Investigation for susceptibility of burbot, *Lota lota* (Walbaum), to IHNV, IPNV, *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, and *Renibacterium salmoninarum*

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Running title Disease susceptibility of burbot

Abstract

In this study, susceptibility and potential carrier status of burbot, *Lota lota,* were assessed for five important fish pathogens. Burbot demonstrated susceptibility and elevated mortality following challenge with infectious hematopoietic necrosis virus (IHNV) by immersion and to *Aeromonas salmonicida* by intraperitoneal (i.p.) injection. IHNV persisted in fish for at least 28 d, whereas *Aeromonas salmonicida* was not re-isolated beyond 17 d post challenge. In contrast, burbot appeared completely refractory to *Flavobacterium psychrophilum* following intramuscular (i.m.) injection and to infectious pancreatic necrosis virus (IPNV) by immersion. However, IPNV was re-isolated from fish following i.p. injection for the duration of the 28 d challenge. *Renibacterium salmoninarum* appeared to induce an asymptomatic carrier state in burbot following i.p. injection, but overt manifestation of disease was not apparent. Viable bacteria persisted in fish for at least 41 d, and bacterial DNA isolated by diagnostic polymerase chain reaction was detected from burbot kidney tissue 90 d after initial exposure. This study is the first to investigate susceptibility of burbot to select fish pathogens and this information will aid in efforts to culture and manage this species.

Keywords: Burbot, IHNV, IPNV, *Flavobacterium psychrophilum*, *Aeromonas salmonicida*, *Renibacterium salmoninarum*

Introduction

Burbot, *Lota lota*, is an emerging species in aquaculture with relatively unknown disease susceptibility. The pernicious effects of aquatic pathogens can be far reaching in aquaculture. Loss of production, spread of a pathogen to new facilities, introduction of a pathogen to wild fish, and further amplification of a pathogen within a watershed are of great concern for fish culturists (Winton 2001). Few tools are available to combat pathogenic disease once established, and preventative vaccines are limited and costly. Effective control is often limited to avoidance, if possible, or containment of an outbreak once identified. It is, therefore, crucial that fishery managers, hatchery managers, and regulatory officials are adequately informed concerning the susceptibility of their stocks in order to make informed management decisions.

Burbot are the only true freshwater species of gadiform (cod), and are a species relatively new to artificial culture. Declining natural populations have been recently observed throughout their circumpolar distribution (Pulliainen, Korhnonen, Kankaanranta and Maeki 1992; Fisher 2000; Paragamian 2000; Paragamian, Whitman, Hammond and Andrusak 2000) which has led to increased attention by fisheries professionals (Paragamian and Bennett 2008; Paragamian and Willis 2000). Concern for wild stocks has initiated the examination of artificial culture, and hatchery protocols are being developed, both in the North America (Jensen, Ireland, Siple, Williams and Cain 2008a; Jensen, Williams, Ireland, Siple, Neufeld and Cain 2008b; Jensen, Zuccarelli, Patton, Williams, Ireland and Cain 2008c) and in Europe (Harzevilli, De Charleroy, Vught, Van Slychen, Dhert and Sorgeloos 2003; Vught, Harzevilli, Auwerx and De Charleroy 2008). Attention is currently focused on conservation aquaculture for supplementation and

reintroduction of this species. However, as aquaculture techniques are developed, commercial production of this freshwater cod could prove possible for the future.

Disease susceptibility of burbot is largely unknown. Manifestations of disease and consequent mortality from an outbreak have not been reported in cultured populations. Burbot have been listed by the United States Department of Agriculture (USDA) as a species susceptible to infection from viral hemorrhagic septicemia virus (VHSV) genotype IVb found in the Great Lakes Region of the United States and Canada (USDA 2007). However, information regarding mortality or the ability of burbot to act as a potential carrier remains unexplored for this or any other viral pathogen. The bacterial pathogen *Yersinia ruckeri* was isolated from burbot in the Mackensie River of Canada by Dwillow, Souter and Knight (1987), although manifestations of disease were not apparent. In the United States, burbot have tested positive for *Renibacterium salmoninarum* DNA by diagnostic polymerase chain reaction (PCR) from specimens collected in Washington and Montana as part of the U.S. Fish and Wildlife Service Wild Fish Health Survey (USFWS-WFHS 2009). Additionally, *Aeromonas salmonicida*, subsp. *salmonicida* and subsp *achromogenes* was also isolated from burbot as part of this survey (USFWS-WFHS 2009). During the captive rearing of wild caught broodstock and progeny (captured in British Columbia) at the University of Idaho, disease investigation conducted at the Washington Animal Disease Diagnostic Laboratory (WADDL) of over 30 mortalities has not observed viral or bacterial disease apart from minimal opportunistic bacterial infection. A potentially unique internal fungal infection in adults has been noted, although transmittion and associated mortality has yet to be determined. Such documentation gives some insight into susceptibility of burbot to these pathogens; however disease manifestations, associated mortality, and potential carrier status remain unknown. Further indications of disease susceptibility will be needed as

conservation aquaculture of this species progresses and concerns for movement and reintroduction are raised. Although documentation of burbot susceptibility to as many potential pathogens as possible would be desirable, certain aquatic pathogens are of particular interest due to their high virulence, ability to spread rapidly in fish populations, and their regulatory status .

Infectious hematopoietic necrosis virus (IHNV) is an endemic rhabdovirus with a worldwide distribution. Host specificity includes most salmonid species (reviewed by Bootland and Leong 1999; Wolf 1988) and large losses of revenue and fish have occurred in the salmonid aquaculturein public and private facilities as a result of this virus (Congleton 1988). IHNV is known to overlap current endemic populations of burbot (McPhail and Paragamian 2000). As far as we are aware, natural epizootics have exclusively been observed in salmonid species; however, some non-salmonids have shown susceptibility by experimental infection. Marine sea bream, *Sparus autrata,* and turbot, *Scophthalmus maximus* showed increased mortality after intraperitoneal (i.p.) injection (Castric and Jeffroy 1991) and white sturgeon, *Acipenser transmontanus,* were shown to carry this virus suggesting that this species could be a potential vector of this pathogen (LaPatra, Jones, Lauda, McDowell, Schneider and Hendrick 1995).

Infectious pancreatic necrosis virus (IPNV) is a ubiquitous birnavirus that causes disease in salmonids worldwide. Currently, the prevalence and impacts associated with IPNV has expanded to incorporate a wide range of host species (reviewed by Reno 1999). To our knowledge, susceptibility of burbot or their ability to act as a reservoir for IPNV has yet to be investigated. Although another gadid, the saltwater Atlantic cod, *Gadus morhua,* has been observed to be a vector for IPNV and susceptibility was demonstrated following cohabitation (Garcia, Urquhart and Ellis 2006).

It is believed that all fish are susceptible to some form of disease caused by *Flavobacterium* spp. (Shotts and Starliper 1999). One of the most significant pathogens in this genus is *F. psychrophilum*, the causative agent of bacterial coldwater disease (CWD) in rainbow trout, *Onchorynchus mykiss*, and other salmonids. CWD has become a significant problem in aquaculture worldwide (Michel, Antonio and Hedrick 1999). Although typically considered a disease of salmonids, susceptibility of non-salmonids has also been well documented (Lehmann , Mock, Sturenberg and Bernardet 1991; Wakabayashi, Toyama and Iida 1994; Iida and Mizokami 1996; Elsayed, Eissa and Faisal 2006; Hallett, Atkinson, Holt, Banner and Bartholomew 2006).

Aeromonas salmonicida is a prevalent pathogen of fish worldwide and most fish species appear susceptible to infection from at least one subspecies of this bacteria (reviewed by Hiney and Olivier 1999). As previously mentioned, A. *salmonicida* subsp. *salmonicida* and subsp. *achromogenes* have been isolated from burbot in the United States (USFWS-WFHS 2009); however, baseline information regarding disease manifestations and/or mortality remains unknown.

Bacterial Kidney Disease (BKD) caused by *Renibacterium salmoninarum* is an important and prevalent disease in salmonid fish (reviewed by Fryer and Sanders 1981). In the Pacific Northwest, USA, its large impact to wild and hatchery salmonid stocks has resulted in it being considered one of the most problematic bacterial pathogens (Wiens and Kaattari 1999). Some non-salmonid species have shown susceptibility to experimental infection with *R. salmoninarum* (reviewed by Evelyn 1993); however, it is thought that under natural conditions it is restricted to host in the family *Salmonidae* (Kent, Traxler, Kieser, Richard, Dawe, Shaw, Prosperi-Porta, Ketcheson and Evelyn 1998; Paclibare, Albright and Evelyn 1988). However, cohabitation with infected salmonid stocks has resulted in the transfer of this pathogen to non-salmonids. Sakai and

Kobayashi (1992) demonstrated that greenling, *Hexagrammos otakii*, and flathead, *Platycephalus indicus*, had antigen for *R. salmoninarum* in waters surrounding the net pen culture of coho salmon, *Oncorhynchus kisutch*, in Japan. Kent *et al.* (1998) found Pacific hake, *Merluccius productus*, obtained from waters around salmonid net pen cultures in British Columbia, Canada, to be positive for *R. samoninarum* by enzyme-linked immunosorbent assay (ELISA). Determining the susceptibility of burbot to *R. salmoninarum* is of important because of the possibility for cohabitation or co-facility habitation of burbot and salmonids in the future.

Given the seriousness and prevalence of these fish pathogens, the lack of information available regarding their ability to infect or persist in burbot, and a developing need for conservation aquaculture for this fish species that requires movement beyond natural migration, the objectives of this study were to assess the ability of these pathogens to produce disease and associated mortality in burbot, and determine if a carrier state can result in burbot for each selected pathogen.

Materials and Methods

Fish and rearing conditions

All fish used in this study were obtained from the University of Idaho's Aquaculture Research Institute (ARI), Moscow, ID, USA. Two fish species, burbot and rainbow trout, were used for each trial to compare burbot pathogen susceptibly to that of a positive control, rainbow trout. Although feeding response and growth rates between these species differed, attempts were made

to use specimens at the most comparable weights available. In trials with *R. salmoninarum*, subadult burbot (age > 2 yr) were utilized as virulence of this pathogen is known to be high in adult salmonids (Wiens and Kaattari 1999). For all other pathogen trials, juvenile burbot (age < 1 yr) were used. Rearing conditions for these two age classes of fish were as follows:

Juveniles

Burbot larvae (less than 0.5 g), following the termination of live feed diets, were maintained in 240 L troughs supplied with de-chlorinated single-pass municipal water at 14 °C. Attempts to transition larvae to artificial diets were made using commercially prepared 600-800 µm weaning diets (Epac CW; INVE Aquaculture, Inc., Salt Lake City, UT, USA) fed *ad libitum* via mechanical belt feeders. Fish were visually graded every few weeks by size to reduce cannibalism. Once fish reached approximately 1 g, feed size was increased to Epac CW 800-1,200 µm. A portion of these fish were transferred to 19 L circular tanks for pathogen challenge trials. Juvenile rainbow trout (less than 1 g) having no previous disease history were maintained in 200 L circular tanks supplied with 3 L min^{-1} de-chlorinated municipal water at 14 °C. A crumbled trout diet (Rangen, Inc., Buhl, ID, USA) was fed once daily to apparent satiation. A portion of these fish were transferred to 19 L tanks for challenge trials. Challenged burbot were maintained in 1 L min^{-1} 15 °C de-chlorinated single-pass municipal water and fed commercially prepared weaning diet (Epac CW 800–1,200 µm) once daily to apparent satiation. Rearing conditions for trout during challenge were identical to those of burbot with the exception that trout were fed Rangen crumbed trout diet.

Subadults

Juvenile burbot were transferred to 200 L circular tanks for grow out. Fish were maintained by feeding Epac 800 -1,200 μm weaning diet once daily to apparent satiation for approximately 6 mo, transistioned to by Epac cod diet (2.0 mm) for 6 mo, and then transitioned to Rangen 5/32 moist pellet trout diet and fed 1-2% body weight day^{-1} for approximately 1 yr. A portion of these fish were transferred to new 200 L tanks for *R. salmoninarum* challenge trials. Challenged fish were maintained by feeding Rangen 5/32 moist pellet trout diet to apparent satiation once daily in 3 L min^{-1} single-pass municipal de-chlorinated water at 14 °C. Challenge conditions were identical for rainbow trout.

Pathogen preparation and quantification

A summary of pathogens used to challenge burbot and rainbow trout in this study can be seen in Table 1. Each pathogen was prepared and quantified for fish challenge as follows:

IHNV

Isolates representing both M and U genotypes of IHNV (Kurath, Garver, Troyer, Emmenegger, Einer-Jensen and Anderson 2003) were propagated on Epithelioma Papulosum Cyprini (EPC; Fijan, Sulimanovic, Bearotti, Musinie, Zwillenberg, Chilmonczyk, Vantherot and DeKinklin 1983) at 15 °C in minimal essential media (MEM) with Earle's salts supplemented with fetal bovine serum and Glutamax, pH to 7.4 (MEM-10; GIBCO® cell culture, Invitrogen Corporation, Grand Island, NY, USA) as described by Anderson *et al*. (2000) with slight modifications. Briefly, cells were infected with each viral isolate at a multiplicity of infection (MOI) of 0.01. Once complete cytopathic effect (CPE) was observed (4 -7 days), culture media was removed, clarified by centrifugation at 5,000 × gravity (*g*) for 5 minutes at 15 °C, and stored at -80 °C. Upon the day of challenge, viral stocks were thawed in a 15 °C water bath and used directly to load syringes for i.p. injection or diluted in hatchery water for waterborne exposure. Quantification of virus was accomplished by plaque assay procedures similar to those of LaPatra, Barone, Jones and Zon (2000) where virus was inoculated in replicate with serial $\log_{10}$ dilutions onto EPC cell culture and allowed to absorb for 60 min. Growth media (MEM-10) supplemented with 1% methyl cellulose was added and after 7 days incubation at 15 °C, cells were fixed with formalin and stained with 1% crystal violet solution. Plaques were counted and IHNV concentrations were determined.

IPNV

Two North American serotypes of IPNV were obtained to represent the West Buxton A1 and Jasper A9 serotypes (Hill and Way 1995). Virus was propagated similar to IHNV using Chinook

salmon embryo (CHSE-214; Lannan, Winton and Fryer 1984) cell cultures. Virus was quantified after 7 days of incubation at 15 °C and titers reported as tissue culture infective dose 50% ($TCID_{50}$) mL^{-1} determined by the method of Reed and Muench (1938).

Flavobaterium psychrophilum

The isolate 259-93 of *F. psychrophilum* was cultured on tryptone yeast extract salts (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulphate, pH 7.2) agar at 15 °C for 5 d. One colony was used to inoculate 20 mL of TYES broth cultured statically at 15 °C for 72 h, from which 2 mL was used to inoculate 250 mL TYES broth and cultured for an additional 72 h. This culture was centrifugation at 6 500 *g* for 15 min at 4 °C and the pellet re-suspended in phosphate buffered saline (PBS) to an optical density (OD) of 0.09 and 0.17 at 525 nm for a low and high challenge dose. Bacteria was quantified following challenge using the drop plate method as described by Chen, Nace and Irwin (2003).

Aeromonas salmonicida

Isolate HNFH-00 of *A. salmonicida* subsp *salmonicida* was cultured on Tryptic Soy Agar (TSA; Becton Dickinson and Company, Sparks, MD, USA) for 48 h at 23 °C. Three colonies were used to inoculate 200 mL Tryptic soy broth (TSB) which was incubated statically at 23 °C for 48 h. This culture was harvested by centrifugation at 1,000 *g* for 15 min at 4 °C and re-suspended in

PBS to an OD of 0.98 at 525 nm for a single challenge dose. Following challenge, stock concentrations were quantified using the drop plate method previously described (Chen *et al.* 2003).

Renibacterium salmoninarum

Isolate CK-90 of *R. salmoninarum* was grown in 100 mL of KDM-2 broth (Evelyn 1977). After 14 d at 15 °C, 10 µL of this culture was used to inoculate slants of KDM-C agar (Daly and Stevenson 1985) and cultured for 17 d. Bacteria were harvested from slants by suspension in PBS through gentle titration. Mixtures were combined, centrifuged at 1 000 *g* for 15 min at 4 °C, and re-suspended in PBS to an OD of 1.75 and 1.0 at 525 nm for high and low challenge doses. Following challenge, bacterial suspensions were stained with 0.1% crystal violet and enumerated using a Petroff-Hausser sperm counting chamber.

Challenge Procedures

IHNV and IPNV immersion

Fish were challenge with IHNV and IPNV by immersion similarly to LaPatra et al (1993c). Triplicate 25-fish groups of juvenile burbot (mean weight 1.6 g) and rainbow trout (mean weight 0.8 g) were exposed to waterborne IHNV at approximately10^5 pfu mL^{-1} or IPNV at

approximately 10^5 $TCID_{50}$ mL^{-1} in an amount of water equaling 10 times the total weight (g) of the fish for 60 min in closed systems with aeration. Each group was then placed in a separate 19-L aquarium. An additional triplicate group of each fish species was mock infected using MEM-10 without virus to act as a negative control.

IPNV injection

The CF-94 isolate of IPNV failed to produce mortality in burbot or rainbow trout by immersion, and therefore an injection challenge was performed. A Buhl-93 isolate of IPNV was incorporated, and smaller burbot (mean weight 0.5 g) were used relative to immersion trials. Rainbow trout of reduced size were unavailable, and therefore fish from previous stocks (mean weight 1.4 g) were used. For both species, triplicate 20-fish groups were anaesthetized by immersion in 100 μg mL^{-1} tricaine methane sulphonate (MS-222; Argent, Redmond, WA, USA). Fish were given 25 μL (burbot) or 50 μL (rainbow trout) suspensions of CF-94 and Buhl-93 viral isolates by intra-peritoneal (i.p.) injection on the left ventral surface just anterior to the anus using a 30-guage needle. Each group was then placed in a separate 19-L aquarium. An additional triplicate group of each fish species was mock infected using MEM-10 without virus to act as a control.

Flavobacterium psychrophilum

Juvenile burbot (mean weight 4.6 g) and rainbow trout (mean weight 4.9 g) were challenged similar to LaFrentz *et al.* (2003). Fish were anaesthetized by immersion in 100 μg mL^{-1} MS-222 and administered 25 μL *F. psychrophilum* 259-93 by intramuscular (i.m.) injection on the left anterior side of the caudal peduncle using a 30-guage needle. Fish were administered bacteria in duplicate 20-fish groups. Each group was then placed in a separate 19-L aquarium and an additional duplicate group of each fish species was mock infected using PBS as a control.

Aeromonas salmonicida

Duplicate 20-fish groups of juvenile burbot (mean weight 3.5 g) and rainbow trout (mean weight 2.1 g) were anaesthetized by immersion in 100 μg mL^{-1} MS-222 and challenged using a standard injection model (Burr S.E., Pugovkin D., Wahli T., Segner H. & Frey J. 2005; Dacanay A., Knickle L., Solanky K.S., Boyd J.M., Walter J.A., Brown L.L., Johnson S.C. & Reith M. 2006) where fish were administered 50 μL *A. salmonicida* HN-00 isolate suspension by i.p injection on the left ventral surface just anterior to the anus using a 30-guage needle. Each group was then placed in separate 19-L aquaria. An additional duplicate group of each fish species was mock infected with PBS to act as a control.

Renibacterium salmoninarum

Challenges involving *R. salmoninarum* were conducted similar to those of Jones and Moffitt (2004). Subadult burbot (mean weight 125 g) and rainbow trout (mean weight 73 g) were anesthetized with MS-222 and administered 150 µL *R. salmoninarum* CK-90 isolate suspension by i.p injection on the left ventral surface just anterior to the anus using a 22-guage needle in duplicate 20-fish groups. Care was taken to continually mix bacterial suspensions during the challenge procedure. Each group was then placed in separate 200 L circular tanks. Low dose and high dose treatments were compared to PBS injected controls for both fish species.

Monitoring and Sampling

Mortalities were recorded and removed daily for a period of 60 d following pathogen exposure for *R. salmoninarum*, and 28 d for all other pathogen trials. Pathogen re-isolation was attempted on a minimum of 20% daily mortality. Re-isolation of pathogen was also attempted at the termination of challenge on 10-15% (original population size) of surviving fish where available. Procedures for viral and bacterial isolation are as follows:

Virus re-isolation

Whole fish were homogenized in MEM-10 supplemented with penicillin-streptomycin-neomycin (PSN) antibiotic mixture (0.05 mg, 0.05 mg, 0.1 mg ml^{-1} respectively; GIBCO®) in a volume equaling 10 times the weight (g) of fish. Homogenates were centrifuged at 3,000 × *g* for 10 min

at 15 °C to pellet debris. Remaining supernatants were forced through 0.45 µm syringe filters (Fisher Scientific®) to remove any bacterial contamination. Filtrate was collected in a 1.5 mL vials to be used directly for quantification assays or stored at -80 °C for later use in reverse transcriptase polymerase chain reaction (RT–PCR). Viral quantification was performed as previously described. If CPE was observed in the cell culture assay, RT-PCR was performed on the original sample filtrate to confirm IHNV or IPNV by standard methods (USFWS and AFS-FHS 2005).

Bacteria re-isolation

Isolation of bacteria was attempted from sampled fish using standard methods (USFWS and AFS-FHS 2005). Briefly, kidney, liver, and spleen tissues were inoculated onto TSA for the isolation of *A. salmonicida* or on KDM-C agar for the isolation of *R. salmoninarum*. Spleen tissue was inoculated onto TYES agar for the isolation of *F. psychrophilum*. Cultures were incubated and resulting colonies were identified using morphological characteristics and Gram staining.

Statistical Analysis

Statistical analysis of data was accomplished using Graphpad Prism 2.01 software. Survival curves were generated to analyze mortality rate by the Kaplan-Meier method (Kaplan and Meier 1958). Curves were compared using the logrank test (Petro and Petro 1972) and differences were considered significant at P-value < 0.05. Cumulative per cent mortality (CPM) was adjusted to

remove background mortality in mock infected controls. This corrected CPM (cCPM) was used to compare treatments to mock infected controls following arcsine transformation (Y = arcsine√Y) within each trial using a Student's t-test. Differences were considered significant at P-value < 0.05. To calculate cCPM, the following equation was used:

$$cCPM_{\text{treatment group}} = CPM_{\text{treatment group}} - \text{mean } CPM_{\text{mock infected groups}}$$

Results

IHNV

Waterborne exposure of IHNV confirmed burbot to be susceptible to infection by this virus (Table 3). A significant increase in mortality rate was observed following immersion with both the RB1 and 220-90 isolates relative to mock infected controls (Figure 1A). Viral titers determined at challenger were 8.8×10^6 and 9.5×10^6 plaque forming units (pfu) mL^{-1} for 220-90 and RB1 isolates respectively. Mortality was first observed 5 days post challenge and continued through the termination of trial. Viral titers ranged from 10^4 to $> 10^6$ pfu $fish^{-1}$ and IHNV was confirmed by RT-PCR. However, no external or internal clinical signs of disease could be distinguished relative to mock infected fish. Surviving burbot yielded viral titers in the range of 10^2 to 10^4 pfu $fish^{-1}$ for isolate 220-90, but virus was not detected in cell culture or by PCR from surviving burbot challenged with the RB1 isolate. Mean cCPM in RB1 and 220-90 challenged burbot was 17 and 24% respectively, which were both significantly higher than mock

infected controls (Table 2). Burbot showed a less severe and more protracted infection to both IHNV isolates than rainbow trout. Challenge with 220-90 resulted in near total mortality in rainbow trout with a mean cCPM of 99%. Mean cCPM was lower in rainbow trout challenged with RB1 at 39%, but still higher than mortality observed in burbot. For rainbow trout, all mortality occurred between 4 and 13 days post challenge and IHNV was confirmed by RT-PCR. Viral titers were > 10^6 pfu $fish^{-1}$ in all samples for 220-90, and ranged from10^5 to >10^6 pfu $fish^{-1}$ in samples for RB1. Rainbow trout surviving challenge with RB1 had viral titers between 10^3 to >10^6 pfu $fish^{-1}$ at 28 d. Only a single rainbow trout survived challenge with 220-90, from which virus was not detected.

IPNV

Challenge with the CF-94 isolate of IPNV by immersion did not appear to induce disease in burbot and was not re-isolated from challenged mortalities or survivors (Table 3). Viral solutions used for challenge were determined to be approximately 1.0×10^7 $TCID_{50}$ mL^{-1} for both isolates used. Mean cCPM was 10%, and not significantly different from mock infected controls (Table 2). The rates of mortality were likewise not significant between infected fish and controls. Mortality in rainbow trout was not observed (Figure 1B); however, viral titers in surviving rainbow trout following challenge ranged from 10^2 to 10^3 $TCID_{50}$ $fish^{-1}$, indicating that a latent viral infection was induced in this species.

Injection challenge with CF-94 and Buhl-93 isolates of IPNV did not induce clinical signs of disease in burbot. Mortality appeared likewise unaffected by viral challenge. However,

severely unstable survival inherent in the culture of this early life stage for burbot resulted in near total mortality in mock infected controls (Figure 1C). Therefore, conclusions could not be made as to the effect of pathogen caused mortality in burbot. Viable virus was cultured from mortalities and the few remaining survivors following challenge (Table 3), indicating this virus' ability to persist in burbot for at least 28 days. However, quantification of virus beyond original challenge levels was not attempted.

Flavobacterium psychrophilum

Injection with isolate 259-93 of *F. psychrophilum* induced severe infections in challenged rainbow trout. The low and high challenge doses were determined at 4.0×10^7 and 9.9×10^7 colony forming units (cfu) mL^{-1} respectively. Lesions developed typical of experimental coldwater disease (CWD), and *F. psychrophilum* was re-isolated from the spleen of all the rainbow trout moralities that were examined. In contrast, burbot exhibited no overt disease manifestations and *F. psychrophilum* was not cultured from challenged mortalities or survivors (Table 3). Mortality rates in high and low dose burbot treatment groups were comparable to mock infected controls (Fiure 1D). Mean cCPM was 2% lower than observed in mock infected fish (Table 2).

Aeromonas salmonicida

Burbot demonstrated a susceptibility to *A. salmonicida* subsp. *salmonicida*. A significant increased mortality was observed following injection with this pathogen at a concentration of 4.0 $\times 10^7$ cfu mL^{-1} (Figure 1E). The mean cCPM was 23%, and was significantly higher than mock infected controls (Table 2). Infected burbot developed lesions around the injection site, and became lethargic with a poor feeding response. Upon necropsy, petechial hemorrhaging was observed on internal organs and peritoneal walls. Histology showed bacteria present in pancreatic and connective tissues, and gram negative, non-motile, bacterial colonies having brown diffusible pigment characteristic of *A. salmonicida* subsp. *salmonicida* were isolate from kidney, liver, and spleen tissues of challenged burbot mortalities (Table 3). Nevertheless, burbot appeared much less sensitive to disease caused by *A. salmonicida* than rainbow trout, as higher and more acute mortality was observed in rainbow trout compared to burbot (Figure 1E). Additionally, this bacteria was cultured from tissues of trout following challenge, whereas *A. salmonicida* was not detected in surviving burbot (Table 3).

Renibacterium salmoninarum

Injection with *R. salmoninarum* did not appear to induce disease or mortality in burbot, although a latent carrier state developed. Mortality during the 60 d trial was minimal (Table 2), and mortality rates were comparable for the high and low challenge doses (1.1×10^{10} and 3.4×10^9 bacteria mL^{-1} respectively) and control groups in burbot (Figure 1F). Conversely, rainbow trout experienced typical manifestations of bacterial kidney disease (BKD) caused by *R. salmoninarum* with high levels of mortality (mean cCPM 50% for both high and low challenge

dose) and significantly increased mortality rates (Figure 1F). *R. salmoninarum* was cultured from liver, kidney, and spleen tissues from a 46% (18/39) of rainbow trout challenge mortalities examined. The single challenged mortality in burbot (high dose treatment) observed at 41 d post challenge, also yielded growth of *R. salmoninarum* in culture from kidney, liver and spleen tissues; demonstrating viable bacteria can persist in these tissues of burbot. This bacteria was not cultured from tissue of any other burbot sampled; however, diagnostic nested PCR of kidney tissues from surviving fish revealed the prescence of *R. salmoninarum* DNA in these tissues up until the termination of the challenged stocks (90 d post infection).

Discussion

It is evident from these trials that burbot are susceptible to the rabdovirus IHNV and can carry viable virus for at least 28 days. Increased mortality was observed in burbot after waterborne challenge with both M and U genotypes of IHNV, and the presence of viable virus was confirmed in fish up to 28 days post exposure. Previous studies have shown a variation in virulence for these genotypes depending on host species. LaPatra, Fryer and Rohovec (1993a) found experimentally infecting 0.4 g rainbow trout with U or M isolates resulted in 30 and 100 CPM, respectively. Similar results were observed in this study with 0.8 g rainbow trout (39 CPM U, 99 CPM M). However, the relative increase in mortality (mean cCPM 17-24%), suggests that burbot are less susceptible to either of these isolates than rainbow trout (mean cCPM 39-99%) and are unlikely a primary target host for this virus. It is interesting to note when considering the ability of burbot to act as a potential vector of IHNV, that in this study, visually asymptomatic

burbot 28 days following challenge with the M genotype isolate were infected with high levels of viable virus (> 10^6 pfu $fish^{-1}$) in some cases. No virus was re-isolated from burbot survivors following challenge with the U genotype isolate. It has been hypothesized that *in vivo* virulence of IHNV is linked to the ability for rapid replication by the virus, thus allowing a kinetic advantage over the host innate immune response (Penaranda, Purcell and Kurath *In press*). Although no attempts were made to evaluate an immune response in this study, it is evident that both IHNV isolates have the potential to quickly overcome the burbot innate immune response as seen by high viral titers in challenged mortalities within five days post infection. However, variations between genotypes become evident in chronic persistence, where burbot appeared to be able to clear (or at least drastically reduce) infection from U while the M isolate continued to persist at high levels This suggests that the M genotype of IHNV has a slightly higher virulence than the U genotype in burbot. Further investigation will be needed to determine the ability of burbot to transmit this virus, however given these current findings burbot should be considered a possible vector for both U the M genotypes of IHNV. Currently the distribution of the U genotype of IHNV overlaps with endemic burbot populations (Garver, Troyer and Kurath 2003) in the United States and Canada, where as the distribution of the M genotype is unconnected. However, if M genotype contact were to occur, the ability for burbot to act as a vector for IHNV could become enhanced.

The susceptibility of burbot to IPNV remains somewhat ambiguous. Although waterborne exposure to IPNV did not increase mortality in burbot, mortality in challenged rainbow trout was likewise unaffected. As mortality and disease manifestations in salmonids is often only observed between one and four months of age (LaPatra *et al.* 2000), it is quite possible that six month old fish challenged in this study were at a size and age that resulted in

limited disease manifestations. Nevertheless, latent infection developed in rainbow trout but not in burbot, suggesting that waterborne exposure alone is insufficient to induce viral infection in burbot. In a second challenge trial with IPNV, fish were administered virus by i.p. injection to bypass the external fish barrier. To our knowledge, mechanisms for *in vivo* entry have not been reported for IPNV, and although waterborne exposure is sufficient to induce infection in many species, it is possible for an alternative route of infection to exist. The size of burbot (mean 0.5 g) was also reduced in an attempt to elicit mortality, and an additional isolate (Buhl-93) representing the A1 serotype with known virulence in rainbow trout (LaPatra, Lauda, Woolley and Armstrong 1993b) was incorporated. Unfortunately, the increased fragility of burbot that accompanies reduced size at this early life stage resulted in extremely high background mortality in mock infected controls and this confounded mortality comparisons. Nevertheless, viable IPNV was re-isolated from mortalities and survivors following the challenge indicating that IPNV can persist *in vivo* for at least 28 days in this species. However, because virus is known to be extremely environmentally stable (Toranzo and Hetrick 1982), it is unclear as to whether virus re-isolated from challenged fish was due to persistence of the original injected virions or actual virus replication in the host. Endpoint viral titers for this study were not determined beyond 10^5 $TCID_{50}$ $fish^{-1}$, and as fish were injected with a similar dose, it remains unclear as to if viral replication was occurring. Further investigation will be needed to determine the full extent of susceptibility of burbot to IPNV. Although waterborne exposure did not yield infection, it may be advisable to consider burbot a possible vector for IPNV given its demonstrated ability to persist *in vivo* in burbot following injection.

Burbot appeared refractory to infection by *F. psychrophilum* following i.m. injection. It is possible that an alternative exposure method, such immersion or anal intubation, could induce

disease. However, such methods have proved less effective than injection to induce acute disease in salmonids (LaFrentz, LaPatra, Call and Cain 2008). Therefore, the failure of *F. psychrophilum* to induce an infection at high injection doses in this trial suggests an overall refractive nature of burbot to this bacterial pathogen and they are unlikely to be a susceptible host.

In trials with *A. salmonicida salmonicida*, burbot showed susceptibility to this pathogen by i.p. injection. Lesions developed, mortality rates increased, and bacteria was re-isolated from internal organs. However, disease manifested to a lesser degree in burbot than observed in rainbow trout. In this trial, rainbow trout surviving initial exposure became asymptomatic carriers, and previous observations have noted such covert infections to be a common occurrence in salmonids (Hiney, Smith and Bernoth 1997). Burbot that survived the challenge cleared the bacteria to below detectible levels, and given the extremely high initial dose (2.1×10^8 cfu fish^{-1}), it is likely that burbot under natural conditions would show signs of disease unless otherwise severely stressed. If an infection were to become established it is likely to result only in a limited disease state and be cleared within a short period following exposure. Further investigation into the ability and duration for burbot to transmit this pathogen is warranted. It should be noted that previous isolation from wild burbot stocks (USFWS-WFHS 2009), suggests that this species has a potential to carry at least two subspecies of this pathogen and therefore additional investigation into the susceptibility of burbot to atypical forms of this pathogen is needed to increase our level of understanding of burbot susceptibility to *A. salmonicida*.

Injection of *R. salmoninarum* into burbot induced an asymptomatic carrier state. Manifestations of disease were not apparent, which is somewhat surprising given the extremely high doses used to challenge fish (1.7×10^9 bacteria fish^{-1}). Rainbow trout, which aren't considered to be particularly prone to disease caused by this bacterium (Sakai, Shizuo and

Kobayashi 1991; Starliper, Smith and Shatzer 1997), experienced total mortality within approximately 40 d after injection. It is therefore unlikely that this pathogen would manifest into disease or produce subsequent mortality in burbot under natural conditions. Even so, burbot were unable to clear this pathogenic bacterium completely. Viable bacteria was cultured from a challenged mortality 41 d post exposure, and bacterial DNA was detected by PCR at 90 d post challenge, indicating that this bacteria can survive for over one month *in vivo* and possibly longer. Previous research has shown that *R. salmoninarum* can survive macrophage phagocytosis and even escape the phagosome to the cellular cytoplasm (reviewed by Wiens and Kaattari 1999). Such intracellular invasion may help to explain the lack of immune clearance of this bacterium in burbot and the persistence of bacterial DNA. Additionally, Hirvela-Koshi, Pohjanvirta, Koshi and Sukura (2006) noted atypical growth and morphological characteristics during subclinical infections, suggesting that when conditions are suboptimal, this bacterium may enter a dormant state similar to that of *Mycobacterium tuberculosis* in subclinical human infections of tuberculosis. Atypical morphology was not observed in the only case where *R. salmoninarum* was cultured from burbot in this study; however, bacterial persistence in some dormant state at relatively low levels could explain the inability to culture this bacteria from other samples from burbot that where positive by PCR. Burbot may prove a useful species for future investigation into subclinical infections of *R. salmoninarum.*

The development of aquaculture methods for a new species brings with it difficulties inherent for dealing with an unknown. Attempting to optimize performance of new species in captivity can be problematic, and protocols for the intensive culture of burbot have only just begun to be developed (Jensen *et al.* 2008a). General observations at the ARI burbot aquaculture rearing facility have noted high losses during early larval rearing of burbot with chronic

mortality persisting well into juvenile development (Jensen, unpublished data). This is not uncommon for highly fecund species possessing a larval stage and transition to artificial diets can be problematic. Techniques are improving, but early life stage rearing of burbot is extremely delicate. Ideally, studies into disease susceptibility would be conducted once fish mortality had stabilized, however the numbers of individuals required to conduct adequate replicated studies currently surpasses available stock populations reaching such stability. Furthermore, the potential mortality and manifestations of disease exclusively associated with young fish, such as seen with IPNV in salmonids (Reno 1999; LaPatra *et al.* 2000), would likely be missed. Therefore, in many of these trials stable population survival was sacrificed (as observed by mortality in the controls) to ensure adequate fish numbers at a size where disease manifestations would most likely be apparent. Since the objectives of this study were to determine gross susceptibility (positive or negative) and potential carrier status of burbot to these pathogens, any underlying mortality did not affect results and conclusions.

Surveillance for normal salmonid pathogens quantifies and defines the risks of movements outside of the existing host range. In this study, we give baseline susceptibility of burbot to five important and prevalent fish pathogens so as to provide a tool for fish health managers addressing concerns of pathogen transfer for this species. Burbot clearly demonstrated a susceptibility to IHNV as well as *A. salmonicida*, athough to a lesser degree than what was observed in rainbow trout. Burbot appeared refractory to CWD induced by *F. psychrophilum* and to infectious pancreatic necrosis (IPN) caused by IPNV following immersion. Burbot did not appear susceptible to BKD caused by *R. salmoninarum*, however a latent carrier state developed. This study lays a foundation for further investigation into the ability of burbot to act as potential

vectors for the pathogens that cause IHN, BKD, and Furunculosis, as well as insight into potential disease concerns related to this species both in future aquaculture or in natural environs.

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Table 1 Summary of pathogen isolates used for challenge. Challenge method indicated for waterborne immersion (W), intraperitoneal injection (i.p.), or intramuscular injection (i.m.); NA = previous citation not available. Challenge dose given as amount of pathogen administed to individual burbot, Lota lota, during challenge; high and low dose are indicated if more than one concentration was used

Pathogen	Isolate	Host species	Location	Year	Citation	Challenge method	Challenge dose (burbot^{-1})
IPNV (serotype A9)	CF-94	Cutthroat trout *Salvelinus fontinalis*	Clark Fork Hatchery, ID, USA	1994	NA	W / i.p.	1.0×10^5 $TCID_{50}$
IPNV (serotype A1)	Buhl-93	Rainbow trout *Oncorhynchus mykiss*	Buhl, ID, USA	1993	(LaPatra *et al.*(1993b)	i.p.	1.0×10^5 $TCID_{50}$
IHNV (genotype U)	RB1	Steelhead trout *Oncorhynchus mykiss*	Round Butte Hatchery, OR, USA	2000	(Anderson *et al.* 2000)	W	9.5×10^4 pfu
IHNV (genotype M)	220-90	Rainbow trout *Oncorhynchus mykiss*	Buhl, ID, USA	1990	(LaPatra *et al.*(1994)	W	8.8×10^4 pfu
Flavobacterium psychrophilum	259-93	Rainbow trout *Oncorhynchus mykiss*	Southern Idaho, USA	1993	Sudheesh *et al.*(2007)	i.m.	1.0×10^6 cfu (low) 2.5×10^6 cfu (high)
Aeromonas salmonicida salmonicida	HN-00	Rainbow trout *Oncorhynchus mykiss*	Hagerman National Hatchery, ID, USA	2000	NA	i.p.	2.1×10^8 cfu
Renibacterium salmoninarum	CK-90	Steelhead trout *Oncorhynchus mykiss*	Dworshak National Hatchery, ID, USA	1990	Jones *et al*. (2007)	i.p.	5.1×10^8 cells (low) 1.7×10^9 cells (high)

Table 2 Cumulative per cent mortality (CPM) ± standard error (SE) and corrected CPM (cCPM) ± SE for burbot, *Lota lota*, and rainbow trout, *Oncorhynchus mykiss*, following pathogen challenge. Asterix (*) indicates significant increase ($P < 0.05$) relative to mock infected controls.

Pathogen	Isolate	Challenge method	Species challenged	CPM	cCPM
IPNV (serotype A9)	CF-94	W	Burbot Rainbow trout	53 ± 4 0	10 ± 4 0
IPNV (serotype A9)	CF-94	i.p.	Burbot Rainbow trout	100 ± 0 0	3 ± 1 0
IPNV (serotype A1)	Buhl-93	i.p.	Burbot Rainbow trout	94 ± 6 7 ± 4	-2 ± 6 7 ± 4
IHNV (genotype U)	RB1	W	Burbot Rainbow trout	60 ± 4 39 ± 5	17 ± 4* 39 ± 5*
IHNV (genotype M)	220-90	W	Burbot Rainbow trout	67 ± 6 99 ± 1	24 ± 6* 99 ± 1*
Flavobacterium psychrophilum	259-93	i.m.	Burbot Rainbow trout	38 ± 2 100 ± 0	-6 ± 2 100 ± 0*
Aeromonas salmonicida salmonicida	HN-00	i.p.	Burbot Rainbow trout	57 ± 3 53 ± 3	23 ± 3* 53 ± 3*
Renibacterium salmoninarum	CK-90	i.p.	Burbot Rainbow trout	5 ± 5 100 ± 0	3 ± 5 50 ± 0*

Table 3 Pathogen re-isolation from challenged burbot, *Lota lota*, and rainbow trout, *Oncorhynchus mykiss*. Re-isolation of appropriate pathogen (+), pathogen was not re-isolated (–), or samples were unavailable (NA) to attempt re-isolation. Challenge methods are indicated for waterborne immersion (W), intraperitoneal injection (i.p.), or intramuscular injection (i.m.)

Pathogen	Isolate	Challenge method	Species challenged	Pathogen re-isolation	
				mortalities	survivors
IPNV (serotype A9)	CF-94	W	Burbot	-	-
			Rainbow trout	NA	+
IPNV (serotype A9)	CF-94	i.p.	Burbot	+	+
			Rainbow trout	NA	+
IPNV (serotype A1)	Buhl-93	i.p.	Burbot	+	+
			Rainbow trout	+	+
IHNV (genotype U)	RB1	W	Burbot	+	-
			Rainbow trout	+	+
IHNV (genotype M)	220-90	W	Burbot	+	+
			Rainbow trout	+	+
Flavobacterium psychrophilum	259-93	i.m.	Burbot	-	-
			Rainbow trout	+	+
Aeromonas salmonicida salmonicida	HN-00	i.p.	Burbot	+	-
			Rainbow trout	+	+
Renibacterium salmoninarum	CK-90	i.p.	Burbot	+	+
			Rainbow trout	+	+

Figure 1 Curves comparing survival rates of burbot, *Lota lota*, and rainbow trout, *Oncorhynchus mykiss,* following challenge with IHNV by immersion (A), IPNV by immersion (B), IPNV by i.p. injection (C), *Flavobacterium psychrophilum* by i.m. injection (D), *Aeromonas salmonicida salmonicida* by i.p. injection (E) and *Renibacterium salmoninarum* by i.p. injection (F). Asterix (*) indicates significant increased mortality rate relative to mock infected controls within each trial, asteries (**) indicates significant increased mortality rate relative to other treatments as well as controls within each trial.

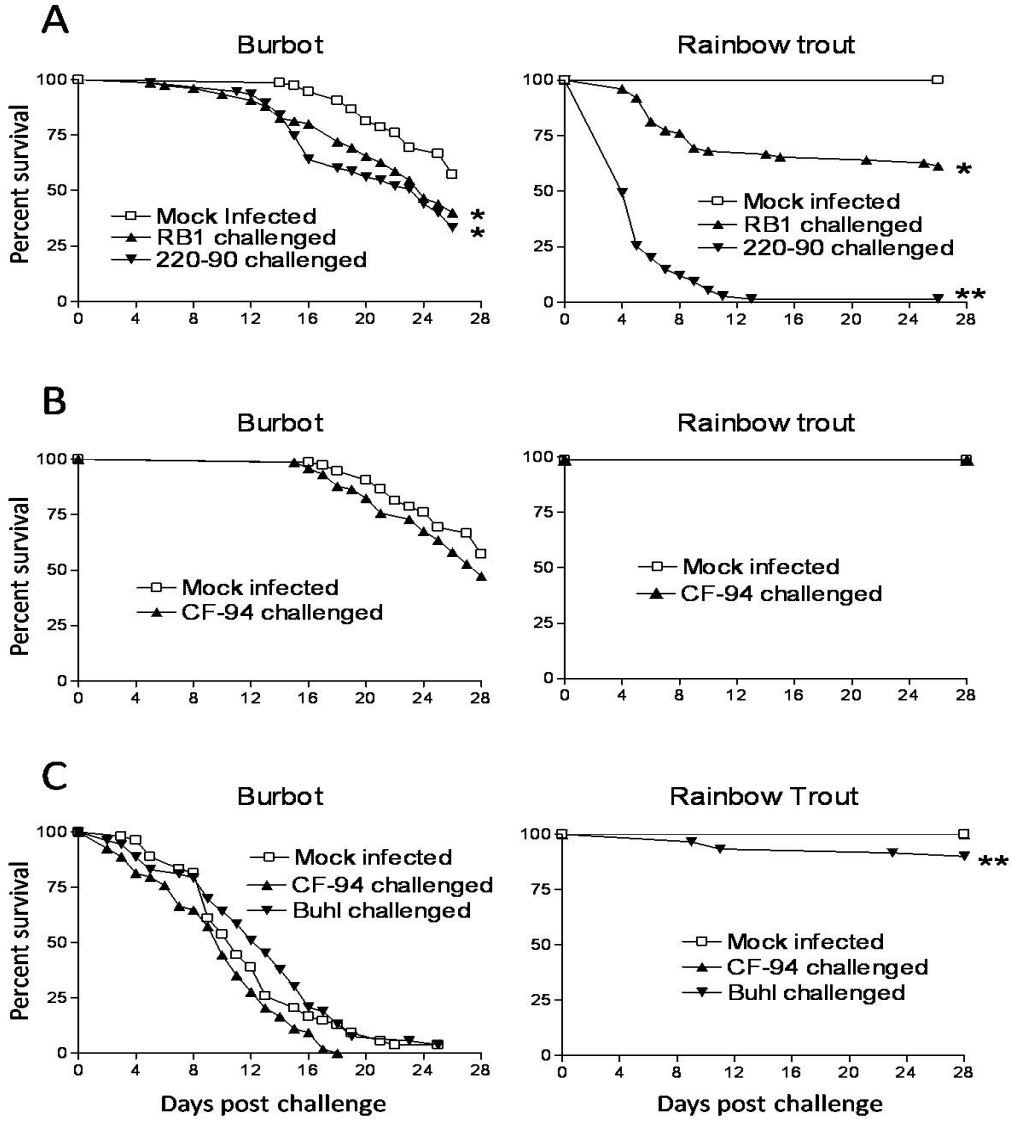

Figure 1 continued