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Appetite of chinook salmon (*Oncorhynchus tshawytscha*) naturally infected with bacterial kidney disease ☆

Juhani Pirhonen [a,*], Carl B. Schreck [a], Ann Gannam [b]

[a] *Oregon Cooperative Fish and Wildlife Research Unit, US Geological Survey and Department of Fisheries and Wildlife, Oregon State University, 104 Nash Hall, Corvallis, OR 97331, USA*

[b] *Abernathy Salmon Culture Technology Center, 1440 Abernathy Road, Longview, WA 98632, USA*

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Abstract

We evaluated the use of feed restriction to decrease mortality and infection rates in yearling chinook salmon (*Oncorhynchus tshawytscha*) naturally infected with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD). Fish were purposely stressed and then fed either full ration, half ration, or fasted. At the termination of the 6-week experiment, feed intake of the fish was evaluated by X-radiography after feeding all groups in excess and the amount of BKD p57 antigen in the kidneys was measured by enzyme linked immonosorbent assay (ELISA) to assess effects of infection on feeding rates. Only a few individuals in each treatment died during the experiment, but the proportion of fish with detectable antigen concentration increased as ration level decreased. Within each treatment, fish with undetectable concentrations of p57 antigen ate significantly more than fish with elevated antigen levels. Exponential regressions were fitted for each ration level describing the decrease of appetite as levels of antigen concentrations increased. The data indicate that even fish that were quite sick as judged from their

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* Corresponding author. Tel.: +1-541-737-1964; fax: +1-541-737-3590.
E-mail address: juhani.pirhonen@orst.edu (J. Pirhonen).

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relatively high antigen concentrations can still feed and that previous food shortage can increase the feed intake to some extent in the sick fish.

Keywords: BKD; p57 antigen; X-ray; Feed intake; Feed restriction; Chinook salmon

1. Introduction

Renibacterium salmoninarum is the causative agent of bacterial kidney disease (BKD). Outbreaks of BKD have only been found in salmonids, Pacific salmon (*Oncorhynchus* spp.) being more susceptible to the infection than Atlantic salmon, *Salmo salar* (Evelyn, 1993). The disease has spread almost worldwide and causes significant losses to salmonid aquaculture operations (Evelyn, 1993; Bruno and Ellis, 1996). In the Pacific Northwest, BKD is a major constraint to the culture of salmonids (Fryer and Lannan, 1993).

Control of BKD by antibiotics or vaccines has proven to be difficult and inefficient (Evelyn, 1993; Fryer and Lannan, 1993). The best control of BKD is obtained by avoidance of the pathogen (Evelyn, 1993) since chemotherapy provides only temporary relief of the disease and commercial vaccines are not yet available. Once fish are infected, avoidance of horizontal transmission of *R. salmoninarum* may be difficult; however, certain hatchery practices might reduce the transmission of this bacterium. For example, Mazur et al. (1993) have shown that after transfer to sea water, infection rates were directly proportional to rearing densities in Atlantic salmon, suggesting that transfer to sea water and holding at low densities reduces disease transmission. Recent experiments with some other bacteria (*Edwardsiella ictaluri*, Wise and Johnson, 1998; *Vibrio salmonicida*, Damsgård et al., 1998b) have shown that restricted feeding levels and even fasting can reduce mortality in channel catfish, *Ictalurus punctatus*, and Atlantic salmon, respectively. The mechanisms behind these reduced infection rates are unknown.

It is generally accepted that many animal diseases decrease the appetite of the infected individual, even though only a limited amount of information is available describing this decrease, especially concerning fish. Néji and de la Noüe (1998) reported an approximate 25% reduction in feed intake of rainbow trout (*Oncorhynchus mykiss*) after infection with *Aeromonas salmonicida*. Damsgård et al. (1998a) reported that Atlantic salmon infected with infectious pancreatic necrosis virus had significantly lower growth rates and feed intake than non-infected fish, and that infected fish can have high virus titres before any changes in appetite or growth could be detected. However, there are few studies of the interaction between the infection and appetite, and we are unaware of information regarding the effects of the level of bacterial infection on feeding rates of fish. As bacterial diseases can be economically disastrous on aquaculture farms, it is important to understand the extent to which infections limit the feed intake, for example when considering chemotherapy through feeding.

In the present experiment, we examined if infection rates and mortality of spring chinook salmon (*Oncorhynchus tshawytscha*) naturally infected with *R. salmoninarum* could be reduced by restricting feeding after handling stress (transferring fish from large

tank into smaller experimental tanks) and how infection rates affect feed intake of individual fish.

2. Materials and methods

2.1. Fish and rearing conditions

The experiment was carried out at the Fish Performance and Genetics Laboratory of Oregon State University between May 21 and June 29, 1999. On May 21 (day 0), groups of 25 yearling spring chinook salmon were transferred into each of nine 0.9 m diameter (volume: 0.5 m^3) round experimental tanks supplied with flowing (6 l min^{-1}) 13°C well water. These chinook salmon had been naturally infected with *R. salmoninarum*, which induced some mortality in this stock of fish during the spring before the start of the experiment. The transfer would have caused some degree of stress in the fish (Schreck, 1981), which was used to exacerbate progression of the disease to allow us to detect possible treatment effects. During the transfer to the experimental tanks, those individuals with any external signs of the disease (e.g. exophthalmia, skin abrasions, and lesions) were discarded. Biomass in each tank was measured by weighing all the fish to calculate the food ration. Three different ration treatments were tested in triplicate: fish were (1) fed to satiation by using a fixed ration amount, (2) fed half of the satiation ration level, and (3) fasted. The fed groups were fed by hand twice a day during the week and once on weekends. Periodically, feeding rates were adjusted according to the appetite of the fish receiving a full ration. Feeding rates were 0.5% and 0.25% body weight per day for the full and half-ration groups, respectively, until day 12 when the ration amounts were doubled to 1.0% and 0.5% because of seemingly increased appetite. Daily ration amount was increased to 1.2% and 0.6% weight per day on day 18, but decreased to 1.0% and 0.5% on day 34. On days 21 and 31, the fasted fish were fed to satiation in the morning to ensure that no fish would die because of fasting.

2.2. Determination of feed intake

The feed was a commercial dry feed (Bio Dry 1000 by Bio-Oregon, Warrenton, OR, USA, containing 52% protein, 18.5% fat, and 8.5% moisture, according to the manufacturer). For the experiments, the feed was ground into powder and repelleted. A small batch of the ground feed was mixed with tiny X-ray dense lead glass beads (0.8% by weight of the feed; ballotini size 9, average diameter 0.355 mm, Jencons, Leighton Buzzard, UK) before repelletising for the estimation of feed intake (Jobling et al. 1993). All the feed had to be repelletised to ensure that the texture, color, and size in regular feed and in feed with ballotinis were identical. On the last day of the experiment (day 39) the fish were fed to satiation in the morning with the X-ray dense feed. Immediately after feeding, all the fish were killed by overdose in tricainemethanesolfonate (MS-222, 200 mg/l, buffered by $NaHCO_3$), X-rayed (Faxitron 804 cabinet X-ray machine, Agfa Structurix D7 film), and weighed (to 0.1 g). The number of ballotinis present in the gut

of each fish was counted from the X-ray plates and the amount of feed eaten by each individual was estimated.

2.3. Assessment of BKD infection level

To quantify BKD infection, after X-raying the fish, kidneys (generally the middle section, but the entire kidney if fish were very small) were stored in microcentrifuge tubes at −20°C until processing. Tissues were thawed, mixed 1:1 (weight:volume) with 0.5% bovine serum albumine (BSA) in phosphate buffered saline (PBS) (pH 8.0) and homogenised with a tissue grinder (Pellet Pestle mixer). Samples were centrifuged (3400 × *g*, 7 min) and the supernatant fluids were analysed for the amount of BKD p57 antigen, a protein produced by *R. salmoninarum*, by enzyme linked immonosorbent assay (ELISA). The ELISAs were performed in 96-well immunoassay plates (Corning). Plates were coated overnight at 4°C with 50 μl $well^{-1}$ of (3 μg ml^{-1}) purified monoclonal antibody (MAb) 4D3 in coating buffer (1.59 g l^{-1} Na_2CO_3, 2.93 g l^{-1} $NaHCO_3$, and 0.20 g l^{-1} NaN3; pH 9.6). Plates were emptied and blocked with 100 μl $well^{-1}$ of 3% BSA, in 1% Tween 20 TBS (6.07 g l^{-1} Trizma base, 0.37 g l^{-1} EDTA, and 8.7 g l^{-1} NaCl; pH 8.0). After 1 h at room temperature, the plates were emptied and standard dilutions of the BKD p57 protein (6, 12 and 24 ng ml^{-1}) and supernatant of tissue samples were loaded onto the plates (50 μl $well^{-1}$) using triplicates for standards (two at the beginning and one at the end of the plate) and duplicates for samples. After 1 h incubation at room temperature, plates were washed three times with 0.5% Tween 20 in PBS using a platewasher (Ultrawash Plus) and 50 μl $well^{-1}$ of biotinylated MAb was added and incubated at room temperature for 1 h. Plates were washed as described above. Horseradish peroxidase conjugated streptavidin (Zymed) was diluted 1:2000 in 0.5% BSA in PBS and 50 μl was added to each well. After 1 h at room temperature and 3 washes, 100 μl of peroxidase substrate (ABTS-H_2O_2) was added to each well. Substrate was prepared by adding 4 μl of 7.5% hydrogen peroxide to each ml of the ABTS-citrate buffer solution [2 ml of 0.08 M ABTS (Sigma) solution added to 100 ml of citric acid (13.88 g l^{-1}) and sodium citrate (10 g l^{-1}) solution, pH 4.0; filter sterilized by 0.22 μm cellulose acetate filter]. The color reaction was developed for 10 min at room temperature and the reaction was stopped by adding 50 μl of 2% SDS to each well. The optical densities at 405 nm were measured on a V_{max} kinetic microplate reader (Molecular Devices) and the concentration of the antigen for each sample was calculated using the standard curve. MAb 4D3, biotinylated MAb, and standards of the BKD p57 antigen (6, 12 and 24 ng ml^{-1}) were all obtained from DiagXotics (Wilton, CT, USA). If the measured value in a sample was below the lowest standard, it was considered undetectable for antigen p57 and therefore negative for the disease. Samples with values above 24 ng ml^{-1} were further diluted until the reading was within the standards. We validated the method by testing for parallelism and corrected the assay to account for temporal drift. In the fasted, half-ration, and full-ration groups, 55, 69 and 54 fish, respectively, were successfully analysed for the antigen. The p57 antigen levels were also measured for all the fish that had died during the study and from 17 individuals that had been sampled at the start of the experiment. As the detection limit

for the p57 antigen was 6 ng ml^{-1}, the individuals with concentrations below this value will be regarded as undetectable while the others as detectable for the antigen.

2.4. Statistical analysis

Possible differences between means were tested by using a nested ANOVA model in cases in which individual responses (feed intake, fish weight, and antigen concentration) were measured. Kruskall–Wallis ANOVA was used to examine group responses (fish weight at the beginning and the proportional difference in the decrease of feed intake after arcsin transformation). A chi-square test was used to analyze the difference in frequencies of fish with detectable and undetectable antigen concentrations between treatments. The relationship between feed intake and p57 antigen concentrations was described by exponential regressions, which were calculated for each treatment of those fish with detectable antigen concentrations (i.e. above 6 ng ml^{-1}). Individual data from different tanks for these calculations were pooled because according to Kruskal–Wallis ANOVA, no differences between the replicates were observed in feed intake and antigen concentrations, except a slight difference ($P = 0.034$) in antigen level in full-ration groups.

3. Results

3.1. Morality and infection rates

Few fish died during the course of the experiment and no differences in mortality were apparent between treatments (perhaps attributable to small sample sizes). In total, 13 fish died: 3 of which were from the non-fed group, 6 from the half-ration group, and 4 from full-ration group. No fish died in one of the non-fed and one of the full-ration tanks, otherwise 1–3 fish died in each tank. All fish that died exhibited very high concentration of p57 antigen (from 6600 to over 100,000 ng ml^{-1}), but stomach fullness for those individuals was not analysed.

At the beginning of the experiment (day 0), all the fish sampled (17) had p57 antigen concentrations below the limit of detection. At the termination of the experiment, a large majority of the fish still exhibited undetectable antigen levels; however, there was a tendency for feed restricted groups to have higher occurrence of elevated antigen concentrations than fully fed group. In fasted, half-ration, and full-ration groups, respectively (tank mean $\pm$ S.D., $n = 3$; analyses have been done and reported by tank, not by individual fish in a tank to be statistically conservative to avoid pseudoreplication; see Hurlbert, 1984), $40 \pm 7\%$, $31 \pm 8\%$, and $24 \pm 6\%$ of the individuals had antigen concentrations above 6 ng ml^{-1}. While these differences did not appear statistically significant, this is likely an artifact of low sample size, since regression analysis (Fig. 1) supports the contention that there is a reverse relationship between ration amount and BKD level.

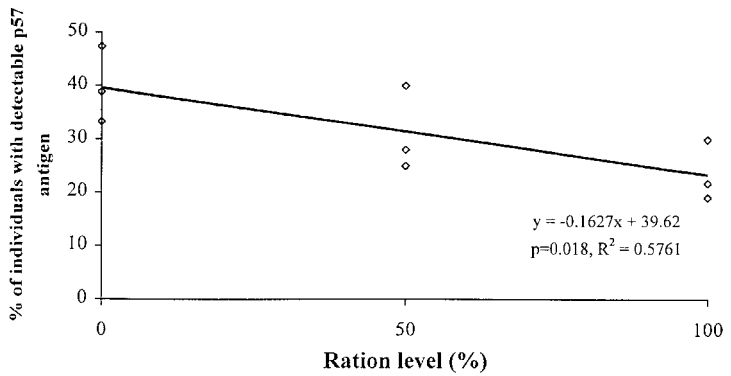

Fig. 1. Percentages of individuals in each tank with detectable p57 antigen in fasted (0%), half-ration (50%), and full-ration (100%) groups. Each data point represents mean value of one tank.

3.2. Fish size and feed intake

Mean weight of the fish at the beginning of the experiment did not differ statistically between the treatments (tank mean ± S.D., $n = 3$, 105.4 ± 9.8, 112.6 ± 17.7, and 124.7 ± 5.0 g for fasted, half-ration, and full-ration groups, respectively). At the termination of the experiment, significant differences ($P < 0.05$) between each treatment were observed (86.0 ± 5.7, 115.4 ± 15.7, and 141.9 ± 3.3 g, respectively). There was no difference in size between fish within groups that had detectable or undetectable level of the p57 antigen.

At the end of the experiment, when all the fish were fed in excess, there were no statistical differences between the treatments in mean feed intake (percentage of body weight) (Table 1), perhaps because of large variation. However, fish having undetectable p57 antigen concentrations in half-ration group ate significantly more ($P < 0.05$) than those in full-ration group. Average feed intake of fish with detectable antigen levels did not differ between treatments (Table 1). Within each treatment, fish with detectable

Table 1
Mean feed intake (percentage of body weight during the last morning feeding when all groups were fed to satiation) of all the individuals and those with undetectable and detectable p57 antigen concentrations at the termination of the experiment in the non-fed, half-ration, and full-ration groups. P-value (based on nested ANOVA) shows the statistical difference between the means below and above 6 ng ml^{-1} within each treatment
Values calculated from tank means, $N = 3$.
Values within columns denoted by different superscripts are significantly different.

Treatment	All data		Undetectable		Detectable		P-value
	Mean	S.D.	Mean	S.D.	Mean	S.D.	
Non-fed	1.45[a]	0.14	1.78[ab]	0.11	1.05[a]	0.17	0.003
Half-ration	1.70[a]	0.18	1.85[b]	0.19	1.01[a]	0.32	0.016
Full-ration	1.18[a]	0.25	1.38[a]	0.12	0.52[a]	0.48	0.023

antigen levels ingested significantly less feed than fish with undetectable levels (Table 1). Both feed restricted groups had proportionally smaller differences in feed intake than the full-ration group between fish with detectable and undetectable antigen levels (tank mean ± S.D., $n = 3$; 41.0 ± 8.1%, 45.6 ± 15.3%, and 62.3 ± 33.8% for fasted, half-ration, and full-ration groups, respectively), however, these results were not significantly different.

Individuals with detectable antigen concentrations had a significant exponential negative relationship between antigen level and feed intake in all the groups (Fig. 2). We observed little variation in the slope of the line obtained from the data in each treatment; however, the smaller the food ration, the higher the y-intercept of the equation. A few of the fish in an advanced stage of the disease (antigen concentration over 1000 ng ml^{-1}) were still eating relatively well (about 1% of body weight) even though the remainder of the individuals at those infection levels fasted (Fig. 2). In half-ration and full-ration groups, the majority of the individuals with detectable antigen concentrations ate less than 1% of body weight, but 54.5% of their counterparts in fasted group ate more that 1% of body weight, and the proportion of non-feeding individuals varied between 19% and 33.3% (Fig. 2). Regardless of the treatment, the majority of the fish with unde-

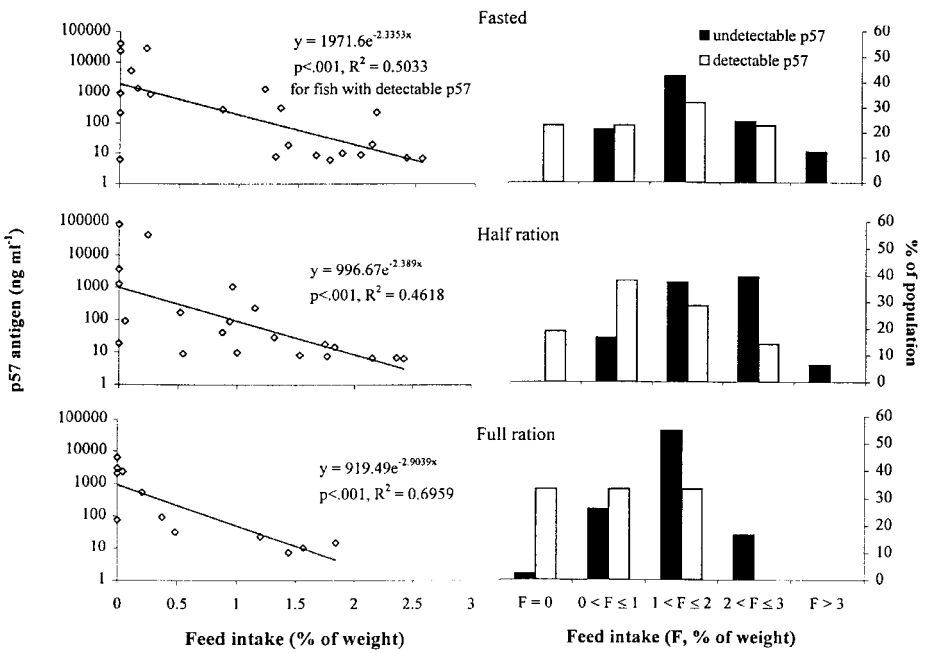

Fig. 2. Left column: relationship between feed intake (percentage of body weight) and p57 antigen concentrations of individual fish with detectable level of antigen at the termination of the experiment, when all the groups were fed in excess. Right column: proportions of individuals at different levels of feed intake calculated for the fish with undetectable and detectable concentrations of the p57 antigen. Data for the triplicates were pooled.

tectable antigen concentrations ate more than 1% of their weight, and among them only one non-feeding individual was encountered (Fig. 2).

4. Discussion

Over the 6-week course of this experiment, between 24% and 40% of the fish in each group had detectable levels of BKD antigen. Infection levels of *R. salmoninarum* were extremely low at the beginning of the experiment because no p57 antigen was detectable. Even though experimental fish were stressed, which is known to exacerbate the susceptibility of fish to pathogens (Schreck, 1996; Barton, 1997), the slow progression of this disease (Evelyn, 1993) likely means that most fish were infected to some extent with *R. salmoninarum* during the experiment, even those reported as ''undetectable''.

Total fasting resulted in highest survival when channel catfish (Wise and Johnson, 1998) and Atlantic salmon (Damsgård et al., 1998b) were infected with *E. ictaluri* and *V. salmonicida*, respectively. In our experiment, there were no statistical differences in proportions of fish with elevated p57 antigen concentrations between the treatments, even though an inverse relationship between ration level and percentage of fish with detectable level of p57 antigen was observed. Mazur et al. (1993) found no effect of feeding level (100% and 67% of satiation) on infection rate by *R. salmoninarum* in chinook salmon transferred to sea water. The difference in the results of ours and those of Damsgård et al. (1998b) and Wise and Johnson (1998) is probably because our fish were already infected with the bacteria at the start of the experiment, and also the mechanism of pathogenesis is different between different species of bacteria.

While it is usually taken for granted that infections reduce feed intake, there is a paucity of research describing the actual change in appetite with increasing level of infection in fish. Damsgård et al. (1998a) found that Atlantic salmon infected with infectious pancreatic necrosis virus had a small but consistent reduction in feed intake, and that individual fish needed a relatively high virus titer before any effects on appetite could be detected as estimated by X-radiography. These findings agree with ours as we found an exponential relationship between feed intake and concentration of p57 antigen in fish with detectable levels of the antigen. While we lack proof, one inference from our findings could be that fish with relatively low levels of infection can still have a good appetite, which then rapidly decreases as the infection progresses. However, while this was a general trend, it was not always the case because some individuals that could be regarded as having an advanced stage of the disease (p57 antigen concentration at least about 1000 ng ml^{-1}) still could eat relatively well, especially in feed restricted groups.

We clearly demonstrated that fish, regardless of the treatment, having detectable antigen concentrations ate significantly less than fish with undetectable antigen concentrations. There was a tendency for fish that had been previously feed-restricted to eat more than their non-restricted counterparts, even though statistical evidence of this was found only in fish with undetectable p57 antigen concentrations. It is possible that these differences would have been more apparent also if fish could have been fed within a more extended time, taking advantage of the facts that fish adjust their stomach capacity

according to meal size (Ruohonen and Grove, 1996) and that periodic feeding to satiation increases the quantity of food eaten per meal through time (Koskela et al., 1997; Pirhonen and Forsman, 1998). Thus, a stronger tendency to eat more after being on a restricted ration likely would have emerged if the test could have been continued. Our observations might be relevant for application of medicated diet, as it seems possible that the fasting of sick fish with slowly progressive disease before administration of medicated ration could increase the probability that also very sick individuals would eat. This suggestion is supported by the observations of Wise and Johnson (1998), that survival of channel catfish suffering from *E. ictaluri* infection was increased when the medicated (as well as non-medicated) feed was offered only every other or every third day instead of every day.

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