Date of Approval: November 19, 2015

Corrected[1]

FREEDOM OF INFORMATION SUMMARY

ORIGINAL NEW ANIMAL DRUG APPLICATION

NADA 141-454

opAFP-GHc2 rDNA construct in EO-1α lineage Atlantic salmon (AquAdvantage Salmon)

Product Identity:

A single copy of the α-form of the opAFP-GHc2 rDNA construct at the α-locus in the EO-1α lineage triploid, hemizygous, all-female Atlantic salmon (Salmo salar) known as AquAdvantage Salmon.

Claim:

Significantly more AquAdvantage Salmon grow to at least 100 g within 2,700 ^{0}C-days than their comparators.

Sponsored by:

AquaBounty Technologies, Inc.

[1] One parenthetical statement sentence was deleted from the caption to Figure 7 on page 111 because it had been added in error, and a conforming change was made by deleteing a word in a preceding paragraph referring to it.

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II. GENERAL INFORMATION

A. File Number

NADA 141-454

B. Sponsor

AquaBounty Technologies, Inc.
Maynard, MA 01754

Drug Labeler Code: 086053

C. Proprietary Name

"AquAdvantage Salmon", *opAFP-GHc2* rDNA construct in the EO-1α lineage of Atlantic salmon

D. Species/Class

Atlantic salmon

E. Indication

Significantly more AquAdvantage Salmon grow to at least 100 g within 2,700 ^{0}C-days than their comparators.

III. BACKGROUND

A. STATUTORY AND REGULATORY AUTHORITY

FDA regulates genetically engineered (GE) animals under the new animal drug provisions of the Federal Food Drug and Cosmetic Act (FD&C Act), 21 U.S.C. § 321, *et seq.* Section 201(g) of the FD&C Act, 21 U.S.C. § 321(g)(1)(C), defines drugs to include "articles (other than food) intended to affect the structure or any function of the body of man or other animals." When, as is the case for AquAdvantage Salmon, the genetic material, or recombinant DNA (rDNA) construct, used to engineer the animal at issue is intended to affect the structure or function of that animal, the rDNA construct meets the definition of a drug in the FD&C Act. As a short-hand, the agency sometimes refers to regulating the article as regulating the GE animal.

B. GUIDANCE FOR INDUSTRY 187; A RISK-BASED APPROACH TO ASSESSING GE ANIMALS

1. Guidance for Industry 187

In 2009, FDA issued Guidance for Industry 187: Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs[2] to describe how the new animal drug provisions of the FD&C Act and FDA's implementing regulations apply with respect to GE animals and to clarify FDA's requirements and recommendations for GE animals and their products. The guidance describes a multi-step approach for submitting and assessing data for an NADA for a GE animal that is cumulative and risk-based. This FOI summary is organized based on the steps outlined in the guidance.

As described in Guidance for Industry 187, FDA used a hierarchical, risk-based, weight-of-evidence approach in the review of data and information submitted in support of this new animal drug application (NADA). This approach is "hierarchical" in that it does not rely on a single "critical" study, but rather on the cumulative weight of the evidence provided by all of the steps in the review. It is risk-based because it examines both the potential hazards (that is, components that may cause an adverse outcome) identified at each step along the hierarchical pathway and likelihood of harm among the receptor populations (that is, those individuals or populations exposed to the GE animal(s) or their product(s)).

Consistent with other FDA reviews of the products of biotechnology, this approach is, in general, "event-based." An event can be defined as the result of an insertion(s) of an rDNA construct that occurs as the result of a specific introduction of the DNA to a target cell or organism. Animals derived from different events, even if they are based on the previously approved construct(s), would require separate evaluations.

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2 http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM113903.pdf

2. Weight-of-evidence evaluation

FDA's weight-of-evidence evaluation of GE animals draws on data from a number of sources. These include the following, listed in rank order (from highest to lowest) of importance in the overall weight-of-evidence evaluation: (1) controlled studies conducted on the specific GE animals being considered for approval; (2) non-controlled studies on these same animals and historical records and data for these animals; and (3) studies reported in the scientific literature investigating these or similar animals (see Table 1). Each source, in turn, is given appropriate deference with respect to its relevance to the risk or hazard identification question under consideration. Irrespective of the source or order of deference given to a given dataset, all of the data and information is evaluated in the context of basic scientific principles and external validity.

Table 1: Weight of Evidence Evaluations for GE Animals

Order of Deference	Description	Potential Modifier/s	Examples
Highest	• Controlled studies • Final structure of rDNA construct • Same lineage animal • Internal validity	• Study quality • Generations of Animals • Relevance to risk question	• Large double blind study • Use of "Good Study Practices" • Full data set • Concurred protocol followed
Intermediate	•Historical retrospective - Same lineage - Same rDNA construct • Hypothesis proposing investigation	• Study quality • Generations from lineage progenitor • Husbandry conditions	• Not a "formal" study - Cage or tank records - Incomplete records - Pilot or dose range study • Summary data only
Lowest	• Studies on GE GH Atlantic salmon that may be descended from the AAS progenitor or similar animals or similar constructs • Different rDNA copy number or event	• Degree of similarity - Related article - Related animal	• GE Atlantic salmon that may be descended from the AAS progenitor • Different species, breeds • Different gene confers same or similar phenotype • Other regulatory elements in rDNA construct
All rest upon the foundation of biological plausibility	• Basic principles of science (biological plausibility) • External validity	• Constrains or elaborates	•Same effect, similar mechanism/mode of action •Effects observed for similar processes • Information related to genotype or phenotype

IV. PRODUCT DEFINITION[3]

Product Identity

A single copy of the α-form of the opAFP-GHc2 rDNA construct at the α-locus in the EO-1α lineage triploid, hemizygous, all-female Atlantic salmon (Salmo salar) known as AquAdvantage Salmon.[4]

Claim

Significantly more AquAdvantage Salmon grow to at least 100 g within 2,700 ^{0}C-days than their comparators.

Limitations for Use

AquAdvantage Salmon are produced as eyed-eggs and grown-out only in physically-contained freshwater culture facilities specified in an FDA-approved application.

For purposes of describing GE Atlantic salmon whose data were considered as part of the weight-of-evidence evaluation, the agency used the following nomenclature:

AquaBounty Technologies salmon (ABT salmon) are any GE Atlantic salmon from the E0-1α lineage irrespective of ploidy, zygosity, or gender (i.e., the set of salmon that includes diploid GE salmon that may be used as broodstock, as well as AquAdvantage Salmon or other triploid GE salmon).

AquAdvantage Salmon (AAS) are the triploid,[5] hemizygous, all-female Atlantic salmon from the E0-1α lineage GE Atlantic salmon subject to this application. They are a subset of ABT salmon.

GH transgenic Atlantic salmon or GH genetically engineered Atlantic Salmon are GE Atlantic salmon that contain a growth hormone (GH) construct, but whose specific lineage is unknown.

opAFP-GHc2 construct refers to the α-form of the *opAFP-GHc2* recombinant DNA construct inserted into Atlantic salmon at the α-locus (i.e., the regulated article), to produce ABT salmon, including those diploid animals that serve as broodstock.

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[3] A glossary of technical terms is available at http://www.fda.gov/downloads/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/UCM466218.pdf.

[4] As shorthand in this document, the product will be referred to as AquAdvantage Salmon (AAS).

[5] With reference to AquAdvantage Salmon, "triploid" means that, based on sampling, at least 95% of released eyed-eggs have three complete sets of chromosomes per cell with a probability of 0.95 (i.e., probability that these eggs are not at least 95% triploid is less than 0.05.)

V. MOLECULAR CHARACTERIZATION OF THE CONSTRUCT

A. Overview

Risk evaluation in the Molecular Characterization of the Construct Step of the Hierarchical Review Process characterized the potential hazard(s) the *opAFP-GHc2* construct might pose. In particular, FDA evaluated the intrinsic properties of the *opAFP-GHc2* construct that might cause harm. The properties that were of most interest in this respect included potentially mobilizeable DNA sequences, or sequences encoding pathogens, toxins (including allergens), or substances likely to perturb the growth control of cells, tissues, or organs, except by explicit design. FDA also evaluated the purity of the construct in order to ensure that unknown sequences were not introduced.

The evaluation of the Molecular Characterization of the *opAFP-GHc2* construct for AAS is presented in five general sections:

1. Source and description of DNA for the inserted construct
2. Construction, including method and intermediate organisms
3. Sequence of the final product
4. Demonstration of promoter function in salmonid cells
5. Components of microinjection syringe

The submitted materials described the construction, intermediate forms, and confirmation of a fish growth regulator (Chinook salmon growth hormone (GH)) under the control of transcriptional regulatory elements derived from ocean pout as well as short synthetic linkers carried in a standard plasmid backbone. These constructs did not contain coding regions clearly derived from known toxins, pathogens, oncogenes, tumor suppressor genes, or sequences derived from transposable elements or retroviruses that would confer transgene mobilization. FDA's evaluation of subsequent portions therefore focused on the Chinook salmon GH gene and gene product, the ocean pout and Chinook salmon-derived regulatory sequences, and the bacterial plasmid backbone that comprised the final *opAFP-GHc2* construct.

B. Evaluation

1. Source and description of DNA

a. Plasmids

An understanding of what plasmids were used to generate various intermediates in the assembly of the rDNA constructs helped inform the agency as to potential hazards associated with the plasmids, as well as identifying the rDNA that was to be the subject of evaluation in subsequent steps. Several closely related, and commonly used bacterial plasmids (pUC9, pUC13, pUC14 and pUC18) were used to generate various intermediates in the assembly of the *opAFP-GHc2* construct used for generation of AAS .

Diploid ABT salmon were tested for pUC origin plasmid DNA sequences (see Molecular Characterization of the GE Animal Lineage, Section VI, below). No unanticipated sequences from these plasmids were found in the EO-1α lineage ABT salmon.

b. Virus or Bacteriophage
No bacterial or eukaryotic viruses or sequences were used; thus, no viruses or viral sequences could be transferred to or propagated in ABT salmon.

c. Inserts
Recombinant DNA inserts from three sources were used for the final construct (*opAFP-GHc2*). These sources included regulatory sequences from ocean pout, the GH coding region from Chinook salmon, and small synthetic linkers to aid in assembly of the inserts and plasmid. This final construction is discussed in detail below (Section V.B.2).

i. Ocean Pout Anti-Freeze Protein (opAFP) Regulatory (non-coding) Sequences
The upstream (5') and downstream (3') regulatory sequences used in the *opAFP-GHc2* construct were obtained from a genomic isolate of a Type III anti-freeze protein (AFP) gene from the ocean pout (op). Hew et al (1998) described the isolation of opAFP, and Du et al (1992b) described the isolation of the opAFP regulatory regions.

ii. Chinook Salmon GH Coding Sequence
The Chinook salmon GH gene was identified and isolated from a complementary DNA (cDNA) library prepared using pituitary gland of Chinook salmon. This cDNA is full-length and encodes a single, mature hormone.

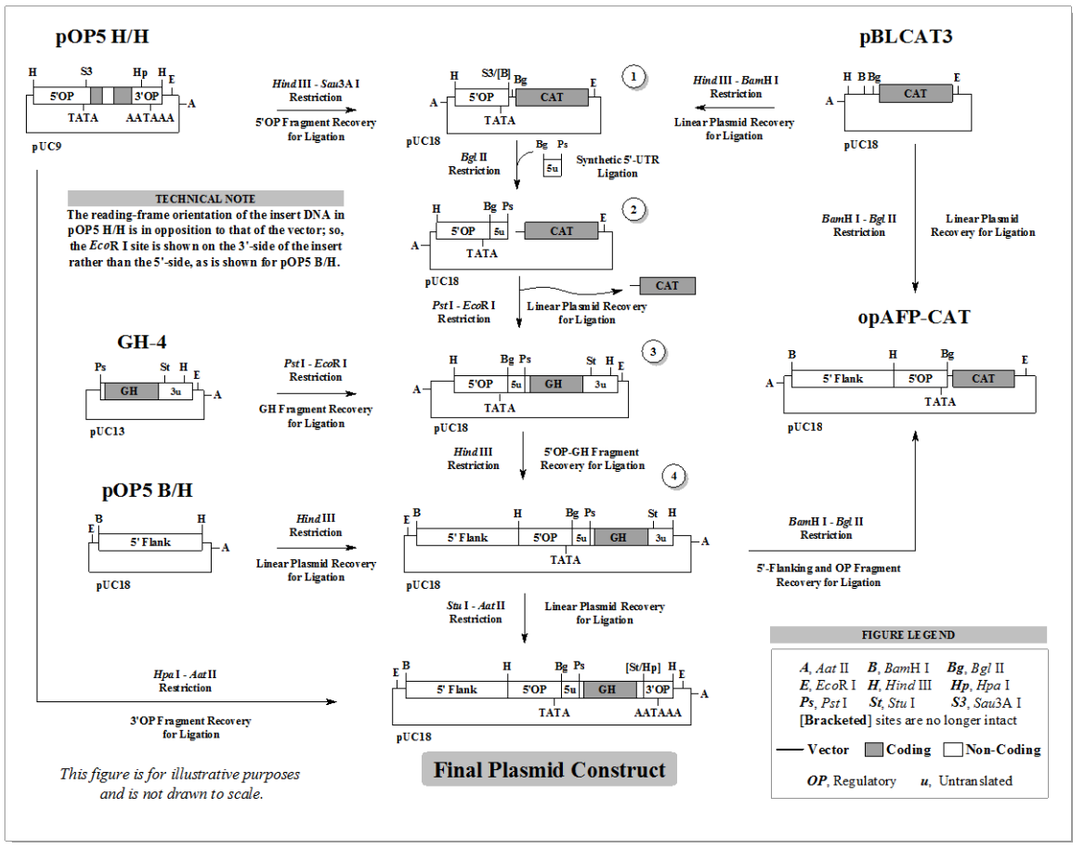
iii. Synthetic linkers
Two synthetic DNAs corresponding to the 5' untranslated regions (UTRs) were prepared using established sequences of the Chinook salmon GH-1 and the ocean pout AFP. These double-stranded DNA strands included 5' Bgl II and 3' Pst I sites, giving rise to 75 bp and 74 bp 5' UTRs, respectively. The GH-1 UTR was used for assembly of the *opAFP-GHc* construct, while the AFP UTR was used for the *opAFP-GHc2* construct. This difference in 5' UTR constitutes the only reported difference between *opAFP-GHc* and *opAFP-GHc2*. The latter construct was employed in producing the founder animal that led to the establishment of the EO-1α lineage of salmon that became AquAdvantage Salmon.

Conclusion: FDA concluded that the data and information describe a standard plasmid backbone, regulatory elements (i.e., the promoter) derived from ocean pout, a fish protein growth regulator (Chinook salmon GH), and synthetic linkers. FDA therefore concluded that these data and information did not indicate that there were any sequence elements in the constructs that contained coding regions clearly derived from known toxins, pathogens, oncogenes, tumor suppressor genes, or sequences derived from transposable elements or retroviruses that would confer mobilization of the construct. Thus, FDA's subsequent evaluation focused on the Chinook salmon GH gene and gene product, the ocean pout and Chinook salmon–derived regulatory sequences, and the bacterial plasmid backbone in the *opAFP-GHc2* construct.

2. Construction, including method and intermediate organisms

a. Assembly of the opAFP-GHc2 Construct
The assembly strategy used for the *opAFP-GHc2* construct used in ABT salmon as well as constructs containing chloramphenicol acetyltransferase gene (CAT; used below to test promoter function in salmonid cells) is presented in Figure 1. The final verified construct (*opAFP-GHc2*) did not contain CAT.

Figure 1. Schematic Summary of the Cloning Strategy Employed in Development of the *opAFP-GHc* and *opAFP-GHc2* Constructs



The multi-step assembly was typical for the time that the construct was assembled and employed routine rDNA procedures. As described in Section V.B.3 below and in the Molecular Characterization of the AAS Lineage, the final *opAFP-GHc2* rDNA construct (shown at the bottom of Figure 1) was verified.

b. Bacterial Hosts of Construct

The plasmids were propagated in, and isolated from *E. coli* K12 strain DH5α. This is a widely used laboratory bacterial strain; *E. coli* K12 is often used as a source of food enzymes and is considered generally recognized as safe (GRAS)[6] for the production of a food processing enzyme (chymosin) by FDA. It therefore poses no toxicological risk in this context.

c. Eukaryotic Cells as Potential Hosts of Construct

The constructs were not propagated or expanded in eukaryotic cells prior to transduction of the fish eggs.

[6] 55 FR 10932 (March 23, 1990).

Conclusion: The construction strategy used techniques generally employed at the time when the construct was generated and was based on protocols cited either in the primary literature or standard laboratory manuals such as Molecular Cloning (Sambrook et al. 1989).

3. Sequence of the final construct

The DNA sequence was determined for the "insert" portion of the construct, not including the plasmid backbone. The sequencing coverage was at least two-fold for the entire insert. Sequencing coverage of the Chinook salmon GH gene and immediately adjacent control regions was eight-to ten-fold, more than sufficient for sequencing the final construct. Chromatograms contained clear, well-defined peaks that were typically readable for greater than 500 bases and the observed sequence was reliable. Finally, the deduced amino acid sequence of the open reading frame of the Chinook salmon GH gene was consistent with published materials.

Conclusion: The sequence determination submission was acceptable and sufficient to support molecular characterization of the *opAFP-GHc2* construct.

4. Demonstration of promoter function in salmonid cells

A series of studies demonstrated that the ocean pout antifreeze type III regulatory regions were functional and that the promoter (or small parts of it) was functional in appropriate salmonid cell types (Du et al., 1992b; Gong and Hew, 1993). This supported the proposed use of the *opAFP-GHc2* construct for this part of the evaluation.

5. Components of microinjection syringe

Linearized DNA dissolved in 2–3 nL of sterile 0.9% NaCl was used in the microinjection to produce the founder animals. The purity of the preparation containing the *opAFP-GHc2* rDNA construct prior to introduction into recipient cells was acceptable.

C. Conclusions

The general information for the molecular construction of the vectors and transgenes injected was internally consistent, with data for the sequence of the insert (but not plasmid) in the injected DNA provided. Evaluation of the submitted data did not identify any specific hazards intrinsic to the *opAFP-GHc2* construct with the possible exception of the growth hormone gene that was present by explicit design.

The data and information submitted are acceptable for the Molecular Characterization of the Construct portion of the hierarchical review of the new animal drug application for AAS.

VI. MOLECULAR CHARACTERIZATION OF THE GE ANIMAL LINEAGE

A. Overview

For the Molecular Characterization of the GE Animal Lineage, FDA evaluated the molecular consequences of the insertion of the *opAFP-GHc2* construct into the EO-1α lineage of AAS to identify potential hazards that might result because of the integration event, and to evaluate the overall stability of the *opAFP-GHc2* construct in the lineage over multiple generations. Specifically, FDA examined the molecular characterization of the *opAFP-GHc2* construct integrated in the genome of AAS. The *opAFP-GHc2* construct was for expression of Chinook salmon GH under the control of an ocean pout promoter in the EO-1α lineage.

The four general hazard identification questions asked about any GE animal at this stage of the hierarchical evaluation are the following:

i. Does the GE animal contain sequences that are likely to pose potential hazards to the animal, humans or animals consuming food from that animal, or the environment?
ii. Is the genotype changing over the life span of the animal or product?
iii. Is the inserted DNA what was expected from the data presented in support of the Molecular Characterization of the Construct section?
iv. Does the GE animal contain other contaminating or hazardous materials such as viruses, cells, or chemicals?

FDA concluded that the data submitted support the Molecular Characterization of the GE Animal Lineage step of the Hierarchical Review Process. FDA identified no hazards, with the possible exception of the growth hormone gene itself, which is present by design, and will be evaluated at a subsequent step of the hierarchical review process.

B. Evaluation

1. Does the GE animal contain sequences that are likely to pose potential hazards, including to the animal, or humans or animals consuming food from that animal?

To evaluate the consequences of the insertion of the *opAFP-GHc2* construct, FDA evaluated data and information that characterized (a) the number of insertion sites, and (b) the insertion site itself (including possible disruption of other genes) and analysis of open reading frames (ORFs) within and around the insertion site

a. Number of Insertion Sites

Using information from the Molecular Characterization of the Construct, assays were designed and conducted to detect the *opAFP-GHc2* construct and the pUC plasmid backbone sequences that could have been (but were not) inserted into the fish genome. This analysis addressed the following:

(i) Whether any plasmid DNA was present in the lineage; and

(ii) The insertion sites present in initial, and production, lines of ABT salmon.

Three methods (Southern analysis, polymerase chain reaction (PCR) amplification, and DNA sequence analysis) were used to characterize the rDNA in the animal(s).

i. Plasmid DNA

The Chinook salmon GH expression construct was released from the bacterial pUC plasmid DNA prior to microinjection of the eggs, but was not removed from the delivery solution (see Section VI B 4 below). It was therefore necessary to determine whether the backbone plasmid DNA was absent from the genome of ABT salmon; if so, no assessment of associated hazard or risk would be necessary. Southern analysis (with appropriate controls) was conducted to determine if the pUC plasmid DNA was present in ABT salmon. No pUC plasmid DNA was detected in any of the F_1 ABT salmon descended from the EO-1α lineage. Because the fish in this study included the progenitors to all of the lines that led to the development of AAS, if no pUC plasmid was present in the progenitors, it could not be present in subsequent generations. The agency therefore concluded that the pUC plasmid DNA is not present in AAS.

Conclusion: pUC plasmid DNA is not inserted into the genomic DNA of AAS and no further consideration of it was necessary.

ii. Number of insertion sites

Multiple methods were used to characterize the number of construct insertion sites. These methods included Southern analysis, PCR amplification, and DNA sequence analysis. These analyses showed that although the initial GE animal contained two insertion events, at the α- and β- loci, the progeny lines developed for production contained one well-characterized construct only at the α-locus.

Southern analysis demonstrated that early generations of ABT salmon contained up to two distinct insertion sites, referred to as the α and β loci. The Southern analysis gave rise to multiple distinct bands that corresponded to these two copies of the *opAFP-GHc2* construct. The sponsor determined that the presence of the α-locus conferred the enhanced growth phenotype while the β-locus did not. The sponsor chose to select for the α-locus and bred the β-locus out of their production lines by conventional back-crossing. Additional Southern analysis data was provided supporting the absence of the β-locus from the lines selected for production use (see Section VI B 2 below).

PCR amplification of the inserted construct in F_2 generation fish, followed by enzymatic digestion of the PCR products was a second method for determining the number of constructs integrated into the salmon genome. Primers were specifically selected to distinguish between the α and β loci, the inserted growth hormone, native salmon growth hormone loci, and single insertion versus multiple insertions per site. These data were consistent with the Southern analysis discussed above. (Note: additional PCR analysis of the α-locus in F_2, F_4 and F_6 generation fish is discussed further in subsequent sections).

DNA sequence analysis was a third method employed for determining copy number and stability of the insert at the α-locus. Primers were designed to specifically anneal to the least

conserved regions of the 5' and 3' genomic flanking regions around the α-locus. This allowed for obtaining better specificity in amplification of the construct and monitoring genomic stability over multiple generations. Eleven other primer pairs were designed to fully sequence the inserted construct at the α-locus. Sequence data were consistent with a single copy of the *opAFP-GHc2* construct at the α-locus. A contract laboratory performed sequencing of the α-locus in accordance with Good Laboratory Practice standards (GLP, 21 CFR Part 58). Each sequencing reaction produced > 600 base pairs (bp) of good coverage. Chromatograms were provided and demonstrate the high quality of these data.

Conclusion: ABT salmon, including AAS, contain a single, well-characterized copy of the *opAFP-GHc2 construct* at the α-locus.

b. Evaluation of Insertion Site

Evaluation of the α-locus focused primarily on site specific effects: (i) disruption of genes at the insertion site, and (ii) generation of a novel open reading frame by the recombination of the *opAFP-GHc2 construct* and genomic DNA.

i. Possible Disruption of Other Genes

The sequence data discussed above showed that the α insert was in a region of repeated DNA (a 35 base repeat), and not in a protein coding region. Additionally and importantly, if, as was expected with non-homologous recombination, part of the chromosomal DNA was deleted when the construct was inserted at the α-locus, it was likely that only part of this 35 base repeat region was lost. Repeated regions like this are quite variable and nonessential, so loss of part of the repeat region was unlikely to adversely affect the resulting fish.

Conclusion: The insertion of the *opAFP-GHc2 construct* at this site is not expected to impact the expression of native genes.

ii. Open Reading Frame (ORF) Analysis

Given the construct is located in a repeat region, there are no open reading frames in the region flanking the insertion site. As such, generation of novel open reading frames across the insert junction is not possible.

Furthermore, as discussed in the Molecular Characterization of the Construct (see Section V, above), the insert only contains sequences derived from ocean pout and Chinook salmon, both of which are commonly consumed as food. FDA did not identify any sequences that are of obvious concern. Food consumption risks were evaluated during the Food Safety step of the Hierarchical Review Process (see Section IX, below).

Conclusion: Because the *opAFP-GHc2 construct* is inserted into a repeat region, there are no putative open reading frames other than those intended in the construct itself. Therefore, no additional risk from novel or altered open reading frames is present.

Overall Conclusion for Risk Question 1: Based on the data and information provided, DNA sequences related to the α-locus are adequately characterized. With the possible exception of the growth hormone gene included by design, review of the data and information did not identify

sequences likely to contain potential hazards, including, but not limited to the target animal, humans, and animals consuming food from that animal.

2. Is the genotype changing over the life span of the animal or product?

FDA assessed the genotypic and phenotypic stability of AAS using a number of approaches. Several of these approaches employed molecular biological analysis, including DNA sequence analysis, Southern analysis, and PCR analysis (described previously). As discussed further below, FDA concluded from its weight-of-evidence analysis, that the *opAFP-GHc2* construct is unchanged and stably maintained at the α-locus over at least seven (7) generations (F_0 to F_6) and multiple lineages of ABT salmon.

The DNA sequence analysis of the α-locus was consistent (other than the rearrangement discussed subsequently) from the test tube through one F_2 and one F_4 generation of ABT salmon. The sequence of the coding region was unchanged in these samples. Additionally, the sequence of the genomic DNA flanking the α-locus (a repeated region) was also unchanged between the F_2 and F_4 generations. From this, FDA concluded that the growth hormone expression insert is stably maintained at this chromosomal position (the α-locus).

Similar to the PCR analysis of F_2 generation fish described above, the sponsor conducted additional PCR analysis of the α-locus in F_2, F_4, and F_6 generation ABT salmon. The sponsor developed detailed procedures and provided supporting data for a series of PCR amplifications that identified the *opAFP-GHc2* construct at the α-locus. Specifically, the sponsor described several primer sets and resulting amplification products corresponding to the 5' and 3' ends of the α-locus (with adjacent chromosomal flanking DNA) as well as the *opAFP-GHc2* growth hormone gene and two different endogenous growth hormone genes. The technique was appropriate, the method was well described, and the results were clear and unambiguous. The sponsor applied this method to samples from a total of 72 F_2, F_4 and F_6 generation fish well dispersed among the lineages being pursued for further development. These data provided evidence that the α-locus is stable over seven (7) generations.

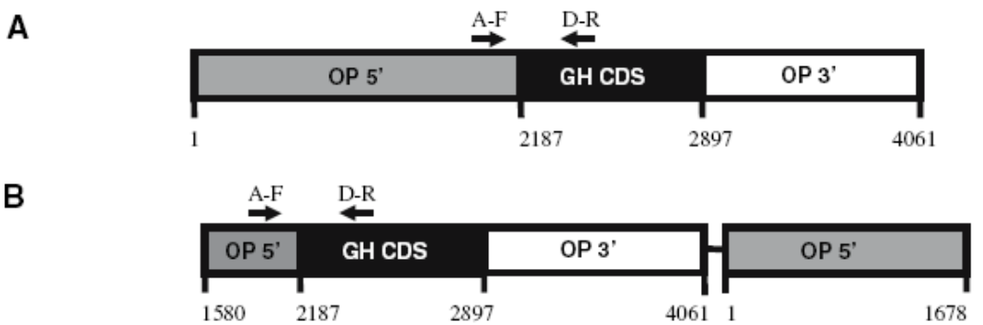
To determine the stability of the α-locus over multiple generations as well as to confirm earlier PCR studies showing that the β-locus was successfully bred out of the later generation fish, a contract laboratory performed additional Southern analysis in accordance with GLP standards. Samples of 22 fish (including controls) dispersed among the lineages under development were subjected to Southern analysis. The analysis and some of the samples used replicate the Southern analysis described above. The results were consistent between studies. Here only the previously identified F_2 generation fish contained the α and β loci where all other F_2, F_4, and F_6 generation fish contained only the α-locus. No negative controls contained an insert.

Conclusion: The α-locus is stable between the founder and the seventh generation (F_0 through F_6). The β-locus was selectively bred out of the lineage and is not present in the lines of fish currently in production.

3. Is the inserted DNA consistent with the data presented in support of the Molecular Characterization of the Construct?

The general structure of the *opAFP-GHc2* construct, stably maintained in ABT salmon, was characterized and consistent with data submitted for the Molecular Characterization of the Construct evaluation. Characterization and analysis of the construct was also provided (see also Yaskowiak et al., 2006). This analysis identified a rearrangement compared with the original construct prior to insertion (Panel A Figure 2). The rearrangement displaced a portion of the far 5' non-coding regions of the insert to the 3' end of the insert (Panel B Figure 2).

Figure 2. Characterization of the *opAFP-GHc2* construct at the EO-1α locus



(A) Structure of the *opAFP-GHc2* construct that was injected into Atlantic salmon eggs to produce the EO-1α strain of transgenic Atlantic salmon. (B) Schematic of the genomically integrated transgene EO-1α. (From Figure 3 in Hobbs and Fletcher, 2008.)

The sponsor hypothesized that the rearrangement identified in the EO-1α lineage occurred during the initial transformation event that resulted in generation of the founder of the EO-1α lineage. The sponsor stated that circularization of the *in vitro* linearized DNA prior to integration of the DNA into the Atlantic salmon chromosome may have resulted in the rearrangement of the elements in the original construct at the α-locus. This explanation provided a reasonable justification for how the final molecular structure of the α-locus arose. The rearrangement moved the far upstream promoter regions (typically enhancer domains) to a downstream location relative to the growth hormone coding region. As detailed in the Molecular Characterization of the Construct evaluation (Section V, Part B.4), the sponsor previously provided data demonstrating that the far upstream regions of the promoter were not required for expression from this promoter. Furthermore, enhancer elements act at a distance and are generally not orientation dependent. This rearrangement is well characterized and requires no further consideration in future evaluations.

Conclusion: With the exception of the well-characterized rearrangement described above, the sequence of the integrated construct was consistent with the sequence of the *opAFP-GHc2* construct. No additional evaluation, above that which would be normally conducted, was required in other steps.

4. Does the GE animal contain other contaminating or hazardous materials such as viruses, cells, or chemicals?

Data and information regarding the preparation and purification of the *opAFP-GHc2* construct DNA for injection into salmon eggs was submitted. The insert was excised from the bacterial plasmid sequences by overnight digestion with restriction endonuclease *Eco*RI, followed by phenol/chloroform extraction and ethanol precipitation. The insert was linearized and released from the plasmid, but not purified to remove the plasmid fragment from the solution. Thus, the GH containing inserts from the plasmids as well as the pUC plasmid sequences were microinjected into the eggs. (As described above in Part VI.B.1.a.i above, pUC plasmid DNA was not incorporated into the genome of the EO-1α lineage salmon being developed for production and so is not a concern.)

The descriptions of the molecular biological methods and procedures included in the submission as described in Section V.B.2 were standard procedures routinely used at the time the work was conducted and do not pose a hazard.

Microinjection of salmon eggs was described in several submissions from the sponsor, and found acceptable. Several publications (Du et al., 1992a; Du et al., 1992b; Fletcher et al., 2001; Shears et al., 1991) supported the conclusion that the methods used in the production of ABT salmon were consistent with the methodology generally in use at the time of injection.

Conclusion: There is no risk from any contaminants or other hazardous materials (with the possible exception of the growth hormone present by design) in the EO-1α lineage.

C. Conclusions

The information provided by the sponsor for the Molecular Characterization of the GE Animal Lineage is consistent and in agreement with the Molecular Characterization of the Construct. The sponsor provided supporting data for the sequence of the injected *opAFP-GHc2 construct* and the molecular stability of the construct over seven generations.

The data submitted support the Molecular Characterization of the GE Animal Lineage for AAS. FDA identified no hazards, with the possible exception of the growth hormone gene itself, which is present by design, and which FDA evaluated at subsequent steps of the hierarchical review process.

VII. PHENOTYPIC CHARACTERIZATION OF THE GE ANIMAL

A. Overview

In this section, FDA characterized the ABT salmon and their derivatives, including AAS, with respect to various aspects of their phenotype in order to make judgments regarding the safety of the *opAFP-GHc2* construct to the target animal. Although a number of lines of GE salmon were generated, the sponsor limited expansion of populations for production only to specific lines derived from the EO-1α lineage progenitor. The information FDA evaluated often contained data that supported other levels of the hierarchical review process (e.g., durability, claim validation); however, the primary focus in this portion of the overall review process was on the data and information evaluated to support the phenotypic characterization of the GE animal, and to draw conclusions regarding animal health.

During the initial phase of review, FDA stated that, as with all data sets, there are some uncertainties. See VMAC Briefing Packet at 21, http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/UCM224762.pdf. At the time FDA performed its preliminary review, which was released in the briefing packet for the VMAC meeting, the primary area of uncertainty was in determining the actual rate of adverse outcomes in grow-out facilities, as the process of selecting animals for the initial sponsor study, which has been referred to as "culling," may have influenced the apparent rate of abnormalities observed. Because of concerns that the culling procedures for the initial study may not have reflected typical acquaculture procedures and may have obscured adverse outcomes, FDA requested and received from the sponsor additional information regarding culling practices, the health of the ABT fish populations at the grow-out facilities, and the potential role that culling could have had in masking adverse outcomes. These data and information submitted to the agency since the preliminary review are found in Section VII B 2, and Tables 8-13 and include information on morbidity and mortality from more than 150,000 ABT salmon and approximately 9,000 non-GE Atlantic salmon from both the PEI and Panama facilities. These new data did not reveal any new abnormalities or altered rates of abnormalities beyond those identified in the initial study, and did not indicate any bias in the initial study's estimation of (i.e., did not mask) rates of morphologic abnormalities, mortality, or morbidity. In addition, FDA directed the sponsor to collect data from the Panamanian grow-out facility to be used as part of a surveillance program in the durability plan (see Genotypic and Phenotypic Durability Plan, Section VI below), and as the basis for determining unexpected and serious adverse events in the post-approval record keeping and reporting requirements (see Letter of Approval, including Appendix A).

FDA's review of the additional data and information submitted since the preliminary review (see subsequent sections), strengthened the agency's conclusions regarding the phenotype of ABT salmon, including AAS, by addressing these uncertainties.

After evaluating all of the data submitted and referring to peer-reviewed publications as appropriate and as described in the agency's weight-of-evidence approach (see Section III B 2), FDA did not identify any significant hazards or risks with respect to the phenotype of AAS as a result of the *opAFP-GHc2* construct. There are no significant adverse outcomes associated with the introduction of the *opAFP-GHc2* construct and the production of triploid monosex (all- female) AAS. Most of the

adverse outcomes that have been observed (e.g., morphological changes) were present in comparators or have been described in the peer-reviewed literature with attribution either to the induction of triploidy or to non-transgenic rapid growth phenotypes. These adverse outcomes occur mostly in the early life stages; their consequences are likely to be small and within the range of abnormalities affecting rapid growth phenotypes of Atlantic salmon. FDA therefore determined that the *opAFP-GHc2* construct is safe for the animal, as shown below.

None of the adverse outcomes noted, which were minimal, are expected to have any implications for food consumption risks (see Food Safety Section); some of these phenotypic changes may affect the fitness of GE animals such that any escapees from containment would be less capable of surviving (see EA, Section 7.3.1.1.2). For example, although AAS would have one key increased fitness attribute (i.e., more rapid growth in the first year) relative to their wild and domesticated non-GE counterparts, in many other respects, their fitness would be reduced (e.g., increased need for food, increased dissolved oxygen utilization, etc.). Further, reports on fitness characteristics of GH transgenic Atlantic salmon indicate that the presence of the EO-1 α construct appears to result in decreased fitness, which would be expected to reduce the chances for survival in the very unlikely event of escape. For a more complete discussion of how phenotypic changes in AAS may affect fitness, see EA, Section 5.2.

FDA applied a risk-based approach to evaluate AAS to address four risk/hazard questions developed for the phenotypic characterization of GE animals:

1. Is there direct or indirect toxicity to the animal?

2. Are there phenotypic characteristics that identify hazards for other steps in the evaluation (e.g., food safety or the environmental assessment)?

3. What are the risks to the user (user safety)?

4. What are the risks to the animal from any components of any biological containment strategy?

B. Evaluation

General husbandry conditions and facilities affect the phenotype of farm-raised animals, including Atlantic salmon. As all ABT salmon (including AAS) are to be raised in contained inland tanks, this section first describes those conditions and facilities, and the effects they may have on the populations of fish that are the subject of this application. For a more specific discussion of the effects of husbandry and tank conditions, refer to the Environmental Assessment (EA) Section 5.

1. General Description of Facilities and Husbandry Conditions

The Prince Edward Island (PEI), Canada facility is the site of broodstock maintenance, breeding, and egg production operations. The Panamanian facility receives triploid, fertilized (eyed) eggs from PEI, hatches the eggs in incubators, and grows the young fish (alevins through fry) in fry

tanks until they reach an average size of at least 25 grams, at which time they are transferred to larger grow-out tanks to reach market size.

Because fish husbandry conditions, particularly those that affect water quality, can affect fish health and phenotype (e.g., morbidity, mortality and stress-related parameters), included in the assessment of phenotype was a consideration of husbandry conditions. ABT and comparator[7] salmon were cultured at PEI under standard conditions for the freshwater (hatchery and smolt production) phase of salmon aquaculture[8]. Water specifications were maintained at the following conditions: pH = 7.3; oxygen > 8 mg/L (range of 11.7 – 17.7 mg/L); carbon dioxide < 20 ppm; ammonia < 0.03 ppm; nitrate < 40 ppm; nitrite < 0.15 ppm; and stocking densities of 10-35 kg/m^3. Temperature ranges were from 12.1 – 14.3°C. As needed, corrective actions were taken to bring water quality within these parameters.

These water specifications were generally applicable to the grow-out facility in Panama as well, though water temperatures in Panama were slightly higher (approximately 15-16°C). The large tanks at the PEI facility are 11.2 m^3 cylindrical tanks using recirculating ground water. The water is adjusted, as necessary, to meet the specific water conditions described above. At the Panama facility, the grow-out tanks have a maximum capacity of 100 m^3, but are operated at a maximum volume of 85 m^3.

Fish were fed to satiety using a commercial salmon diet of appropriate composition and pellet size for the applicable production phase. Records were maintained for water quality parameters and tank feed amounts.

The fish stocking densities at both locations were kept within the range of 10 – 35 kg/m^3, a range representative of commercial freshwater salmon aquaculture conditions to maintain optimal water quality and growth conditions, with water flow that supports complete turnover of tank capacity approximately once an hour.

Conclusion: FDA noted that although some of the culture conditions (e.g., water temperature, pH, alkalinity, etc.) at the Panama facility likely differed from the facility at PEI as a result of differences in, among others, water source, facility design, and environmental factors due to geographic location, the general husbandry conditions for ABT salmon were consistent with commercial freshwater aquaculture conditions. FDA concluded that the general husbandry conditions present no identifiable hazards or safety concerns to the salmon or the environment beyond those seen for commercial freshwater aquaculture. (See EA, Section 5.4.1). Husbandry and rearing conditions will continue to be monitored through post-approval records and reports (see Approval Letter, Appendix A).

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[7] Throughout this section, unless otherwise specified, the term “comparator” refers to non-GE farm-raised Atlantic salmon of a similar, but not identical, genetic background as ABT salmon, including, depending on the study, both diploid and triploid fish.

[8] Once they reach smolt size, Atlantic salmon are normally transferred to seawater and reared to market size in open water net pens, however, the entire lifecycle of ABT salmon occurs in contained freshwater facilities.

a. Potential Biasing Influence of Culling

In general, fish culling practices at the PEI facility were and are consistent with established procedures in the management of fish inventory and animal husbandry (Freshwater Institute/Atlantic Salmon Federation, 2013)[9]. ABT removed fish, especially at early life stages (e.g., eyed eggs, fry or smolts) due to factors including space constraints and inventory management, i.e., separation of fast-growing individuals from slow-growing individuals, selection of broodstock, and maintenance of the appropriate level of biomass for the contained life-stage of the salmon. Because of space limitations, extensive culling was performed at the PEI facility on what was described by the sponsor as an *ad hoc* basis. Excess inventory of eggs and early-life stages were removed at several different time periods: (1) between egg fertilization and hatching; (2) after hatching and before separation; (3) at the time slow- and fast-growing fry were separated (> 5g); and (4) after PIT tagging during the grow-out phase to maintain the < 35 kg/m^3 biomass:water ratio. Typically, no data were collected on the excess inventory of fish; therefore, morbidity and malformation information were not available for those discarded fish.

Culling may have introduced uncertainties with respect to baseline data used to assess fish health, especially the rate of abnormalities, prior to data collection for fish presented as part of phenotypic characterization and animal health/safety evaluation. Issues related to culling procedures at the PEI facility and the potential impact on study outcomes are discussed in Section m. It is possible that the culling of eggs or fry were from crosses exhibiting high occurrences of malformation, morbidity and/or mortality (which would be the expectation of a hatchery attempting to select for the most fit and fastest growing offspring), would tend to skew the population of the fish remaining in the facility after culling toward one with lower prevalence of those malformations or rates of death, thereby biasing the data.

The subsequent discussion in this section addresses whether the potential for bias from culling has influenced conclusions on rates of morbidity, mortality, and malformations, including additional data submitted by the sponsor. After review of these data and other information subsequently submitted by the sponsor, described in section m, the agency determined that any bias in the initial study's estimation of rates of morphologic abnormalities, mortality, or morbidity that might have been introduced into the earlier studies by standard commercial culling practices did not skew (i.e., did not mask) these rates to an apparently lower level.

b. Disease Status

During the third quarter of 2009, a disease outbreak later determined to be infectious salmon anemia (ISA) occurred at the PEI facility. Prior to this, Canadian authorities had considered the PEI facility "disease free" for many years based on periodic inspections and testing. The ISA outbreak was first detected in fish in the grow-out area (GOA) and later spread to fish in parts of the early rearing area (ERA). Once the presence of the infectious salmon anemia virus (ISAV) was

[9] The exception is described at Section VII A, where the sponsor selected (culled) fish to to generate the appropriate number of animals for the sponsor-initiated study for the initial phenotypic characterization.

confirmed, ABT notified the Canadian Department of Fisheries and Oceans (DFO) and, shortly thereafter, the Canadian Food Inspection Agency (CFIA)[10].

After FDA learned of this outbreak in late December 2011, the agency requested all pertinent information from the sponsor. The sponsor provided relevant records, including full access to primary records during a formal inspection in June of 2012 (see Appendix F of the EA).

The sponsor addressed the ISA outbreak at the PEI facility by implementing standard mitigation strategies appropriate for this disease (e.g., extirpation of all affected fish, and implementation of an ISA detection and monitoring program). All fish displaying any characteristic of poor health or high viral load, most of the broodstock, and other non-essential fish were culled from the facility. In the GOA, only asymptomatic AquAdvantage broodstock and a few non-GE females were retained, while the ERA was completely depopulated and decontaminated. Subsequently, quarantine areas were constructed within the GOA to house and isolate important broodstock that had potentially been exposed to ISAV. The ERA and GOA have also been permanently and physically separated into two distinct, biosecure facilities. Ultraviolet (UV) lights were installed to disinfect both the incoming well water and the recirculated water within both the ERA and GOA. Ozone treatment was added to disinfect water recirculated within the ERA (see EA Section 5.4).

All year-classes of ABT fish produced since the 2009 ISA outbreak have tested negative for ISAV when assayed using the most sensitive, quantitative, real time polymerase chain reaction (qPCR) diagnostic assay available. Since November 2009 all mortalities in the GOA have been necropsied and examined for signs of ISAV and no mortalities with clinical signs of ISAV have been reported to the agency. Samples of fertilized eggs, fry, and blood from fish in the ERA have been collected periodically since the ERA was depopulated and decontaminated in October 2009. No ISAV positive samples (fry, whole blood, or mortalities) have been detected by any method in the ERA during that time.

The sponsor, as confirmed by FDA inspection (see discussion below), has collected samples of water entering tanks and facility effluent monthly since October 2009 and tested for the presence of ISAV using qPCR. None of the water or effluent samples has ever tested positive for ISAV since October 2009.

CFIA conducted an inspection of the PEI egg production facility in May 2013 and assessed the risk of introduction of disease for a number of exposure pathways and pathogens, including viruses causing ISA, epizootic erythropoietic necrosis (EHN), infectious pancreatic necrosis (IPN), infectious hematopoeietic necrosis (IHN), and viral hemorrhagic septicemia (VHS), among

[10] The Government of Canada has developed a National Aquatic Animal Health Program (NAAHP) to bring Canada into compliance with international aquatic animal health management standards. Anyone who owns or works with aquatic animals and knows of or suspects a reportable disease is required by Canadian law to notify the Canadian Food Inspection Agency (CFIA). CFIA and DFO share responsibilities for federal components of NAAHP. CFIA is responsible for aquaculture health surveillance, and is responsible for certification of the health status of aquatic animal exports with respect to the risk of introduction or movement of an aquatic animal disease into a receiving country.

others. The overall introduction of risk was found to be minimal for ISA, EHN, IHN, and VHS (these pathogens received the lowest possible rating), and acceptable for all other pathogens. The facility report concluded that the facility's biosecurity plan was adequate and no further mitigation measures were required to address pathogens of concern (see EA Section 5.4.2). In addition, Canada requested no amendments for the facility's biosecurity plan.

As of late November 2012, no Schedule II diseases or disease agents, as described in Canada's Fish Health Protection Regulations, have been detected in fish or eggs from either the ERA or the GOA of the PEI facility since before the ISA outbreak in 2009. Negative results have been found in all subsequent inspections of each area, including several inspections conducted by the DFO Fish Health Unit in 2010, 2011, and 2012 that specifically tested for ISAV and Schedule II pathogens. The most recent Fish Health Certificates issued by DFO for the ERA and GOA specifically list the following pathogens as "not detected"[11]:

- Viral hemorrhagic septicemia virus
- Infectious hematopoietic necrosis virus
- Infectious pancreatic necrosis virus
- *Aeromonas salmonicida*
- *Yersinia ruckeri*
- *Myxobolus cerebralis*
- *Ceratomyxa shasta*

c. Inspections and Site Visits

FDA personnel conducted an inspection of the sponsor's facility at PEI in 2008, examining all available records at the location. Inspectors found the facility acceptable with no objectionable items communicated to the sponsor to which they needed to provide a formal response. [12]

Staff from the FDA Center for Veterinary Medicine and the National Oceanic and Atmospheric Administration conducted a site visit of the Panama facility in late 2009 (see EA, Appendix F for more details) primarily to verify that there was acceptable physical containment at the facility. In addition, the water quality and rearing conditions at the facility and the general health of the fish in residence were also examined. Nothing was observed that would indicate an issue of concern with respect to the facility or the fish therein.

FDA conducted a second inspection of the PEI facility in June of 2012 with the primary goals of evaluating the record-keeping, diagnosis and extirpation of the incidence of ISAV, and ensuring

[11] The pathogen listed as "Other filterable replicating agent" is shown as "Detected" on the certificates. This is *not* due to any actual detection of filterable replicating agents within the PEI facility, but rather due to a Canadian regulatory technicality related to the outside source of wild stocks that was used to supply the ABT facility. This outside source did have a detection of a filterable replicating agent at one time, which requires a carry-over to Fish Health Certificates for all facilities supplied by that source. It should be noted that the filterable replicating agent is not one of current pathological concern and does not affect the export status of the facility.

[12] Objectionable items are formally communicated to sponsors at the end of an inspection with an FDA Form 483. For more information, see FDA form 483 frequently asked questions *at* *http://www.fda.gov/ICECI/Inspections/ucm256377.htm*.

adherence to conditions of physical containment. Details of that inspection are found in Appendix F of the EA. As a result of the inspection, FDA concluded that (1) the results of the diagnostic evaluations are consistent with ISAV presence; (2) appropriate biosecurity measures were taken in response to the outbreak, including installation of UV and ozone water treatment systems; and (3) physical containment was secure. See Appendix F of the EA for additional information on this inspection.

FDA notes that the occurrence of ISAV, a viral disease that is known to occur in aquaculture facilties, and that was detected in the PEI grow-out facility, does not impact the safety or effectiveness of the *opAFP-GHc2* construct. Nonetheless, under the conditions established in the approved application, FDA will require notification of the putative or confirmed presence of infectious disease in either the PEI broodstock or Panama grow-out facilities.

2. Evaluation of Data and Information Relevant to Phenotypic Characterization

A broad range of sources of data and information were drawn from for the weight-of-evidence evaluation of the ABT phenotypes. These included the following data and information: (1) controlled studies conducted on ABT salmon; (2) other non-controlled studies on ABT salmon; (3) historical hatchery records and data for ABT or GH Atlantic salmon; and (4) studies reported in the scientific literature investigating GE salmon. In general, comparator fish were non-GE, diploid Atlantic salmon of a closely related strain, unless otherwise indicated.

In the following, data and information used for the evaluation of phenotype are described on an endpoint basis (a-i), with deference applied to studies as described previously for the weight-of-evidence evaluation. These endpoints included the following:

a. general health observations;
b. size, weight and related measurements (e.g., feed consumption and rate of weight gain);
c. physical examinations: behavioral and physical abnormalities;
d. overall mortality and morbidity;
e. clinical pathology assessments (clinical blood counts and chemistry panels);
f. macroscopic and microscopic evaluation (other than gross morphology);
g. disease resistance;
h. smoltification and seawater survival; and
i. other phenotypic characteristics.

These areas of observation/data summary are referenced, by letter, below in the descriptions of data/information sources. Nutritional (compositional) and hormonal data for adult AAS were considered as part of the FDA evaluation of food safety (see Food Safety, Section IX below).To the extent possible, numbers of ABT salmon (including AAS) and comparator salmon (or the number of crosses from which ABT or comparator salmon were derived) that contributed to a particular evaluation were provided.

a. General Observations on ABT Salmon from the Animal Safety Study and Supporting Observations

The sponsor's animal safety study evaluated the effect of the integrated *opAFP-GHc2 construct* on the health of ABT salmon. The primary objective of the designed target animal safety study was to

perform a comprehensive animal safety evaluation on weight-matched diploid (2n) and triploid (3n) ABT salmon, and non-GE comparator diploid and triploid farm-raised Atlantic salmon, with equal numbers of male and female fish (n=6 per gender, n=12 per group) in each of these four groups (n=48 for study). Enrollment of fish and initiation of the study occurred when the whole body weight of individual fish in each group ranged from 1,000-1,500 g. In addition, age-matched diploid and triploid non-GE comparators were included in the study (n = 3 per gender, n=6 per 2n or 3n group), as described below.

This well-controlled study provided the focal point for subsequent data summaries addressing phenotypic characterization and animal health, had a prequalification phase, and included the following endpoints, which are listed below:

a. general health observations;
b. size, weight and related measurements (e.g., feed consumption and rate of weight gain);
c. physical examinations: behavioral and physical abnormalities;
d. overall mortality and morbidity;
e. clinical pathology assessments (clinical blood counts and chemistry panels);
f. macroscopic and microscopic evaluation (other than gross morphology).

FDA also evaluated other data and information related to ABT salmon (e.g., historical data with or without non-GE comparators, other studies) and published literature as part of the overall weight-of-evidence evaluation of AAS.

Facility personnel and independent veterinary professionals specializing in aquatic species (including Atlantic salmon) assessed the general health and behavior of all of the fish.

Prequalification Assessment
Initially, measurements to establish baseline morphological rankings of external appearance were conducted during the pre-qualification phase of this study; these are summarized in Table 1. Study fish were selected from a pre-qualification phase of candidate fish, during which fish showing clear signs of morbidity were excluded. Once candidate fish reached 15 - 30 g in weight, each fish was given a passive integrated transponder (PIT) and a unique fish identification number. A total of 645 fish were considered as candidates in the pre-qualification phase, with 194 diploid (2n) and 167 triploid GE fish, and 187 diploid and 97 triploid non-GE comparator fish. All 645 candidate fish were subject to morphological evaluation of external appearance (summarized in Table 1 below). In addition, these candidate fish were randomized using a pre-generated list that assigned a code to either “include” or “exclude” each fish by PIT-tag number; fish with the code of “include” were subject to further screening for identifying fish for the designed animal safety study.

Results: In this prequalification study, the triploid ABT salmon group had the lowest total frequency of morphological changes (10.2%), while triploid non-GE Atlantic salmon had the highest total percentage of malformations (33.1%), as reflected in Rank Scores 2, 3, and 4 (Table 2).

The study fish were observed at the PEI facility at four separate time points following pre-enrollment qualification of the fish from each study group described above and summarized in Table 1. Subsequent assessments were made for feeding activity, behavior, posture, and position in

the water column, coloration, external lesions, morbidity, mortality, and other abnormal clinical signs. Results for external appearance are summarized in Table 2. No health abnormalities were observed and the fish were regarded as in good health and of normal behavior.

Conclusion: ABT salmon showed no general health or behavioral abnormalities relative to comparator fish.

Table 2. Percentage of Scores by Rank for External Appearance in the Phase I Pre-Qualification Selection for Salmon in the Animal Safety Study (2007 Year-Class)

Ploidy & Group			**ABT Salmon**					**Non-GE Salmon**		
Rank	**N**	**1**	**2**	**3**	**4**	**N**	**1**	**2**	**3**	**4**
2n - Included	97	82.5	16.5	0	1.0	94	88.3	11.7	0	0
2n - Excluded	97	80.4	15.5	4.1	0	93	78.5	21.5	0	0
2n - Total	194	81.4	16.0	2.1	0.5	187	83.4	16.6	0	0
3n	**N**	**1**	**2**	**3**	**4**	**N**	**1**	**2**	**3**	**4**
3n - Included	85	90.6	8.2	0	1.2	49	59.2	28.6	8.2	4.1
3n - Excluded	82	89.0	6.1	4.9	0	48	75.0	8.3	10.4	6.3
3n - Total	167	89.8	7.2	2.4	0.6	97	67.0	18.6	9.3	5.2

Ranking scale for morphologic changes: 1 = none; 2 = slight; 3 = moderate; 4 = severe
2n = diploid; 3n = triploid; inclusion criteria were as described above.

b. Size, Weight, and Related Parameters

Understanding the design and interpreting the results of the animal safety study required accommodating both the effects of the intended rapid early life stage growth of the GE fish, and general salmon seasonal spawning behavior. AAS are intended to grow more rapidly in early life than their comparators. This different growth rate results in harvesting fish at either the same age but different sizes, or at about the same size (e.g., “market size”), but at different ages. Because salmon spawning is seasonal and once-yearly, in the latter case, this implies harvesting at different times of the year.

The design used for the animal safety study incorporated harvesting fish for the main comparison groups when the individual fish reached 1,000 – 1,500 g; these fish experienced different growth conditions up to, and at the time of, harvest. For example, fish harvested in February likely experienced shorter days and exposure to colder water with potentially lower microbe burdens than fish harvested in late summer. To address the differences in growth conditions, the study design included a smaller group of “satellite control” fish (non-GE Atlantic salmon of respective ploidy), referred to as SAT or satellite controls, which were harvested at the same time as the ABT salmon, and were therefore age-matched comparators.

Fish in the animal safety study were fed to satiety using appropriate size-adjusted feeding rates using a commercial salmon diet, an accepted practice for freshwater aquaculture.

Fish enrolled in the animal safety study were evaluated for size by measuring overall body weight, fork length, condition factor (body weight/[fork length]^3, and gonadosomatic index (gonad weight/body weight x 100). Statistical analysis of the data showed no differences among study groups with one exception. The body weight of diploid ABT salmon and triploid ABT salmon was much greater than that of corresponding satellite controls. This was expected given that the *opAFP-GHc2 construct* was intended to result in accelerated early life-stage growth. By design, body weight of ABT salmon was similar to the weight-matched, non-GE comparators.

A more detailed presentation of growth comparisons between AAS and their non-GE comparators is provided under Claim Validation (see Section XI).

Conclusion: There were no observed adverse effects on size, body weight, or related growth indices in AAS relative to comparator fish. Growth rates, as expected, were greater in ABT salmon, including AAS, containing the *opAFP-GHc2 construct*.

c. *Observations of Phenotype: Behavioral and Physical Abnormalities*

i. Observations from the animal safety study

Specific physical evaluation of adult (1,000-1,500 g) fish enrolled in the animal safety study occurred on three distinct levels. First, fish were observed during the study for avoidance and feeding behavior, posture-position in the water column, and any other observed behavioral or physical abnormalities. Throughout the pre-enrollment phase, no abnormal behavior was observed for either ABT salmon or comparator groups.

Second, a gross external examination was conducted on each fish enrolled in the animal safety study. This examination included nine specific observations, and included photographs of each fish. Individual fish were then given a rank score (1 to 4 as described in Table 3). The results are presented in Table 2.

Table 3. Results of Gross External Examinations: Number & Rank of Abnormal Findings

Feature	*ABT Salmon*		*Comparators*		*Satellite Controls*	
	Diploid	***Triploid***	***Diploid***	***Triploid***	***Diploid***	***Triploid***
Number of Fish	12	12	12	12	6	6
Jaw	1	1	0	1	0	0
Operculum	2	0	0	0	0	0
Gills	0	10	4	12	0	4
Fin Structure	0	3	1	3	0	1
Vertebral Column	1	0	1	1	0	1
Eyes-Cornea	0	0	1	0	1	0
Skin	0	0	0	0	0	0
Color-Markings	0	0	0	0	0	0
Other: Cranium	0	0	1	0	0	0
Total Findings	4	14	8	17	1	6
Fish Without Findings	9	2	6	0	5	2
Counts for Overall Rank Scores *- Rank 1*	9	2	6	0	5	2
Counts for Overall Rank Scores *- Rank 2*	3	10	6	11	1	4

Feature	*ABT Salmon*		*Comparators*		*Satellite Controls*	
	Diploid	***Triploid***	***Diploid***	***Triploid***	***Diploid***	***Triploid***
Counts for Overall Rank Scores *- Rank 3*	0	0	0	1	0	0
Counts for Overall Rank Scores *- Rank 4*	0	0	0	0	0	0

Rank 1 = no change compared with a perfectly-formed Atlantic salmon
Rank 2 = slight change
Rank 3 = moderate change typical of farm-raised salmon
Rank 4 = severe change that could affect commercial viability or fitness

From Table 3, the frequency of external abnormalities was similar in ABT salmon vs. comparator salmon; frequency of abnormalities was associated more with triploidy regardless of the genotype of the fish (GE or non-GE).

Nine internal organs were examined post-mortem in the animal safety study. Following sorting into "normal" or "abnormal" groups, samples were taken from the abnormal group for histopathology and other microscopic testing. The weight and size index (the ratio of organ weight to body weight) was determined for the gastrointestinal tract, heart, liver, and gall bladder. No differences were found between ABT salmon and their appropriate age- or size-matched comparators.

As previously described, in addition to evaluating data from the target animal safety study and as part of its weight-of-evidence evaluation, FDA evaluated extensive data and information gathered from the sponsor's facilities with respect to phenotype and animal health (see ensuing discussions), including sponsor-provided summaries of historical data addressing the health of ABT salmon in several consecutive year-classes (i.e., 2003-2007; 2001-2006; 2010, 2011, 2012, from the PEI facility, and 2008, 2010, 2011, and 2012 from the Panama facility). FDA's evaluation of these data and information are described below.

ii. Observations from the 2003-2007 Year-classes

Data on abnormalities/irregularities from 2003-2007 year-class fish from the PEI facility were summarized by ploidy (diploid or triploid) and lineage (ABT salmon or non-GE comparators). The number of fish included in this evaluation was as follows: 8,349 diploid (2n) ABT salmon; 2,003 triploid (3n) ABT salmon; 2,866 diploid (2n) non-GE comparator fish; and 748 triploid (3n) non-GE comparator fish. Data on abnormalities/irregularities were summarized by rank scores (i.e., (1): none observed; (2): slight-moderate; and (3): severe). For purposes of combining all of these year-classes, frequencies of slight and moderate levels were collapsed into a single rank score (Rank Score 2) (see footnote to Table 4), and are summarized in Table 4 below. Table 4 provides results on abnormalities/irregularities (as reflected in rank scores) on a large number of fish over several year-classes and potentially varied rearing conditions (as impacted by year or season).

The data indicate that, in general, irregularities decrease over time, in both rate and severity for the diploid and triploid ABT salmon. With the exception of the 2006 and 2007 year-classes, this trend is also observed in the comparator non-GE Atlantic salmon. Most of the irregularities observed were classified as "2", slight to moderate. With an exception in triploid GE fish in year-

class 2005, fish with rank scores of 3 (severe) were infrequent (see discussion of 2005 year-class below).

Table 4. Percentage of Irregularities By Rank in Diploid (2n) and Triploid (3n) Fish for the 2003-2007 Year-Classes of ABT Salmon and Non-GE Salmon

Ploidy & Year-Class	ABT Salmon				Non-GE Salmon			
	N	1	2	3	N	1	2	3
2n - 2003	1327	42.1	57.9	0.1	215	94.4	5.6	0
2n - 2004	2368	91.7	8.1	0.2	627	100	0	0
2n - 2005	1586	17.2	70.4	12.5	816	98.7	1.0	0.4
2n - 2006	1276	61.4	36.1	2.4	544	97.8	2.2	0
2n - 2007	1792	95.2	4.3	0.5	664	85.1	14.4	0.6
3n -2003	1165	39.1	59.3	1.6	233	80.7	19.3	0
3n - 2004	328	36.0	61.0	3.1	92	96.7	1.1	2.2
3n - 2005	38	7.9	42.1	50	82	89.0	9.8	1.2
3n - 2006	289	72.3	27.0	0.7	148	66.2	33.1	0.7
3n - 2007	183	92.4	7.1	0.6	193	28.5	71.5	0

Rank scores: 1 = no irregularity; 2 = slight-moderate irregularity; 3 = severe irregularity
Note: Scoring for the 2007 year-class used a 4 point scale rather than the 3 point scale used earlier. In order to make the results comparable all year-classes, totals for fish with slight and moderate irregularities (Ranks 2 and 3 on the 4 point scale) were combined for the 2007 year-class.

Triploidy has been associated with an increased level of abnormalities in Atlantic salmon, commonly manifested as lower jaw malformations (Benfey, 2001; O'Flynn et al., 1997). Although there were more (>30%) slight-to-moderate irregularities in triploid ABT salmon than in their non-GE comparators in three of the five year-classes (also seen in animal safety study), similar results were observed in the diploid ABT salmon and their non-GE comparators. Therefore, induction of triploidy may not fully explain the occurrence of increased irregularities.

Examination of the rate of irregularities over time indicates that there was a large reduction in the percentage of slight-moderate abnormalities in the triploid ABT salmon. In 2004, modifications were made to the PEI facility intended to improve conditions and animal health, which likely contributed to the improved morphology of all fish. For example, slight-to-moderate irregularities in the 2007 year-class were only 7.1%, substantially lower than the range of 42.1 to 61.0% found in the 2003 to 2005 year-classes.

Interestingly, the decrease in irregularities was not as notable in non-GE comparators. For example, the incidence of irregularities in triploid non-GE salmon was highest in the 2006 and 2007 year-classes (33.1% and 71.5%, respectively). This high rate is likely not due to the induction of triploidy alone (in which case the rate of irregularities in ABT salmon should also have been elevated). Rather, it is likely a function of the underlying genetics of the broodstock families used in the breeding crosses. (For each breeding, a “wild-type” or non-GE parent is used to ensure hemizygous triploid fish). A family (genotype) effect has previously been observed on

survival and other performance measures of diploid and triploid Chinook salmon (Johnson et al., 2004).

The 2005 year-class presents an abnormally high rate of irregularities in triploid ABT salmon relative to their non-GE comparators or to ABT salmon in other year-classes. Although the reasons are not entirely clear, some of the differences may be attributable to sample size; the rates in 2005 were based on a very small sample size (there were only 38 triploid ABT salmon). In contrast, the sample size ranged from 183 to 1,165 triploid ABT salmon for other year-classes. The number of diploid ABT salmon in the 2005 year-class was more consistent with other year-classes and exhibited a lower rate of irregularities. Therefore, the smaller sample size may have contributed to the frequency of irregularities noted in triploid ABT salmon for the 2005 year-class. The concomitant increased rate of irregularities in the diploid cohort, albeit at a lower frequency, does not provide a full explanation for the increase.

Further examination of the entire data set indicated that with the exception of the 2005 year-class, non-marketable severe irregularities were not demonstrably higher in ABT salmon compared with non-GE comparators. The sponsor indicated that changes in incubation procedures might have been responsible for these effects. More recent summary data for the 2006 and 2007 year-classes (see also Table 3) supported this contention and did not indicate elevated levels of severe abnormalities in diploid or triploid ABT salmon compared to either diploid or triploid non-GE comparator salmon. Although not common, extreme rates of severe abnormalities in a given year-class, cross, or geographic location have been reported in the literature (Sadler et al., 2001). FDA therefore concluded that the 2005 year-class was an outlier with respect to severe abnormalities.

In response to FDA's request, the sponsor provided a short white paper addressing the occurrence and origin of morphological irregularities in salmonids and summarizing data presented in several other submissions on ABT salmon. As part of FDA's weight-of-evidence evaluation, the agency subsequently performed its own literature search to confirm the results of the white paper and as to form its own conclusions regarding the range of abnormalities found in commercial salmonid aquaculture, as well as the source of those abnormalities. The results of these reviews are discussed below.

Many factors and/or conditions have been associated with developmental abnormalities in salmon, including deficiencies in phosphorus and vitamin C, excess vitamin A, high or variable temperatures during early growth phases, exposures to certain drugs (e.g., oxytetracycline), contaminants in feeds (e.g., heavy metals, insecticides, PCBs), and some parasites (Vågsholm, 1998). Skeletal and jaw malformations are reportedly quite common (up to 80%, as discussed below) in salmon and trout reared on commercial farms, and may result in decreased productivity due to decreased survival, growth or consumer rejection. Supporting the ubiquity of observation of skeletal abnormalities in farm-raised Atlantic salmon, a recent study proposes a classification system that describes 20 different types of vertebral column malformations in Atlantic salmon that are repetitively observed under farming conditions (Witten et al., 2009).

The frequency of deformities in farm-raised fish seems to vary widely depending on a number of factors, including factors such as genetics, local husbandry conditions, or level of examination. 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 malformations in 80% of salmon at commercial sites in Chile (Roberts et al., 2001).

Importantly, published data on commercial farming operations are not widely available and fish farmers are not generally open to sharing this type of information. The background occurrence of malformations in fish used as controls in various studies is generally less than 5% (Ørnsrud and Waagbø, 2004). This appears to be a reasonable gross estimate of what might be expected in wild populations and cultured populations not subject to disease or environmental stressors (e.g., poor water quality, contaminants or nutritional deficiencies). Therefore, a background rate of malformations of approximately 5% would not be unexpected.

A study in Norway found a frequency rate of deformed Atlantic salmon (percent of individuals with one or more deformed vertebrae) that ranged from 6.6% to 17.1% (Fjelldal et al., 2009). In this study, neither genetic background, smolt quality, or off season smoltification was found to be an important factor in the etiology of vertebral malformations in farm-raised Atlantic salmon. In contrast, a 2009 study of Chinook salmon (Evans and Neff, 2009) found a very high variability in the overall frequency of spinal deformities between different families within the same fish population, with spinal deformities affecting up to 21% of the offspring within susceptible families; however, the overall frequency of malformations when looking across families in the two fish populations that were examined was less than 1%.

Certain abnormalities seem to be associated with the induction of triploidy. In a review article on the physiology and behavior of triploid fishes, Benfey (1999) stated that although in general, triploids have similar, if not identical morphological characteristics as diploids, several specific morphological differences and abnormalities have been associated with triploidy in fish. The most frequently described gross anatomical abnormality in triploid fish was lower jaw deformities in triploid Atlantic salmon, which might be linked to rapid growth rates in seawater. The two other abnormalities described in triploid Atlantic salmon, cataracts and changes in erythrocyte size, might be due to nutritional deficiencies. Sadler *et al*. (2001) described a gill filament deformity syndrome which was found at a much higher frequency in triploids (in up to 60% of triploid smolts) than in diploids.

There is some controversy in the literature as to whether it is the triploid condition itself, or the process by which it is induced (e.g., pressure or heat shock) which causes abnormalities in fish (Piferrer et al., 2009). Evidence for both causes has been presented and, in many cases, it has been impossible to separate the effects of the two. Even the specific process by which triploidy is produced may have an effect. For example, in one recent study with rainbow trout, Haffray *et al.,* (2007) found that triploidy induced by temperature shock produced morphological anomalies in fry at a higher rate than triploidy produced by pressure shock (11.7% vs. 2.8%), which in turn produced abnormalities at a rate not much different from that in diploids (1.9%). (Pressure shock is the method used to produce triploid ABT salmon.)

Regardless of the delineation of underlying causes of morphological irregularities in farm-raised Atlantic fish, the frequency of irregularities noted with ABT salmon were comparable to frequencies noted in published literature for farm-raised, non-GE salmonids.

Conclusion with respect to physical and behavioral abnormalities: Analyses of the behavior and gross external abnormalities of market size (1,000 – 1,500 g) ABT salmon show no demonstrable differences from the comparator fish populations.

d. Overall Mortality and Morbidity

During the target animal safety study, 25 of 645 candidate fish were removed from consideration due to non-viability, morbidity, or mortality. Ten ABT salmon (three diploid and seven triploid) and fifteen size-matched non-GE comparators (10 diploid and 5 triploid) comprised these "for-cause" removals from the study, for an overall removal rate of 3.88%. Numbers of "for cause" removals were similar between ABT and comparator non-GE salmon as well as between diploids and triploids.

Of the 25 fish removed "for cause," 22 were subjected to histopathological analysis[13]. The analysis of these 22 fish showed small inflammatory changes in both ABT and comparator non-GE salmon. These changes were regarded as normal and typical findings in Atlantic salmon in aquaculture. No other abnormalities were identified.

Mortality observations at all life stages have been recorded at the PEI facility since 1996 (2001-2006 year-classes presented below).

For the 2001-2006 year-classes, mortality (as percent survival to first feeding) was summarized by ploidy (diploid/2n or triploid/3n) and lineage (ABT salmon or non-GE comparators). The number of different crosses that contributed to each "average" percent survival was reported, but not a count of the individual fish that contributed to percent survival. A total of 220 crosses were observed: 96 diploid (2n) and 59 triploid (3n) GE crosses; 42 diploid (2n) and 23 triploid (3n) non-GE comparator crosses.

Data were summarized as an average percent survival (among crosses in a particular group), and as a range in percent survival values (among crosses in a particular group).

Although survival to first feeding varied significantly from year to year, and sometimes between different spawning crosses in the same year, in general, survival at this stage was similar between ABT and non-GE comparators (Table 5). Low survival, when it occurred, was attributed primarily to fungi and opportunistic bacteria, and as a result, offspring of both ABT and non-GE comparator salmon periodically required treatment with drugs such as formalin, chloramine-T and salt. The sponsor reported that survival in the early rearing area improved since the facility upgraded to combi-tanks[14] in 2004, however, the data first reflect improved survival in 2006 when rates ranged from 70-95% across GE and non-GE crosses.

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[13] Two fish (one diploid and one triploid non-GE comparator) were accidentally frozen at PEI and therefore were not suitable samples for histopathology analysis. One of the 23 sent for analysis was delayed; by the time the fish was investigated the cells had already deteriorated to the point that histopathological analysis was impossible.

[14] The sponsor reported that the design of the previous tanks used for rearing of early life stages may not have allowed for a thorough cleaning resulting in a chronic fungal problem.

Table 5. Average (%) Survival to First-Feeding for ABT Salmon (TX x SC) and Non-GE Diploid (SC x SC) and Triploid (SC ♂ x SC ♀ PS (3n)) Salmon from the 2001-2006 Year-Classes*

Spawning Year	ABT Salmon Crosses			Non-GE Crosses	
	TX ♂ x SC ♀	SC ♂ x TX ♀	TX ♂ x SC ♀ **PS** (3n)	SC ♂ x SC ♀	SC ♂ x SC ♀ **PS** (3n)
2001	75 (37-95) n=14	nd	70 (37-94) n=13	67 (22-95) n=9	58 (20-92) n=4
2002	71 (60-81) n=8	nd	21 (10-38) n=6	72 (45-88) n=9	45 (10-87) n=5
2003	42 (2-82) n=29	nd	44 (2-86) n=25	46 (8-84) n=8	41 (18-88) n=9
2004	54 (31-73) n=8	24 (4-50) n=3	50 (33-58) n=4	59 (13-89) n=7	57 (52-62) n=2
2005	48 (12-90) n=10	49 (26-64) n=8	37 (4-85) n=3	17 (6-31) n=5	nd
2006	70 (10-98) n=12	86 (53-97) n=4	95 (91-98) n=8	95 (94-96) n=4	94 (92-95) n=3

* Abbreviations: ***TX***, Treated (ABT salmon); ***SC***, Sponsor Control (comparator non-GE); ***PS*** *(3n)*, pressure shocked (i.e., triploid); ***nd***, no crosses set up. Columns two through five indicate average percentage survival & range of percentage survival *(Min-Max)* for number of crosses *(n)*

Other researchers found elevated mortality in non-GE triploid salmon prior to the start of first feeding (Benfey, 2001; O'Flynn et al., 1997; Atlantic salmon) and in early development (Johnson et al., 2004; Chinook salmon) relative to diploid comparators. This type of elevated mortality was not observed in triploid ABT salmon, possibly due to genetic selection in the diploid broodstock, and is consistent with the findings of Johnson *et al.* (2004), whose results suggest that improvement of triploid performance (e.g., survival, growth) may be possible by selecting specific combinations of high-performance diploid broodstock for the production of triploids over several generations.

The sponsor also provided a large amount of data on the entire 2004 breeding season's fish. That year, approximately 19,000 ABT and 6,000 non-GE fry were grown. Pre-smolt size fish exhibited similar numbers of developmental irregularities (0.3% and 0.5% for ABT and comparator salmon, respectively). Mortalities were higher among non-GE comparators in these early stages (8.7% for ABT salmon versus 18.5% for comparator salmon), and mortalities were similar among animals once they reached larger grow-out weights (3.5% for ABT salmon versus 2.0% for comparator salmon).

The sponsor made observations on growth, mortality, and morbidity on year-classes of fish reared at the PEI and Panama facilities which were unavailable at the time of the original review of phenotypic characterization. These data were submitted to the agency to provide updates on these observations after the initial submissions, and support the original conclusions regarding animal health, and presented below. They also helped clarify uncertainty related to items such as mortality, morbidity, culling, and planned population reductions and their effects on study results. Results are provided in the sections below.

PEI Facility

For year-classes 2010, 2011, and 2012 at the PEI facility, data summaries are presented on survival of AAS to the eyed-egg stage (Table 6). Table 6 contains a summary of mortality and abnormalities (rank score) for the 2010 and 2013 year-classes of AAS. For the 2010 year-class, abnormalities/rank scores were assessed on approximately 900 randomly-selected AAS fry averaging 22.8 g. For the 2012 year-class, abnormalities/rank scores were assessed on approximately 1,100 randomly-selected AAS fry averaging 17.9 g.

Survival to the eyed-egg stage ranged from ~40-60% across the three year-classes (Table 6), and is comparable to similar data reported for earlier year-classes.

Table 6. Percentage survival to eyed egg stage for the 2010, 2011, and 2012 year-classes for AAS at the PEI facility

Year-Class	# of Eggs Fertilized	# Eggs Remaining	% Survival to Eye
2010	497,313	203,266	40.9
2011	161,112	95,901	59.5
2012	34,919	18,763	53.7

Table 7. Mortality and percentage rank scores for 2010 and 2012 year-classes for AAS at the PEI facility

Year-Class	Start Number	# of Mortalities	# of Irregular Mortalities	# of Fish Assessed	% Rank 1	% Rank 2	% Rank 3
2010	905	63	17	842	91.3	8.7	0
2012	1,086	25	10	1,061	91.0	9.0	0

Panama Facility

For the reporting of mortality rates at the Panama facility for the year-classes discussed below, the sponsor summarized and presented mortalities on both a per month and overall period basis. These data are summarized below for the overall time periods as specified in the applicable tables.

Mortality of AAS and non-GE comparator Atlantic salmon reared at the Panama facility from the 2008 year-class (2009-2010 production cycle) are presented in Table 8. For this year-class, AAS and non-GE comparator Atlantic salmon were reared in the same tanks prior to June 2009, and thus the mortality was presented for a mixed population. After July of 2009, AAS and comparators were reared in separate tanks. To distinguish between the genotypes, fish were first separated into lower and upper size modes for presumptive separation. To confirm, blood was collected for PCR analysis for presence/absence of the *opAFP-GHc2 construct* in the respective groups of fish. Note that mortality at the time of initial acclimation was ~ 16%, after which it (July 2009 through December, 2010) was less than 10% for AAS and comparators.

Ranks scores for irregularities are summarized in Table 9. 98-99% of AAS and comparators had a rank score of 1 (no irregularity), with the remainder with rank scores of 2-4 reflecting mild, moderate, and severe irregularity. The distribution of rank scores was similar between genotypes.

Table 8. Mortality of GE and non-GE salmon reared at the Panama facility from the 2008 year-class

Description	Total Fish (n)	Mortality (n)	Mortality (%)
Apr/May/Jun, 2009			
Mixed GE/non-GE[1]	87,727	13,755	*15.7%*[2]
Jul 2009 – Dec 2010[3]			
GE	44,038	4,279	9.7%
Non-GE	8,566	680	7.9%

[1] Shipped as mixed population, GE status determined after receipt, acclimation, and early growth.
[2] Reflects acclimation loss, issues with water temperature, dissolved oxygen, and tank design
[3] Separated based on GE status; used upper/lower mode body weights for initial sorting, then confirmed at study termination on a sampling of fish with PCR test on blood.

Table 9. Summary of percent irregularities (as indicated by Deformity Rank (%)) for the 2008 Year-Class of AAS

Group	# of Fish Assessed	1	2	3	4
Sponsor Control	600	99.2	0	0	0.5
AAS	1400	98.2	1	0	0.4

Table 10 summarizes the mortality, culling and planned population reduction (to optimize stocking density) for AAS reared at the Panama facility (2010 year-class, 2011-2013 production cycle). Eyed-eggs experienced a 38% mortality in transit due to shipping delays. For the period of June, 2011 to September, 2013, mortality and culling (for cause) rates were a very low, 12 and 9.4%, respectively. Planned population reduction (for purposes of optimizing stocking density) was 74.2% over this time period.

Table 10. Mortality, Culling, and Planned Population Reduction in the 2010 Year-Class of AAS in Panama

Description	Total (n)	Mort (n)	Mort (%)	Cull (n)	Cull (%)	Reduce (n)	Reduce %)
Arrival[1]	35,096	13,335	38.0				
Jun 2011 – Sep 2013	21,761	2,607	12.0%	2,051	9.4%	16,144	74.2%

[1] Problems with shipping logistics caused a large number of mortalities in transit.

For the 2010 year-class (2011-2013 production cycle, see Table 11), nearly 97% of AAS had no irregularity (rank score = 1), while 2.2% had mild-moderate irregularity (rank score = 2), and 1.1% had severe irregularity (rank score = 3).

Table 11. Summary of Percent Irregularities (as indicated by Rank Score (%)) for the 2010 AAS Year-Class in Panama

	# of Fish Assessed	1	2	3
AAS	1,500	96.7	2.2	1.1

For the 2011 year-class (2012 production cycle, see Table 12), mortality rate during the first part of the 69 day study period was 37.7%. This was the sponsor's first test of incubating eyed-eggs in a closed, recirculating incubation system. Water chemistry imbalances may have led to the high rate of mortality. During the second part of the study period, mortality was 3.4%, and culling was 1.3%.

Table 12. Mortality and Culling of 2011 AAS Year Class in Panama

Description	Total (n)	Mortality (n)	Mortality (%)	Culled (n)	Culled (%)
Total	38,643	15,884	41.1%	500	1.3%
Mar/Apr – Eggs/Sac Fry		14,565	37.7%	500	1.3%
May/June - Fry		1,319	3.4%	-	-

Mortality, culling, and planned population reduction are summarized in Table 13 for the 2012 year-class (2013-2015 production cycle) for AAS reared at the Panama facility. Based on low overall mortality rates for this year-class, it appears that the sponsor resolved issues for their closed, recirculating incubation systems noted for the previous year-class. Mortality and culling rates were 12.3 and 2.9% for the March to September, 2013 period. Planned population reduction to optimize stocking density for this time period was 29%. It was also noted that of the mortalities removed from the tanks containing fish from this year-class, 8.2% displayed irregularities. Of the > 21,000 observations on fish taken at twice monthly observation time points, irregularities were noted at 0.2% of the observations. At the end of the production cycle, 2% of 280 randomly-selected AAS for observation displayed irregularities.

Table 13. Mortality, Culling, and Planned Population Reduction in the 2012 AAS Year-Class in Panama

Description	Total (n)	Mort (n)	Mort (%)	Cull (n)	Cull (%)	Reduce (n)	Reduce (%)
Mar – Sept	16,176	1,982	12.3%	473	2.9%	4,700	29.0%

Results from the year-classes of AAS reared to date at the Panama facility once husbandry conditions were established (e.g., water quality, temperature, and oxygen concentration were appropriately regulated) indicate that rates of mortality, culling, and irregularity (as depicted with rank scores) were generally low, and were within the same range as, or below, those observed in earlier year-classes of ABT salmon reared at the PEI facility. These data also help address earlier questions of uncertainty with respect to mortality, rates of culling, and irregularities. In addition, the sponsor provided information on recent rates of morphological abnormalities, and morbidity and mortality observed in AAS at the Panama facility that will serve as part of the baseline for post-approval requirements (see Approval Letter, Appendix A). The information in Tables 13, 14, 15, and 16 provides the basis for the information in Section 5 of the new animal drug labeling required for AAS.

Table 14 provides rates of morphological irregularities in pre-smolt AAS from the 2010 and 2012 year-classes; numbers are based on a sampling and assessment of three batches (~20-60 g) of fish.

Table 14: Rates of Morphological Irregularities in Pre-Smolt AAS (2010 and 2012 year-classes)

Moderate Irregularities				Severe Irregularities[1]			
% Spinal	% Jaw	% Operculum	% Other	% Spinal	% Jaw	% Operculum	% Other
0 – 4.3	0.1 – 1.4	0 – 5.6	0 – 2.5	0 – 0.4	0	0	0

[1] Severe irregularities are those which could have a significant impact on viability or render the fish unfit for commercial sale.

Based on data from the 2008-2012 year-classes of AAS, Tables 15 and 16 present reported typical ranges for mortality and morbidity, respectively, on a monthly basis for different life-stages.

Table 15: Mortality Observed in AAS by Life-Stage on a Monthly Basis[1]

Life Stage	Approximate Time Period Post First Feeding in Months	Range of % Mortality on a Monthly Basis
Eggs and yolk-sac fry	0	2.2 to 5.1
Fry from first feeding to 5 g size	~1 to 2	1.0 to 9.8
Pre-smolt	~3 to 5	0.1 to 4.0
Juveniles	~6 to 11	0.1 to 1.8
Late juveniles to adults	~12 to 24	0.2 to 5.2

[1] Non-representative high values due to one-time events or atypical conditions have been excluded.

Table 16: Morbidity Observed in AAS by Life-Stage on a Monthly Basis[1]

Life Stage	Approximate Time Period Post First Feeding in Months	Range of % Morbidity on a Monthly Basis
Eggs and yolk-sac fry	0	0
Fry from first feeding to 5 g size	~1 to 2	0 to 0.4
Pre-smolt	~3 to 5	0 to 3.2
Juveniles	~6 to 11	0 to 2.2
Late juveniles to adults	~12 to 24	0 to 3.4

[1] Observations made on fish that were eventually discarded, which included moribund fish with actual or presumptive disease conditions, those with gross morphological irregularities, and fish of inappropriate size.

Conclusion: There were no consistent differences in mortality and morbidity between ABT salmon, including AAS, and non-GE comparator Atlantic salmon in the animal safety study, the large-scale historical retrospective data evaluation, or results obtained on subsequent year-classes of fish reared at the PEI and Panama facilities. Uncertainties regarding differences in animal health between the Canada and Panama facilities have been significantly reduced, with approximately equal survival and animal health in both locations once husbandry conditions were established. Further, uncertainties regarding the influence of early culling have been resolved. Because of concerns that the culling procedures for the initial study may not have reflected typical aquaculture procedures and may have obscured adverse outcomes, the agency requested and received from the sponsor additional information regarding culling practices, the health of the ABT fish populations at the grow-out facilities, and the potential role that culling could have had in masking adverse outcomes. These data and information (Tables 8-13) include information on morbidity and mortality from more than 150,000 ABT salmon and approximately 9,000 non-GE Atlantic salmon from both the PEI and Panama facilities. These new data did not reveal any new abnormalities or altered rates of abnormalities beyond those identified in the initial study, and did not indicate any bias in the initial study's estimation (i.e., did not mask) of rates of morphologic abnormalities, mortality, or morbidity.Rates of morphological abnormalities, mortality and morbidity have been submitted and establish the expected rate of such events for use in determining adverse events that must be reported as part of the post-approval reporting requirements (see Approval Letter, Appendix A).

e. Clinical Pathology Assessments

As part of its determination of the safety of the *opAFP-GHc2* construct on the phenotype and, therefore, the safety of the ABT salmon, including AAS, FDA was concerned about the extent to which introduction of the *opAFP-GHc2* construct would adversely affect the hematology and serum chemistry values of the resulting ABT salmon. Analysis of the clinical pathology values from the animal safety study indicated that all the of the differences between diploid ABT

salmon or triploid ABT salmon, including AAS, and non-GE comparators can be explained by triploidy, seasonality, growth conditions at the time of harvest, or a combination of these factors. In other words, no clinically relevant differences in the serum chemistry or hematology values for AAS as compared with contemporaneous non-GE Atlantic salmon are clearly attributable to the *opAFP-GHc2* construct.

An additional overarching consideration in interpreting the clinical pathology data collected in this study was the well-known effect of triploidy, including increasing cell size with resulting effects on other parameters (Benfey, 1999; Cal et al., 2005; Dorafshan et al., 2008). For example, erythrocyte counts are generally lower for triploid fish than for diploid fish, with corresponding decreases in packed cell volume (PCV), hematocrit, and hemoglobin widely reported in triploid fish relative to diploid comparators.

The available clinical pathology data for Atlantic salmon, while quite extensive relative to available data for other fish species, is relatively limited compared to similar data for terrestrial species. Serum chemistry and hematology are not assays routinely conducted by aquaculture facilities so the historical data from the literature for these clinical pathology assessments are primarily useful for understanding the breadth of the values considered "normal" under a variety of growth and aquaculture conditions. There are also notable gaps in published data and the range of clinical pathology values that have been reported.

Among the cited references in the animal safety study report, one is particularly relevant to this evaluation. Cogswell *et al.* (2001) have previously published the hematology values of diploid and triploid growth hormone (GH) GE Atlantic salmon. They report that triploid erythrocytes are significantly longer and proportionately thinner than diploid erythrocytes for both GE and non-GE genotypes. The authors speculated that GE fish may produce erythrocytes with higher surface area to volume ratio in response to their elevated metabolic rates. No other major hematological differences were observed between GE and non-GE salmon of the same ploidy.

In the sponsor's designed animal safety study, samples obtained from each enrolled animal were analyzed for clinical chemistry and complete blood count parameters. These data included hemoglobin, hematocrit, platelet count, neutrophils, lymphocytes, monocytes, glucose, sodium, potassium, chloride, alanine aminotransferase, aspartate aminotransferase, total bilirubin, creatine kinase, total protein, albumin, globulin, albumin/globulin ratio, calcium, inorganic phosphorus, cholesterol, and osmolality. The results from these studies are found in Appendix 1.

Overall, the range of values for the various parameters was comparable for the ABT salmon, including AAS, and non-GE comparators [15] in the study. In several cases, however, as illustrated by the figures in Appendix 1, there appeared to be differences in the values for specific subgroups of the fish. Often these differences were identified by statistical analyses when focusing on the comparison between the GE triploids and the non-GE diploids. Several analytes were identified as exhibiting a statistically significant difference ($p < 0.05$) when evaluating the

[15] The term "comparator" refers to non-GE Atlantic salmon of a similar, but not identical, genetic background as AAS, including both diploid and triploid salmon. These are appropriate controls for these studies given the inherent constraints of a selective breeding program.

effect of the *opAFP-GHc2* construct; other statistically significant differences were also identified, but were not related to the GE status of the fish. For example, the hematocrit and platelet count were at higher levels in at least some non-GE diploid fish compared with the non-GE triploid fish.This "triploidy effect" is expected and has been well documented in the literature as discussed above.

Analysis of the hematology values in Appendix A showed a difference in the relative level of lymphocytes and neutrophils for the market-sized diploid ABT salmon as compared to all of the other market-sized salmon in this study. The levels of the lymphocytes in the market-sized diploid ABT salmon were within the range of levels reported in the literature and are comparable to the levels for satellite controls sampled at the same time. FDA concluded that these differences reflect differences in growth conditions at the time of sampling and are not attributable to the GE status of the salmon.

Similarly, initial comparisons of the protein values (albumin, globulin, total protein and albumin:globulin ratio) for the market size fish identified a statistically significant difference between ABT salmon and non-GE salmon. These differences were small, and unlikely to be biologically relevant. Furthermore, the values determined for the market size ABT salmon, including AAS, were comparable to published values, as well as the values determined for the age-matched satellite control non-GE salmon. Similar apparently statistically significant differences were initially identified for calcium, cholesterol, phosphorous, and total bilirubin, but were not reflective of a difference attributable to the test article (i.e., the *opAFP-GHc2* construct) when the age-matched comparators and historical data were considered.

Aspartate aminotransferase (AST) was identified as being statistically significantly different when non-GE salmon were compared with the ABT salmon; its levels are, however, lower in the ABT salmon than in the non-GE comparators. Elevation of AST is often used clinically as an indicator of tissue damage, so this difference is not likely to be indicative of a health problem in the ABT salmon. Further examination of the AST values in the comparators indicated that the statistical difference was likely influenced by very high AST level in one of the market-sized non-GE fish (of the SC-3n group), and thus the statistical difference is not attributable to the GE construct.

Finally, there was a statistically significant difference between the glucose level of the market size ABT salmon and non-GE market size comparators. Examination of the graphically presented data showed that the overall values were not grossly different between the groups, but that the glucose values for the ABT salmon tend to be a bit lower than for the non-GE comparators. The glucose values reported here were lower than the values reported in the literature. This could reflect a difference in handling of the fish (fasting, sedation, tank conditions), or the samples (glucose levels are typically reduced in samples that are not processed immediately as the blood cells in the samples will metabolize the glucose). Some of the values were so low for all of the market size fish groups (<40 mg/dl) that an artifactual source such as a longer holding time seems likely. Physiological values this low would likely result in observable behavior deficiencies manifested in swimming or feeding behaviors, which were not noted, or compromised growth, which also was not the case.

An alternative explanation based on reports in the published literature (see discussion below in Part B 1 k, Other Phenotypic Characteristics) rests on the observation that GH transgenic Atlantic salmon have a higher metabolic rate than non-GE comparators. A higher metabolic rate would be consistent with lower serum glucose levels or increased utilization of glucose in samples held before processing due to residual metabolic processes in the blood cells contained in the samples. FDA concludes the statistical difference in reported glucose values is unlikely to represent a clinically relevant finding with respect to the health of the ABT salmon.

Of primary interest with respect to hematology and serum chemistry data was the potential influence of the GE status of the study population. With respect to the clinical pathology values obtained in the animal safety study, there were differences between ABT salmon and non-GE salmon that can be explained by ploidy, seasonality, growth conditions at the time of harvest, or a combination of these factors. In other words, no clinically relevant differences in the serum chemistry or hematology values for ABT salmon in comparison to non-GE Atlantic salmon comparators are clearly attributable to the *opAFP-GHc2 construct* in ABT salmon.

An additional overarching consideration in interpreting the clinical pathology data collected in this study is the well-known effect of triploidy, including increasing cell size with resulting effects on other parameters (Benfey, 1999; Cal et al., 2005; Dorafshan et al., 2008). For example, erythrocyte counts are generally lower for triploid fish than for diploid fish, with corresponding decreases in packed cell volume (PCV), hematocrit, and hemoglobin widely reported in triploid fish relative to diploid comparators.

Cogswell *et al.* (2001) have previously published the hematology values of diploid and triploid GH transgenic Atlantic salmon that the sponsor produced. These authors reported that erythrocytes were significantly longer and proportionately thinner in triploid vs. diploid Atlantic salmon in both GE and non-GE genotypes. The authors speculated that GE fish may produce erythrocytes with higher surface area to volume ratio in response to their elevated metabolic rates. No other major hematological differences were observed between GE and non-GE salmon of the same ploidy.

Conclusion: FDA determined that there were no clinically relevant differences in the serum chemistry or hematology values for ABT salmon compared with contemporaneous comparator non-GE salmon that are clearly attributable to the GE construct.

f. Macroscopic and Microscopic Evaluation (other than gross morphology)

Observations for fish in the 2007 year-class were collected as part of the animal safety study, Table 17 summarizes significant lesions.

Macroscopic (gross) observations

Table 17. Prevalence of Selected Gross Observations

Ploidy & Group	Diploid SP-CON		Diploid SAT-CON		Diploid ABT Salmon		Triploid SP-CON		Triploid SAT-CON		Triploid ABT Salmon	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Number Examined	6	6	3	3	6	6	6	6	3	3	6	6

Ploidy & Group	Diploid SP-CON		Diploid SAT-CON		Diploid ABT Salmon		Triploid SP-CON		Triploid SAT-CON		Triploid ABT Salmon	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Gill Arch Structural Abnormalities	2	2	-	-	-	-	5	6	2	-	5	5
Gill Arch Thickening/ Opacity	2	2	-	-	-	-	3	5	1	1	1	2
Fin Abnormalities	1	-	-	-	-	-	-	3	-	1	2	1
Heart Abnormalities	-	-	1	-	-	-	-	1	1	1	-	1
Jaw Erosions	-	-	-	-	3	1	-	-	-	-	-	-

SP-CON = Sponsor control, SAT-CON = Satellite control

Gill and Fin Abnormalities

There were a wide variety of gross observations that involved various organ systems; none of these observations were substantitally more prevalent among ABT salmon as compared to either size-matched (SP-CON) or age-matched (SAT-CON) non-GE controls of the same ploidy, except for gill arch structural abnormalities, which were elevated in the triploid ABT salmon compared to the triploid age-matched controls (Table 17). Gill arch abnormalities were more prevalent in triploid salmon than in diploid salmon of the same comparator group. Gill filaments (primary lamellae) were truncated (shortened, incompletely developed) or absent; deformities of the gill arch itself, with or without scarring; and gill thickening or opacity were noted. Structural gill arch abnormalities (truncated or absent filaments and gill arch deformities) were most often correlated microscopically with truncated or absent filaments, whereas gill arch thickening was correlated with increased segmental hyperplasia of the lamellar (secondary lamellae) epithelium. There was no observed effect on fin structure attributable to the GE status of the salmon in this study. Triploid salmon exhibited a higher prevalence of fin abnormalities than diploid salmon, regardless of the presence of the *opAFP-GHc2* construct. Erosions, shortening, twisting (torsion), nodules and bifurcation were observed. These lesions were distributed among a variety of fin types (pectoral, dorsal, pelvic, caudal) among different salmon. Except for a fin nodule that was correlated with an epidermal cyst (Male, Diploid, SP-CON), and a shortened, twisted dorsal fin that was correlated with a skeletal deformity (Female, Triploid, SP-CON), the study pathologist was unable to associate specific microscopic changes with macroscopic fin lesions (in four instances, fin tissues with gross findings were not available for microscopic examination).

Heart abnormality

There was no observed effect on heart structure attributable to the GE status of the salmon in this study. Loss of pyramidal profile (cardiac shape abnormalities) of the heart was noted in some fish. The prevalence was higher among triploids. No microscopic correlates were observed.

Jaw erosions

Jaw erosions were noted exclusively in male (three of six) and female (one of six) diploid ABT salmon. No jaw erosions were noted among triploid fish irrespective of GE status.

Microscopic observations

Focal inflammation

Foci of inflammation, which were generally minimal to mild, were observed in a variety of tissue types. Inflammation was most frequently characterized as granulomatous, consisting of chiefly macrophages in spherical nodular aggregates, with or without multinucleated giant cells or central areas of necrosis. Other types of inflammatory lesions (acute, chronic active, necrogranulomatous, pyogranulomatous) were less regularly observed. The most commonly affected sites for inflammation were the abdominal mesentery, cranium, and trunk kidney (Table 18). Etiologic agents were not evident in any of the lesions. The prevalence of focal inflammation was higher among diploid salmon than triploid and higher among diploid (and to a lesser extent triploid) ABT salmon compared with either size-matched (SP-CON) or age-matched (SAT-CON) controls.

Table 18. Prevalence of Inflammation in Various Tissue Types[a]

Group	Diploid SP-CON		Diploid SAT-CON		Diploid ABT Salmon		Triploid SP-CON		Triploid SAT-CON		Triploid ABT Salmon	
Sex	M	F	M	F	M	F	M	F	M	F	M	F
Number Examined	6	6	3	3	6	6	6	6	3	3	6	6
Body wall	1	1	-	-	-	-	-	-	-	-	-	-
Cranium	-	3	1	-	4	1	-	-	-	-	-	1
Distal Intestine	-	-	-	1	-	-	-	-	-	-	-	-
Eye	1	-	-	-	1	1	-	-	-	-	-	-
Gall Bladder	-	1	-	-	-	-	-	1	-	-	-	-
Head Kidney	-	-	1	-	-	-	1	-	-	-	1	-
Heart	-	-	-	-	-	2	-	1	-	-	1	-
Liver	1	2	-	-	1	1	1	1	1	-	-	1
Spleen	-	-	1	1	1	-	1	2	2	-	1	-
Stomach	1	-	-	-	-	-	-	-	-	-	-	-
Swim Bladder	-	-	-	1	1	1	1	1	-	-	-	-
Testes	-	na	-	na	-	na	-	na	1	na	-	na
Trunk Kidney	1	1	2	-	2	4	1	1	1	1	3	1
Number Examined	1	1	0	0	3	2	1	0	1	1	3	1
Abdominal Mesentery	1	1	-	-	3	2	1	-	1	1	3	-
Number Examined	1	1	1	0	1	1	1	0	0	0	2	0
Pancreas	1	1	1	-	1	1	1	-	-	-	2	-
Number Examined	0	0	0	0	1	0	0	0	0	0	0	0
Urinary Bladder	-	-	-	-	1	-	-	-	-	-	-	-

[a] Based on number of animals affected per group; na= not applicable
SP-CON = Sponsor control, SAT-CON = Satellite control

Gill lesions
Gill lesions were more prevalent among triploid salmon than diploid salmon of the same comparator group, specifically between triploid ABT salmon and the triploid age-matched controls (SAT-CON), but not for the triploid size-matched controls (SP-CON). The lesions included structural abnormalities of the gill filaments along with increased segmental lamellar epithelial hyperplasia. The structural abnormalities were truncated or absent gill filaments. In some instances the abnormal filaments also demonstrated inflammation. Gill filament absences occurred most frequently at the apex of the gill arch. Little, if any, inflammation of the gill arch was noted.

Information on the gill morphometry of GH transgenic Atlantic salmon was reported by Stevens and Sutterlin (1999). Although these salmon were GH transgenic Atlantic salmon produced by the sponsor, it was unclear from the information provided how closely related they are to the ABT or AAS. The authors found that many of the morphological features of the respiratory system of these transgenic salmon were larger than those of similarly-sized comparator salmon. For example, the gill surface area available for respiratory exchange in the transgenic salmon was found to be about 1.24 times that of comparator salmon, and due largely to a relatively uniform increase in the length of each gill filament. The authors reported that there were no obvious differences between the two groups of salmon in overall gill morphology when viewed with a dissecting microscope.

Ectopic mineralization
Soft tissue mineralization affecting multiple tissue types was more prevalent in triploid SAT-CON and triploid ABT salmon compared to SP-CON triploid and diploids (both SP-CON and ABT). Among affected fish, females generally had a higher prevalence of mineralization than males. The most commonly affected sites were the eye, heart, liver, and trunk kidney. Most instances were graded as minimal, although some lesions were mild to moderate. A few mineralized lesions of the urinary tract were noted grossly at necropsy.

Hepatocellular vacuolization
Hepatocellular vacuolization ranged from minimal to moderate and tended to be higher in triploid salmon as compared to diploid salmon. Vacuolization was characterized by single or multiple, variably-sized, discrete, round, sharply-defined spaces within the hepatocyte cytoplasm. Larger vacuoles displaced the nucleus toward the periphery of the cell.

Discussion of the results
The experimental design of the animal safety study adequately addressed the situation for age-matched vs. size-matched comparators; limitations on this study included the effects of seasonality and small sample size. The effect of seasonality may be an explanation for differences in the leukocyte profile, specifically lymphocytes and neutrophils, particularly among ABT salmon and SAT-CON diploid Atlantic salmon (as noted in the previous discussion).

Historical data on macroscopic/microscopic observations

Macroscopic and microscopic observations (other than gross morphology of the musculoskeletal system as described above) were examined for ABT salmon from the 2001-2005 year-classes.

Observations included the 2001-2005 year-classes, and were collected as part of routine health evaluations of the broodstock development program. As fish were found dead, moribund, or culled, they were subject to necropsy and diagnostic histopathology and bacteriology.

Among the necropsy and histopathologic findings, spontaneous skeletal deformities were noted, including dorsoventral and lateral deviations of the vertebral column. In addition, malformations of the head, primarily lower jaw, were observed. These macroscopic observations have been described above in this evaluation by year-class and include diploid and triploid ABT salmon and non-GE comparator Atlantic salmon. Information from 2006 and 2007 year-classes was summarized together with information from 2003-2005 year-classes with respect to the rate of irregular external appearance in Table 3 above.

Microscopic observations from earlier generations of diploid and triploid ABT salmon and non-GE salmon comparators document a variety of inflammatory and degenerative lesions that are mostly consistent with diseases of intensively-reared fish. The data and information provided were assembled from fish production records and reflect a variety of crosses and husbandry conditions over several years. The range and severity of histopathologic lesions, morphologic diagnoses, and etiologic diagnoses do not appear to indicate a difference in frequency between GE and non-GE salmon. Although this would not be considered an adequate and well-controlled study due to the variability of husbandry conditions, numbers of fish crosses, and long time course, this information is nonetheless considered as part of FDA's weight-of-evidence evaluation, and contributes to our understanding of the effect of the *opAFP-GHc2 construct* on ABT salmon. The information provided encompasses a large number of animals over many generations and year-classes, so there is a level of inferential value for this information to the general situation of rearing of ABT salmon containing the *opAFP-GHc2 construct*. These observations are generally consistent with those of the animal safety study.

Of the macroscopic and microscopic lesions observed, most appeared to be associated with the induction of triploidy. Morphologic abnormalities of the axial skeleton, fins, opercula, and gills have been documented among a variety of finfish in the literature and in triploid Atlantic salmon in particular. Gill abnormalities, often accompanied by skeletal, jaw, and opercular malformations, have been most commonly reported for triploid Atlantic salmon in the literature; this result was also observed in both triploid ABT salmon and triploid non-GE comparator Atlantic salmon.

Microscopic lesions of the gills are extremely well correlated to gross observations and are consistent with those described in the literature for triploid fish. Ectopic mineralization, seen with higher prevalence among triploids in this study, has not previously been documented in the literature. Hepatocellular vacuolation in many species, including fish, is a reflection of lipid metabolism within the body (Wolfe and Wolfe 2005). Many factors influence the deposition and mobilization of lipid stores that might ultimately result in hepatocellular vacuolation: triploidy may have contributed to these findings as in some cases triploids have smaller livers than diploids (Benfey et al. 1988).

An increased prevalence of minimal to mild focal inflammation in various tissue types in ABT salmon seemed to be higher among diploid Atlantic salmon than triploid, and higher among diploid ABT salmon and to a lesser extent triploid ABT salmon compared with either size-

matched or age-matched controls. Although focal inflammation may presage immunodysfunction, seasonality or other factors might confound such implications.There was no other evidence that the health status of ABT salmon was compromised in any other way (for a discussion of disease resistance and immunocompetence, see Section j below).

Microscopic observations from earlier generations of diploid and triploid ABT salmon and non-GE comparators document a variety of inflammatory and degenerative lesions that are mostly consistent with diseases of intensively-reared fish. The data and information evaluated as part of FDA's weight-of-evidence evaluation were assembled from fish production records and reflect a variety of crosses and husbandry conditions over several years. The range and severity of histopathologic lesions, morphologic diagnoses, and etiologic diagnoses do not appear to indicate a difference in frequency between GE and non-GE fish. Although these observations do not constitute an adequate and well-controlled study, this information was nonetheless considered as part of FDA's weight-of-evidence evaluation, and contributed to the overall understanding of the effect of the *opAFP-GHc2* construct on ABT salmon, including AAS.

Uncertainties had initially been indicated regarding the effect of culling practices for reasons including the size of the broodstock facility, as previously discussed. The information provided in these observational studies, along with the additional data obtained more recently (see above Tables 8-13), encompasses a large number of animals over many generations and year-classes. The inferential value and direct evidence from these observations and studies contributed to decreasing the uncertainty associated with the culling practices to provide additional confidence that culling did not substantially mask underlying abnormalities, morbidity, or mortality.

Conclusions: Macroscopic observations of gill, fin, and heart abnormalities were most likely attributable to the induction of triploidy, rather than to the presence of the *opAFP-GHc2* construct. Jaw erosions were observed exclusively in male and female diploid ABT salmon, albeit in a study of limited size. Enhanced growth phenotype is the most likely cause of this trait.

Microscopic observations of gill lesions and ectopic mineralization were most likely associated with the induction of triploidy. The increased prevalence of focal inflammation is most likely due to the presence of the *opAFP-GHc2* construct.

FDA concluded that, although the presence of the *opAFP-GHc2 construct* appears to have increased the prevalence of jaw erosions and focal inflammation in adult fish, these findings are of low magnitude and not likely to be problematic to fish in a production setting, where food is provided and foraging is not required.

g. Disease Resistance

A limited study on salmon (weighing ~ 20 g) was performed to determine if the presence of the *opAFP-GHc2 construct* alters the disease resistance of the ABT salmon to furunculosis (*Aeromonas salmonicida*) compared to size-matched non-GE Atlantic salmon. Although there was an earlier peak in the mortality of the ABT salmon following challenge (days 12-15) relative to the comparators (days 14-21), overall there was no obvious difference in mortality profiles between the two according to the study investigators.

Additional information regarding an outbreak of ISAV at the PEI facility is found in Section VII.

An analysis of general mortality data for ABT salmon, including AAS, and non-GE Atlantic salmon at both the PEI and Panama facilities over the period from 2005 through 2012 (as previously discussed) show that there are similar rates of morbidity and mortality between the two groups for the several year-classes of fish examined, indicating that ABT salmon, including AAS, do not appear to have altered susceptibility to disease.

Aside from the information presented for ABT salmon, limited data exist on disease resistance in other GE fish. Jhingan et al. (2003) have studied resistance to the bacterial pathogen *Vibrio anguillrium* in diploid and triploid GH transgenic coho salmon (*Onchorhymcus kisutch)*. They found that resistance (as measured by cumulative mortality) was not affected in the transgenic fish relative to their non-transgenic counterparts when they were infected at the fry stage, but was lower in transgenic fish when infected near smolting (i.e., transgenic fish had higher mortality rates). Vaccination against vibriosis provided equal protection to both transgenics and non-transgenic fish. Triploid fish showed a lower resistance to vibriosis than their diploid counterparts.

Conclusion: The limited available information does not indicate a significant change in disease resistance of ABT salmon relative to non-GE comparators.

h. Smoltification and Seawater Survival

AAS are not approved for grow-out in seawater; nonetheless, some preliminary studies were conducted by the sponsor to determine whether diploid ABT salmon could undergo smotification and survive if transferred to seawater. These pilot studies suggest that survival of diploid ABT salmon was consistent with commercial experience with non-GE Atlantic salmon over the body-weight range examined (Table 19). Survival was very high (≥ 98%) when the fish weight at transfer was at least 150 grams. Comparable data for triploid ABT salmon were not available, but there have been reports in the literature that the survival rate of triploids in saltwater is lower than that of diploids (Benfey, 2001; Galbreath and Thorgaard, 1995; O'Flynn et al., 1997).

Table 19. Survival of Diploid ABT Salmon Fry-Smolt Following Transfer to Seawater

Year-Class	Transfer Date	Transfer Weight	Survival
1999	Nov 2000	30 g	85%
2001	Dec 2002	57 g	80%
2002	Oct 2003	161 g	98%
2005	Jul 2006	150 g	99%

Additional data on smolt development in Atlantic salmon genetically engineered with growth hormone have been published by Saunders *et al*. (1998); these findings are consistent with the data described above for ABT salmon. GE Atlantic salmon that approached smolt size (16 cm) were able to survive for greater than 96 hours following direct transfer from freshwater to full strength seawater with a salinity of 35%, while their normal, non-GE siblings were smaller (<10 cm) and survived less than 24 hours following a similar transfer to seawater. In addition, GE salmon exposed to various temperature-photoperiod conditions were able to complete the

smoltification process under conditions that would inhibit or delay completion of smolting in non-GE Atlantic salmon. After transfer to seawater, GE salmon exhibited satisfactory survival and growth for an additional 4 months (when observations were terminated).

Conclusion: The limited available information indicates that diploid ABT salmon of smolt size survive and grow normally following transfer from freshwater to seawater, indicating that basic aspects of the physiology of the these salmon have not been altered, and that the presence of seawater would not act as physical barrier to survival and establishment. Information on smoltification for triploid GE salmon is currently lacking.

i. Other Phenotypic Characteristics

A wide variety of additional phenotypic characteristics of GH transgenic Atlantic salmon[16, 17] have been studied and reported in the scientific literature by investigators at ABT and academic research institutions in Canada. These characteristics include feed consumption, foraging and predator avoidance (Abrahams and Sutterlin, 1999), gill morphology (Stevens and Sutterlin, 1999), gut morphology (Stevens et al., 1999), myogenesis and muscle metabolism (Levesque et al., 2008), metabolic rate (Cook et al., 2000b), respiratory metabolism and swimming performance (Stevens et al., 1998). In general, these studies found that pre-smolt GH transgenic Atlantic salmon had higher rates of myogenesis, muscle metabolism, and oxygen consumption than non-GE comparators, as well as altered morphology of some body structures. These findings have been associated with rapid growth phenotypes, independent of how that phenotype was established. Because this information is limited to scientific literature investigating GH transgenic Atlantic salmon, but not necessarily ABT salmon or AAS, it is given less weight than the controlled studies presented previously in this section. In many cases, these studies are the only sources addressing these phenotypic characteristics.

Although information is limited for AAS specifically, studies have shown that oxygen consumption in older juvenile and adult GH transgenic Atlantic salmon was higher than in non-GE comparators (Abrahams & Sutterlin, 1999; Cook *et al*., 2000a; Cook *et al*., 2000b; Deitch *et al*., 2006). In contrast, oxygen consumption of eyed embryos, newly hatched larvae (alevins), and first-feeding juveniles (fry) in the GH transgenic salmon was similar to that of non-GE Atlantic salmon (Moreau, 2011; Moreau, *et al*., 2014). Moreover, the timing of early life history events was similar.

Stevens *et al.* (1998) found that pre-smolt GH transgenic Atlantic salmon have a higher oxygen uptake during routine culture conditions and during forced swimming activity relative to similar sized comparators. Overall, the oxygen uptake of GE fish was 1.7 times that of comparators over the course of a day. These fish also had a higher critical oxygen concentration. (Critical oxygen concentration is the concentration in water at which oxygen uptake by fish becomes limited by the oxygen supply (i.e., the concentration threshold where the oxygen uptake rate starts to decrease)). The critical concentration for GE fish was 6 mg/L vs. 4 mg/L in comparator fish. This

[16] Unless stated otherwise in the referenced studies, salmon are assumed to be diploid.
[17] These were GH transgenic Atlantic salmon produced by the sponsor, however, from the information provided it is not possible to determine whether or not they were in fact early generations of ABT salmon.

higher critical oxygen level for GE fish has potential implications for growers and for the establishment and survival of fish if they somehow escape from grow-out facilities.

Cnaani et al. (2013) compared responses to fasting and acute hypoxia of the following groups of Atlantic salmon (derived from St. John's strain): (1) wild-type non-GE diploid Atlantic salmon, (2) ABT salmon, including AAS, and (3) triploid non-GE Atlantic salmon. To monitor responses to stress due to fasting or acute hypoxia, blood was collected for measurement of the following stress response related endpoints: hematocrit, cortisol, glucose, pH, pCO_2, pO_2, Ca^{2+}, K^+, Na^+, and Cl^-. The diploid non-GE Atlantic salmon maintained homeostasis more effectively than the diploid ABT salmon containing the *opAFP-GHc2* construct or triploid non-GE Atlantic salmon, exhibiting smaller changes in stress-response endpoints. The lower tolerance to stress of the ABT salmon, including AAS, may have implications with respect to aquaculture practices and fitness in the wild.

Particularly in regions where water temperatures are elevated[18], water oxygen levels may be below the critical level; any escaped fish will likely be adversely affected and may not survive for extended periods of time. In addition, Stevens *et al.* (1998) cautioned that future growers of growth enhanced salmon should be prepared to either deliver more water or more oxygen in the water per unit of biomass of GE fish compared to that required by non-GE salmon. Based on the information reviewed, AAS may have reduced tolerance for low dissolved oxygen content.

GH transgenic Atlantic salmon have been found to have much higher rates of feed consumption than non-GE comparators, and were more willing to feed in the presence of predators (Abrahams and Sutterlin, 1999). In terms of gut morphology, one group has reported that GH transgenic Atlantic salmon have more (and longer) intestinal folds and a larger digestive surface area than size-matched non-GE comparators (Stevens et al., 1999). Most morphological features of the intestine and of the pyloric caeca of GE salmon were larger than those of comparator salmon. However, the animal safety study specifically addressed the observations of Stevens *et al.* and did not report any significant macroscopic or microscopic differences between ABT salmon and non-GE comparators with respect to gut morphology.

Growth rates, body composition, and feed digestibility/conversion efficiency have been studied in pre-smolt (8 - 55 g) GH transgenic Atlantic salmon by investigators from the sponsor and the Atlantic Veterinary College (Cook et al., 2000a). In this study, GH transgenic Atlantic salmon exhibited a 2.62 to 2.85-fold greater rate of growth compared to non-GE fish over the body weight ranges examined. In addition, gross feed conversion efficiency in pre-smolts was improved by approximately 10% relative to non-GE comparator fish. Body protein, dry matter, ash, lipid and energy were significantly lower in the GH transgenic Atlantic salmon pre-smolts relative to comparators, while moisture content was significantly higher.

Tibbetts et al. (2013) conducted a study similar to that of Cook et al (2000a), using fish at starting weights of ~ 96 g. In this study, the researchers compared diploid and triploid non-GE

[18] Oxygen solubility in water is inversely proportional to water temperature. Salmonids in general have higher oxygen requirements than most other fish and thus require lower water temperatures so that oxygen levels are not limiting.

salmon and ABT salmon of mixed gender. Similar to the results of Cook et al. (2000a), Tibbetts et al. (2013) showed enhanced growth rates and feed conversion in ABT salmon vs. non-GE salmon; this was applicable in both the diploid and triploid fish. With respect to whole body composition, ash and crude protein percentages increased, while lipid and gross energy percentages decreased, in ABT salmon vs. non-GE salmon. Whole body composition was not influenced by ploidy. Composition of filets (ash, lipid, and crude protein percentages) did not differ between ABT salmon and non-GE salmon. Similarly, ploidy had not effect on these composition measurements. (A detailed compositional analysis of ABT salmon is specifically addressed under food safety evaluation (see Section IX below)). In addition, Tibbetts et al. reported on the growth and nutrient utilization of GE AquAdvantage relatives (both diploid and triploid) fed a practical grower diet (see following section for a description of results related to growth). This study included a skeletal bone analysis, as well as an appearance assessment conducted using a ranking system (1 = no obvious skeletal disorder, marketable; 2 = minor skeletal disorder, marketable; and 3 = major skeletal disorder, unmarketable). The overall occurrence of major skeletal disorders (rank = 3) was low (<4%) in all salmon regardless of ploidy or whether or not the fish contained the GH transgene. Triploid salmon had a slightly higher prevalence of major skeletal disorders (2.9% for nontransgenics; 3.7% for transgenics) than diploids (0.3% for nontransgenics; 0.9% for transgenics). These results are very similar to those presented by Fjelldal and Hansen (2010) for vertebral deformities in diploid and triploid non-GE Atlantic salmon underyearling smolts (triploids 1-3%; diploids 0–1%) and suggest that triploidization has a greater effect than transgenesis on the malformation rate, although neither had a substantial effect on producing skeletal disorders that would make the salmon `unmarketable.

A comprehensive, comparative examination of the cardiorespiratory physiology of post-smolt from the fifth generation of GH transgenic salmon was conducted by Deitch *et al.* (2006). In this study, GH transgenic salmon had an 18% lower metabolic scope, 25% higher standard oxygen consumption, and 9% reduction in critical swimming speed relative to size-matched non-GE comparators. This decreased metabolic capacity/performance occurred despite the 29% larger heart and increased cardiac output in GH transgenic salmon. Because gill surface area was the only cardiorespiratory parameter that was not enhanced in these salmon, it was suggested that gill oxygen transfer may have been limiting.

Conclusion: A number of phenotypic characteristics have been reported to have been altered in GH transgenic salmon relative to non-GE comparators. Many of these changes, for example increased growth rate, are the intended and expected effects of introduction of this GH construct into the salmon and are desirable (see Section XI, Claim Validation); others are expected as the result of a rapid growth phenotype. None of these changes, as observed in GE Atlantic salmon, would be expected to adversely affect the animal health or safety of AAS under normal conditions of commercial grow-out if adequate water oxygen levels are maintained. Some of the reported changes would potentially make these fish less fit and less likely to survive if they were to escape from grow-out facilities.

j. Monosex (All-Female) Population

Although the product definition for AAS is limited to a triploid monosex (all-female) population, many of the studies that have been conducted to date have included mixed populations of both males and females. In most of the early studies, no attempt was made to determine the gender

of the fish. In the animal safety study, where fish were identified by gender, it was possible to evaluate the effects of the *opAFP-GHc2* construct by gender. Other than gender-specific characteristics, no differences in gross morphologic or microscopic lesions were apparent in that study.

In order to ensure an all-female population, the sponsor employs a gynogenesis method described in detail in the EA (Section 5.3.1). The adequacy of the gynogenesis process and overall production plan was reviewed as a part of the Durability Plan assessment (see Section VIII). Based on the information provided by the sponsor and considering the physiologic mechanisms of the gynogenesis process, the phenotypic characteristics of a mixed gender population adequately represented the range of phenotypic characteristics expected in a monosex (all female) population.

k. *Impact of Additional Studies on Uncertainties Associated with Culling Procedures*

Fish culling practices at the PEI facility followed established procedures in the management of fish inventory.[19] Although culling is part of typical procedures for broodstock facilities, culling may represent an uncertainty with respect to certain baseline data (e.g., health abnormalities) prior to data collection for fish presented as part of phenotypic characterization and animal health/safety evaluation. Removal of fish, especially at early life stages (e.g., eyed eggs, fry or smolts) was performed due to space constraints/inventory management, slow vs. fast growth, presence of moderate/severe health abnormalities, and selection of broodstock.

Culling of slow growing fish is part of best management practices in commercial, land-based aquaculture operations (Freshwater Institute/Atlantic Salmon Federation, 2013). This is applicable to commercial grow-out facilities and to broodstock facilities, such as the PEI facility. In space-constrained facilities, fish removal is a part of normal inventory control, and biosecurity and pathogen control are very important considerations. Slow growth is a common reason for fish removal, as are other health-related considerations such as the control of clinical disease/illness, reduction of pathogen amplification and external fungal infections, and removal for irregularities/deformities. In broodstock facilities, an additional criterion for keeping or removing fish relates to the selection of breeding animals for the propagation of desirable genetics. Thus, use of culling for genetic selection at a broodstock facility (e.g., PEI facility) is likely a primary distinction from a grow-out facility (e.g., Panama facility). Although culling practices at PEI may have influenced the baseline values for certain health abnormalities, the general criteria for fish removal apply equally to the GE and non-GE fish at the facility. Although there is no way to definitively establish the impact of selective breeding/culling on the phenotype of animals, its use in the selection for desirable and against undesirable traits is an accepted practice in food animal species in general, and aquaculture species in particular (Hulata, 2010).

Results from the Panama facility for later year-classes of fish helped address issues related to culling of fish and planned reductions in populations. Data on these management procedures

[19] The exception is described at Section VII A where the sponsor selected (culled) fish to to generate the appropriate number of animals for the sponsor-initiated study for the initial phenotypic characterization.

from three year-classes (2010, 2011, and 2012) confirmed that under the proposed commercial conditions, rate of culling was generally low (< 10% of fish). In addition, because culling is a normal commercial practice, these data are not expected to be biased or differ substantially from what would be expected to occur in a commercial setting. Because of concerns that the culling procedures for the initial study may not have reflected typical acquaculture procedures and may have obscured adverse outcomes, FDA requested and received from the sponsor additional information regarding culling practices, the health of the ABT fish populations at the grow-out facilities, and the potential role that culling could have had in masking adverse outcomes. These data and information submitted to the agency since the preliminary review are found in Section VII B 2, and Tables 8-13 and include information on morbidity and mortality from more than 150,000 ABT salmon and approximately 9,000 non-GE Atlantic salmon from both the PEI and Panama facilities. These new data did not reveal any new abnormalities or altered rates of abnormalities beyond those identified in the initial study, and did not indicate any bias in the initial study's estimation of (i.e., did not mask) rates of morphologic abnormalities, mortality, or morbidity. Given these observations, FDA concluded that it is not likely that culling had any substantive influence on animal health evaluations for either facility. Further, because culling is a normal commercial practice, these data are not expected to be biased or differ substantially from what would be expected to occur in a commercial setting.

The issue of culling and any impact on the occurrence of health abnormalities will be monitored as part of an overall post-approval surveillance and reporting program. Sponsors of all approved NADAs are subject to these requirements, and include such observations as shipment and disposition of product(s), adverse health events, etc. Required observations at the Panama site relative to culling, morbidity/health abnormalities, mortality, etc. represent one segment of the sponsor's post-approval commitments, which are detailed in the Post-Approval Reporting Requirements of this Summary and the posted Approval Letter (http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/ucm466214.htm) that describes the conditions established in the approved application.

C. Addressing the Risk Questions

Risk Question 1: Is there direct or indirect toxicity to the animal

The overall weight-of-evidence evaluation of the data and information in ABT's submission indicates that there is no significant impact of the *opAFP-GHc2* construct on the overall health, morbidity, mortality, or incidence of malformations in ABT salmon, including AAS. Some minimal effects were noted in fish bearing the *opAFP-GHc2* construct in the form of an increased frequency of skeletal malformations, and increased prevalence of jaw erosions and multisystemic, focal inflammation. These results were not confirmed by the sponsor's data from the grow-out site in Panama or from the more recent Tibbetts et al. (2013) study, neither of which shows any significant diffences in malformations in AAS compared to non-GE fish. There was also evidence from the scientific literature of minimal effects in the form of increased cardiac output and reduced tolerance to low dissolved oxygen (DO) concentrations. These effects of the *opAFP-GHc2* construct are likely to impact the overall fitness of AAS in the natural environment (See EA Section 7.3.1.1.2). The consequences of these effects to AAS in a production setting are likely to be small and within the range of abnormalities affecting rapid growth phenotypes of Atlantic salmon.

Conclusion: The phenotypic characterization of AAS has been adequately addressed according to the risk-based, hierarchical system employed for the evaluation of GE animals. There is sufficient information to support the safety of the *opAFP-GHc2* construct to the AAS. No significant adverse outcomes were noted as the result of the incorporation of the *opAFP-GHc2* construct; therefore FDA concludes that no significant hazards or risks have been identified with respect to the phenotype of ABT salmon, including the AAS subset.

Risk Question 2: Are there phenotypic characteristics that provide hazard identification for other steps in the evaluation?

No hazards were identified specifically for the genotypic and phenotypic durability, environmental and food/feed safety, or claim validation evaluations. With respect to the environmental safety evaluation, several phenotypic changes were identified that may result in decreased fitness (e.g., increased oxygen requirements, decreased critical swimming speed, lower metabolic scope, etc.). These changes are expected to impact survival and establishment should any AAS escape from commercial production facilities. These issues are described in more detail in the EA Section 7.3.1.1.2.

Conclusion: No phenotypic characteristics have been identified that would provide hazard identification for other steps in the evaluation based on the product definition.

Risk Question 3: What are the risks to the user (user/handler safety)?

No data/information indicated that there were any additional risks to handler safety above that of commercially farm-raised Atlantic salmon.

Conclusion: There is no risk to user/handler safety associated with AAS.

Risk Question 4: What are the risks from any components of any biological containment strategy?

Induction of triploidy in ABT salmon increased risk of gill, fin, and heart abnormalities, and ectopic mineralization. The severity of these effects was generally minimal and not expected to have a significant consequence in a production setting. Any reduction in growth characteristics with induction of triploidy, often reported in the scientific literature, was more than compensated for by the increased growth rates in ABT salmon vs. non-GE comparators (see Claim Validation, Section XI).

The effects of triploidy on AAS are no different from those observed with non-GE comparators. Induction of triploidy is a common aquaculture technique regularly used in practice.

Conclusion: There are no risks to AAS from triploidy that are not already present in triploid-based aquaculture systems.

D. Conclusions

FDA conducted a weight-of-evidence evaluation of the phenotype of ABT salmon, including AAS, drawing on data from a number of sources. This evaluation has used four sources of data and information. As discussed in the weight-of-evidence section, the agency placed the most emphasis

on controlled studies conducted on the specific animals being considered for approval. FDA also considered other non-controlled studies, as well as historical hatchery records and data for these animals. Finally, FDA evaluated studies reported in the scientific literature investigating these same animals or their relatives.

Based on the weight-of-evidence evaluation for phenotypic characterization of AAS, FDA has made the following conclusions:

- The phenotypic characterization of ABT salmon, including AAS, was adequately addressed according to the risk-based, hierarchical system for the evaluation of GE animals.
- There was sufficient information to support the safety of the *opAFP-GHc2* construct to the ABT salmon, including AAS.
- No unique adverse outcomes were noted as the result of the incorporation of the *opAFP-GHc2* construct; FDA concludes that no significant hazards or risks have been identified with respect to the phenotype of ABT salmon, including AAS.
    - The sponsor submitted additional data that minimized FDA's concern regarding uncertainties associated with early culling rates.
- Because of increased metabolic demands of the rapid growth phenotype, drug labeling includes recommendations for ensuring that the animals are fed to satiety and that dissolved oxygen is carefully monitored to provide optimal growth conditions.
- Any adverse outcomes that have been noted (e.g., jaw malformations, increased metabolic demand) will likely render ABT salmon, including AAS less fit in a competitive environment.
- Sufficient data and information exist to provide a baseline for expected rates of severe unexpected outcomes to be used as baselines for determining post-approval reporting categories.
- Based on the information in this evaluation and considering the physiologic mechanisms of the gynogenesis process, the phenotypic characteristics of a mixed gender population adequately represent the range of phenotypic characteristics expected in a monosex (all female) population.

No significant hazards were identified and the phenotype was stable over multiple generations. Although no specific hazards have been identified, we note that alterations in some of the phenotypic characteristics reported in the scientific literature for GH transgenic salmon may alter fitness characteristics that are not relevant to growth specified under the conditions of use (see Approval Letter) and have been considered in the EA (See Section 7.3.1.1.2).

VIII. GENOTYPIC AND PHENOTYPIC DURABILITY

A. Overview

This section evaluates the genotypic and phenotypic durability of the lineage of AAS containing the *opAFP-GHc2* construct at the α- locus as well as the plan to ensure that AAS post-approval continue to meet requirements for safety and effectiveness and quality characteristics and meets the identity it was found to meet prior to approval.

FDA posed three risk questions as part of the genotypic and phenotypic durability evaluation:

i. Is the genotype changing over the lifespan of the animal or product such that it would affect the risks associated with the product?

ii. Is the phenotype changing over the lifespan of the animal or product such that it would affect the risks associated with the product?

iii. Is there a plan in place to ensure that over time the phenotype and genotype will not change, or if it does, are there procedures in place to provide for either a remedy or a risk assessment of the new animal?

Under the review process described in Guidance 187, the initial levels of review primarily identify and characterize potential hazards associated with the GE animal and successive levels of review consider any hazards that have been previously identified. Evaluations at the earlier levels did not identify hazards that impact the durability assessment or plan. Therefore, no risk questions beyond those stated above need to be addressed in this section. The Phenotypic Characterization step (see Section VII above) identified some areas of uncertainty, and recommendations involved addressing these uncertainties through a post- approval surveillance program at the Panamanian facility. We incorporated this program as a part of the durability plan for AAS as discussed below.

After evaluating data submitted by ABT, FDA concludes that the data submitted support the Genotypic and Phenotypic Durability portion of the hierarchical review of AAS, and that the proposed Durability Plan is acceptable.

B. Evaluation

1. Is the genotype changing over the lifespan of the animal or product such that it would affect the risks associated with the product?

FDA conducted the genotypic durability assessment during its evaluation of the Molecular Characterization of the GE Animal Lineage (see Section VI above). From that evaluation, FDA concluded that the genotype did not change and was durable over seven generations.

Conclusion: The genotype did not change between generations, was durable, and did not change in any manner that would impact the other risk questions in this or other steps of the hierarchical review process for AAS.

2. Is the phenotype changing over the lifespan of the animal or product such that it would affect the risks associated with the product?

FDA conducted the phenotypic durability assessment during the Phenotypic Characterization step (see Section VII above). From that evaluation, and the Claim Validation studies on the 2009 and 2010 year-classes (see Section XI), FDA concluded that the phenotype has not changed over multiple generations.

3. Is there a plan in place to ensure that over time the phenotype and genotype will not change, or if it does, are there procedures in place to provide for either a remedy or a risk assessment of the new animal?

A durability plan consists of several components. First, the plan contains a list of one or more characteristics critical to the durability of the final product, as well as methods, testing schedules, and specifications for each of these characteristics. Second, the plan discusses procedures that will be carried out in the event that an individual test result does not meet its specification. Third, the plan includes a commitment from the sponsor to withhold or withdraw from the market any product that does not meet all of the durability specifications. Finally, additional tests and monitoring procedures may be included as a part of the durability plan in order to address uncertainties from other steps of the product's evaluation. Many of these components comprise the conditions established in the approved application and are described in Appendix A of the Approval Letter.

In this section, the proposed characteristics that were evaluated as a part of the durability plan are presented, then the overall testing schedule and production plan, followed by a detailed evaluation of each testing method and its validity. FDA then evaluated procedures for out-of-specification results and the sponsor's withdrawal commitment. Finally, the record keeping and reporting schedule that comprise the post-approval requirements are presented.

Proposed tests, schedules, methods, and specifications are described in Table 20.
The sponsor submitted the following: (1) a list of characteristics critical to the durability of the final product and methods, testing schedules, and specifications for each of these characteristics; (2) procedures to be carried out in the event that an individual test result does not meet its specification; and (3) a commitment to withhold or withdraw from the market any product that does not meet the durability specifications.

Table 20. Summary of Durability Plan

Characteristic	Sample Matrix	Method	Testing Parameters
Presence of the *opAFP-GHc2 construct*	Blood	PCR	(1) Individual testing of homozygous females used for generation of broodstock (ABT salmon), and (2) Each broodstock fish during the first three production cycles after approval
opAFP-GHc2 construct Stability	Blood	PCR	(1) Individual testing of homozygous females used for generation of broodstock, and

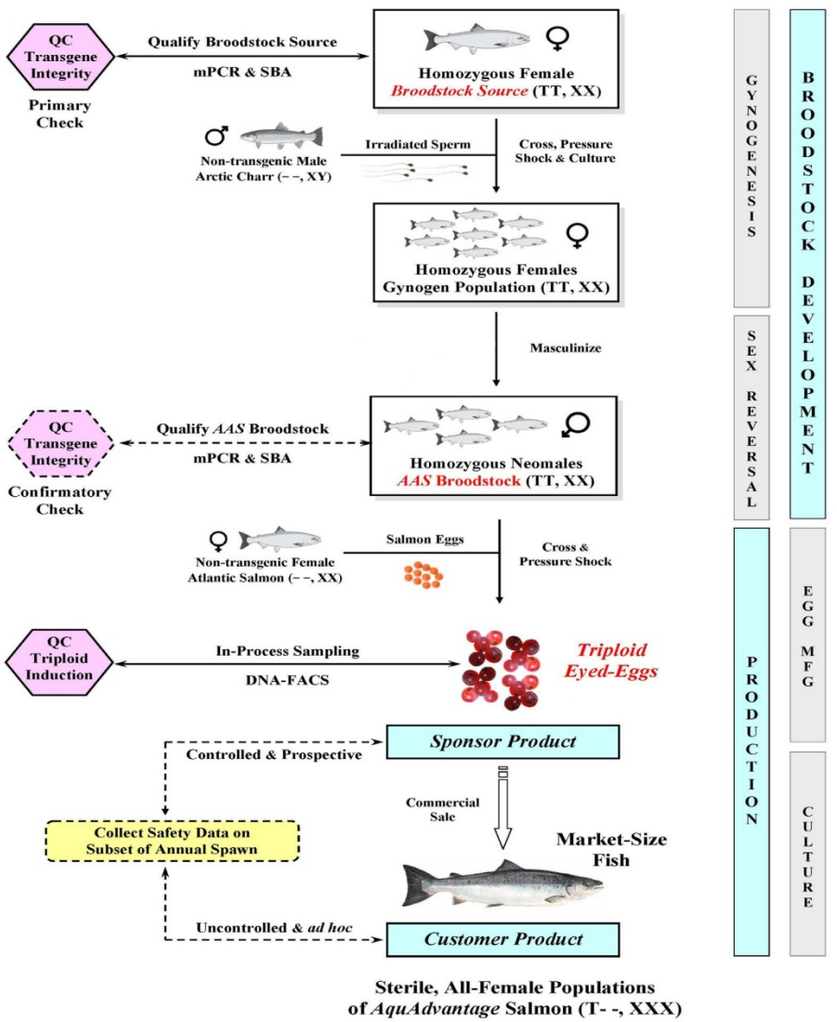
Characteristic	Sample Matrix	Method	Testing Parameters
			(2) Each broodstock fish during the first three production cycles after approval
Copy Number	Blood	Southern Blot or PCR	(1) Individual testing of homozygous females used for generation of broodstock, and (2) Each broodstock fish during the first three production cycles after approval
Triploidy	Eyed-eggs	FACS	Composite sampling from multiple egg batches in a single 23L upwelling chamber; based on sampling, the number of triploid eggs in each lot must be sufficient to provide a high expectation that the lot consists of at least 95% triploid eggs.

a. *Testing Schedule and Production Plan for Broodstock*

The sponsor proposed a two-phased testing schedule for qualifying broodstock ABT salmon into their production program based on their overall plan for the production of AAS (the "production plan"). The first phase covered the three production cycles after approval. During this phase, all broodstock and the homozygous females used to generate the broodstock will be individually qualified. In the second phase (after the third production cycle), assuming no out-of-specification results during phase one, the testing schedule will be reduced to include only the homozygous females used to generate future broodstock. In the unexpected event that there is an out-of-specification result during phase one, the duration of that phase will be reconsidered.

The sponsor proposed to follow a specific production plan. The overall plan is presented in Figure 3.

Figure 3. Graphical representation of the AquAdvantage Salmon production plan.



Females homozygous for the *opAFP-GHc2 construct* are used as the broodstock source for AAS. These fish are pre-qualified via confirmation of construct presence and construct stability via multiplex qualitative PCR and for construct copy number using Southern blot. Eggs from qualified homozygous females are then subjected to gynogenesis, resulting in an all-female gynogen offspring population.

Gynogenesis is a procedure commonly used in modern salmonid aquaculture. Briefly, irradiated sperm are introduced to eggs, followed by a pressure treatment to result in diploid “twin” offspring. In this case, the sponsor uses Arctic char milt that has been irradiated so that no Arctic char DNA is present in the gynogen population. In the event that the milt irradiation was not successful, the offspring would be an Arctic char/Atlantic salmon hybrid. These fish are readily identifiable by their differential markings and phenotypic appearance. As such, these hybrid fish

can be easily removed early in the production process without extensive testing. Therefore, only offspring that appear to be Atlantic salmon will be gynogens of the homozygous females; these animals will be used in subsequent steps in the production plan. The all-female gynogen population is then masculinized through the application of 17-methyltestosterone, so that they produce milt instead of viable eggs. Milt from these "neomales," homozygous for the *opAFP-GHc2 construct*, are then used to fertilize the eggs from non-GE Atlantic salmon to produce an all-female hemizygous population of GE fish. Use of 17-methyltestosterone for this procedure is fairly common in modern aquaculture.

Because these steps are so far removed from the production of food, given that 17-methyltestosterone is only used as part of the process to generate the neomales to produce an all female population, the use of this compound was not considered to pose a food consumption risk to the human consumer of the marketed AAS product.

During the first three production cycles after approval, the resultant homozygous neomale broodstock fish will be subjected to confirmatory testing for *opAFP-GHc2 construct* presence, stability, and copy number. These homozygous, neomale gynogens comprise the commercial broodstock for AAS.

ABT broodstock salmon, upon sexual maturity, are then out-crossed with non-GE Atlantic salmon females. Collection of the milt from neomale broodstock is a terminal procedure, as these fish lack seminal vesicles for normal spawning. Each neomale, therefore, may only be used for one production cycle. Milt from a single fish can fertilize approximately 50,000 eggs.

Fertilized eggs are then subjected to pressure shock treatment in order to render the offspring triploid, with two copies of the genome coming from the non-GE female and one copy from the homozygous neomale gynogen. Female triploid salmon are effectively reproductively incompetent, providing additional environmental and intellectual property safeguards. Samples from individual triploid batches are combined to assess the rate of triploid induction via fluorescence-activated cell sorting (FACS) analysis.

Conclusion: No hazards have been identified in the production plan. A possible source of uncertainty is the impact of gynogenesis on the durability of the inserted *opAFP-GHc2* construct. The additional testing of all broodstock during the first three production cycles should be sufficient to determine the impact of gynogenesis on the genetic durability of the ABT *opAFP-GHc2* construct in broodstock and resulting AAS (see Approval Letter, Appendix A). The proposed production plan is acceptable.

b. Presence and Stability of Inserted opAFP-GHc2 construct

Testing for the presence of the inserted *opAFP-GHc2 construct* and verification of its location is conducted following the PCR method outlined below.

PCR Procedure

The method takes advantage of standard multiplex Polymerase Chain Reaction (PCR) using sets of primers, whose composition is based on the sequence of the *opAFP-GHc2* construct and the regions flanking the site of integration in the ABT salmon genome. The sponsor's PCR assay allows reliable differentiation between the AAS and their non-GE Atlantic salmon counterparts

and provides confirmation of the presence of the approved *opAFP-GHc2* construct in the fis. Samples from both GE and non-GE fish generate two DNA amplicons corresponding to the endogenous growth hormone gene. Samples from the ABT salmon amplify an additional DNA fragment, which is unique for the approved *opAFP-GHc2 construct*. The PCR assay uses additional primer pairs to amplify DNA regions at the 5' and 3' junctions of the EO-1α insertion site and verify that the *opAFP-GHc2 construct* remains in its originally-approved location and orientation within the genome.

Reference Standards and Controls
The PCR method includes one reference standard and several controls. The specified reference standard is a commercially available 100 bp DNA ladder. Each PCR assay contains two control samples: a positive control from a known ABT salmon, and a negative control from a known non-GE salmon. The positive control produces a band consistent with the presence of the transgene in the sample. The negative control does not have the transgene, and therefore will not show this band.

Additionally, the method includes a no-template control. This assay should not show any bands unless the sample has been contaminated. Therefore, this control determines whether the entire assay has been compromised via the introduction of contaminating DNA.

Each individual reaction in the assay also contains its own internal control. The primers amplify amplicons from endogenous growth hormone in all fish irrespective of their GE status. The presence of these DNA fragments in the PCR product mixture confirms the quality of the genomic DNA and PCR conditions.

Together, these controls and standards provide evidence that the assay was conducted properly and its results are valid.

PCR Method Validation
Validation of a durability indicating method considers method accuracy, precision, specificity, linearity, range, limits of detection and quantitation, ruggedness, and whether the method is capable of detecting a durability failure. The extent to which these factors are considered is dependent on the type of method and its application.

The method used to determine the presence or absence of the inserted *opAFP-GHc2* construct in the fish and whether the construct remains at the original EO-1α site of integration is based on the polymerase chain reaction (PCR). The relative ease of performing a PCR assay and the likelihood of its success primarily depend on the complexity of the DNA molecule being analyzed, the efficiency of the primer-DNA template hybridization, and the stringency of the PCR conditions. Provided that the primers are appropriately designed and the conditions of the reaction are specified, they will only bind to, and therefore amplify, the unique target sequence of DNA. In the event that there are mismatches between the primer and target sequences, the PCR reaction will likely not take place. The qualitative nature of the method provides a binary output provided that the conditions of the reaction are suitable – either the reaction occurred because the target sequences are present, or the reaction did not occur because the target sequence is absent or sufficiently changed so as to render the kinetics of primer binding

inefficient under the method conditions. Therefore, linearity and range are not at issue for this type of method.

Limits of detection and quantitation are important measures for quantitative methodologies. Although quantitative PCR methods do exist and such considerations would be evaluated for those methods, the method at issue here is a qualitative PCR. Practical limits of detection do exist for even qualitative PCR, and given that the sponsor will have ready access to sample DNA, this is not a concern for this particular method. Similarly, with respect to qualitative PCR, methodologies are extraordinarily rugged and robust under the specified conditions of the reaction. Even significant deviations from the specified conditions often have little or no effect on the result.

PCR methods are also durability indicating. For a method to be durability indicating, it should be able to detect changes in the sequence, arrangement, or location of the *opAFP-GHc2* construct. The types of changes we are interested in are gross changes, such as large deletions, insertions, duplications, or rearrangements; more subtle changes, such as single nucleotide changes, are not *per se* durability failures. In the unlikely event that a change in the DNA sequence that is not detectible using qualitative PCR methods results in a safety or effectiveness issue, post-approval product monitoring should identify such events and lead to removal of broodstock fish with such issues.

The proposed method includes specific primers to be used and the conditions under which the reaction is to be conducted. The sponsor also provided to FDA the specific reaction conditions and methods for extracting the sample DNA; FDA determined that the primer sequences and specified reaction conditions were appropriate for determining the presence and stability of the inserted *opAFP-GHc2 construct* in ABT broodstock salmon.

Conclusion: The qualitative PCR method is acceptable as a method for determining the presence and stability of the inserted *opAFP-GHc2 construct* at the α-locus.

c. Copy Number

The qualitative PCR method employs the Southern blot technique to identify the number of constructs and their respective integration sites in the animal genome. A contract testing laboratory performed Southern blot analysis using GLP standards. The method is capable of distinguishing insertion of the *opAFP-GHc2* construct at the α-locus versus other locations in the genome.

Southern Blot Procedure

Briefly, genomic DNA is extracted from blood samples using standard DNA extraction techniques. DNA quality is confirmed via agarose gel electrophoresis and ethidium bromide staining. Qualified DNA samples are digested singly with *Pst* I and *Eco*R I restriction enzymes. Digestion of each sample is confirmed by agarose gel electrophoresis. The remaining digestion mixture is resolved by electrophoresis followed by transfer onto nylon membranes. A specific DNA fragment is radioactively labeled and used to probe the nylon membrane. Autoradiograms for the *Pst* I and *Eco*R I digests are obtained by exposing film for specific periods of time.

If the *opAFP-GHc2* construct is present at the expected α-locus, the Southern blots will show specific banding patterns in each of the two digests.

Reference Standards and Controls
The Southern blot method includes molecular weight standards and two controls. Molecular weight standards are a commercially available mixture of *Hin*d III digested λDNA and *Hae* III digested φX174 DNA. Each autoradiogram contains two controls: a negative (non-GE salmon genomic DNA) and a positive (non-GE salmon genomic DNA with added *opAFP-GHc2* construct). Negative control lanes are expected to reveal no bands on the film, whereas positive control lanes should exhibit the specific banding pattern consistent with appropriate insertion of the *opAFP-GHc2* construct.

Southern Blot Validation
As discussed above with respect to the PCR validation, validation of a durability indicating method considers several factors depending on the type of method and its application. The described method is intended to find all inserted copies of the *opAFP-GHc2* construct within the salmon genome. Accuracy, precision, and specificity of a Southern blot method depend on the design of the probe and the region of genomic DNA that is being probed. The validity of the probe is confirmed during each blot through the use of the positive and negative control samples.

Similar to a PCR method, this technique is qualitative. Linearity, range, and limits of quantitation are therefore not a concern. Although not quantitative, the method is stability-indicating. Genomic events that would lead to a durability failure are detectable with this method, and include deletions (loss of the *opAFP-GHc2* construct), duplications (appearance of additional copies of the construct in other genomic locations), mobilizations (movement of the construct to another site), and concatamerizations (addition of a new copy at an existing site of insertion). Each of these four events will lead to changes in the banding patterns on the exposed film.

It is important to note that although this method will be able to detect a durability failure as a result of mobilization, duplication, concatamerization and loss of the gene, it is not able to distinguish between a hemizygous (having just one allele with the inserted *opAFP-GHc2* construct) and a homozygous (two copies of the allele) fish. This inability is not a significant regulatory concern for several reasons. First, the original hemizygous fish were bred to homozygosity using an appropriate breeding scheme. Each fish's zygosity was determined through observation of the phenotype of its out-crossed offspring; a homozygous fish would produce a population of fish who were all fast-growing whereas a hemizygous fish would produce a bimodal population with respect to growth rate. The sponsor maintains records of this backcrossing for each fish in its lineage. The gynogens of a homozygous fish should be homozygous; therefore it is reasonable to assume that a confirmatory Southern blot can be interpreted to mean that the animal is homozygous.

Second, the post-market surveillance program (discussed in detail below) will identify bimodal distributions of fish at the Panamanian facility, thereby alerting the sponsor of a durability failure. The risk of a durability failure due to a hemizygous broodstock does not represent a safety concern. Furthermore, in the unlikely event that such a failure occurs, FDA will be notified

through the post-approval reporting requirements that some of the fish did not meet the product's rapid growth claim.

The sponsor also developed and validated a quantitative PCR method as an alternative to the Southern blot method. This quantitative PCR method has the same capabilities as the Southern blot method and, in addition, will distinguish between one and two copies of the inserted construct allele.

Conclusion: The Southern blot method is acceptable as a method for determining copy number stability in the EO-1α lineage.

d. Triploidy

Triploidy is one method of producing reproductively incompetent fish for use in aquaculture and fishery management, and is widely accepted as the most effective method for this purpose (Benfey 1999). An animal is triploid when it contains three copies of its genomic chromosomes, as opposed to the usual two copies. In the case of certain species of fish, triploidy results in fish that are similar in most respects to diploids when examined at the whole animal level with the exception of rendering the fish incapable of reproducing (especially in female fish, such as the AAS). The impact of triploidy on the AAS is discussed at length in the evaluation of the Phenotypic Characterization (see Section VII). The sponsor uses triploidy as a method for genetic containment, for both environmental risk management as well as protection of intellectual property.

The sponsor submitted results from a study designed to validate the process used to induce triploidy in AAS, which are described below.

Induction of Triploidy

In five successive weeks, ten 1-to-1 crosses were established between 10 non-GE female Atlantic salmon and milt from eight ABT salmon males hemizygous for EO-1α. Two crosses were generated in each of five successive weeks. Bulk fertilized eggs from each cross were apportioned by volume into four samples of approximately 1,500-3,000 eggs. To induce triploidy, fertilized eggs were placed in a 750 mL pressure vessel and subjected to a specific pressure for a specified period of time. Following pressure treatment, eggs were water hardened, disinfected, and incubated until sampling for ploidy count. When the treated eggs reached the eyed stage, approximately 350 eggs were randomly sub-sampled to estimate triploidy rate.

Percent Triploidy Results: The percentage of triploid eggs from each induction was estimated and a lower 95% confidence bound was estimated using exact binomial methods rather than methods based on the assumption of normality. The pooled percentage ploidy for crosses was estimated assuming that all samples were combined. Results are provided in Table 21. The counts made from samples within a cross provide an estimate of within cross variability and the counts made for all crosses provide an evaluation of the robustness of the method.

Of the 20 samples, 14 samples had a lower 95% confidence bound greater than 99% with 19 samples having a lower 95% confidence bound greater than 98%. The estimated sample average

percent of triploidy for the 5 crosses ranges from 99.7%-99.9%. For the triploidy rate estimates within a cross, the minimum range was 99.7-100% and the maximum range was 98.9-100%. The tight ranges indicate that the induction method has low variability within a given cross. Further, these results have been confirmed in additional validation studies using high-capacity pressure chambers, in which the percentage of triploids for 10 independent crosses (n = 200 eggs per cross) also averaged 99.8%, with 100% triploidy in six crosses and 99.5% triploidy in the other four crosses. See Section 5 3 2 3.

Conclusion: The method provides triploid rates higher than 98% for most inductions.

Table 21. Summary analysis of triploid induction for multiple batches of multiple crosses

Cross Code	% Triploid	Lower 95% CB[1]	Average % Triploid (a – d)
ABF08-AS10PSa	100	99.2	99.9
ABF08-AS10PSb	99.7	98.6	
ABF08-AS10PSc	100	99.2	
ABF08-AS10PSd	100	99.2	
ABF08-AS15PSa	99.4	98.2	99.7
ABF08-AS15PSb	100	99.2	
ABF08-AS15PSc	100	99.2	
ABF08-AS15PSd	99.4	98.2	
ABF08-AS43PSa	100	99.2	99.7
ABF08-AS43PSb	98.9	97.4	
ABF08-AS43PSc	100	99.2	
ABF08-AS43PSd	100	99.2	
ABF08-AS45PSa	100	99.2	99.9
ABF08-AS45PSb	99.4	98.2	
ABF08-AS45PSc	100	99.2	
ABF08-AS45PSd	100	99.2	
ABF08-AS54PSa	99.7	98.7	99.9
ABF08-AS54PSb	100	99.2	
ABF08-AS54PSc	100	99.2	
ABF08-AS54PSd	100	99.2	
		Average for all 5 Crosses	99.8

[1] CB = Confidence Bound. Average % Triploid represents the mean of cross codes ending in a through d for each distinct “AS” group.

Post-Approval Sampling Plan

To evaluate the triploidy process in production batches, eggs were placed in a 3 L vessel for pressure treatment and concomitant triploidy induction. In order to accommodate larger volumes of eggs, a number of such “batches” are combined in a 23 L upwelling chamber (referred to as a “lot”), which ultimately contains 100,000 to 200,000 eggs. Fertilized eggs are subject to an increasing flow of water that facilitates thorough mixing. From this chamber, pooled samples are taken for analysis and analyzed by FACS analysis to determine ploidy.

The overall ability of the sampling plan to serve as an appropriate and discriminatory process control depends on the specific acceptance criteria. As an initial matter, the release testing is aimed at controlling for false positive results, namely the commercial release of eggs that, in fact, do not meet a certain minimum requirement of triploidy.

The minimum level of triploidy acceptable for AAS has been set to assure that, based on sampling, at least 95% of released eggs are triploid with a probability of 0.95 (i.e., the probability that these eggs are not at least 95% triploid is less than 0.05).

The proposed sampling procedure consisted of the following four steps:

Step 1: Determine the proportion of 200 sample eggs that are triploid.

Step 2: If the proportion of triploid eggs ≥ p1, release the lot; if not, sample 700 additional eggs.

Step 3: Determine the proportion of 700 additional eggs that are triploid and estimate proportion of all 900 eggs that are triploid.

Step 4: If the combined proportion of triploid eggs ≥ p2 release the lot. Otherwise, destroy the entire lot.

The release specifications used as the Step 2 and Step 4 criteria were estimated using a series of simulations (see Table 22 below). In each simulation 100,000 eggs were generated, with a specified true proportion (true p) of triploid eggs (the remainder being diploid). From each simulation 200 eggs were randomly selected (Step 1) and the proportion of triploid eggs was compared to p_1 (Step 2). If the criterion were not met, an additional 700 eggs were selected (Step 3) and the proportion of triploid eggs in the combined sample of 900 eggs was compared to p_2. Table 18 shows the proportion of the 10,000 simulations that passed the p_1 and p_2 criteria.

Table 22. Simulation (10,000) of the sampling plan (chamber size = 100,000 eggs) and Probability of Lot Being Accepted

True p	$p_1 = 0.98$	$p_2 = 0.975$	$p_2 = 0.964$	$p_2 = 0.950$
0.90	0.0000	0.0000	0.0000	0.0000
0.91	0.0000	0.0000	0.0000	0.0000
0.92	0.0004	0.0004	0.0004	0.0009
0.93	0.0010	0.0010	0.0010	0.0088
0.94	0.0070	0.0070	0.0076	0.1177
0.95	0.0261	0.0261	0.0432	0.5376
0.96	0.0925	0.0963	0.3189	0.9432
0.97	0.2796	0.3641	0.8652	0.9993
0.98	0.6257	0.9090	0.9993	1.0000
0.99	0.9500	0.9998	1.0000	1.0000

Due to controlling for an overall 5% false positive rate that released batches contain at least a true 95% proportion of triploid eggs, specific criteria were identified to meet these constraints. Cells in the Table 16 were identified where the true p was 0.95 and the proportion of simulations that met the release criteria was ≤ 0.05. Of the simulated sampling schemes, a Step 2 criterion (p_1) of 0.98 and a Step 4 criterion (p_2) of greater than or equal to 0.964 (shaded cells above) are, therefore, appropriate.

Ninety-five percent of triploid eggs in a lot was considered to be a reasonable production target. A system process control strategy was developed to ensure that, based on sampling, fewer than 5% of the lots released would contain less than 95% triploid eggs. Cells in Table 16 were identified where the true proportion of triploid eggs in a lot was less than 0.95 (95%) and the proportion of simulations (lots) that met the release criteria was <=0.05. Of the simulated sampling schemes, a Step 2 criterion (p1) of 0.98 and a Step 4 criterion (p2) of greater than or equal to 0.964 (shaded cells above) are, therefore, appropriate. Because the information in Table 21 supports the expectation that the actual production of triploid eggs in a lot is at least 98% and the probability of a lot with less than 95% triploid eggs being produced is actually close to 0.03 in most cases (Table 22), this is considered an acceptable production strategy.

Conclusion: The proposed sampling plan and method for determining egg ploidy is acceptable for the qualification of production lots of AAS eggs.

e. Out-of-Specification Procedures

The sponsor has further committed to retesting any test samples or production lots found to be out-of-specification (OOS). All OOS results will be investigated to determine the cause of the result or, in the case of a triploidy failure, will result in destruction of a production lot. Any confirmed OOS results will result in the disqualification of that animal from the broodstock or the destruction of that production lot. Should the EO-1α lineage fall out of specification, the sponsor commits to procedures for the regeneration of the line, including maintenance of cryogenically preserved milt at two distinct locations.

The conditions established in the approved application, as described in the Approval Letter, Appendix A, reiterate this procedure for each lot.

Conclusion: The out-of-specification procedures are adequate and acceptable.

f. Post-Approval Safety Surveillance

The sponsor provided information on its plans for post-approval surveillance with respect to animal safety (i.e., mortality, morbidity, morphology) (See Phenotypic Characterization). These have been summarized in Table 20, and are conditions established in the approved application as described in Appendix A.

In the post-approval record-keeping and reporting requirements found in Appendix A of the Approval Letter, and Section 7.4.1.2 of the EA ploidy testing will continue to be conducted on all composite batches of fertilized eggs intended to be sold or distributed. As previously described in Post Approval Plan, (Section VIII B 3 d) if, based on sampling, triploidization in these eggs does not exceed 95% (based on the statistical 95% lower confidence limit as described previously), the entire batch of eggs must be destroyed. (We note again that during method validation testing, the lowest effectiveness observed for triploidization in an individual batch of eggs was 98.9% and the mean was 99.8%). Because the testing methodology used for verifying triploidy results in egg destruction, it is be impossible to ensure 100% triploidy in all of the eggs actually used for grow-out through testing.

In addition, for the PEI broodstock facility, assessments of morbidity-mortality and morphology will be conducted on the annual spawn during the early-life stages. Assessments will include evaluation of morphologic irregularities in a predetermined number of randomly selected animals (fry and juveniles) prior to selection of any group of fish for grow-out or culling.

Conclusion: The overall surveillance approach is acceptable and addresses concerns about collecting animal safety data under commercial grow-out conditions and on early-life stages of fish. Relevant portions have been incorporated into the Summary of Required Records and Reports (Appendix A) of the Approval Letter.

C. Conclusions

The information the sponsor provided supports a finding that (1) both the genotype and phenotype of AAS are durable, and (2) the sponsor has in place an acceptable plan to ensure the future durability of the EO-1α lineage of fish. Appendix A of the Approval Letter incorporates requirements of this plan.

IX. FOOD AND FEED SAFETY

A. Overview

In this step of the hierarchal review process, FDA evaluated the data and information submitted in support of a food[20] safety assessment of triploid, all female, GE salmon containing the *opAFP-GHc2* construct, AquAdvantage Salmon (AAS). **ABT salmon** are any GE Atlantic salmon from the E0-1α lineage (including AAS), irrespective of ploidy or gender, and can serve as the source of the broodstock fish. **AAS** are a subset of the ABT salmon, characterized by triploid, hemizygous, all female Atlantic salmon from the E0-1α lineage GE Atlantic salmon and are the particular set of salmon subject to this new animal drug application. FDA has carefully evaluated the data generated from AAS and ABT salmon to determine the food safety of the AAS that are the subject of the current new animal drug application.

This step included information and conclusions drawn from prior steps of the AAS evaluation, as well as data and information evaluated for the identity, composition, level(s) of expression product from the o*pAFP-GHc2* construct, and other potential downstream hazards that may be influenced by the expression product, and allergenicity. This evaluation meets FDA's statutory and regulatory requirements for demonstrating food safety (21 U.S.C. 360b(d)(2), 21 CFR 514.1(b)(8)), as described by Guidance for Industry 187: *Regulation of Genetically Engineered Animals with Heritable Recombinant DNA Constructs*, and is consistent with the Codex Alimentarius Commission's *Guideline for the Conduct of Food Safety Assessment of Foods Derived from Recombinant-DNA Animals* (CAC, 2008). Appendix 3 includes the evaluation of the analytical method used as the regulatory method (i.e., the method used to detect the presence of the *opAFP-GHc2* construct in food). Safe or safety is defined for food additives at 21 CFR 570.3(i) to mean that there is "a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty

[20] For the purposes of this evaluation, "food" refers to human food and animal feed.

the absolute harmlessness of the use of any substance." This same standard is applicable to the food safety review of new animal drugs.

The primary risk question considered in FDA's evaluation was whether there were any risks of direct or indirect effects associated with the consumption of edible products derived from AAS. The conclusions of this assessment are provided in the context of food safety. Accordingly, the most appropriate way in which to consider the primary risk question is to determine whether there is any difference between food from AAS and other Atlantic salmon, and whether food from AAS is as safe as food from other Atlantic salmon. To this end, FDA conducted a weight-of-evidence evaluation of the data and information provided in support of a food safety assessment.

Direct effects, for the purposes of this food safety evaluation, are defined as those that arise from consumption of edible products from the GE animal, including consumption of the o*pAFP-GHc2* construct or its gene expression product (i.e., the Chinook salmon growth hormone). Because nucleic acids, including DNA, are present in the cells of every living organism, including every plant and animal used for food by humans and animals, and do not raise a safety concern as a component of food, nucleic acids are presumed to be generally recognized as safe (GRAS) for food consumption (57 FR 22984, 22990, May 29, 1992). Accordingly, there is no direct food consumption risk associated with exposure to the o*pAFP-GHc2* construct itself. Evaluation of direct food consumption effects was therefore focused on effects associated with consumption of the Chinook salmon growth hormone in food derived from AAS.

Indirect effects, for the purposes of this evaluation, are those effects that can be attributed to the o*pAFP-GHc2* construct or its gene product perturbing the physiology of the animal. These could alter the composition of food and may pose an increased risk compared to consumption of food from appropriate non-GE Atlantic salmon comparators (see later discussion).

The distinction between direct and indirect effects is not always clear-cut, and it may be that the evaluation of certain effects can fit into either or both categories. For the purposes of this evaluation, FDA made the distinctions found in Table 23, which are discussed in more detail later in this section.

Table 23. Characterization of Potential Direct and Indirect Effects

Direct Effects	Indirect Effects
- Alterations from gene expression product (Chinook salmon growth hormone) including - Alterations in levels of hormones associated with the somatotropic axis, including IGF1 - Allergenicity of the gene expression product	- Alterations in the composition of edible tissues - Alterations in the endogenous allergenicity of edible tissues

B. Is AquAdvantage Salmon an Atlantic salmon?

The first step in determining whether any changes in composition introduced as the result of the introduction of the *opAFP-GHc2* construct, or if AAS was more allergenic than other Atlantic salmon, was to determine whether AAS is Atlantic salmon.

An empirical confirmation that AAS is, in fact, an Atlantic salmon can be accomplished by referring to the FDA Regulatory Fish Encyclopedia (RFE). The RFE is a searchable compilation of data in several formats that assists with the accurate identification of fish species that was developed by FDA scientists at the Seafood Products Research Center (SPRC, Seattle District), and the Center for Food Safety and Applied Nutrition (CFSAN) to help federal, state, and local officials and purchasers of seafood identify species substitution and economic deception in the marketplace (available at http://www.fda.gov/Food/FoodScienceResearch/RFE/). Data in the RFE includes high-resolution photographs of the whole fish and marketed products (fillets and steaks), tissue protein patterns determined by isoelectric focusing electrophoresis gels, and mitochondrial DNA sequence patterns determined by DNA barcoding.

The following FDA study evaluated ABT salmon tissue using the RFE standardized approach for fish species identity based on isoelectric focusing gel patterns.

Comparison *of Growth-Hormone Transgenic Fish Atlantic Salmon Salmo salar Edible Tissue with the FDA/CFSAN RFE Standard for Non Transgenic Fish. FDA/Center for Veterinary Medicine Office of Research. Report dated 3 December 2004*.

The goal of this study was to determine whether there were differences in the IEF and 2-dimensional gel (2D gel) electrophoresis fingerprints between non-GE Atlantic salmon and ABT salmon. PCR was used to verify the presence or absence of the *opAFP-GHc2 construct*, based on a probe sequence obtained from the sponsor.

ABT salmon and non-GE Atlantic salmon samples were obtained as blinded samples from the sponsor and stored at -80°C. The sponsor also provided identified samples to use as controls. Reference samples of Atlantic salmon were purchased from a local market and served as internal controls. These samples were also stored at -80°C.

Protein was extracted from the samples and the extracts were used for IEF and 2D gel analyses. Sample identification and the presence of the *opAFP-GHc2* construct were confirmed by the sponsor. The IEF and 2D gel results showed no appreciable differences in banding patterns (see Appendix 3). The finding of identical IEF banding patterns confirmed that the ABT salmon, including AAS, met the standard of identity for Atlantic salmon under the criteria developed for the RFE.

Some differences were noted in the intensity of some of the bands on the 2D gels among all tissues assayed, including within controls. This likely indicated differences in gene expression among the salmon samples. This is expected, as slight gene expression changes within individual animals is a consequence of natural genetic and epigenetic variations. Based on this rationale, these differences in banding intensity are not relevant to the confirmation of the identity of the ABT Salmon, including AAS, as Atlantic salmon.

The following FDA study evaluated AAS muscle-skin tissue using the RFE standardized approach for fish species identity based on mitochondrial DNA barcoding. [*AquAdvantage Barcode Analysis dated 1 November 2012.]*

The goal of this study was to determine whether the new DNA barcode species identification analysis used in all FDA regional laboratories would identify AAS as Atlantic salmon. Frozen AAS skin-on filet was obtained from the sponsor. Two subsamples were used for DNA barcode analysis (Handry et al., 2011), and CFSAN's SOP for FDA Analysis: DNA Based Fish Identification (Barcoding) Method: Version 2: November 2011.
http://www.fda.gov/Food/FoodScienceResearch/DNASeafoodIdentification/ucm237391.htm#SOP

FDA found that AAS matched two FDA reference standards for Atlantic salmon based on the cytochrome *c* oxidase 1 mitochondrial gene currently used by the agency for species identification.

Conclusion: FDA has determined that AAS meets FDA's standard for identity for Atlantic salmon under the criteria established for the RFE by both IEF and mitochondrial DNA bar coding.

C. Hazard Characterization for Food Safety Determination

1. Characterization of Direct Food Consumption Hazards

Based on the hazard identification steps, the only direct hazards identified were those related to the expression product of the introduced *opAFP-GHc2* construct (i.e., Chinook salmon growth hormone) and endogenous substances in the salmon that could be altered as the result of changes in growth hormone expression (e.g., insulin-dependent growth factor 1 (IGF1)). In order to characterize this potential hazard, the sponsor submitted studies that were conducted to measure the levels of the Chinook growth hormone and select other hormones in ABT salmon and non-GE comparator salmon.

a. Analysis of Gene Expression Product

The following section begins with a discussion of information on the gene expression product available from peer-reviewed journals, and continues with studies that the sponsor performed to address this particular issue. The peer-reviewed study is addressed first because it provides a framework for consideration of potential hazards that may be found in AAS.

i. Peer-Reviewed Publication: Du, S.J., A. Gong, G.L. Fletcher, M.A. Schears, M.J. King, D.R. Idler, and C.L. Hew (1992) Growth Enhancement in Transgenic Atlantic Salmon By The Use Of An "All Fish" Chimeric Growth Hormone Gene Construct. Biotechnology (NY). 10(2):176-181.

The developers of the lineage of growth hormone (GH) transgenic Atlantic salmon that ultimately became AAS published a report in 1992 in which selected plasma hormone levels were measured. The GH transgenic Atlantic salmon used in this study were derived from the same parental animals from which the EO-1α lineage was eventually derived. This study reported on 500 GE and non-GE Atlantic salmon, resulting from Atlantic salmon eggs injected with the ocean pout antifreeze protein (AFP) promoter-Chinook salmon GH construct in November, 1989. The 500 Atlantic salmon were selected for PCR analysis with the 200 heaviest (largest) GH transgenic Atlantic salmon selected. All salmon weighing more than eight grams (n=14) had blood samples drawn and were tagged; 36 additional fish (weighing more than five grams) were also bled and tagged. Of these total 50 fish, six were shown to contain the construct in both their red blood cells and in their scales. (One additional fish appeared to be a mosaic, containing the construct in its scales, but not in its

red blood cells, and was eliminated from further analysis). Body weight and plasma levels of growth hormone and triiodothyronine (the thyroid hormone often referred to as T3) were determined. Control salmon derived from non-injected eggs of approximately the same age were also weighed and plasma concentrations of the same two hormones were analyzed.

Body weights and plasma concentrations of growth hormone and T3 measured on January 12, 1991 are abstracted from Table 1 of the publication and provided below in Table 24.

Table 24. Body Weight and Plasma Concentrations of Growth Hormone and T3

	GH (ng/ml)	T3 (ng/ml)	Fish Weight (g)
GH Transgenic Atlantic salmon	39.9 ± 14.8 (5)	1.1 ± 0.5 (5)	*47.3 ± 9.5 (6)*
Non-GE siblings & P-value	28.2 ± 8.8 (7) NS	2.8 ± 0.5 (5) <0.05	*9.48 ± 0.6 (43)* *<0.01*
Controls & P-value	20.5 ± 7.97 (5) NS	1.9 ± 0.1 (3) NS	*10.4 ± 0.6 (10)* *<0.01*

Growth hormone (GH) and triiodothyronine (T3) measured from the five largest GH transgenic Atlantic salmon, the largest non-GE siblings, and five blood samples pooled from two control salmon derived from non-injected eggs. Values presented as mean ± standard error. Statistical comparisons were made between the GH transgenic Atlantic salmon and their non-GE siblings or the controls (method of analysis not reported).

Mean plasma growth hormone concentrations did not differ statistically between the GH transgenic Atlantic salmon used in this study and either comparator (non-GE siblings or controls). Mean plasma T3 concentration in the GH transgenic Atlantic salmon was statistically different from their non-GE siblings, but not statistically different from the farm control salmon. The authors noted that plasma GH levels ranged from 9.5 to 91.4 ng/ml in the GH transgenic Atlantic salmon, with the largest salmon having the lowest concentrations. Similarly, the three largest GH transgenic Atlantic salmon had much lower plasma T3 concentrations (0.26 ± 0.18 ng/ml) compared to the two smaller GE Atlantic salmon (2.34 ± 0.14 ng/ml).

The authors noted that GH enhances conversion of thyroxine (T4) to triiodothyronine (T3) in eels, and speculated that a similar action may occur in salmon. They proposed that there might be a negative feedback loop between GH and T3 that diminished T4 production, and that decreased T3 levels may serve as an indicator of expression of the GH from the inserted *opAFP-GHc2 construct*. They further proposed that the ocean pout promoter provided tissue specific expression in the liver, facilitating more efficient interaction between GH and the GH receptors in liver cells. This, in turn, may permit very low GH expression (with little or no increase in plasma concentration) to stimulate expression of IGF1, resulting in increased growth. Possible support for the proposed mode of action is provided in the publication by Hobbs and Fletcher (2008).

Regardless of the mechanism, the authors concluded that there was no correlation between blood plasma GH levels and growth rates or the presence of the transgene (the *opAFP-GHc2 construct*).

ii. The sponsor's Study: Determination of IGF1, GH, T3, T4, 11-Keto Testosterone, Testosterone, and Estradiol in Salmon Tissue. CTBR Bio-Research Inc. Canada. Project Number 42361. Study Report AAS-HFS-001. Report dated 26 July 2004.

The purpose of this Good Laboratory Practice (GLP)-compliant study was to determine the concentration of IGF1, GH, estradiol, testosterone, T3, T4, and 11-ketotestosterone in salmon muscle and skin. Tissue samples were taken from a total of 73 diploid and triploid market-size salmon (10 farm-raised control, 33 sponsor control, and 30 ABT salmon). Validation information for each of the assay methodologies and calibration data for the performance of the actual assays were provided. Calibration curve parameters, calculated concentrations for the standards, and calculated concentrations for the quality control samples were provided.

Table 25 provides the summary of units, limits of quantitation, and limits of detection for the assays.

Table 25. Assay Parameters for Selected Hormones in Salmon Tissue

		Assay parameters* Concentration in assay specific units		**Assay parameters* Concentration (ng/mL)**		**Amount per gram of tissue (ng/g)***	
Analyte	**Tracer**	**$LLOQ_{assay}$**	**$ULOQ_{assay}$**	**$LLOQ_{assay}$**	**$ULOQ_{assay}$**	**$LLOQ_{tissue}$**	**LOD_{tissue}**
IGF1	^{125}I-IGF1†	1,500.0	50,000.0	1.500	50.00	3.27	2.18
GH	^{125}I-GH†	2,500.0	20,000.0	2.500	20.00	10.40	6.24
Estradiol	^{125}I-estradiol†	17.5	1,800.0	0.0175	1.80	0.018	a
Testosterone	^{125}I-testosterone‡	36.4	1,018.3	0.459	10.18	0.46	a
T3	^{125}I-T3‡	36.4	584.0	0.364	5.84	0.36	a
T4	^{125}I-T4#	1.5	15.0	15.000	150.00	15.00	a
11-keto testosterone	11-keto testosterone-acetylcholin-esterase†	18.9	850.2	0.019	0.85	0.019	a

* LLOQ=lower limit of quantitation, ULOQ=upper limit of quantitation, LOD=limit of detection
† Assay units are pg/mL
‡ Assay units are ng/dL
# Assay units are µg/dL
a = not determined

The mean concentration of IGF1, growth hormone, estradiol, testosterone, T3, T4, and 11-keto testosterone for the farm control, sponsor control, and "treated" (i.e., GE) fish (each respective group pooled for gender and ploidy) are summarized in Table 26.

Table 26. Summary of Contractor's Hormone Analysis

Variable	**Group**	***N***	**mean**	**std**	**min**	**max**
Estradiol	GE	20	0.36	0.375	0.02	1.32
Estradiol	SControl	22	0.38	0.439	0.02	1.85
Estradiol	FControl	5	0.04	0.019	0.02	0.06
Growth hormone	GE	0	<LOQ	n/a	n/a	n/a
Growth hormone	SControl	0	<LOQ	n/a	n/a	n/a

Variable	Group	*N*	mean	std	min	max
Growth hormone	FControl	0	<LOQ	n/a	n/a	n/a
IGF1	GE	6	10.26	4.971	3.97	18.43
IGF1	SControl	11	7.34	2.818	3.56	12.24
IGF1	FControl	0	<LOQ	n/a	n/a	n/a
11-keto testosterone	GE	29	86.21	92.490	20.76	389.52
11-keto testosterone	SControl	33	71.42	87.302	21.00	380.53
11-keto testosterone	FControl	10	55.27	30.357	23.17	101.97
T3	GE	26	0.85	0.312	0.44	1.59
T3	SControl	28	0.84	0.270	0.41	1.57
T3	FControl	10	1.31	0.505	0.73	2.01
T4	GE	2	19.65	0.426	19.35	19.95
T4	SControl	2	19.96	3.746	17.32	22.61
T4	FControl	2	18.52	1.320	17.58	19.45
Testosterone	GE	25	1.06	0.476	0.46	2.21
Testosterone	SControl	30	1.17	0.692	0.55	3.35
Testosterone	FControl	10	1.01	0.646	0.52	2.68

N = number of fish sampled with values above LOQ
SControl = Sponsor control (non-GE fish)
FC = Farm-raised fish control (non-GE fish)

Growth hormone was below the limit of quantitation in all samples, whether in the treated (GE), sponsor control, or farm-raised control groups. Even if slightly increased but undetected levels of Chinook salmon growth hormone are present, however, they do not pose a food consumption risk to humans because fish growth hormone does not bind to mammalian growth hormone receptors, nor does it have any biological activity in mammalian systems (Liu et al., 2001, Souza et al., 1995; also see Table 1 of Appendix 4 for lack of degree of homology).

Treated (GE) salmon did not have statistically different concentrations of estradiol, testosterone, 11-ketotestosterone, T3, or T4 when compared to sponsor control fish.

Initial evaluation of the results suggested that there may have been an increase in the level of IGF1 in the GE fish compared to sponsor control fish. A further evaluation of the data showed that the most apparent potential differences were between the mature diploid sponsor control and the mature diploid GE salmon. The individual values are reproduced in Table 27.

Table 27. IGF1 Levels in Mature Diploid Salmon
Individual results (ng/g) (LOQ = 3.27 ng/g)

Sponsor control	6.191	6.980	7.642	8.784	9.485	10.928	12.235
GE salmon	<LOQ	3.971	6.350	10.527	10.718	11.578	18.428

Summary Statistics	N*	Mean	Std. Dev.	Min > LOQ	Max
Sponsor control	7	8.892	2.167	6.191	12.235

Summary Statistics	N*	Mean	Std. Dev.	Min > LOQ	Max
GE salmon	7	9.263	5.251	3.971	18.428

*concentrations below the LOQ were included as the LOQ value

Although there did not appear to be a statistically significant difference between the mean IGF1 level for ABT and non-GE salmon, the range of values for ABT salmon exceeded that of the non-GE salmon by more than 10%. As part of the heuristic[21] method applied to assessing data and information, FDA established the paradigm of assessing the biological relevance of any measurement if it exceeded the comparator range by 10% or more[22].

One possible explanation for the observed difference in concentrations could be due to differences in body weights of the sampled fish with constant hormone levels. This did not appear to be the case, as body weights were shown to be similar across groups as seen in Table 28.

Table 28. Range of Body Weights

Group	Range of Body Weights (g)
Farm control diploid Atlantic salmon	3,972 - 5,786
Farm control triploid Atlantic salmon	3,938 - 6,604
Sponsor control diploid Atlantic salmon	2,748 - 6,896
Sponsor control triploid Atlantic salmon	2,133 - 4,286
ABT diploid Atlantic salmon	2,867 - 5,813
ABT triploid Atlantic salmon	2,061 - 5,865

Because the IGF1 levels of the mature diploid ABT salmon results exceeded the IGF1 levels of the mature diploid sponsor control salmon results by more than 10%, FDA conducted a margin of exposure assessment (MOE) in order to determine whether the observed differences are biologically relevant.

iii. Margin of Exposure for IGF1

IGF1 is an endogenous hormone that is closely linked with growth hormone expression and circulating levels (Frost and Lang, 2003). It has been considered as a potential hazard for human consumption following increased growth hormone levels in food producing animals (Juskevich and Guyer, 1990; USFDA, 1993). Although growth hormone levels were not shown to be significantly different in the ABT salmon compared to non-GE fish, in order to ensure that the other potentially hazardous constituents along the somatotropic axis (i.e.,

[21] A heuristic method is one that is modified based on experience in order to learn or provide feedback. In this context, the heuristic method employed was to choose a 10 % exceedance of the reference range as the trigger that initiated a closer look to determine whether that exceedance was biologically relevant.

[22] The 10% exceedance is a level FDA customarily uses for further investigation to determine whether there is a biological significance beyond the statistical signficance. (FDA, 2008. Animal Cloning, A Risk Assessment. http://www.fda.gov/downloads/animalveterinary/safetyhealth/animalcloning/ucm124756.pdf) . This additional evaluation does not imply that beyond a 10% difference there is an *a priori* safety concern.

IGF1) were not sufficiently elevated to constitute a food consumption hazard, FDA performed a margin of exposure assessment (MOE).

MOE assessments are often performed to determine whether exposures to a particular substance or component of the food(s) under consideration fall within the range of daily exposures or are different from those in the comparator group and, if so, whether the difference is expected to result in an adverse outcome.

MOE assessments are best performed considering both maximum likelihood and plausible upper-bound estimates of exposure. Maximum likelihood estimates consider central tendencies of intake estimates (i.e., medians or means), while plausible upper-bound limits often take the form of 95th percentile intake estimates. Both are useful in coming to conclusions regarding population exposures and characterizing the potential for substances in food to pose hazards, as they take into account level of intake for "average" and "high end" consumers.

In general, for purposes of this assessment, FDA used conservative (health protective) assumptions and defaults when data were lacking, or where inferences regarding direct or proportional intake needed to be made. For example, because there are no reliable data on the intake of Atlantic salmon, and no GH GE Atlantic salmon have been marketed, one of the key assumptions FDA made in the initial MOE evaluation was that all of the fish consumed were Atlantic salmon, and that all of those salmon were ABT salmon. The agency assumed that all of the salmon consumed contained IGF1 at the maximum concentration identified in the one outlier mature diploid salmon presented in Table 29. Subsequent analyses considered less than the upper-bound estimates by using less conservative assumptions.

The results of these analyses are found in Table 29; narrative descriptions of the information, data, and assumptions used follow immediately.

Daily human consumption of non-tuna finfish has been estimated to be 300 g per day for the 95th percentile eaters of finfish in the United States (USDA 2002). The agency made the conservative (health protective) assumption that all of the finfish consumed were salmon, and adjusted that consumption value for the fraction of salmon consumed estimated to be Atlantic salmon (approximately 2/3) or 200 g per day (Knapp et al., 2007).

The upper bound for IGF1 consumption may then be estimated assuming that all salmon contain the maximum tissue levels detected in the mature diploid sponsor control Atlantic salmon and mature diploid ABT salmon[23]. The incremental increase calculated from the difference in residue concentrations between the mature diploid ABT salmon and the mature diploid sponsor control Atlantic salmon was also determined. This difference is the MOE between IGF1 in non-GE Atlantic salmon and diploid ABT salmon and is presented in Table 26.

[23] These assumptions were made to ensure that the analysis was "conservative"; that is, health protective, by chosing values that would tend to overestimate rather than underestimate exposures.

In estimating the "natural background" levels of IGF1, FDA chose teenaged boys as the most "sensitive" population based on their biological sensitivity to the effects of IGF1 due to their rapid growth and development, and their tendency to consume adult portions of food despite a lower body weight (Ungemach, 1998)[24].

The results of this analysis are found in Table 29.

Table 29. Margin of Exposure Estimates of IGF1 in ABT Salmon

Description	Calculation	Value/Outcome
Daily non-tuna finfish consumption for the 95th percentile eater[1]	-	300 g/day
Assuming all consumed non-tuna finfish are salmon, consumption corrected for fraction of Atlantic salmon	2/3 * 300g/day = 200 g/d	200 g/d
Upper bound of IGF1 residue found at the maximum level in sponsor control mature diploid Atlantic salmon	-	12.235 ng/g of muscle
Upper bound IGF1 residue in mature diploid ABT salmon	-	18.428 ng/g of muscle
Daily consumption of IGF1 from Atlantic Salmon	200 g/d * 12.235 ng/g =2,447 ng/d ~ 2.4 µg/d	2.4 µg/day
Daily consumption of IGF1 from ABT salmon	200 g/d *18.428 ng/g =3,686 ng/d ~ 3.7 µg/d	3.7 µg/day
Incremental increase in daily consumption of IGF1I	3.7 µg/d – 2.4 µg/d =1.2 µg/d	1.2 µg/day
Calculated total serum burden of IGF1 in 50 kg teenager[3]	1,220,000 ng = 1,220 µg	1,220 µg/person
Margin of Exposure (MOE)	Total serum burden/ Dietary contribution	-

[24] In the evaluation of the safety of bovine somatotropins, the 41st Joint Food and Agriculture Organization of the United Nations/ World Health Organization Expert Committee on Food Additives (JECFA) considered the daily production of IGF1 in different age groups, and estimated by calculation the total serum burden for a 15 kg child (50,000 ng), a 60 kg adult (714,000 ng) and a 50 kg teenager (1,220,000 ng), considering the mean IGF1 concentration in plasma, and assuming blood volume to be 5% of body weight.

Description	Calculation	Value/Outcome
MOE for mature diploid (non GE) Atlantic salmon	1,220 µg/2.4 µg/day = 508	508-fold (fractionally 2.0 x 10^{-3})
MOE for mature diploid ABT salmon	1,220 µg/3.7 µg/day =330	330-fold (fractionally 3.0 x 10^{-3})

[1]US Department of Commerce (2002)
[2]Knapp et al (2007)
[3]Ungemach (1998)

Comparisons to other sources of IGF1 from other finfish or food producing animals are also appropriate to provide some context for this analysis. This information is summarized in Table 30.

Table 30. IGF1 levels in Various Foods

Species	Source (tissue)	units	Range	Mean
Chinook salmon[1]	Plasma	ng/ml	5-35	Intentionally Blank
Coho salmon[2]	Plasma	ng/ml	7-13	Intentionally Blank
Coho salmon[3]	Plasma	ng/ml	10-15	Intentionally Blank
Gilthead Bream[4]	Plasma	µg/L	36-100[5]	Intentionally Blank
Bovine[6]	Raw milk	ng/ml	Intentionally Blank	5.6 ± 0.56
Bovine[6]	Pasteurized milk	ng/ml	Intentionally Blank	8.2 ± 0.35
Bovine[6]	Raw bulk milk	ng/ml	1.27-8.10	4.32 ± 1.09
Homo sapiens[6]	Milk	ng/ml	1 d post partum 17.6 2 d 12.8 3 d 6.8 6-8 wk 13-40	19
Chum salmon[7]	Plasma	ng/ml	Depends on maturity/sex/month: varies between 16.5 and 100	Intentionally Blank
Rainbow trout (O.kiss)[8]	Plasma	ng/ml	Function of temperature/time Lowest value 11.2 Highest 33.6	Intentionally Blank
Japanese beef cattle[9]	Plasma	ng/ml	*Intentionally Blank*	Preweaning 11.7± 3.6 Postweaning 50.5 ± 2.1
Homo sapiens[10,11]	Plasma	nmol/L	Meat-eaters 29.3-32.7 Vegetarians 29.5-32.9 Vegans 25.5-28.6	Intentionally Blank
Polish Holstein[12]	Plasma	ng/ml	698-1024	Intentionally Blank

[1]Beckman B.R., K.D. Shearer, K.A. Cooper, and W.W. Dickhoff (2001) Relationship of insulin-like grown factor-I and insulin to size and adiposity of under-yearling Chinook salmon. *Comp. Biochem. Physiol.* Part A 129:558.
[2]Shimizu M., P. Swanson, and W.W. Dickhoff (1999) Free and Protein-bound insulin-like Growth Facor-I and IGF-binding proteins in plasma of Coho salmon. *Gen. Comp. Endocrinol.* 115:398.

[3] Pierce A. L., B.R. Beckman, K.D. Shearer, D.A. Larsen, and W.W. Dickhoff (2001). Effects of ration on somatotropic hormones and growth in Coho salmon. Comp. Biochem Phys. 128:255/
[4]Perez-Sanchez J., H. Marti-Palanca, and S.J. Kaushik (1995) Ration size and protein intake affect circulating Growth Hormone concentration, Hepatic Growth Hormone binding and plasma Instulin-like Growth Factor-I immunoreactivity in a marine teleost, the Gilthead sea bream. *J. Nutr.* 125:546.
[5]Fish were fed several experimental diets; no information was provided on how these diets differed from standard commercial diets.
[6]Juskevich, J.C. and C.G. Guyer (1990) Bovine Growth Hormone: Human Food Safety Evaluation. *Science* 249:875.
[7]Onuma T.A., K. Makineo, H. Katsumata, B.R. Beckman, M. Ban, H. Ando, M.A. Fukuwaka, T. Azumaya, P. Swanson, and A. Urano (2010) Changes in the plasma levels of insulin-like growth factor-I from the onset of spawning migration upstream migration in chum salmon. *Gen. Comp. Endocrinol.* 165:237.
[8]Gabillard J.C., C. Weil, P.Y. Rescan, I. Navarro, J. Gutierrez, and P.Y. Le Bail (2003) Effects of environmental temperature on IGF1, IGF2 and IGF type I receptor expression in rainbow trout. *Gen. Comp. Endocrinol.* 133:233.
[9]Suda Y., K. Nagaoka, K. Nakagawa, T. Chiba, F. Yusa, H. Shinohara, A. Nihei, and T. Yamagishi (2003) Change of Plasma insulin-like growth factor-1 concentration with early growth in Japanese beef cattle. *Animal Sci J.* 74:205.
[10]Allen, N.E., P.N. Appleby, G.K. Davey, R. Kaaks, S. Rinaldi, and T.J. Key (2002) The associations of diet with serum insulin-like growth factor I and its main binding proteins in 292 women meat-eaters, vegetarians and vegans. *Cancer Epidemiol. Biomarkers Prev.* 11:1441.
[11]Crowe F.L., T.J. Key, et al. (2009) The association between diet and serum concentrations of IGF-I, IGFBP-1, and IGFBP-3 in the European Prospective investigation into cancer and nutrition. *Cancer Epidimiol Biomarkers Prev.* 18:1333.
[12]Maj A. and M. Snochowski, E. Siadkowska, B. Rowindska, P. Lisowski, D. Robakowska-Hyzorek, J. Oprzadek, R. Grochowska, K. Kochman, and L. Zweirzchowski (2008) Polymorphism in genes of growth hormone receptor (GHR) and insulin-like growth factor-1 (IGF1) and its association with both the IGF1 expression in liver and its level in blood in Polish Holstein-Friesian cattle. *Neuro. Endocrinol. Lett.* 29:981.

Results

The calculated estimate of the upper bound dietary exposure to IGF1 via the consumption of non-GE Atlantic salmon and ABT salmon was 2.4 and 3.7 µg per day, respectively.

The MOE for dietary consumption of **Atlantic salmon (non-GE)** is 1,220 µg/2.4 µg per day, which yielded a **508-fold margin of exposure**, equivalent to approximately two one-thousandths (0.002) of the total serum burden. The MOE for dietary consumption of IGF1, assuming that IGF1 was present at the **maximum concentration recorded from the mature diploid ABT salmon** cohort is 1,220 µg/3.7 µg/d, yielding a **330-fold margin of exposure,** which corresponded to approximately 0.003 of the total serum burden.

Finally, calculation of the incremental increase in IGF1 exposure from the maximum estimated GE salmon intake relative to IGF1 exposure from the study comparator yielded only 1.2 µg per day or 0.001 of the total serum burden.

Conclusions:

The only direct food consumption hazards FDA could identify were those related to the expression product of the introduced *opAFP-GHc2* construct (i.e., Chinook salmon growth hormone) and endogenous substances in the salmon that could be altered as the result of changes in growth hormone expression (e.g., insulin-dependent growth factor 1 (IGF1)). In order to characterize this potential hazard, the sponsor submitted studies that were conducted to

measure the levels of the Chinook growth hormone and select other hormones in ABT salmon and non-GE comparator salmon.

No additional food consumption risks were identified for the consumption of growth hormone or hormones associated with the somatotropic axis for diploid ABT salmon compared to non-GE salmon. Risk is a function of both hazard and exposure, and the conclusion on food consumption risks was based on the MOE evaluation in the context of non-GE Atlantic salmon.

As supporting information, Du *et al.* demonstrated that plasma growth hormone concentrations did not differ statistically between the GH transgenic Atlantic salmon (genetically engineered to contain the same construct as the ABT salmon) and either age-matched non-GE siblings or pooled control samples from age-matched siblings. Mean plasma T3 concentrations in the GH transgenic Atlantic salmon were statistically different from and lower in the GH transgenic Atlantic salmon compared to non-GE siblings but were not lower than T3 concentrations in the pooled controls. The highest plasma GH levels correlated with the largest GH transgenic Atlantic salmon while an inverse correlation was shown for mean plasma T3 concentrations.

From the sponsor's study, mean levels of estradiol, testosterone, 11-ketotestosterone, T3, and T4 were not different in the ABT salmon than levels in comparator non-GE salmon (Table 22). Growth hormone was below the limit of quantitation in all samples, whether in the treated (GE), sponsor control, or farm control groups. No additional food consumption risks were identified for the consumption of growth hormone or hormones associated with the somatotropic axis for diploid ABT salmon, including IGF1, compared to non-GE salmon.

Further, even if there were increases in the amounts of Chinook or Atlantic salmon growth hormone, which are normally occurring, the increases would not likely effect any biologically meaningful interactions with human growth hormone receptors due to interspecies differences that result in an inability of salmonid growth hormone to bind to mammalian (including human) growth hormone receptors (see Figure 1 in Appendix 4 and the references cited therein). This lack of biological interaction is likely to also be true for mammalian and avian food producing species that could possibly eat animal feed made up of ABT salmon (see Appendix 4), and would pose no additional food consumption risks for humans or animals consuming edible products from these fish.

FDA concludes that even if the expression of IGF1 were present at the highest levels measured, and even if expected high consumers of salmon ate nothing but ABT salmon containing this likely upper bound level of IGF1, the margin of exposure to this endogenous component of food would be well within levels of exposure from other dietary sources of IGF1, and poses no additional risk.

AAS differ from diploid ABT salmon in ploidy as well as being limited to hemizygous female salmon. FDA has concluded that the assessment of food consumption risks for the consumption of growth hormone and other hormones of the somatropopic axis based on diploid ABT salmon represents a conservative estimate of the consumption risk for these potential hazards provided through consumption of AAS.

b. Potential Allergenicity of Gene Expression Product

There are two major questions with respect to the allergenicity of food from an rDNA organism: (1) the potential allergenicity of the newly expressed protein(s) present in the food, and (2) the endogenous allergenicity of the food from the recipient organism. FDA considered the potential allergenicity of the gene expression product, Chinook salmon growth hormone (see below), as it qualified as a direct food consumption hazard. FDA's consideration of the allergenicity of food from the recipient organism is addressed under section C2 below, "Characterization of Indirect Food Consumption Hazards."

The Codex rDNA Animal Guideline describes a conservative (health protective) approach to determining whether a newly expressed protein present in a food from an rDNA organism is likely to pose an allergenic risk. This assessment strategy includes the following three main components:

- allergenicity of the gene source;
- structural similarity to known allergens; and
- resistance to proteolytic degradation (2008).

i. Consideration of Allergenicity of Gene Source

In general, the initial step in assessing the allergenicity of a newly expressed protein in a food from a GE organism involves information regarding the history of allergic reaction of humans to the source (i.e., organism) from which the transferred gene is isolated. Transfer of a gene from an allergenic source may create a new risk for those individuals allergic to the gene source because those individuals may experience allergic reactions to foods from the GE organism which contain the transferred gene in addition to allergic reactions to the allergenic source and products derived from it (Nordlee et al., 1996).

In the case of ABT salmon, the introduced growth hormone gene was isolated from Chinook salmon (*Oncorhynchus tshawytscha*). Chinook salmon are finfish, and finfish are one of the eight major food allergens in the U.S. (FALCPA, 2004; Hefle et al., 1996). Although salmon growth hormones have not to-date been identified as allergenic proteins, each allergenic food contains multiple allergenic proteins, many of which have not been identified or fully characterized (Gendel, 1998). Therefore, FDA made the conservative (health protective) assumption that the transferred Chinook growth hormone was a putative salmon allergen. It is important to note, however, that individuals allergic to Chinook salmon also would likely be allergic to Atlantic salmon (*Salmo salar*). Because salmon present a hazard to salmon-allergic individuals, salmon-allergic individuals will likely avoid consumption of all salmon, including AAS.

ii. Analysis of Structural Similarity of Gene Product to Known Allergens

In general, approaches to assessing the allergenicity of a newly expressed protein in food from a GE organism recommend comparing the structure of the gene product to that of known allergens in order to evaluate potential IgE cross-reactivity. The Codex rDNA Animal Guideline provides guidance on how to conduct protein database searches to detect any significant amino acid sequence homologies, defined as greater than 35% identity in a segment of 80 or more amino acids, or other scientifically justified criteria (CAC, 2008). The

Codex rDNA Animal Guideline also discusses searching stepwise contiguous identical amino acid segments as these may represent linear IgE-binding epitopes.

To evaluate the potential cross-reactivity of the Chinook salmon growth hormone with known allergen protein sequences, FDA conducted searches of the AllergenOnline database version 10 (released January 2010) (www.allergenoline.org) and the Structural Database of Allergenic Proteins (updated April 16, 2010) (www.fermi.utmb.edu/SDAP/) using deduced peptide sequences from GenBank (protein ID AAT02409.1 and AAW22586.1). These searches revealed no amino acid sequence identities of greater than 35% in segments of 80 amino acids with any entries in either database. In addition, there were no matches of eight or more contiguous amino acids with any entries in either database. Because this search, using the Codex rDNA Animal Guideline, did not identify significant amino acid similarities, it is unlikely that the introduced growth hormone in AAS or other ABT salmon will pose an allergenic risk greater than that posed by non-GE salmon.

iii. Resistance to Proteolytic Degradation

A number of food allergens have been shown to be resistant to degradation by pepsin (Astwood et al., 1996). Because of this correlation between allergenic potential and resistance to pepsin digestion, newly expressed proteins in foods from rDNA organisms are typically assessed for resistance to pepsin.

For ABT salmon, the newly expressed protein is the native Chinook salmon growth hormone. There is no scientific rationale to suggest an altered resistance to pepsin when the protein is expressed in Atlantic salmon rather than in Chinook salmon. For this reason, FDA found the pepsin resistance assay to be unnecessary.

Conclusion: The expression of Chinook salmon growth hormone in ABT salmon (including AAS) does not present a new risk of allergic reaction to salmon allergic individuals and is unlikely to cause allergic cross-reactions.

c. Summary of and Conclusions from the Identification and Characterization of Direct Food Consumption Hazards

Only growth hormone and other hormones associated with the somatotropic axis (IGF1, estradiol, testosterone, 11-ketotestosterone, T3, and T4) were identified as potential hazards for the consumption of ABT salmon in food.

Du *et al*. demonstrated that mean plasma growth hormone concentrations did not differ statistically between the GH transgenic Atlantic salmon (genetically engineered to contain the same construct as the ABT salmon) and either age-matched non-GE siblings or pooled control samples from age-matched siblings. Mean plasma T3 concentrations in the GH transgenic Atlantic salmon were statistically different from and lower in the GH transgenic Atlantic salmon compared to non-GE siblings but not when compared to the pooled controls. The highest plasma GH levels correlated with the largest GH transgenic Atlantic salmon while an inverse correlation was shown for mean plasma T3 concentrations.

Mean concentrations of estradiol, testosterone, 11-ketotestosterone, T3, and T4 were not different in the ABT salmon compared with comparator non-GE salmon (Table 25). The apparent difference in IGF1 in mature diploid ABT salmon compared to sponsor control non-GE salmon was small. Analysis of concentrations of the IGF1 via the MOE evaluation showed that even the highest measured concentrations would not be biologically significant in the background of the existing systemic and dietary hormonal milieu for the consumer. No differences were observed in concentrations of growth hormone in edible tissues at the level of quantitation for the analytical method.

Further, even if there were increases in the amounts of normally occurring Chinook or Atlantic salmon growth hormone, they would not likely effect any biologically meaningful interactions with human growth hormone receptors due to interspecies differences in the ability of these substances to bind to homologous receptors in mammals or to cause physiological changes via such binding. This lack of biological interaction is likely to also be true for mammalian and avian food producing species that could possibly eat animal feed containing ABT salmon (see Appendix 4).

FDA concluded that the expression of Chinook salmon growth hormone in ABT salmon, which include AAS, does not present a new risk of allergic reaction to salmon allergic individuals and is unlikely to cause allergic cross-reactions. No direct food consumption hazards were identified.

2. Characterization of Indirect Food Consumption Hazards

Indirect food consumption hazards and any indirect food consumption risks are those that might arise as the result of changes that occur following the insertion of the *opAFP-GHc2* in AAS.

Based on the responses to the risk questions posed in the other steps of the hierarchical review (molecular characterization of the construct, molecular characterization of the construct in the GE animal lineage, phenotypic evaluation), FDA identified no indirect food hazards.

a. Compositional Analysis

Compositional analyses are a longstanding and well-established approach for assessing the safety of novel foods. Compositional analyses permit an assessment of potential indirect effects that may result from the insertion of an rDNA construct into the genome of an rDNA organism that may impact the safety of foods from that organism. These analyses typically include an assessment of the levels of key nutrients, constituents in the particular food that may have a substantial impact in the overall diet, as well as key toxicants where applicable. Such analyses allow for an assessment of potential nutritional and toxicological risk that may result from changes in significant compositional constituents in the food (CAC, 2008).

An indirect effect of the genetic engineering that could result in a GE animal may be a change in the composition of the edible tissues. Such a change may pose a hazard to humans by altering the expected nutritional composition of the food, or it may serve as a signal that an underlying change in the metabolism or physiology of the animal has occurred that may pose a toxicological hazard to humans. Either of these may pose nutritional or toxicological risks via the consumption of edible products from the animal.

The sponsor's approach to address potential indirect toxicity associated with the AAS was to evaluate compositional differences between the ABT salmon and non-GE Atlantic salmon. Potential adverse outcomes with respect to consumption of ABT salmon addressed in this section included biologically relevant changes in the proximate, vitamin, mineral, amino acid or fatty acid composition of edible tissues from ABT salmon that might suggest toxicological or nutritional concerns compared with levels of these analytes in comparator non-GE Atlantic salmon. A compositional analysis study was provided by the sponsor.

i. A Single-Blind, Comparator-Controlled, Quantitative Analysis of the Composition of Muscle Skin from Diploid and Triploid Atlantic Salmon (Salmo salar) Modified Transgenically with the AquAdvantage Gene Cassette (opAFP-GHc). Covance Laboratories Inc., Wisconsin. Covance Study Identification 7352-100. Study Report AAS-HFS-001. Report dated 22 January 2003.

A total of 144 market-sized (2.0 to 7.5 kg) Atlantic salmon were included in the study: diploid and triploid ABT salmon (referred to as "treated", "TX" by the sponsor) and non-GE Atlantic salmon from the sponsor's facility (referred to as "sponsor control", "SC" by the sponsor); and non-GE diploid and triploid Atlantic salmon from commercial farms in Maine and Canada (referred to as "farm control", "FC" by the sponsor). TX and SC salmon were bred and reared in the sponsor's facilities; these salmon were not raised in the same tank, but were distributed in different tanks according to their ploidy and the presence/absence of the *opAFP-GHc2 construct*. TX and SC salmon were fed one of three different diets; MCO, MCAB, or MCA (described subsequently). Husbandry conditions, including diets, of FC salmon were proprietary and therefore not available to the sponsor. Salmon were collected for the study at two different times (in October 2001 and June 2002).

Salmon were screened visually for general health status and traits relevant to commercial marketability, including skin and fin condition, color and markings, and general body morphology. Because of differences in rates of growth to market size, TX and control (SC and FC) fish that were weight-matched may not have been age-matched.

Screening, harvesting, measurements, necropsy, genotype and ploidy analyses were performed by the sponsor for TX and SC salmon. For FC salmon, screening, harvesting, measurements and necropsy were performed by the salmon farm; the sponsor performed genotype and ploidy analyses. Blind-coded salmon fillets were frozen and stored at -70ºC. Frozen samples were shipped to two different testing laboratories for compositional analysis and hormone analysis.

Tissue samples from a total of 73 salmon were analyzed for proximates, mineral, vitamin, amino acid and fatty acid content. See Section IX C 2 a iii, below, for selection criteria. Validation information for each of the assay methodologies was provided. Precision and accuracy results for these analyses are provided. Table 31, below, provides the lower limit of quantitation for the analytes. This study was conducted in compliance with GLPs.

Table 31. Lower Limit of Quantitation ($LLOQ_{assay}$) for Analytes for Compositional Analysis

Analyte	$LLOQ_{assay}$
Total (individual) amino acids	0.010 g/100 g (0.010%)
Ash	0.1%
Total carbohydrate	0.10%
Fat by Soxhlet Extraction	0.10%
Fatty acids as triglycerides	0.004%-0.020%*
Folic acid	0.06 µg/g
Free fatty acids by titration	0.01%
Calcium	1.00 mg/100g
Copper	0.0250 mg/100g
Iron	0.100 mg/100g
Magnesium	1.00 mg/100g
Manganese	0.0150 mg/100g
Phosphorus	1.00 mg/100g
Potassium	5.0 mg/100g
Sodium	5.00 mg/100g
Zinc	0.0200 mg/100g
Moisture	0.1%
Niacin	0.3 µg/g
Pantothenic Acid	0.4 µg/g
Protein	0.1%
Selenium	0.030 ppm
Vitamin A	50.0 IU/100g
Vitamin B1	0.01 mg/100g
Vitamin B2	0.2 µg/g
Vitamin B6	0.07 µg/g
Vitamin B12	0.0012 µg/g
Vitamin C	1.0 mg/100g

* The lower limit of quantitation for fatty acids was dependent upon the amount of fat extracted from the sample.

ii. Summary of the Compositional Analysis Results of Study AAS-HFS-001

The arithmetic mean and standard deviation and the maximum and minimum values of compositional analytes (proximates, vitamins, minerals, amino acids and fatty acids) for the farm control (FC), sponsor control (SC) and ABT salmon (TX) are summarized in Tables 32-36 below. Analysis of each group is pooled for gender and ploidy.

Table 32. Results of Analysis of Proximate Analytes in ABT (TX), Non-GE Sponsor Control (SC) and Non-GE Farm Control (FC) Salmon

Analyte	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
Carbohydrate	FC	9	0.46	0.357	0.1	1
Carbohydrate	SC	22	0.37	0.167	0.1	0.6
Carbohydrate	TX	16	0.38	0.335	0.1	1.3
Ash	FC	10	1.13	0.164	0.9	1.4
Ash	SC	33	1.18	0.160	0.8	1.4
Ash	TX	30	1.14	0.218	0.7	1.6
Moisture	FC	10	64.4	2.068	61.1	68
Moisture	SC	33	69.3	1.990	64.1	75.2
Moisture	TX	30	65.2	3.249	57.4	73.7
Protein	FC	10	18.85	0.610	18.2	19.9
Protein	SC	33	20.16	0.965	15.7	21.4
Protein	TX	30	19.13	1.341	16.3	21.6
Total fat	FC	10	15.17	2.106	11.2	18.9
Total fat	SC	33	9.14	1.686	4.5	14.8
Total fat	TX	30	14.42	4.123	3.6	24.1

Table 33. Results of Analysis of Vitamins* in ABT (TX), Non-GE Sponsor Control (SC) and Non-GE Farm Control (FC) SalmonAnalyte

	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
Folic acid	FC	10	0.29	0.142	0.15	0.58
	SC	33	0.25	0.092	0.13	0.5
	TX	30	0.22	0.073	0.09	0.41
Niacin	FC	10	88.89	4.375	80.7	96.4
	SC	33	88.66	8.231	63.5	100
	TX	30	97.46	9.164	80.7	118
Pantothenic acid	FC	10	13.40	5.469	5.75	21.6
	SC	33	13.12	2.460	9.09	17.1
	TX	30	11	2.177	6.89	14.8
Vitamin B1	FC	10	0.06	0.014	0.05	0.1
	SC	33	0.08	0.012	0.06	0.11
	TX	30	0.07	0.012	0.04	0.09
Vitamin B12	FC	10	0.03	0.008	0.02	0.05
	SC	33	0.03	0.007	0.02	0.04
	TX	30	0.03	0.008	0.01	0.04
Vitamin B2	FC	10	1.01	0.089	0.86	1.2
	SC	33	1.13	0.143	0.83	1.49
	TX	30	1.08	0.101	0.90	1.28
Vitamin B6[§]	FC	10	6.56	0.593	5.76	7.67
	SC	33	7.20	0.739	4.86	8.72
	TX	30	7.67	0.791	6.50	10.21
Vitamin C	FC	10	2.77	1.069	1.6	4.5
	SC	33	3.98	1.311	1.8	7.5
	TX	30	2.98	0.780	1.6	4.6

*Vitamin A was below the limit of quantitation in all samples and was not included in the evaluation.
[§] Vitamin B6 concentrations are reported as the free base form. See Appendix 6. Analyte refers to FC, SC, and TX each.

Table 34. Results of Analysis of Minerals in ABT (TX), Non-GE Sponsor Control (SC) and Non-GE Farm Control (FC) Salmon

Analyte	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
Calcium	FC	10	31.49	4.310	25	37.7
	SC	33	30.03	6.260	17.6	43.5
	TX	30	27.57	6.531	16.1	43.4
Copper	FC	10	0.06	0.014	0.04	0.08
	SC	33	0.07	0.014	0.05	0.11
	TX	30	0.08	0.050	0.04	0.33
Iron	FC	10	0.52	0.338	0.29	1.43
	SC	33	0.48	0.082	0.37	0.74
	TX	30	0.52	0.233	0.33	1.65
Magnesium	FC	10	25.56	0.789	24.5	26.8
	SC	30	26.96	1.388	21.9	28.9
	TX	30	24.69	2.265	20.5	29.3
Manganese	FC	10	0.03	0.012	0.02	0.06
	SC	33	0.03	0.045	0.02	0.28
	TX	30	0.03	0.008	0.02	0.06
Phosphorous	FC	10	260.7	3.683	254	267
	SC	33	268.3	13.452	219	285
	TX	30	256.4	17.136	214	291
Potassium	FC	10	375.5	9.606	361	386
	SC	33	393.8	21.760	300	422
	TX	30	368.6	24.795	311	409
Selenium	FC	10	0.20	0.018	0.18	0.23
	SC	33	0.18	0.015	0.14	0.21
	TX	30	0.17	0.011	0.14	0.20
Sodium	FC	10	32.47	2.266	29.2	36.2
	SC	33	35.81	4.322	28.8	47.9
	TX	30	32.53	6.323	25.4	52.6
Zinc	FC	10	0.57	0.096	0.45	0.74
	SC	33	0.52	0.071	0.42	0.73
	TX	30	0.51	0.075	0.39	0.7

Table 35. Results of Analysis of Amino Acids in ABT (TX), Non-GE Sponsor Control (SC) and Non-GE Farm Control (FC) Salmon

Analyte	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
Alanine	FC	10	1.09	0.044	1.04	1.17
	SC	33	1.17	0.061	0.92	1.27
	TX	30	1.10	0.083	0.96	1.26
Arginine	FC	10	1.06	0.037	1.02	1.13
	SC	33	1.15	0.058	0.90	1.24
	TX	30	1.09	0.075	0.93	1.25
Aspartic acid	FC	10	1.78	0.068	1.7	1.89
	SC	33	1.94	0.099	1.51	2.08
	TX	30	1.82	0.134	1.54	2.08
Cysteine	FC	10	0.21	0.010	0.20	0.23
	SC	33	0.23	0.011	0.19	0.25
	TX	30	0.22	0.014	0.19	0.25
Glutamic acid	FC	10	2.44	0.082	2.33	2.55
	SC	33	2.63	0.127	2.04	2.79
	TX	30	2.44	0.194	2.09	2.82
Glycine	FC	10	0.93	0.044	0.89	1.04
	SC	33	1.02	0.052	0.82	1.08
	TX	30	0.94	0.056	0.84	1.04
Histidine	FC	10	0.51	0.024	0.48	0.55
	SC	33	0.55	0.034	0.42	0.61
	TX	30	0.53	0.036	0.44	0.61
Isoleucine	FC	10	0.85	0.037	0.80	0.91
	SC	33	0.92	0.053	0.70	1.01
	TX	30	0.88	0.059	0.75	0.99
Leucine	FC	10	1.40	0.050	1.34	1.48
	SC	33	1.52	0.077	1.17	1.63
	TX	30	1.42	0.109	1.21	1.64
Lysine	FC	10	1.64	0.054	1.55	1.71
	SC	33	1.77	0.088	1.37	1.89
	TX	30	1.66	0.118	1.42	1.88
Methionine	FC	10	0.54	0.021	0.52	0.58
	SC	33	0.59	0.033	0.48	0.65
	TX	30	0.56	0.039	0.47	0.64
Phenylalanine	FC	10	0.72	0.029	0.69	0.77
	SC	33	0.79	0.040	0.62	0.85
	TX	30	0.74	0.052	0.64	0.85
Proline	FC	10	0.67	0.034	0.62	0.75
	SC	33	0.73	0.039	0.57	0.8
	TX	30	0.68	0.047	0.59	0.77
Serine	FC	10	0.76	0.027	0.73	0.81
	SC	33	0.81	0.055	0.63	0.92
	TX	30	0.76	0.077	0.63	0.89
Threonine	FC	10	0.76	0.035	0.71	0.82

Analyte	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
	SC	33	0.83	0.045	0.64	0.9
	TX	30	0.79	0.060	0.68	0.93
Tryptophan	FC	10	0.17	0.006	0.16	0.18
	SC	33	0.19	0.016	0.13	0.21
	TX	30	0.18	0.014	0.15	0.21
Tyrosine	FC	10	0.62	0.025	0.6	0.67
	SC	33	0.68	0.036	0.53	0.74
	TX	30	0.65	0.049	0.54	0.75
Valine	FC	10	0.99	0.049	0.93	1.08
	SC	33	1.07	0.063	0.81	1.17
	TX	30	1.01	0.072	0.88	1.15

Table 36. Results of Analysis of Free Fatty Acids and Fatty Acids* in ABT (TX), Non-GE Sponsor Control (SC) and Non-GE Farm Control (FC) Salmon

Analyte	Physiological Name[1]	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
Arachidic	20:0	FC	10	0.03	0.005	0.02	0.03
		SC	32	0.01	0.005	0.01	0.02
		TX	30	0.03	0.008	0.01	0.04
Arachidonic	20:4 (*n*-6)	FC	10	0.08	0.01	0.07	0.1
		SC	33	0.06	0.016	0.03	0.12
		TX	30	0.09	0.027	0.03	0.17
Docosahexaenoic	22:6 (*n*-3)	FC	10	1.46	0.234	1.06	1.78
		SC	33	0.96	0.186	0.52	1.58
		TX	30	1.42	0.355	0.4	2.26
Docosapentaenoic	22:5 (*n*-3 or 6)	FC	10	0.44	0.073	0.36	0.57
		SC	33	0.27	0.097	0.12	0.66
		TX	30	0.5	0.146	0.18	0.89
Eicosadienoic	20:2 (*n*-6)	FC	10	0.05	0.009	0.03	0.06
		SC	33	0.04	0.01	0.02	0.06
		TX	30	0.06	0.023	0.01	0.1
Eicosapentaenoic	20:5 (*n*-3)	FC	10	1.17	0.199	0.86	1.44
		SC	33	0.59	0.196	0.29	1.37
		TX	30	1.1	0.346	0.26	2.07
Eicosatrienoic	20:3 (*n*-3)	FC	9	0.02	0.006	0.01	0.03
		SC	29	0.01	0.004	0.01	0.02
		TX	27	0.03	0.011	0.01	0.04
Eicosenoic	20:1 (*n*-9)	FC	10	0.91	0.106	0.79	1.16
		SC	33	0.46	0.114	0.22	0.64
		TX	30	0.53	0.176	0.18	0.77
Free fatty acids	Variable	FC	9	0.04	0.026	0.01	0.09
		SC	33	0.07	0.028	0.03	0.13
		TX	28	0.09	0.033	0.03	0.17
Gamma linolenic	18:3 (*n*-6)	FC	10	0.03	0.005	0.02	0.04
		SC	33	0.02	0.004	0.01	0.03
		TX	30	0.03	0.007	0.01	0.04
Heptadecanoic	17:0	FC	10	0.04	0.007	0.02	0.04
		SC	33	0.02	0.006	0.01	0.04
		TX	30	0.04	0.011	0.01	0.06
Linoleic	18:2 (*n*-6)	FC	10	0.67	0.105	0.43	0.78
		SC	33	0.51	0.097	0.28	0.68
		TX	30	0.74	0.311	0.14	1.2
Linolenic	18:3 (*n*-3)	FC	10	0.18	0.049	0.12	0.24
		SC	33	0.13	0.039	0.07	0.21

Analyte	Physiological Name[1]	Group	N	Arithmetic Mean	Standard Deviation	Minimum	Maximum
		TX	30	0.23	0.128	0.025	0.42
Myristic	14:0	FC	10	0.75	0.111	0.51	0.92
		SC	33	0.4	0.091	0.19	0.74
		TX	30	0.66	0.196	0.15	1.18
Oleic	18:1 (*n*-9)	FC	10	2.88	0.437	2.2	3.68
		SC	33	2.01	0.328	1.11	2.69
		TX	30	3.3	1.085	0.74	4.98
Palmitic	16:0	FC	10	1.91	0.333	1.17	2.21
		SC	33	1.07	0.262	0.48	2.05
		TX	30	1.79	0.549	0.41	3.39
Palmitoleic	16:1 (*n*-7)	FC	10	0.98	0.138	0.75	1.21
		SC	33	0.56	0.137	0.26	1.06
		TX	30	0.89	0.265	0.23	1.7
Pentadecanoic	15:0	FC	10	0.05	0.008	0.03	0.06
		SC	33	0.03	0.007	0.01	0.05
		TX	30	0.04	0.012	0.01	0.07
Stearic	18:0	FC	10	0.39	0.067	0.25	0.46
		SC	33	0.24	0.061	0.11	0.48
		TX	30	0.42	0.131	0.11	0.82

*Caprylic, capric, pentadecenoic, heptadecenoic, erucic and lignoceric acids were below the limit of quantitation in all samples; lauric, myristoleic, and behenic acids were below the limit of quantitation in all but one sample. These analytes were not included in our evaluation.
[1]Physiological name is biochemical nomenclature for both lipid number, degree of saturation, and omega number.

iii. Analysis of Study Results

(a) General Approach

Characteristics of individual fish, e.g., sex or season of harvest (time of catch), may have an impact on their composition. The comparisons of interest are between TX, SC, and FC salmon with consideration of ploidy. If, in general, the relative differences among TX, SC, and FC salmon are the same for both ploidies, then ploidy is not a consideration and comparisons among groups can be made ignoring ploidy. Variability among fish within groups is considered when making the comparisons and inclusion of fish with different characteristics broadens the inference.

Although it may be important to try to identify toxicologically or nutritionally significant compositional differences between TX and SC salmon, it is equally important to identify such differences between TX salmon and salmon normally consumed by humans, such as FC salmon. Considering all these factors, FDA determined that comparing the composition of TX salmon to either or both SC and FC control salmon groups is appropriate for assessing

whether or not TX salmon have important compositional differences from biologically relevant comparator salmon.[25]

Initially, the arithmetic means of values for each analyte derived from samples from TX fish were compared to the respective means from samples from the SC and FC groups. If the arithmetic mean from the TX fish were equal to or between the arithmetic means of SC and FC groups, i.e., SC ≤ TX ≤ FC, the results for TX salmon were considered to be similar to "control salmon" (SC and FC salmon) results. If the arithmetic mean for the TX salmon fell outside the range of either the SC or FC group, the minimum and maximum values (extreme values) for the TX salmon were compared to the range of values from the SC and FC salmon. If these extreme values from the TX salmon did not fall outside the range of values from the SC and FC salmon, the results for TX salmon were considered to be similar to "control salmon" results. If TX salmon were not considered similar to "control salmon", individual values were compared and if the individual values for the TX salmon were not more than 10% beyond the range of values for the individual "control salmon," the values for the TX salmon were considered to be within normal biological variability and thus similar to the "control salmon."

Following this evaluation, FDA performed a statistical analysis in which the TX salmon were not considered similar to "control salmon." The statistical analysis took into consideration the variability among the fish in each group to test for differences in means. Selected data were analyzed using analysis of variance (ANOVA) with group (FC, SC, TX), ploidy (diploid, triploid), and the group-by-ploidy interaction included in the model as fixed effects. If the group-by-ploidy interaction was considered significant, this indicated that generally the mean results among the groups differed in some way. In this case, the TX group mean was compared to the FC and SC group means separately within ploidy. If the group-by-ploidy interaction was not considered significant, and the group effect was considered significant, the TX group mean was compared to the FC and SC group means without regard to ploidy. Note that the analysis results were interpreted with the understanding that the estimated p-value may be under-estimated because comparisons are generated after examination of the data. However, for exploratory analyses, this is an acceptable strategy. Results of the statistical analyses are provided in Appendix 5.

(b) Results of FDA's Analysis

(i) Analysis of Results of Proximates, Vitamins, Minerals and Amino Acids

Based on the comparison of arithmetic means and extreme values, the following analytes from TX salmon were considered to be similar to those for SC and FC (comparator) salmon:

- **proximates** - carbohydrate, ash, moisture, protein and total fat;
- **vitamins** – pantothenic acid, vitamins B1, B12, B2 and C;

[25] Additional details regarding this study are available in the briefing packet document prepared for the 2010 VMAC meeting http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/UCM224762.pdf.

- **minerals** – potassium, selenium and sodium; and
- **amino acids** – alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine and valine.

For the remaining analytes (**vitamins** - folic acid, niacin and vitamin B6; **minerals** - calcium, copper, iron, magnesium, manganese, phosphorous and zinc; and **amino acid** – serine) that did not meet the criteria identified above, the individual values from the TX salmon were again compared to the individual values from the two comparator samples using a 10% exceedance range.

In all of these 11 analytes, at least one value exceeded the range of values for control salmon by at least 10%; five analytes had one value from TX salmon that exceeded the non-GE range by more than 10% (i.e., calcium, copper, iron, manganese and serine). The remaining six analytes had between two and eight values from TX salmon that exceeded the comparator range by 10% or more (i.e., folic acid, niacin, vitamin B6, magnesium, phosphorous and zinc).

From the statistical analyses, analytes for which no statistical difference was detected when ploidy was considered as a variable for each group included calcium, copper, manganese, serine, vitamin B6, and zinc. When results from analytes from all TX salmon were compared to results from all the comparator groups (SC and FC salmon), four were not statistically significantly different from either SC or FC salmon (calcium, copper, manganese and zinc); two, potassium and serine, were statistically significantly different from SC salmon but not from FC salmon; and one (vitamin B6) was statistically significantly different from both SC and FC salmon (see discussion of vitamin B6 below).

From the statistical analyses, analytes for which the results were affected by ploidy were iron, phosphorous, folic acid, magnesium and niacin. When these analyte results for *diploid* TX salmon were compared to results for diploid SC and FC salmon, three were not statistically significantly different from either group of control salmon (iron, magnesium, and phosphorous), and two were statistically significantly different from both SC and FC salmon (folic acid and niacin). When these analyte results for *triploid* salmon were compared to results for triploid SC and FC salmon, two were not statistically significantly different from either group of control salmon (folic acid and iron), three were statistically significantly different from SC salmon but not from FC salmon (niacin, magnesium, and phosphorous).

Conclusions for proximate, vitamin, mineral, and amino acid analytes.
Of the proximate, vitamin, mineral and amino acid analytes in this study, only three analytes were present at levels in TX salmon that were statistically significantly different from levels in control salmon (SC and FC): *vitamin B6* (when diploid and triploid salmon were considered together for TX, SC and FC salmon), *folic acid* (when diploid salmon were compared in TX, SC and FC salmon) and *niacin* (when triploid salmon were compared in TX, SC and FC salmon). Based on all previous criteria including statistical analysis, FDA concluded that the levels of all proximate, vitamin, mineral and amino acid analytes in TX salmon except vitamin B6 are similar to levels in one or more appropriate groups of control salmon.

(ii) Vitamin B6

The levels of Vitamin B6 in the ABT salmon were statistically different from both the comparator groups. In order to determine whether there was any biological relevance to this statistical difference, FDA performed a MOE assessment using the same assumptions as for the analysis of IGF1 (Table 30: MOE for IGF1; Table 37: MOE summarizes the analysis for Vitamin B6).

Margin of Exposure for Vitamin B6

Vitamin B6 concentrations were slightly elevated in the TX salmon samples: this elevation was determined to be statistically significant. Prior to conducting any further analyses to determine whether this statistically significant elevation had any biological implications, we performed two assessments: (1) visual inspection of the data to determine the distribution of values among the diploid and triploid TX samples to determine the source of the elevation, and (2) a MOE on the most elevated level to ascertain whether if all of the fish consumed contained that highest level of vitamin B6 observed in the on outlier diploid ABT salmon, exposures would pose a hazard.

We used the most extreme value as the intrinsic exposure level in the MOE assessment. The same assumptions for consumption values were used in the MOE for vitamin B6 as were used for the MOE analysis of IGF1 (Table 30; MOE IGF1).The margin of exposure between Vitamin B6 in non-GE Atlantic salmon and diploid ABT salmon are presented in Table 37.

Table 37. Margin of Exposure Estimates of Vitamin B6 in ABT Salmon

Description	Calculation	Value
Daily non-tuna consumption for the 95th percentile eater[1]		300 g/day
Assuming all consumed non-tuna finfish are salmon, consumption corrected for fraction of Atlantic salmon	(2/3) * 300 g/day	200 g/day
Plausible upper bound of Vitamin B6 concentration observed at maximum level in sponsor control mature diploid Atlantic salmon (µg of Vitamin B6/g total weight)		8.7 µg/g
Plausible upper bound of Vitamin B6 concentration observed at maximum level in mature diploid ABT salmon (µg of Vitamin B6/g total weight)		10.2 µg/g
Daily consumption of Vitamin B6 from non-GE Atlantic salmon	200 g/day * 8.7 µg/g	1.74 mg/day
Daily consumption of Vitamin B6 from ABT salmon	200 g/day * 10.2 µg/g	2.04 mg/day
Recommended maximum level of daily Vitamin B6 intake[2,3]		100 mg/day
Margin of Exposure (MOE) for mature diploid (non-GE) Atlantic salmon	(100 mg/day)/(1.74 mg/day)	57.5 fold

Description	Calculation	Value
MOE for mature diploid ABT salmon	(100 mg/day)/(2.04 mg/day)	49.0 fold

1 US Department of Commerce (USDOC), Technology Administration, NTIS, Springfield, VA 22161 (2002) Foods Commonly Eaten in the United States. Quantities Consumed per Eating Occasion and In A Day, 1994-1996. PB2005110468.
2 Institute of Medicine (IOM) of the National Academies. Food and Nutrition Board (2001) Dietary Reference Intakes: The National Academies Press. Washington, DC
3 World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) (2004) Vitamin and Mineral Requirements in Human Nutrition. 175-179. Geneva, Switzerland.
Comparisons to other sources of B6 from finfish and other common protein sources are also appropriate to put this analysis into context. This information is summarized in Table 38.

Table 38. Vitamin B6 Concentration Found in Commonly Eaten Protein Sources[1] (Reported as mg/100 g Tissue Mass)

Source	Mean	Minimum	Maximum
ABT Salmon*†	0.77	0.65	1.02
Non-GE Sponsor Control Salmon*†	0.72	0.49	0.87
Atlantic Salmon (farm and wild)‡	0.73	0.64	0.82
Chinook Salmon (farm and wild)‡	0.43	0.4	0.46
Chum Salmon (farm and wild)‡	0.55	0.38	0.94
Coho Salmon (farm and wild)‡	0.6	0.55	0.66
Sockeye Salmon (farm and wild)‡	0.23	0.19	0.28
Rainbow Trout (farm and wild)‡	0.51	0.41	0.62
Tuna‡	0.81	0.46	1.04
Flatfish‡	0.28	0.16	0.42
Beef‡	0.46	0.36	0.56
Pork‡	0.49	0.21	0.75
Poultry‡	0.54	0.25	1.47
Milk‡	0.05	0.04	0.05

[1]U.S. Department of Agriculture, Agricultural Research Service (2009) USDA National Nutrient Database for Standard Reference, Release 22. Nutrient Data Laboratory Home Page, http://www.ars.usda.gov/nutrientdata
*ABT and Sponsor Control Salmon Vitamin B6 concentrations calculated and reported as free base form. See Appendix 6. †Indicates Sponsor-Provided Data. ‡Indicates USDA Nutrient Database-provided information.

Results

The recommended maximum level of daily nutrient intake (UL) for vitamin B6 is **100 mg/day** for healthy male adults aged 13 and above (IOM, 2001; WHO-FAO, 2004). Healthy, non-pregnant women aged 19 and above are also advised to consume no more than **100 mg/day** of vitamin B6 (IOM, 2001; WHO-FAO, 2004).

FDA calculated the upper bound dietary consumption level of vitamin B6 from sponsor control mature diploid non-GE salmon, and mature diploid ABT salmon to be **1.74 mg/day** and **2.04 mg/day** respectively.

The MOE for dietary consumption of vitamin B6 was therefore reported as the index number of the maximum level of daily intake divided by the upper bound dietary

consumption level [i.e., (100 mg/ day UL)/(2.12 mg/day)]. For sponsor control non-GE salmon, the MOE yielded **57.5 fold less than the maximum** allowable consumption level. For mature diploid ABT salmon, the MOE was calculated as **49.0 fold less than the maximum** allowable consumption level.

Conclusions
FDA investigated the statistically significant difference in mean vitamin B6 levels using a MOE assessment. Even if the highest level of vitamin B6 observed in the diploid ABT salmon were to be found in all ABT salmon, which include AAS, the MOE assessment indicated that it would still be well within the upper bound recommended daily intake for vitamin B6. Therefore, FDA finds that vitamin B6 provides no additional food consumption hazard compared to non-GE Atlantic salmon.

(iii) Fatty Acids and Free Fatty Acids
Comparison of TX, SC and FC means and ranges
Based on the comparison of arithmetic means and extreme values, the following fatty acids for TX salmon were considered to be present at similar levels in TX and control salmon: arachidic, docosahexaenoic, eicosanoic, eicosapentaenoic, gamma linolenic, heptadecanoic, myristic, palmitic, palmitoleic and pentadecanoic fatty acids.

For the remaining fatty acids not considered to be present at similar levels in TX and control salmon according to the criteria described above (for proximates, vitamins, minerals, and amino acids), the individual values for the TX salmon were compared to the individual values from the two comparator samples using a 10% exceedance range as the trigger for further evaluation, as described previously.

The values for one fatty acid, eicosatrienoic acid, were entirely within the range of controls (or within 10%); these were considered to be similar to results in control salmon by the criteria described in Section b.*i*. However, the remaining eight fatty acid analytes in TX salmon appeared to be dissimilar from those in control salmon: the levels of three analytes (the free fatty acids, arachidonic acid, and docosapentaenoic acid) were more than 10% beyond the range of control values, and for the remaining five fatty acid analytes (stearic acid, oleic acid, linoleic acid, linolenic acid and eicosadienoic acid), 8-16 measurements in the TX group were more than 10% beyond the range of controls values. Eicosadienoic, linoleic, linolenic, oleic, arachidic, docosahexaenoic, docosapentaenoic, eicosopentaenoic, palmitoleic, palmitic, and stearic acids and free and total fatty acids therefore were statistically analyzed.

From the statistical analyses for eicosadienoic, linoleic, linolenic, oleic and palmitic fatty acid analytes, the group-by-ploidy interaction was not considered statistically significant but the group effect was. Mean comparisons were made among groups for these fatty acid analytes. For eicosadienoic, linoleic, linolenic and oleic fatty acids, the TX mean was statistically significantly different from the SC mean. For palmitic acid, no statistically significant differences were found between TX mean and the FC or SC mean.

From the statistical analyses for arachidic, docosahexanoic, docosapentaenoic, eicosapentaenoic, palmitoleic, palmitic and stearic and total and free fatty acid analytes, the

group-by-ploidy interaction was considered significant and mean comparisons were made within ploidy. For diploid salmon, statistically significant differences were found between the TX mean and the SC mean for docosahexanoic, docosapentaenoic, eicosapentaenoic and stearic fatty acids and total fatty acids, and between the TX mean and both the SC and FC means for arachidic, palmitic and palmitoleic fatty acids. For diploid salmon, no statistically significant difference was found between the TX mean and either SC or FC mean free fatty acids. For triploid salmon, statistically significant differences were found between the TX mean and the SC mean for arachidic, docosahexanoic, docosapentaenoic, eicosapentaenoic, palmitic and palmitoleic fatty acids and total fatty acids and between the TX mean and both the SC and FC means for stearic acid and free fatty acids.

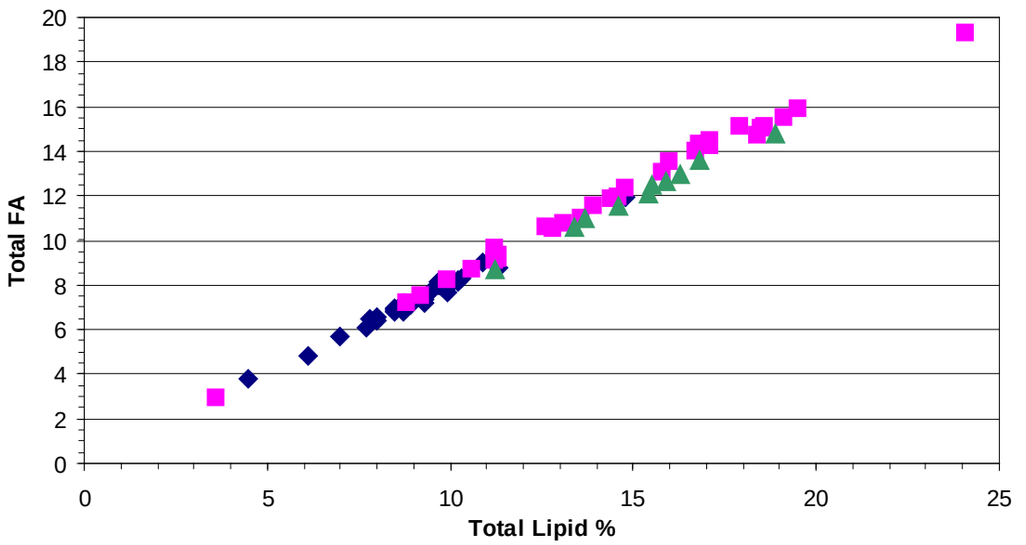
Of the fatty acid analytes in this study, only four were present at levels in TX salmon that were statistically significantly different from levels in both SC and FC control salmon – fatty acids arachidic, palmitic and palmitoleic (when diploid salmon were compared in TX, SC and FC salmon) and stearic acid (when triploid salmon were compared in TX, SC and FC salmon). Thus, based on all previous criteria, including statistical analysis, FDA concluded that the levels of all fatty acid analytes in TX salmon were similar to levels in one or more appropriate groups of control salmon.

Comparison of dietary fat, total fat levels and levels of free fatty acids
In this study, the variation in total fat levels within each group of salmon was fairly broad, with arithmetic mean values of 14.4%, 15.2% and 9.1% and standard deviations of 4.12, 2.11 and 1.69 for TX, FC and SC salmon, respectively.

Although the arithmetic mean total fat content for TX salmon was similar to that of the FC salmon – which was the basis for concluding that total fat levels in TX salmon were similar to those for control salmon - it was higher than the total fat content of the SC salmon. Nonetheless, the total fat level arithmetic means for all of the salmon in this study were within the 7% - 19% range for total lipids as reported in scientific literature for wild and farm-raised Atlantic salmon (Hamilton et al., 2005; Torstensen et al., 2005).

The amount of total fatty acids in fish is generally directly proportional to total lipid deposition (Ikonomou et al., 2007); this relationship is also observed in Figure 4 (generated using the sponsor's data). Thus, because the level of total lipids in TX salmon is higher than in SC salmon, it is reasonable to expect that the levels of some fatty acids in TX salmon will be proportionately higher as well. This pattern is particularly evident for the following fatty acids: eicosadienoic, linoleic, linolenic, oleic, docosahexanoic, docosapentaenoic, eicosapentaenoic and stearic and total fatty acids; for these fatty acid analytes, levels in TX salmon are similar to levels in FC salmon but are higher than levels in SC salmon.

Figure 4. Total Fatty Acids vs. Total Lipid %



Relationship between total fatty acids and total lipids as a percentage of body weight (Blue Diamonds: Sponsor Controls; Pink Squares: ABT salmon; Green Triangles: Farm Controls).

The effect of dietary lipid levels and their origin (plant versus animal) in the diet of fish on the fatty acid composition of fish has been extensively studied (Friesen et al., 2008; Kennedy et al., 2005; Polvi and Ackman, 1992; Torstensen et al., 2005). The composition and amount of total fat and fatty acids in salmon vary from species to species and are, in general, a function of the overall composition of their feed and, in particular, of the amount of dietary fat and fatty acids in the diet.

Most of the ABT and sponsor control salmon participating in the study were fed the Moore-Clark Orion (MCO) commercial grower diet. Smaller groups of the fish were fed the Moore-Clark Atlantic (MCA) and the Moore-Clark Atlantic Broodstock (MCAB) commercial diets. ABT provided FDA with the composition of these diets. All three commercial diets can be considered “high energy” diets due to their high protein content (MCO 37%, MCA 43% and MCAB 46%) and their high crude fat content (MCO 36%, MCA 32%, and MCAB 25%). The other control group used in this study, farm-raised fish (FC), was purchased from a commercial Atlantic salmon farm facility. The farm facility that raised the FC considered the information regarding the feed formulation used at that facility proprietary and did not provide it, limiting the extent to which comparisons could be made.

Generally, fish on a “high energy” diet exhibit elevated total lipid levels. The sponsor compared the protein and fat content of two of the diets fed to TX and SC salmon with the fat levels in the salmon during the three months before they were killed. (No TX salmon were fed the third diet for the three months before they were killed.) TX fish fed both diets appear to have higher percentages of total fat than SC salmon, even when the diets had identical fat content.

As noted previously, all three diet formulations used for TX and SC salmon contained plant based oils as their main source of lipids. Because TX and SC fish were fed similar diets during the three months prior to collection, observed differences in their total fat content cannot be attributed to different levels of energy, protein, or total fat in their diets or to differences in sources of dietary fats in the diets (e.g., animal versus plant).

Because Atlantic salmon are an important source of lipids and, more specifically, of polyunsaturated omega-3 and omega-6 fatty acids in human diets, consumers may be concerned that consuming ABT salmon may result in lower intakes of omega-3 and omega-6 fatty acids or a change in the ratio of these fatty acids. Examination of omega-3/omega-6 ratios showed that they were virtually identical across the TX, SC, and FC groups and are similar to the ratios found in scientific literature for farm-raised Atlantic salmon (which constitute almost the entirety of the consumption of Atlantic salmon in the United States (Seafood Health Facts 2014; http://seafoodhealthfacts.org/pdf/seafood-choices-salmon.pdf)). These data, along with data from published literature, are presented in Table 39.

Table 39. Mean Omega-3 and Omega-6 Fatty Acid Levels in ABT Salmon and Farm-Raised Atlantic Salmon (as % of the wet weight)

Fatty Acid	Degree of saturation	Farm raised†	Sponsor control†	ABT†	Farm-Raised[1‡]	Farm-Raised[2‡]	Farm-Raised[3‡]	Wild caught[4‡]	Farm-Raised[4‡]
Oleic	18:1	2.88	2.011	3.299		0.465	1.05		
Linoleic[¶]	18:2	0.668	0.507	0.743	0.303	0.162	0.194	0.067	0.65
a-Linolenic[§]	18:3	0.178	0.131	0.232	0.066	0.031	0.103	0.05	0.181
g-Linolenic[¶]	18:3	0.03	0.019	0.027				0.003	0.014
Arachidonic[¶]	20:4	0.084	0.055	0.092	0.037	0.025	0.029	0.03	0.091
Eicosenoic	20:1	0.913	0.455	0.534		0.144	0.275		
Eicosadienoic[¶]	20:2	0.053	0.039	0.059			0.029	0.017	0.063
Eicosatrienoic[§]	20:3	0.021	0.012	0.024				0.009	0.024
Eicosapentaenoic[§]	20:5	1.174	0.593	1.095	0.324	0.225	0.326	0.414	1.08
Docosapentaenoic	22:5	0.436	0.266	0.5				0.12	0.519
Docosahexaenoic[§]	22:6	1.46	0.961	1.422	0.623	0.568	0.932	0.629	1.57
w-3/w-6 ratio		3.9	3.2	3.6				10.4	4.1

[§] Omega - 3 fatty acids
[¶] Omega - 6 fatty acids
[1] Blanchet et al. (2005)
[2] Kennedy et al (2005)
[3] Bell et al (2002)
[4] Hamilton et al (2005)
†Indicates Aqua Bounty-provided data
‡Indicates Scientific Literature-provided data

Conclusions for Fatty Acids

Based on the data and information evaluated FDA concluded that:

- The levels of any individual fatty acid in ABT salmon are similar to those of the comparators;

- Total fat content for ABT salmon is similar to those for comparator salmon and within the 7-19% range for total lipids as reported for wild and farm-raised Atlantic salmon;
- ABT salmon offer a balanced diet of omega-3 and omega-6 fatty acids, similar in quantity and ratio to that provided by the FC controls and by farm-raised Atlantic salmon currently consumed; and
- ABT salmon are not materially different from other Atlantic salmon with respect to omega-3 and omega-6 fatty acid levels and the ratio of omega-3 to omega-6 fatty acids.

Composition Conclusions

Based on all previous criteria including statistical analyses, FDA concluded that levels of all analytes in ABT salmon are similar to levels in appropriate comparator salmon (e.g., either the sponsor controls, farm-raised salmon, literature reports, or some combination of the three) and do not differ from other Atlantic salmon.

Any differences observed for analytes are the result of normal biological variation, and are highly unlikely to be associated with toxicological or nutritional hazards to humans consuming ABT salmon.

The statistically significant difference in mean vitamin B6 levels was investigated using a MOE assessment. Even if the highest observed level of vitamin B6 observed in the diploid ABT salmon were to be found in all ABT salmon, the MOE assessment indicates that it would still be well within the upper bound recommended daily intake for vitamin B6.

For fatty acid analytes, values found in the ABT salmon are consistently more similar to the farm-raised control values than to the sponsor control values, and are proportional to total fat levels in these three groups of salmon; these differences do not appear to be due to differences in fat content or protein source of the ABT and sponsor control salmon diets.

ABT salmon offer a balanced diet of omega-3 and omega-6 fatty acids, similar to that provided by the FC controls and by farm-raised Atlantic salmon.

Finally, FDA concluded that ABT salmon are not materially different from other Atlantic salmon with respect to omega-3 and omega-6 fatty acid levels and the ratio of omega-3 to omega-6 fatty acids.

Because AAS are a triploid, hemizygous female subset of ABT salmon, FDA has concluded that compositional data derived from ABT and non-GE salmon support the same conclusion that AAS do not differ from non-GE salmon.

b. Endogenous Allergenicity

i. Context

The Food Allergen Labeling Consumer Protection Act of 2004 (P.L. 282) (FALCPA, 2004) identifies eight major foods or food groups that are allergenic: milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat, and soybeans. These eight foods are believed to account for 90 percent of documented food allergies in the U.S. and most serious reactions to foods in the U.S. (FALCPA, 2004; Hefle et al., 1996). Each of these eight major allergenic foods contains multiple allergenic proteins, many of which have not been fully characterized (Gendel, 1998).

Food allergies affect more than 1-2% but less than 10% of the U.S. population (NIAID, 2010). Although there are numerous scientific publications on food allergy, there are many uncertainties with respect to diagnosis, best practices for management and prevention, mechanisms of sensitization, and allergenic thresholds that will elicit responses from sensitive individuals (CFSAN, 2006; Chafen et al., 2010).

There are a great number of uncertainties when attempting to assess potential changes in the levels of allergens in commonly allergenic foods, including salmon. FDA is unaware of any data that may exist on the natural variation in the levels of endogenous allergens in salmon or other finfish that are currently consumed in the U.S. Tools to assess endogenous allergen levels are limited. Human sera containing specific IgE are often used for this purpose; however, the utility of these studies is limited because there is typically little or no information available regarding the allergic history of the donors. This is important because the presence of specific IgE in sera does not necessarily correlate with a clinically relevant food allergy (Chafen et al., 2010); therefore, the allergic history of the donor should be taken into account when interpreting data from such a study. In addition, because of the relatively small numbers of individuals represented in such assays, these *in vitro* studies may not reflect the responses of a general population. Finally, there is no consensus in the scientific and medical communities regarding the magnitude of increase in endogenous allergens in an allergenic food that would present an additional risk to public health (Goodman et al., 2008), especially considering that individuals who are allergic to a particular food would likely avoid that food.

Because finfish are one of the eight major food allergens in the United States (FALCPA, 2004; Hefle et al., 1996; Sampson, 2004) one potential indirect hazard that may result from the insertion of the *opAFP-GHc2 construct* at the α- locus is an alteration in the endogenous levels of allergens in ABT salmon due to insertional mutagenesis. In particular, the question was asked whether the edible tissue from ABT salmon is more allergenic than the non-GE comparator. FDA evaluated this question (see discussion below).

ii. Sponsor Study: A Comparator-Controlled Immunochemical Study of the Allergenic Potency of Muscle-Skin from Diploid and Triploid Atlantic Salmon (Salmo salar) Modified Transgenically with the AquAdvantage Gene Construct opAFP-GHc2). Testing Facility: IBT Reference Laboratory. Kansas. Study Report AAS-HFS-003. Report dated 22 March 2006.

The purpose of this study was to examine potential quantitative and qualitative changes in allergens in salmon muscle and skin from market-size, diploid and triploid ABT salmon vs. non-GE Atlantic salmon. This study was conducted in compliance with GLPs.

Overall study design:
Market-sized (2.0 to 7.5 kg) diploid and triploid ABT salmon (treated group: TX_D and TX_T) and non-GE diploid Atlantic salmon (sponsor control group: SC_D) were included in the study. From the available pools of each type fish, six fish were selected non-systematically by net capture for a total of 18 fish. Sex and maturity were not considered for selection, therefore distribution of those characteristics was not uniform within or between groups.

Salmon were screened visually for general health status and traits relevant to commercial marketability, including skin and fin condition, color and markings, and general body morphology. Because of differences in rates of growth to market size, ABT and control fish may not have been age-matched.

Harvest, measurements, necropsy, genotype, and ploidy determinations were performed by the sponsor. Blind-coded salmon fillets packed on dry ice were shipped to a testing laboratory, which homogenized the samples under liquid nitrogen. A representative subsample of each frozen salmon-fillet homogenate (FSFH) was shipped on dry ice to IBT Reference Laboratory (IBT) for testing.

The sponsor subsequently unmasked the identities of all 18 samples to facilitate use of control FSFH in further analyses. IBT performed aqueous extractions of a subsample of each FSFH, and extracts were stored at -70°C. Separate aqueous extracts from the same FSFH samples were used in the allergen potency and allergen identity assays due to insufficient quantity of extracts. IBT also performed total protein determination of extracts as well as allergen potency and identity assays.

Total protein concentration from salmon skin-muscle extracts was determined using the Micro BCA™ Protein Assay Kit (Pierce Chemical Company), in accordance with GLPs. Validation information for assay methodology was provided and the limit of detection of the assay was < 2 total protein concentration from salmon skin-muscle extracts, determined using the Micro BCA™ Protein Assay Kit (Pierce Chemical Company), in accordance with GLPs. Validation information for assay methodology was provided and the limit of detection of the assay was < 2 µg/ml; the lower limit of quantitation (LLOQ) was 3.1 µg/ml. All extracts were normalized to 2 mg/ml prior to further analysis.

iii. Fluorescent Enzymatic Immunoassay (FEIA)

IBT developed an inhibition assay to determine relative allergenic potency (RP) of FSFH extracts based on the ImmunoCAP system, a commercial reagent and equipment system for clinical diagnostic testing of human sera for specific IgE by Pharmacia Diagnostics AB (now Phadia AB).

Instrumentation, methods, reagents and salmon-allergen standard used in the FEIA were developed and validated for the commercial use of the ImmunoCAP system by Pharmacia Diagnostics AB. The assay was conducted in accordance with GLPs.

IBT used this FEIA as the basis for the development of an inhibition assay to determine the allergenic potency of muscle-skin extracts from ABT salmon compared with extracts from

sponsor control non-GE salmon. Briefly, soluble salmon allergen in FSFH extracts is used to inhibit binding of highly salmon-reactive IgE pooled sera to the solid phase bound salmon standard f41 which is derived from the muscle of Atlantic salmon. This assay provides a quantitative determination of inhibition of salmon-specific IgE binding which is then used to calculate the potency of salmon allergen in muscle-skin from ABT salmon relative to that in a control extract, comprised of equal volumes of all six sponsor control non-GE FSFH extracts.

Individual human sera with salmon-specific IgE of greater than or equal to Class 3 by ImmunoCAP scoring guide (greater than or equal to 3.5 kU/l, with individual sera ranging from 4.8 – 98.60 kU/l) were obtained commercially and pooled. IBT determined binding characteristics of salmon-specific IgE pool by ImmunoCAP for use in the FEIA inhibition assay. Individual human sera negative for salmon-specific IgE (less than 0.10 kU/l) were obtained from IBT's sera bank and pooled.

Each of the 18 individual FSFH extracts was run in the FEIA inhibition assay six times. Validation information for assay methodology was provided.

Percent inhibition was calculated and plotted against the log of the reciprocal sample dilution. These data were used to generate an inhibition curve, from which the allergenic potency in U/ml at 40, 50, and 60 % inhibition was calculated for each FSFH sample and pooled FSFH control. Relative potency was estimated using the percent inhibition of pooled FSFH control.

FDA's evaluation[26] of the study indicated the following notable concerns:

1. The number of samples per group was limited. Fish were included irrespective of sex or maturity so that these were not distributed uniformly within or between the different groups. Because fish were included irrespective of sex or maturity, results may not necessarily be representative of AAS that will be marketed for consumption.
2. Farm-raised salmon were not included as a control. Because farm-raised salmon were selected for rapid growth, inclusion of this group would have provided a control for potential effects related to a rapid growth phenotype. In addition, farm-raised salmon could have provided additional information regarding the natural variability levels of endogenous allergens in salmon currently consumed in the U.S.
3. The study protocol stated that *"FSFH subsamples deriving from homogenization of the blind-coded left fillets would be sent . . . for immunochemical analysis extracts identified only by the UFID [universal fish identification number] originally provided . . . by [the sponsor].*" The sponsor unmasked the identities of all 18 samples to facilitate use of control FSFH in further analyses by the testing laboratory. Blinding the identities of the samples could have provided some bias control in the outcomes of both FEIA and Western blot analyses.

[26] Additional details regarding this study and FDA's evaluation are available in the VMAC Briefing Packet, http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/UCM224762.pdf.

FDA found the sponsor's analysis and acceptance criteria of relative potency based on FDA Center for Biologics Evaluation and Review's (CBER) Guidance for Reviewers: Potency Limits for Standardized Dust Mite and Grass Allergen Vaccines: A Revised Protocol (November 2000) (CBER's Allergen Vaccine Guidance) (Available at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Allergenics/ucm071931.htm), to be irrelevant for food safety assessment.

FDA conducted its own analysis of the data. FDA considered allergenic potency (in U/ml) at 40, 50, and 60% inhibition in lieu of RP values for two reasons. First, the agency wanted to compare directly the allergenic potency of GE diploid and triploid fish vs. sponsor control fish. This direct comparison was not possible using RP values that had been normalized using the pooled control FSFH, which was comprised of equal volumes of all six sponsor control FSFH extracts. Second, the agency was unable to determine how RP values for individual FSFH and pooled control FSFH extracts were calculated from allergenic potency at 40, 50 and 60% inhibition.

In each of the six assays for each FSHS, FDA estimated the allergenic potency at 40, 50, and 60% inhibition. Although the differences between values provided an indication of measurement error and assay sensitivity, they did not provide information about variability that may exist between fish, which was one of the agency's concerns. For initial evaluation, the mean allergenic potency for the six assay runs was estimated at 40, 50, and 60% inhibition. Additionally, using analysis of variance, FDA determined that the difference between the fish type (SC_D, TX_D and TX_T) was consistent whether measured at 40, 50, or 60 % inhibition. Because of this consistency, the final evaluation used the mean allergenic potency from all six assay runs estimated at all inhibition levels.

Initial evaluation of the results suggested that there may be an increase in the relative allergenic potency in the GE diploid salmon compared to sponsor control salmon. Given that salmon is often consumed as one individual fish fillet per serving rather than a mixture of many fish, FDA also considered the allergen level in individual fish in addition to group means.

Table 40 contains a summary of mean allergenic potency data.

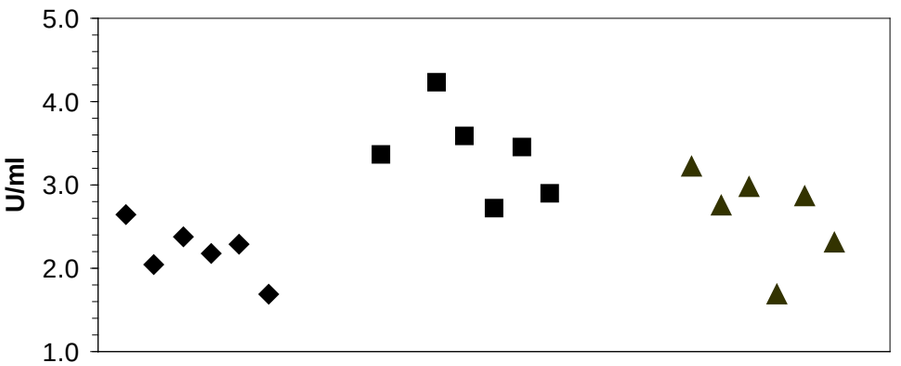
Table 40. Summary of Mean Allergenic Potency of Salmon Extracts

UFID	Group	Ploidy	Mean
234	SC	Diploid	2.65
202	SC	Diploid	2.04
206	SC	Diploid	2.38
222	SC	Diploid	2.17
231	SC	Diploid	2.29
212	SC	Diploid	1.69
204	TX	Diploid	3.36
210	TX	Diploid	4.23
215	TX	Diploid	3.57
223	TX	Diploid	2.71

UFID	Group	Ploidy	Mean
225	TX	Diploid	3.44
219	TX	Diploid	2.90
221	TX	Triploid	3.22
230	TX	Triploid	2.75
207	TX	Triploid	2.99
208	TX	Triploid	1.70
227	TX	Triploid	2.88
232	TX	Triploid	2.31

Although confidence in the data describing the diploid ABT salmon are low, these data indicated that four diploid GE fish had mean allergenic potency greater than 3.00 U/ml, with one fish having a mean allergenic potency value of 4.23 U/ml. Only one triploid GE salmon had a mean allergenic potency value greater than 3.00 U/ml. Figure 5 depicts the mean allergenic potency of individual fish.

Figure 5. Mean Allergenic Potency of Individual Fish



Black Diamonds: Sponsor Control Diploids; Black Squares: ABT diploid salmon; Black Triangles: ABT triploid salmon

Initial evaluation suggested that there may be an increase in the allergenic potency in the GE diploid salmon compared to sponsor control salmon. Allergenic potency (U/ml) data were analyzed using analysis of variance (ANOVA) with type included in the statistical model as a fixed effect. Pairwise comparisons of means for each of the TX groups were made to the mean for the SC_D group. P-values less than 0.05 indicate a statistically significant difference. There was no evidence of a statistically significant difference between the mean allergenic potency U/mL for sponsor control diploid (SC_D) fish compared to the triploid GE (TX_T) fish. A statistically significant difference existed between the mean allergenic potency U/mL for sponsor control diploid (SC_D) fish compared to the diploid GE (TX_D) fish. Table 41 summarizes the statistics for the mean allergenic potency per group.

Table 41. Least Squares Mean Allergenic Potency of Salmon Fillets per Group

Group*	Least Squares Mean Allergenic Potency	Standard Error	P-value from Test of Difference from Mean SC_D
SC_D	2.21	0.196	-

Group*	Least Squares Mean Allergenic Potency	Standard Error	P-value from Test of Difference from Mean SC_D
TX_D	3.37	0.196	0.0008
TX_T	2.64	0.196	0.1388

* SC_D = sponsor control non-GE diploid; TX_D = GE diploid; and TX_T = GE triploid.

Conclusion: The allergenic potency of triploid ABT salmon is not significantly different from that of sponsor control diploid salmon. There are insufficient data and information to draw a conclusion on the relative allergenic potency of diploid ABT salmon (see Appendix 2). The agency notes, however, that individuals allergic to salmon are likely to avoid all salmon.

iv. Western Blot

The secondary objective of the study was to determine if any qualitative changes occurred in the major salmon allergen parvalbumin (*Sal s1*), due to the insertion of the *opAFP-GHc2 construct* at the α-locus in ABT salmon. In this arm of the study, aqueous extracts from FSFH were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). This study was technically flawed and FDA was unable to draw any meaningful conclusions regarding the relative levels of parvalbumin between ABT salmon and their comparators.[27]

v. Further Consideration of Allergenicity of GE Salmon

One potential indirect hazard that may result from the insertion of the *opAFP-GHc2* construct at the α- locus is a possible increase in the endogenous levels of allergens in ABT salmon due to insertional mutagenesis in a region of the genome that may act as a regulator of the expression of one or more of these proteins. Although the previous study attempted to address this point, its various technical deficiencies make it difficult to determine whether the allergenicity of salmon, or the prevalence of any known endogenous protein that has been implicated in allergic responses (i.e., parvalbumin) have changed, thereby somehow increasing the allergenicity of the fish.

Others attempted to address this issue. Nakamura *et al.* (2009) compared the allergenicity of growth hormone (GH) transgenic and non-transgenic amago salmon (*Oncorhynchus masou ishikawae*). Western blots using antibodies against frog parvalbumin and fish type-I collagen and 22 individual fish-allergic sera demonstrated no differences between GH-transgenic and non-transgenic amago salmon with respect to the amount of binding to known or suspected allergens. In this study, the rapid growth phenotype of a GH-transgenic salmon did not confer additional allergenicity as measured by *in vitro* IgE binding.

In 2011, FDA consulted with Dr. Dean Metcalfe, Chief, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health on general scientific matters related to endogenous allergens in foods known to be allergenic (see Appendix 2).

[27] Additional details regarding this study and FDA's evaluation are available in the VMAC Briefing Packet, http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/UCM224762.

In this consultation, Dr. Metcalfe provided a general overview of the standard approach to assessing the potential allergenicity of foods derived from recombinant DNA organisms. He noted that the level of allergen at which individuals experience allergic reactions is largely variable for all major allergenic food groups, including fin fish, and therefore a threshold has not yet been established for an allergic reaction to any of the major allergenic foods. He also noted that the major "treatment" for a food allergy is avoidance of the food causing the allergic reaction.

With respect to transferring a gene from one species in an allergenic food group (e.g. fin fish) to another closely-related species, Dr. Metcalfe observed that for people who know they are allergic to fin fish, there would be no new risk as they are already likely practicing food avoidance. With respect to the endogenous allergenicity of fin fish, although different species of fin fish show up to 100-fold differences in the level of the major allergen, there is no apparent public health impact because individuals who are allergic to one species of fin fish generally avoid consuming all species of fin fish. Thus, small changes in the levels of endogenous allergens would likely have little or no public health impact. Use of a five-fold increase in the level of endogenous allergens in an allergenic food could serve as a signal that an additional evaluation for possible public health impact would be warranted, not because a five-fold increase would cause a public health problem, but because it would provide a useful "flag" to investigate whether one would result. In Dr. Metcalfe's opinion, increases in endogenous allergen levels of less than five-fold in this setting would not be expected to result in an adverse effect on public health.

Based on this consultation, FDA identified no additional concerns regarding the allergenicity of food from AAS beyond those associated with food from non-GE Atlantic salmon.

Allergenicity conclusions

Triploid ABT salmon pose no additional allergenic risk than non-GE comparator Atlantic salmon. Insufficient data and information were available from which to draw a conclusion regarding possible additional allergenic risk posed by diploid ABT salmon.

Because AAS are an all female hemizygous subset of the triploid ABT salmon group (that include male triploid ABT salmon), the conclusions for the triploid ABT salmon also apply to AAS.

c. Summary of and Conclusions from the Identification and Characterization of Indirect Food Consumption Hazards

Based on all previous criteria including statistical analyses, FDA concluded that levels of all analytes in ABT salmon are similar to concentrations in appropriate comparator salmon (i.e., either sponsor controls, farm-raised salmon, literature reports, or some combination of the three).

FDA has concluded that any differences observed for analytes are the result of normal biological variation and are highly unlikely to be associated with toxicological or nutritional hazards to humans consuming ABT salmon.

The statistically significant difference in mean vitamin B6 levels was investigated using a MOE assessment. Even if the highest concentration of vitamin B6 observed in the diploid ABT salmon was found in all ABT salmon, the margin of exposure assessment indicated that it would still be well within the upper bound recommended daily intake for vitamin B6. FDA therefore found there is no food consumption hazard due to vitamin B6.

For fatty acid analytes, values found in the ABT salmon were consistently more similar to the farm-raised control values than to the sponsor control values, and were proportional to total fat levels in these three groups of salmon; these differences did not appear to be due to differences in fat content or protein source of the ABT and sponsor control salmon diets.

ABT salmon offer a balanced diet of omega-3 and omega-6 fatty acids, similar to that provided by the FC controls and by farm-raised Atlantic salmon.

FDA concluded that ABT salmon are not materially different from other Atlantic salmon with respect to omega-3 and omega-6 fatty acid levels and ratio of omega-3 to omega-6 fatty acids.

Triploid ABT salmon pose no additional allergenic risk than control Atlantic salmon. Insufficient data and information were available from which to draw a conclusion regarding possible additional allergenic risk posed by diploid ABT salmon. FDA again notes, however, that individuals allergic to salmon are likely to avoid consuming all salmon, including ABT salmon.

Because AAS are an all-female hemizygous subset of the ABT salmon group, the conclusions for the triploid ABT salmon also apply to AAS.

D. Characterization and Summary of Food Consumption Risks

FDA conducted a weight of evidence evaluation of the data and information presented in this application to assess the food safety of AAS. Primary deference was given to controlled studies submitted by the sponsor; data and information from the scientific literature were also considered for both the identification of potential hazards and for providing comparisons.

Because no food consumption hazards were identified, there are no food consumption risks.

After evaluating the previous reviews in the hierarchical risk-based approach including the molecular characterization of the GE animal lineage, the phenotypic characterization, genotypic and phenotypic durability, and food safety, FDA has not identified any unique animal feed safety issues with respect to the potential introduction of byproducts from AAS into animal feed.

E. Analytical Method for a Tolerance

A tolerance was not considered to be needed for residues resulting from insertion of the *opAFP-GHc2 construct* into the AAS. Consequently, there was no need for the development of an analytical method for the tolerance because no hazard was identified.

F. Analytical Method of Identity

An analytical method for the purpose of identity of the GE animal containing the approved construct resulting from the insertion event and lineage evaluated for the NADA is described in Appendix 3.

G. Conclusions for Food Safety

ABT salmon meets the standard of identity for Atlantic salmon as established by FDA's Reference Fish Encyclopedia. All other assessments of composition have determined that there are no meaningful differences in food composition between ABT salmon and other Atlantic salmon. No biologically relevant differences were observed in the general (e.g., proximates, including total protein and total fat) or detailed (e.g., specific amino acids, vitamins, fatty acids, ratios of fatty acids, including omega-3 and omega-6 fatty acids) composition of food from AAS and farm-raised Atlantic salmon.

Based on a careful evaluation of data generated from ABT salmon, FDA concluded that food from the hemizygous, female triploid ABT Salmon (AAS) is as safe as food from non-GE Atlantic salmon, and that there is a reasonable certainty of no harm from consumption of food from AquaAdvantage Salmon (AAS). FDA identified no animal feed consumption concerns.[28]

[28] Additional details regarding FDA's evaluation, including the conclusion that uncertainties regarding food consumption risks for AAS are no greater than those for non-GE salmon are available in the VMAC Briefing Packet, http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/VeterinaryMedicineAdvisoryCommittee/UCM224762.

X. ENVIRONMENTAL SAFETY

The EA and Finding of No Significant Impact have been posted on the agency's website at http://www.fda.gov/downloads/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/UCM466218.pdf; http://www.fda.gov/downloads/AnimalVeterinary/DevelopmentApprovalProcess/GeneticEngineering/GeneticallyEngineeredAnimals/UCM466219.pdf.

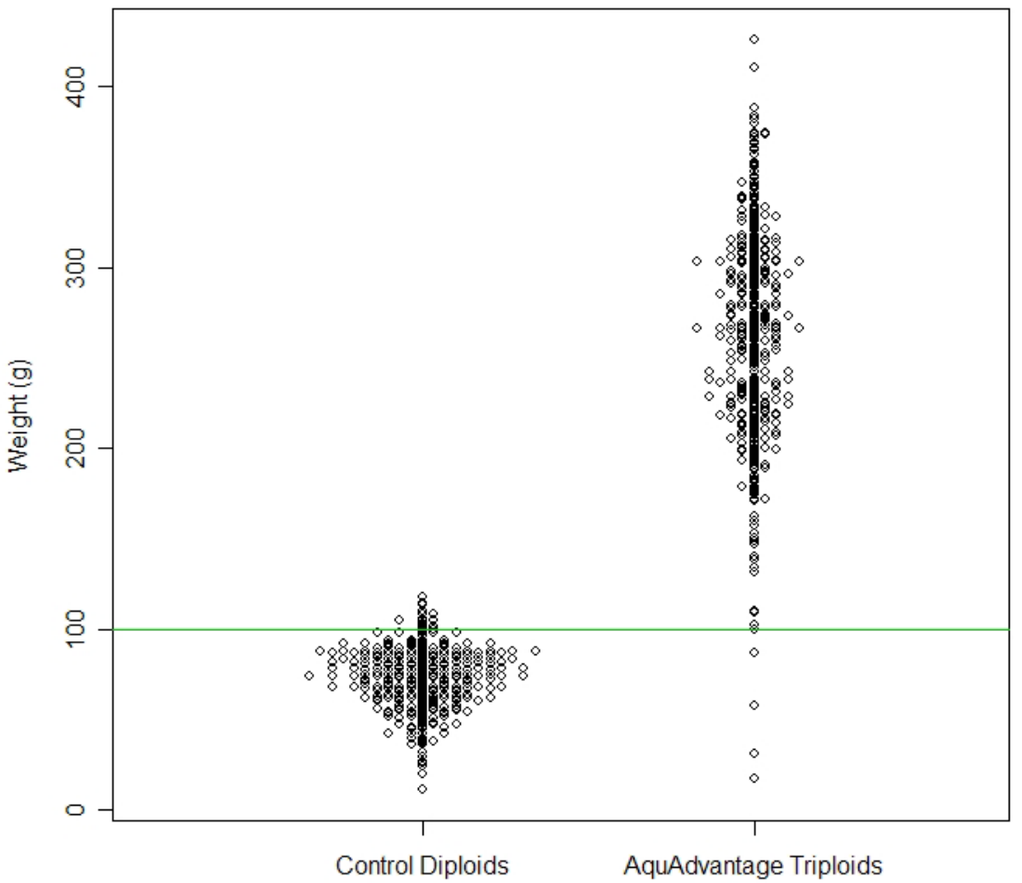
XI. CLAIM VALIDATION

The Claim Validation step focused on whether AAS met those characteristics claimed in the Product Definition. The description and the statements provided in the Product Definition served as the basis of the Claim Validation evaluation and determined the data requirements for this step. In addition, the Claim Validation evaluation drew upon data and conclusions from other steps of the review process. In the Product Definition of this application, the sponsor claimed that "Significantly more AAS grow to at least 100 g within 2700 ^{0}C-days than their comparators." Generally speaking, this meant that AAS reached 100 g, a size of importance to the aquaculture industry, sooner than farm-raised non-GE Atlantic salmon.

Data provided by the sponsor demonstrated that when compared to farm-raised diploid non-GE Atlantic salmon (1) AAS grew to a mean body weight of at least 100 g within 2700 ^{0}C-days of first-feeding, and (2) a greater proportion of AAS grew to at least 100 g within 2700 ^{0}C-days after first-feeding under normal freshwater commercial aquaculture conditions.

The data presented in support of this claim were based on the fish from the 2007 year-class. The summary of these data is presented in the Figure 6 below.

Figure 6. Weight of AquAdvantage Salmon and Comparators at 2700 deg C days



Statistical review of the data showed that AAS significantly increased body weights and the proportion of fish weighing more than 100 g at 2700 degree-days. A summary of results is presented in Table 42 below.

Table 42. Comparisons of Comparator Diploid Salmon with AquAdvantage Salmon

	Number of Fish	Mean Weight (g)	Standard Error of Weight	Number of Fish Weighing > 100 g	Percent of Fish Weighing > 100 g
Control Diploids	306	72.6	1.02	15	4.9
AquAdvantage Triploids	369	261.0	3.29	364	98.6

AAS weighed significantly more than diploid control salmon (261.0 g versus 72.6 g, respectively; $p < 0.0001$). Additionally, the percentage of AAS exceeding 100 g at 2700 degree-days (98.6%) is significantly greater than diploid control salmon (98.6% versus 4.9 %, respectively; $p < 0.0001$). FDA concluded that

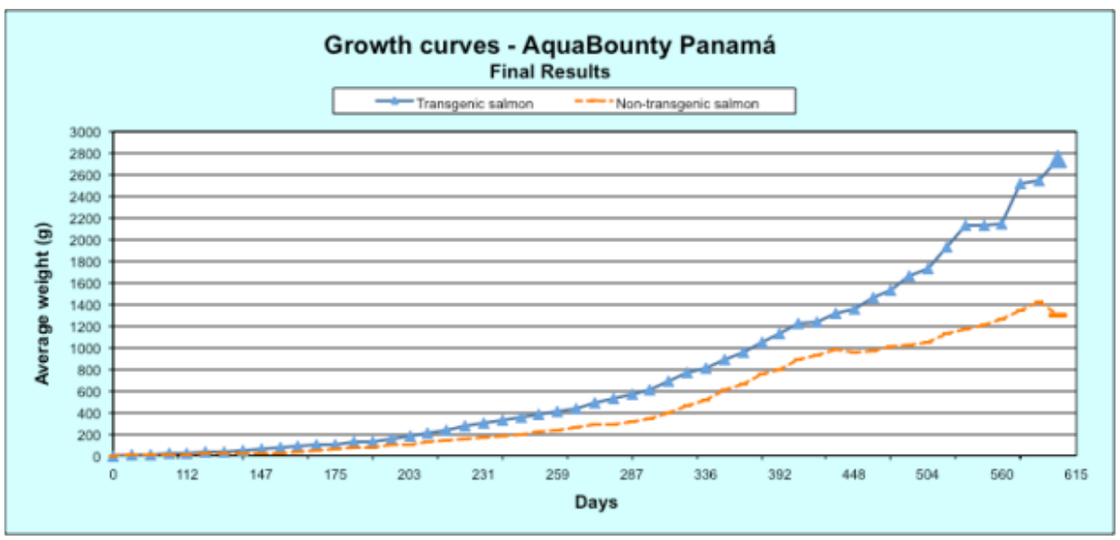
the *opAFP-GHc2* rDNA construct inserted into the genome resulted in increased growth rate in AAS vs. their non-GE comparators.

In addition to the adequate and well-controlled study presented above, in a later submission, the sponsor submitted data on the growth of AAS in Panama that provided further evidence in support of the claim, shown in Figures 7 and 8 below.

AAS were shipped from PEI to Panama in April 2009 to begin a commercial field trial. Presumptive AAS and non-GE half-siblings from all-female, hemizygous triploid crosses were reared together until they could be separated visually, based upon their size. Morphological and body weight assessments of a random sample (n = 200 fish per tank) of the fish enrolled in this production field trial were completed in December. The presumptive determination of genotype was verified by PCR analyses from blood collected from study fish at the end of the study.

The results from this study are provided in Figure 7 below. Under the management conditions at the Panama grow-out facility, AAS outperformed their non-GE counterparts in terms of growth, thereby confirming the results reported above for the designed claim validation study.

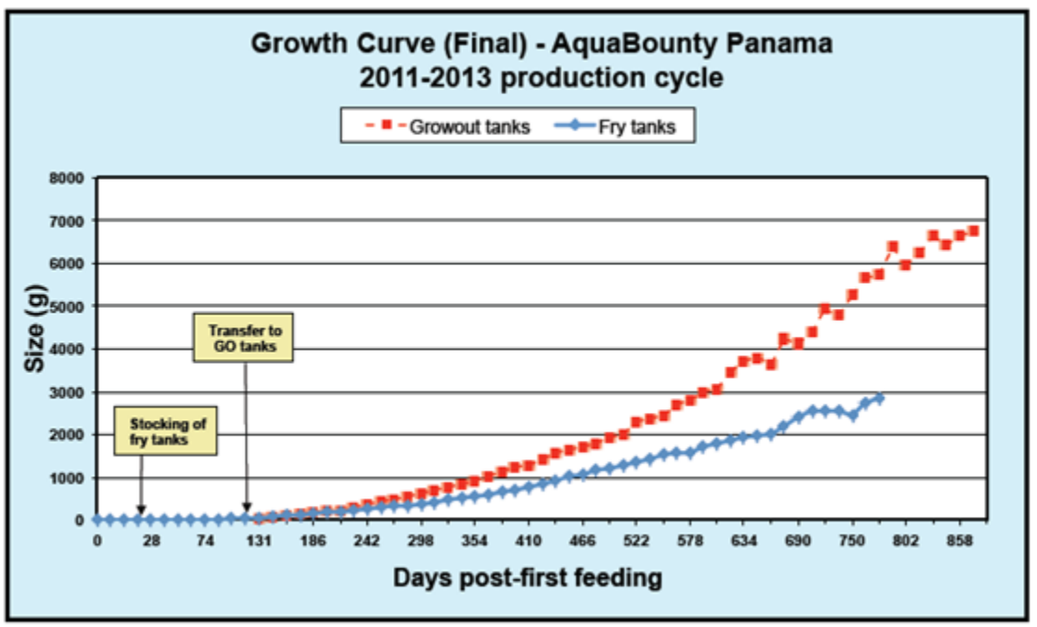
Figure 7: Growth curves for AAS and non-GE comparator (Non-transgenic salmon) fish from the 2008 year-class (2009-2010 production cycle) at the Panama facility[29].



[29] The following parenthetical statement was deleted: (n = 600 non-GE fish reared in three grow-out tanks; n = 1,400 GE fish reared in seven grow-out tanks; genotypic sampling was conducted on 200 fish per grow-out tank); the word “grow-out” in the second full paragraph which refers to the deleted statment in the caption to Figure 7 was also deleted.

A second set of data on the growth of AAS in Panama (2011-2013 production cycle, 2010 year class) evaluated growth rates in fry tanks and grow-out tanks (Figure 8). The AAS reared in grow-out tanks reached market size (~5-6 kg) in roughly two years (720-730 days). The slower growth rate of the AAS reared in the fry tanks illustrates the importance of maintaining the appropriate biomass in grow-out facilities. In addition, growth rates of GE fish reared in grow-out tanks for this production cycle were similar to those seen for GE fish from the previous production cycle (see Figure 7 above). Non-GE fish (sponsor controls) were not reared at the Panama facility beyond those from the 2008 year class.

Figure 8. Growth curves of GE fish from the 2010 year class (2011-2013 production cycle) reared in fry and grow-out tanks at the Panama facility



XII. FDA CONCLUSIONS

FDA concludes that the data submitted in support of this NADA satisfy the requirements of section 512 of the Federal Food, Drug, and Cosmetic Act and 21 CFR Part 514, and reflect the recommendations in Guidance 187. The data demonstrate that the *opAFP-GHc2* rDNA construct in AAS is safe and effective for the approved claim:

> Significantly more AquAdvantage Salmon grow to at least 100 g within 2700^{o} C-days than their comparators.

~ finis~

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XIV. APPENDICES

Appendix 1 - Phenotypic Characterization[30]

Key to Identification of Fish Groups for all Graphs:
SAT-2n= diploid non-GE age matched comparators
SAT-3n= triploid non-GE age matched comparators
SC-2n = diploid non-GE size matched comparatos
SC-3n = triploid non-GE age matched comparators
TX-2n = diploid GE; TX-3n = triploid GE

Note that the monocytes counts were zero (0) for all samples. Data are not presented graphically here as the figure would appear blank for all groups.

Hematology Values[31]

Figure 1. Hemoglobin

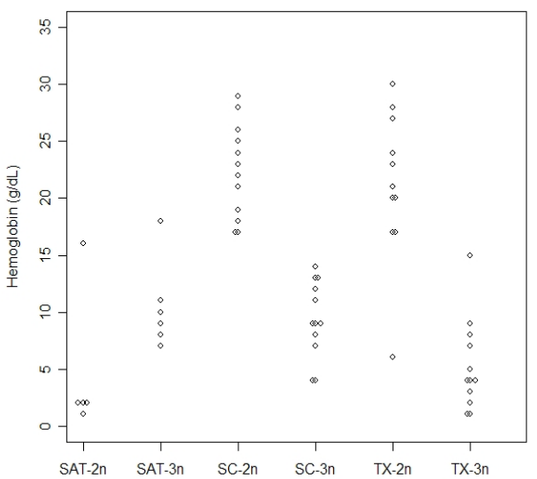


Figure 2. Hematocrit

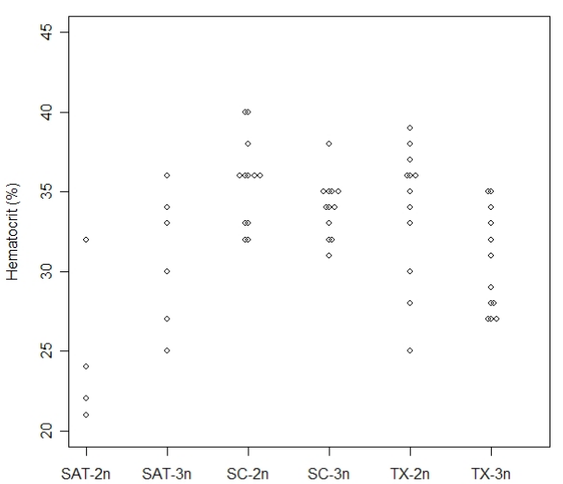


Figure 3. Platelets

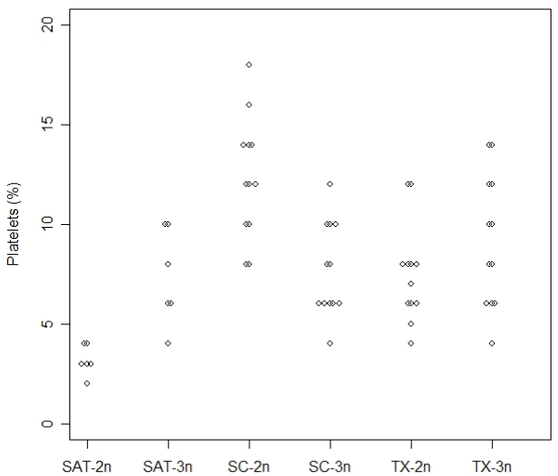


Figure 4. Neutrophils

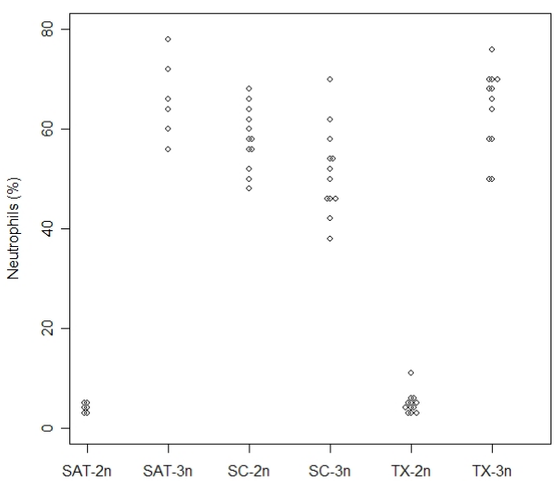
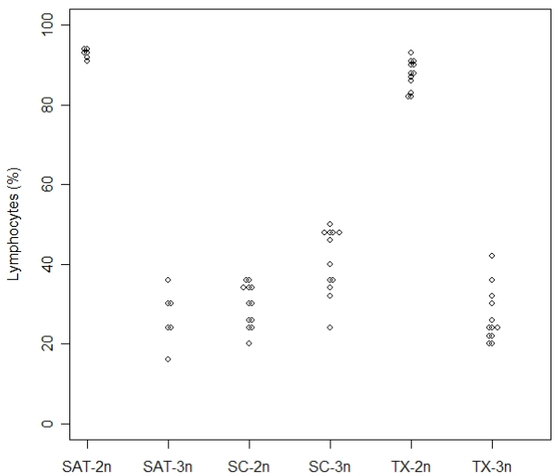


Figure 5. Lymphocytes



Biochemistry Panel Values - Protein

Figure 6. Total Protein

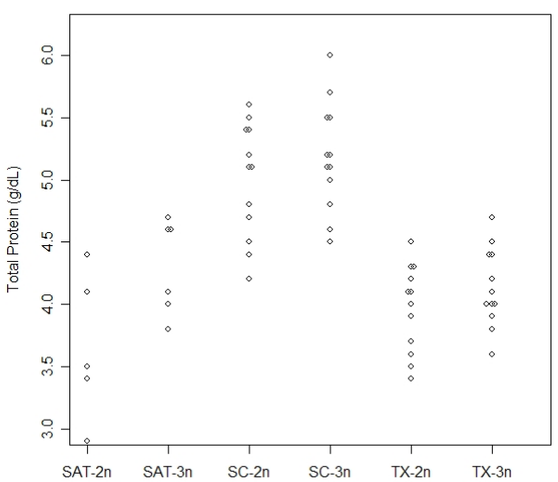


Figure 7. Albumin

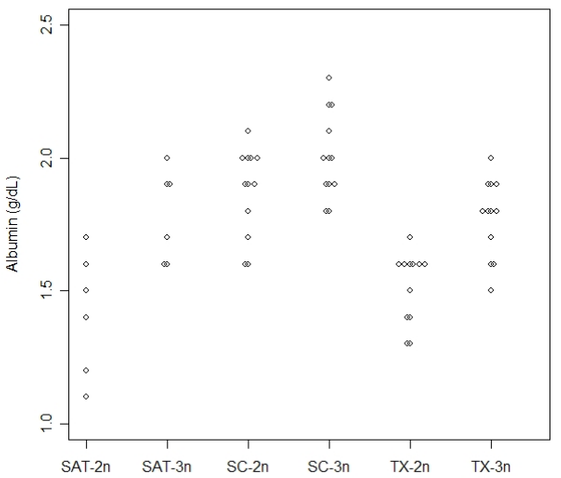


Figure 8. Globulin

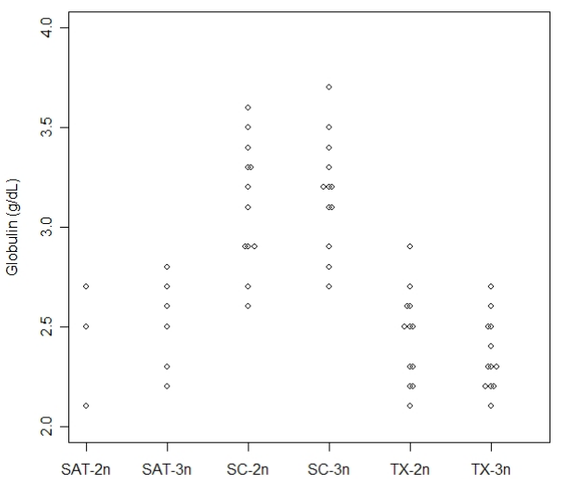
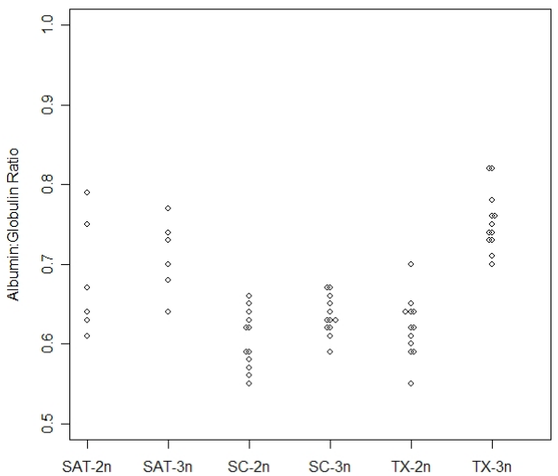


Figure 9. Alubmin:Globulin Ratio



Biochemistry Panel Values - Electrolytes

Figure 10. Sodium

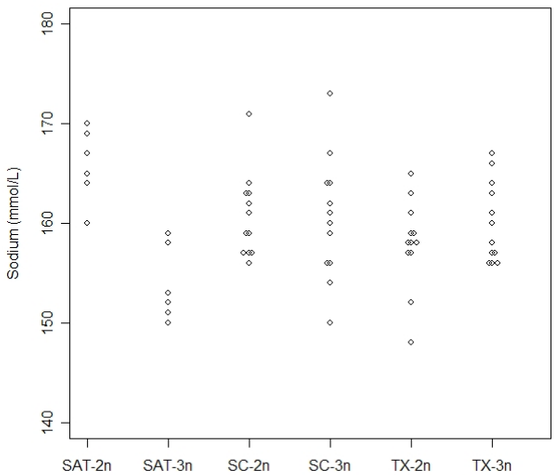


Figure 11. Chloride

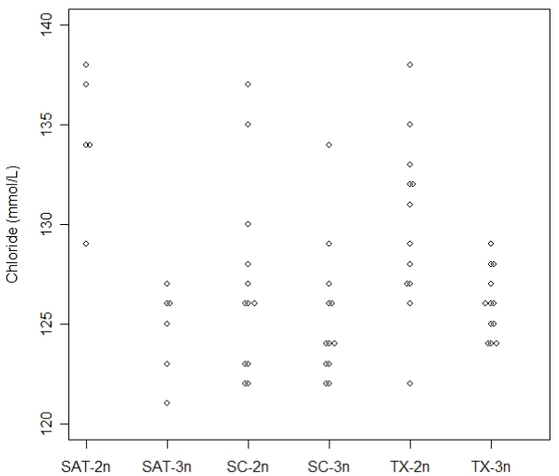


Figure 12. Potassium

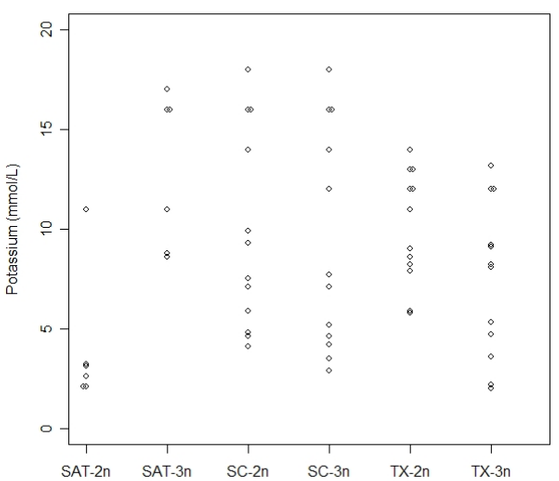


Figure 13. Calcium

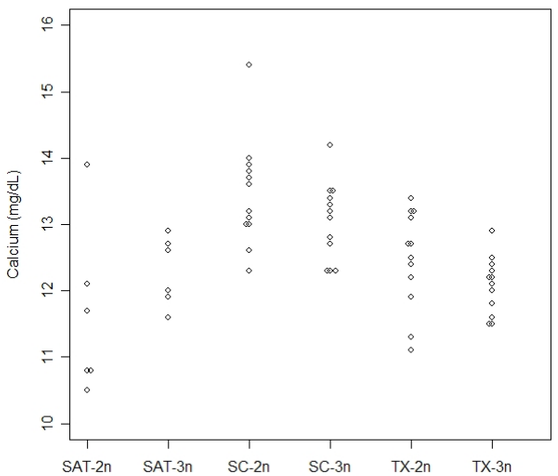


Figure 14. Phosphorous

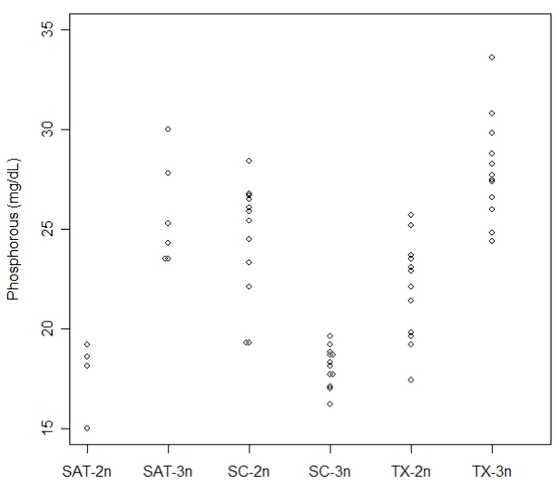
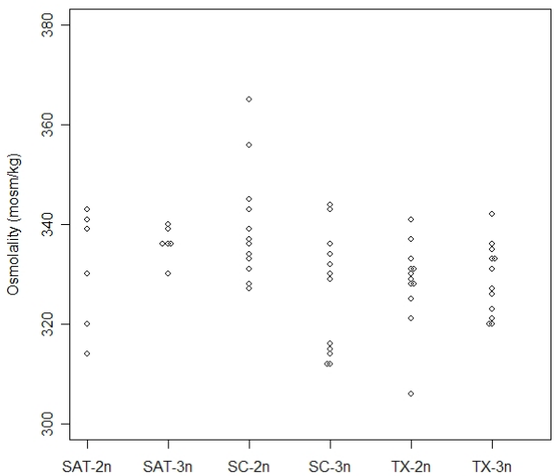


Figure 15. Osmolarity



Biochemistry Panel Values – Enzymes and Metabolites

Figure 16. Alanine Aminotransferase

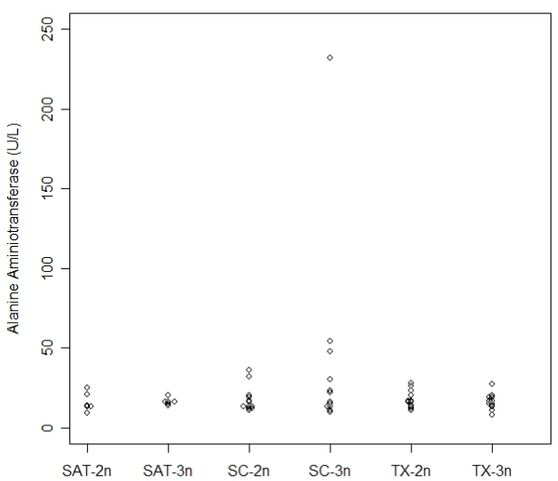


Figure 17. Aspartate Aminotransferase

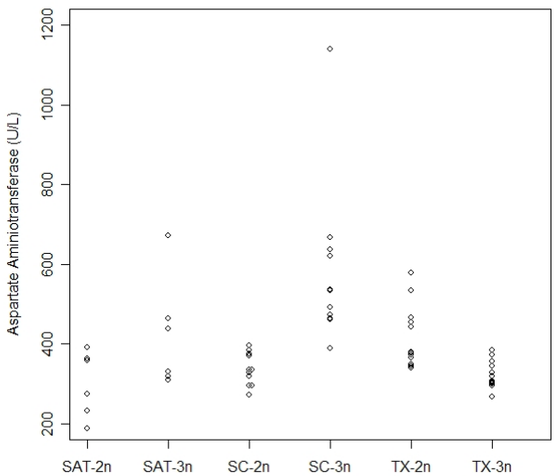


Figure 18. Total Bilirubin

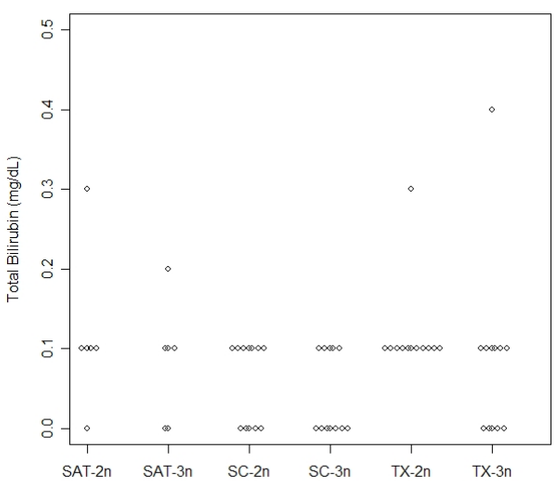


Figure 19. Creatine Phosphokinase

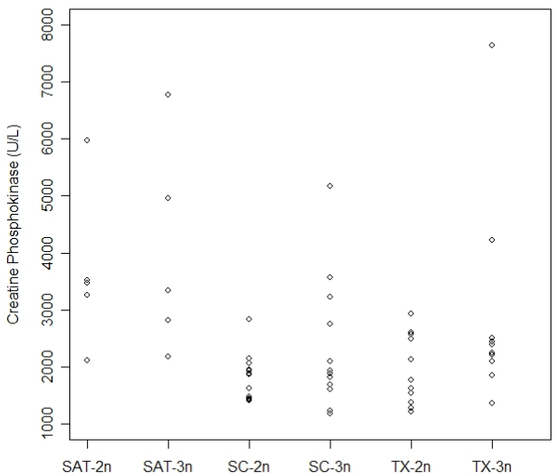


Figure 20. Cholesterol

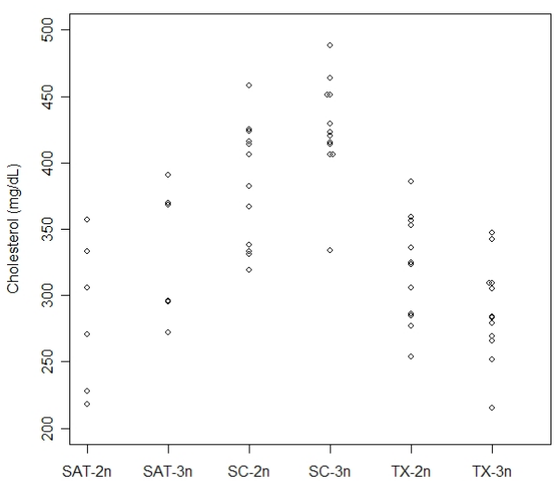
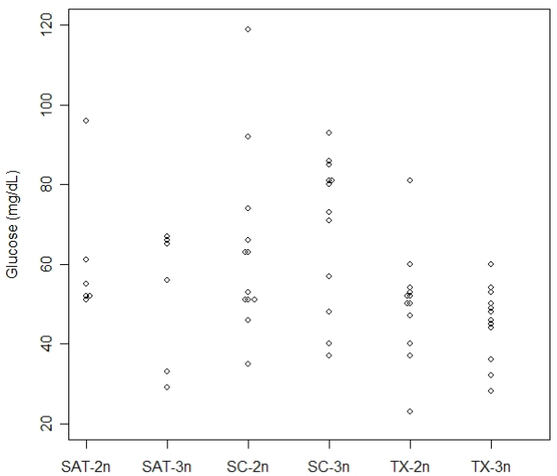


Figure 21. Glucose



Appendix 2 – Consultation with Dr. Dean Metcalfe

I. Letter from FDA to Dr. Dean D. Metcalfe

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
Rockville MD 20857

Dean D. Metcalfe, M.D.
National Institute of Health
National Institute of Allergy and Infectious Diseases
Laboratory of Allergenic Diseases
Bldg. 10, Rm. 11C207
MS 1881
Bethesda, MD 20892-1881

March 25, 2011

Dear Dr. Metcalf:

As part of concluding our review of an application for the approval of AquAdvantage Salmon (AAS), the first genetically engineered (GE) animal intended for human consumption, FDA's Center for Veterinary Medicine (CVM) has sought the advice of various scientific experts. In addition to the recommendations of the Veterinary Medicine Advisory Committee (VMAC), we are seeking additional advice from scientific experts within the U.S. government on specific technical matters.

Given that the AAS is the first GE animal intended for human consumption, our food safety experts have carefully reviewed the data and information presented by the sponsor in support of food safety. An important question that we have considered involves the assessment of the potential increased allergenicity of food from the AAS. As you know, fin fish are one of the eight foods believed to account for 90 percent of food allergies and most serious allergic reactions to food in the U.S., and one of the questions that has been raised is whether these salmon pose an additional allergenic risk relative to non-GE Atlantic salmon.

As a result, we would like to consult with you on general scientific matters related to endogenous allergens in foods already known to be allergenic particularly as these matters relate to the allergenicity of salmon, including AAS. Given your extensive knowledge and expertise in the field of allergy including food allergy and particularly your familiarity with the scientific issues associated with the allergenicity of foods derived from GE organisms, we would welcome your advice on issues that have arisen in the course of our review process.

These issues *may* be phrased as the following questions:

1. Is there value in attempting to measure the changes in the endogenous allergenicity of foods known to be allergenic (e.g., salmon and other fin fish)?
2. What are the public health implications and biological relevance of such information given the large variability in the amounts of endogenous allergens present in allergenic foods such as salmon and other fin fish?
3. Would small changes in the quantity of endogenous allergens in foods known to be allergenic (such as salmon and other fin fish) likely have any impact on public health?
4. Based on what is known about endogenous allergens in foods; is it possible to predict what magnitude of change in such endogenous allergens could reasonably be expected to pose a public health impact?

In our consideration of these issues, we have generated or consulted a set of materials including CVM's Briefing Packet from the AAS VMAC meeting, references cited in the allergenicity assessment in the Briefing Packet, and several references that were published after the completion of the allergenicity assessment. We have provided these for you to review, should you find them helpful.

After you have had a chance to consider these issues, we would like to schedule a meeting with you for an in-depth discussion. We will contact you in the next week or two to schedule a mutually convenient time. In order to maximize our interactions with you, and minimize intrusions on your very busy schedule, we propose that we prepare a draft Memorandum of Conference of that meeting, recording the salient points of the discussion, which we would make available for you as a starting point for a record of our consultation. Our intent is to make that Memorandum a part of the record of CVM's review of the submission, and in the event of an approval, it will be posted on our website as part of the Freedom of Information summary.

Thank you in advance for your assistance. Please do not hesitate to contact me if you have any questions.

Sincerely yours,

Kathleen M. Jones

Kathleen M. Jones, PhD
Animal Biotechnology Interdisciplinary Group

cc: B. Dunham/CVM Center Director
T. Forfa/CVM Deputy Director
A. Charo/FDA Office of Policy
L. Rudenko/Animal Biotechnology Interdisciplinary Group

II. Memorandum of Conference with Dr. Dean Metcalfe

MEMORANDUM OF CONFERENCE
April 21, 2011

Summary

Background
As part of concluding our review of an application for the approval of AquAdvantage Salmon (AAS), FDA's Center for Veterinary Medicine's (CVM) Animal Biotechnology Interdisciplinary Group (ABIG) has sought the advice of various scientific experts. In addition to the recommendations of the Veterinary Medicine Advisory Committee (VMAC), we sought additional advice from scientific experts within the U.S. government on specific technical matters.

Given that AAS is the first GE animal intended for human consumption, our food safety experts carefully reviewed the data and information presented by the sponsor in support of food safety. An important question that we considered involved assessing the potential for increased allergenicity of food from the AAS. Fin fish are one of the eight foods believed to account for 90 percent of food allergies and for most serious food allergic reactions in the U.S. One of the questions that have been raised is whether these salmon pose an additional allergenic risk compared to non-GE Atlantic salmon.

We consulted with Dean D. Metcalfe, M.D., of the National Institute of Allergy and Infectious Diseases on general scientific matters related to endogenous allergens in food known to be allergenic. Dr. Metcalfe is Chief of the Laboratory of Allergic Diseases, Chief of the Mast Cell Biology Section of the LAD, a former president of the American Academy of Allergy, Asthma, and Immunology, and a former chair of the American Board of Allergy and Immunology. Dr. Metcalfe is an internationally recognized expert in allergy, including food allergy. Importantly, Dr. Metcalfe is also well versed in scientific issues associated with the allergenicity assessment of foods derived from GE organisms, having served as an invited expert and elected chair of the Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Consultation on Allergenicity of Foods Derived From Biotechnology (January 2001).

As part of this consultation, we provided Dr. Metcalfe with a set of materials including CVM's Briefing Packet from the AAS VMAC, references cited in the allergenicity assessment in the Briefing Packet, several references that were published after the completion of the allergenicity assessment and several specific technical questions, particularly as these matters may relate to the allergenicity of salmon, including AAS. These materials served to guide a discussion between Dr. Metcalfe and Drs. Kathleen Jones and Larisa Rudenko of CVM's ABIG on April 21, 2011. We did not ask Dr. Metcalfe to provide an opinion on the safety of food from AAS, or the allergenicity of AAS. This memorandum summarizes the key points of the discussion.

In April 2011, Dr. Dean Metcalfe, an allergy specialist from NIH, met with two FDA staff involved in the review of the AquAdvantage Salmon. The meeting was held to discuss general scientific matters related to foods known to be allergenic. Dr. Metcalfe provided a general overview of the standard approach to assessing the potential allergenicity of foods derived from recombinant DNA organisms. He noted that the level of allergen at which individuals experience allergic reactions is largely variable for all major allergenic food groups, including fin fish, and therefore a threshold has not yet been established for an allergic reaction to any of the major allergenic foods. He also noted that the major "treatment" for a food allergy is avoidance of the food causing the allergic reaction.

With respect to transferring a gene from one species in an allergenic food group (e.g., fin fish) to another closely-related species, Dr. Metcalfe observed that for people who know they are allergic to fin fish, there would likely be no new risk as they are already likely to be practicing food avoidance. With respect to the endogenous allergenicity of fin fish, although different species of fin fish show up to 100-fold differences in the level of the major allergen in fin fish, there is no apparent public health impact because individuals who are allergic to one species of fin fish generally avoid consuming all species of fin fish. Therefore, small changes in the levels of endogenous allergens would likely have little or no public health impact. Use of a five-fold increase in the level of endogenous allergens in an allergenic food could serve as a signal that an additional evaluation for possible public health impact would be warranted, not because a five-fold increase will cause a public health problem, but because it would provide a useful "flag" to investigate whether one would result. In his opinion, increases in endogenous allergen levels of less than five-fold in this setting would not be expected to result in an adverse effect on public health.

General Comments

Dr. Metcalfe provided an overview of the standard approach to assessing the potential allergenicity of foods derived from recombinant DNA organisms.(CAC, 2008). This approach focuses on the allergenic potential of any newly expressed protein(s) in food. In Dr. Metcalfe's opinion, this component of the allergenicity assessment is critical for the protection of allergic individuals who experience allergic reactions when exposed to the source of a transferred gene, because these Individuals may be unaware of the presence of a newly expressed protein resulting from expression of the transferred gene in food derived from the rDNA organism. In Dr. Metcalfe's professional judgment,the expression of a protein from a known allergenic food group, such as fin fish, in a closely related fin fish species would not be anticipated to pose a new allergenic risk because individuals allergic to fin fish would likely already be practicing food avoidance.

Specific Questions

Question 1. Is there value in attempting to measure the changes in the endogenous foods known to be allergenic (e.g., salmon and other fin fish)?

In Dr. Metcalfe's opinion, there is some value in attempting to measure changes in endogenous allergens in foods known to be allergenic because of the intrinsic value of such scientific data. Any new data generated would add to existing knowledge on food allergy and provide additional insight into thethreshold for the elicitation of food allergic reactions.

Question 2. What are the public health implications and biological relevance of such information given the large variability in the amounts of endogenous allergens present in allergenic foods such as salmon and other fin fish?

Dr. Metcalfe discussed the current state of knowledge regarding food allergy and the foods responsible for the majority of allergic reactions in the U.S. Food allergic reactions are caused by aberrant immune responses to certain proteins found in some foods, not to the "food" itself. Although most individuals can safely consume a variety of foods, allergic individuals develop specific immune proteins known as Immunoglobulin E (IgE) that bind to specific proteins in allergenic foods and mediate a number of allergic symptoms,ranging from uncomfortable itching and/or hives to life-threatening anaphylaxis. Each allergenic food generally contains multiple allergenic proteins,some of which have been identified and characterized (for example, tropomyosins in crustacean shellfish and ovomucoid in eggs), while others have not (Gendel, 1998). Food allergies are not the same as other adverse reactions to food such as food intolerances. For example, allergy to milk is mediated by an immune response to certain proteins including caseinsin milk, whereas lactose intolerance is the inability to break down the sugar lactose from milk products due to an enzyme deficiency. Although both conditions may cause adverse reactions to milk products, lactose intolerance can be treated by enzyme

supplementation. The "treatment" for a food allergy is avoidance of the food causing the allergic reaction.

Fin fish comprise one major allergenic food group with fin fish allergies appearing to be more common in adults than in children. Fin fish allergy is less common in the U.S. than peanut, milk, shellfish, or egg allergy (Chafen et al., 2010) and consequently, relative to the other major allergenic food groups, there are fewer

publications in the scientific literature that address fin fish allergy. Allergic cross-reactivity between different species of fin fish exists (Van Do *et al.,* 2005; Griesmeier *et al.,,* 2010), meaning that an individual allergic to one species of fin fish may be allergic to other species of fin fish, although monosensitivity (allergic reactivity to only a single species of fish) has been reported (Kelso et al.,1996; Asero *et al,* 1999; Ebo *et al.,* 2010; Kuehn *et al.,* 2011). Cross-reactivity is likely due to the presence of parvalbumin, the major allergen in fish (Lindstrom *eta!.,* 1996; Van Do *et al.,* 2005; Griesmeier *et al..,* 2010). Several studies have shown a correlation between the quantities of parvalbumin present in particular species of fish and the overall allergenicity of the species (Griesmeier *eta/.,* 2010; Kuehn *eta/.,* 2010). Although there is an apparent correlation between the level of parvalbumin in fin fish species and the overall allergenicity of that species, the level of allergen at which individuals experience allergic reactions is largely variable for fin fish. This is the case for all major allergenic food groups, and consequently, a threshold for an allergic reaction for any of the major allergenic foods has not yet been established with certainty.

Question 3. Would small changes in the quantity of endogenous allergens in foods known to be allergenic (such as salmon and other fin fish) likely have any impact on public health?

Dr. Metcalfe discussed ranges of thresholds for the elicitation of allergic reactions, including reported lowest observed adverse effect levels (LOAEL) (the lowest amount shown to cause an allergic reaction) for major allergenic foods, and uncertainties associated with such thresholds (CFSAN, 2006; Madsen et al., 2009). The range of published LOAELs for fin fish is 1-100 mg protein which is higher than that for peanut, tree nuts, egg or milk (CFSAN, 2006). In other words, it would generally take a higher dose of fin fish protein to elicit an allergic response in a susceptible individual compared to the dose of food protein required to elicit a response in individuals susceptible to peanuts, tree nuts, eggs, or milk.

In order to determine whether changes in the quantity of endogenous allergens could have a public health impact, it is important to have a general understanding about The background levels of these allergens. In the case of fin fish, parvalbumin is the Major protein responsible for causing allergic reactions based on existing studies. Relatively few studies have been published on the prevalence of parvalbumin in fin fish. One study revealed that two highly allergenic fin fish, cod and whiff, contained 20-30 times the amount of parvalbumin as the lower allergenic swordfish (Griesmeier *et al.,* 2010). Another study quantified parvalbumin in eight different fin fish species (Kuehn *et al..,* 2010), and showed that the parvalbumin levels varied over 100-fold between herring and tuna. Using reactivity to IgE from the sera of fish allergic individuals as a measure of allergenicity, this study showed that cooking and processing may also significantly impact the allergenic properties of fin fish.

Although different species of fin fish show up to 100 fold differences in the level of the major allergen in fin fish, there is no apparent public health impact because individuals who are allergic to one species of fin fish generally avoid consuming all species of fin fish.

In theory, large changes in the quantity of endogenous allergens could have a public health impact because individuals with relatively high thresholds to particular food allergens who consume relatively small doses of the allergens could be unaware of their underlying allergic status. A significant increase in the quantity of allergens

present per serving of food (and consequently the quantity of allergens ingested per eating occasion) could adversely impact these individuals. Because the current approach to treating food allergies is avoidance of particular foods responsible for eliciting allergic responses, allergic individuals with known food allergies should theoretically be unaffected by a large increase in endogenous allergens within foods to which they are known to be allergic.

Because of the wide variation in allergen levels in different types of fin fish, in Dr. Metcalfe's opinion, small changes in the levels of endogenous allergens would be expected to have little or no public health impact.

Question 4. Based on what is known about endogenous allergens in foods, is it possible to predict what magnitude of change in such endogenous allergens could reasonably be expected to pose a public health impact?

There is currently insufficient information to predict what magnitude of change in endogenous allergens could be expected to pose a public health impact. One of the areas of regulatory science that could benefit from additional research would be additional investigations into the magnitude of change for a particular class of allergenic foods that could serve either as a signal further investigation. Based on existing knowledge and the previous discussion, in Dr. Metcalfe's professional opinion, a five-fold increase in the level of endogenous allergens in an allergenic food could serve as a signal that additional evaluation for possible public health impact would be warranted. This does not imply that a five-fold increase will cause a public health problem, but rather that such an increase would provide a useful "flag" to investigate whether one would result. Increases in endogenous allergen levels of less than five-fold would not be expected to result in an adverse effect on public health.

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Concurrence Table	
{Individual and Role)	**Signature and Date**
Kathleen Jones, PhD Toxicologyand Food Safety Larisa Rudenko, PhD, DABT	Kathleen Jones 8-19-2011 8/18/2011

Toxicology and Risk Assessment

cc: Document Control Unit, for the administrative file of:

Attachment 1
Jones, Kathleen

From: Metcalfe, Dean (NIH/NIAID) [E] [DMETCALFE@niaid.nih.gov]

Sent: Thursday, June 23, 2011 1:34 PM

To:Jones, Kathleen

Cc:Austin, Sarah J (NIH)

Subject:
RE:FDA Consult with NIH/OM Reviewed

Attachments: MOC-AIIergenicityfish OM ED 21June2011.doc

Kathleen – Attached is a very slightly edited version which has my approval. I am satisfied it accurately reflects our discussion.

Dean D. Metcalfe, M.D.
Chief, Laboratory of Allergic Diseases
NIAID, NIH
Building 10, Room 11C205
10 Center Drive- MSC1881
Bethesda, MD 20892-1881
Phone: 301-496-2165
Fax; 301-480-8384
Email dmetcalfe@niaid.nih.gov

Appendix 3 – Regulatory Method

FOI SUMMARY FOR AQUADVANTAGE SALMON
REGULATORY METHOD

AquAdvantage Salmon have an enhanced growth phenotype when compared to non-genetically engineered (non-GE) Atlantic salmon which allows them to grow faster. However, at market size the AquAdvantage Salmon are not phenotypically distinguishable from non-GE fish. The FDA requires that animals of species that are traditionally consumed as food must have an analytical method designed to detect the presence of the *opAFP-GHc2 construct* in tissues or edible products from these GE animals.

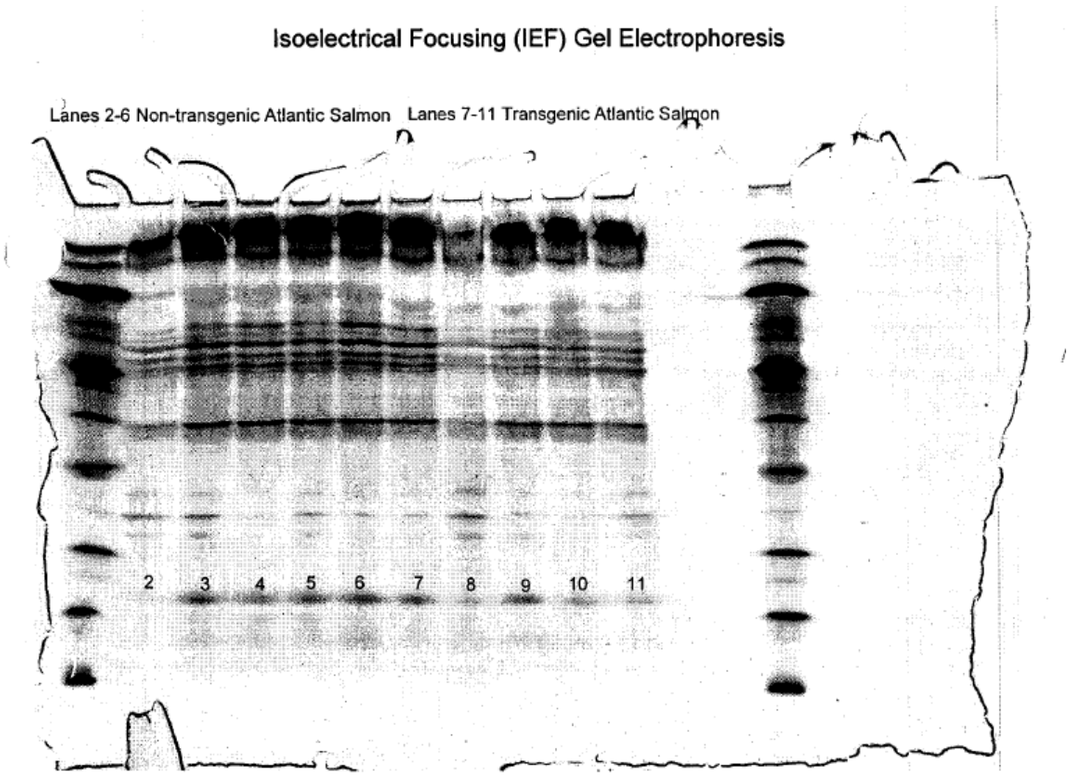
A multiplex polymerase chain reaction (PCR) procedure used by the sponsor to confirm both the genotype of the AquAdvantage Salmon and the location of the integration site for the *opAFP-GHc2 construct* at the α- locus was provided to FDA. This is the method the sponsor proposes to use for routine genotyping and surveillance of product durability and hence it is their proposed Regulatory Method. Some aspects of this multiplex PCR method were modified by FDA to achieve a robust, repeatable, accurate, molecular regulatory method.

The regulatory method is capable of discriminating between the AquAdvantage Salmon and their unmodified, non-GE counterparts. The sponsor's proposed PCR assay uses three primer pairs. The first primer set provides confirmation of the presence of the approved *opAFP-GHc2 construct* in the fish. Samples from both AquAdvantage Salmon and non-GE fish will generate two DNA amplicons corresponding to the endogenous growth hormone gene. However, samples from AquAdvantage Salmon will also amplify an additional DNA fragment unique to the *opAFP-GHc2 construct*. PCR assays using two additional primer pairs amplify DNA regions at the 5' and 3' junctions of the *opAFP-GHc2 construct* as further evidence in support of the integration event in the EO-1α lineage. These two primer sets provide additional assurance about the genetic nature of the fish samples and help identify these fish as the AquAdvantage Salmon containing the *opAFP-GHc2 construct* at the α- locus.

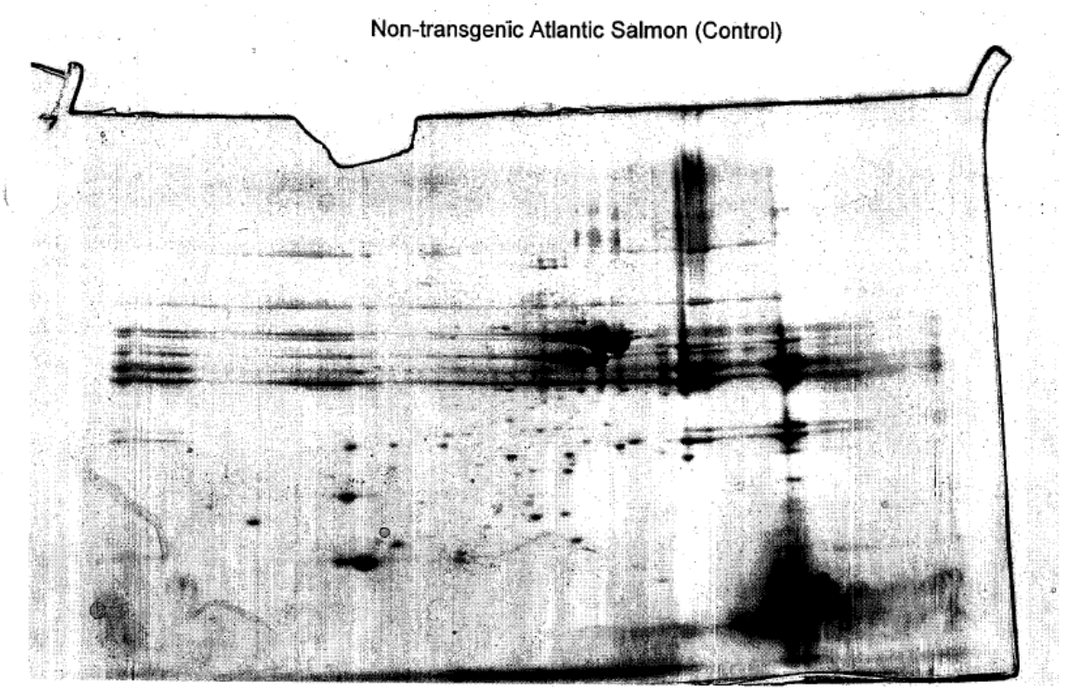
In addition to being able to distinguish AquAdvantage Salmon from non-GE fish in a mixed population this method is also capable of (1) identifying edible tissue from AquAdvantage Salmon, (2) identifying a durability failure, and (3) discriminating between the AquAdvantage Salmon and a knock-off (other GE fish containing similar constructs that are not AquAdvantage Salmon).

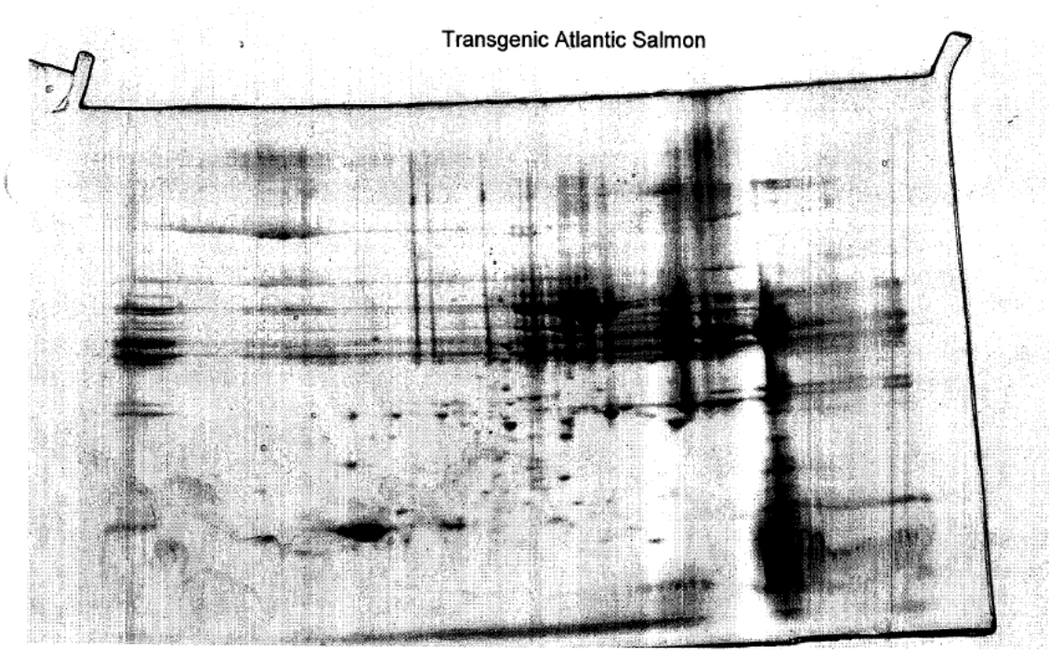
We conclude that this method is suitable for the purposes proposed by the sponsor and is capable of determining genotype and confirming rDNA integration at the described α- locus. Further, the FDA modified method has been peer-validated at the agency's Office of Regulatory Affairs (ORA) district laboratory. This validated PCR method meets the agency's requirements for a Regulatory method to identify the presence of the *opAFP-GHc2 construct* in fish and is available from the Center for Veterinary Medicine, FDA, 7500 Standish Place, Rockville, MD 20855.

Appendix 4A – Figures from the Regulatory Fish Encyclopedia Study

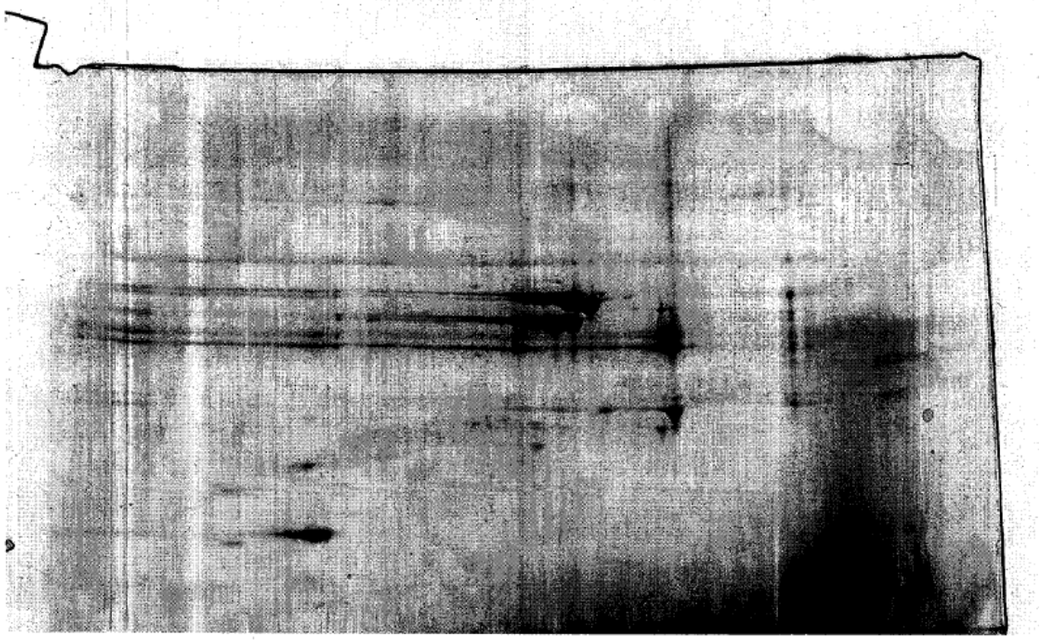


2-Dimensional Gel Electrophoresis




Transgenic Atlantic Salmon

Store bought Atlantic Salmon (Reference Sample)



Appendix 4b – Bar Coding Study

FLAG Compliance Sample

ANALYST WORKSHEET	1. PRODUCT: AquAdvantage GMO Salmon		2.. SAMPLE NUMBER: NA
3. SEALS: ☒ NONE ☐ INTACT ☐ BROKEN	4. DATE REC'D: 10/23/12	5. RECEIVED FROM: Dawn Runighan (Aqua Bounty)	6. DISTRICT OF LABORATORY: CFSAN

7. DESCRIPTION OF SAMPLE
1 frozen skin-on filet of salmon (0.75 kg)

8. NET CONTENTS	9. LABELING
☒ NOT APPLICABLE ☐ NOT DETERMINED EXAMINED ☐ DECLARE/UNIT AMOUNT FOUND % OF DECLARED	___ ORIGINAL(S) SUBMITTED ___ COPIES SUBMITTED ☒ NONE

10. SUMMARY OF ANALYSIS

CHAIN OF CUSTODY:

Cooler box containing dry ice and one AquAdvantage GMO salmon filet (0.75 kg) was received at FDA CFSAN College Park, MD (Fed Ex tracking # 8381 3398 0686 0426) by Research Biologist Jonathan Deeds on 10/23/12. Sample was contained in a plastic whirl pac bag labeled as "AAS Fish ID# ABF10-AS3PS.128 SR#1466". Two 10 mg sub-samples were taken using a sterile scalpel and forceps, placed in separate sterile microcentrifuge tubes, and transferred to Biologist Sara Handy for DNA Barcode analysis on 10/23/12. Entire sub-sample was used for analysis. The remaining filet was placed in secure -80C freezer at CFSAN.

REASON FOR ANALYSIS:

Confirmation that new DNA Barcode species identification analysis recently transferred to all FDA regional field laboratories would identify AquAdvantage GMO salmon as Atlantic Salmon (*Salmo salar*).

RESULTS SUMMARY.

The AquAdvantage GMO salmon filet was found to match (>98% Pairwise Identity Score) two FDA reference standards for Atlantic Salmon (*Salmo salar*) based on the cytochrome *c* oxidase 1 (COI) mitochondrial gene currently used for species identification by the FDA.

Detailed results are below.

11. RESERVE SAMPLE
Remaining tissues from AquAdvantage salmon filet labeled as "AAS Fish ID# ABF10-AS3PS.128 SR#1466" are stored in -80C freezer SMSB#32 located in BEL04-D.

12.a. ANALYST SIGNATURE Jonathan Deeds, Ph.D. Research Biologist	13. WORK-SHEET CHECK	a. BY John H. Callahan
b.		b. DATE 11/01/12
c.		14. DATE REPORTED 11/01/12

FORM FDA 431 (5/84) PREVIOUS EDITION IS OBSOLETE PAGE 1 OF 5 PAGES
ATTACHMENTS

Appendix 5 – Species Specificity of Growth Hormone

With the intent of determining the ability of growth hormone to bind to homologous growth hormone receptors and potentially activate them physiologically across vertebrate species we performed a survey of existing scientific literature; the relevant studies are cited in the following text or in the references at the end of this Appendix. Based on the results of this search we conclude that Growth Hormone (GH) binding efficiency decreases as one compares up the phylogenetic tree, meaning that the GH of lower vertebrates do not bind the Growth Hormone Receptor (GHR) of higher vertebrates with sufficient affinity to activate the signaling cascade that causes the somatotropic effects associated with GH activity. Thus, fish GH would not activate human GHR and would therefore be physiologically ineffective.

Initial interest in the ability of cross species activity of GH resulted from an attempt to use porcine pituitary extracts against dwarfism in humans in the 1950s. These pituitary extracts were effective in animals but not in humans. Porcine GH shows *in vivo* cross species reactivity when administered subcutaneously or intramuscularly to rats (wild type and hypophysectomized), and dogs but not in hypophysectomized monkeys or human cells. GH derived from bovine, ovine, porcine and whale pituitaries were ineffective in humans.[1,2] Further, porcine pituitary extracts when used in humans did not stimulate growth, increase plasma free fatty acid levels, decrease plasma alpha-amino nitrogen, impair glucose tolerance or cause hyperinsulinaemia in GH deficient children.[1]

In rats recombinant porcine GH (rpGH) was not orally active and showed no effect when orally administered and was not bioactive across the GI tract. rpGH did not bioaccumulate in the serum or cause an immune reaction. rpGH when administered at 4 mg/kg (considered a high dose in rats) by oral gavage caused no treatment related toxicity and the conclusion was that rpGH being a protein was subject to degradation by digestive enzymes in the gastrointestinal tract. Subcutaneous administration of pGH and rpGH in both wild type and hypophysectomized rats led to stimulation of growth.[1] Similarly, bovine GH is effective when administered subcutaneously to rats and has no significant effect via the oral route .[2]

Injecting pGh or rpGH in pigs resulted in increased serum GH levels but not in physiologically significant increased muscle GH concentrations when measured at 27 hours post-treatment.[1] The peak serum GH levels after intramuscular injection of pGH were seen at 6 hours and the mean half life of pGH was 4 minutes (fast phase) and 38 minutes (slow phase). Serum GH levels in untreated pigs was in the range of 1.6 to 7 ng/ml.[1]

Species specificity is related to several features of both the GH molecule and its cognate receptor. Most non-primate GH differ from each other by zero to four amino acids in the mature full length peptide of 190 amino acids[3] while human and rhesus GH differ from non-primate GH by 59-63 amino acids a difference of ~33% ([3] and see Table 1 below), indicating a large evolutionary shift in GH protein sequence with potential for significant secondary and tertiary structure effects that could affect function. Porcine GHs (both pGH and rpGH) are 66% similar to human GH they still do not bind sufficiently to human GHR as determined by their inability to displace bound ^{125}I-hGH *in vitro*[1]. Fish GH is 34% identical to human GH (see Table 1) and does not bind human GHR effectively enough to cause its activation. Bacterially expressed recombinant Dolphinfish GH had no mitogenic activity *in vitro* on cloned hGH receptor expressing cells. However, both recombinant common carp and recombinant dolphinfish GH stimulated rabbit GHR expressing cells[5]. Competitive binding assays with purified

recombinant proteins and homologous GHRs provided the following results: IC_{50} dophinfish GH = 3.06 nM, gilthead seabream ~2 nM and K_a values of 0.77, 1.30 and 0.52 nM^{-1} for dolphinfish, common carp and gilthead seabream GH respectively[5]. Ruminant placental lactogen shows some *in vivo* effect in fish but is not as efficient a somatotrophic agent as homologous fish GHR[5]. Human GH has a high affinity for non-primate GHRs but non-primate GHs have ~3000X lower affinity for human GHR than human GH[3,4]. Essentially this results in an inability of non-primate GHs to stimulate human GHR[3].

The molecular basis for this species specificity is a change in human GH amino acid sequence at position 171 from histidine to aspartic acid (Asp). This Asp residue at position 171 is shared among all primates and therefore believed to have occurred in a common ancestor of simians. Tarsiers, lemurs and non-primates share a histidine at this position, along with all other vertebrates from cartilaginous fish to mammals, indicating that this is the ancestral form of the protein.[3,4] Additionally, human GHR has a Leucine (Leu) to Arginine (Arg) change at position 43 which causes an unfavorable charge repulsion/steric hindrance between Arg43 and non-primate GH His170 resulting in the decreased binding efficiency and inability for receptor activation[4]. The Asp at 171 of human GH forms a favorable salt bridge with Arg43 of human GHR allowing efficient binding and activation.[3] Thus, non-primate GHs have little to no binding and activation potential for human GHR, especially if ingested via the oral route.

The *opAFP-GHc2 construct* contains the Chinook salmon (*Onchorhynchus tshawytscha*) GH1 open reading frame including both the 188 amino acid coding region and the 22 amino acid signal peptide. Sequences were aligned using the European Bioinformatics Institute (EBI) online tool EMBOSS (http://www.ebi.ac.uk/Tools/emboss/align) which uses the Needleman-Wunsch algorithm to produce a full path matrix upon implementing a Pairwise Sequence Alignment. Our analysis used the default settings for protein alignments - a Blosum62 matrix and Gap penalties of 10.0 (Open) and 0.5 (Extend).

Table 1: Percentage identity and similarity between derived amino acid sequences of Growth Hormone1 (GH1) coding regions of various vertebrate species.

Genbank Accession Numbers for the amino acid sequences used are listed below. All the sequences were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/sites/gquery).
Homo sapiens GH1 Accession#: NM_000515.3
Bos taurus GH1 Accession#: NP_851339.1
Mus musculus GH1 Accession#: NP_032143.1
Gallus gallus GH1 Accession#: NP_989690.1
Danio rerio GH1 Accession#: NP_001018328.2
Salmo salar GH1 Accession#: AY614010.1
Salmo salar GH2 Accession#: EU621899.1
Onchorhynchus tshawytscha GH1 Accession#: EU621900.1
Onchorhynchus tshawytscha GH2 Accession#: EU621901.1

Identity / Similarity	Chinook GH1	Chinook GH2	Salmo GH1	Salmo GH2	Danio GH1	Gallus GH1	Mus GH1	Bos GH1	Human GH1
Chinook GH1		93.8% 97.1	95.2 99.0	94.3 98.1	62.3 79.2	37.7 57.4	36.1 53.9	35.7 52.7	34.4 50.9
Chinook GH2			96.7 97.6	97.6 98.6	63.2 79.7	37.7 55.6	34.7 52.0	35.7 50.4	33.5 50.0
Salmo GH1				97.1 98.1	63.2 79.7	38.6 57.4	36.1 53.9	36.6 52.2	33.9 50.0
Salmo GH2					62.9 79.3	37.7 56.1	36.1 53.9	36.6 51.8	33.9 50.0
Danio GH1						41.1 59.4	39.9 57.3	40.2 57.5	35.2 52.2
Gallus GH1							74.2 88.9	72.0 84.9	56.4 73.6
Mus GH1								82.9 88.9	66.5 78.0
Bos GH1									66.7 76.7
Human GH1									

References

1. Porcine somatotropin (WHO Food Additives Series 43). 52nd Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). World Health Organization- IPCS, Geneva, 2000. www.inchem.org/documents/jecfa.jecmono/v43jec06.htm

2. Juskevich, J. and Guyer, C. G. (1990). Bovine Growth Hormone: Human Food Safety Evaluation. *Science.* 249: 875-884.

3. Liu, J. –C., Makova, K. D., Adkins, R. M., Gibson, S., and Li, W. –H. (2001). Episodic Evolution of Growth Hormone in Primates and Emergence of the Species Specificity of Human Growth Hormone Receptor. *Molecular Biology and Evolution*. 18(6): 945-953.

4. Souza, S. C., Frick, G. P., Wang, X., Kopchick, J. J., Lobo, R. B., and Goodman, H.M. (1995). A Single arginine residue determines species specificity of the human growth hormone receptor. *Proc. Natl. acad. Sci. USA.* 92: 959-963.

5. Paduel, A., Chapnik-Cohen, N., Gertler, A., and Elizur, A. (1999). Preparation and Characterization of Recombinant Dolphin Fish (Coryphaena hippurus) Growth Hormone). *Protein Expression and Purification*. 16: 417-423.

Appendix 6 – Statistical Analysis Results for Composition

Results from the statistical analysis of selected analytes are provided below. Data were analyzed using analysis of variance with group (FC, SC, TX), ploidy (diploid, triploid) and the group-by-ploidy interaction (group*ploidy) included in the model as fixed effects. The analysis results are interpreted with the understanding that the estimated p-value may be under estimated (too small) because comparisons are generated after the data were examined. However, for exploratory analyses, this is an acceptable strategy.

If the group-by-ploidy interaction (group*ploidy) is considered significant, the TX group mean was compared to the FC and SC group means separately within ploidy. If the group-by-ploidy interaction was not considered significant, and the group effect was considered significant, the TX group mean was compared to the FC and SC group means without regard to ploidy.

Analyte	Effect	Probability*
calcium	group	0.1447
	ploidy	0.6262
	group*ploidy	0.8451
copper	group	0.6270
	ploidy	0.8828
	group*ploidy	0.7579
manganese	group	0.7421
	ploidy	0.3552
	group*ploidy	0.4949
potassium	group	0.0001
	ploidy	0.6698
	group*ploidy	0.1314
serine	group	0.0016
	ploidy	0.4425
	group*ploidy	0.1224
vitamin b6	group	0.0002
	ploidy	0.0442
	group*ploidy	0.1697
zinc	group	0.1094
	ploidy	0.5896
	group*ploidy	0.4116
folic acid	group	0.0756
	ploidy	0.0195
	group*ploidy	0.0277
iron	group	0.6575
	ploidy	0.3233
	group*ploidy	0.3109
magnesium	group	<0.0001

Analyte	Effect	Probability*
	ploidy	0.0466
	group*ploidy	0.0065
niacin	group	<0.0001
	ploidy	0.0977
	group*ploidy	0.0223
phosphorous	group	0.0039
	ploidy	0.4593
	group*ploidy	0.0489

* Probability associated with the F-test for the specified effect.

Analyte	Group*	Least Squares Mean	Standard Error	P-value**
calcium	FC	31.490	1.9787	
	SC	30.057	1.0937	
	TX	27.573	1.1424	
copper	FC	0.064	0.0110	
	SC	0.069	0.0061	
	TX	0.075	0.0063	
manganese	FC	0.028	0.0099	
	SC	0.033	0.0055	
	TX	0.027	0.0057	
potassium	FC	375.500	6.8889	0.3911
	SC	394.244	3.8080	<0.0001
	TX	368.633	3.9773	
serine	FC	0.761	0.0196	0.7779
	SC	0.811	0.0108	0.0006
	TX	0.755	0.0113	
vitamin b6	FC	8.002	0.2691	0.0001
	SC	8.736	0.1488	0.0086
	TX	9.318	0.1554	
zinc	FC	0.568	0.0243	
	SC	0.515	0.0135	
	TX	0.509	0.0141	

* Group: FC = farm control, SC = sponsor control, TX = transgenic.
** The P-value associated with comparing the TX mean to the FC or SC mean.

Analyte	Ploidy	Group*	Least Squares Mean	Standard Error	P-value**
folic acid	diploid	FC	0.366	0.0397	0.0009
		SC	0.272	0.0209	0.0391
		TX	0.207	0.0229	
	triploid	FC	0.212	0.0397	0.7145
		SC	0.231	0.0229	0.9589
		TX	0.229	0.0229	
iron	diploid	FC	0.65	0.088	0.1159
		SC	0.46	0.046	0.7180
		TX	0.49	0.051	
	triploid	FC	0.39	0.088	0.1065
		SC	0.49	0.051	0.3965
		TX	0.55	0.051	
magnesium	diploid	FC	26.100	0.7244	0.6799
		SC	26.706	0.3818	0.0973
		TX	25.753	0.4182	
	triploid	FC	25.020	0.7244	0.0988
		SC	27.260	0.4182	<0.0001
		TX	23.620	0.4182	
niacin	diploid	FC	86.280	3.3301	0.0001
		SC	91.033	1.7551	<0.0001
		TX	102.527	1.9226	
	triploid	FC	91.500	3.3301	0.8157
		SC	85.813	1.9226	0.0181
		TX	92.400	1.9226	
phosphorous	diploid	FC	263.000	6.2586	0.8686
		SC	265.167	3.2986	0.4938
		TX	261.800	3.6134	
	triploid	FC	258.400	6.2586	0.3139
		SC	272.067	3.6134	0.0001
		TX	251.067	3.6134	

Analyte	Effect	Probability*
eicosdienoic acid	group	<0.0001
	ploidy	0.6500
	group*ploidy	0.2720
linoleic acid	group	0.0002
	ploidy	0.9317
	group*ploidy	0.3109
linolenic acid	group	0.0001
	ploidy	0.5104
	group*ploidy	0.5594
oleic acid	group	<0.0001
	ploidy	0.3675
	group*ploidy	0.1397
arachidic acid	group	<0.0001
	ploidy	0.6600
	group*ploidy	0.0099
docosahexaenoic acid	group	<0.0001
	ploidy	0.5783
	group*ploidy	0.0073
docosapentaenoic acid	group	<0.0001
	ploidy	0.9796
	group*ploidy	0.0968
eicosopentaenoic acid	group	<0.0001
	ploidy	0.5967
	group*ploidy	0.0241
free fatty acids	group	0.0045
	ploidy	0.7525
	group*ploidy	0.1044
palmitoleic acid	group	<0.0001
	ploidy	0.5604
	group*ploidy	0.0296
palmitic acid	group	<0.001
	ploidy	0.5154
	group*ploidy	0.0032
stearic acid	group	<0.0001
	ploidy	0.6767
	group*ploidy	0.0097
total fatty acids	group	<0.0001
	ploidy	0.5646
	group*ploidy	0.0207

Analyte	Group*	Least Squares Mean	Standard Error	P-value**
eicosdienoic acid	FC	0.05	0.005	0.2852
	SC	0.04	0.003	<0.0001
	TX	0.06	0.003	
linoleic acid	FC	0.67	0.068	0.3403
	SC	0.51	0.038	<0.0001
	TX	0.74	0.039	
linolenic acid	FC	0.18	0.028	0.1004
	SC	0.13	0.016	<0.0001
	TX	0.23	0.016	
oleic acid	FC	2.88	0.234	0.1245
	SC	2.00	0.129	<0.0001
	TX	3.30	0.135	

Analyte	Ploidy	Group*	Least Squares Mean	Standard Error	P-value**
arachidic acid	diploid	FC	0.03	0.003	0.0916
		SC	0.01	0.001	0.0014
		TX	0.02	0.002	
	triploid	FC	0.02	0.003	0.2258
		SC	0.01	0.002	<0.0001
		TX	0.03	0.002	
docosahexaenoic acid	diploid	FC	1.44	0.116	0.3061
		SC	1.04	0.061	0.0065
		TX	1.30	0.067	
	triploid	FC	1.48	0.116	0.6451
		SC	0.86	0.067	<0.0001
		TX	1.55	0.067	
docosapentaenoic acid	diploid	FC	0.41	0.052	0.2325
		SC	0.30	0.027	<0.0001
		TX	0.48	0.030	
	triploid	FC	0.46	0.052	0.3464
		SC	0.22	0.030	<0.0001
		TX	0.52	0.030	
eicosopentaenoic acid	diploid	FC	1.13	0.116	0.3192
		SC	0.67	0.061	0.0006
		TX	1.00	0.067	

Analyte	Ploidy	Group*	Least Squares Mean	Standard Error	P-value**
	triploid	FC	1.22	0.116	0.8663
		SC	0.50	0.067	<0.0001
		TX	1.19	0.067	
free fatty	diploid	FC	0.05	0.014	0.3447
		SC	0.07	0.007	0.9515
		TX	0.07	0.008	
	triploid	FC	0.03	0.014	0.0002
		SC	0.07	0.008	0.0655
		TX	0.09	0.008	
palmitoleic acid	diploid	FC	0.97	0.086	0.0952
		SC	0.61	0.046	0.0051
		TX	0.80	0.050	
	triploid	FC	0.98	0.086	0.9575
		SC	0.51	0.050	<0.0001
		TX	0.97	0.050	
palmitic acid	diploid	FC	2.11	0.173	0.0118
		SC	1.16	0.091	0.0021
		TX	1.59	0.100	
	triploid	FC	1.71	0.173	0.1752
		SC	0.96	0.100	<0.0001
		TX	1.99	0.100	
stearic acid	diploid	FC	0.43	0.041	0.2595
		SC	0.26	0.022	0.0008
		TX	0.37	0.024	
	triploid	FC	0.36	0.041	0.0367
		SC	0.21	0.024	<0.0001
		TX	0.46	0.024	
total fatty acid	diploid	FC	15.20	1.283	0.1483
		SC	9.76	0.676	0.0017
		TX	13.03	0.741	
	triploid	FC	15.14	1.283	0.6574
		SC	8.39	0.741	<0.0001
		TX	15.80	0.741	

Appendix 7 – Vitamin B6 – Explanation of Conversion Factor

In this review, we report vitamin B6 concentrations in free base form. The sponsor used a standard yeast-based biochemical assay to measure the protonated ('salt') form of vitamin B6 (pyroxidine HCl). In order to be able to compare vitamin B_6 levels across studies, a conversion was required. We consulted with Darryl Sullivan of Covance Inc. on the conversion issue.

Vitamin B6 in peer-reviewed scientific literature is usually reported as the free base form (pyroxidine) and measured via HPLC. Conversion from salt to free base is accomplished by multiplying the salt form of Vitamin B6 by a coefficient of 0.823. This calculation accounts for the removal of HCl from the molecule.[32] We therefore refer to vitamin B6 in its free base form throughout this review.

[32] Pyroxidine HCl ($C_8H_{11}NO_3$-HCl) molecular weight is equal to 205.64 g/mol. Free base pyroxidine ($C_8H_{11}NO_{3)}$ has a molecular weight of 169.24 g/mol. The difference between the two forms is simply calculated as 205.64/169.34 = 0.823.