Differential virulence mechanisms of infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*) include host entry and virus replication kinetics

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Host specificity is a phenomenon exhibited by all viruses. For the fish rhabdovirus infectious hematopoietic necrosis virus (IHNV), differential specificity of virus strains from the U and M genogroups has been established both in the field and in experimental challenges. In rainbow trout (*Oncorhynchus mykiss*), M IHNV strains are consistently more prevalent and more virulent than U IHNV. The basis of the differential ability of these two IHNV genogroups to cause disease in rainbow trout was investigated in live infection challenges with representative U and M IHNV strains. When IHNV was delivered by intraperitoneal injection, the mortality caused by U IHNV increased, indicating that the low virulence of U IHNV is partly due to inefficiency in entering the trout host. Analyses of *in vivo* replication showed that U IHNV consistently had lower prevalence and lower viral load than M IHNV during the course of infection. In analyses of the host immune response, M IHNV-infected fish consistently had higher and longer expression of innate immune-related genes such as *Mx-1*. This suggests that the higher virulence of M IHNV is not due to suppression of the immune response in rainbow trout. Taken together, the results support a kinetics hypothesis wherein faster replication enables M IHNV to rapidly achieve a threshold level of virus necessary to override the strong host innate immune response.

**INTRODUCTION**

Infectious hematopoietic necrosis virus (IHNV) is an enveloped, negative-sense, single-stranded RNA virus belonging to the family *Rhabdoviridae*. It causes acute systemic infection in a wide variety of salmonid fish and can lead to significant mortality. North American isolates of IHNV are subdivided into three major genogroups named U, M and L, based on phylogenies of the 303 nt variable ‘mid-G’ region of the virus glycoprotein (Kurath *et al.*, 2003). In the Columbia River basin, where the geographical ranges of U and M IHNV overlap, general patterns of differential host specificity have been observed. The majority of IHNV isolates from rainbow trout (*Oncorhynchus mykiss*) belong to the M genogroup, whilst isolates from sockeye salmon (*Oncorhynchus nerka*) are mostly U genogroup (Garver *et al.*, 2003).

These trends of host association observed in the field correlate with the host-specific virulence of various IHNV isolates in experimental challenge studies that were reported before the U and M genogroups were defined (LaPatra *et al.*, 1990, 1993; Yamamoto & Clermont 1990). Rainbow trout were more susceptible to IHNV strains now known to be in the M genogroup, whilst kokanee salmon (landlocked *O. nerka*) were more susceptible to strains now presumed to be U. More recently, this host-specific virulence of U and M IHNV was clearly demonstrated by Garver *et al.* (2006). In rainbow trout, immersion challenge with three M genogroup isolates caused high mortality, whilst challenge with three U genogroup isolates resulted in low mortality. Conversely, high virulence of U isolates and low virulence of M isolates were observed in sockeye and kokanee salmon.

Purcell *et al.* (2009b) recently investigated the virus–host dynamics that contribute to host-specific virulence of U and M IHNV in sockeye salmon. In sockeye salmon, both virus types entered the host, replicated and spread, but the more virulent U IHNV replicated significantly faster, and to higher levels relative to M IHNV. Both virus types induced a type I interferon (IFN) response, but U IHNV continued to replicate in the presence of this response.
whereas M IHNV was cleared to below detectable levels. In rainbow trout, where the U and M virulence pattern is reversed relative to that of sockeye salmon (i.e. high virulence with M, low virulence with U), the basis for host-specific virulence remains to be elucidated. In this study, we looked at two key stages of the IHNV infection cycle – virus entry into fish hosts and subsequent in vivo replication – to identify differences between representative strains of U and M IHNV that might explain the genogroup-specific trend in virulence. In addition, differences in the level and types of host immune-response genes induced by U and M IHNV infection in rainbow trout were assessed.

**METHODS**

**Virus strains and propagation.** The 220-90 isolate of IHNV from rainbow trout isolated in Hagerman Valley, ID, USA, in 1990 (LaPatra et al., 1994; Kurath et al., 2003) was the representative strain for M genogroup IHNV. The BLK94 isolate from sockeye salmon isolated in Baker Lake, WA, USA, in 1994 (Emmenegger & Kurath 2002) was the representative strain for U genogroup IHNV. These strains were selected because they are highly pathogenic in rainbow trout and sockeye salmon, respectively (Garver et al., 2003). They are referred to hereafter as M and U IHNV. Viruses were propagated at 15 °C in an epithelioma papulosum cyprini (EPC) fish cell line (Fijan et al., 1983). Virus titre was determined by a standard plaque assay (Batts & Winton, 1989).

**Fish.** Juvenile rainbow trout from the research division of Clear Springs Food, ID, USA, were kindly provided by Dr S. LaPatra. Fish were reared at 15 °C in pathogen-free water and fed daily (1.5 % body weight) with a semi-moist pelleted diet (Bioproducts). All experiments were conducted at 15 °C.

**Comparison of U and M IHNV virulence in immersion and injection challenges.** Duplicate groups of 20 juvenile rainbow trout (mean weight 1.8 g) were challenged with U or M IHNV by either immersion or intraperitoneal (i.p.) injection. For immersion challenge, fish were held in 1 l tanks and water circulation was resumed in the 4 l tanks. I.p. challenge was performed by injecting 2 × 10⁴ p.f.u. per fish (low dose) or 5 × 10⁵ p.f.u. per fish (high dose) of U or M IHNV in 50 µl PBS into the peritoneal cavity of fish anesthetized with buffered tricaine methanesulfonate (MS-222, 50 µg ml⁻¹).

Fish in control groups (mock-challenged) were exposed to virus-free medium (immersion experiments) or PBS (injection experiments) instead of the challenge virus. Fish were observed for signs of infectious haematopoietic necrosis disease and mortalities were recorded daily for 30 days after infection. Virulence was assessed by comparing the final cumulative percentage mortality (CPM) and mortality rates among fish exposed to U and M IHNV. Significant differences in final CPM were determined by using a χ² test with Bonferroni correction, and mortality curves were estimated with the Kaplan–Meier method and compared with a log-rank test (SPSS, version 11.5).

**Studies of U and M IHNV in vivo replication kinetics.** Three separate experiments to compare the in vivo replication kinetics of U and M IHNV were conducted in rainbow trout using different fish sizes and routes of infection (Table 1).

### Table 1. Summary of conditions used for the three experiments to define U and M in vivo replication kinetics and the host response in rainbow trout

<table>
<thead>
<tr>
<th>Kinetics experiment</th>
<th>Mean fish weight (g)</th>
<th>Fish stage/age</th>
<th>Challenge conditions</th>
<th>Virus dose (per fish)</th>
<th>Sampled tissues</th>
<th>Sampled time points post-infection</th>
<th>Holding conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4</td>
<td>Juvenile/~3 months</td>
<td>Immersion, 12 h batch</td>
<td>2 × 10⁶ P.I.U. ml⁻¹</td>
<td>Whole fish</td>
<td>0, 1, 2, 3, 7, 10</td>
<td>Individual fish in separate beakers, static water, flowing water, in batch, flowing water</td>
</tr>
<tr>
<td>2</td>
<td>71</td>
<td>Subadult/~9 months</td>
<td>Immersion, 1 h batch</td>
<td>2 × 10⁵ P.I.U. ml⁻¹</td>
<td>Anterior kidney</td>
<td>0, 25, 35</td>
<td>In batch, flowing water, in batch, flowing water</td>
</tr>
<tr>
<td>3</td>
<td>116</td>
<td>Subadult/~1 year</td>
<td>I.p. injection</td>
<td>2 × 10⁶ P.I.U.</td>
<td>Anterior kidney</td>
<td>34, 45</td>
<td>30, 45</td>
</tr>
</tbody>
</table>

Different numbers of fish were used in the three treatment groups due to different levels of anticipated mortality. Fish were collected from the three treatment groups 12 h after immersion infection and I.P washing. Three anterior kidney samples from each fish were collected immediately before virus challenges.
For kinetics experiment 1, juvenile rainbow trout were challenged by immersion in a static suspension of 2 x 10^5 p.f.u. U or M IHNV ml^-1 in 1 l water for 12 h with aeration. After the exposure period, fish were moved to a separate bucket with circulating water for 1 h to wash out excess virus. Fish were then separated into individual beakers of 400 ml static water and held for 10 days. Five fish per virus group were randomly sampled immediately after the end of the 12 h immersion infection and 1 h wash (day 0) and subsequently at 1, 2, 3, 7 and 10 days post-infection. Two mock-challenged fish were also sampled at each time point. Half (200 ml) of the static water was replaced at days 4 and 8 post-infection to maintain water quality, and dead fish were collected daily.

For kinetics experiment 2, immersion challenge of subadult rainbow trout was conducted with a 1 h static exposure period at a dose of 2 x 10^5 p.f.u. ml^-1. Fish groups were then held in batches and anterior kidney tissue was sampled from six or seven fish per group at 3, 7 and 14 days post-challenge. Anterior kidney from seven unhandled fish was collected immediately before virus infection to serve as pre-challenge samples.

In kinetics experiment 3, i.p. challenge of subadult rainbow trout was performed by injecting 2 x 10^5 p.f.u. U or M IHNV per fish in 50 ml PBS into the peritoneal cavity of fish anaesthetized as described above. Anterior kidney was sampled from five fish per group at 1, 2, 3, 7, 14 and 35 days post-challenge. Anterior kidney from three pre-challenged fish was collected immediately before virus infection.

Sampling in juvenile rainbow trout was done by euthanizing the fish with an overdose of buffered MS-222 and placing them in individual whirl pack bags. Subadult rainbow trout were euthanized as above and approximately 50 mg anterior kidney was aseptically dissected from each fish and snap-frozen in liquid nitrogen. Samples were stored at -80 °C until RNA extraction.

**RNA extraction and quantitative RT-PCR (RT-qPCR) assay for quantification of viral load.** RNA was extracted from whole-fish homogenates of juvenile fish using a guanidinium thiocyanate protocol [4 ml denaturing solution (g fish)^-1] modified from the method of Anderson et al. (2000), as described previously (Troyer et al., 2008). For subadult fish kidney samples, RNA was extracted using an RNeasy RNA extraction kit (Qiagen) with in-column DNase I treatment following the manufacturer's instructions. Total RNA was quantified by spectrophotometry (SpectraMax Plus; Molecular Devices).

cDNA was synthesized from 1 μg total RNA using oligo(dT) and random hexamer primers, as described previously (Purcell et al., 2004). After synthesis, cDNA reactions were diluted to a final volume of 100 μl, and 5 μl was used for quantitative PCRs (qPCRs). The RT-qPCR assay for IHNV viral load used the G-946F and G-1066R primers and G-1003T probe described by Purcell et al. (2006b). U and M in vitro transcripts, reverse-transcribed simultaneously with the fish RNA samples, were used to create an absolute standard curve based on the estimated molecular mass of the RNA transcribed from the U or M IHNV glycoprotein genes (Purcell et al., 2006a). Samples were considered positive if two replicate wells reached the RT-qPCR threshold by 38 cycles (C_T ≤ 38).

Viral load data were calculated as gene copies (μg RNA)^-1 and reported as the geometric mean of all sampled fish at a given time point. For geometric mean calculations, fish or kidney samples having no detectable viral load (C_T >38) were assigned a value of 10 IHNV copies (1 log), the detection limit of the U and M in vitro transcripts used as standards for the IHNV RT-qPCR assay.

**Host immune gene expression assays.** Expression level of the Mx-1 gene was determined in whole fish or anterior kidney tissues using the same cDNA synthesized for viral load quantification. In addition, expression changes of 11 other key innate and adaptive immune response genes were evaluated in the anterior kidney samples of subadult rainbow trout challenged with U or M IHNV by i.p. injection (kinetics experiment 3). The primer and probe sequences used for real-time RT-qPCR are provided in Supplementary Table S1 (available in JGV Online). Our standard RT-qPCR methodology using an ABI 7900HT (Applied Biosystems) has been described in detail previously (Purcell et al., 2004). As the purpose of this part of the study was to compare relative levels of gene expression rather than absolute quantities, cDNA preparations previously known to have high levels of the genes of interest were used as standards to assess PCR efficiency for each assay plate. This standard was assigned an arbitrary value and then diluted serially in tenfold increments to generate the standard curve used to extrapolate gene copy numbers in the samples. The expression levels of each immune-response gene were normalized against the expression level of a housekeeping gene, acidic ribosomal phosphoprotein P0 (ARP) (Pierce et al., 2004; Purcell et al., 2004). Analysis and calculation of fold change relative to mock-infected control groups has been previously described (Purcell et al., 2004). To assess statistical significance, we used a Mann–Whitney U-test (two experimental groups) to compare groups (SPSS, version 11.5). P values <0.05 were considered significant.

**RESULTS**

Role of host entry in differential virulence of U and M genogroup IHNV in rainbow trout

To determine the role of virus delivery route on virulence of U and M IHNV, we compared the mortality induced by these two virus types in juvenile fish following challenge by immersion or i.p. injection. M IHNV caused high mortality (93–100 % final mean CPM), regardless of infection route (Fig. 1). Consistent with previous data (Garver et al., 2006), immersion challenge with U IHNV resulted in low mortality (18 % final mean CPM). i.p. challenge with U IHNV led to increased mortality, with the low-dose and high-dose injection groups resulting in 45 and 83 % final mean CPM, respectively.

![Fig. 1. Virulence of U and M IHNV in immersion and i.p. injection challenges of juvenile rainbow trout. Bars show final CPM in duplicate groups of 20 fish over a 30-day challenge period. Letters denote statistically significant groups (P<0.05) within each challenge route.](image-url)
Kinetics experiment 1: U and M IHNV in vivo replication and host response in juvenile rainbow trout following immersion challenge

In the first kinetics experiment, juvenile fish challenged by immersion were separated into individual isolation units to eliminate the possibility of secondary rounds of infection. No viral load was detectable by RT-qPCR in the mock-challenged fish sampled at each time point (results not included on graphs). All fish sampled in the M-infected group were IHNV positive, whilst the prevalence of U-positive fish was consistently lower at all sampling time points (Fig. 2a). In fish challenged with M IHNV, the mean viral load increased from the first sampling, immediately after the 12 h virus challenge and 1 h wash and peaked at day 2 post-challenge. M viral load then gradually declined but was still present in all fish at day 7, after which there were no fish left for sampling due to high mortality. By comparison, growth of U IHNV was delayed, with minimal increase in viral load during the 10-day infection period. Mean viral load in U-positive fish was consistently lower by 1–2 logs relative to M IHNV. U IHNV was cleared below the detectable level in most fish (four of five) by day 10. The U IHNV-infected group had lower mortality than the M-infected group during the 10-day sampling period (data not shown).

The host innate immune response was measured in this study by examining expression of the interferon-stimulated gene (ISG) Mx-1, as a proxy marker. Induction of this gene has been strongly correlated in previous studies with protective early anti-rhabdoviral immunity in fish and fish cell lines (Caipang et al., 2003; McLauchlan et al., 2003; Purcell et al., 2006b; Saint-Jean & Perez-Prieto, 2006). As not all fish in the U-infected group had detectable viral load, mean Mx-1 expression (reported as fold change relative to the mock control) is shown for all of the sampled fish (i.e. both virus positive and negative, Fig. 2b) and also for the virus-positive fish only (Fig. 2c), to avoid any potential bias in gene expression analysis due to a difference in the infection status of the host.

Mx-1 gene expression levels were quantitatively similar between the mock-, U- and M-challenged groups immediately after the end of the 12 h challenge and 1 h wash (day 0, Fig. 2b, c). Mx-1 expression was significantly upregulated in the M-infected group by day 1, peaked at day 3, and remained elevated up to the last available sampling time point for this viral group (day 7). The U-infected group showed significant Mx-1 upregulation only at day 3, and decreased to the level of the mock control by day 7.

Kinetics experiment 2: U and M IHNV in vivo replication and host response in anterior kidney sampled from subadult rainbow trout following immersion challenge

In published experimental challenges of rainbow trout, size-dependent susceptibility to IHNV has been observed, with mortality generally being lower in larger fish (LaPatra et al., 1990; Bergmann et al., 2003). Virus immersion challenges were conducted in larger fish to examine whether the differences in the replication kinetics of, and host response to, U and M type viruses observed in whole juvenile fish samples would be consistent in individual kidney tissue samples from larger trout. No viral load was detectable in the anterior kidney samples of pre-challenged (unhandled) fish (Fig. 3a) or in the mock controls sampled at each time point (not shown). As in the previous experiment, all fish challenged by immersion with the M
virus were positive for viral load in the anterior kidney at days 3, 7 and 14. Although decreasing mean viral load was evident from days 3–14, M virus was still present at the end of the sampling period. Viral load was initially detectable in all U-challenged fish at day 3, but was cleared in all but one fish by days 7 and 14. Mean viral load in U-positive fish was consistently lower (1–2 logs) than in M-positive fish at all sampling time points. As expected, mortality in subadult rainbow trout during the 14 day sampling period in both virus groups was lower than in juvenile rainbow trout (data not shown). However, as in immersion challenge of small fish, higher mortality was observed in M- compared with U-infected groups.

Both U- and M-challenged groups had significant upregulation of Mx-1 gene expression in the anterior kidney relative to the mock control at all sampling time points (Fig. 3b, c). Mx-1 gene expression was tenfold higher in the M infection relative to the U infection at day 3, decreased by day 7 in both groups and was at comparable levels at day 14.

**Kinetics experiment 3: U and M IHNV in vivo replication and host response in anterior kidney sampled from subadult rainbow trout following i.p. injection challenge**

Differences in the ability of U and M IHNV to enter host fish could potentially affect the kinetics of in vivo viral load levels and subsequent host responses in fish challenged by immersion. To assess viral replication without any entry component, a third virus challenge was conducted by i.p. injection in subadult rainbow trout. This facilitated the delivery of equal amounts of U or M virus to each fish, and initiated more controlled and synchronous infections.

No viral load was detectable by RT-qPCR in kidney tissues of the pre-challenged fish (Fig. 4a) or in mock-injected fish sampled at each time point (not shown). All M-challenged fish were positive for IHNV, with a rapid increase in mean viral load by day 1 and a peak at day 2. After day 2, M IHNV load decreased gradually, but remained at relatively high levels in all sampled fish through to day 14. IHNV prevalence in the U-infected group was consistently lower than in the M-infected group except at day 2, when all sampled U-challenged fish showed detectable viral load in their anterior kidney. After an initial increase on day 1, there was minimal increase (if any) in mean U viral load.
throughout the infection period, with virus clearance resulting in below detectable levels in most fish by day 7. Mean viral load in U-positive fish was consistently lower than in M-positive fish by 1.5–3 logs at all sampling time points, and there was no detectable viral load by day 35. No mortality resulted from the U challenge, whilst high mortality was observed in the M challenge (data not shown). No M-infected fish were left for sampling after day 14.

The Mx-1 host response differed slightly following injection challenge compared with the immersion challenge. At day 1, only M-infected fish had significant Mx-1 upregulation relative to the mock control. At day 2, both M- and U-infected groups had comparable Mx-1 upregulation. Thereafter, Mx-1 levels remained elevated in the M-infected group, albeit with a slight decrease at day 7, until the last available sampling time point (day 14). Mx-1 expression in U-infected fish had decreased by day 3 and was significantly less than in M-infected fish on days 3, 7 and 14. At day 14, the mean Mx-1 level for all five fish in the U group was comparable to the mock-infected group (Fig. 4b), although Mx-1 was slightly but significantly elevated in the one U-positive fish (Fig. 4c).

**Other host responses to U and M IHNV in subadult rainbow trout following i.p. injection challenge**

To get a more comprehensive picture of the rainbow trout immune response to acute U or M IHNV infection, we looked at the expression patterns of additional key innate and adaptive immune-response genes in kidney tissue from injection-challenged fish in kinetics experiment 3. In rainbow trout, type I IFN has been shown to upregulate ISGs such as Mx-1 and to mediate early antiviral protection against IHNV (Ooi et al., 2008). In the present study, the inducible short transcript form of group I type I IFN (IFN-IS) (Purcell et al., 2009a) was upregulated to significant levels at days 2, 3 and 7 in the M-infected group and at day 2 in the U-infected group (Fig. 5a). Except for a slight induction at day 3 in the M-infected group, no significant increase in the long transcript form of IFN-1 (IFN-L) was observed (Fig. 5b), consistent with the known constitutive expression of this transcript form (Purcell et al., 2009a).

In addition to Mx, a large number of virus-induced genes (Vigs) are known to be induced by type I IFN in rainbow trout (Boudinot et al., 1999, 2001; O’Farrell et al., 2002). The expression pattern of Vig-1 was found to be very similar to that of Mx-1 (compare Fig. 4c with Fig. 5c). In the M-infected group, significant Vig-1 upregulation relative to the mock control was observed at day 1 and increased at day 2 to high levels that persisted through to the final sampling on day 14 (Fig. 5c). High Vig-1 upregulation was observed at days 2 and 3 in the U-infected group and levels on day 2 were comparable to levels in the M-infected fish. U-infected group Vig-1 levels declined on days 7 and 14 but remained significantly higher than the mock-control fish, returning to basal levels by day 35. At all sampling time points except day 2, Vig-1 expression was higher in the M- than in the U-challenged group.

Similar to its mammalian counterpart, rainbow trout tumour necrosis factor (TNF)-α is an important macrophage-activating factor that has been shown to induce the expression of a number of genes including another pro-inflammatory cytokine, interleukin (IL)-1β, and the chemokine IL-8 (Zou et al., 2003). For a modest induction at day 2 in the M-infected group, TNF-1α was not significantly upregulated in either the U or M challenge groups (Fig. 5d). For IL-1β (Fig. 5e) and IL-8 (Fig. 5f), higher expression (relative to the mock control) was observed from days 1 to 7 in the M-infected group, but only at day 2 in the U-infected group.

IL-12 is a key pro-inflammatory cytokine that bridges both innate and adaptive immunity. In mammals, it is known to induce the efficient production of IFN-γ, one of the key cytokines in defining Th1 immune responses in natural killer and T cells (Chan et al., 1992; Kubin et al., 1994; Murphy et al., 1994). The short transcript form of IL-12 β-chain (IL-12S) was only significantly upregulated in the M group at day 2 (Fig. 5g), whilst IFN-γ (Fig. 5h) was significantly induced at comparable levels in the U and M groups at days 2 and 3 post-infection.

The co-stimulatory molecules CD28 and CTLA-4 were used as markers for resting and activated T cells of rainbow trout, respectively. In addition, CD8z, a co-receptor of cytotoxic T cells, was used as a marker to evaluate cytotoxic T-lymphocyte activity in the U- and M-infected fish. CTLA-4 was upregulated at days 2 and 7 post-infection in the M-infected group and at day 14 in the U-infected group (Fig. 5j). Correspondingly, CD28 downregulation was observed in both U- and M-infected groups at days 2, 3 and 7 post-infection, with the level being more downregulated in the M-infected group at day 7 (Fig. 5i). CD8z levels were also slightly downregulated at days 2 and 7 in the M-infected group and at day 3 in the U-infected group (Fig. 5k).

**DISCUSSION**

From the point of view of a pathogen, each stage in the infection cycle represents a barrier that it must overcome in order for successful infection to occur. Virulence is one of a number of possible outcomes of this continuous host–pathogen interaction throughout the infection cycle. For IHNV, the virus must be able to gain entry efficiently into the fish host, traffic into target organs (kidney and spleen), replicate in susceptible cells and be shed into the water in order to facilitate transmission and initiate subsequent rounds of infection, whilst simultaneously encountering, controlling and/or evading the host’s corresponding immune responses. Differences in the ability of U and M IHNV to overcome any of these stages could influence the
outcome of an infection and thus determine genogroup-specific virulence.

We hypothesized that the difference in virulence between U and M IHNV during immersion challenge could be due to a differential ability to gain entry into the fish. Infection by i.p. injection circumvents the external physical barriers and mucosal immune responses that IHNV normally encounters during the natural waterborne route of infection via skin, gills, gastrointestinal tract and fin bases (Yamamoto & Clermont, 1990; Drolet et al., 1994; Hellick et al., 1995; Ellis, 2001; Harmache et al., 2006). A dose-dependent increase in mortality in the U-infected fish challenged by i.p. injection (compared with immersion) was observed, suggesting that inefficiency in gaining entry into the host does contribute to the lower virulence of U IHNV in rainbow trout. Mortality in the U IHNV i.p. challenges, however, did not reach the same high levels of mortality induced by M IHNV either by immersion or injection, indicating that there must be other stages/factors in the infectious cycle, subsequent to entry, that play a role in the genogroup-specific virulence.

In the next three experiments, we focused on two of the key components of the infectious cycle: in vivo replication of U and M IHNV, and the corresponding fish host immune response, using Mx-1 as a proxy marker for the IFN-mediated response. The IHNV challenges in juvenile rainbow trout (kinetics experiment 1) were novel compared with typical batch challenge studies in that we eliminated the possibility of fish-to-fish virus transmission by holding individual fish in isolated beakers immediately after the challenge exposure. In addition, viral load was determined from whole-fish samples rather than individual tissues, thus reflecting the total virus production in each fish. In subadult rainbow trout, on the other hand, infected fish were held in batches and viral load was quantified from the anterior kidney, the main target organ of IHNV where the virus is likely to be most concentrated (Amend et al., 1969; Wolf, 1988; Yamamoto & Clermont, 1990; Yamamoto et al., 1990; Romero et al., 2005). The general trends of higher viral load, higher virus prevalence and faster replication kinetics in M- relative to U-challenged fish were consistent in the three experiments, regardless of challenge conditions, fish life stage and sampled tissue. This pattern of higher viral load in M infection could be a consequence of an inherently faster virus replication rate or higher virus production per replication cycle, or both. Differences in the ability of U and M IHNV to spread and...
Traffic into the main target organs and establish systemic infection could also explain the difference in measured viral load, particularly at the early sampling time points. As in previous observations in sockeye salmon (Purcell et al., 2009b), Mx-1 expression mirrored viral load in rainbow trout. This suggests a lagging immune response rather than a causal factor that controls viral load. Similarity in expression patterns of Mx-1 and Vig-1 indicated that the trend in transcriptional regulation for U and M IHNV is consistent among fish ISGs. Upregulation of the inducible form of type I IFN, IFN-1S, was not as dramatic as that of ISGs Mx-1 and Vig-1, which is not surprising given that IFN is an upstream component of the IFN-mediated signalling pathway. This overall trend in host immune response to U and M suggested that the lower level of immune response was sufficient to control the relatively lower level of viral load, resulting in lower fish mortality and eventual virus clearance. In the case of M IHNV, however, the virus continued to replicate in the presence of a strong IFN-mediated response. The decreasing M viral load at later times after infection (days 3–14) suggested that M IHNV is susceptible, at least to some degree, to the host innate burden, and thus can probably be attributed to the continuous availability of M viral components that can constantly trigger the host immune system. It is also possible that this markedly higher and longer elevation could contribute to the higher virulence of M IHNV in rainbow trout via immunopathogenesis.

Infection with IHNV is known to rapidly induce a strong host innate IFN response (Trobridge et al., 1997a, b; Purcell et al., 2004; Landis et al., 2008; Purcell et al., 2009b) and, as in other aquatic rhabdoviruses, IHNV is reported to be sensitive to the effects of IFN (Eaton, 1990; Rogel-Gaillard et al., 1993; Ooi et al., 2008). In U IHNV infection, it appeared that the lower level of immune response was sufficient to control the relatively lower level of viral load, resulting in lower fish mortality and eventual virus clearance. In the case of M IHNV, however, the virus continued to replicate in the presence of a strong IFN-mediated response. The decreasing M viral load at later times after infection (days 3–14) suggested that M IHNV is susceptible, at least to some degree, to the host innate
immune responses, but that this response was not sufficient to clear the infection.

For an acute virus such as IHNV, viral control and/or clearance are mediated mainly by the host innate response, and induction of the adaptive immune response will be more important in the protection of survivors against subsequent IHNV infection. Similar to previous studies (Purcell et al., 2009a), upregulation of IFN-γ and another Th1-associated molecule, IL-12, in the U or M groups was transient and did not persist into the adaptive immune phase.

In coldwater fish such as salmonids, a significant adaptive immune response is usually not achieved until several weeks after infection (Lorenzen & LaPatra 1999). However, stimulation of adaptive-response-associated genes has also been observed as early as 7 days after DNA vaccination with an IHNV glycoprotein gene (Purcell et al., 2006b). It is possible that transcriptional regulation occurs in the adaptive immune cells very early, even if their functional activity occurs much later in the infection. Downregulation of CD28, occurring simultaneously with the upregulation of CTLA-4, suggested T-cell activation (Bernard et al., 2006), whilst the initial decrease in the expression levels of cytotoxic T-cell marker CD8α in the anterior kidney, which is the major haematopoietic tissue in trout, could be due to the migration of these activated immune cells to other sites of IHNV infection.

In summary, these studies have identified fish host entry and virus replication as two components of the IHNV infection cycle that play a significant role in the differential virulence of U and M virus in rainbow trout. Consistent with data obtained by Purcell et al. (2009b) in sockeye salmon, the primary difference between the U and M viral infections was the faster in vivo kinetics and higher level of replication of the high-virulence virus (M IHNV in rainbow trout), which was able to persist even in the presence of a relatively high Mx-1 response. These results fit well with the kinetics hypothesis presented by Purcell et al. (2009b), which links high virulence to rapid replication of IHNV. The virus takes advantage of the fact that the host innate immune system requires induction to become active. This lag gives the rapidly replicating M virus a head start to quickly achieve a threshold level of virus (within 2–3 days of infection) that can outrun the trout IFN-mediated antiviral response, and hence win the virus–host race. This simple and efficient strategy of escaping the IFN-induced response by multiplying extremely fast has also been reported in influenza (Grimm et al., 2007). It should be noted that the M-type virus group is thought to have evolved from an ancestral U-type virus in the unique conditions of the rainbow trout industry (Troyer et al., 2000; Kurath et al., 2003). As a consequence, it appears to have lost virulence for its original sockeye salmon host. By adaptation, the M genogroup has probably gained virulence for rainbow trout by some change that facilitated rapid replication, providing a strategy to withstand the host immune response.

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