Re: Update on Science Review 2009 Fraser Sockeye

I have serious concerns regarding a number of unsubstantiated assumptions in the Disease section of this document. I am limiting my critique to this section, as this is my area of expertise. My comments are presented in italics below. CMW

Excerpt from the Update on Science Review 2009 Fraser Sockeye:
Section 2. Disease

1. Disease

Rationale: Research by the DFO Genomics lab identified a powerful gene expression signature consistent with the presence of a viral infection in >75% of sockeye salmon returning to the Fraser River in 2005 (the brood year for the 2009 returns). In 2006 returns, individuals carrying this putative anti-viral signature (present before they entered the river) suffered 30-60% higher mortality during their river migration than "healthy" individuals.

It would be easier to interpret the importance of these findings if more complete data were presented (i.e. inclusion of sample numbers, probability values and confidence intervals). It would also benefit the reader to know when these samples were collected and whether samples were obtained for single or multiple runs of fish.

I strongly object to the designation ' healthy' or the converse designation 'unhealthy'. These terms reflect the researcher's bias and are very misleading. Health is based on the ability of an individual to satisfy its needs and maintain homeostasis despite changes within its immediate environment. PSM sockeye may not survive long enough to fulfill the biological imperative of reproduction, but they are among the very low percentage of sockeye salmon that survive to maturation and return to freshwater.

The anti-viral signature may raise the suspicion of a viral infection, however this is still unproven. Further, an infection does not always result in disease. The virulence of the pathogen, amount of infectious particles on exposure, route of exposure, immunocompetence of the host, etc., all contribute to the outcome of an infectious process. Consider the herpes virus that causes cold sores – in North America the estimated human prevalence of infection is up to 80%, with 85% of the infected group having subclinical infections only (don't get cold sores but carry the virus and are capable of transmitting it periodically). It would be inappropriate to call someone with a cold sore 'diseased' without other signs of illness and even a further stretch to label a carrier who's never had a cold sore 'unhealthy'. And for the Fraser Sockeye, we still don't know that a virus is involved.

The process of senescence compromises the immune system of the fish and microorganisms that are normally of low virulence will be detected in much higher than normal levels. Pathogen detection should not be interpreted as a guarantee of clinical relevance. One cannot assume that a detected microorganism is causing disease unless there is pathology to support the assertion. Examinations of spawning Fraser Sockeye by the Aquatic Animal Health Section at PBS have routinely detected a number of infectious

micro-organisms which potentially compromise the well being of the fish. Common pathogens identified include: viral- IHNV; bacterial- Aeromonas, Renibacterium, Piscirikkettsia, Flavobacterium, Pseudomonas; parasitic- Parvicapsula, Loma, Ichthyophtheirus, Epitheliocystis, Myxobolus, Cryptobia, Ceratomyxa, Anisakis, Philonema, Kudoa; etc.). It is not uncommon for a brood fish to have multiple pathogens detected, each contributing an unknown amount of harm. The clinical signs attributable to each of these common pathogens are well described. To my knowledge, no pathology has been identified in PSM sockeye salmon to suggest the involvement of an unknown, unidentified viral disease agent. Additionally, to my knowledge, no fish with the anti-viral signature by microarray have been submitted for a full diagnostic screening; relying on histology only, and neglecting the complementary diagnostic tests of microscopy, bacteriology, virology, serology, serum biochemistry, etc.

It is difficult to critique the microarray data based on research summaries alone. Dr Miller-Saunders should be encouraged to publish this work ASAP in a peer-reviewed journal to allow critical review by the wider scientific community. Based on the limited information I have seen, insufficient work has been done to draw any legitimate conclusions from the presence of the antiviral signature. One must be very careful interpreting results from global gene expression profiles. It is unclear whether the roles of the highly expressed salmon genes are known, or if their functional assignments are being inferred from their putative mammalian homologs. The researcher is also assuming that these genes are transcribed, translated and undergo post-translational modifications resulting in functional proteins that are providing an anti-viral immune response. Additional efforts are required to elucidate the relevance of the increased expression of the putative anti-viral signature.

The anti-viral signature has been observed in gill, liver and brain tissue. Within the last several months, brain dissections of sockeye showing the anti-viral signature revealed the presence of highly vascularized lesions in the optic lobe.

Gross morphologic changes in dissected sockeye brains have been described as: nylon webbing, 1-3mm pink mass within the ventricle, highly vascularised lesions in the optic lobe. The researcher has made the assumption that these findings are abnormal. These gross findings have been described in this document and elsewhere as 'lesions', 'anomalies' or 'tumours', again descriptive terms reflecting bias. Three examiners (one DFO and two independent, external pathologists) failed to discern a mass lesion or any indication of either a neoplastic or inflammatory process in any of 12 brains submitted for histologic examination, despite 8 of the 12 brains having had grossly observed masses.

It needs to be determined whether or not the gross morphologic changes noted are artefacts associated with dying (out-of-water asphyxiation is the presumed method of killing used for fish sampled during the test fisheries and by Dave Patterson's group). While this method of killing is convenient and a harvest-fisheries' standard slaughter method, it is slow, inhumane and may affect the gross appearance of the brain. A comparison needs to be made with sockeye euthanized by anaesthetic overdose to

determine if the gross findings are consistent regardless of the method of killing. Ideally, this comparison should also include a control group of specific pathogen free sockeye reared on disinfected water. Additionally, the amount of elapsed time between death and dissection should be standardized.

Interspecies brain morphology can be highly variable in fish – if the researcher intends to continue making conclusions based on brain morphology, it might be advisable to consult with a comparative anatomist. Further, if a protocol could be established for its collection, examination of the cerebrospinal fluid for cell type and number and protein content might serve as a less subjective indicator of the health of the nervous system as compared to gross brain morphology.

In 2009, the prevalence of these lesions was 70% in the ocean, 50% upon river entry, and <30% at spawning, indicative of mortality en route to spawning grounds. Similar reductions in lesion incidence (55% to <20%) were observed in 2008.

Provided that 1) the lesions are genuine clinical findings and not artefacts of collection, and 2) the prevalence estimates are based on sufficiently robust sample numbers to provide statistical confidence that the prevalence differences detected among the different points in the spawning migration are not attributable to chance alone:
mortality en route is one interpretation; other interpretations are healing, pathogen clearance, combined effects of higher water temperatures and progressing senescence speeding death and limiting the development of peri-mortem changes, etc.

Out-migrating smolts of southern BC stocks of Sockeye, Coho and Chinook salmon collected in the summers of 2008 and 2009 expressed the same anti-viral signature and a high incidence of brain lesions, with levels declining significantly in the first few months in the ocean (from 40-50% June to 10% Sept) in all three species. Importantly, these genomic signatures and brain anomalies were present before salmon left their natal rearing areas within the Fraser River, suggesting that the purported disease agent responsible for the anti-viral signature observed in both smolts and adults was transmitted in the freshwater rearing environment. Molecular assays have not yielded positives for any known viral pathogens. However, viral arrays pointed to the presence of a virus in the retrovirus family.

Microarrays can be extremely useful tools in the study of host-pathogen interactions. Valuable insights into the molecular determinants of resistance to known pathogens have been gained through controlled experiments. But global gene expression analysis is not suitable for surveillance pathogen screening. Embarking on a "retrovirus bug-hunt" based on the consistent presence of the anti-viral signature and the viral array results is not good science. I am concerned that using highly sensitive molecular diagnostic assays for surveillance of novel pathogens may not be appropriate due to the high risk of obtaining false positive results. Retroviruses are extremely common. Pathogenic teleost retroviruses have been characterized, however, the presence of a retrovirus does not necessarily result in disease.

Likelihood: Disease is a factor that could impact sockeye at any or all life history stages. Fraser sockeye salmon are known to carry a variety of viral, bacterial and parasitic agents that can cause disease. Based on the work of the DFO Genomics lab, it appears that there may be a disease agent that remains unidentified. It is anticipated that with climate change, significant change to the physical and biological environment will negatively impact sockeye host-pathogen relationships, as well as increase levels of stress experienced by the fish. It is likely that under these conditions naturally occurring pathogens may cause disease with effects at both the individual and population levels.

The very significant reduction in prevalence of brain lesions both in the first few months in the ocean and en route to spawning grounds may be indicative of lesion-associated mortality. If so, the levels of mortality required to bring prevalence levels down by over 30% would be sufficient in magnitude to account for large-scale losses in the ocean. However, the possibility that lesions could regress must also be considered.

Next Steps: Since the meeting, the brain lesions have been examined histologically, and it appears that they are hemorrhagic lesions. Hemorrhagic lesions can result from head trauma, leukemia, viral brain infections, and diseases that disrupt blood coagulation. The powerful gene signatures associated with brains containing these lesions is not consistent with head trauma induced from handling/collections. Spores of *Myxobolus* sp. (believed to be *M. arcticus*), were also observed in 11 of 12 brains examined histologically (including those negative for lesions). This parasite is known to occur in sockeye salmon and may have a significant negative impact on swimming performance of sockeye smolts. A gene expression study of a related species *Myxobolus cerebralis* (agent of whirling disease) identified the up-regulation of many interferon-regulated genes that can also be upregulated in viral infections. However, the tissue distributions for Myxobolus parasites of salmon (nervous system, cartilage, skin, and muscle are not consistent with the tissue involvement associated with the anti-viral signature (brain, gill, liver and NOT muscle); hence at this time we do not believe that there is a link between the signature and this parasite. More detailed histological studies of brain and other tissues are currently underway by Dr. Mike Kent of Oregon State University.

The most important next steps are to 1) identify the purported novel viral pathogen and develop a molecular screening assay based on its DNA sequence, 2) conduct challenge studies to establish infectivity, 3) conduct smolt holding studies to assess associations of this and other diseases with mortality, and environmental conditions that may impact virulence, and 4) conduct epidemiological studies to establish pathogen distribution.

Please also consider that my criticisms extend to the piecemeal manner of pathogen surveys and disease investigations of Fraser River sockeye that we have conducted to date. For example, while there has been a large amount of data collected on the incidence and severity of Parvicapsula minibicornis kidney infestations and it is generally accepted that this parasite may be an important contributory stressor influencing pre-spawning mortalities, it has not been demonstrated that even severe infections with this parasite compromise either the osmoregulatory ability or survival of

the fish. This is a common weakness in the Fraser Sockeye pre-spawning mortality investigation research. I advocate collaboration with a fish health veterinarian in the design and interpretation of future research into the health of the Fraser Sockeye. A medical education encourages looking beyond pathogen detection and focuses on determining the clinical significance of the pathogen to the host. There are a number of fish health veterinarians with specialized post-graduate training that would be excellent resources for future work; Dr Gary Marty (BC MAL, board certified pathologist), Dr John Lumsden (OVC, board certified fish immunopathology) and Dr Sonja Saksida (BC-CAHS, epidemiology) to name a few.

I wanted to end this saying it is not my intent to criticize the work of the genomics group. I think the anti-viral signature results are intriguing, especially if proven to be a reliable marker for spawning success. I hope this work is pursued to figure out what it means. However, it would be folly to continue to over-interpret these findings or to use them to develop the future departmental direction in the investigation of Fraser Sockeye health.

Suggested Next Steps for Disease Investigation:

1. *Develop a comprehensive, co-ordinated and consistent sampling effort to assess the health status of Fraser River sockeye populations*
   - *sample Sockeye of all lifestages of interest:*
     fry, migrating smolts, immature adults in SW and mature adults in FW
   - *on an individual fish basis collect samples for a complete diagnostic screening profile, including:*
     serum biochemistry, CBC Hct and TP, virology, cytology, bacteriology, serology, histology, genomics
     Note: bissecting tissue samples for multiple tests may not be possible for smaller sized fish; increased numbers will need to be sacrificed.
   - *consult in-house expertise and a veterinarian to design the study based on current knowledge (select diagnostic tests / tissues to be collected / cell lines / media, etc.)*
   - *provide training and adequate supervision for sampling technicians to ensure sample quality, consistency and prevention of cross contamination between samples*
   - *consult with a statistician for power analysis to determine appropriate sample numbers to detect statistical significance with a predetermined level of confidence*
   - *continuing the sampling and screening effort for at least one cycle or generation*
   - *consult with a veterinarian to interpret clinical significance of findings*

2. *Run validation work to facilitate interpretation of the gross brain necropsy findings*
   - *investigate effects of various methods of killing (out-of-water asphyxiation, blunt cranial trauma, TMS overdose; Aquacalm sedation followed by TMS overdose) on gross and microscopic brain appearance*

   - *consult a comparative anatomist to aid interpretation of results if indicated*

3. *Consider investigating subclinical disease states by stress testing (temperature stress and/or administration of an immunosuppressive steroid dose) a group of juvenile sockeye which have been identified with the anti-viral signature through nonlethal gill biopsy; considerations: quarantine facilities with effluent disinfection, selection of control groups (signature-negative sockeye, SPF sockeye reared on disinfected water if available)*

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