Hypothetical Case Study: Confined release of Atlantic salmon resistant to furunculosis in Peru

1. GENERAL INFORMATION

Salmon are fish that belong to the family of *Salmonidae*. Within the Salmonidae family are the genus *Salmo*, which contains Atlantic salmon, and the genus *Oncorhynchus*, which contains Pacific salmon, trout, and char. A common characteristic of salmonids is the presence of an adipose fin just in front of the tail on the dorsal (top) side. In general, these are all bony fish that are caught commercially from the wild, sought after by recreational anglers, and farmed intensively because of their desirable taste and nutritional characteristics (FDA, 2010).

Atlantic salmon is susceptible to numerous bacterial, viral, and fungal diseases. One of the more common bacterial diseases is furunculosis. Caused by *Aeromonas salmonicida*, furunculosis can be a problem in both the freshwater and marine life stages of Atlantic salmon and it is extremely widespread. It can be treated in hatchery populations through the administration of antibacterial medicated feed and/or intraperitoneal (IP) injections. Control measures include commercial vaccines and surface disinfection of eggs with iodophore. Furunculosis can be a source of significant mortality in wild populations if river water temperatures become unusually high for extended periods (Fay *et al*., 2006).

Vaccination programmes as well as antibiotics have kept the problem under some control. However, side effects of vaccinations have raised a series of ethical and welfare questions (Koppang *et al*., 2008). Hence, inherent resistance in the fish against furunculosis would be preferable in order to reduce medication and side effects from immunoprophylactic procedures.

2. HOST ORGANISM DATA

Please refer to the accompanying salmon biology document.

3. RECEIVING ENVIRONMENT

3.1. Fishing and aquaculture in Peru

Peru's ocean fishing industry has always been a key component of the country's economy, especially because of the economic importance of the anchovy fisheries (the world largest single species fishery) and the related fishmeal and fish oil industry (Evans & Tveteras, 2011).

Peru's marine resources are amongst the richest in the world. The country's coastline is dominated by a cold current known as the Peruvian or Humboldt Current that flows from south to north, with waters that are extremely rich in oxygen and nutrients as a result of the intense upwelling (Sánchez Durand and Gallo Seminario, 2009).

In Peru, with the exception of the fishmeal industry, the fisheries and aquaculture are mostly dominated by the small-scale sector. In general terms, the Peruvian fishery industry consists of two completely different sectors:

the pelagic or industrial fishery and the demersal fishery (which includes the small-scale or artisanal fishery). The pelagic fishery, a large-scale and relatively modern operation, provides the raw material to the fishmeal and fish oil processing industries and accounts for almost 92 % of the catch by volume and approximately 91 % of the value of fish products exports. The amount of fish for human consumption is small in comparison (see Table 1 and Figure 1).

Table 1. Fish landings, processing and consumption in Peru in 2005 (Sánchez Durand & Gallo Seminario, 2009)

	Landings*	Processing**	Domestic consumption	Export
Total	**9 400**	**2 444**	**565**	**2 492**
Reduction fisheries	8 629	2 221	127	2 280
Fishmeal		1 931	66	2 001
Fish oil		290	60	279
Human consumption	772	223	438	212
Fresh fish	312		323	
Frozen fish	322	146	55	180
Canned fish	89	55	46	12
Cured fish	48	22	14	20

*Fish landings are measured in wet weight, while domestic consumption and export are measured in product weight.

**Volume of processed product does not equal the totals of domestic consumption and export, as part of the export volume in 2005 consisted of 2004 production.

The Peruvian aquaculture sector is relatively small and young compared to many other countries with aquaculture industries (notably the neighbour Chile), but because of biological conditions and plentiful access to water resources (see Figure 2), fresh and marine, this sector is growing quickly. At the governmental level, the sector has been mainstreamed for development, with projects partnering with international organisations scheduled to take place between 2010 and 2021. However, undercapitalisation means that most enterprises in this sector are small-scale for now (Evans & Tveteras, 2011).

The aquaculture industry is dominated by a few species, mainly scallop, shrimp and trout (see Figure 3). In scallop production there are only a handful large firms that have managed to close the production cycle. The scallop sector is dominated by artisanal activities that are purely extractive or a combination of culture and extractive practices. Shrimp is the most developed and capitalised part of aquaculture in Peru, and is located in the Northern region bordering to Ecuador because this region offers good biological and climatic conditions for shrimp farming.

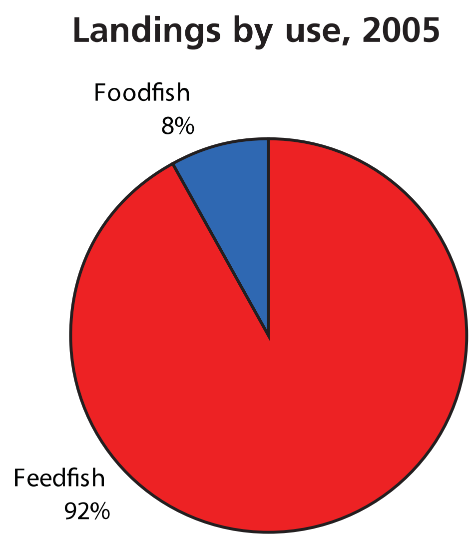

Figure 1. Uses of fish
(Sánchez Durand & Gallo Seminario, 2009)

Next follows trout farming that takes place in the Andes Mountains. The trout farming industry consists of a large number of small-scale producers and only a few large integrated companies that are involved in export activities. The main challenge facing a sector like trout is commercialisation, as the small-scale farmers have working capital that barely covers the inputs, and there are few resources left to devote to the sales of their products (Evans & Tveteras, 2011).

Salmon aquaculture in Peru is almost non-existent. In fact, in 2011 the world production of farmed salmon was mainly concentrated in Norway, Chile, UK, North America and New Zealand/Tasmania (Marine Harvest, 2012).

Figure 2. Peru, lakes and rivers (The Human Potential Peru, 2013).

3.2. Consumption

Even if Peru is one of the major world fishing countries, fish consumption per capita is only at the average level of the Latin America. As a source of protein, Peruvians prefer chicken. The Peruvian government is currently promoting a change in dietary practices to include more fish. This resulted in increases, albeit small, in the per capita consumption of fish: from 18 kg fish/capita in 2005 to 22 kg fish/capita in 2012 and expected to reach 26 kg fish/capita in 2016 (Gestion, 2012).

3.3. Biotic interactions

Both rainbow trout (*Oncorhynchus mykiss*) and Brook trout (*Salvelinus fontinalis*) (both are introduced, non-native species), and the electric eel (*Anguilla electrica*) are freshwater fishes known to prey upon Atlantic salmon in Peru (Mongabay.com, 2010). Birds known to prey on salmon in Peru are ospreys (*Pandion haliaetus*), shearwaters (*Puffinus* spp.) and cormorants (*Phalacrocorax* spp.) (BirdLife International, 2013). Larger animals such as otters and seals can prey on salmon as well, the latter in marine estuaries.

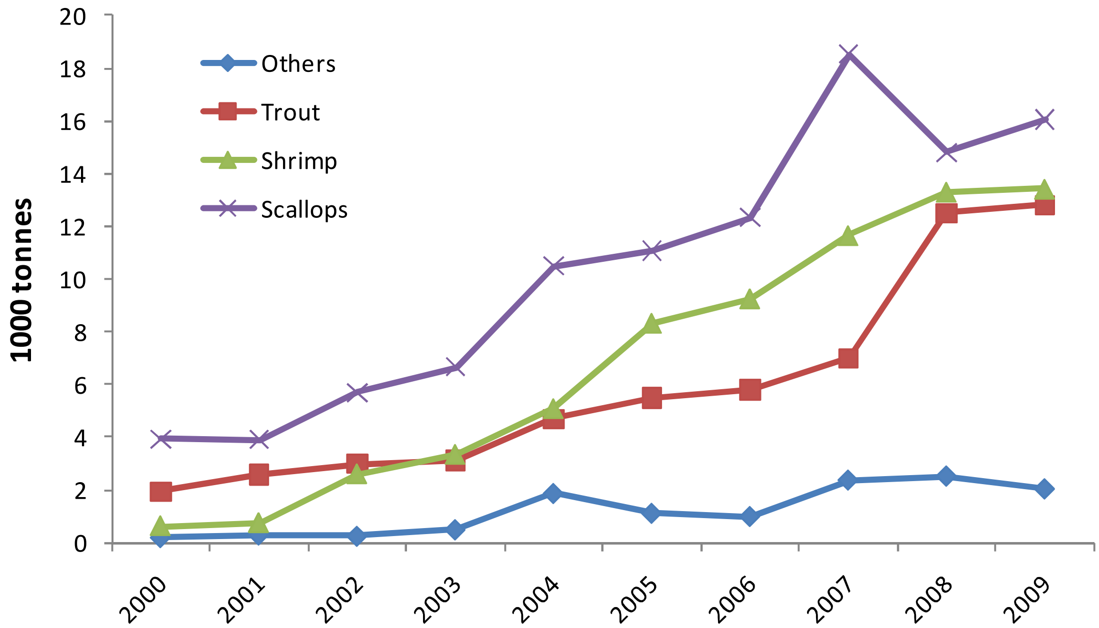

Figure 3. Aquaculture production in Peru (Ministry of Production, cited by Evans and Tveteras, 2011).

Salmon itself is also a significant predator. Salmon may opportunistically consume small fish to supplement their primary foraging base of macro-invertebrates. In addition, anadromous rainbow smelt are known to be a favoured spring prey item of Atlantic salmon kelts (Fay *et al*., 2006).

Hybridisations with closely-related species, such as trout, are possible. Hybridisation between native Atlantic salmon and introduced brown trout was found to occur at a mean frequency of 0.9 % in Atlantic salmon populations in eastern North America, 0.3 % reported for salmon populations in the British Isles, and both are higher than frequencies observed in salmon populations in Sweden. Hybridisation between species will be more frequent where one species is introduced than in areas where both are native (Verspoor, 1998). It should be noted, however, that Atlantic salmon x Brown trout hybrids are functionally sterile (Galbreath & Thorgaard, 1995), and for that reason wild populations of hybrids do not exist in nature.

Salmon can be affected by an array of pests and diseases as reviewed in the biology document, and in particular furunculosis. Furunculosis is caused by the bacterium *Aeromonas salmonicida* subsp. *salmonicida*. This bacterium is intracellular and so may avoid some host immune defences after infection. During infection, the bacterium produces extracellular toxins that may play a significant role in the pathogenesis of the disease.

Figure 4. Salmon with furunculosis (DAFF, 2012).

There are four other subspecies of *A. salmonicida* cause a different disease syndrome. Gross pathological signs include: haemorrhages on the skin, mouth and fin bases (mainly of paired fins); darkening of body colour and pale gills bloody discharge from nares and/or vent exophthalmos (popeye); haemorrhages in muscle and internal organs; enlarged spleen and focal necrosis of the liver; stomach filled with mucus, blood and sloughed epithelial cells; congested intestine, and; death without any clinical signs other than darkening of the skin (DAFF, 2012).

Furunculosis is highly contagious and affects fish of all ages although it cannot grow at 37 °C, and thus cannot infect humans. The disease is one of the most commercially significant salmonid diseases, occurring in freshwater and marine salmonid aquaculture in all countries except Australia and New Zealand. The disease must be controlled on farms by medication or vaccination. Outbreaks typically occur at temperatures above 10 °C, however outbreaks can occur in very young fish and at temperatures as low as 2–4 °C. Disease may be precipitated by endogenous (e.g. smoltification, spawning) and exogenous (e.g. temperature fluctuations, poor water quality) stressors. Rainbow trout is relatively resistant to the disease, but is still considered susceptible (DAFF, 2012).

Horizontal transmission of the bacteria occurs via the water column, but also through direct fish-to-fish contact and animal vectors (birds and invertebrates such as sea lice) (DAFF, 2012).

4. PRODUCT DESCRIPTION

Salmon aquaculture presents risks of increasing disease outbreaks, proliferating possible disease transmission routes in the environment, and decreasing the immunity of wild fish to disease. Transmission of pathogens and diseases from aquaculture to vulnerable wild fish can occur through populations that are infected at the hatchery source, through contact with the wild. Furunculosis is one of the main risks. As stated previously, vaccination programmes as well as antibiotics have kept the problem under some control. However, for vaccinations, autoimmunity similar to the mouse model of adjuvant oil-induced lupus was commonly found in vaccinated farmed Atlantic salmon (Koppang *et al*., 2008), while antibiotics increase the risk of antibiotic resistance in humans. Both methods also add to the production costs of farmed salmon. Thus developing a GM salmon resistant to furunculosis could provide inherent resistance to the disease and an amortisation of costs in the medium term.

The present GM salmon has been engineered to express two specific alleles of the major histocompatibility complex (MHC), one for MHC I and other for MHC II genes. These alleles were identified in a population of Chinook salmon grown in Canada where a strong correlation between its presence and resistance to infection with *A. salmonicida* was shown.

5. TRANSGENE DONOR ORGANISMS

5.1. Chinook salmon (Oncorhynchus tshawytscha)

It is the largest of the salmonid fish with recorded lengths up to 1.6 metres and weights of nearly 60 kg. In natural circumstances, young and adult fish spend most of their life at sea, returning to their natal streams to spawn. Both males and females usually die after spawning. Mature, ripe males develop a very prominent hooked lower jaw, and colouring of both males and females darkens to black on some parts of the body. Body condition deteriorates markedly as spawning time nears. Carnivorous, they feed on small fish such as galaxiids, crustaceans, molluscs and insects, and their natural habitat is the cool, fast-flowing streams of western North America and the ocean. For farming, deep, cool, volcanic lakes were found to generate the best results, but the shallower lakes supported good populations as well (DEPI, 2010).

6. PRODUCT CHARACTERISATION

6.1. Host organism

Atlantic salmon, *Salmo salar.*

6.2. Trait

A central component of the immune system in vertebrates, MHC genes are involved in the recognition of pathogens and initiation of the immune response. They are the most polymorphic genes in the vertebrate genome and this high level of variability is thought to be a product of natural selection for the ability to respond to a wide range of pathogens i.e. individuals that are heterozygous at MHC loci can recognise and respond to a wider range of infectious disease organisms than homozygous individuals. Challenge experiments have shown associations between specific MHC alleles and resistance or susceptibility to bacterial (furunculosis) and viral (infectious salmon anaemia virus) diseases. The Atlantic salmon is one of the few species in which convincing evidence of specific MHC/disease relationships has been demonstrated (Garcia de Leaniz *et al*., 2007).

MHC contains some of the most polymorphic genes known to date and encode polypeptides which recognise and bind both self and foreign peptides and present them to T cells. Generally, foreign peptides produced by the degradation of intracellular pathogens are bound by MHC class I molecules and presented to cytotoxic T cells. Alternatively, foreign peptides derived from extracellular pathogens are bound by MHC class II molecules and presented to helper T cells. Some cross-talk between MHC class I and MHC class II pathways also exist.

Some pathogens may escape recognition by certain MHC molecules since their peptides are not presentable by the MHC. Alternatively, resistance to pathogens may be derived through efficient presentation of certain peptides by specific MHC alleles. In Atlantic salmon, MHC consists of only one expressed MHC class I locus and one classical class II locus, hence the number of peptide-presenting alleles is limited to two per animal within each MHC class. Also, MHC class I and class II loci reside on different linkage groups in Atlantic salmon; it has been shown that exon shuffling may play a role in creating new MHC I alleles. How the given MHC-peptide combinations affect the T-cell repertoire remains to be established. It was found that fish with combined MHC class I and class II genotypes performed significantly better and were more resistant against furunculosis, compared with single allele (Kjøglum *et al*., 2007).

6.3. Introduction of trait and gene construct

The initial animal was generated by micro-injecting non-recombinant DNA, composed of entire MHC I and MHC II alleles from Chinook salmon, into the fertilised eggs of wild Atlantic salmon. Subsequent selection and breeding for eight generations led to the establishment of the animal line intended for commercial purposes.

The MHC gene was isolated from a genomic DNA library of a Chinook salmon line that exhibited a high degree of resistance to furunculosis. The gene was cloned in a pUC series plasmid and cut using the same blunt-ended endonuclease *Swa*I (ATTT/AAAT), therefore the DNA fragment used for transformation did not carry any plasmid-derived sequence. No virus or other pathogen-derived vectors or sequences were used in the process, with *E. coli* K12 strain DH5α used to maintain and produce the plasmids used.

After microinjection, the initial transgenic animal was identified by PCR analysis, but was likely to be a mosaic of GM and non-GM cells. This animal was then used to breed a second generation, from which a second animal was selected for further breeding after confirming the presence of the cisgene by microinjection.

Southern analysis demonstrated that this second animal contained two distinct insertion sites. Subsequent breeding led to the intentional separation of the two insertions in the offspring, and the animal line finally selected was confirmed to contain a single insert.

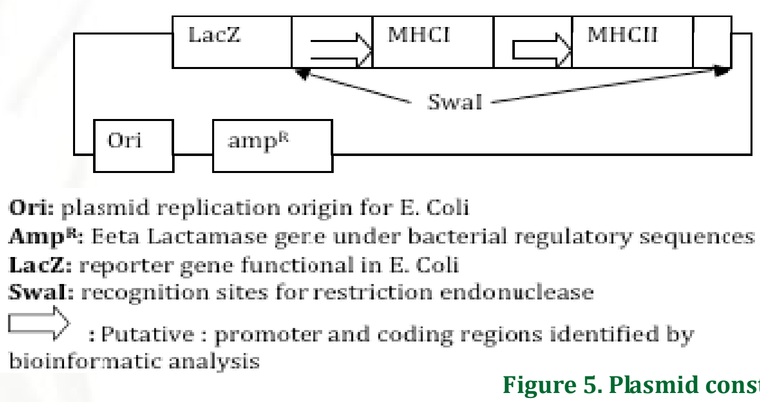

Figure 5. Plasmid construct

The DNA sequence of the inserted MHC genes and immediately adjacent flanking regions were determined in the fourth generation. No mayor rearrangements or mutations in the coding region of the gene were found. The flanking regions were determined to be non-coding after bioinformatics analysis. The MHC regulatory and coding regions were identified by bioinformatic analysis through homology search against known salmon MHC genes and other fish genes. The expression of the proteins was confirmed by Western-blot, and estimated in levels within the normal range of expression of endogenous MHC genes. To this end, the protein was also expressed in E. coli and monoclonal antibodies were obtained by standard procedures. The antibodies finally used for the study were checked for non-reactivity against the preexisting MHC molecules in the Atlantic Salmon breed used. Southern analyses were repeated after four generations and the same results were obtained.

The animal intended for commercial use was challenged for furunculosis using standard procedures, where it showed an increased resistance to the disease.

6.4. GM salmon

The gross anatomy, histopathology, and clinical chemistry of male and female, diploid and triploid GM salmon and non-GM comparator Atlantic salmon were evaluated in an identity-masked, controlled study. Triploidy is a common aquaculture technique regularly used as a biological reassurance in case of fish escapes. With the exception of jaw abnormalities, triploid salmon are usually morphologically identical to diploids. Verification is therefore generally done by measuring red blood cell dimensions

The incidence of abnormalities was similar, with the number of abnormal findings being greater for triploid fish of both lines, especially with regard to irregularities in gill structure. The observed abnormalities are within the frequency range and severity commonly noted in cultured salmonids. An examination of several internal organs, as well as relative organ weights, revealed no differences between GM and non-GM salmon or between diploid and triploid salmon.

Fish were observed during the study for avoidance and feeding behaviour, as well as posture-position in the water column, finding no differences between GM and non-GM salmon. GM salmon was found to have the same rates of feed consumption as comparators, and the same willingness to feed in the presence of a simulated predator.

Almost all of the values for haematology and serum chemistry parameters of GM Salmon were consistent with published values that represent the normal range for Atlantic salmon. A few statistically significant differences that were observed are believed to be related to the effect of triploidy on red cell number and size, and unavoidable limitations in study design.

Aspartate aminotransferase (AST) was statistically significantly higher in non-GM fish. Elevation of AST is often used clinically as an indicator of tissue damage, so this difference is not likely to be indicative of health problems in the GM salmon.

Finally, there was a statistically significant difference between the glucose level of the GM and non-GM comparator fish. Examination of the presented data showed that the overall values were not grossly different, but the glucose values were slightly lower than the values reported in the literature. This could reflect a difference in handling of the fish or the samples.

The critical oxygen concentration for GM salmon was found to be normal (5 mg/L). 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. This has implications for the establishment and survival of fish if they somehow escape from grow-out facilities. Particularly in areas where water temperatures are elevated, 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.

6.5. Information on the proposed activity

GM salmon were initially grown in a contained laboratory as triploid populations with eyed-eggs in order to bluk up numbers for field production. The lowest effectiveness for inducing triploidy observed for an individual batch of eggs was 99 %.

Atlantic GM salmon, upon sexual maturity were out-crossed with non-GM Atlantic salmon females. Fertilised eggs were then subjected to pressure shock treatment in order to render the offspring triploid, with two copies of the genome coming from the non-GM female and one copy from the homozygous GM male. Female triploid salmon are effectively reproductively incompetent, while spermatozoa in milt from male triploid salmon is very dilute and aneuploid, which usually leads to death at the embryonic stage of progeny. Besides, triploids tend to have a poorer grow-out performance and survival than diploids, and are prone to the development of a characteristic lower-jaw deformity that affects growth. All of this provides additional environmental safeguards. Samples from individual triploid batches were combined to assess the rate of triploid induction via fluorescence-activated cell sorting (FACS) analysis.

After confirming the effective induction of triploidy in the eggs, they are then to be shipped to the grow-out facility, where they are to be reared to market size and harvested for processing. The contained release is proposed for cages in a lake situated in the Mid-Northern mountains of Peru, where the temperature is adequate

Figure 6. Proposed location for the release.

for this species. The release site lies in the margins of the "Laguna Carpa", which is located at 3,200 metres above sea level, 25 km away from the Tantamayo city, which is the capital of the Huamalies province (Figure 6). The lake is fed by snow melting, and it drains down to the eastern lowlands into agricultural fields where the small water currents raise temperature and eventually disappear without reaching the sea or other water basins.

The general husbandry conditions will be consistent with commercial freshwater salmon aquaculture conditions, including a pellet-based artificial diet diets commercially manufactured for salmonids. In particular the Canadian Fish Health Protection Standards will be implemented regarding periodic analysis for the most relevant diseases.

A durability plan will be implemented, which includes checking for the presence of the cisgene and triploidy-induction effectiveness in each fish batch.

Geographical and geophysical containment is provided by the location of the grow-out sites: in addition to the lake drain-off not reaching the ocean, the environment downstream of the grow-out site is inhospitable to Atlantic salmon due to high water temperatures and poor habitat. Biological containment is facilitated through the production of triploid fish, which greatly reduces its chance of reproduction.

The facilities are located in an area where floods, storms, earthquakes, and other natural disasters are virtually absent. Also, the location is quite far away from large cities and local populations are small in numbers and scattered.

Figure 7. Cage for salmon aquaculture

There will be at least one person permanently stationed at the facilities, along with security measures to control the normal movement of authorised personnel and to prevent unauthorised access to the site by humans and potential terrestrial predators (e.g. dogs) that could potentially carry fish offsite.

Shipping of Altantic salmon from the laboratory to the grow-out facility will occur via ground routes that do not run along water streams, and only cross a few streams at identified crossings. The transport vehicle will be accompanied with a second support vehicle.

Initial phases of rearing the fish will occur in a series of three tanks of small size (from 1.5 to 8 m^3) for rearing fry and fingerlings (juveniles). The water for these tanks will be pumped from the lake. Tanks have internal, central pipes to control the water height which drain water through a slotted (0.9 cm) rigid PVC drainage plate. The drainage plate and slots serve as the primary form of physical containment for the fish in these tanks. The water outlet goes through a series of plastic sedimentation chambers for wastes, and then through three mechanical fine mesh metal screens (12 - 5 - 1 mm) to further prevent fish from escaping, prior to entering a drainage canal that returns the water into the lake.

Finally, after the fingerlings reach a mean weight of approximately 100 g, they will be transferred to the grow-out cages (similar to those depicted in Figure 7), which are equipped with a bi-layer screen that can be adjusted in accordance with the size of the fish to prevent escapes. Fish mortalities will be deposited in 1-m deep, on-site burial pits. Individual fish will be separated by a layer of caustic lime and the pit will be filled with soil once it has reached a depth of 0.5 m of dead fish.

7. ADDITIONAL ENVIRONMENTAL SAFETY CONSIDERATIONS

The lake is fed by snow melting, and it drains down to the low lands into agricultural fields where it disappears - it does not reach the sea.

There is no salmon in the lake; trout, although introduced in some regions in Peru, is not yet found in this lake. The high altitude lakes are typically nutrient poor and not particularly rich in in plankton or other species but they contain many endemic fish and molluscs.

People living nearby typically fish and eat their catches. A campaign will be implemented and, in any case of escape from the cages, they can recognise salmon, report and deliver the captured fish to the grow-out facility.

The temperature of the lake is fairly constant throughout the year at approximately 18 °C. However, the discharging rivers increase in temperature as they drop in elevation. In the lower reaches, the water temperature is in the range of 26 to 28 °C, which is above the temperature stress lethal limit for Atlantic salmon, commonly reported to be at 23 °C. At this water temperature the dissolved oxygen content is below the lethal limit for salmon. Finally, feeding is reported to stop between 20-22 °C, likely due to temperature stress.

Although there is no water connection between the lake and the ocean, and the lake is on the eastern side of the Andes mountains. Based on evidence from escapes of conventional Atlantic salmon from sea cages in Chile, it is estimated that although conventional diploid Atlantic salmon can reproduce and compete with wild Pacific salmon, biological risks to wild populations is low and both species do not interbred easily. If a GM salmon were to have improved resistance to disease or parasites, in theory it could out-compete its non-GM counterparts, however the habitat area of Pacific salmon does not include the coastal line of Peru.

8. FOOD SAFETY

8.1. Toxicity and allergenicity

The introduced gene was isolated from Chinook salmon which is a finfish; finfish are one of the eight major allergenic foods according to Codex Alimentarius. Although the major salmon allergen is parvalbumin and salmon MHC proteins have to-date not been identified as allergenic proteins, allergenic foods may contain different allergenic proteins with may have not been identified or characterised to the same extent. Under the cautionary assumption that the transferred Chinook protein could be a putative allergen, it is important to note that individuals previously allergic to Chinook salmon will also likely be allergic to Atlantic salmon and/or avoid all salmon products. Regarding "*de novo*" allergenicity, the transferred MHC genes are very similar to the Atlantic salmon MHC genes in terms of sequence and expression levels, hence it is not likely that it would increase the risk of causing novel allergies.

The MHC protein sequences have also been submitted to a Codex Alimentarius-compliant bioinformatic allergenicity analysis (potential homology to known allergens) which was negative.

The newly-expressed protein is the native MHC protein from Chinook salmon, therefore it is present in foods derived from Chinook salmon which have a long history of safe use. 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, the pepsin resistance assay was considered unnecessary. For similar reasons, a toxicity analysis was also considered not necessary.

Regarding indirect food consumption hazards, in the case of GM animals the Codex Alimentarius suggest that it is important to assess the health status of the animal intended for food production. The GM salmon under consideration does not show an increased susceptibility to diseases or other health problems compared to conventional salmon, neither will it be grown using different drugs or chemical products. Hence, no increased or different risks in comparison with conventional salmon, are expected.

8.2. Nutritional characteristics

With regards to nutritional assessment, key components and proximates (vitamins, minerals, amino acids, fatty acids, total protein and carbohydrate) of edible tissues were selected according to their relevance to suggest putative toxicological or nutritional concerns, and a compositional analysis was performed. The values were obtained from GM and non-GM triploid salmon bred under three different husbandry conditions chosen to be consistent with ordinary salmon pool-based aquaculture practices.

For all key components, with the exception of one, there were no statistically significant differences between the GM and non-GM salmon grown under the same husbandry conditions, excepting one. The amount of vitamin B6 was found to be significantly higher in GM salmon compared to the control group, a fact for which no explanatory hypothesis is available. However, the value found is within the range of literature for salmonids (i.e. considering a wider range of species and husbandry conditions). Although Vitamin B6 has a recommended maximum level for daily intake, it could not be reached even considering an exposure assessment based on populations with high daily consumption of salmon (e.g. Japan, Norway, countries that may be also comprise future consumers of exported products).

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