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Innate and adaptive immune responses in migrating spring-run adult chinook salmon, *Oncorhynchus tshawytscha*



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ABSTRACT

Adult Chinook salmon (*Oncorhynchus tshawytscha*) migrate from salt water to freshwater streams to spawn. Immune responses in migrating adult salmon are thought to diminish in the run up to spawning, though the exact mechanisms for diminished immune responses remain unknown. Here we examine both adaptive and innate immune responses as well as pathogen burdens in migrating adult Chinook salmon in the Upper Willamette River basin. Messenger RNA transcripts encoding antibody heavy chain molecules slightly diminish as a function of time, but are still present even after fish have successfully spawned. In contrast, the innate anti-bacterial effector proteins present in fish plasma rapidly decrease as spawning approaches. Fish also were examined for the presence and severity of eight different pathogens in different organs. While pathogen burden tended to increase during the migration, no specific pathogen signature was associated with diminished immune responses. Transcript levels of the immunosuppressive cytokines IL-10 and TGF beta were measured and did not change during the migration. These results suggest that loss of immune functions in adult migrating salmon are not due to pathogen infection or cytokine-mediated immune suppression, but is rather part of the life history of Chinook salmon likely induced by diminished energy reserves or hormonal changes which accompany spawning.

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1. Introduction

Wild animals faced with limited resources must carefully balance their energetic demands, including devoting resources to generating effective immune responses. Spring run Chinook salmon, *Oncorhynchus tshawytscha*, represent an extreme example of animals faced with limited resources. Adult salmon migrate from the ocean into freshwater streams where they complete their life cycle by spawning and then die [1]. Upon transitioning to freshwater, salmon stop feeding and live primarily off fat reserves, which dwindle during the migration; in our study system the period of

fasting and starvation in freshwater is about 11 months in duration. In addition to a physically demanding migration, extremely energetically costly maturation of gametes by females and proliferation of gametes by males, competition-related costs at the spawning grounds, and costs associated with final sexual maturation that precedes spawning, salmon must also contend with freshwater pathogens that they have either never encountered, or encountered in the distant past as juveniles [2]. To survive until spawning, salmon must utilize energetic resources to successfully mount immune responses against such pathogens.

While it is generally accepted that immune responses wane in the maturing adult up to spawning, the exact details remain poorly characterized. Researchers have demonstrated that increased cortisol levels can negatively affect antibody production and anti-bacterial responses [3,4]. Likewise, elevated testosterone levels associated with sexual maturation in both males and females [5] are known to suppress immune function [6,7]. However, recent findings based on mRNA transcript expression of migrating salmon

Abbreviations: rkm, river kilometers; TGFβ, transforming growth factor beta; TSP, tryptic soy broth; FPGL, Fish Performance and Genetics Lab; ln, natural log; DOY, Day of the Year; n.s., not statistically significant.

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suggest that immune responses may remain intact. Schouten et al. demonstrated that antibody heavy chain transcripts in Sockeye salmon, *Oncorhynchus nerka*, remain relatively constant during the migration, with perhaps a slight reduction post-spawning morbidity [8]. Other studies have observed elevated immune-response transcripts in Sockeye Salmon, possibly induced by viral infection, which were predictive of pre-spawning mortality [9].

In addition to the hormonal changes in migrating adult salmon, increased exposure to novel, freshwater pathogens has the potential to alter immune responses. Certain pathogens may diminish either adaptive or innate immune responses, leaving animals more susceptible to infection. Others may shift the immune response to favor a particular type of a response that can either be protective or detrimental to the infected host. For instance, infection with *Nanophyetus salmincola*, one of the most prevalent pathogens in our study system [10], results in increased susceptibility to fish when challenged with bacteria [11]. It is therefore important to understand what role freshwater pathogens play in alteration of immune responses during the adult migration and spawning.

In this report we sought to quantify the immune response profile of wild adult Chinook salmon during the migration to spawning grounds. Adaptive immune responses were quantified by measuring the levels of heavy chain immunoglobulin RNA transcripts while innate immune responses were determined by measuring the ability of plasma to kill laboratory strains of bacteria. We also quantified the pathogen burden and profile in each fish collected. Immunosuppressive cytokine RNA transcripts levels were also quantified. The data set was then examined for patterns linking pathogen burden, immune response and time spent in the river.

2. Materials and methods

2.1. Fish collection and holding

Hatchery-origin adult Chinook salmon were obtained from traps at various points in the Willamette River system (Fig. 1). Collection sites have been previously described [12]. Willamette Falls, the first collection point fish pass in the migration is located 32.6 river kilometers (rkm) from the mouth of the Willamette River or 206 rkm from the mouth of the Columbia River. The next set of collection sites were situated on different tributaries of the Willamette River and included Foster Dam (418 rkm from the mouth of the Columbia), Dexter Dam (491 rkm), and the Minto Fish Collection Facility (413 rkm). Samples were collected immediately following euthanasia via anesthetic (AQUI-S) overdose. Fish carcasses were held on ice for necropsy within that same day. Some fish collected at Willamette Falls early in the run were transported to the Oregon State University Fish Performance and Genetics Laboratory (FPGL), Corvallis, Oregon and held in cool, pathogen free water until spawning [12]. Other samples were taken from fish spawned at the Oregon Department of Fish and Wildlife's Willamette Hatchery; these fish had been transported upstream to the hatchery from Dexter Dam.

All work was done in conformance with OSU Institutional Animal Care and Use Committee (ACUP 4438). The use of AQUI-S 20E (AquaTactics Fish Health and Vaccines) was conducted under INAD protocol 11–741 (from USFWS-AADAP). Adult salmon were sampled under Endangered Species Act Take permits W1-10-UI200, W1-11-UI200, W1-12-UI200 issued by NOAA-Fisheries and appropriate state scientific collection permits issued by Oregon Department of Fish and Wildlife (ODFW).

2.2. RNA isolation, cDNA synthesis, and quantitative PCR

Small (~0.1 g) sections of the anterior kidney were collected

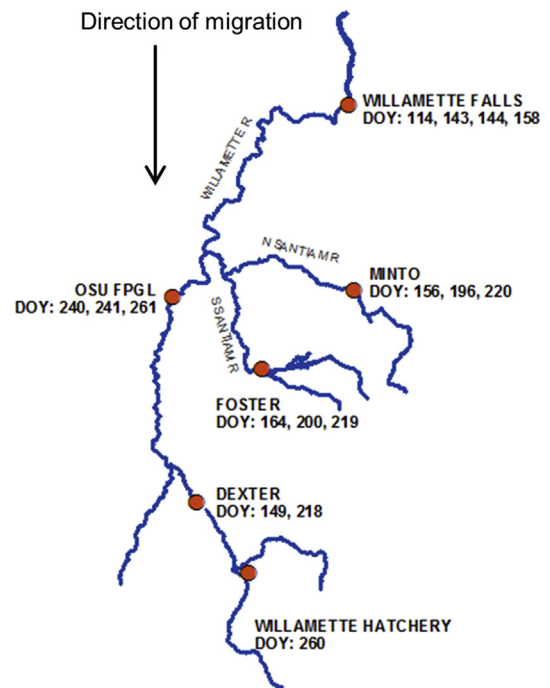


Fig. 1. Locations and Day of Year (DOY) for specimen collection. The map shows locations where fish were collected and the sampling time as indicated by the day of year. Additionally, some fish collected from Willamette Falls were transported and held at the Oregon State Fish Performance and Genetics Laboratory (FPGL) and sampled after spawning on the indicated day of the year. Other fish were collected by ODFW and held at Willamette Falls Fish Hatchery until spawning. The direction of migration indicates where fish enter the Willamette River and the initial direction of migration.

from specimens and immediately placed in 500 μ l RNA later (Life Technologies) and stored at -80°C upon return from field collection sites. RNA was isolated from tissue using Nucleospin RNA isolation kits (Clontech) following the manufacturer's recommendations. Briefly, samples were thawed and transferred to 2 ml microtubes containing 1.4 mm ceramic beads (OMNI International) and 300 μ l RA1 buffer (Clontech) containing 10 μM DTT. Tissues were disrupted using an OMNI Bead Rupter 24 at a speed of 5.65 m/s for 45 s, twice, with a 30 s delay between cycles. The resulting mixture was microfuged to pellet insoluble material and the supernatant was mixed with 300 μ l 100% ethanol. The manufacturer's recommended procedure was followed for the remaining RNA isolation procedure. RNA was reverse transcribed into cDNA using EcoDry Premix kits containing a poly-dT primer (Clontech) according to the manufacturer's recommendation. Primers for secreted and membrane-bound heavy chain μ , tubulin, TGF β and IL-10 have been previously described [8,13]. Primers specific for secreted heavy chain tau (IgH tau) were designed using rainbow trout messenger RNA for IgT heavy chain (AAW66981.1). The forward primer sequence is 5'-ACGTCTACATCATGTGGA-3' and the reverse primer is 5'TCACATGAGTTACCCGTG-3'. All primer sets were tested in a preliminary PCR reaction using a randomly selected cDNA and amplified with Fidelity Taq (Affymetrix) by a 40 cycle PCR reaction with a 30 s 95°C melt, a 15 s 55°C anneal, and a 30 s 68°C extension. PCR reactions were examined by agarose gel electrophoresis to confirm products were the correct size. PCR products were purified using PCR purification kit (Clontech) and Quantified by Qubit fluorometric quantitation (Life Technologies). Ten-fold serial dilutions of the purified PCR product were used to establish a standard curve which was included as a control in quantitative PCR measurements to calculate the transcript levels. Quantitative PCR experiments were conducted utilizing Fast SYBR Green

reagents (Applied Biosystems) and analyzed on a StepOnePlus real-time PCR system set to fast analysis. Included in all analysis was a no cDNA control. Cycle threshold values of samples falling within the range of the standard curve were subsequently converted to copy number and normalized against tubulin transcripts.

2.3. Bacterial killing assay

The bacterial killing assay measures the ability of plasma components to prevent the growth of bacteria *in vitro* and is commonly used to measure innate immune responses in a variety of wild animal samples [14,15]. Salmon plasma samples collected and frozen in the field were thawed and filtered through a 0.22 μm filter. Gentamycin-resistant *Escherichia coli* cells were plated on Luria–Bertani agar plates containing 10 $\mu\text{g}/\text{ml}$ gentamycin and cultured for 1–2 days. Five colonies were collected using the BBL Prompt system (BD) and vortexed in the provided sterile saline solution. The bacterial solution was diluted 1:50 in sterile PBS and 30 μl (approximately 15,000–20,000 CFU) were distributed to wells of a 96 well plate. Fifty microliters of filtered plasma diluted 1:25 or 1:50 in sterile PBS was added to the wells and the plate was incubated at room temperature for 30 min. Tryptic soy broth (TSP, 100 μl) containing 10 $\mu\text{g}/\text{ml}$ gentamycin was added to the wells and the plate was incubated at 37 $^{\circ}\text{C}$. The absorbance of the plate at 600 nm was measured at the start of the experiment, then again 5 h later and at every hour thereafter for a total of 13–15 h until all curves had plateaued. Absorbance was plotted as a function of time and the data was fitted with sigmoidal curve and the time to 50% absorbance was calculated using Prism (Graph Pad) software. The experiments were repeated three times and the average time to 50% absorbance was calculated.

2.4. Histological evaluation scoring system

A complete necropsy was conducted on all fish used for immunological assays. Heart, brain, gills, liver, spleen, posterior kidney, gonads, pyloric caeca, and lower intestine were collected and fixed in 10% neutral buffered formalin. Tissues were processed for histology in which four hematoxylin and eosin stained slides were prepared from organs of each fish as follows: 1) liver, kidney, spleen and heart (ventricle); 2) cross sections of the lower intestine, stomach and several (5–8) pyloric; 3) hind brain and anterior spinal; and 4) four primary gill filaments, including a region of gill arch. Severity of the common infections and lesions were scored as described below.

2.4.1. *Parvicapsula minibicornis*

Both the glomeruli and tubules of the kidney were assessed for infection. Ten random fields of each tissue were evaluated at X 400 magnification and scored for both the presence and severity of infection as follows: 1) three or fewer glomeruli infected, with light infections; 2) most glomeruli infected, light to moderate, with absent or mild glomerulonephritis; and 3) indicating heavy infection with essentially every glomeruli infected or prominent glomerulonephritis. For tubule infections, parasites were confined to the lumen and were not associated with pathological changes and were scored as: 1) rare infections, 2) light infection in several tubules, and 3) heavy infections in which most tubules were replete with parasites. With two sites of infection, fish could receive a maximum score of 6.

2.4.2. *Ceratonova shasta*

Intestinal infections were evaluated by first examining tissues at lower magnification (X 100) and infections were then confirmed by X 400 examination of specific regions. Infections

were scored as follows: 1) rare infections with parasites confined to the epithelium; 2) moderate infections, parasites observed in about half the fields of view, with occasional extension into the lamina propria; or 3), prominent, with regions of massive infection, extending through lamina propria and often into the muscularis. Extraintestinal infections by *Ceratonova shasta* were evaluated by examination of all the other organs, and scored 0–3 based on severity of infection with 3 indicating the most severe. A fish could receive a maximum score of 6 for *C. shasta* (3 intestinal and 3 extraintestinal).

2.4.3. *Brain Myxobolus sp*

A *Myxobolus sp.* was observed in the hind brain and spinal cord, which was comprised of aggregates (pseudocysts) of mature myxospores. These were scored as follows: 1) rare, small aggregates of about 5–10 myxospores detected; 2) focal regions of infection, occupying <25% of the tissue; or 3) extensive infection, often occupying large regions (e.g., >50%) of the tissue. The maximum potential score is 6, 3 for hind brain and 3 for the spinal cord.

2.4.4. *Nanophyetus salminicola*

Metacercariae consistent with *N. salminicola* were counted in kidney, heart and gill sections. Scores for each organ were as follows based on evaluation of one section: 1) one or two parasites; 2) 3 to 10 parasites, or 3) > 10 parasites. As three organs were counted, potential scores for *N. salminicola* were zero to nine.

2.4.5. *Gill metacercariae*

The entire gill tissue present on the slides, and metacercariae consistent with either *Apophallus sp.* or *Echinostomatidae* were counted and scored using the same criteria for *N. salminicola*.

2.4.6. *Bacterial kidney disease*

Chronic inflammatory lesions consistent with bacterial kidney disease, caused by *Renibacterium salmoninarum* was assessed in the kidney, spleen and liver. Severity was scored as 1, 2 or 3 based on extent of these granulomatous lesions in these three organs, with a total potential score of 9.

2.4.7. *Furunculosis*

Aggregates of bacterial bacilli consistent with *Aeromonas salmonicida* were evaluated for six organs; liver, kidney, spleen, heart, gut and gill. Scores were based on numbers and size of aggregates and extent of associated necrotic changes as follows; 1) rare, small aggregates < approximately about 15 μm diameter; 2) multiple aggregates present, with minimal tissue damage; or 3) large, coalescing aggregates of bacilli with prominent associated necrosis.

2.4.8. *Definitions and statistical analyses*

We calculated 13 infection severity indices by summing the category specific scores, described above. Total pathogen load per fish was obtained by adding the scores (0–3) for each of the seven pathogens as well as the types of tissues infected. The number of heavily infected organs was defined as the total number of species of pathogens and organs with severity scores at maximum (3). The number of moderate to heavily infected organs was estimated as the total number of organs and pathogens with severity scores at moderate (2) or heavy (3). We also summed pathogen-specific severity scores for each organ and the organ-specific scores for each pathogen.

We examined the relation between the active and innate immune responses and severity of infection by calculating Pearson correlations for all pairs of variables. We also examined pairwise plots to identify potential non-linearity. The relations between immune response and all combinations of day of year, specimen

collection location, fish sex, and severity of infection were evaluated using multiple linear regressions. Changes in pathogen loads through time were evaluated by calculating Pearson correlations between severity of infection scores and day of year and by examining pairwise plots for fish that were not held at the FPGL. These fish were withheld from the analysis because they were collected early in the run and maintained in cool, pathogen free water and therefore not reflective of the pathogen burden that would be associated with fish sampled from the river. Similarly, spawned fish collected from Willamette Hatchery were not included in the analysis as they too would not be reflective of the pathogen burden associated with fish sampled from the river. All relations were considered statistically significant at $\alpha = 0.1$ level. Linear regression assumptions were evaluated by examining residuals. When necessary, data were natural log (ln)-transformed to meet statistical assumptions. All statistical analyses were conducted using R software [16].

3. Results

3.1. Alterations to the immunoglobulin heavy chain transcripts during adult salmon migration

To determine both the on-going antibody production and the potential of fish to develop an antibody response at different locations and times during the migration, we measured the levels of mRNA transcripts encoding the heavy chain of IgM in the anterior kidney. The anterior kidney of teleosts is a primary immune tissue and is the site of hematopoiesis. A variety of immune cells are localized to the anterior kidney including macrophages and long-lived plasma cells [17,18]. Both the membrane bound (Fig. 2A) and secreted forms (Fig. 2B) of *IgH mu* in the anterior kidney were quantified by qPCR and normalized to the level of tubulin transcripts. In both data sets, there was fairly severe heteroscedasticity and to meet the assumptions of a linear regression, the data were

log transformed (Fig. 2C, and D). Linear regression analysis of log transformed transcript levels indicated a statistically significant ($p < 0.0001$) decrease for both membrane-bound and secreted forms of *IgH mu* through time. Further analysis revealed that female fish expressed lower levels of both membrane bound and secreted forms of *IgH mu* ($p < 0.01$). These data suggest that both the ability to generate and sustain an antibody response is diminished as adult fish approach spawning.

In addition to IgM, we also tested levels of the mucosal-associated immunoglobulin IgT. Transcript levels of the heavy chain (*Igh tau*) were detected at very low levels in the anterior kidney and were below the detection limit in most fish tested. Transcript levels were quantified in approximately 1/3 of the fish and plotted as a function of time. Loss of secreted *Igh tau* through time was not observed during the migration (Fig. 3A). Interestingly, levels of *Igh tau* and *IgH mu* were not intercorrelated ($p > 0.1$), suggesting salmon were mounting a predominant IgM or IgT response and that high levels of antibody production of one type did not necessarily indicate a generalized increase in production of immunoglobulins (Fig. 3B).

3.2. Diminished innate, anti-bacterial, immune responses during migration

To determine if innate immune responses were impaired in adult migrating salmon, we tested the ability of collected plasma to prevent the growth of a laboratory strain of *E. coli*. Salmon are not readily infected with *E. coli* making it unlikely that they would have mounted an antigen-specific immune response against the bacteria. This would limit the anti-microbial properties of plasma to the antibody-independent complement pathways as well as anti-microbial peptides and proteins produced by the liver and secreted into the blood. In general, growth curves followed classical bacterial exponential growth and the time to 50% growth was calculated for each sample (Fig. 4A). To determine the “killing”

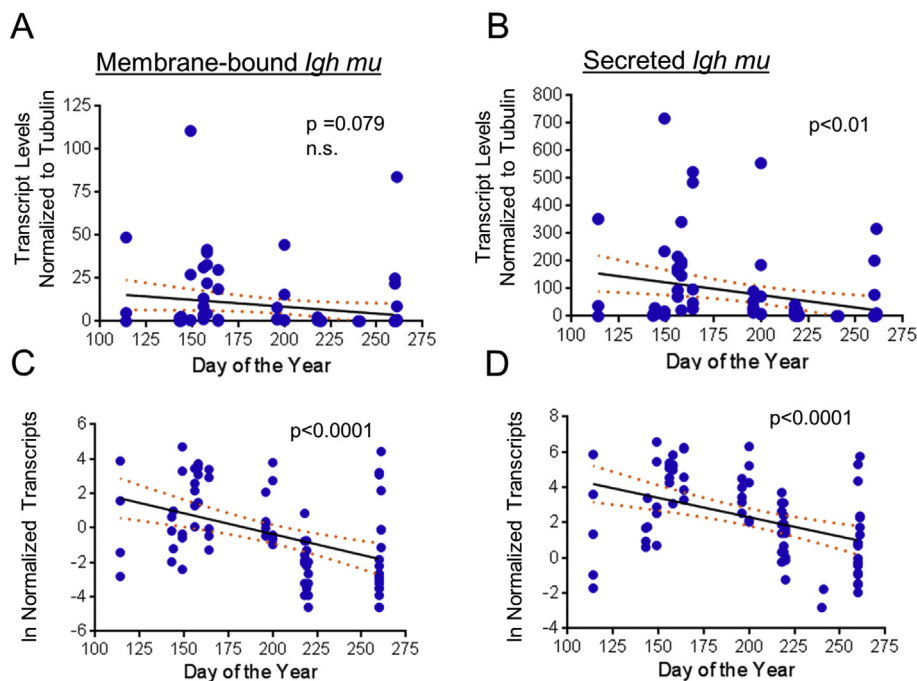


Fig. 2. Heavy chain *IgH mu* expression pattern in migrating adult salmon as a function of day of the year. A and C. Membrane-bound heavy chain *mu* transcripts were determined by quantitative PCR. The number of copies of transcript per microliter were normalized to tubulin levels. Heavy chain transcript expression for each fish was compared to the DOY that the fish was collected and sampled. B and D. Similar to (A) except transcripts encoding secreted *IgH mu* were quantified. C and D. The natural log (ln) of each normalized *IgH mu* transcript are plotted as a function of day of the year. A linear regression with 95% confidence intervals is plotted and the slope for each plot is statistically different from zero ($p < 0.0001$).

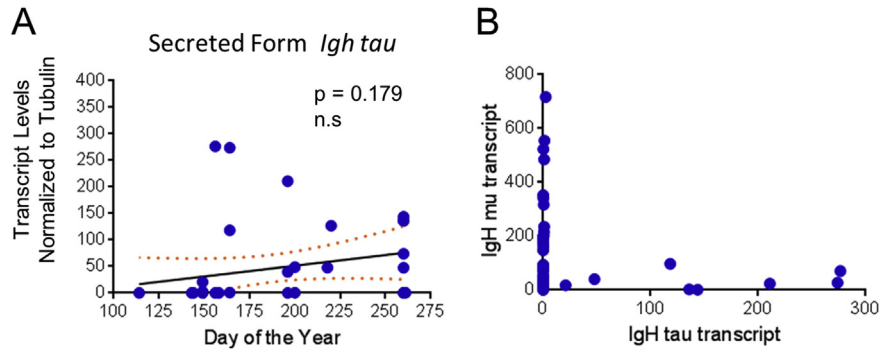


Fig. 3. Immunoglobulin Heavy Chain tau transcripts in migrating adult salmon as a function of day of the year. A. Transcripts encoding secreted forms of the immunoglobulin heavy chain tau in the anterior kidney were quantified and plotted as a function of day of the year. Linear regression analysis determined the slope of the line was not statistically different from zero. B. Secreted Igh tau (x-axis) and mu (y-axis) transcripts were compared in each fish sampled.

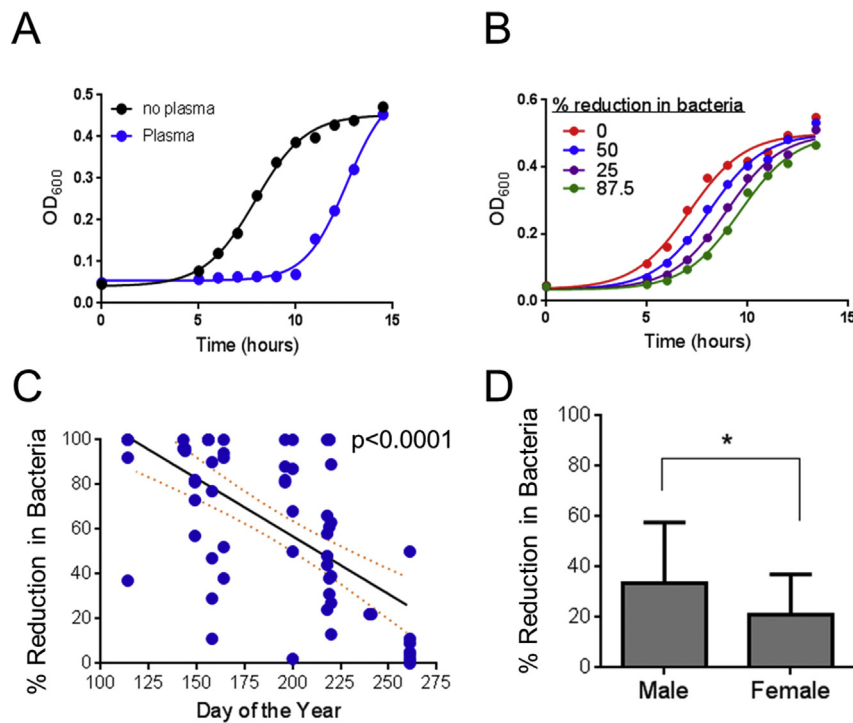


Fig. 4. Plasma-killing of bacteria diminishes during the run. A. Example of *E. coli* growth kinetics in the absence of (Black) and presence of (Blue) plasma. A sigmoidal growth curve was fitted to the data and the time to 50% growth calculated. B. Example growth curves for different dilutions of bacteria used to establish a standard curve for determining reduction in bacterial growth. C. Average bacterial killing values for all plasma samples diluted 1:25. The time to 50% growth was used to determine the % reduction in bacteria as a result of plasma incubation. Values for each sample were plotted as a function of the day of the year for which the sample was collected. Linear regression analysis reveals a slope statistically different from zero ($p < 0.0001$). D. Comparison of plasma bacterial killing from male fish and female fish ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

capability of plasma, we compared the time to 50% growth in plasma-treated samples to growth obtained by in cultures of diluted bacteria (Fig. 4B). In this manner we could infer the reduction in bacteria in plasma treated samples.

Linear regression analysis of the killing capability of plasma diluted at 1:25 demonstrated a significant negative relation between killing capability and DOY (Fig. 4C, $P < 0.0001$). These data suggest that the innate anti-bacterial components of plasma are diminished during migration, especially late in the run and after spawning. Further reducing the plasma concentration in the microbial growth reaction yielded a similar pattern, but allowed for better resolution of the innate effector functions of plasma. The killing capability of plasma from male fish was significantly greater than female fish ($P < 0.05$).

3.3. Fish collected at Willamette Falls have slightly altered immune response patterns

Immune parameters measured may vary as a function of location in the river where fish were collected. Linear regression analysis of natural log transformed secreted Igh mu transcripts indicated that levels increased significantly ($P < 0.001$) as a function of DOY for fish collected at Willamette Falls, whereas levels decreased significantly through time for the other locations (Fig. 5A). Additionally, migrating fish collected at Willamette Falls appear to have lowered levels of innate anti-bacterial responses than fish collected at other locations (Fig. 5B, $P < 0.01$). No other immune parameter measured exhibited significant location or time dependent variation.

3.4. Pathogen profiles do not account for diminished immune responses

One possible explanation for the increase in innate immune responses in fish between Willamette Falls and fish captured later in the run at upstream sites is the increased exposure to freshwater pathogens. In general, pathogen load increased in time in fish that were not being held at the FPGL (Fig. 6A). Of the seven pathogens species examined, only *Myxobolus* spp. infection severity was not significantly positively related to DOY ($P = 0.91$). Linear regression analysis indicated that the anti-bacterial killing capability of plasma was significantly greater when fish were infected with *Parvicapsula minibicornis* (Fig. 6B, $P < 0.05$). A representative image of *P. minibicornis* infection of the kidney shows presporogonic forms in the glomerulus (Fig. 6C) and in the tubules (Fig. 6D). Note the severe glomerulonephritis associated with the infection. No other significant relationship between immune measures and the other 12 infection severity indices was observed. These data indicate that certain pathogenic infections may slightly elevate innate immune responses, but in general pathogens do not drive the rapid loss of innate immunity in the run up to spawning.

3.5. Systemic anti-inflammatory cytokine transcripts are not altered during migration

Immunosuppression is often mediated by the cytokines IL-10 and TGF β , which can function in many ways to dampen immune responses. IL-10 transcripts have been detected in multiple fish species [19], including Rainbow Trout and salmon [13,20], and IL-10 is believed to function as an immunosuppressant in other teleost

species [19]. TGF β is known to suppress immune responses in mammals [21] and may repress immune responses in teleost species [22,23]. To determine if the loss of immune responses in migrating salmon was due to systemic IL-10 and/or TGF β induction, we measured the transcripts of both cytokines in the anterior kidney. Levels of IL-10 transcript remained relatively constant as a function of DOY (Fig. 7A), whereas levels of TGF β slightly decreased (Fig. 7B, $P < 0.05$). Because neither cytokine was upregulated, these data indicate that the migrating salmon did not actively suppress immune responses through cytokine-mediated signaling.

4. Discussion

Our study supports the general paradigm that salmon are “immunosuppressed” as they approach the end of their life cycle, but the exact cause of diminished immune responses is not known and multiple factors may be involved. Increased expression of immunosuppressive hormones, diminished energy reserves, or pathogen infection may diminish ongoing immune responses which could explain the data presented here. It is worth considering these data in light of the different possible pathways of immunosuppression.

Migrating salmon undergo massive hormonal changes as the spawn approaches. Cortisol levels increase in fish, which is known to suppress immune function [4,24,25]. Testosterone, another known immunosuppressant [6], increases at spawning, and is higher in female fish. The diminished immune responses in females reported here may be due to increased testosterone. In mammals, testosterone is known to act directly on T lymphocytes to increase expression of the cytokine IL-10 [26,27]. Cortisol may also increase production of IL-10 in leukocytes [28–30]. IL-10 is a critical mediator of immunosuppression [31], but here we find that IL-10 levels do not change as a function of time in the anterior kidney, suggesting that the alteration in hormone levels as spawning approaches does not result in a systemic increase in IL-10 expression. Therefore, altered hormone levels are likely to be acting in an IL-10 independent manner.

Immune responses have energetic costs and balancing the energy required to fight off infectious agents with other life history traits is important for wild animals, particularly those with limited ability to acquire exogenous energy by feeding. Exactly how the immune response is impacted by loss of caloric intake is not completely understood. Spring run Chinook salmon face an incredibly energetically demanding task, a several hundred kilometer migration against the river flow to complete their life cycle and successfully spawn. Remarkably, they can do this without consuming food during the freshwater migration, which can last as long as 8–9 months. In the absence of food consumption, it is highly likely that the immune response must change. Indeed, Martin et al. noted loss of immune gene transcripts in the liver of juvenile Atlantic salmon faced with a four week starvation [32]. As Chinook salmon cease feeding when they have returned to freshwater to spawn, it is likely that they have a concurrent reduction in their ability to mount immune responses. However, at this time these fish face additional pressures from freshwater pathogens that are prevalent during the warmer summer months. Here and in other studies [12], we have documented an increase in pathogen burdens during the migration up to spawning in the autumn. Therefore, salmon must devote some energetic resources to combating pathogenic infections if they are to survive until spawning.

Previous work indicated that both innate and adaptive immune responses can be limited during times of energy reduction. Memory B cell responses in mice of the *Peromyscus* genus are diminished if food consumption is limited by 30% [33,34]. Innate immune

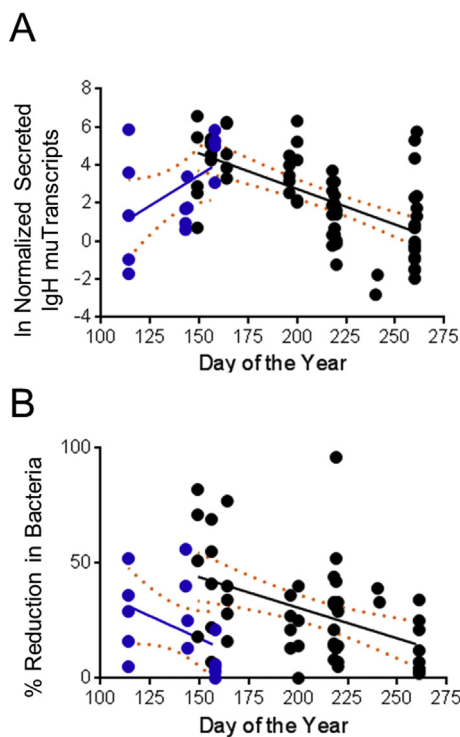


Fig. 5. Immune responses vary at Willamette Falls collection site. A. Transcripts encoding secreted IgH mu from fish collected at Willamette Falls (blue) and from all other locations (black) were plotted as a function of Day of the Year. B. Bacterial killing capacity of plasma was plotted as a function of Day of the Year for fish collected at Willamette Falls (blue) and from all other collection sites (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

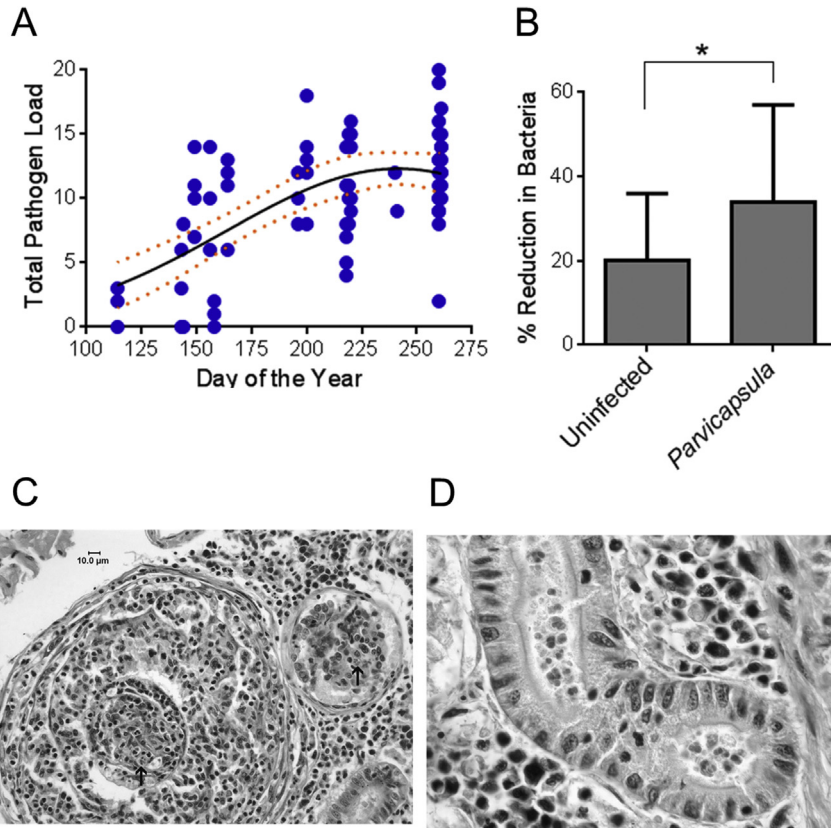


Fig. 6. Pathogen burden increases in migrating salmon and *P. minibicornis* infection enhances innate immune responses. A. Overall pathogen burden increases in salmon as a function of time. B. Bacterial killing capability of plasma diluted 1:50 was compared between *P. minibicornis* infected and uninfected fish. C. *P. minibicornis* in the kidney presenting with severe glomerulonephritis in glomerulus (arrows = parasites in presporogonic stages in the capillaries). D. Sporogonic forms of *P. minibicornis* in the lumen of renal tubules.

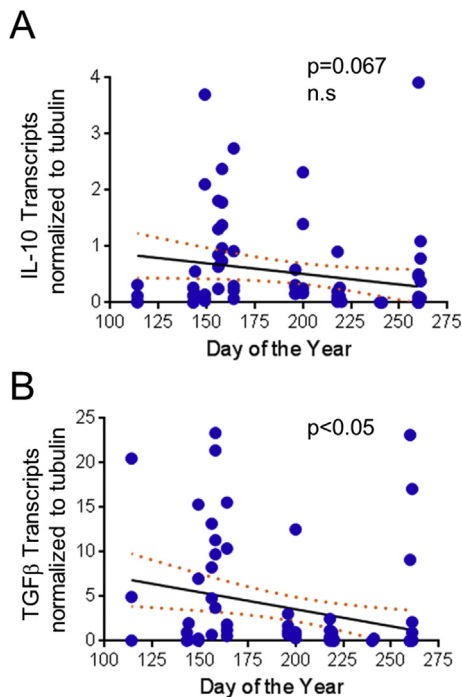


Fig. 7. IL-10 and TGFβ transcript levels do not increase during salmon migration. Transcripts of IL-10 (A) and TGFβ (B) in the anterior kidney were quantified in each sample as a function of day of the year. The linear regression with 95% confidence intervals is plotted and does not significantly deviate from zero for IL-10 levels, but significantly decreases for TGFβ.

responses relating to wound healing were significantly reduced in tree lizards with limited energy resources during reproduction [35,36]. In other instances, innate anti-bacterial responses are diminished in African Cape Buffalo during the dry season when food availability is decreased [37]. Here, we demonstrate that both innate and adaptive immune responses are diminished during the migration, but the effect is more pronounced on the innate, anti-bacterial immune responses. Plasma collected late in the run or after fish have spawned has a marked reduction in its capability to prevent a laboratory strain of *E. coli* from proliferating. This suggests that the sum total of anti-bacterial compounds in plasma, such as complement proteins, C-type lectins, anti-microbial peptides etc., are greatly reduced or even absent as fish near the end of their life cycle. Indeed most plasma samples from fish collected late in the season were indistinguishable from PBS-only controls, suggesting a near complete loss of bacterial killing ability.

In contrast to the loss of innate immune responses, transcripts encoding heavy chain immunoglobulins were only slightly, though significantly, reduced through time, and were present in fish even after spawning. Given that immunoglobulin molecules are long-lived in teleost species [38], these slight reductions in secreted IgM transcripts may actually have no bearing on the levels of functional antibody proteins present in the animal and may overestimate the loss of adaptive immune function, at least in terms of antibody production. The continued presence of antibody may help protect fish returning to their native spawning grounds as the presence of long-lived immunoglobulin producing plasma cells will be generating antibodies directed against pathogens that juvenile fish were exposed to [2].

Perhaps the most inexplicable finding presented here is the

continued presence of transcripts encoding membrane-bound immunoglobulins, essentially the antigen specific component of the B cell receptor. The anterior kidney of salmonids is known to contain not only long-lived plasma cells, but also proliferating and mature B cells, which would express membrane-bound IgM [18]. Our data suggest that even after spawning, B cell generation, though diminished from earlier time points, is still occurring in Chinook salmon. Why fish would continue to produce and/or maintain B cell populations at the end of their life cycle and under starving conditions is unknown, but this phenomenon has also been observed in a Sockeye salmon [8]. Teleost B cells are known to phagocytose and kill bacteria [39], and perhaps their continued production is not to maintain the potential for antibody production, but to provide a source of leukocytes cells capable of innate defenses.

Pathogens can often dampen immune responses. Because pathogen burdens increase as spawning approaches we hypothesized that certain pathogens may act to suppress the immune system. However, our extensive analysis showed no such signature of pathogen mediated suppression; and indeed the only partial pattern which emerged suggested that infection with *P. minibicornis* increased innate immune responses. The glomerular stages of this myxozoan are associated with profound glomerulonephritis (Fig. 6C), and perhaps the inflammatory nature of the infection is correlated with the increase in innate immune response. Glomerular changes have been reported previously in Sockeye salmon infected with *P. minibicornis* [40,41], but not to the extent observed in Chinook salmon in our study. We therefore suggest that loss of functional immune responses is not due to pathogen infections, but is a normally occurring physiological process in migrating salmon and results in increased pathogen loads.

The loss of immune responses, primarily innate immune responses, may be beneficial to the salmon during this phase of their life, as it allows for conservation of energy so as to achieve successful spawning. Moreover, Chinook salmon and other Pacific salmon species, are semelparous, and all die shortly after spawning in the fall. Therefore, it is not essential for them to eliminate these severe infections as they are destined to die within a short period of time. Indeed, these pathogens that infect the fish near the scheduled end of life of the salmon likely co-evolved with their hosts. This is exemplified by *Ceratomyxa shasta* in adult salmon. This parasite continues to develop and sporulate after Chinook salmon die, which was the first report of this life cycle strategy within the Phylum Myxozoa [42].

Not all fish survive until spawning and in some years, the rates of pre-spawning mortality can be as high as 90% in the Willamette River watershed [12]. The exact causes of pre-spawning mortality are not well understood, but previous work has suggested that pathogen accumulation may play a role. Interestingly, increased pre-spawning mortality in Sockeye salmon in the Fraser River is correlated with elevated immune gene expression [9]. Perhaps the increased energetic effort in used by these fish to combat a putative viral infection leaves them depleted of energy necessary to complete the run and successfully spawn. Though it seems paradoxical, a dampening of immune responses maybe advantageous to adult migrating salmon.

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