

Effluent from the grow-out facility in Panama is also subjected to highly redundant containment, inclusive of screened sumps, before being released into a series of four sedimentation ponds. The considerable water-exchange rates available (25-50 fold daily) are sufficient to dilute attendant fish wastes, and suspended solids are trapped in the sedimentation ponds, which are dredged every 18-24 months (as may be required), before flowing into the local watershed. Aggregate waste generation will be less than is typical of a commercial salmon farm due to the relatively low biomass of the grow-out facility.

3.3.3 Disposal of processing wastes

Wastes generated during fish processing in Panama will be disposed of per applicable law.

3.4 Labeling, Packaging, and Shipping

The product entering commerce from the production site on PEI will be limited (as a condition of US product approval) to eyed-eggs, which are the life-stage most efficiently, effectively, and safely transported.

The product will be packaged in a manner consistent with, but more rugged than, the Styrofoam egg crate typical of industry practice. *AquAdvantage* eyed-eggs be packed in a hard-plastic "Igloo" cooler containing alternating trays of eggs and wet-ice; the cooler will be bound with packing straps and further secured in a heavy-cardboard shipping container.

A bilingual Product Label printed on tear- and water-resistant paper will be affixed to both the egg crate and shipping container; this Label provides a summary of product definition, purpose, intended use, warnings, and cautions-instructions of immediate importance to the end-user. A bilingual Package Insert comprising detailed handling recommendations and data regarding performance, food safety, animal safety, transgene characterization, and environmental considerations will also be included. The shipment will be identified as a "Live Animal Product" that is "Not for Resale." The following additional warnings (or facsimile thereof) will also appear on the Product Label: [3]

- These fish must be reared in land-based, highly contained systems that prevent their release into the environment;
- These fish cannot be reared in conventional cages or net pens deployed in open bodies of water; and,
- Morbid or dead fish should be disposed of in a manner consistent with local regulations.

Product prepared for shipment will be transported by car (or truck) to a local international airport by ABT staff, where direct control will be assumed (through prior arrangement) by a freight-forwarder. The freight-forwarder will arrange, manage, and personally monitor air-freight shipment of the Product to Panama (inclusive of permits & customs requirements), where control will be returned to ABT personnel waiting on the ground.

4.0 AFFECTED ENVIRONMENT

This EA is specific to production of eyed-eggs of *AquAdvantage* Salmon at a specific site on PEI and grow-out of *AquAdvantage* Salmon at a specific site in Panama. Consideration of the affected environment therefore focuses on these two locations from the perspective of their potential impact upon the global commons and uninvolved foreign nations. Effects at the local level remain under the jurisdiction of local authorities.

4.1 Egg-Production Site

Production of eyed-eggs will occur at a land-based, freshwater aquaculture facility on Prince Edward Island.

4.1.1 Physico-chemical properties

The climate at the production facility is generally damp with an average yearly rainfall of 87 cm and an average yearly snowfall of 340 cm; average temperature is -7ºC in January and 19ºC in July. The nearest location for which climate data are available is shown in Table 2: average values for minimum and maximum daily temperatures by-month have ranged from -16.6 to 13.5ºC and -3.3 to 23.2ºC, respectively, over the past 30 years.

[3] The Product Label, Package Insert, and deployment thereof are under review by FDA at the time of this writing; the Sponsor intends to satisfy recommendations for any alternative or additional warnings or advisements, or means of their incorporation or display on the marketed Product, that may derive from said review.

Table 2. Weather Data for the Production Site Environment *

Month	Avg Daily Temp (ºC)		Avg Rainfall	
	Min	Max	Amt (cm)	Rain Days
Jan	-12.6	-3.3	10.6	18.8
Feb	-12.4	-3.3	8.6	16.1
Mar	-7.1	0.9	9.2	16.0
Apr	-1.4	6.7	8.8	15.4
May	4.0	14.1	9.8	14.7
Jun	9.6	19.6	9.3	12.8
Jul	13.8	23.2	8.6	12.4
Aug	13.5	22.6	8.7	11.3
Sep	9.1	18.0	9.5	13.7
Oct	3.8	11.8	10.9	15.0
Nov	-1.1	5.7	11.1	17.5
Dec	-8.1	-0.1	12.3	20.6

* Abbreviations: ***Amt***, amount; ***Avg***, average; ***Max***, maximum; ***Min***, minimum. Values are based on monthly averages for the 30-year period 1971-2000. Mean number of rain days = mean number of days with at least 0.2 mm of precipitation, including both rain and snow.

During the spring, summer and fall, temperatures in the waters adjacent to the facility are suitable for salmon survival; however, water temperatures during the winter months are typically very low, with surface ice being common. The temperature of local estuarine waters ranges from -2 to 2ºC in the winter, with a typical ice cover of 0.3-0.6 m. The ice cover limits the growth of marine life by acting as a barrier to both oxygen and light. Salmon would tend to avoid these conditions by either a) remaining in fresh water (i.e., rivers or lakes) where minimum water temperatures do not fall below 0°C, or b) migrating offshore to ocean waters where such low temperatures and ice can be avoided. Consequently, local coastal conditions would be inhospitable to salmonids during the coldest periods of winter. Salinity in the water adjacent to the facility varies with the tide, distance from the outflow, and time of year. Despite these variations, the water remains quite saline, with values exceeding 21 ppt (and up to ~30 ppt) being common.

No natural disasters (e.g., hurricanes, fires, or earthquakes) of significance have occurred, or are known to occur, in the environs of the manufacturing facility on PEI.

4.1.2 Biological/ecological properties

The local environment has numerous shallow bays, broad estuaries, and short rivers that contain an abundance of favorable habitat for diadromous fishes. Fish common to the area include the following: mackerel; herring; eel; gaspereau (e.g., alewife & blueback herring); silverside; smelt; and, salmonids. The salmonid group comprises the following: Atlantic salmon (*Salmo salar*); brook trout (*Salvelinus fontinalis*), which is native to the region; and, rainbow trout (*Oncorhynchus mykiss*), which was introduced into the region in 1925. Commercially important crustaceans include lobster and snow crab; bivalves (e.g., mussels, oysters, soft-shelled & bar clams, quahogs) are also fished commercially.

Between 1971 and 1985, the estimated abundance of 1-SW Atlantic salmon in North America fluctuated between 0.8-1.7MM fish annually; between 1995 and 2006, the estimated abundance declined to about 0.4-0.7MM fish. This prompted the closure of all commercial fisheries for Atlantic salmon in the Gulf Region of Canada (which includes New Brunswick, Nova Scotia, and Prince Edward Island) in 1984, which was expanded to all of eastern Canada in 2000. The most severe declines in abundance have been reported in the 32 rivers of the Inner Bay of Fundy, where Atlantic salmon have been designated as "endangered" by the Committee on the Status of Endangered Wildlife in Canada and listed under the Species at Risk Act. The factors contributing directly to reduced marine survival remain largely unknown, while the factors in fresh water include acid rain and poaching.

Barriers to migration and over-exploitation have contributed to the elimination of natural salmon runs in the environs of the production site; the chief limitation to recovery is stream sedimentation caused by agriculture and other land-use activities. Restocking and habitat enhancement have been attempted with limited success; however, as a practical matter, no wild salmon populations remain, and future returns of salmon to local rivers are dependent on hatchery stocking of smolts raised semi-naturally in open impoundments.

4.2 Grow-Out Site

The land-based, grow-out site is located at high elevation in Panama adjacent to a river within a major watershed that flows from north to south into the Pacific Ocean. Dams associated with three operational hydro-electric facilities divert a significant portion of the aggregate water flow from the river for power generation, returning effluent to the watershed further downstream. During the 4-5 month dry season, ~100% of the water flow in the river may be diverted for this purpose. Water diversion occurs through canals that provide a poor habitat for salmonids because of a low gradient and high sedimentation rate, which results in a poor bottom substrate and low food availability (see further discussion below). Four additional hydro-electric facilities are currently planned for the watershed. These existing (and planned) facilities, and the water diversion structures (i.e., dams & canals) associated with them, constitute a significant, but not complete, barrier to fish migration to the Pacific Ocean.

4.2.1 Physico-chemical properties

Air and water temperatures were determined at a series of points along the course of the local river from its highland origins (Point 11) to its lowland return to the Pacific Ocean (Point 1) in September 2009. These values, which are shown in Table 3, vary little from month-to-month and are representative of year-round conditions.

[End of Page]

Table 3. Air & Water Temperatures in the Local River Adjacent to the Grow-Out Facility *

		Temp (°C)	
Point	**Elev** (m)	**Air**	**Water**
1	13	28.9	26.4
2	91	31.9	28.1
3	250	29.4	26.0
4	347	28.6	25.8
5	649	24.3	22.6
6	995	21.6	19.3
7	1024	21.6	19.0
8	1086	21.7	20.7
9	1278	20.7	18.8
10	1792	17.2	15.1
11	1850	18.1	15.8

* Abbreviations: ***Elev***, elevation; ***Temp***, temperature.

The watershed, and rivers and streams discharging into it, receive average-annual rainfall of 398 cm, 91.8% of which occurs during the rainy season. During the dry season, precipitation is markedly less, but streams and rivers do not go dry. Generation of hydro-electric power continues to dominate water use (> 93%), followed by agricultural and industrial demands. As shown in Table 4, average-monthly air temperatures at higher elevation in the watershed range from 16.8 to 19.6ºC over the course of the year (minimum: 11.6-14.8ºC; maximum: 23.3-28.8ºC; World Meteorological Society). Data collected over a period of nine years for the region indicate that average-daily temperature ranges from 17.6 to 20.6ºC regardless of the month of the year (WorldClimate).

Table 4. Weather Data in the Higher-Elevation Vicinity of the Grow-Out Facility *

Temp (ºC)	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
Avg	18.9	18.9	19.2	19.6	19.2	18.9	16.8	18.2	18.5	18.2	18.1	19.0
Min	11.6	13.2	13.7	14.8	13.1	14.0	13.8	14.2	13.9	14.3	13.6	12.4
Max	23.3	27.9	27.7	28.8	28.1	26.5	26.1	27.7	27.4	28.2	27.0	26.0
Humid. (%)	56.5	59.6	60.0	64.1	80.0	78.5	77.6	83.2	84.0	85.3	82.8	58.9
Rain (d; cm)												
Days with	1	5	4	9	21	17	24	30	24	25	18	10
Days without	30	24	27	21	10	14	7	1	6	6	12	21
Total mo^{-1}	0.4	1.9	2.9	9.1	104.0	32.9	78.8	101.3	79.6	89.7	53.9	8.2
Total yr^{-1}	0.4	2.3	5.2	14.3	118.3	151.2	230.0	331.3	413.8	503.5	557.4	569.6

* Data from a private weather station in the immediate vicinity of the facility. Abbreviations: ***Avg***, average; ***d***, days; ***Humid***, humidity; ***Max***, maximum; ***Min***, minimum.

Data recorded at two locations near sea level also show very little variation during the year. As shown in Table 5, average-monthly minimum and maximum daily temperatures ranged from 18.8-21.6ºC and 31.9-36.3ºC, respectively, over 30 years for which data are available.

Table 5. Weather Data for the Near Sea-Level Locations *

Month	Avg-Daily Temp (ºC)		Avg Rainfall	
	Min	Max	Amt (cm)	Rain Days
Jan	18.8	34.5	3.3	2.8
Feb	19.3	35.6	1.9	1.7
Mar	19.9	36.3	3.6	3.2
Apr	21.1	36.3	10.3	6.7
May	21.6	33.8	29.7	16.3
Jun	21.5	32.5	32.3	16.3
Jul	21.2	32.7	29.0	15.4
Aug	20.9	32.4	34.0	18.1
Sep	21.1	32.0	40.7	19.9
Oct	21.1	31.7	40.1	21.3
Nov	20.7	31.9	30.0	15.7
Dec	19.3	33.1	7.7	6.4

* Abbreviations: ***Amt***, amount; ***Avg***, average; ***Max***, maximum; ***Min***, minimum. Data are the aggregate monthly averages for the 30-year period from 1971 to 2000. Average number of rain days = average number of days with at least 0.1 mm of rainfall.

In addition to temperature, other physical and chemical parameters affect the likelihood of survival and propagation of fish and wildlife in the major rivers of the watershed. Values for these chemical and physical parameters are presented in Table 6.

Table 6. Chemical & Physical Parameters in the Major Rivers of the Watershed *

Parameter	Units	Upper	Mid-basin	Lower
Avg Annual Rainfall	(cm)	300	300	600
Avg Annual Rainfall Volume	(m^3)	1.43	5.54	50.8
Avg Water Temperature	(ºC)	14-15	24.9 - 25.2	23.6 - 25.8
Dissolved Oxygen Content	(mg/L)	7.6 - 8.4	7.0 - 7.2	7.8 - 8.0
Transported Sediment	(Ton/yr)	1058	*na*	116,000
Turbidity	(NTU)	1.6 - 23.0	1.4 - 4.0	1.4 - 6.0
Total Solids	(mg/L)	74.1 - 80.6	90.0 - 45.1	84.6 - 117.0

* Abbreviations: ***Avg***, average; ***na***, not available; ***NTU***, nephelometric turbidity units.

The upper part of the local river has favorable conditions for establishing salmonid populations: temperature, DO, and turbidity are all within their tolerances. However, these conditions change in the mid- and lower-parts of the river where temperatures exceed the lethal limit (~23ºC) identified by Stead and Laird (2002). High sedimentation loads downstream further diminish the quality of the local environment for salmon survival.

No natural disasters (e.g., hurricanes, fires, or earthquakes) of significance have occurred, or are known to occur, in the environs of the grow-out facility in Panama.[4]

4.2.2 Biological/ecological properties

A diversity of macroinvertebrates exist in the local river, including mayflies, stoneflies, and other organisms that would be prey for salmon; these macroinvertebrates, however, are not abundant. Predators would include birds, especially kingfishers and herons, and mammals, especially nutria (*Myocastor coypus*), a large semi-aquatic rodent. There are few natural predatory fish in the area. Freshwater tarpon (*Tarpon prochilodus)* occur in the warmer waters of the lower basin, and a population of rainbow trout that were introduced in the upper basin could prey on salmon. These rainbow trout were intentionally stocked beginning in 1925, and are reported to constitute an established, naturally reproducing population (Welcomme, 1988); however, their abundance has not been well documented. In the upper-basin, vegetation on the river banks is scarce, and the substrate tends to consist of medium to very large round stones.

The natural physiography of the river basin reflects the high volume of water that flows through it during the rainy season; there are no areas of waterfalls or natural barriers to fish passage. However, the river has been, and will continue to be, used for hydro-electric energy generation. Although fish can navigate the upper part of the river, a large dam presents an obstacle to fish passage, especially during the dry season.

In short, while conditions in the immediate vicinity of the grow-out site could potentially support the earlier life stages of salmonids, physical barriers, sub-optimal habitat, and lethally-high water temperatures would be likely to prevent the long-term survival and establishment of Atlantic salmon in the river downstream.

4.3 Disposal Sites

The disposal of moribund or dead fish, fish wastes, and fish-processing wastes is described in §3.3; there are no properties of the affected environment that would cause the potential environmental impact of this disposal to differ from that of non-transgenic salmon.

5.0 POTENTIAL HAZARDS

The major difference between *AquAdvantage* Salmon and their non-GE counterparts is an increased rate-of-growth that is most evident during their first year of life. Observations in many fish species, including Atlantic salmon, show that larger body size may offer a number of advantages in securing mates and limited resources (Muir & Howard, 2002); however, such advantage could be offset by the diminished reproductive success observed for farmed Atlantic salmon vis-à-vis their wild-type counterparts (Fleming *et al*., 2000). In

[4] A 100-year flood that did occur in the general area of the Panama site damaged some bridges, roads, and buildings adjacent to the local watershed; however, the grow-out facility incurred no damage whatsoever, since it is sited at higher elevation than the associated flood plain; no problems of significance to aquaculture operations occurred as a result.

addition, relatives of *AquAdvantage* Salmon have been shown to exhibit reduced predator-avoidance response, increased predation mortality, and reduced tolerance of low DO, all of which would reduce their viability in natural environments (Abrahams & Sutterlin, 1999). The complexity of these fitness components and their interactions, and the additional influence of environmental factors on potential ecological risk, makes the prediction of hazards and risks that may be posed by GE salmon a difficult undertaking.

Muir (2004) has stated that the environmental risk of GE fish results from a chain of events: escape; followed by spread; followed by harm, such that the weakest link defines the upper-limit of risk. If the probability of any of these links can be shown to be near zero, it is not necessary to quantify all of the risks. A number of questions are pertinent when considering the hazards of GE salmon (Muir, 2004; Kapuscinski *et al*., 2007), especially since populations of wild Atlantic salmon have been declining.

- Are GE salmon able to escape into the environment?
- If an accidental escape occurred, could GE salmon survive in the surrounding environment and compete with wild salmon (and escaped domesticated, non-GE salmon), or otherwise impact natural or ecological resources of global importance?
- Could the rDNA construct be transmitted to wild salmon, escaped domesticated, non-GE salmon, or other species?
- Could GE salmon breed successfully with populations of wild salmon (and escaped domesticated, non-GE salmon)?
- Could the offspring resulting from these matings adversely affect the population of Atlantic salmon or other ecological resources of global importance?

The potential hazards addressed in this EA center on the likelihood and consequences of *AquAdvantage* Salmon escaping, becoming established in the environment, and spreading to other areas. These hazards must be addressed for the production of eyed-eggs, grow-out to market size, and disposal (i.e., of fish & fish wastes).

5.1 Likelihood of Escape

In general, fish and insects are among the groups of organisms with a high degree of mobility and significant capacity to escape captivity and become feral (NRC, 2002). Fish have life stages that can be difficult to contain, and are impossible to re-capture for all practical purposes. They can be highly mobile if the aquatic environment is sufficiently hospitable. In general, and compared to other farmed animals (such as poultry, dairy cattle, or sheep), fish pose a higher level of concern for escaping into the wild.

As discussed in §2.4.2.1, the estimated escape rate of salmon from sea cages is about 1%. Sea cages, or net pens, have a direct connection with the aquatic environment. For *AquAdvantage* Salmon, both the production of eyed-eggs and the grow-out of the fish are conducted in land-based facilities with redundant containment measures, with point-to-point control of shipping and land-based materials transfer. These measures are discussed in detail in §6.0. The use of land-based facilities and containment measures would reduce

any escape to much less than 1%. Breakdown of these measures is substantially decreased in likelihood by the following: the Sponsor's commitment to containment; use of experienced, properly-trained staff operating under established plans and procedures; automated monitoring of culture conditions and unauthorized intrusion; passive and active measures to ensure physical security; redundant back-up power generation; and, the historical absence of natural disasters that could render these measures ineffective.

5.2 Likelihood of Establishment

The risk assessment paradigm involves the integration of the probability of exposure with the probability of harm resulting from exposure. In evaluating the environmental concerns associated with GE organisms, the NRC (2002) stated that exposure must constitute more than release or escape in order to constitute a hazard; the NRC defined exposure, more specifically, as the establishment of a GE organism in the community. The NRC also identified the following three variables as being important in determining the likelihood of establishment: (1) the effect of the transgene on the fitness of the animal for the ecosystem into which it is released; (2) the ability of the GE animal to escape and disperse into diverse communities; and, (3) the stability and resiliency of the receiving community. The components of fitness include all of the attributes of phenotype that affect survival and reproduction. For example, a transgene could improve the adaptability of an organism to a wider range of environmental conditions, or allow it to obtain nutrition from previously indigestible sources. A stable receiving community has an ecological structure and function that is able to return to the initial equilibrium following a perturbation; resiliency is a measure of how fast that equilibrium is re-attained (Pimm, 1984). The overall concern is a product of these three variables, not the sum; thus, if the risk of any one of the variables is negligible, the overall concern would be very low (NRC, 2002).

AquAdvantage Salmon exhibit enhanced growth. Enhanced growth and productivity is a trait that has been developed in many domesticated farm animals through both selective breeding and genetic engineering. In selective breeding, the resulting phenotype occurs due to the cumulative effect of change in allelic frequencies in many genes, the distribution of effects ranging from small to large for which selection occurs over many generations. This process tends to produce an organism that has a greater overall degree of fitness than one produced by genetic engineering, in which case only one or a few genes with relatively large effects are introduced into a single founder generation. Experience with GE animals to date tends to support the notion that domesticated animals subjected to transgenesis to enhance phenotype might exhibit a greater reduction in fitness than their selectively bred counterparts; these findings suggest that GE organisms developed for commercial production have a low probability of establishment (NRC, 2002).

There is evidence that oversized, hatchery-reared salmonids can socially dominate and sometimes displace smaller wild-conspecifics through increased aggressive behavior or increased competition for food and space (Bachman, 1984; Nickelson *et al*., 1986; Vincent, 1987). This could potentially result in declines in species diversity and disruption of the ecosystem. However, there is also evidence that survival rates of artificially propagated and hybrid strains of salmonid fishes are often lower than those of naturalized salmonids,

and this trend is consistent with reduced predator avoidance behavior by these groups (Negus, 1999; Fleming *et al*., 2000; McGinnity *et al*., 2003).

In order for escapees to survive, the accessible ecosystem must meet their needs for food, habitat, and environmental cues for reproduction. The existing presence of conspecifics or species closely related to the GE escapee in accessible ecosystems indicates that a suitable environment does exist (Kapuscinski *et al*., 2007). Atlantic salmon, brook trout, and rainbow trout do occur in the vicinity of the production site on PEI, which indicates that the environment is generally suitable for survival. However, Atlantic salmon do not occur at the grow-out site in Panama. Artificially introduced populations of rainbow trout do exist as a result of previous stocking; and, large trout have been observed, which is significant because they constitute a known and formidable predator of salmon fry, fingerlings, and juveniles. Adult rainbow trout present in the adjacent watershed would prey on any smaller salmon that manage to escape from the grow-out site; and, although the presence of these rainbow trout indicates that the environment is suitable for salmonids, the average water temperature downstream exceeds the lethal-maximum that Atlantic salmon can tolerate.

As Kapuscinski and Brister (2001) have noted, even if the escaped fish were sterile, a type of pseudo-establishment could occur if successive waves of large numbers entered the environment, with each wave replacing the former as it dies off. This scenario implies release of large numbers, which will not be pertinent to either the egg-production or grow-out sites for *AquAdvantage* Salmon due to the redundant containment measures employed.

According to Muir (2004), escaped salmon tend to starve before they learn to seek natural prey rather than feed pellets, a limitation that would be exacerbated by the low abundance of such prey in the environment at the grow-out site. For *AquAdvantage* Salmon more specifically, additional factors would further reduce the likelihood of their establishment, including: an increased metabolic rate that reduces their tolerance for low DO (which is characteristic of warm water temperatures); and, reduced swimming ability and predator avoidance that increases their predation mortality. These attributes suggest that *AAS* would not be particularly fit for the environment in Panama, even if they were to escape.

The environment at the production site on PEI is such that eggs released inadvertently or broodstock that managed to escape could survive (in theory), except during the coldest months of the year; the true likelihood of survival, however, is very small: eyed-eggs must be incubated under controlled conditions in order to hatch, and their maturation in the local estuarine environment must be considered extremely unlikely; broodstock would face environmental-climatological impediments to survival that remain considerable, one clear indication being the substantial failure of intentional efforts to re-establish Atlantic salmon in their native habitat. In fact, as noted by the Council on Environmental Quality and Office of Science and Technology Policy (***CEQ-OSTP***), farmed Atlantic salmon have not established themselves successfully in the wilds of North America (CEQ-OSTP, 2001), despite the fact that they are reared commercially on both coasts.

5.3 Likelihood of Spread

The spread of GE fish would depend upon how many escaped and survived, their characteristics, and their reproductive potential. The very low likelihood of their escape and survival has been discussed. The reproductive potential of escapees is based upon their survival rate and fertility, and environmental conditions affecting reproduction in the affected ecosystem. For example, highly domesticated fish may be ill-equipped to mate in the wild due to the effects of captivity, such as being used to artificial diets and being raised at a high stocking density (Kapuscinski *et al*., 2007).

Although the reproductive potential of escaped *AquAdvantage* Salmon is essentially nil, given that the populations to be grown-out are sterile females, the use of triploidy to eliminate reproductive risk is not perfect. Some *AAS* may escape the induction of triploidy, thereby remaining reproductively capable, since the induction process, albeit greater than 99% effective on average, cannot totally eliminate the possibility (*see*, §6.1.2). Of countervailing benefit is the fact that the production of all-female populations of *AquAdvantage* Salmon can be accomplished with 100% efficiency, since the process of gynogenesis offers that guarantee based upon reproductive biology. The production of females is preferred, since triploid males, although sterile, can engage in spawning behavior with diploid females in the wild, thereby leading to their reduced reproductive success.

Even if they were not sterile, mature *AquAdvantage* Salmon escaping into the watershed near the grow-out site would not encounter conspecifics or even closely-related species with which to interbreed. Rainbow trout (*Oncorhynchus mykiss*) do exist; and, although Atlantic salmon are known to interbreed with brown trout (*Salmo trutta*), they are not reported to interbreed with rainbow trout (Teufel *et al.*, 2002; Hindar, 1993). High water temperatures in the lower reaches of the watershed would preclude the spread of any escapees into the Pacific Ocean, which does not have indigenous populations of salmonids.

5.4 Consequences of Potential Escape, Establishment, and Spread

Evaluating the consequences of the potential escape, establishment, and spread of *AAS* is a considerable challenge. There are numerous factors, both genetic and environmental, that can influence the ability of *AquAdvantage* Salmon to affect the environment should they escape, survive, and spread; these factors may have positive or negative impacts, which are further complicated by their mutual interaction. However, per the analogy of Muir (2004), it is not necessary to quantify the consequences (or harm, or effects) if the probability leading to the harm (the exposure) is zero or close to zero.

The environmental risk posed by GE organisms is similar to that of introduced species. As discussed by Kapuscinski and Hallerman (1991), ecological impacts of GE individuals would be related to their fitness, interactions with other organisms, role in ecosystem processes, or potential for dispersal and persistence. In some respects, *AquAdvantage* Salmon have increased fitness attributes relative to their wild and domesticated counterparts, but in other respects, their fitness is reduced. Natural selection would act upon these fitness attributes in the environment, but the outcomes are not easily predicted.

With respect to their interactions with other organisms, *AAS* would be expected to occupy the same ecological niche as wild and domestic Atlantic salmon, competing for food, shelter, and other resources. However, because *AquAdvantage* Salmon are all sterile females, they will be unable to reproduce or contribute their genes to conspecifics. In marine waters, Atlantic salmon do not have a singularly important role in nutrient cycling and energy flow; therefore, impacts on ecosystem processes are unlikely. Finally, the potential for dispersal and persistence of *AAS* is very low due to the redundant means of biological, physical, physico-chemical, geographical, and geophysical containment being employed, as described in §6.0. The scale and frequency of introductions of GE fish into a particular environment would have a large influence on the potential ecological risk. Any introductions would have to involve a critical mass that could offset natural mortality, and be of sufficient frequency in proper season to allow for establishment. Kapuscinski and Hallerman (1991) have stated: "*Although surprising outcomes cannot be ruled out a priori, low ecological risk may be a reasonable conclusion in situations where phenotypic and ecological attributes of transgenic individuals raise concerns, but the scale and frequency of their introductions are so small that their chances of becoming established in the natural setting are extremely low*."

A report by the Ecological Society of America (***ESA***; Snow *et al*., 2005) identified six major environmental concerns associated with GE organisms. These processes and their potential ecological consequences, which remain largely undocumented to date, are presented in Table 7. These concerns are further considered in §8.0 for *AquAdvantage* Salmon when produced and grown-out as described in this EA.

Table 7. Primary Environmental Concerns Regarding GE Organisms *

Process	Potential Ecological Consequences
GE organisms persist without cultivation	GE organisms that are able to spread and maintain self-sustaining populations could disrupt biotic communities and ecosystems, leading to a loss of biological diversity.
GE organisms interbreed with related taxa	Incorporation of transgenes could result in greater invasiveness or loss of biodiversity, depending upon the amount of gene flow from generation to generation and the transgenic trait(s).
Horizontal gene flow	The transfer of genes through nonsexual means is common in some microbes but rare in plants and animals. Ecological consequences would depend on amount of gene flow and the transgenic trait(s).
Changes in viral disease	In GE virus-resistant organisms, recombination between viral transgenes and invading viruses could lead to increased virulence of a disease and undesirable effects on wild hosts in natural habitats.
Non-target and indirect effects	Loss of biodiversity, including species of conservation concern, may occur, as well as altered community or ecosystem function, including reduced biological pest control, reduced pollination, altered soil carbon and nitrogen cycling, and secondary pest outbreaks.
Evolution of resistance	Resistance to pesticides (including pesticide-producing plants) can lead to greater reliance on chemicals and other pest control methods that are damaging to the environment, including unregistered pesticides under emergency exemptions. This applies to insects, weeds, and other pests.

* After Snow *et al*., 2005. **Note:** Few GE organisms have been released into the environment, so little documentation of these potential ecological consequences actually exists at the present time.

6.0 STEPS TO MITIGATE HAZARDS

As was stated previously, it is not necessary to quantify the consequences of the escape, establishment, and spread of GE salmon if the probability of escape leading to the exposure (i.e., establishment & spread) is zero or close to zero. Therefore, the use of measures to ensure that the exposure is nil is considered the best means of reducing the risk. Measures for containment of *AquAdvantage* Salmon (i.e., preventing exposure) are discussed in this section.

That 100% containment can be achieved by any single method is difficult to guarantee. Thus, several different methods are used simultaneously to provide redundancy and ensure that it is highly unlikely that GE salmon can escape. These measures are as follows: biological containment; physical containment (including physico-chemical containment & operations management); and, geographical/geophysical containment.

6.1 Biological Containment

Biological containment can serve as a barrier by either a) preventing any possibility of reproduction at the site, thus avoiding risk of escape of gametes, embryos, or larval stages, or b) greatly reducing the possibility of reproduction or survival of the GE organisms if they accidentally escape.

6.1.1 Production of all-female eggs

The eyed-eggs that are produced are 100% female. As described in §3.1.1, this is accomplished by fertilizing eggs from non-GE female salmon with milt from GE neomale broodstock (i.e., genotypic females) produced via gynogenesis. Since no true male fish are involved, monosex populations of *AAS* can be produced for grow-out, thereby preventing *AquAdvantage* **x** *AquAdvantage* reproduction outside of the production facility.

6.1.2 Induction of triploidy in all-female eggs

One of the important means of biological containment is the sterility of the fish. Thus, even if some fish were to escape the grow-out facility and survive in the environment, they would not be able to reproduce. The induction of triploidy is the only accepted method currently available for sterilizing fish on a commercial scale.

Triploid fish have three sets of chromosomes in their somatic cells, rather than the two sets in their normal diploid state. According to Benfey (2001), triploidy has two fundamental effects on fish physiology: 1) the size of the somatic cells increases to accommodate the extra genetic material, but the number of cells decreases so that triploids are no larger overall than diploids; and, 2) gametogenesis and gonadal development is so severely impaired that triploids are sterile. Other than their sterility, a comprehensive review of the literature conducted by Benfey (1999) reveals remarkably little difference between triploids and diploids on a whole-animal level. However, triploid salmon cannot be assumed to be identical to diploids, as some differences do occur, as summarized by Benfey (2001): poor performance under conditions of low oxygen availability and/or high oxygen demand; jaw abnormalities, which have been observed by a number of investigators; and, somewhat

poorer performance relative to diploids, especially when grown in sea water. Piferrer *et al*. (2009) summarized data showing that triploid Atlantic salmon did not show significant differences in growth relative to diploids in fresh water for the first nine months or in sea-cages until the onset of sexual maturation; however, adult triploids did grow faster than diploids.

Triploidy is generally induced by either thermal or hydrostatic pressure treatment of the eggs within the first hour after fertilization. Hydrostatic pressure treatment is more easily controlled and therefore preferred (Benfey, 2001); this is the method used to generate triploid *AquAdvantage* Salmon. Treatment for five minutes at 300ºC-min after fertilization has been used successfully to induce triploidy in five year-classes of Atlantic salmon in New Brunswick, Canada (O'Flynn *et al*., 1997). The preferred method for verification of effective induction is flow cytometry, because it is rapid and yields unambiguous results (Benfey, 2001). This process is the same as that used during the production of eyed-eggs at the production facility. Following pressure treatment, the eggs are water-hardened. The very high efficiency of the induction process (> 99%) ensures that very few (if any) diploid fish with reproductive potential are shipped to Panama for grow-out (*see*, §6.1.2.2).

6.1.2.1 Reliability of inducing triploidy

The use of triploidy greatly reduces, but does not eliminate, all environmental risks that are dependent upon reproductive capacity. The assurance of risk-mitigation by this particular measure is complicated by several factors: its reliability; its effectiveness in inducing sterility; residual spawning behavior in sterile males; and, the survivability of sterile triploids should they be released in sufficient numbers to compete with diploid conspecifics of other species (CEQ-OSTP, 2001). The first three factors are addressed below, while overall survival ability of sterile triploids has been addressed in §5.0.

The major variables influencing the effectiveness of pressure shock in inducing triploidy are the following, in order of decreasing importance: timing; intensity; and, duration of shock (Felip *et al*., 1997). Method optimization for effective induction varies by species (Piferrer *et al*., 2009). Laboratory-scale efficiencies of 100% that have been reported for Atlantic salmon (Benfey & Sutterlin, 1984) are not necessarily attainable on a commercial scale (McGeachey *et al*., 1995).

A study was conducted to determine the effectiveness of the conditions used for the induction of triploidy at the PEI production facility (Sponsor submission to CVM). One-to-one crosses were established with eggs from non-GE female Atlantic salmon and milt from *AquAdvantage* Salmon males hemizygous for EO-1α. The fertilized eggs from each cross were apportioned into five replicate groups: one diploid control group that was not subjected to pressure shock, and four treated replicates that were pressure shocked (9500 psi for five minutes at 300ºC-min post-fertilization). Ploidy analysis was performed on a sub-sample of 350 eyed-eggs collected from each of the treated replicates from five different crosses using flow cytometry; the efficiency of triploid induction was determined for a total of 20 independent pressure-shocked groups. The results indicated that conditions used in the production facility can reliably produce batches of eggs that are on average 99.8% triploid (range: 98.9 to 100%).

For quality control, effectiveness of triploid induction in a statistically-appropriate sample of eyed-eggs from the production stream will be confirmed using established methods and procedures that require strict performance of controls and interpretability of analysis. Composite sampling of individual upwelling chambers, which comprise multiple batches of pressure-shocked eggs, will be conducted routinely. The acceptance criterion is such that the likelihood of releasing a batch of eyed-eggs that are not at least 95% triploid is less than 0.05. Individual upwelling chambers that fail to meet test criteria will be re-tested and destroyed upon confirmed failure.[5]

6.1.2.2 Effectiveness of triploidy in inducing sterility

The degree of functional sterility in triploids varies depending upon the species and sex (Kapuscinski, 2005), and appears to be more complete in triploid females than triploid males (Thorgaard & Allen, 1992). In reviewing data on approximately 26 fish and shellfish species being investigated in Japan, Arai (2001) noted that triploid males exhibit more gonadal development than females and display secondary sex characteristics. Lee and Donaldson (2001) have reported that triploid coho salmon (sex not stated) in Japan and older triploid fish (of unidentified species) have sometimes been found to be fertile. In research with Arctic charr (*Salvelinus alpinus*), few of the triploid females developed ovaries, fecundity was low, and the fertilized eggs from the triploid females did not hatch (Gillet *et al*., 2001); overall growth and survival of the triploids and diploids in this 443-day study was not different.

Since triploidy is not 100% effective in inducing sterility in GE fish, particularly in males, the combination of this technique with the production of monosex fish, especially when all females are produced, is more reliable for biological containment (Donaldson & Devlin, 1996). As stated by Mair *et al*. (2007), "…*the production of all-female triploids combines the benefit of almost-guaranteed sterility of any escapees with the reduced risk of disruption of spawning in natural populations that might arise with triploid males*." Arai (2001) has stated, "*All female triploids can be used for effective biological containment of transgenic fish, so as to protect wild populations from contamination with genetically modified fish*."

6.1.2.3 Residual spawning behavior

Sterile male Atlantic salmon are still capable of exhibiting spawning behavior with fertile diploid females, which could lead to the decreased reproductive success of the latter. However, this situation cannot occur with the *AquAdvantage* Salmon produced for grow-out, since they are all females. There is no evidence to indicate that triploid females could cause reproductive interference with native conspecifics. In fact, a study of the controlled release of micro-tagged triploid and diploid groups of Atlantic salmon (both mixed-sex and

[5] Quality control is dependent upon the statistically-appropriate sampling of large populations; samplings are chosen in such a way that the measure of effectiveness determined is a probable *minimum* value for induction efficiency. Actual efficiencies may, in fact, be 100% or very close to that value, since the probability of an alternative (i.e., non-triploid) outcome under effective induction conditions is exceedingly low. Proof of 100%-efficient induction is an unrealistic benchmark that would require analysis of *every* egg regardless of the production-scale used, the impracticality of which is made worse by the fact that the analysis requires destruction of the egg itself.

all-female groups) on the western coast of Ireland found that the return rate of triploid salmon, both to the coast and fresh water, was substantially reduced compared to diploid salmon (Cotter *et al*., 2000a). In addition, and of direct relevance to triploid *AquAdvantage* females, the triploid females in this study had severely immature ovarian development (Murphy *et al*. 2000) and abnormal gonadal steroid and gonadotropin hormone profiles (Cotter *et al*., 2000b). The authors concluded that their reduced rate-of-return, and inability to produce viable offspring, demonstrate the potential for triploidy as means of eliminating the genetic interaction and reducing the general impact of escaped farmed fish on wild populations.

6.2 Physical Containment

Physical containment refers to measures implemented on-site, such as the use of mechanical devices, either stationary or moving (e.g., tanks, screens, filters, covers, nets, etc.), or the use of lethal temperatures or chemicals to prevent uncontrolled escape. For example, treatment with 10-15 mg/L chlorine for 15-30 minutes is effective in killing fish in fresh water (ABRAC, 1995). An important component of physical containment is the implementation of policies and procedures to ensure that the devices and chemicals are used as prescribed (Mair *et al*., 2007). Security measures are also needed to prevent unauthorized access, control movement of authorized personnel, and prevent access by predators.

The potential for accidental escape could derive from any of the following components of the water system: influent water and makeup water; effluent and draw-down water; and, waste slurries collected when filters are backwashed, screens scrubbed, or rearing units cleaned by siphoning (ABRAC, 1995). In addition, it is important that all equipment that comes in contact with live GE animals is properly cleaned and drained after each use.

6.2.1 Containment for egg production

A number of measures have been implemented to provide physical containment of the GE salmon at the production facility on PEI. In general, means of physical containment comprise entrapment of animals at the immediate source of housing for cultivation (i.e., via tank covers or nets), and redundancy in screening and filtration of water flows into which fish could gain access. These measures, which are employed at multiple levels of the containment strategy, are summarized in Table 8; a schematic of the containment system is provided in Figure 8.

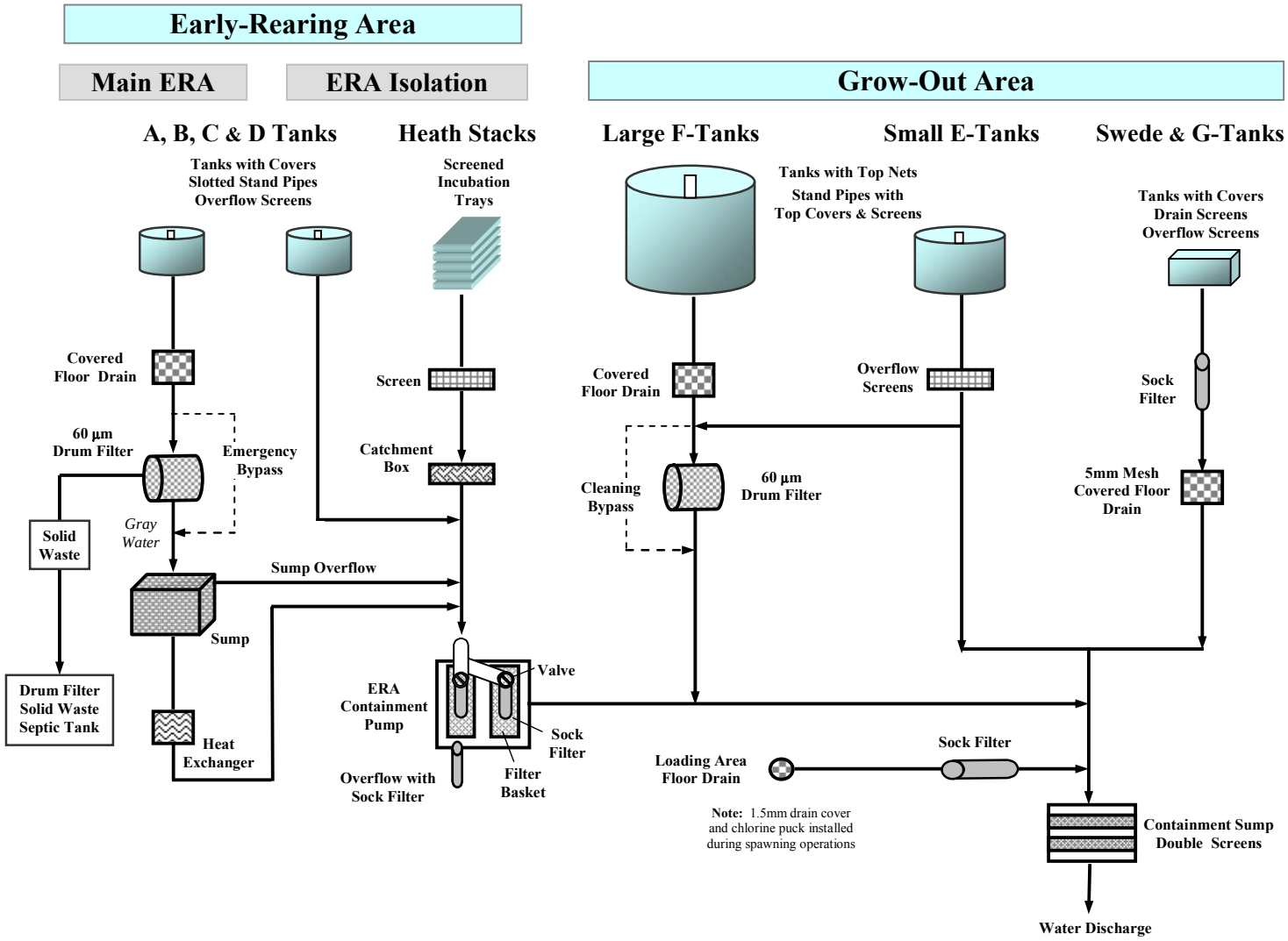
[End of Page]

Table 8. Key Components of Physical Containment at the Production Facility

Purpose	Feature or Component
Primary containment	
To prevent escape through rearing unit or incubator water overflow	Perforated metal screens on tank bottoms
	Screens on stand pipes, top and bottom (where appropriate for size of fish to be contained)
	Incubator tray screens
To prevent escape over the side of a tank or incubator	Screened tank overflows Cover nets Jump fences Tank covers Incubator tray screens
To prevent downstream passage of newly fertilized eggs and/or gametes	Chemically lethal environment (chlorine puck) in spawning area drain
	Perforated metal drain cover in spawning area
	Closed septic system
Secondary containment	
To prevent entry of fish into drains	Floor drain covers, solid or mesh
	Incubator-stack catchment box
	Waste de-watering sieve box
To prevent downstream passage of fish within the drains	Barrier screens within drains
	Drum filter
Tertiary and Quaternary containment	
To prevent downstream passage of fish within the drains	Barrier screens within drains of various sizes & locations
	Double screens within the sump
	Mesh filter on drum-filter gray water
	Heat exchanger
Waste treatment	
Sock filters, containment screens, basket-sieve for straining waste material from the ERA tanks	
Chlorine kill solution (5 mL Javex containing 0.52 grams sodium hypochlorite per liter of water)	
Chlorine pucks	

[End of Page]

Figure 8. Schematic Summary of Containment Measures at the Production Facility



6.2.2 Containment for grow-out

Physical containment to prevent the escape of fish at the grow-out facility is provided by the use of screens wherever water flows out of the system. Security is provided by surrounding the fry tanks and grow-out tanks with netting and fencing topped with barbed-wire to deter human or animal intrusion. An additional level of physical containment is provided by several downstream hydro-electric plants, which also serve to prevent passage of any escaped fish to downstream riverine areas or the Pacific Ocean (*see*, §4.2). These measures are summarized in Table 9; a schematic of the containment system is provided in Figure 9.

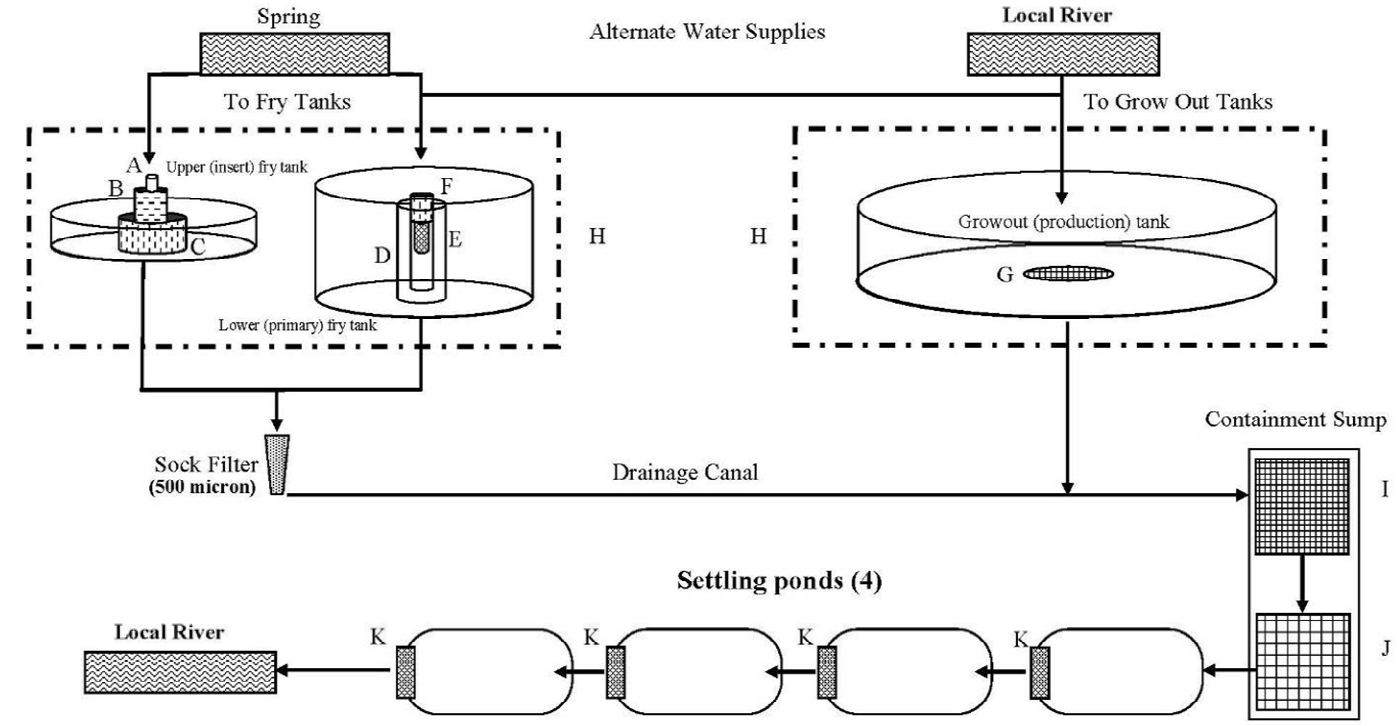
In summary, a minimum of 11 sequential physical barriers are in place between the fry tanks and local river, confining *AAS* to the site; seven of these barriers are installed following outflow from the grow-out tanks. In addition, netting prevents the fish from being actively removed from containment by predators or passively removed in the event of any overflow of the water level.

[End of Page]

Table 9. Key Components of Physical Containment Measures at the Grow-Out Facility

Purpose	Feature or Component
Primary containment	
To prevent escape from the fry tanks via water	Center standpipe cut below tank rim to ensure water level is always below rim
	Netting stretched taut over top of tank to prevent fish from escaping even if tank was overflowing
	Collar-sleeve screens inserted into top of standpipes to prevent fish from entering standpipe by swimming
	Metal screen inside standpipe at base of basket screen impedes fish that entered standpipe (by jumping) from leaving the tank
	Rigid circular plastic screens surrounding the center standpipes
	Porous gravel floor around each tank allows downward percolation of overflow water but traps any fish in the overflow
To prevent escape from the fry tanks by avian predators	The building is covered and sealed by netting
	Netting stretched taut over the top of each tank
To prevent escape from the grow-out tanks via water	A single external (so no fish can jump into it) standpipe cut below tank rim to ensure water level is always below rim
	A 1 cm thick, rigid PVC slotted drain plate affixed by screws to the only drain in the tank
	Porous gravel floor around each tank allows downward percolation of overflow water but traps any fish in the overflow
To prevent escape from the grow-out tanks by avian predators	Each tank is entirely covered by netting stretched over and around the tank on a rigid support structure
	Netting stretched taut over the top of each tank
Secondary containment	
To prevent escape from fry tanks into drains	Sock filter (500 μm) on the terminal end of the only drain pipe receiving effluent from the fry tanks
To prevent escape from grow-out tanks into drains	Sealed metal cage (affixed to ground) through which all effluent from grow-out tanks must pass before entering drain canal
To prevent escaped fish from passing through the drain canal to the sedimentation ponds	Concrete structure and containment sump through which all water must pass
	Rigid metal screen affixed to bottom of containment sump through which all water must pass
To prevent escaped fish from passing from one sedimentation pond to another	Rigid metal screens on the outlet of each pond
To prevent escaped fish from entering the river from the drain canal	Four sedimentation ponds in series, each with its own outlet screen
Tertiary and Quaternary containment	
To prevent unauthorized personnel from entering the fish rearing area	The project is in a very remote location
	The project is built on the opposite side of the river from the road
	A narrow pedestrian bridge crosses the river, with access controlled by a locked metal fence
	Tall barbed wire security fence completely surrounding the perimeter of the fish rearing tanks, with locked entry gates
	Permanent presence of aggressive dogs

Figure 9. Schematic Summary of Containment Measures at the Grow-Out Facility



A	Interior Stand Pipe	I	Screen Basket (6 mm)
B	Interior Stand Pipe Screen (1.0, 1.5 mm)	J	Screen Plate (12 mm)
C	Exterior Stand Pipe Screen (1.5 mm)	K	Outlet Screen (12 mm)
D	Exterior Stand Pipe		
E	Basket Screen (3, 6 mm)		
F	Top Screen (3, 6, 12 mm)		
G	Slotted Drainage Plate (0.9 cm)		
H	Security Fence		

6.2.3 Redundant, multi-level strategy

The containment measures described above for the sites of egg production and grow-out include strictly physical measures (e.g., screens, covers, filters), as well as physico-chemical measures (e.g., chlorine). In addition, a strong operations management plan is in place at both sites, comprising policies and procedures that meet the recommendations for an integrated confinement system for GE organisms (Kapuscinski, 2005), as summarized in Table 10.

Table 10. Implementation of an Integrated Confinement System for *AquAdvantage* Salmon *

	Use at Production & Grow-Out Sites	
Recommended element	**Production**	**Grow-Out**
Commitment by top management	✓	✓
Written plan for implementing backup measures in case of failure, including documentation, monitoring, and remediation	✓	✓
Training of employees	✓	✓
Dedication of permanent staff to maintain continuity	✓	✓
Use of standard operating procedures for implementing redundant confinement measures	✓	✓
Periodic audits by an independent agency	✓	✓
Periodic internal review and adjustment to allow adaptive modifications	✓	✓
Reporting to an appropriate regulatory body	✓	✓

* After Kapuscinski, 2005.

6.3 Geographical/Geophysical Containment

Environmental conditions in the geographic settings of the aforementioned sites afford additional means of containment of any escaped fish, given that these conditions are generally inimical to their survival, growth, and reproduction. Unanticipated natural events such as floods and hurricanes must also be considered in evaluating the effectiveness of site location in ensuring containment. In that regard, and as previously stated, no such natural disasters have occurred, or are known to occur, in proximity to the facilities on PEI or in Panama.

6.3.1 Environmental conditions at egg production and grow-out sites

Hatchery-reared Atlantic salmon do inhabit the ocean waters immediately surrounding the production site, even though the local environment does provide suitable habitat for some life stages during part of the year. The climate is temperate, with warm summers and cold winters.

The grow-out site is located in a predominantly tropical environment adjacent to a major watershed that drains into the Pacific Ocean at a latitude inhospitable to Atlantic salmon: water temperatures in the lower reaches of the watershed (≥ 25°C) exceed those known to be lethal to Atlantic salmon, thereby providing a thermal barrier to their seaward migration.

6.3.2 Site conditions *vs*. life-stage requirements

Open waters in proximity to the production facility are saline. Salmon eggs and fry are adapted to freshwater conditions and would be adversely affected by escape into a saltwater environment. The extreme temperature conditions during the winter months at this location would be lethal to salmonids of all developmental stages. However, during the remainder of the year, the local environment would not be inhospitable to escaped smolt, juvenile or adult GE salmon, which have adapted to salt water and could survive.

Open waters in proximity to the grow-out facility are fresh water. Fry and later life-stages could potentially survive in the immediate vicinity of the site, although predation by trout could occur. However, escaped GE salmon would not survive seaward migration due to the high water temperatures, poor habitat, and physical barriers (i.e., multiple hydro-electric plants) encountered downstream.

7.0 JURISDICTIONAL AND REGULATORY ISSUES

The product that is the subject of this EA (i.e., triploid eyed-eggs from the EO-1α line of Atlantic salmon bearing a single copy of the stably integrated α-form of the opAFP-GHc2 gene construct at the α-locus) will be produced on Prince Edward Island for grow-out in Panama. Regulatory approval of this product is being sought from CVM/FDA through an NADA. As specified by 21 CFR 25.20(m), NADAs are agency actions requiring the preparation of an EA.

7.1 Effects on the Global Commons

In accordance with Executive Order 12114, "*Environmental Effects Abroad of Major Federal Actions*," FDA regards the consideration of environmental risks abroad, including those to other countries and the global commons, as being a part of the NEPA analysis (21 CFR 25.60). The global commons comprises those parts of the earth beyond national boundaries, principally the open ocean and living resources therein, and those parts held in-common, such as the atmosphere. These policies and regulations require that this EA consider environmental impacts to the global commons. As was discussed in §6.0, the multiple and redundant physical, physico-chemical, biological, and geographical-geophysical levels of containment in place at both the egg-production and grow-out sites serve to mitigate any unacceptable risks potentially posed by the product at those sites and in the immediate environment surrounding them. Since the risks of escape, establishment, and spread of *AquAdvantage* Salmon are negligible at and around the associated facilities, there are no risks beyond these sites that would extend to the global commons.

7.2 Effects on Foreign Nations Not a Party to This Action

Executive Order 12114 also requires that Federal agencies examine the potential effect of their actions on the environment of any foreign nation that is not participating with the US in a given activity or otherwise involved in the associated action. The egg-production and grow-out sites for *AquAdvantage* Salmon identified in this EA are located in Canada and Panama, respectively, which could affect countries not participating or otherwise involved in the review and approval of the *AAS* NADA. However, as was discussed in §6.0, the redundant containment being employed at these sites effectively precludes *AquAdvantage* Salmon from impacting the local or coastal waters of those countries.

7.3 Threatened and Endangered Species

For Atlantic salmon in North America, endangered species listings include those populations in the Inner Bay of Fundy (Government of Canada, 2009) and Gulf of Maine (Fish and Wildlife Service [***FWS***], 2009). The recognized decline in wild salmon stocks has prompted NASCO to adopt the so-called Williamsburg Resolution, which is designed to minimize impacts of aquaculture introductions, transfers, and transgenics on the wild stocks of Atlantic salmon (NASCO, 2006). NASCO "*Guidelines for action on transgenic salmonids*" (Resolution, Annex 5) states that, "*while there may be benefits from the introduction of such salmonids if, for example, they could not interbreed with wild stocks...*," specific steps should be taken to ensure protection of the wild stocks. These steps include the following: notification of any proposal to permit the rearing of transgenic salmonids and provision of details of the method of containment and other measures to safeguard the wild salmon stocks; utilization of all possible actions to ensure that the use of transgenic salmonids, in any part of the NASCO Convention area, is confined to secure, self-contained, land-based facilities; consultation with the salmon farming industry; improvement of knowledge on the potential impacts of transgenic salmonids on wild salmon stocks and their habitat; and, examination of the trade implications associated with transgenic salmonids.

AquAdvantage Salmon pose no special risks to endangered wild stocks of Atlantic salmon. In fact, because they are sterile, even if they should escape they would be unable to mate with their wild counterparts. In this regard, *AquAdvantage* Salmon pose *less* risk to populations of wild salmonids than do escaped domesticated, non-GE salmon, which are known to interbreed with wild populations.

8.0 RISK ASSESSMENT

Ecological risk assessment "*evaluates the likelihood that adverse ecological effects may occur or are occurring as a result of exposure to one or more stressors*" (EPA, 1992). Inherent in this definition is that both exposure and effects are required components of risk, i.e., Risk = Exposure **x** Effects. Muir (2004) has presented a modification of this concept for the risk assessment of GE organisms, wherein exposure comprises two parts: 1) the probability of the organism escaping into the wild, dispersing, and becoming feral; and, 2) the ability of the transgene to spread into the wild population once it has been introduced by an escaped animal. As described previously and summarized in this section, redundant

measures will be taken to ensure that the probability of escape and establishment (part 1) and the ability of transgene spread (part 2) are essentially zero. With essentially zero exposure, the risk is essentially zero. A report by the ESA (Snow *et al*., 2005) has proposed six major environmental processes that may be associated with GE organisms. In Table 11, each of these processes and their potential ecological consequences, which remain largely undocumented to date, are presented vis-à-vis their prospective applicability to *AquAdvantage* Salmon.

Table 11. Risk of Environmental Impact of GE Organisms *

Process	Potential Ecological Consequence	Risk Associated with *AAS*
Persistence without cultivation	Transgenic organisms able to spread and maintain self-sustaining populations could disrupt biotic communities & ecosystems, leading to a loss of biological diversity.	*AAS* are all sterile females unable to reproduce; a self-sustaining population cannot be established. NO SIGNIFICANT RISK.
Interbreeding with related taxa	Incorporation of transgenes could result in greater invasiveness or loss of biodiversity, depending on particular transgenic trait and gene flow from generation to generation.	*AAS* are all sterile females unable to breed with wild Atlantic salmon or related taxa. NO SIGNIFICANT RISK.
Horizontal gene flow	Non-sexual gene transfer is common in some microbes but rare in plants & animals; ecological consequence would depend on particular transgenic trait and gene flow.	Integrated transgene in *AAS* is incapable of being passed thru non-sexual means. NO SIGNIFICANT RISK.
Change in viral disease	In virus-resistant transgenic organisms, genetic recombination could lead to increased virulence of viral disease and undesirable effects on natural hosts.	rDNA construct used for *AAS* had no viral component; this type of recombination is not possible. NO SIGNIFICANT RISK.
Non-target & indirect effects	Loss of biodiversity, altered community or ecosystem function, reduced biological pest control, reduced pollination, and altered soil carbon and nitrogen cycling.	*AAS* escape minimized by redundant containment; low probability of establishment due to poor fitness and reproductive incapacity; likelihood of further spread is nil. NO SIGNIFICANT RISK.
Evolution of resistance	Pesticide resistance leading to greater reliance on damaging chemicals or other controls for insects, weeds, and other pests.	Not applicable for fish. NO SIGNIFICANT RISK.

* Process and General Consequence information derives from Snow *et al*., 2005.

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8.1 Mitigation of Risks at Each Stage of Product Life Cycle

A key way to manage risks associated with the use of GE fish in aquaculture is through the application of confinement measures designed to minimize the likelihood of their causing harm to the environment (Kapuscinski, 2005). The three primary aims of confinement cited by Mair *et al*. (2007) are listed below along with the measures used for production, grow-out, and disposal of *AquAdvantage* Salmon:

- **Limit the organism:** Prevent the fish from entering and surviving in the receiving environment;

  AquAdvantage Salmon are prevented from entering the environment by the use of redundant physical and physico-chemical barriers at the sites of egg production and grow-out. They are further prevented from surviving in the receiving environment because of geographic and geophysical issues. At the production site, escaped early-life stages that are adapted to fresh water would encounter salt water, and during part of the year, lethally cold temperatures. At the grow-out site, escapees would encounter lethally warm temperatures, poor habitat, a series of hydro-electric facilities, and likely predation by an established population of rainbow trout.

- **Limit (trans)gene flow:** Prevent gene flow from the GE fish during production or following escape;

  Gene flow from *AquAdvantage* Salmon is prevented because the fish are triploid females incapable of reproduction, among themselves or with wild fish, should they escape and survive.

- **Limit transgenic trait expression:** It is likely that the expression of the trait, not the transgene itself, poses the hazard.

  The enhanced growth rate of *AquAdvantage* Salmon is readily expressed under the optimum conditions provided in a commercial environment; however, in the wild, the absence of readily available food (to which they are accustomed) and the consequent depletion of energy reserves decrease the likelihood of effective exploitation of their inherent growth capacity.

8.2 Redundant Mitigation Measures

No single containment measure can be assured of 100% effectiveness. Therefore, optimum containment is dependent upon the deployment of a number of independent measures in series; three to five separate measures have been recommended (ABRAC, 1995). The NRC (2002) recommended simultaneous use of multiple, redundant containment strategies for GE fish. By combining containment measures with different strengths, attributes, and modes-of-action, the compromise of aggregate containment by the failure of a single measure becomes increasingly unlikely. GE fish are considered to pose little risk to native populations if they are adequately contained (Wong & Van Eenennaam, 2008; Mair *et al*., 2007).

Biological, physical and geographical/geophysical means of containment will be used to mitigate the potential environmental risk of *AquAdvantage* Salmon. Each method has different strengths and weaknesses, but the combination results in a very high level of effectiveness. Biological containment includes the production of entirely female, triploid fish with very limited (or no) capacity to breed with wild fish; in and of itself, this technique is considered very effective (Mair *et al*., 2007; Arai, 2001). Physical and physico-chemical means of containment comprise the use of additional, multiple, and redundant measures at the production and grow-out sites that will effectively prevent escape. The reliability of these measures is further ensured by adherence to a strong management operations plan that includes staff training, appropriate policies and procedures, and routine audits and inspections. In addition, geographical/geophysical containment is provided by the specific location of the aforementioned sites. Although Atlantic salmon can survive on PEI, the immediate environs of the production facility is inhospitable to early-life stages due to the salinity of the local waters. The environment downstream of the grow-out site is inhospitable to all life-stages due to the high water temperatures, poor habitat, predation risk, and physical barriers that diminish the likelihood of survival and establishment in the receiving stream.

8.3 Uncertainties in the Risk Assessment

Sources of uncertainty in any risk assessment include those regarding the true value of a given parameter, its inherent variability, and our limitations in understanding the input variables comprising it. Uncertainty can be reduced by obtaining or generating more information, but variability is a natural phenomenon that cannot. For quantitative risk characterizations, safety factors are often used to deal with uncertainty. This approach is typically used for chemical and drug assessments, when concentration in the environment can be estimated and compared to the concentration predicted to cause effects. The risk assessment for *AquAdvantage* Salmon is by necessity more qualitative. In more qualitative risk assessments, professional judgment is used to estimate the degree of uncertainty.

Uncertainty in this risk assessment is addressed qualitatively for the three potential hazards identified: the likelihood of escape; the likelihood of establishment; and, the likelihood of spread. There is little uncertainty in the evaluation of the likelihood of escape, as the measures used at the sites of egg production and grow-out are known with considerable specificity. Somewhat greater uncertainty exists in the evaluation of the likelihood of establishment, since detailed information is not available on every environmental factor that could affect the survival of escaped *AquAdvantage* Salmon at the aforementioned sites. Even if such information were available, the interactions of contingent environmental factors and biology of the organism would still engender a degree of uncertainty. Nevertheless, sufficient information exists to provide an acceptable degree of confidence in the evaluation of the likelihood of establishment; and, little uncertainty exists with regard to the likelihood of spread, since any escaped *AquAdvantage* Salmon would not have the capacity to reproduce and populate the surrounding environment.

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9.0 CONCLUSIONS

As discussed in §5.0, a number of questions are pertinent when considering the potential environmental risks of GE salmon. Information relevant to these questions has been presented in this EA, and the answers can be given as follows:

- Are GE salmon able to escape into the environment?

  The likelihood of escape is extremely small due to the multiple containment measures at the sites of egg production and grow-out.

- If an accidental escape were to occur, could GE salmon survive in the surrounding environment and compete with wild salmon (& escaped domesticated, non-GE salmon)?

  If an accidental escape occurred, environmental conditions at the sites in question are such that survival of the organisms would be highly unlikely.

- Could the rDNA construct be transmitted to wild salmon, escaped domesticated, non-GE salmon, or other species?

  The rDNA construct cannot be transmitted to wild or domesticated salmon, or other species.

- Could GE salmon breed successfully with populations of wild salmon (& escaped domesticated, non-GE salmon)?

  The GE salmon are all-female, triploid fish that cannot reproduce among themselves or with wild or escaped domesticated, non-GE salmon.

- Could the offspring resulting from these matings adversely affect the population of Atlantic salmon?

  There will be no offspring since there will be no matings.

Overall, the production, grow-out and disposal of *AquAdvantage* Salmon under the conditions described in this EA will not result in significant effects on the environment.

10.0 DOCUMENT PREPARATION

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