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Risk Assessment and Mitigation of AquAdvantage Salmon

Anastasia Bodnar

Aqua Bounty Technologies, Inc. has recently applied for deregulation of their AquAdvantage salmon—salmon that have been genetically engineered to grow faster than wild-type salmon. The salmon have the potential benefit of providing high-quality animal protein without putting additional pressure on declining wild fish stocks. However, these salmon present some potential risks that warrant examination. First, effects on the health and welfare of the animals must be determined. Second, if genetically engineered salmon were to escape and become established in the wild, native salmon populations or other aspects of the ecosystem could be adversely affected. Third, this genetically engineered trait or some part of the development or rearing process might have health consequences for consumers. These risks must be fully addressed before deregulation can be considered.

The science behind the salmon

In 1989, the founder animal of the AquAdvantage salmon line was created by injecting an Atlantic salmon (*Salmo salar*) egg with a gene construct (termed opAFP-GHc2; **Fig. 1a**) that contained a promoter and termination region from the antifreeze gene of the ocean pout (*Zoarces americanus*) and a growth hormone gene from Chinook salmon (*Oncorhynchus tshawytscha*). The ocean pout antifreeze promoter was previously shown to be constitutive, or continually expressing, in salmon[1], in contrast to the native growth hormone promoter in salmon, which only expresses in response to certain environmental cues, such as day length and temperature[2].

The Chinook and Atlantic salmon growth hormone genes are very similar. A BLAST comparison of the mRNA for each (GenBank S50867.1 and X14305.1, respectively) showed 90% (1013/1126) of the nucleotides were identical and only 6% gaps (70/1126). A comparison of the protein sequences revealed 95% (198/210) of the amino acids were identical, 98% (205/210) of the amino acids were similar, and 0% gaps.

Figure 1a.

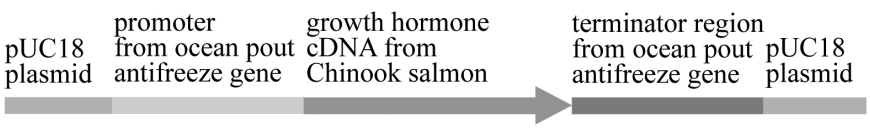

Figure 1b.

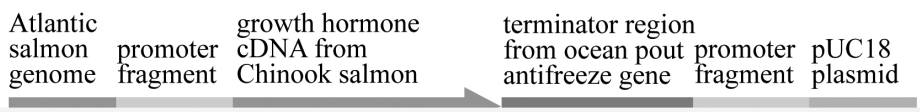

Figure 1a. Gene construct, termed opAFP-GHc2, used to develop AquAdvantage salmon as integrated in the pUC18 plasmid. Figure **1b.** Gene construct as integrated into the salmon genome, termed EO-1α[3]. Not to scale.

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Virginia Tech
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Editor:
Ruth Irwin
rirwin@vt.edu

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A single copy of the construct was integrated into the Atlantic salmon genome. The genomic sequence flanking the insert on both sides consisted of a 35bp repeat, and there was no evidence of mutational effects due to insertion[3]. During transgene integration, a rearrangement of the construct took place (termed EO-1α; **Fig. 1b**), which resulted in the integration of a small fragment of the plasmid into the salmon genome. This fragment did not contain any coding sequences. The promoter was rearranged such that part of the promoter was integrated downstream of the termination region. There is evidence that the truncated promoter has reduced expression compared to the full promoter in salmon[4], but the truncated promoter remains functional. The founder animal was backcrossed to wild-type Atlantic salmon, and the EO-1α gene sequence was identical in the second and fourth generations, indicating that the insertion is stable[3].

Animal growth, health, and welfare

Salmon have a wide variability of phenotypes that allow them to adapt to a variety of environmental conditions. This phenotypic plasticity means that even genetically similar fish may have very different phenotypes when exposed to different environments. For example, Coho salmon (*Oncorhynchus kisutch*) overexpressing growth hormone from sockeye salmon (*Oncorhynchus nerka*)[5] show different phenotypes, depending on environment.

Transgenic fish fed to satiation in hatchery conditions grew almost three times longer than controls, while transgenic fish in a simulated natural environment grew to be only 20% longer than controls[6], as shown in **Figure 2**. AquAdvantage salmon are significantly larger than wild-type siblings under hatchery conditions ($p<0.0001$)[7], as shown in **Figure 3**. It is not expected that AquAdvantage salmon would attain such large sizes in a non-hatchery environment.

Many studies have found that overexpression of growth hormone can result in changes in a variety of traits, including behavior, swimming ability, and body structure in salmon. Body malformations found in salmon and carp overexpressing growth hormone can mean the fish might not be able to swim as fast as wild-type fish[8]. However, these problems may not be due to the presence of the transgene. For example, vertebral malformation in wild-type salmon may result from a variety of causes, including fast growth rate[9]. Malformations may also be due to the triploid induction process, as described below.

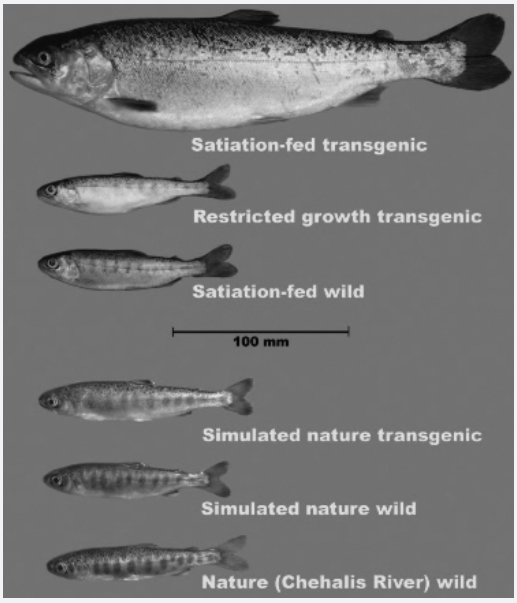

Figure 2. Wild-type Coho salmon and salmon overexpressing growth hormone that have been exposed to different environments[6].

The first generations of AquAdvantage salmon had a higher incidence of body malformations than wild-type controls, but later generations had rates similar to control salmon[7]. Aqua Bounty Technologies, Inc. has submitted to the Food and Drug Administration (FDA) Center for Veterinary Medicine[7] ten years of data indicating no difference in

animal health and welfare between AquAdvantage and wild-type salmon, but that information is not publically available.

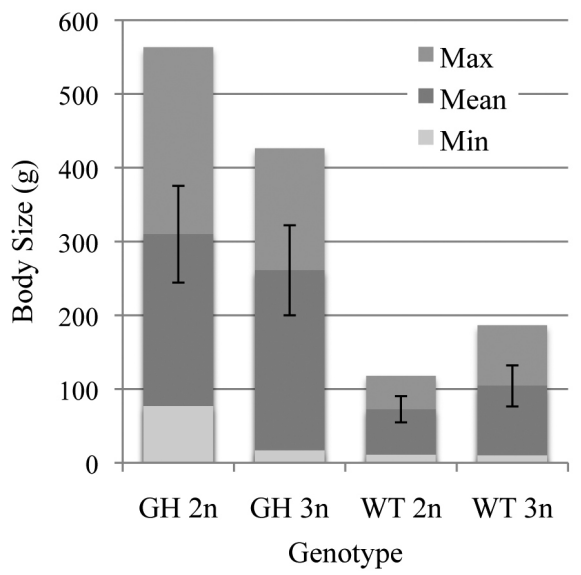

Figure 3. Mean body size with standard deviation, maximum, and minimum body size in grams of four groups of salmon: diploid and triploid AquAdvantage salmon expressing transgenic growth hormone, and diploid and triploid wild type salmon. N = 309, 369, 306, and 464, respectively[7].

Preventing escape

On 25 August 2010, Aqua Bounty Technologies, Inc. submitted an environmental assessment[7] for AquAdvantage salmon to the FDA's Center for Veterinary Medicine as part of their request for deregulation. The request is isolated to one specific egg production facility and one specific fish production facility, not for unconditional deregulation. At these facilities, Aqua Bounty plans to use many redundant systems, including biological, physical, and environmental, to prevent the release of genetically engineered salmon into the environment.

Biological containment

One of the most effective measures that will be used to prevent AquAdvantage salmon from breeding with wild salmon is the use of triploid fish. Most wild-type fish are diploids, having two copies of each chromosome, while triploids have three copies. Triploid fish do not produce gametes and therefore are sterile. Triploidy can be induced by treating fertilized fish eggs with pressure, temperature, or chemicals. The treatment itself can have a negative effect, as shown by experiments comparing triploid and diploid fish that had the same treatment to non-treated diploids and triplods that had been produced with other methods[10]. Exact treatment parameters can be adjusted to reduce negative effects and increase the incidence of triploid induction for each fish species and variety.

Pressure treatment is used to produce triploid AquAdvantage salmon. This treatment is successful at creating 98.9% or more triploids, with 1.1% or fewer eggs remaining diploid[11]. Testing of each batch of eggs will be conducted, and any batch that contains 5% or more diploids will be destroyed[7,11]. Any diploid individuals are capable of reproduction, so the possibility of their escape must be controlled with other measures.

Triploid fish of many species have been used for at least ten years in countries around the world in commercial fisheries and recreational fishing areas to prevent farmed or stocked fish from breeding with wild fish. Trout, carp, and salmon are commonly stocked as triploids and can reach very large sizes, as in **Figure 4**[12]. Larger body size and higher quality meat result because the animals do not undergo the stress of reproduction[10,13].

Figure 4. 2006 record grass carp weighing 59 pounds, 12 ounces caught by Mark Kronyak of Middletown, New Jersey[12].

Triploids cells are larger than diploids because of the increase in the amount of DNA in the nuclei. This results in an increased cell size, although overall body size is not larger compared to pre-reproduction age diploids. Having a larger cell size means the cell surface area available for gas exchange is decreased relative to the volume of the cell, compared to wild-type cells, resulting in increased oxygen demand of triploid fish compared to diploids[10].

Triploid and diploid fish in most species are not distinguishable from one another until after maturity, when diploids will divert energy to reproduction and triploids use that energy to grow in size. Triploid Chinook salmon are phenotypically indistinguishable from and have a gene expression pattern very similar to diploids, except when under extreme stress conditions. Reduced immune function of triploids may be due to the pressure treatment

or due to abnormal gene interactions arising from the third complement of genes[14]. Triploid salmon overexpressing growth hormone have reduced size and growth rate relative to diploid salmon overexpressing growth hormone, but the growth rate of both is higher than that of wild type controls[5,7].

Triploid females have a complete loss of reproductive ability, but some triploid males retain the ability to produce sperm. To avoid the possibility of producing any male eggs, all fish used to produce sperm for AquAdvantage salmon egg production are neomales. There is great flexibility of gender in many fish species such that genetically male fish may develop into females and vice versa when in the presence of certain hormones. In the case of AquAdvantage salmon, genetic females that are homozygous for the EO-1α gene are induced with 17-methyltestosterone to produce male gonads, a fairly common procedure in modern aquaculture and in fish reproduction research. Sperm produced by these fish are used to fertilize wild-type Atlantic salmon eggs, resulting in all female fish that each have one copy of the EO-1α gene[7,11]. The possibility that a male fish will be produced with this method is zero, because no male sex chromosomes are involved.

Physical containment

Because a small percentage (1.1% at most) of AquAdvantage salmon could be diploids capable of reproduction, additional containment methods are necessary. Physical containment at the egg and fish production facilities will provide multiple layers of security. These include on-facility living quarters for security personnel, security cameras, and 8' chain link fencing around each property, among other measures. Numerous filters, nets, and other containment devices reduce the likelihood of escape to less than 1%. At the egg production facility, chlorine is used in the drainage area to kill eggs that escape filters[7]. Because all farmed salmon have a reduced ability to survive in the wild, and 98% or more of AquAdvantage salmon are sterile, the likelihood that escaped animals could interfere with the natural ecosystem by becoming established in the environment or breeding with sexually compatible fish nearby is extremely small. Animals that might escape the redundant means of containment would face environmental conditions that make survival unlikely.

Environmental containment

The land-based, fresh water egg production facility is located at Prince Edwards Island, Canada. Historically, Atlantic salmon inhabited the fresh bodies of water in this area, but no wild salmon populations remain, due to overexploitation, barriers to migration, and acid rain. In the winter, temperatures in bodies of water near the facility are too low for salmon, although spring and summer temperatures are hospitable[7]. The barriers to migration would prevent escaped animals from moving out to sea during the summer. In addition, relatively high salinity in the nearby river would further reduce the likelihood of survival for animals which are acclimatized to fresh water.

The land-based fish production facility is located at a high altitude in Panama near a river that drains to the Pacific ocean. Much of the river water (up to 100% in the 4 – 5 month dry season) is used for power generation, and the canals that control water flow to power generation facilities are not suitable for salmon. In addition, the dams provide a physical barrier to movement downstream. If animals were able to navigate these barriers, the river closest to the facility does have conditions that are favorable to salmon, but in the lower parts of the river the water temperatures are lethal to salmon[7]. While the areas near the facility could sustain young salmon for a short time, escape to the Pacific ocean is highly unlikely.

Human health

General concerns with AquAdvantage salmon, or any other genetically engineered organism intended for human consumption, include increased allergenicity and unintended changes in the composition of edible tissues. Wild-type salmon is a known allergen, so AquAdvantage salmon is expected to cause allergic reactions in individuals that are allergic to salmon. The amino acid sequence of the Chinook growth hormone is unlike the sequence of known protein allergens[11]. Nonetheless, additional analysis of allergenicity would be useful. However, allergenicity studies conducted by Aqua Bounty Technologies Inc. were determined to be unsatisfactory by the Food and Drug Administration's Center for Veterinary Medicine, due to small sample size and inappropriate statistical analysis. A reanalysis of the data by FDA CVM found that the allergenic potency of triploid salmon expressing EO-1α was not significantly different from the control, although additional testing is needed to determine the allergenicity of diploid salmon expressing EO-1α[11]. Diploid amago salmon (*Oncorhynchus rhodurus*, also known as *Oncorhynchus masou ishikawae*) expressing growth hormone did not

have increased allergenicity compared to control salmon[15].

The carbohydrate, ash, moisture, protein, total fat, vitamin, mineral, amino acid, and fatty acid composition of the edible tissue of AquAdvantage salmon was compared to control salmon by Aqua Bounty Technologies, Inc. The only tested compound that exceeded the range of values found in the controls was vitamin B6 (0.77 and 0.72 mg per g tissue in AquAdvantage and control, respectively), but the amount of vitamin B6 is less than that found in tuna (0.81 mg/g), another commonly consumed finfish[11]. Consumption of normal amounts of AquAdvantage salmon is unlikely to result in daily intake of vitamin B6 that exceeds the recommended maximum amount of 100 mg/day. Omega 3 and omega 6 fatty acids are found at similar amounts in AquAdvantage and control salmon[11].

A specific concern with AquAdvantage salmon is the possibility of increased hormone content in edible tissues. The growth hormone content in both AquAdvantage salmon and non-genetically engineered control salmon is below the lower limit of quantitation (10.40 ng/g of tissue), while amounts of estradiol, testosterone, 17-ketotestosterone, T3, and T4 were not significantly different in the two groups. The only statistically different concentration was for insulin-like growth factor 1 (IGF-1), with a mean of 7.34 ng/g in the control group and 10.26 ng/g in the test group[11].

The overall amount of IGF-1 present in AquAdvantage or wild-type salmon is similar to or lower than the amount found in other animal products. For example, milk from cows treated with growth hormones, milk from cows not treated with growth hormones, and organic milk were found to have 3.12, 2.04, and 2.73 ng IGF-1 per mL of milk, respectively[16]. Beef cattle were found to have greater than 275 ng IGF-1 per mL of blood[17] without application of growth hormone. For comparison, adult human males that consume 60.1 g of protein daily have 168 ng of IGF-1 per mL of blood and adult human males that consume 81.7 g of protein daily have 200 ng of IGF-1 per mL of blood[18]. Consumption of normal amounts of AquAdvantage salmon would result in dietary amounts of IGF-1 that are no greater than a normal diet containing other animal foods.

The sequence and structure of IGF-1 varies by species such that fish IGF-1 is unlikely to react at a biologically significant level with mammalian IGF-1 receptors. The IGF-1 protein sequences for human (NM_001111283.1) and Atlantic salmon (NM_001077828.1) are quite dissimilar. A BLAST comparison of the protein sequences found 64% (90/141) of amino acids were identical and 76% (106/141) were similar. Contrast this with a BLAST comparison of human and bovine (EF432852.2) IGF-1 protein sequences, which are 96% (129/135) identical and 96% (129/135) similar.

A comparison of the binding activity of IGF-1 proteins from a variety of species to human IGF-1 receptors showed that salmon IGF-1 is two to three times less effective at binding than mammalian or marsupial IGF-1; however, salmon IGF-1 is better able to bind to sheep IGF-2 receptors than human IGF-2[19]. Further testing is needed to determine the interspecies interactions of IGF-1 and IGF-2 proteins and receptors. It is worth noting that, while consumption of bovine IGF-1 does cause elevated IGF-1 levels in humans, dietary IGF-1 is degraded, indicating that bovine IGF-1 does not directly contribute to increased human IGF-1 levels[20]. Fish IGF-1 can be expected to be similarly degraded. Human IGF-1 levels increase with increasing dietary protein, whether that protein is from animal or vegetable sources[18].

Conclusions

The FDA considers the EO-1α gene sequence in AquAdvantage salmon as an animal drug rather than regard the salmon as a novel food[21]. This approach has advantages and disadvantages, but all available evidence suggests that AquAdvantage salmon are within the normal range of wild-type triploid fish for all characteristics except growth rate, with few exceptions. Similar increases in body weight can be achieved with injection, oral application, or controlled release of a variety of compounds, including growth hormone and IGF-1[5]. A major disadvantage to considering the EO-1α gene sequence in AquAdvantage salmon as an animal drug is that it has led to consumer distrust and confusion. Even triploidization itself has led to some consumer concern[10], indicating that efforts to educate consumers on the risks and benefits of the technology may be helpful. Another disadvantage of considering the EO-1α gene sequence as an animal drug is that it allows AquAdvantage to keep some experimental results confidential to protect their intellectual property. Even though the FDA has access to those results, the withholding of data from the public has only served to increase distrust of AquAdvantage salmon and of the FDA itself.

Widely circulated fears about risks of AquAdvantage salmon do not seem to be based on the available research. Based on the research, animal health and welfare is not different from that of other triploid, hatchery reared fish. Animal welfare issues as well as sustainability issues related to fish farming are important and should be considered, but these issues affect all fish farming and are

not unique to AquAdvantage salmon. Human health risks are no greater than that posed by other meats and animal products. Additional tests could be conducted, such as larger scale allergenicity testing of AquAdvantage salmon, but the available research does not indicate that such tests are likely to find significant differences from wild-type salmon. Further research of the potential effects on human health of dietary IGF-1 from different species would be useful, but this question is not unique to AquAdvantage salmon. Long- or short-term studies in which AquAdvantage salmon are fed to test animals are not scientifically necessary, because of a lack of evidence that the edible tissue is different from that of wild-type salmon, but feeding studies comparing AquAdvantage salmon to commonly eaten salmon species may be needed to assuage consumer concerns.

The available research and the containment measures proposed by Aqua Bounty indicate that the environmental risks of AquAdvantage salmon are minimal. However, despite all containment efforts, less than 1% of AquAdvantage salmon could escape from the rearing facility and, on average, 1.1% of the salmon will be diploids. The possibility that one diploid AquAdvantage salmon could escape from the facility and survive climactic, physical, and ecological barriers is extremely unlikely, amounting to less than 0.01% of all fish reared or 1 fish in 10,000. Reproductive age for Atlantic salmon depends on latitude, such that reproductive age is 50 weeks at the latitude of Prince Edwards Island[22]. An escaped fertilized egg may meet a favorable environment for survival, but is unlikely to survive to 50 weeks. Breeding age of Atlantic salmon at the latitude of the hatchery facility in Panama is not known because Atlantic salmon do not survive at low latitudes where water temperature is so high. Still, if escape were to happen and the escapee reached reproductive age, what would the result be?

The salmon reproductive process requires complex mating and nesting behavior as well as fresh running water with a gravel bed. A sexually compatible male must be present at the time of spawning[22]. In the waters near the egg and fish rearing facilities, neither sexually compatible males nor gravel beds are available[7]. However, even though attempts to reintroduce salmon and other species to the rivers near the egg facility and rainbow trout in the rivers near the fish rearing facility have failed[7], future attempts may be successful and river bed conditions may change. Hybridization between some trout and salmon species is possible, but generally produce sterile offspring[23]. Research is needed to determine the survivability and fertility of Atlantic salmon and rainbow trout hybrids. The energy investment in reproduction is so high for female Atlantic salmon that there is a 60% or higher probability of death, post-spawning[22].

All AquAvantage salmon carry only one copy of the EO-1α gene sequence, so if an escaped diploid AquAvantage salmon reached reproductive age and found a suitable mate, only one-half of her offspring would carry the gene sequence. Those that carried the EO-1α gene sequence would, according to available research, be at a disadvantage compared to their siblings that did not carry the gene. Salmon overexpressing growth hormone under wild conditions display decreased swimming speed, which results in higher death rates due to the decreased ability to swim away from predators and decreased ability to catch prey[8]. Any advantage that EO-1α-carrying progeny might have over wild-type fish in size and growth rate would likely be cancelled out by negative effects, and the gene would either be eliminated from the wild population by natural selection or remain at a very low gene frequency. Studies in near natural environments on the survival rates of fish overexpressing growth hormone compared to wild-type fish, as well as on dynamics of mixed populations, are needed.

The final question about AquAdvantage salmon is how additional salmon on the market will affect the wild salmon fishing industry, the farmed salmon industry, and the tax revenues to the states that support those industries. These industries and their representatives have expressed concern that AquAdvantage salmon will lead to the decline of wild-caught salmon, due to the escape of farmed salmon and increased competition in the marketplace. Neither of these issues are specific to AquAdvantage salmon, but are concerns related to all fish farming. For example, domesticated fish have less genetic diversity than wild fish, so there is concern that accidental releases of large numbers of domesticated fish could cause decreased ability to adapt in wild populations[24]. Because of fewer controls against escape, fish farming as it exists today could be considered more risky for wild populations than AquAdvantage salmon. As for increased market competition, voluntary labeling such as "wild caught" and "not genetically engineered" would allow different products to prove themselves in the marketplace.

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Anastasia Bodnar
Doctoral Candidate in Genetics and Sustainable Agriculture
Iowa State University
anastasia@geneticmaize.com
http://geneticmaize.com

Editor's note:

On September 19 and 20, 2010, the Veterinary Medicine Advisory Committee of the US Food and Drug Administration convened two days of meetings intended to 1) orient participants on the scientific issues and regulatory constraints, and 2) consider issues regarding the safety and effectiveness of the new animal drug application concerning AquAdvantage salmon produced by AquaBounty Technologies, Inc. At the close of the meeting, the committee chairman reported that the majority of the expert panel concluded that the AquAdvantage salmon is safe; however, they recommended further research to add weight in areas where data are relatively sparse. Consumer protection organizations called for more research on the potential for any allergy risk posed by consuming AquAdvantage salmon.

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