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Spatial and Temporal Variation of Whirling Disease Risk in Montana Spring Creeks and Rivers

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Abstract
Spring creeks are important spawning and rearing areas for wild trout, but the stable flows, cool temperatures, and high nutrient levels that characterize these unique habitats may also make them highly susceptible to establishment and proliferation of the whirling disease pathogen Myxobolus cerebralis. We evaluated the spatial and temporal dynamics in whirling disease risk by using sentinel rainbow trout Oncorhynchus mykiss fry in nine different spring creeks and their conjoining rivers or reservoirs in Montana over a 20-month period. Whirling disease risk was high in five of the seven pathogen-positive spring creek study sites; at these sites, prevalence levels exceeded 90% and over 50% of sentinel fry had moderate to high infection severity scores. Spring creeks generally had higher disease prevalence and severity than paired river or reservoir sites. Fine sediment levels varied widely among springs creeks with high and low whirling disease risk, and we found no significant association between fine sediment level and infection severity. The low risk measured for some spring creeks was likely attributable to the pathogen invasion being in its early stages rather than to environmental characteristics limiting the severity of infection. High whirling disease risk occurred over a wide range of temperatures at spring creek sites (4.5–13°C) and river sites (1.7–12.5°C). There was an unusual seasonal cycle of infection in spring creeks, with peak infection levels occurring from late fall to early spring and declining to near zero in late spring to early fall. The low infection risk during spring suggests that spring-spawning trout would be at a low risk of infection, even in spring creeks with otherwise high disease severity. In contrast, fry of fall-spawning trout may be much more susceptible to infection in spring creek environments.

Groundwater-fed streams, or “spring creeks,” are important for wild trout populations as the consistent cool temperatures, high nutrient levels, and stable discharge provide near-optimum conditions for spawning, growth, and rearing. In particular, spring creeks provide critical spawning areas, drawing large numbers of spawners from long distances, thereby contributing to recruitment over a large geographic area (Kiefling 1978; Decker-Hess 1987; Clancy 1988).

The same characteristics that make spring creeks unique and valuable as trout habitat may also make them highly susceptible to establishment and proliferation of Myxobolus cerebralis, the nonindigenous myxosporean parasite that causes whirling disease in salmonids. Whirling disease has spread rapidly throughout the USA over the past 20 years (Bartholomew and Reno 2002) and has been associated with significant declines in some highly valued fisheries for wild rainbow trout Oncorhynchus mykiss in the western USA (Vincent 1996; Nehring 2006; McMahon et al. 2010). Myxobolus cerebralis has a complex, two-host life cycle involving salmonids and the aquatic oligochaete Tubifex tubifex (Wolf and Markiwick 1984; Hedrick

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et al. 1998; Kerans and Zale 2002). Trout that are younger than 9 weeks of age are the most susceptible to disease from infection by the free-floating triactinomyxon stage (Ryce et al. 2004) that is released from the oligochaete host (Wolf and Markiw 1984). *Myxobolus cerebralis* attacks cartilage in the cranial and skeletal regions of trout and, at high infection rates, produces the eponymous “whirling” behavior, cranial and skeletal deformities, blackened tails, and high mortality (Hedrick et al. 1998; Baldwin et al. 2000; MacConnell and Vincent 2002; Ryce et al. 2004), ultimately leading to sharp declines in survival and recruitment of age-0 fish (Vincent 1996; Nehring 2006; McMahon et al. 2010). Disease severity in young trout depends on pathogen abundance (Markiw 1992; Vincent 2002; Ryce et al. 2004) and species-specific differences in susceptibility (Hedrick et al. 1999; Thompson et al. 1999; Vincent 2002). Infection also requires spatiotemporal overlap between triactinomyxon release and the narrow window of high infection sensitivity in young trout (Downing et al. 2002; MacConnell and Vincent 2002; Pierce et al. 2009). Factors that have been associated with a high risk of whirling disease infection include many common features of spring creek environments (Burckhardt and Hubert 2005), such as an abundance of fine sediments, which are favored as habitat by the *T. tubifex* host (Hiner and Moffitt 2001; Sandell et al. 2001; Burckhardt and Hubert 2005; Krueger et al. 2006); water temperatures of 10–15°C (El-Matbouli et al. 1999; Baldwin et al. 2000); low flows (MacConnell and Vincent 2002; Hallett and Bartholomew 2008); low gradient (Anlauf and Moffitt 2008); anthropogenic disturbances that increase sedimentation and organic inputs, thereby increasing *T. tubifex* density (Zendt and Bergersen 2000; Kaeser et al. 2006; Granath and Moffitt 2008); and the presence of disease-resistant brown trout *Salmo trutta*, which serve as an infection reservoir for sustained parasite production (Nehring 2006).

Whirling disease was first confirmed in Montana in 1994 after sharp declines in Madison River rainbow trout (Vincent 1996). The discovery prompted initiation of a statewide program to monitor parasite distribution and infection risk by using caged sentinel fish (Baldwin et al. 1998). *Myxobolus cerebralis* is now widespread in many western Montana drainages (Vincent 2000; McGinnis 2007); however, little is known about the role spring creeks have in the spread of whirling disease. Over 100 spring creeks occur in western Montana, where they typically originate in the agricultural lands of river valley bottoms; many spring creeks are important spawning and rearing areas for wild trout (Decker-Hess 1987). Few spring creeks in Montana have been tested for *M. cerebralis* presence, but the potential for spring creeks to be foci of whirling disease infection appears to be high. In a survey of *M. cerebralis* in trout of the Salt River drainage, Wyoming, spring creeks had the highest incidence of infection among all stream types examined (Isaak and Hubert 1999).

The purpose of our study was to investigate the occurrence of *M. cerebralis* in Montana’s spring creeks and to determine factors that may affect infection severity. We also measured seasonal dynamics of whirling disease risk in relation to timing of the vulnerable early fry rearing period because wide temporal variation in infectivity has been observed, even in rivers with very high infection risk (Sandell et al. 2001; Downing et al. 2002; MacConnell and Vincent 2002). We hypothesized that whirling disease infection risk would be of higher magnitude and of longer duration in spring creeks than in adjoining river systems due to habitat and thermal characteristics that support high pathogen production.

**METHODS**

*Study sites.*—Whirling disease risk was estimated for nine spring creeks within eight different drainages; each spring creek was paired with its confluent river or reservoir (Figure 1). Sites were chosen to represent a diversity of spring creeks across southwestern Montana. Site selection criteria included (1) previous detection of the pathogen in the spring creek or nearby river and (2) known importance of the spring creek as a spawning tributary. Sampling was conducted over a 20-month period from January 2000 to August 2001. Spring creeks ranged from 0.5 to 9.7 km in length, with discharge ranging from 0.1 to 1.4 m³/s (Table 1). At all sites, sentinel rainbow trout were exposed

<table>
<thead>
<tr>
<th>Spring creek</th>
<th>River or reservoir</th>
<th>Length (km)</th>
<th>Mean width (m)</th>
<th>Discharge (m³/s)</th>
<th>Surface fines (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anceny Spring Creek</td>
<td>Gallatin River</td>
<td>0.5</td>
<td>8.8</td>
<td>–</td>
<td>39.3</td>
</tr>
<tr>
<td>Ben Hart Spring Creek</td>
<td>East Gallatin River</td>
<td>4.8</td>
<td>10.5</td>
<td>0.7–1.0</td>
<td>48.5</td>
</tr>
<tr>
<td>Blaine Spring Creek</td>
<td>Madison River</td>
<td>8.3</td>
<td>11.1</td>
<td>0.9–1.0</td>
<td>40.3</td>
</tr>
<tr>
<td>Clark Canyon Spring Creek</td>
<td>Clark Canyon Reservoir</td>
<td>0.8</td>
<td>4.5</td>
<td>0.1–0.2</td>
<td>42.0</td>
</tr>
<tr>
<td>Kleinschmidt Creek</td>
<td>Blackfoot River</td>
<td>4.8</td>
<td>4.1</td>
<td>0.4–0.5</td>
<td>30.0</td>
</tr>
<tr>
<td>Mitchell Slough</td>
<td>Bitterroot River</td>
<td>9.7</td>
<td>14.6</td>
<td>0.9</td>
<td>33.0</td>
</tr>
<tr>
<td>Nelson Spring Creek</td>
<td>Yellowstone River</td>
<td>3.2</td>
<td>19.6</td>
<td>1.1–1.4</td>
<td>52.0</td>
</tr>
<tr>
<td>Rock Creek</td>
<td>Blackfoot River</td>
<td>4.8</td>
<td>5.2</td>
<td>0.6</td>
<td>13.3</td>
</tr>
<tr>
<td>Willow Springs Creek</td>
<td>Jefferson River</td>
<td>1.6</td>
<td>8.5</td>
<td>0.4</td>
<td>57.0</td>
</tr>
</tbody>
</table>
FIGURE 1. Locations of spring creek study sites and associated river drainages in southwestern Montana, USA.
twice in the spring (April and May) of 2000 and 2001 and once in the fall (October) of 2000 to assess the spatial distribution of whirling disease risk over a wide geographic area. Spring and fall sampling periods were chosen because they coincided with the periods of peak infectivity identified in previous studies of seasonal infection cycles in Montana and Wyoming streams (Downing et al. 2002; Murcia et al. 2006). Three spring creeks (Ben Hart Spring Creek, Nelson Spring Creek, and Willow Springs Creek) and their conjoining rivers (East Gallatin, Yellowstone, and Jefferson rivers, respectively) were sampled monthly to assess the seasonal dynamics of whirling disease risk.

**Whirling disease risk.**— Sentinel fish exposures were used to measure relative abundance of the infective, free-floating *M. cerebralis* triactinomyxon parasite and the resultant effect on whirling disease severity in salmonids. Use of uninfected rainbow trout fry as a biological filter to determine prevalence (percent infected) and severity of infection has been a standard test for measuring whirling disease risk (Baldwin et al. 2000; Sandell et al. 2001; Downing et al. 2002; Krueger et al. 2006). Sentinel fish were held in cages consisting of a wire-mesh cylinder (0.5-m diameter; 0.6 m deep). Sixty juvenile rainbow trout, which were obtained from certified whirling-disease-free hatcheries, were measured (mm TL) and placed into each cage. In total, 167 sentinel cage exposures were conducted during the study. Six strains of rainbow trout (Arlee, Eagle Lake, Madison, Erwin, Shasta, and Fish Lake) were used for sentinel exposures; each of these strains has high to very high susceptibility to whirling disease (Vincent 2002; Wagner et al. 2006). Sentinel fish were similar in strain, age, and size within each sampling period, but these factors differed among sampling periods due to limitations in year-round availability of a particular strain during the study. Sentinel fish ranged from 29 to 53 mm TL and from 24 to 119 d posthatch. Ryce et al. (2004, 2005) showed that sentinel rainbow trout older than 63 d and larger than 40 mm had a diminished infection severity and fewer clinical signs of disease than younger, smaller individuals when exposed to similar numbers of *M. cerebralis* in the laboratory. To evaluate the possible confounding effect of sentinel fish size and age differences on infection severity, we examined infection severity (percentage of sentinel fish with histology scores ≥3; see below) in relation to the size and age of fish from the two spring creek study sites with the highest infection severity and longest time series of sentinel cage data (Willow Springs Creek and Ben Hart Spring Creek). We hypothesized that there would be a negative association between disease severity and size or age if either of these factors had a significant effect on infection severity among test fish.

Sentinel fish were held in cages for 10 d and then were transferred to laboratory aquaria at either the Whirling Disease Laboratory in Pony, Montana (Vincent 2002), or the Wild Trout Research Laboratory at Montana State University, Bozeman (Ryce et al. 2004); the choice of laboratory site depended on tank availability. Fish were reared in the tanks for 80–89 d at 10–13°C to allow for full development of *M. cerebralis* spores (Baldwin et al. 1998). Test fish were fed a standard commercial trout diet twice daily to satiation. At the end of the rearing period, the rainbow trout were sacrificed with an overdose of tricaine methanesulfonate (MS-222). Heads were removed and fixed in a 10% solution of buffered formalin for 72 h prior to transfer into individually marked, sterile plastic bags containing 70% alcohol. At the Washington Animal Disease Diagnostic Laboratory (Pullman, Washington), the heads of the fish were sectioned, and infection severity was scored by using the MacConnell–Baldwin histology rating system (Baldwin et al. 2000; Vincent 2002) based on a lesion severity scale of 0 (no infection) to 5 (severe infection). For the first 6 months of the study (January–June 2000; 80 of the total 294 exposure groups), 50 fish from each 60-fish exposure group were randomly selected and scored histologically. For the final 14 months of the study, the sample size was decreased to 30 fish/exposure group in order to accelerate the time-consuming and costly scoring process. To assess the effect of the reduced sample size, 32 exposure groups were randomly selected and mean disease severity scores were compared between sample sizes of 30 and 50 randomly selected heads. The mean difference in histology scores between sample sizes was low (0.04), and we did not detect significant differences between the paired sample scores (Wilcoxon’s signed rank test: *P* = 0.32); therefore, subsequent analyses were performed on the reduced data set for the remainder of the study.

The study design involved deploying sentinel cages in each spring creek and the conjoining river or reservoir into which it flowed. Generally, at least two cages were placed in each spring creek (the exception was that Willow Springs Creek had only one cage from January to June 2000) along protected stream margins, the typical habitat of age-0 trout fry (Downing et al. 2002). At least one cage was simultaneously placed in each conjoining river or reservoir upstream of the spring creek’s mouth to avoid the influence of spring creek water. The number of months sampled was not equal for all sites due to cage loss from high or variable flows during cage deployment in rivers. Generally, only one sentinel cage was placed within a particular location due to the significant time and cost associated with deploying replicate cages in close proximity. To assess the degree of precision in estimation of disease severity with only one sentinel cage at a site, we compared mean histology scores from replicate sentinel cages that were placed in close proximity (1–7 m apart) at four different study sites during 20 sampling periods. Mean histology scores differed by 0.40 or less between replicate cages, and we did not detect significant differences in disease severity among paired exposure groups (Wilcoxon paired-sample test: *P* = 0.29).

**Spawning and emergence timing.**—Redd counts were conducted within the three intensively sampled spring creeks one to two times per month from February 2000 to September 2001 to compare the timing of spawning and fry emergence in relation to whirling disease risk. For each spring creek, counts were conducted in sections with a high percentage of spawning gravels by walking along the banks while wearing polarized...
M. cerebralis highest susceptibility of young trout to Vincent 2002) was determined as the 9-week interval after the susceptibility window (Downing et al. 2002; MacConnell and 1997). Channel width was measured at transects (Ben Hart Spring Creek, Nelson Spring Creek, and Willow Springs Creek).

Timing of fry emergence from redds was estimated by using the estimated date of redd construction in combination with published values for the accumulated number of thermal units (degree-days, °C) required for emergence: 589 thermal units for rainbow trout emergence (Downing 2000) and 727 thermal units for brown trout emergence (Crisp 1988). The number of degree-days was calculated by summing mean daily water temperatures (°C) obtained from thermographs. The “disease susceptibility window” (Downing et al. 2002; MacConnell and Vincent 2002) was determined as the 9-week interval after the estimated peak in fry emergence, coinciding with the period of highest susceptibility of young trout to M. cerebralis infection (Ryce et al. 2004).

Temperature.—Water temperature was measured at 30-min intervals during each sentinel exposure period by using electronic thermographs. Thermographs were also used to record hourly temperature in the three intensively studied spring creeks (Ben Hart Spring Creek, Nelson Spring Creek, and Willow Springs Creek).

Habitat characteristics.—The length of each habitat type (pool, riffle, and glide) was measured along the entire length of each spring creek by following the procedures of Overton et al. (1997). Channel width was measured at transects (n = 6–10) positioned at systematic intervals of 50–500 m, depending on the overall length of the spring creek. Percent aquatic vegetation was visually estimated within a 10-m band upstream and downstream of each transect. Percent surface fines (<2 mm), the preferred habitat of T. tubifex (Krueger et al. 2006), was measured at each transect by using a Wolman pebble count together with a surface fines grid (Overton et al. 1997). Pebble counts were performed by walking heel to toe across the stream transect until 100 samples were obtained. A surface fines grid was randomly tossed once upstream and once downstream at each transect. Mean surface fines calculated from each method were highly correlated (Pearson’s product-moment correlation coefficient r = 0.86, P = 0.003), so only the Wolman surface fines results are reported herein.

Statistical analysis.—Metrics that were used to summarize disease severity for each exposure group included (1) prevalence, or the total percentage of sentinel fish showing signs of infection; (2) mean histology score; and (3) the percentage of fish with histology scores of 3 or greater. The percentage of histology scores greater than or equal to 3 was considered a measure of high disease severity potential because fish with these infection severity scores exhibit significant cartilage damage, numerous lesions and granulomas, a higher incidence of clinical signs of disease, and reduced performance and survival (Baldwin et al. 1998; Ryce et al. 2004, 2005). Declines in wild rainbow trout populations have been observed when 50% or more of the sentinel fish demonstrate infection severity scores of 3 or higher (Vincent 2000; McMahon et al. 2010).

Disease severity differences in paired spring creek and river or reservoir sites were analyzed by comparing infection prevalence, mean histology scores, and the proportion of fish with high disease severity by using the Wilcoxon paired-sample test (Daniel 1990). When multiple cages were sampled at a site, we used disease severity data from the cage with the highest lesion score as a measure of infection risk for use in paired comparisons. Simple linear regression was used to evaluate the association between percent surface fines and infection severity. Linear and nonlinear relationships between temperature and disease severity were evaluated using the curve-fitting program in SigmaPlot (SigmaPlot 2008), with the highest r^2 values used to select the best-fitting models. Pearson’s product-moment correlation was used to test for a possible negative correlation between disease severity and the size and age of sentinel fish. All tests were performed at an α level of 0.05.

RESULTS

Spatial and Temporal Variation in Whirling Disease Risk

Infection severity in sentinel fish varied widely among the 17 study sites (Table 2). Myxobolus cerebralis was not detected at three of the study sites (Anceny Spring Creek, Clark Canyon Spring Creek, and the Bitterroot River) and was detected at very low levels (infection prevalence ≤12%) at four other sites (Mitchell Slough, Nelson Spring Creek, Gallatin River, and Yellowstone River). In contrast, seven study sites had a high whirling disease risk (Rock Creek, Ben Hart Spring Creek, Blaine Spring Creek, Kleinschmidt Creek, Willow Springs Creek, Madison River, and East Gallatin River), as evidenced by 50% or more of the sentinel fish having histology severity scores of 3 or higher (i.e., indicating moderate to severe infection risk). Sentinel fish that were exposed in the Blackfoot River, Jefferson River, and Clark Canyon Reservoir exhibited intermediate levels of infection risk.

Disease prevalence and severity were generally higher in spring creeks than in conjoining rivers or reservoirs (Table 2). Three spring creeks had significantly higher mean histology scores than their paired river sites, and four spring creeks had a significantly greater proportion of high disease severity than their paired river sites. Disease severity in three spring creeks (Kleinschmidt Creek, Rock Creek, and Willow Springs Creek) reached very high levels (77–97% of sentinel fish had histology scores ≥3), whereas only one river site (Madison River) had a disease severity of this magnitude.

Monthly exposure sampling of several study sites revealed a distinct seasonal pattern in infectivity. In both Willow Springs Creek and Ben Hart Spring Creek, infection severity was highest
TABLE 2. Paired comparisons of sentinel rainbow trout infection ratings for spring creeks and their conjoining river or reservoir sites during April 2000–August 2001. For a given pair of sites, asterisks indicate the significantly higher values of percent infected, mean histology score, or mean percentage of sentinel fish with histology scores of 3 or greater (Wilcoxon paired-sample test: \( P \leq 0.05 \)).

<table>
<thead>
<tr>
<th>Spring creek and paired river or reservoir</th>
<th>Mean (range) percentage infected</th>
<th>Mean (range) histology score</th>
<th>Mean (range) percentage with histology score ( \geq 3 )</th>
<th>Number of months sampled (total number of cage exposures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anceny Spring Creek</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Gallatin River</td>
<td>0.6 (0–3)</td>
<td>0.02 (0–0.1)</td>
<td>0.6 (0–3)</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Ben Hart Spring Creek</td>
<td>62.2 (6–100)</td>
<td>1.5 (0.1–2.8)</td>
<td>25.9 (0–54)*</td>
<td>20 (62)</td>
</tr>
<tr>
<td>East Gallatin River</td>
<td>52.9 (0–96)</td>
<td>1.23 (0–2.8)</td>
<td>19.2 (0–60)</td>
<td>18 (29)</td>
</tr>
<tr>
<td>Blaine Spring Creek</td>
<td>51.0 (4–93)</td>
<td>1.2 (0–2.6)</td>
<td>19.1 (0–50)</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Madison River</td>
<td>87.6 (83–100)</td>
<td>2.6 (1.8–3.8)*</td>
<td>53.1 (28–93)*</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Clark Canyon Spring Creek</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5 (10)</td>
</tr>
<tr>
<td>Clark Canyon Reservoir</td>
<td>53.3 (26–68)*</td>
<td>0.9 (0.4–1.6)*</td>
<td>11.6 (7–25)*</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Kleinschmidt Creek</td>
<td>89.5 (76–100)*</td>
<td>3.4 (2.3–4.0)*</td>
<td>74.1 (53–86)*</td>
<td>5 (9)</td>
</tr>
<tr>
<td>Rock Creek</td>
<td>81.0 (76–90)*</td>
<td>2.7 (2.0–3.9)*</td>
<td>53.6 (38–77)*</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Blackfoot River</td>
<td>31.0 (2–76)</td>
<td>0.8 (0–2.3)</td>
<td>10.8 (0–43)</td>
<td>4 (6)</td>
</tr>
<tr>
<td>Mitchell Slough</td>
<td>0.3 (0–2)</td>
<td>0.04 (0–0.02)</td>
<td>0</td>
<td>5 (8)</td>
</tr>
<tr>
<td>Bitterroot River</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3 (4)</td>
</tr>
<tr>
<td>Nelson Spring Creek</td>
<td>1.5 (0–6)</td>
<td>0.02 (0–0.1)</td>
<td>0</td>
<td>19 (38)</td>
</tr>
<tr>
<td>Yellowstone River</td>
<td>2.0 (0–12)</td>
<td>0.03 (0–0.2)</td>
<td>0</td>
<td>12 (19)</td>
</tr>
<tr>
<td>Willow Springs Creek</td>
<td>64.7 (0–100)*</td>
<td>2.2 (0–4.1)*</td>
<td>42.2 (0–97)*</td>
<td>20 (35)</td>
</tr>
<tr>
<td>Jefferson River</td>
<td>9.1 (0–45)</td>
<td>0.1 (0–0.9)</td>
<td>1.6 (0–10)</td>
<td>19 (25)</td>
</tr>
</tbody>
</table>

from November to May, declined to very low or undetectable levels during June–September, and increased sharply again in October (Figure 2); this pattern occurred during both years of the study. In conjoined river sites (Jefferson and East Gallatin rivers), peak infectivity occurred during October–November and May–June, whereas infectivity was very low during other months of the year. The East Gallatin River showed substantial year-to-year variation in infectivity, with low severity during the first year of the study and high severity during the second year; in the second year, infectivity followed a seasonal pattern similar to that observed in the spring creeks, with high infectivity during November–June and very low infectivity during July–September.

Relationship to Spawning and Emergence Timing

Overall, 314 redds were observed in Willow Springs Creek, 163 were observed in Ben Hart Spring Creek, and 327 were observed in Nelson Spring Creek during the 20-month survey period. Spawning generally occurred over extended periods at all sites (Figure 3). Rainbow trout spawning periods ranged from as early as January to as late as July at some sites, with peak spawning occurring from about early March to early May. Brown trout spawning began as early as September, peaked in mid-November, and extended through January. Infection risk was low during the postemergence disease susceptibility window for spring-spawning rainbow trout (1 June–15 August) and was very high during the susceptibility window for fall-spawning brown trout (1 March–15 May; Figure 3).

Relationship between Infection Risk and Habitat Features

**Temperature.**—Temperature ranged from about 7°C to 12°C in spring creeks and from 1°C to 19°C in river sites during exposure sampling (Figure 2). Infection risk varied widely with temperature at spring creek sites and river sites (Figure 4). Among spring creeks that tested positive for *M. cerebralis*, there was a significant curvilinear relationship between temperature and infection risk, with peak infection severity occurring at 6–8°C. However, high infection risk occurred at temperatures as low as 4.5°C and as high as 13°C. There was a significant negative linear relationship between infection severity and temperature at Willow Springs Creek, the spring creek with the highest infection severity, with severity peaking at about 7°C and declining sharply to low levels at temperatures above 10°C. For river sites, there was no significant relationship \(( P > 0.22)\) between temperature and infection severity. Infection was detected at a wide range of temperatures (0.1–17°C), with high severity occurring mostly in the temperature range of 7.5–12°C but also at temperatures as low as 1.7°C (Figure 4).

**Habitat characteristics.**—Spring creek study sites comprised a high percentage of glide habitat (mean = 71%), fine sediment (mean = 39%), and abundant rooted aquatic vegetation (mean = 59%). There was no significant association between disease severity and percent fine sediment for all nine spring creeks combined \( (r^2 = 0.02, P = 0.73; \text{Table 1})\) or for the subset
Variation in Whirling Disease Risk

FIGURE 2. Seasonal changes in Myxobolus cerebralis infection severity (total percentage of sentinel rainbow trout showing signs of infection [% total infection]; and the percentage of sentinel fish with moderate to severe infection [% histology scores ≥3]) and changes in water temperature for the two intensively sampled spring creeks (Willow Springs Creek and Ben Hart Spring Creek) and their conjoining river sites (Jefferson and East Gallatin rivers, respectively) over the 20-month study period (January 2000–August 2001). Data for Nelson Spring Creek and the Yellowstone River are not shown because infection was very low (total infection <1.2%).
FIGURE 3. Numbers of newly observed spawning redds of rainbow trout (black bars) or brown trout (gray bars) during each survey period in Ben Hart Spring Creek, Nelson Spring Creek, and Willow Springs Creek, 2000 and 2001. Solid line refers to the percentage of sentinel rainbow trout with moderate to severe infection (histological [histo] scores ≥ 3; note that moderate to severe infections were absent among sentinel fish in Nelson Spring Creek). Horizontal lines indicate periods of peak fry emergence from redds (solid lines = rainbow trout; dashed lines = brown trout); vertical arrows frame the disease susceptibility window, the 9-weeks-posthatch period when young trout are most susceptible to *Myxobolus cerebralis* infection and disease.

FIGURE 4. Percentage of sentinel rainbow trout with moderate to severe *Myxobolus cerebralis* infection (histology [histo] scores ≥ 3) in relation to average water temperature during the exposure period for (A) the five high-infection spring creeks (Ben Hart Spring Creek, Blaine Spring Creek, Kleinschmidt Creek, Rock Creek, and Willow Springs Creek; n = 103 exposure groups); (B) Willow Springs Creek, the site with the highest infection severity (n = 26 exposure groups); and (C) the river or reservoir exposure sites (n = 90 exposure groups). Lines represent the best fit of the data (highest r² value); no curve had a significant r² value for panel C (all P > 0.19).
of five spring creeks with high infection risk (Ben Hart Spring Creek, Blaine Spring Creek, Kleinschmidt Creek, Rock Creek, and Willow Springs Creek; $r^2 = 0.00, P = 0.98$; Table 2). Percent surface fines ranged from 13% to 57% among the five high-risk sites and from 33% to 52% among the four low-risk sites.

**Size and Age Effects**

Larger size or greater age of sentinel rainbow trout was not associated with a significant decline in infection severity (Figure 5). The percentage of sentinel rainbow trout with moderate to severe infection was equal between the largest (53 mm) or oldest (108 d posthatch) sentinel fish (>85% with histology scores ≥3) and smaller or younger sentinel fish, indicating that the size and age differences among sentinel fish did not have a strong influence on the observed patterns of infection severity.

**DISCUSSION**

Examination of whirling disease risk by using sentinel rainbow trout fry confirmed that *M. cerebralis* was common across a subset of Montana spring creeks but indicated that infection severity varied substantially among sites. Overall, *M. cerebralis* was detected in seven of nine spring creek study sites, and five of the seven pathogen-positive spring creeks exhibited infection severity levels that have been correlated with significant declines in recruitment of age-0 rainbow trout (i.e., >50% of sentinel fry with moderate to high infection severity scores; Vincent 2000; McMahon et al. 2010). Spring creeks generally had higher disease prevalence and severity than their paired river or reservoir sites. Seven of the eight river or reservoir sites tested positive for the pathogen, but only two sites had infection risk levels that approached the peak infectivity observed during disease exposures in the spring creeks.

High spatial variation in *M. cerebralis* prevalence and abundance, both among and within drainages, has been noted for many other systems (e.g., Baldwin et al. 1998; Hiner and Moffitt 2001; Sandell et al. 2001; Downing et al. 2002; Thompson et al. 2002; Granath et al. 2007; Pierce et al. 2009). Such variation has been hypothesized to be a function of several factors, including how long the parasite has been in a drainage and the degree of spore production by infected fish (Kerans and Zale 2002; Krueger et al. 2006); the amount of fine sediment habitat available for *T. tubifex* (Zendt and Bergersen 2000; Hiner and Moffitt 2001; Krueger et al. 2006); and differences in the strain of *T. tubifex*, as the strains vary markedly in their production of infective triactinomyxons (Kerans et al. 2004; Beauchamp et al. 2005; Lodh et al. 2011). We did not find a strong association between infection severity and fine sediment level. We suspect that the very low infectivity levels in Anceny Spring Creek, Clark Canyon Spring Creek, Mitchell Slough, and Nelson Spring Creek were attributable to *M. cerebralis* having not yet invaded those systems or the invasion being in its early stages; we do not believe that environmental characteristics were responsible for preventing the spread of *M. cerebralis* in those creeks. At all four sites, fine sediment levels were high and similar to those at the five highly infected spring creeks. Moreover, Anderson (2004) found that *T. tubifex* densities in Nelson Spring Creek, our very-low-infection study site, were two to four times higher than those in highly infected Ben Hart Spring Creek and Willow Springs Creek, which indicates that *T. tubifex* density was not limiting the development of high infectivity in our low-severity spring creeks (see also Alexander et al. 2011). We know of no systematic evaluation of *T. tubifex* lineages within spring creeks, but the *T. tubifex* lineages that are known to be highly susceptible to *M. cerebralis* infection (lineages I and III) are common in shallow depositional areas with abundant fine sediments (Dubey and Caldwell 2004; Beauchamp et al. 2005;
Lodh et al. 2011), and such habitat conditions are common in spring creeks.

Study results did not support our hypothesis that fish in spring creeks are exposed to M. cerebralis infection for a longer portion of the year than fish in rivers. In both environments, the duration of infectivity was about 9 months on an annual basis. However, the timing of disease risk in spring creeks was generally different from that observed in rivers. In both of the highly infected spring creeks that were sampled monthly (Willow Springs Creek and Ben Hart Spring Creek), whirling disease risk was highest in the late fall through early spring. For example, in Willow Springs Creek, very high disease risk (>80% of sentinel fish with moderate to severe lesions) was observed during November–February, but the risk then dropped to zero during July and August. The timing of infectivity was surprising, as most previous studies of rivers have shown that whirling disease risk is very low to zero during winter and early spring (Thompson and Nehring 2000) and that peak infection risk occurs during relatively brief time windows in late spring and early fall (Downing et al. 2002; Murcia et al. 2006) or during summer (Thompson and Nehring 2000; Sandell et al. 2001). Seasonal infection risk in our river sites also generally followed this latter pattern, but we did observe an unexpectedly high infection risk in the East Gallatin River during winter and spring months (January–June).

High seasonal variation in whirling disease infection risk has been largely attributed to seasonal temperature changes. Temperature affects development rate and spore production of M. cerebralis in both hosts (El-Matbouli et al. 1999; Blazer et al. 2003; Kerans et al. 2005). El-Matbouli et al. (1999) found that peak triactinomyxon release from T. tubifex under controlled laboratory conditions occurred at 10–15°C. Evaluation of sentinel fish to determine infection risk in rivers has also generally shown that infectivity peaks during the spring and fall within this same temperature range (Baldwin et al. 2000; Downing et al. 2002; MacConnell and Vincent 2002). However, in the present study, high infection risk occurred over a much broader range of temperatures in both spring creek sites and river or reservoir sites. High infection risk in spring creeks (>40% of sentinel fish with moderate to severe lesion scores) occurred at temperatures as low as 4.5°C and as high as 13°C, and peak infection occurred at 6–8°C. In river study sites, we observed high infection risk over a wide range of temperatures (1.7–12.5°C). Moreover, we detected moderate levels of infection (43% of sentinel fish infected, with 10% showing moderate to severe infection) even at very cold temperatures (0.1°C in the Jefferson River, December 2000). Although winter sampling for infection risk has been limited, observations from other studies concur with our findings of the potential for high infectivity during winter and early spring at low temperatures (Monson and Johnson 2001; Nehring et al. 2003; Lukins 2004).

We hypothesize that the unusual timing of peak winter infection risk we observed in spring creeks and the more common spring pulse of infection risk in rivers were a result of different thermal accumulation rates. Triactinomyxons are produced by T. tubifex approximately 1,350 degree-days after ingestion of myxospores (range = 1,300–1,456 degree-days; Markiw 1986; Kerans et al. 2005). Interestingly, the estimate of 1,350 degree-days equates to a back-calculated myxospore input timing of mid- to late August in the spring creek and river study sites, corresponding to the period in which heavily infected age-0 rainbow trout would likely begin dying and releasing myxospores (Kerans and Zale 2002). For spring creeks, warmer winter temperatures accelerate spore development within the T. tubifex host, resulting in a midwinter peak in triactinomyxon release, whereas in non-spring-fed rivers the depressed winter temperatures would prolong spore development and delay triactinomyxon release until the rapid rise in spring temperature. Wide variation in infectivity responses among different systems therefore suggests that the timing of infection risk is a site-specific response based on differences in thermal accumulation and myxospore input (Kerans and Zale 2002; Kerans et al. 2005) and differences in discharge (Hallett and Bartholomew 2008).

The lack of overlap between high infection risk and the disease susceptibility window for rainbow trout fry suggests that spring-spawning trout would be at a low risk of infection, even in spring creeks with high infection risk during other time periods. During the period of highest infection susceptibility for rainbow trout fry in Ben Hart Spring Creek and Willow Springs Creek (1 June–15 August), the incidence of moderate to severe lesions in sentinel fish was 0–10% compared with 40–97% during winter to early spring months. This contrasts markedly with other studies of surface-water-fed streams, which demonstrated a strong temporal overlap between high infectivity and high vulnerability for rainbow trout and other spring-spawning trout (Downing et al. 2002; MacConnell and Vincent 2002; Pierce et al. 2009). In contrast, our results showed that in spring creeks, fall-spawning brown trout had a much higher risk of whirling disease infection than did spring-spawning rainbow trout. Although brown trout are highly resistant to whirling disease, they can still become infected and produce myxospores (Hedrick et al. 1999; Baldwin et al. 2000), thus serving as a source of spores for infection of more susceptible species. Two fall-spawning species that may be susceptible to whirling disease infection in spring creeks are the brook trout Salvelinus fontinalis and kokanee O. nerka. Both species use spring-fed sites for spawning (Curry et al. 1995; Garrett et al. 1998), and both are also susceptible to whirling disease (Vincent 2002). Brook trout numbers have declined markedly in recent years in Kleinschmidt Creek (Ron Pierce, Montana Fish, Wildlife, and Parks, personal communication), a spring creek that produced one of the highest infectivity levels among sentinel rainbow trout in our study (see also Kaeser et al. 2006).

Our findings have several important implications for the monitoring and management of whirling disease. The discovery of high infectivity over a wide range of months and temperatures indicates that effective monitoring of infection risk will require careful tailoring of infection severity measurement in relation to the timing of disease susceptibility windows on a site-by-site basis. From a risk assessment perspective (Bartholomew et al. 2005), the very high infectivity we observed at low water
temperatures does not support the inference that colder water associated with winter, higher elevations, or higher latitudes imparts protection from severe impacts of whirling disease (Schisler and Bergersen 2002; Arsan et al. 2007).

The high spawning and rearing usage of spring creeks, in combination with their high susceptibility for severe whirling disease infection, indicates that these sites are important “hot spots” for the proliferation and intensification of the disease. We only sampled a small subset of the numerous spring creeks in Montana, and more extensive evaluation of infection risk is warranted. Detailed examination of *T. tubifex* lineages in spring creeks would be a useful component of such infection risk assessments. Anthropogenic disturbances that increase sedimentation and organic enrichment are common in spring creeks (Burckhardt and Hubert 2005; our personal observations) and would likely serve to intensify whirling disease impacts (Zendt and Bergersen 2000; McGinnis 2007). Research is needed to test the idea that reducing sedimentation by use of habitat restoration practices can decrease *T. tubifex* habitat and reduce whirling disease infectivity in these key trout spawning and rearing areas (e.g., Thompson 2011).

ACKNOWLEDGMENTS

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Effect of Dietary Supplementation with Achyranthes aspera Seed on Larval Rohu Labeo rohita Challenged with Aeromonas hydrophila

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ARTICLE

Effect of Dietary Supplementation with Achyranthes aspera Seed on Larval Rohu Labeo rohita Challenged with Aeromonas hydrophila

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Abstract
Larval rohu Labeo rohita were fed four different diets: three of the diets contained Achyranthes aspera (prickly chaff-flower) seeds at 0.10% (D1), 0.25% (D2), or 0.50% (D3); the fourth diet was a control diet (D4; no A. aspera supplementation). After 70 d, the rohu were injected intraperitoneally with live Aeromonas hydrophila. Mortality of fish was recorded for 7 d. In the D4 group, the first mortality was observed within 12 h of exposure, whereas in the D1–D3 treatment groups, mortality was first observed at 24 h postexposure. In the D4 group, 50% of fish died within 72 h of exposure, whereas in the D3 group, 10–15% mortality occurred between 72 and 84 h. The cumulative mortality rate was 50% for D4, 40% for D1, 35% for D2, and 15% for D3. Total tissue protein level in the larvae was higher for the D2 and D3 groups than for the other groups. Glutamic oxaloacetic transaminase, glutamate pyruvate transaminase, and thiobarbituric acid reactive substance levels were significantly lower in D3 larvae than in the other groups, whereas lysozyme and nitric oxide synthase levels were significantly higher in D3 larvae compared with the other groups. Dietary supplementation with A. aspera seeds at the 0.50% level provided protection against oxidative stress, prevented tissue damage, and enhanced disease resistance in rohu larvae.

Larval mortality is one of the major problems encountered in freshwater pond culture systems, resulting in a great economic loss every year. This phenomenon is quite common with the larvae of Indian major carps. Larval mortality caused by bacterial infection is a major management and economic problem for the aquaculture industry and may be due to a lack of immune competence in fish at this early stage (Swain et al. 2002). Innumerable diseases are caused by bacterial pathogens. The Gram-negative bacterium Aeromonas hydrophila, a ubiquitous and heterogeneous organism, produces a disease known as motile aeromonad septicemia in fish that are held under stressful conditions or that are already infected by other pathogens. Aeromonas hydrophila is thus a scourge of freshwater fish farming worldwide and is considered a major economic problem (Fang et al. 2000). Aeromonas hydrophila causes various diseases, such as hemorrhagic septicemia, infectious dropsy, and fin rot disease, in freshwater fish species in India (Sudhakaran et al. 2006). Fish respond to infectious agents in both nonspecific and specific ways, but they depend on the nonspecific mechanisms to a much greater extent. The enhancement of innate immunity may improve the health status of fish (Harikrishnan et al. 2011). Therefore, the identification and implementation of measures for preventing diseases caused by this fish pathogen are more important than treating the disease after an outbreak has occurred.

Immunostimulants have the ability to increase disease resistance capacity by enhancing nonspecific and specific defense mechanisms (Rao et al. 2006; Divyagnaneswari et al. 2007). Application of natural immunostimulants has several advantages as they are biocompatible, biodegradable, cost effective, and environmentally friendly. Seeds of Achyranthes aspera (prickly chaff-flower; Amaranthaceae) have shown immunostimulatory properties in carps (Rao et al. 2004; Chakrabarti and Rao 2012). The rohu Labeo rohita is an economically important carp in India, contributing about 40% of the aquaculture production (Sahu et al. 2007). Production of disease-free rohu fingerlings is
required for sustainable aquaculture development. In the present investigation, rohu larvae were fed diets containing seeds of *A. aspera* and were challenged with *A. hydrophila* to determine the effect of dietary supplementation on disease resistance. The age-specific dose of *A. aspera* for rohu was determined.

**METHODS**

*Culture of fish.*—Rohu larvae (age = 4 d) were procured from a fish farm. The initial weight of larvae was 1.0 ± 0.01 mg (mean ± SE). Larvae were acclimated in outdoor cement tanks and were cultured in a recirculating system starting on day 17. The recirculating system consisted of glass aquaria that held the fish and one 55-L glass aquarium that contained a filter (Sharma and Chakrabarti 1998). The stocking density was 125 larvae per 15-L aquarium. Larvae were cultured under four different feeding regimes (with 3 replicates/feeding treatment): three of the diets contained seeds of *A. aspera* at 0.10% (diet 1 [D1]), 0.25% (D2), or 0.50% (D3); the fourth diet served as the control (D4; no *A. aspera* supplementation). Experimental diets (40% protein) were prepared by using *A. aspera* seeds along with fish meal, wheat flour, cod liver oil, and vitamin and mineral premixes (Table 1). *Achyranthes aspera* plants were grown in a plot near the laboratory; ripe seeds were collected and air dried, and the outer covers of the seeds were removed manually. The seeds were then ground, and the powder was added to the diet. Crude protein, crude fat, and ash in the feedstuffs were estimated according to standard methods (AOAC 1984). The gross energy in the feedstuffs was estimated by direct calorimetry. Feed was provided to the larvae at the rate of 5% of body weight per day.

*Challenge of fish.*—*Aeromonas hydrophila* (Microbial Type Culture Collection [MTCC] Number 1739) was cultured in nutrient broth (Himedia, India) for 24 h at 37°C. The culture broth was centrifuged at 3,000 × g for 10 min. The supernatant was discarded, and the pellet was resuspended in phosphate-buffered saline (pH 7.4); the optical density of the solution was adjusted to 1.5 at 456 nm. This bacterial suspension was used for the challenge test.

After 70 d of culture, survival of rohu in each treatment group was recorded; survival was 81 ± 0.5% (mean ± SE) in the D1 group, 86 ± 1.1% in the D2 group, 88 ± 1.2% in the D3 group, and 79 ± 1.3% in the D4 group. Fish from all four groups were then injected intraperitoneally with 10 µL of live *A. hydrophila* (3 × 10^6 CFU) suspended in phosphate-buffered saline. A 0.30- × 8-mm (30-gauge × 0.3125-in) needle (Hindustan Syringes and Medical Devices Ltd.) was used for injection. Mortality of fish was recorded at 12-h intervals, and observation for mortalities continued for 7 d postchallenge. At 7 d postexposure, the fish were collected after 12 h of fasting and the final number of surviving individuals was recorded. Weights of individual fish were measured, and tissue samples were collected from the surviving fish for various biochemical assays.

*Sample preparation.*—Fish were euthanized with tricaine methanesulfonate (MS-222; Sigma, USA). The scales, head, tail, and bones were removed, and the remainder of the fish was used for tissue analyses. The dissection was performed under a dissecting microscope by using forceps. Tissue samples were collected from all surviving fish, and samples were pooled for each aquarium; thus, three pooled samples were collected for each feeding scheme. The samples were preserved at −80°C. One-hundred milligrams of tissue were homogenized with 1 mL of chilled phosphate buffer and then spun down at 10,000 × g for 10 min, and the supernatant was immediately used for assay.

*Assay methods.*—Total tissue protein was measured in accordance with the method of Lowry et al. (1951). Absorbance was recorded at 750 nm using a Synergy HT microplate reader (BioTek, New York).

Tissue glutamic oxaloacetic transaminase (GOT) and glutamate pyruvate transaminase (GPT) were assayed by using diagnostic kits (Siemens Healthcare Diagnostics Ltd., Baroda, India). The absorbance was recorded at 340 nm; GOT and GPT levels were expressed in IU per liter.

**Table 1.** Composition of experimental diets supplemented with *Achyranthes aspera* seeds (D1–D3; supplementation rate is given in parentheses) and the control diet (D4) fed to rohu larvae.

<table>
<thead>
<tr>
<th>Ingredient or component</th>
<th>D1 (0.10%)</th>
<th>D2 (0.25%)</th>
<th>D3 (0.50%)</th>
<th>D4 (0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredients (g/kg of diet)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry fish powder</td>
<td>583.3</td>
<td>583.3</td>
<td>583.3</td>
<td>583.3</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>401.7</td>
<td>400.2</td>
<td>397.7</td>
<td>402.7</td>
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<tr>
<td>Cod liver oil</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Vitamin–mineral premix</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td><em>Achyranthes aspera</em> seeds</td>
<td>1.0</td>
<td>2.5</td>
<td>5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Proximate analysis (% dry matter basis)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude protein</td>
<td>45.0</td>
<td>45.0</td>
<td>45.0</td>
<td>45.625</td>
</tr>
<tr>
<td>Crude fat</td>
<td>7.1</td>
<td>7.6</td>
<td>7.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Ash</td>
<td>7.0</td>
<td>7.1</td>
<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td>Energy value (cal/g)</td>
<td>3.6</td>
<td>3.6</td>
<td>3.6</td>
<td>3.7</td>
</tr>
</tbody>
</table>
Thiobarbituric acid (TBA) reactive substances were measured by following the method of Ohkawa et al. (1979), in which malondialdehyde (MDA)—the end product of lipid peroxidation—reacts with TBA. One gram of tissue was homogenized in 9 mL of KCl (1.15%), and the sample was incubated at 100°C for 1 h in acid medium containing sodium dodecyl sulfate (0.45%) and 0.6% TBA. After cooling, the sample was centrifuged and the absorbance of the supernatant was measured at 532 nm. The standard was prepared by using 1,1,3,3-tetramethoxy propane, and the result was expressed as micromoles of MDA per milligram of protein.

Lysozyme was determined via the method of Siwicki (1989). Micrococcus lysodeikticus (MTCC Number 106) was cultured in Staphylococcus broth (Himedia) containing 1% D-glucose. After a 24-h incubation period, the culture was centrifuged and the bacterial pellet was lyophilized. Lysozyme was determined by incubating 10 µL of supernatant (obtained from tissue) with 1 mL of M. lysodeikticus (20 mg/100 mL) suspended in acetate buffer (0.02 M; pH 5.5) for 60 min at room temperature. After adding the sample, the initial absorbance at 450 nm was noted immediately, and final absorbance was taken after 60 min of incubation. The standard curve was prepared by using hen-egg lysozyme.

Nitric oxide synthase was measured by using the method of Lee et al. (2003). A 100-µL quantity of supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethenediamine in 5% phosphoric acid) and was incubated at room temperature for 10 min. The absorbance was recorded at 540 nm using a microplate reader. The nitrite concentration was determined from the nitrite standard curve and was expressed as moles per milligram of tissue.

Statistical analysis.—Data are reported here as mean ± SE. One-way ANOVA and Duncan’s multiple range test (Montgomery 1984) were used to determine the differences among various treatments. The cumulative mortality rate of rohu was compared by using the nonparametric log-rank significance test (Mantel 1966). Statistical significance was tested at α levels of 0.10 and 0.05.

RESULTS AND DISCUSSION

In the D4 (control) group, the first mortality was observed within 12 h of exposure to A. hydrophila, whereas in groups that were given the A. aspera seed-supplemented diets (D1–D3), the first mortality was recorded at 24 h postexposure (Figure 1). In the D4 group, 45% of fish died within 48 h of exposure to the bacterial pathogen. Among the fish that received A. aspera supplemented diets, mortality within 48 h of exposure was 25% in the D1 group, 20% in the D2 group, and 5% in the D3 group. In the D4 group, 50% of the fish died within 72 h postexposure, but no mortality was observed thereafter. Rohu larvae in the D3 group exhibited 5–10% mortality between 72 and 84 h postexposure; this indicated enhanced resistance of the D3 larvae to bacterial infection in comparison with the other groups.

After 7 d postchallenge, the total cumulative mortality was 50% in the D4 group, 40% in the D1 group, 35% in the D2 group, and 15% in the D3 group. An inverse relationship was found between the level of A. aspera seed supplementation in the diet and the rohu larval mortality rate. The log-rank significance test was performed to compare the survival distributions among the different treatment groups (Table 2). Results indicated a significant difference in survival distribution between the D3 and D4 groups at the $P < 0.05$ level, while significant differences were observed at the $P < 0.10$ level for the comparison of D2 versus D4 and the comparison of D1 versus D3. Supplementation with A. aspera seeds at 0.50% (i.e., D3 treatment) reduced larval mortality by 35% in comparison with larvae that received the control diet (D4). All dead fish exhibited the symptoms of infection, such as hemorrhage on the skin and a swollen abdomen. Similar results were obtained by Rao et al. (2006) after subjecting rohu fingerlings to A. hydrophila challenge. In the present study, the effect of A. aspera seeds was evaluated for the early developmental stage of rohu. Sahu et al. (2007) reported that feeding mango Mangifera indica kernel to rohu fingerlings

<table>
<thead>
<tr>
<th>Comparison</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 versus D4</td>
<td>1.3</td>
<td>0.263</td>
</tr>
<tr>
<td>D2 versus D4</td>
<td>3.1</td>
<td>0.079*</td>
</tr>
<tr>
<td>D3 versus D4</td>
<td>6.3</td>
<td>0.012**</td>
</tr>
<tr>
<td>D1 versus D2</td>
<td>0.4</td>
<td>0.534</td>
</tr>
<tr>
<td>D1 versus D3</td>
<td>3.6</td>
<td>0.056*</td>
</tr>
<tr>
<td>D2 versus D3</td>
<td>2.5</td>
<td>0.117</td>
</tr>
</tbody>
</table>
TABLE 3. Effect of dietary supplementation with *Achyranthes aspera* seeds on average weight and total tissue protein content (mean ± SE) of rohu larvae that were challenged with *Aeromonas hydrophila* (D1–D3 = test diets; D4 = control diet; supplementation rate is given in parentheses). Within a row, means (*n* = 3) with different letters are significantly different (*P* < 0.05).

<table>
<thead>
<tr>
<th>Variable</th>
<th>D1 (0.10%)</th>
<th>D2 (0.25%)</th>
<th>D3 (0.50%)</th>
<th>D4 (0%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average weight (mg)</td>
<td>132 ± 0.02 x</td>
<td>142 ± 0.01 y</td>
<td>159 ± 0.03 z</td>
<td>111 ± 0.02 w</td>
</tr>
<tr>
<td>Total protein (mg/mg of tissue)</td>
<td>0.096 ± 0 y</td>
<td>0.121 ± 0 z</td>
<td>0.126 ± 0.001 z</td>
<td>0.095 ± 0 y</td>
</tr>
</tbody>
</table>

at a rate of 5 g/kg of diet stimulated immunity and made the fish more resistant to *A. hydrophila* infection.

Final average body weight was significantly higher (*P* < 0.05) in rohu that received D3 than in the other treatment groups. Total tissue protein level was higher in the D2 and D3 groups than in the other two groups (Table 3). Supplementation with *A. aspera* seeds enhanced the nutritional value of the diet. Fatty acids, a number of oleanolic acid and bisdesmosidic triterpenoid saponins, ecysterone, and various amino acids have been reported to occur in *A. aspera* seeds (Hariharan and Rangaswamy 1970). Ecysterone reportedly increases protein synthesis in skeletal muscle (Goerlich-Feldmann et al. 2008). The presence of a higher level of tissue protein may have influenced the survival rate of rohu larvae that received the supplemented diets.

Glutamic oxaloacetic transaminase is usually found in a variety of tissues (e.g., liver, muscle, kidney, etc.), and GPT is normally found in liver. Elevated levels of GOT and GPT indicated the degree of tissue damage in the rohu larvae. The GOT level was significantly higher (*P* < 0.05) in the D4 larvae than in the other groups. The GPT level was highest in the D4 group, but there was no significant difference (*P* > 0.05) between the D4 and D1 groups (Figure 2). Infection of rohu larvae with *A. hydrophila* caused a stressful condition, whereas the seed-supplemented diet reduced the degree of cell damage.

The level of TBA reactive substance was significantly lower (*P* < 0.05) in the D3 group than in the other groups (Figure 3). The next-lowest level was observed in the D2 group. Lipid peroxidation is a well-established mechanism of oxidative damage caused by reactive oxygen species such as superoxide (O$_2^−$), OH, and hydrogen peroxide (H$_2$O$_2$), and the measurement of MDA provides a convenient index of lipid peroxidation (Devasena et al. 2001). Lipid peroxidation may bring about protein damage by its end products, MDA and 4-hydroxynonenal (Bhor et al. 2004). Fish tissue contains large quantities of polyunsaturated fatty acids that are essential for membrane function (Martinez-Alvarez et al. 2005). Lipid peroxidation is the process of oxidative degradation of polyunsaturated fatty acid, and its occurrence in biological membranes impairs membrane function and structural integrity and causes the inactivation of several membrane-bound enzymes (Goel et al. 2005). In the present study, the dietary dose of *A. aspera* seeds showed an inverse relationship with the degree of lipid peroxidation.

Lysozyme and nitric oxide synthase levels were significantly higher (*P* < 0.05) in the D3 group than in the other diet groups (Figure 4). Lysozyme is a cationic enzyme that attacks the β-1,4-glycosidic bond between N-acetylmuramic acid and N-acetylg glucosamine in the peptidoglycan of bacterial cell walls. This enables lysozyme to lyse certain Gram-positive bacteria and, in conjunction with complement, even some Gram-negative bacteria (Alexander et al. 1992). Fish lysozymes possess a high potential for bactericidal or bacteriolytic activity and play an
important role in the biodefense system against Gram-positive and Gram-negative bacteria (Saurabh and Sahoo 2008). Nitric oxide synthases are a family of enzymes that catalyze the production of the cellular-signaling molecule, nitric oxide, which plays a vital role in many biological processes. The inducible isoform of nitric oxide synthase produces a large amount of nitric oxide as a defense mechanism. The young fish use innate mechanisms during the first weeks or months of their development, and this may find application for the defense of farmed fish against pathogens at an early age (Rombout et al. 2005).

In conclusion, we found that dietary supplementation with A. aspera seed enhanced the growth of rohu, provided protection against oxidative stress, and prevented tissue damage; it also enhanced the resistance of larvae against the pathogen A. hydrophila. The A. aspera seeds improved the overall health status of the fish. Incorporation of A. aspera seeds at 0.50% proved effective for this early developmental stage of rohu. Achyranthes aspera has immense potential for supporting the production of healthy fish.

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ARTICLE

Effects of Garlic and Ginger Oils on Hematological and Biochemical Variables of Sea Bass *Dicentrarchus labrax*

Sevdan Yılmaz* and Sebahattin Ergün
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Abstract
This study was conducted to investigate the effects of garlic and ginger oils on hematological and biochemical health characteristics of sea bass *Dicentrarchus labrax*. Fish were exposed to garlic oil (0.01 or 0.02 mL/L), ginger oil (0.01 or 0.02 mL/L), or a combination of the two oils (each oil at a concentration of 0.005 or 0.01 mL/L) for 96 h via bath immersion. Results showed that the red blood cell count, hematocrit (%), hemoglobin (Hb) concentration (g/dL), mean corpuscular volume (µm³), mean corpuscular Hb (pg), and mean corpuscular Hb concentration (%) were not significantly affected by herb oil exposure. However, some changes in biochemical variables were observed. Sea bass exposed to the 0.005-mL/L garlic oil–ginger oil mixture exhibited a significant increase in serum glucose. Serum total protein and albumin levels decreased in sea bass that were exposed to a garlic oil–ginger oil mixture (0.005 or 0.01 mL/L) or to garlic oil at 0.02 mL/L. Serum globulin levels decreased and triglyceride levels increased in sea bass exposed to 0.02-mL/L garlic oil or to the 0.01-mL/L mixture. The serum lipase level decreased and the cholesterol level increased in fish that were exposed to 0.02-mL/L garlic oil. In summary, ginger oil at 0.01–0.02 mL/L can be used without negative effects, while the garlic oil or garlic oil–ginger oil mixture should be applied at a concentration below 0.005 mL/L for bath immersion of sea bass. This is the first study to examine how garlic oil and ginger oil exposure via bath immersion affects the hematological and biochemical status of sea bass.

The sea bass *Dicentrarchus labrax* is one of the most economically important fishes cultured in the Mediterranean countries. Intensive culture has been introduced in order to support worldwide demand for fish. Many aspects of fish farming activities, such as improper handling, harsh grading procedures, high stocking density, and poor water quality, can cause stress in fish, making them more prone to diseases. Various antibiotics, vaccines, immunostimulants, probiotics, and chemotherapeutants are applied in fish farming to prevent disease and stress. However, antibiotics and chemicals are not environmentally sustainable, as they produce undesirable side effects and residues. As a result, some medicinal plants containing antibacterial, antifungal, antiviral, and antiparasitic properties are being investigated as alternatives to hazardous synthetic products in fish farming. These herbs are able to improve growth, stimulate feed consumption, enhance immune responses and disease resistance, and prevent stress in fish (Citarasu 2010).

There is now a growing interest in the application of herbs as immunostimulants in fish culture. Garlic is an herb found virtually throughout the world, and it has been used to treat rheumatoid arthritis, the common cold, diabetes, malaria, and tuberculosis for over 4,000 years (Sasaki 2006). Moreover, garlic can generate antioxidant, antimicrobial, antifungal, antiviral, and antiparasitic effects and is able to boost the immune system (Harris et al. 2001). Ginger is another herb that has been used as nourishment and medicine; it contains antiemetic, anticancer, antiplatelet, antimicrobial, antiparasitic, antioxidant, anti-inflammatory, analgesic, hepatoprotective, and immunestimulating properties (Duke et al. 2002).

Although medicinal plants are natural alternatives to chemicals, side effects are sometimes associated with the use of medicinal plants. Such adverse effects are possibly related to toxic constituents, excessive doses, and allergic reactions (Bandaranayake 2006). Similarly, therapeutic synthetic chemicals,
such as copper sulfate, chloramine, potassium permanganate, formalin, acetic acid, and hydrogen peroxide, have been used for fish disease treatment, but high concentrations can result in toxic effects (Straus and Tucker 1993; Altinok 2004; Straus 2008; Taylor and Glenn 2008).

Hematological and biochemical variables are among the most significant physiological indicators of fish health, stress, and welfare (Blaxhall and Daisley 1973; Campbell 2004). Previous studies have shown that starvation (Echevarria et al. 1997), seasonal changes (Kavadias et al. 2003), an improper culture environment (Coz–Rakovac et al. 2005), high stocking density, confinement, and harvest (Vazzana et al. 2002; Roncarati et al. 2006; Di Marco et al. 2008) can negatively affect fish hematological and biochemical characteristics. Synthetic chemicals, such as hydrogen peroxide, formalin, trimethoprim, and sulfamethoxazole, have also been observed to cause alterations in the hematological and biochemical characteristics of sea bass (Roque et al. 2010; Yildiz and Ergunol 2010; Yildiz and Altunay 2011). In contrast, medicinal herbs will generally not affect health variables if the proper dosage is applied (Bandaranayake 2006). Therefore, medicinal herbs have a high potential for application in the aquaculture industry. Various studies have reported that ginger and garlic can be used as alternative components in the diets of fish and other animals to attain growth improvement and immunological enhancement (Cololini et al. 1998; Dügenci et al. 2003; Aly et al. 2008; Diab et al. 2008; Hashemi and Davoodi 2011). However, no previous studies have evaluated a bath immersion application of garlic oil and ginger oil in terms of its effects on hematological and biochemical blood variables of sea bass. Thus, the objective of the present study was to determine the effects of garlic oil, ginger oil, and garlic oil–ginger oil mixtures on hematological and biochemical characteristics in sea bass that were exposed to these herbs via bath immersion.

**METHODS**

**Experimental set up and design.**—Garlic oil and ginger oil (extracted by steam distillation) were obtained from a local market (Harman Business). Healthy sea bass (mean weight \(25.70 \pm 2.05\) g) were obtained from a local fish farm (Ida Gıda) in Çanakkale, Turkey. The experiment was conducted in triplicate for each treatment group. Two-hundred-ten fish were randomly allocated to 140-L, recirculating, cylindroconical fiberglass tanks (10 fish/tank) and were allowed to acclimate for 15 d. The fish were fed a commercial diet twice per day during the acclimation period. The aeration of tanks was facilitated with an air pump. During the 96-h bath immersion treatment, feed was withheld from the fish. The exposure concentrations were determined with a previously characterized assay (data of S. Yılmaz and S. Ergün [paper read at the International Veterinary Medicine Students Scientific Research Congress, 2010]). The experiment involved seven exposure treatments: (1) control (C; no herb oil exposure); (2) a mixture of garlic oil at a concentration of 0.005 mL/L and ginger oil at 0.005 mL/L (M5 treatment); (3) a mixture of garlic oil at 0.01 mL/L and ginger oil at 0.01 mL/L (M1 treatment); (4) garlic oil at 0.01 mL/L (G1 treatment); (5) garlic oil at 0.02 mL/L (G2 treatment); (6) ginger oil at 0.01 mL/L (GR1 treatment); and (7) ginger oil at 0.02 mL/L (GR2 treatment).

During the experiment, water quality characteristics (mean ± SE) were as follows: temperature was 23 \(\pm\) 1°C, dissolved oxygen concentration was 7.4 \(\pm\) 0.1 mg/L, pH was 8.5 \(\pm\) 0.1, salinity was 28.2 \(\pm\) 0.2‰, and conductivity was 45.2 \(\pm\) 0.4 mS/cm. The experiments were performed in accordance with the fish research guidelines from the animal ethics committees at Çanakkale Onsekiz Mart University, Çanakkale.

**Blood collection.**—At the end of the therapeutic bath immersion, 5 fish from each tank were anaesthetized with clove oil at 20 mg/L (Mylonas et al. 2005). Blood samples from all fish (15 fish/group) were collected from the caudal vein by using a syringe. The blood samples were added to tubes containing EDTA (BD Microtainer, UK). Blood samples were then taken for hematological analysis. Blood serum was separated by centrifugation (4,000 \(\times\) g for 10 min) in plastic biochemistry tubes (Kima-vactest, Italy) and was stored at \(-20^\circ\)C until use in biochemical analysis (Bricknell et al. 1999).

**Hematological analysis.**—Red blood cell (RBC) count (\(\times\) 10\(^6\) per mm\(^3\)), hematocrit (Hct;%), and hemoglobin (Hb) concentration (g/dL) were determined by using the method of Blaxhall and Daisley (1973). The RBC count was obtained with a Thoma hemocytometer using Dacie’s diluting fluid. The Hct value was determined using a capillary Hct tube. The Hb concentration was determined by spectrophotometry (540 nm) via the cyanmethemoglobin method. Mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC) were calculated with the following formulae (Bain et al. 2006):

\[
\text{MCV} (\mu m^3) = \frac{\left[(\text{Hct, %}) \times 10\right]}{(\text{RBC, } \times 10^6 \text{ per mm}^3)}
\]

\[
\text{MCH} (pg) = \frac{\left[(\text{Hb, g/dL}) \times 10\right]}{(\text{RBC, } \times 10^6 \text{ per mm}^3)}
\]

and

\[
\text{MCHC} (\%) = \frac{\left[(\text{Hb, g/dL}) \times 100\right]}{(\text{Hct, %})}
\]

**Biochemical analysis.**—Biochemical indices in serum, including glucose (GLU), total protein (TP), albumin (ALB), globulin (GLO), bilirubin (BIL), creatinine (CRE), blood urea nitrogen (BUN), uric acid (UA), lipase (LIP), triglyceride (TRI), and cholesterol (COL), were determined using bioanalytic test kits (Bioanalytic Diagnostic Industry Co.) and were measured with a Shimadzu spectrophotometer (PG Instruments, UK).

**Statistical analyses.**—Values of all measured variables are expressed as mean ± SE. Statistical significance was determined by one-way ANOVA; when differences between treatments were found, Tukey’s test (in SPSS version 17.0) was used to compare means. Differences were considered significant at \(P\)-values less than 0.05.
TABLE 1. Hematological variables (mean ± SE; n = 15 fish/group) in sea bass that were exposed (via bath immersion) to different concentrations of ginger oil and garlic oil (C = control, no herb oil exposure; M5 = a mixture of garlic oil at a concentration of 0.005 mL/L and ginger oil at 0.005 mL/L; M1 = a mixture of garlic oil at 0.01 mL/L and ginger oil at 0.01 mL/L; G1 = garlic oil at 0.01 mL/L; G2 = garlic oil at 0.02 mL/L; GR1 = ginger oil at 0.01 mL/L; GR2 = ginger oil at 0.02 mL/L). Variables are red blood cell count (RBC), hematocrit (Hct), hemoglobin (Hb) concentration, mean corpuscular volume (MCV), mean corpuscular Hb (MCH), and mean corpuscular Hb concentration (MCHC). None of the hematological variables differed between treatment groups (P > 0.05).

<table>
<thead>
<tr>
<th>Variable</th>
<th>C</th>
<th>M5</th>
<th>M1</th>
<th>G1</th>
<th>G2</th>
<th>GR1</th>
<th>GR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (× 10^6 per mm³)</td>
<td>4.50 ± 0.12</td>
<td>4.70 ± 0.30</td>
<td>4.62 ± 0.21</td>
<td>4.59 ± 0.19</td>
<td>4.51 ± 0.14</td>
<td>4.50 ± 0.09</td>
<td>4.52 ± 0.10</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>55.10 ± 0.20</td>
<td>58.02 ± 0.29</td>
<td>56.30 ± 1.60</td>
<td>55.15 ± 1.91</td>
<td>55.10 ± 1.66</td>
<td>52.10 ± 1.00</td>
<td>51.20 ± 1.90</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>10.02 ± 0.05</td>
<td>10.40 ± 0.10</td>
<td>10.11 ± 0.31</td>
<td>10.00 ± 0.23</td>
<td>10.01 ± 0.13</td>
<td>9.55 ± 0.26</td>
<td>9.80 ± 0.12</td>
</tr>
<tr>
<td>MCV (µm³)</td>
<td>122.44 ± 4.10</td>
<td>123.43 ± 2.71</td>
<td>121.86 ± 2.01</td>
<td>120.15 ± 2.09</td>
<td>122.17 ± 2.50</td>
<td>115.78 ± 1.32</td>
<td>113.27 ± 2.09</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>22.27 ± 0.09</td>
<td>22.13 ± 0.12</td>
<td>21.88 ± 0.15</td>
<td>21.79 ± 0.19</td>
<td>22.19 ± 0.10</td>
<td>21.22 ± 0.17</td>
<td>21.68 ± 0.16</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>18.18 ± 0.21</td>
<td>17.41 ± 0.27</td>
<td>17.96 ± 0.25</td>
<td>18.13 ± 0.50</td>
<td>18.17 ± 0.25</td>
<td>18.33 ± 0.61</td>
<td>19.14 ± 0.30</td>
</tr>
</tbody>
</table>

RESULTS

Hematological Variables

The effects of garlic oil, ginger oil, and garlic oil–ginger oil mixture exposures on sea bass hematological variables are presented in Table 1. The RBC count (P = 0.850), Hb concentration (P = 0.269), Hct (P = 0.705), MCV (P = 0.965), MCH (P = 0.169), and MCHC (P = 0.309) in the treatment groups did not vary significantly from the values observed for the control group.

Biochemical Variables

Relative to the value for the control group, serum GLU (P = 0.004) was significantly higher in sea bass that were exposed to the M1 and G2 treatments (Table 2). On the other hand, the M5, G1, GR1, and GR2 treatment groups showed lower GLU levels than the control group.

Serum TP (P = 0.011) and ALB (P = 0.005) levels in fish that were exposed to GR1 and GR2 were significantly higher than the control values, whereas these two variables were lower in the M5, M1, and G2 treatment groups than in the control group. Relative to the control, serum GLO (P = 0.019) levels were higher in sea bass from the GR1 and GR2 groups but were lower in fish from the G2 and M1 groups. The BIL (P = 0.200), CRE (P = 0.679), BUN (P = 0.401), and UA (P = 0.901) levels in sea bass were not significantly affected by exposure to garlic oil and ginger oil. Serum LIP (P = 0.002) levels were significantly higher in sea bass that were exposed to the control, M5, M1, G1, GR1, and GR2 were higher than the LIP level observed for the control, and LIP in the G2 group was lower than that in the control group. The TRI (P = 0.003) levels were significantly higher in fish that were exposed to the M1 and G2 and were significantly lower in fish that were exposed to the M5, G1, GR1, and GR2. The COL (P = 0.005) levels in the M5, G1, GR1, and GR2 treatment groups were lower than the control level, whereas COL in the G2 treatment group was higher than that in the control group.

DISCUSSION

Hematological variables have often been suggested as useful indicators of stress or disease in fish (Campbell 2004; Harikrishnan et al. 2011). Changes in RBC count, Hct value, Hb value, and erythrocyte indexes are important for detecting the health status of organs (Baustua 2005). In this study, garlic oil and ginger oil did not produce any undesired effects on hematological characteristics.

TABLE 2. Changes in biochemical variables (mean ± SE; n = 15 fish/group) in sea bass that were exposed to different concentrations of ginger oil and garlic oil (see Table 1 for definition of treatment codes). Variables are glucose (GLU), total protein (TP), albumin (ALB), globulin (GLO), bilirubin (BIL), creatinine (CRE), blood urea nitrogen (BUN), uric acid (UA), lipase (LIP), triglyceride (TRI), and cholesterol (COL). Within a row, means with differing letters are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Variable</th>
<th>C</th>
<th>M5</th>
<th>M1</th>
<th>G1</th>
<th>G2</th>
<th>GR1</th>
<th>GR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLU (mg/dL)</td>
<td>121.10 ± 1.12 y</td>
<td>100.05 ± 1.06 x</td>
<td>131.10 ± 1.12 z</td>
<td>110.03 ± 3.15 x</td>
<td>132.10 ± 1.30 z</td>
<td>98.02 ± 0.56 w</td>
<td>86.10 ± 1.00 v</td>
</tr>
<tr>
<td>TP (g/dL)</td>
<td>6.45 ± 0.04 x</td>
<td>6.10 ± 0.03 w</td>
<td>5.85 ± 0.04 v</td>
<td>6.33 ± 0.05 x</td>
<td>5.70 ± 0.01 u</td>
<td>6.85 ± 0.02 y</td>
<td>7.20 ± 0.03 z</td>
</tr>
<tr>
<td>ALB (g/dL)</td>
<td>1.40 ± 0.01 x</td>
<td>1.24 ± 0.03 w</td>
<td>1.09 ± 0.02 u</td>
<td>1.46 ± 0.02 x</td>
<td>1.11 ± 0.02 v</td>
<td>1.54 ± 0.03 y</td>
<td>1.76 ± 0.02 z</td>
</tr>
<tr>
<td>GLO (g/dL)</td>
<td>5.05 ± 0.05 x</td>
<td>4.86 ± 0.03 x</td>
<td>4.76 ± 0.01 w</td>
<td>4.87 ± 0.04 x</td>
<td>4.59 ± 0.01 w</td>
<td>5.31 ± 0.01 y</td>
<td>5.44 ± 0.02 z</td>
</tr>
<tr>
<td>BIL (g/dL)</td>
<td>0.13 ± 0.02 z</td>
<td>0.11 ± 0.03 z</td>
<td>0.14 ± 0.05 z</td>
<td>0.11 ± 0.02 z</td>
<td>0.12 ± 0.06 z</td>
<td>0.12 ± 0.07 z</td>
<td>0.14 ± 0.04 z</td>
</tr>
<tr>
<td>CRE (mg/dL)</td>
<td>0.14 ± 0.03 z</td>
<td>0.12 ± 0.05 z</td>
<td>0.14 ± 0.02 z</td>
<td>0.11 ± 0.06 z</td>
<td>0.12 ± 0.07 z</td>
<td>0.11 ± 0.03 z</td>
<td>0.10 ± 0.08 z</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>9.05 ± 0.05 x</td>
<td>10.10 ± 0.12 z</td>
<td>9.50 ± 0.21 z</td>
<td>9.34 ± 0.17 z</td>
<td>10.05 ± 0.11 z</td>
<td>9.90 ± 0.13 z</td>
<td>9.43 ± 0.18 z</td>
</tr>
<tr>
<td>UA (mg/dL)</td>
<td>1.90 ± 0.02 z</td>
<td>1.90 ± 0.01 z</td>
<td>1.90 ± 0.01 z</td>
<td>1.90 ± 0.06 z</td>
<td>1.90 ± 0.01 z</td>
<td>1.90 ± 0.07 z</td>
<td>1.90 ± 0.07 z</td>
</tr>
<tr>
<td>LIP (U/L)</td>
<td>13.10 ± 0.51 v</td>
<td>21.20 ± 0.54 x</td>
<td>19.91 ± 0.10 w</td>
<td>27.91 ± 0.20 y</td>
<td>9.14 ± 0.09 u</td>
<td>28.10 ± 0.19 y</td>
<td>33.21 ± 0.16 z</td>
</tr>
<tr>
<td>TRI (mg/dL)</td>
<td>320.00 ± 3.10 x</td>
<td>290.04 ± 1.25 u</td>
<td>345.10 ± 2.35 y</td>
<td>296.11 ± 2.01 u</td>
<td>391.14 ± 2.32 z</td>
<td>333.03 ± 1.32 w</td>
<td>300.01 ± 2.10 v</td>
</tr>
<tr>
<td>COL (mg/dL)</td>
<td>360.01 ± 1.10 y</td>
<td>321.12 ± 1.19 u</td>
<td>360.52 ± 1.31 y</td>
<td>347.25 ± 1.07 x</td>
<td>386.12 ± 1.01 z</td>
<td>344.04 ± 1.40 w</td>
<td>331.30 ± 1.39 v</td>
</tr>
</tbody>
</table>
An increase in GLU is one of the well-known stress indicators in fish (Morgan and Iwama 1997). The present study demonstrated that GLU level decreased with an increasing dosage of ginger oil. In addition, GLU decreased when garlic oil and the garlic oil–ginger oil mixture were used at a low concentration. Previous studies have also observed that garlic and ginger were associated with a reduction in serum GLU in fish (Shalaby et al. 2006; Sahu et al. 2007; Immanuel et al. 2009; Metwally 2009a). In contrast, our study indicated that blood GLU concentration was not reduced by exposure to a high dose of either garlic oil or the garlic oil–ginger oil mixture; these results suggest that garlic oil exposure is not useful for providing stress relief to sea bass. Serum TP is an important nonspecific immune variable (Magnadottir 2006). Its concentration in fish is less stable than that in mammals, and stress can cause a reduction in the plasma TP for a few days (Satchell 1991). For this reason, an increase in TP level will enable the fish to be stronger and more tolerant of stressful conditions (Satchell 1991). Although the garlic oil–ginger oil mixtures negatively affected TP in sea bass during the present study, fish serum TP increased with an increase in the dose of garlic oil. Similarly, the addition of garlic extracts to fish feed resulted in an increased TP level in fish plasma (Düngenci et al. 2003; Immanuel et al. 2009). Garlic oil exposure at 0.01 mL/L (i.e., G1 treatment) did not affect TP, but the 0.02-mL/L exposure (G2 treatment) produced a decrease in TP relative to the control value. When added to fish feed, the fresh, oil, and powdered forms of garlic increased the TP in the blood of Nile tilapia Oreochromis niloticus, even at a low level (0.25%) of garlic oil (Metwally 2009a). In a previous study (Shalaby et al. 2006), the highest TP level was detected in the blood of Nile tilapia when garlic powder was added to the feed at 10 g/kg; however, as the garlic dose in the feed increased, the TP levels gradually decreased.

The function of the proteins that bind the sex steroids and transport them throughout the body via the bloodstream is very crucial. These proteins include ALB and sex-hormone-binding GLO, which are the major plasma proteins in fish (Gunter et al. 1961). Fish plasma containing ALB was verified by electrophoretic mobility, fatty-acid-binding features, and molecular mass (Davidson et al. 1988). However, some xenobiotics may cause adverse impacts on hormone transport by interfering with the binding of native hormone to high-affinity binding α-globulins, such as sex-hormone-binding GLO (Janz and Weber 2000). Transferrin is another serum protein that is responsible for the transport of iron (Fange 1986). In our study, exposure to ginger oil increased the serum ALB and GLO levels in sea bass. Similarly, ginger extract was shown to increase the ALB and GLO levels in Mozambique tilapia Oreochromis mossambicus (Immanuel et al. 2009). Sahu et al. (2007) reported that the ALB and GLO values in fish treated with different immunostimulants were always greater than the control values. In contrast to our observations for ginger oil exposure, the ALB and GLO levels increased in sea bass that were exposed to the garlic oil–ginger oil mixture or to the higher garlic oil concentration.

Bilirubin, a dominant bile pigment, results from the breakup of the Hb of dead RBCs (Cornelius 1991). An increase of BIL in the blood may arise from the failure of bile secretion, excessive Hb destruction, or the liver’s inability to process the Hb in an active way (Schwartz 1997). In general, high BIL levels are caused by problems with the RBCs, the liver, or bile ducts. In this study, serum BIL levels in sea bass were not affected by exposure to herb oils; this result implies that there were no abnormalities in liver functions after the herb oils were applied. Previous studies have demonstrated that garlic and ginger generate a healing effect in unhealthy animals. For example, in diabetic rats that exhibited an increased BIL level, the administration of garlic juice was able to reduce the BIL to a normal state (El-Deer et al. 2005). Other studies reported that serum BIL was significantly increased in rats that were exposed to mercury chloride and bromobenzene (Vitalis et al. 2007; El-Sharawy et al. 2009); in contrast, rats that were fed a diet supplemented with ginger at 10% had decreased BIL levels, and ginger also displayed a liver-protecting effect against these toxic substances. Moreover, ginger, which is well known as a medicinal plant, displays a healing effect on liver diseases (Treadway 1998).

In the present study, exposures to garlic oil and ginger oil did not cause changes in the CRE, BUN, or UA level in sea bass. Little is known about the physiological effects of CRE, BUN, and UA in fish; however, these variables have been used for gill and kidney disease diagnosis (Adams and Greeley 2000; Campbell 2004). Fish lipid mobilization is possibly facilitated by LIP activity (Sheridan 1989). Plasma LIP is mainly formed in the pancreas and plays an important role in the digestion of fat (Brix 2002). Wagner and Congleton (2004) reported a negative correlation between serum LIP and serum activities of TRI and COL in juvenile Chinook salmon Oncorhynchus tshawytscha; their results generally agree with those of the present experiment. Triglycerides, phospholipids, and COLs are major lipid classes, and plasma lipid levels are affected by diet types and stress levels (McDonald and Milligan 1992). In our study, TRI and COL levels in sea bass decreased with an increase in the exposure concentration of ginger oil. However, TRI and COL increased when garlic oil or the garlic oil–ginger oil mixture was applied at a high concentration. Garlic oil decreased the blood TRI and COL levels in Nile tilapia; this phenomenon was attributed to the fact that garlic could induce LIP to break up the TRIs (Metwally 2009a, 2009b). Ginger oil was also shown to decrease the TRI and COL levels in Mozambique tilapia (Immanuel et al. 2009), possibly in a manner similar to that described for garlic oil.

In conclusion, ginger oil at 0.01–0.02 mL/L can be applied to sea bass via bath immersion, whereas garlic oil or a garlic oil–ginger oil mixture should be applied at a concentration below 0.005 mL/L. This study is the first to examine the effects of garlic oil and ginger oil exposures via bath immersion on the hematological and biochemical status of sea bass. Further study
is needed to determine the optimal application frequency of these herb oils and to assess their impacts on infection with parasites or other diseases. The proper dosages of these herb oils did not cause adverse effects in sea bass, and our results can be applied to the treatment of diseases or the enhancement of immunity in fish.

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COMMUNICATION

Myxobolus neurotropus Infecting Rainbow Trout in Alaska, a New Geographic Record

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Abstract

The Alaska Department of Fish and Game fish pathology laboratory received a rainbow trout Oncorhynchus mykiss from the Alaska Peninsula that was suspected of having whirling disease based on the display of aberrant swimming behavior and a deformed spine. We tested for Myxobolus cerebralis using standard pepsin–trypsin digest and molecular procedures, which yielded negative results. However, many oval shaped myxospores were observed in brain smears and were confirmed to be those of the morphologically similar M. neurotropus based on a diagnostic assay using PCR. The known geographic distribution of this parasite includes Idaho, Washington, Utah, Oregon, California, and now, Alaska. Whether this species is an emerging parasite is not known because it was only described a few years ago. Given the severe infection found in this rainbow trout, perhaps the considerable displacement of neurological tissues and subsequent pressure on peripheral nerves could have contributed to the spinal curvature and accompanied abnormal swimming. Conversely, the M. neurotropus infection may have been incidental and the spinal deformity may have actually been due to one of several nonspecific developmental or congenital causes. Further studies on geographic distribution and impact on host fitness will probably determine the importance of this species to fish health.

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established in Alaska (Moles and Jensen 1999), has distinctly pyriform-shaped spores easily separated from the *Myxobolus* sp. in our study. The two species that are most similar in size and shape to the parasite from the current case are *M. neurobius* and *M. neurotropus*. The former is not considered to occur in salmonids from North America (McDonald and Margolis 1995), and the latter was recently described from a rainbow trout in Idaho (Hogge et al. 2008a).

The application of molecular techniques has improved the identification of morphologically similar *Myxobolus* species infecting salmonids (Andree et al. 1999; Ferguson et al. 2008; Hogge et al. 2008a, 2008b; Urawa et al. 2009, 2011). However, published diagnostic methods based on PCR are reported for only *M. cerebralis* and *M. neurotropus*. Therefore, in addition to standard diagnostic tests used in parasite identification such as light microscopy, site of infection, and host species, we used PCR to confirm both the absence of *M. cerebralis* and the presence of *M. neurotropus* in the rainbow trout submitted for diagnosis. This finding extends the known geographic range of *M. neurotropus* in rainbow trout.

**METHODS**

**Necropsy.—**This fish was caught from Margot Creek in the Katmai National Park (Figure 1). A limited necropsy was performed including direct fluorescent antibody testing of kidney tissues for *Renibacterium salmoninarum* (Short et al. 2009) and screening of head cartilage for *M. cerebralis* using pepsin–trypsin digest (PTD) as described below. Brain and other tissue squashes were stained with either malachite green or Diff-Quick (Siemens Healthcare Diagnostics, Newark, Delaware). Fish age was determined from scales in accordance to Minard and Dye (1998). The decapitated head and remaining carcass was radiographed with a Ducon-I Videx radiography unit (Summit Industries, Chicago, Illinois) and the image was evaluated for the presence of spinal deformity.

**Pepsin–trypsin digest.—**A triangular wedge of the head was taken posterior to the eye; the top portion measured 2 cm and encompassed all layers of the head and cranium. The wedge was split in half for defleshing and the remaining half was re-frozen at \(-80^\circ\mathrm{C}\). Cartilage from the defleshed wedge was chopped with a sterile razor blade and weighed. This

---

**FIGURE 1.** Geographic location of the rainbow trout from Margot Creek (box with asterisk) on the Alaska Peninsula that was submitted for diagnostic evaluation.
bone–cartilage material was digested by the PTD method used for frozen tissue described in MacConnell (2002). Seven pepsin digest slides were examined by wet mount for spores at 400× and found negative prior to proceeding with the trypsin digest (Hauck and Landin 2009). Ten slides containing material from the PTD pellet were examined for spores at 400× and 1,000× magnification; five of these were stained with malachite green and reexamined. An aliquot of the pellet was placed in formalin, and the remainder was frozen for PCR testing.

**Histology.**—A small section of the brain and surrounding tissue were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard histological methods.

**Spore enumeration and measurement.**—The spinal cord was measured from the decapitated fish, sectioned into four equal lengths, and dissected from the surrounding tissue. Each segment of spinal cord was weighed and placed into separate beakers with 0.25% trypsin solution for digestion at 37°C (Lom and Dykova 1992). The digested material was filtered and pelleted by centrifugation. Each pellet was resuspended in 5 mL of phosphate-buffered saline and spores were counted with a hemocytometer according to Peters (2009). The length and width of 20 spores, and polar capsules from eight spores, were measured using a Jenoptik digital camera and ProgRes CapturePro software (JENOPTIK Optical Systems, Easthampton, Massachusetts).

**Molecular analyses.**—Genomic DNA was obtained using the Qiagen DNeasy tissue kit rodent tail protocol (Qiagen, Valencia, California) from 25-mg samples of the PTD pellet, the defleshed material, and cartilage of the archived half wedge. Additionally, a small portion of the anterior spine was extracted after verifying microscopically the presence of myxospores. Quantitative PCR (qPCR) was performed on these samples for *M. cerebralis* using a Taqman assay (Kelley et al. 2004) with modifications of 4 µL extracted DNA and a 20-µL final reaction volume. Reactions were run in triplicate on a Bio-Rad iQ5 thermocycler with optical system software version 2.0 using the following amplification conditions: 50°C for 2 min, 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 60 s.

A species-specific diagnostic PCR assay for *M. neurotropus* was applied to the same DNA extracted samples using primers CH260L and CH260R in a 50-µL reaction volume with amplification conditions specified in Hogge et al. (2008a). Reaction products were visualized by electrophoresis on 2% agarose gel stained with 1% ethidium bromide.

**RESULTS**

**Necropsy**

Findings from the examination of the fish (46 cm fork length) included the following: a 9-cm superficial gash on the right lateral side; a cloudy left cornea; heavy infestation of copepods (*Salmincola* sp.) on the gills and at base of pelvic, pectoral, and anal fins; slight kyphosis in the area between head and dorsal fin, as well as a dorsal ventral compression between the dorsal and caudal fin (Figure 2A); no gross abnormalities of internal organs; no abnormal spleen squash; a spent egg skein; free and encysted nematodes on stomach, gut, air bladder, and egg skein. Kidney tissue was negative for *R. salmoninarum* as determined by direct fluorescent antibody testing. Fish age was estimated to be approximately 7 years. Radiographs indicated no obvious inflammation or ankylosing of vertebrae, but kyphosis of the vertebral column occurred (Figure 2B).

Brain and spinal cord smears demonstrated few to many myxospores that were round to oval in shape (Figure 3A). Previously frozen spores had an average measurement of approximately 11 × 10 µm (range = 10–14 × 9–13 µm; Table 1). Spores had ovoform polar capsules of nearly equal size (Figure 3B) of approximately 5 × 3 µm (range = 4–7 × 3–4 µm) containing filaments with 6–8 turns. Morphology corresponded best to that of *M. neurotropus* (Table 1). The spinal cord weighed approximately 1 g and had an estimated infection intensity of 746,000 spores, the majority of which resided in the anterior segments.

**Screening and Confirmatory Testing**

Evaluation of PTD wet mounts and stained slides using bright-field microscopy did not reveal any spores; however, histological sections of brain tissue stained with hematoxylin and eosin revealed multifocal areas containing aggregates of spores.

*M. neurotropus* DNA was detected by PCR in the spinal cord and defleshed material of the Margot Creek rainbow trout producing a 260 bp amplicon visualized by gel electrophoresis. All other extracted samples tested negative for the presence of DNA of this parasite. All samples tested by qPCR were negative for *M. cerebralis* DNA.

**DISCUSSION**

We documented that *M. neurotropus* occurs in rainbow trout from Alaska based on spore morphology, infection profile, and confirmatory molecular testing of an individual specimen. This expands the geographic range of this species and the diversity of neurotropic myxozoan parasites in this region. The parasite was recently described from a rainbow trout in Idaho, where it also infected cutthroat trout *O. clarkii*, bull trout *Salvelinus confluentus*, Chinook salmon *O. tshawytscha*, and sockeye salmon *O. nerka* (Hogge et al. 2008a). Rainbow trout from Washington and cutthroat trout from Utah have also tested PCR-positive for *M. neurotropus* (Hogge et al. 2008a). Earlier reports of a *Mxyobolus* sp. infecting brains of rainbow trout in California (Hedrick et al. 1991) and various salmonids in Oregon (Lorz et al. 1989) are consistent with *M. neurotropus* based on morphology and tissue tropism. It is unknown whether this species is an emerging parasite, because definitive identification using molecular methods was developed only a few years ago. *Mxyobolus arcticus* was recorded from extensive surveys of spawning sockeye salmon in streams and coastal lakes throughout Alaska, but *Mxyobolus* spp. were not detected in the 50
samples from Margot Creek (Moles and Jensen 1999). Our discovery of *M. neurotropus* in Alaska was a result of multi-faceted testing, where the pepsin–trypsin digest was negative for *M. cerebralis*, yet histological sections and stained brain smears contained spores resembling those of *M. cerebralis*. This finding led to molecular testing for both *M. cerebralis* and *M. neurotropus*.

Molecular characteristics, such as small subunit (SSU) rDNA sequence, have recently been used to differentiate morphologically similar neurotropic species in salmonids. Yet some species are composed of genetically identical strains that differ in spore morphology, pathogenicity, and target host species (Urawa et al. 2011). Therefore, a combination of genetic and phenotypic differences, such as tissue tropism or statistically different myxospore size, has been used to separate these species (Ferguson et al. 2008; Hogge et al. 2008a; Urawa et al. 2009). Furthermore, actinospore morphology may also be an important taxonomic characteristic (Urawa et al. 2011). In several states where
whirling disease has been established, *M. neurotropus* spores are often present during routine screening, causing concern for misdiagnosis of *M. cerebralis* (Hogge et al. 2004). This confusion led to the development of a diagnostic PCR to distinguish between these two morphologically similar *Myxobolus* species (Hogge 2008a). In Alaska whirling disease has not been detected nor any stage of the parasite visualized. However, Arsan et al. (2007) detected low levels of *M. cerebralis* DNA by qPCR in juvenile rainbow trout heads collected from the now decommissioned Elmendorf Hatchery. Although spores were not visualized, regular PCR and DNA sequencing was used to confirm the presence of DNA in samples. Consequently, the biological significance of these molecular data is unclear, since neither spores nor clinical disease were ever observed in the test-positive fish and a 1-year sentinel study of rainbow trout fry (ADFG 2008) exposed to the hatchery water supply were test-negative as determined by nested and qPCR. These

negative tests for *M. cerebralis* were considered evidence that the parasite had not become established at the site of initial detection of suspect DNA. Until unequivocal evidence determines otherwise, *M. cerebralis* remains an exotic pathogen for the state of Alaska. Nonetheless, the presence of morphologically similar spores of *M. neurotropus* was cause for concern, and diagnostic PCR became a necessary tool for parasite identification.

It is uncertain whether the extensive infection in our case was associated with the observed clinical signs. The whirling behavior and spinal deformity of this fish prompted the sample submission, yet there are many causes for these nonspecific clinical signs in fish. In general, spinal deformity may be due to genetic, infectious, traumatic, neoplastic, dietary, and environmental (e.g., pollution) factors (Grimmett et al. 2011). Hogge et al. (2008a, 2008b) used histopathology and found no host response to occur with *M. neurotropus*. Unfortunately, the fish in our study was frozen several times and tissues taken for histology were autolyzed, therefore limiting histological interpretation. However, we did find myxospores throughout the spinal cord and posterior brain, and verified the findings of Hogge and Johnson (2008) that more spores occurred in the anterior spine. Remarkably, the fish in our study had a high infection intensity of nearly 750,000 myxospores in the spinal cord alone, suggesting a well-established infection. Although the rainbow trout exhibited some degree of kyphosis, radiographs of the spine indicated no abnormalities of the vertebrae, despite spinal curvature. Neurological infections of myxozoans can result in deformities, altered swimming, or both, owing to damaged nerves and deterioration in muscle control (Egusa 1985; Langdon 1990; Lom et al. 1991; Longshaw et al. 2003). The mechanism of whirling behavior from *M. cerebralis* involves compression from damaged cartilage of the lower brain stem and spinal cord during initial stages of infection (Rose et al. 2000). This injury disturbs normal functions controlled by the central nervous system including muscle tone affecting swimming behavior and skin pigmentation of the posterior body.

Research on the pathology of chronic *M. neurotropus* infections may provide more definitive information on changes in neural tissues or motor function. The mature age of the fish from Margot Creek further supports the view that exposure to the parasite may have been long term. Continued documentation of the geographic distribution of *M. neurotropus* in Alaska and elsewhere, and its association with host fitness, would be valuable in determining prevalence and any relationship with clinical abnormalities. Additionally, areas that are positive for *M. neurotropus* may be important for future *M. cerebralis* screenings, because co-infections by both *Myxobolus* species have been documented in fish from several western states. Thus, understanding the environmental conditions that support the interactions of hosts and parasites in these systems may improve targeting of high risk sites for surveillance programs.
<table>
<thead>
<tr>
<th>Species</th>
<th>Host</th>
<th>Locality</th>
<th>Infection site</th>
<th>Shape</th>
<th>Spore Length/Width/Thickness</th>
<th>Polar capsule Length/Width/Number of coils</th>
<th>Material</th>
<th>Reference</th>
<th>GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. arcticus</em></td>
<td>Oncorhynchus nerka, O. kisutch, Salvelinus malma, S. neiva</td>
<td>Kamchatka, Russia</td>
<td>HB, SC</td>
<td>Pyriform</td>
<td>(14.3–16.5) (7.6–7.7) NR</td>
<td>(6.6–9.0) (2.5–3.5) NR</td>
<td>NR</td>
<td>Pugachev and Kholodkov (1979)</td>
<td>NA</td>
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<tr>
<td><em>M. arcticus</em></td>
<td>O. nerka</td>
<td>British Columbia</td>
<td>HB</td>
<td>Pyriform</td>
<td>(14.1–16.1) 9.7 (8.3–10.2) NR</td>
<td>NR</td>
<td>NR</td>
<td>Ferguson et al. (2008)</td>
<td>EU346378</td>
</tr>
<tr>
<td><em>M. cerebralis</em></td>
<td>O. mykiss</td>
<td>Germany</td>
<td>Head cartilage</td>
<td>Ovoid</td>
<td>9.0 (7.2) NR</td>
<td>4.5 NR</td>
<td>NR</td>
<td>Hofer (1903)</td>
<td>NA</td>
</tr>
<tr>
<td><em>M. cerebralis</em></td>
<td>O. mykiss</td>
<td>West Virginia</td>
<td>Head cartilage</td>
<td>Round-ovoid</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Andree et al. (1999)</td>
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<tr>
<td><em>M. farionis</em></td>
<td>Salmo trutta fario</td>
<td>Spain</td>
<td>Brain, SC</td>
<td>Oviform-pyriform</td>
<td>9.2 (6.0–7.5) 4.7 (4.5–5.0) NR</td>
<td>4.9 (2.4) 4.9 (4.5–5.5) (2–2.8) NR</td>
<td>Fresh</td>
<td>Gonzalez-Lanza and Alvarez-Pellitero (1984)</td>
<td>NA</td>
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<tr>
<td><em>M. fryeri</em></td>
<td>O. kisutch, O. mykiss, O. clarkii</td>
<td>Silet River, Oregon</td>
<td>Peripheral nerves</td>
<td>Ovoid-pyriform</td>
<td>12.9 (11.1–14.8) 8.6 (7.2–10.1) 7.2 (6.4–7.7)</td>
<td>6.9 (5.9–8.1) 2.8 (2.0–3.3) 8–10 Fresh</td>
<td>Ferguson et al. (2008)</td>
<td>EU346370</td>
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<tr>
<td><em>M. kisutchi</em></td>
<td>O. kisutch</td>
<td>Minter Creek, Washington</td>
<td>SC</td>
<td>Spherical-oval</td>
<td>(7–8.5) (6.5–7.0) (3.5–3.8)</td>
<td>(3.8–5.5) NR NR NR</td>
<td>Fixed</td>
<td>Yasutake and Wood (1957)</td>
<td>NA</td>
</tr>
<tr>
<td><em>M. kisutchi</em></td>
<td>O. kisutch</td>
<td>Minter Creek, Washington</td>
<td>Brain, SC, optic nerve</td>
<td>Oval</td>
<td>9.0 (8.2–10.1) 7.7 (7.0–8.5) 6.1 (5.4–6.7)</td>
<td>3.9 (3.4–4.2) NR 5–6 Fresh</td>
<td>Hoge et al. (2008b)</td>
<td>EF431919</td>
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<td><em>M. murakamii</em></td>
<td>O. masou ishikawae</td>
<td>Oze River, Japan</td>
<td>Lateral line nerves</td>
<td>Oval</td>
<td>11.3 (9.2–10.9) 7.1 (6.2–7.8)</td>
<td>5.1 (4.0–5.5) (2.4–3.9) 5–8 Fresh</td>
<td>Urawa et al. (2009)</td>
<td>AB469984</td>
<td></td>
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<td><em>M. neurobius</em></td>
<td>S. trutta, Thymallus thymallus</td>
<td>Germany</td>
<td>Nervous system</td>
<td>Ovoid</td>
<td>(10–12) 8.0 (6.0)</td>
<td>(6.0–7.0) NR</td>
<td>NR Fixed in section</td>
<td>NA</td>
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<tr>
<td><em>M. neurobius</em></td>
<td>S. trutta</td>
<td>Midtunelva River, Norway</td>
<td>SC</td>
<td>Spherical</td>
<td>9.2 (8.2–10.1) 7.2 (6.2–7.8) 5.6 (4.7–7.0)</td>
<td>4.8 (4.3–5.5) (2.2–2.5) 6–8 Fresh</td>
<td>Urawa et al. (2009)</td>
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<td><em>M. neurotropus</em></td>
<td>O. mykiss, O. clarkii, O. nerka, Salvelinus confluentus</td>
<td>Duncan Creek, Idaho</td>
<td>Brain, SC</td>
<td>Oval-circular</td>
<td>11.8 (11.2–13.5) 10.8 (10.4–12.3) 8.8 (8.4–9.1)</td>
<td>5.8 (5.0–6.9) 3.7 (2.4–3.9) 6–8 Fresh</td>
<td>Fresh</td>
<td>Hoge et al. (2008a)</td>
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<td><em>M. neurotropus</em></td>
<td>O. mykiss</td>
<td>Magot Creek, Alaska</td>
<td>Brain, SC</td>
<td>Oval-circular</td>
<td>10.9 (9.6–13.6) 10.4 (8.6–12.8) 8.2 (8.1–8.4)</td>
<td>5.2 (4.4–6.5) 3.5 (2.7–4.4) 6–8 Fresh</td>
<td>This study</td>
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COMMUNICATION

Spring Viremia of Carp Virus in Minnehaha Creek, Minnesota

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Abstract

Spring viremia of carp virus (SVCV) causes a highly contagious and serious disease of freshwater cyprinid fishes, generating significant economic and ecological impacts throughout the world. The SVCV is therefore listed as a notifiable pathogen by the International Organization for Animal Health. In June 2011, a significant mortality event of wild common carp Cyprinus carpio occurred in Minnehaha Creek near its confluence with Mississippi River Pool 2 in Minneapolis, Minnesota. Clinical signs of moribund fish included hemorrhagic lesions in the skin, eyes, and internal adipose tissue. The SVCV was isolated from pooled kidney and spleen of the fish. Rhabdovirus particles were seen upon examination of infected cell culture fluid by electron microscopy. The virus was confirmed to be SVCV subtype Ia by reverse transcription PCR and sequencing. This is the first report of SVCV within the state of Minnesota and the ninth documented case in North America.

The spring viremia of carp virus (SVCV) is a highly contagious and pathogenic rhabdovirus of freshwater fish. As the name of the virus suggests, the common carp Cyprinus carpio (including its ornamental variant, the koi) and other carp species act as the primary host, although other freshwater fishes are known to be naturally susceptible (Fijan 1999; Ahne et al. 2002). In addition, an SVCV-like virus has been reported from penaeid shrimp in Hawaii but not from finfishes there (Johnson et al. 1999). In carps, the disease often presents with petechial or focal hemorrhaging of the skin, muscles, visceral fat, and internal organs; degeneration of gill tissue; ascites of the peritoneal cavity, frequently containing blood; exophthalmia; and an inflamed intestine and vent (Fijan et al. 1971; Ahne and Wolf 1977; Negele 1977; Ahne et al. 2002). However, there are no pathognomonic lesions of this disease, and when mortality is acute, the clinical signs may be absent altogether.

Epizootic events in both wild and farmed fish have been reported from around the world, including Europe, the Middle East, South America, Asia, and North America (Alexandrino et al. 1998; Ahne et al. 2002; Goodwin 2002). In the United States, SVCV was first reported in 2002 from a koi farm in North Carolina (Goodwin 2002) and from common carp in Cedar Lake, Wisconsin (Dikkeboom et al. 2004). Seven subsequent outbreaks have occurred throughout the country over the last 9 years (Table 1). The SVCV has primarily been limited to temperate climates with cool temperatures of 15–20°C. During prolonged periods of warmer temperatures, the hosts effectively clear the virus; however, these fish do harbor antibodies after infection (Fijan et al. 1971; Fijan 1976; Dixon et al. 1994; Goodwin 2002). This report documents the first isolation of SVCV in Minnesota and represents the ninth documented case in North America.

METHODS

Case history.—On June 12, 2011, 200–300 dead or moribund common carp were observed in Minnehaha Creek (Hennepin County, Minnesota), which flows from Lake Minnetonka...
TABLE 1. Isolation of the spring viremia of carp virus in North America. In all cases, the virus was isolated in cell cultures and confirmed by reverse transcription PCR (USFWS = U.S. Fish and Wildlife Service).

<table>
<thead>
<tr>
<th>Year</th>
<th>State</th>
<th>Water body</th>
<th>Host species</th>
<th>GenBank accession number</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>North Carolina</td>
<td>Koi farm</td>
<td>Koi</td>
<td>DQ227501</td>
<td>First isolation in USA</td>
</tr>
<tr>
<td>2002</td>
<td>Wisconsin</td>
<td>Cedar Lake</td>
<td>Common carp</td>
<td>DQ227500</td>
<td>First isolation in wild fish</td>
</tr>
<tr>
<td>2003</td>
<td>Illinois</td>
<td>Calumet Sag Channel</td>
<td>Common carp</td>
<td>DQ227502</td>
<td>Apparently healthy fish</td>
</tr>
<tr>
<td>2004</td>
<td>Washington</td>
<td>Private pond</td>
<td>Koi</td>
<td>DQ227503</td>
<td>Pond was depopulated</td>
</tr>
<tr>
<td>2004</td>
<td>Missouri</td>
<td>Koi distribution center</td>
<td>Koi</td>
<td>DQ227504</td>
<td>Lot contained fish from Minnesota and Illinois; the sources tested negative.</td>
</tr>
<tr>
<td>2006</td>
<td>Ontario, Canada</td>
<td>Hamilton Harbor, Lake Ontario</td>
<td>Common carp</td>
<td>EF194065</td>
<td>Apparently healthy fish</td>
</tr>
<tr>
<td>2007</td>
<td>Wisconsin</td>
<td>Mississippi River, Pool 8</td>
<td>Common carp</td>
<td>JQ666282</td>
<td>Source: USFWS LaCrosse Fish Heath Center</td>
</tr>
<tr>
<td>2008</td>
<td>Ohio</td>
<td>Pleasant Hill Reservoir</td>
<td>Bluegill</td>
<td>JQ666284</td>
<td>Source: USFWS Lamar Fish Health Center</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Largemouth bass</td>
<td>JQ666283</td>
<td>Source: USFWS Lamar Fish Health Center</td>
</tr>
<tr>
<td>2011</td>
<td>Minnesota</td>
<td>Mississippi River, Pool 2</td>
<td>Common carp</td>
<td>JQ247697</td>
<td>Current study</td>
</tr>
</tbody>
</table>

through numerous water bodies and ultimately connects to Mississippi River Pool 2. The mortality event was isolated to a stretch from the Minnehaha Creek waterfall to the confluence with the Mississippi River, a distance of approximately 1,200 m. Two mature moribund fish were collected by hand on June 15, 2011, and were submitted to the Minnesota Department of Natural Resources (MDNR) Pathology Laboratory, St. Paul. Hemorrhagic lesions were observed in the skin, eyes, and internal adipose tissue of both fish (Figure 1). The necropsy, bacteria isolation, and initial virus isolation were performed at the MDNR Pathology Laboratory. Electron microscopy and molecular analysis were performed at the Minnesota Veterinary Diagnostic Laboratory (MVDL).

**Bacteriology.**—Aseptic swabs of the kidney and eye were cultured onto trypticase soy agar plates for bacterial isolation. The resulting cultures were identified using API 20E strips (bioMerieux, La Balme, France).

**Virology.**—Kidney and spleen were collected from each fish and were pooled for virus isolation using procedures recommended by the American Fisheries Society Fish Health Section (USFWS and AFS-FHS 2007). Briefly, the tissue was homogenized as a 1:10 (weight : volume) suspension in Hanks balanced salt solution (Cellgro, Manassas, Virginia). The homogenized sample was centrifuged at 2,360 × g for 15 min at 4°C. The supernatant (in 1-mL quantities) was transferred to another tube with an equal amount of an antibiotic incubation medium, was vortexed briefly to mix, was incubated for 24 h at 4°C, and was then re-centrifuged at 2,360 × g for 15 min. The supernatant was inoculated in the epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) followed by incubation at 25°C. The isolate was further confirmed by negative and positive contrast electron microscopy and reverse transcription (RT) PCR.

**Electron microscopy.**—For virus screening with negative contrast electron microscopy, infected EPC cells were placed in 1 mL of double-distilled water and then centrifuged at 2,900 × g for 10 min. The supernatant was re-centrifuged for 10 min at 210 kPa (30 psig) using an airfuge (Beckman Coulter, Brea, California). The supernatant from this centrifugation was discarded, and the pellet was re-constituted in 10 µL of double-distilled water. The suspension was placed on Formvar-coated copper grids and stained with 1% phosphotungstic acid (Electron Microscopy Sciences, Hatfield, Pennsylvania) for 1 min.

For cell ultrastructure, the infected EPC cells were fixed in 0.166-M cacodylate-buffered, 3% glutaraldehyde with 1% tannic acid solution (Electron Microscopy Sciences), followed by a second postfixation treatment in 1% osmium tetroxide (Electron Microscopy Sciences). Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate (Electron Microscopy Sciences). These sections were observed under a JEOL 1200 EX II transmission electron microscope (JEOL Ltd., Tokyo, Japan). Images were obtained using a Veleta 2k × 2k camera with iTEM software (Olympus SIS, Munster, Germany).

**Reverse transcription PCR and sequencing.**—The RT-PCR was performed according to the method suggested by Dixon (2009). Briefly, viral RNA was extracted from the infected cell culture supernatant by using the Qiagen Viral RNA Mini Kit...
FIGURE 1. Adult common carp infected with the spring viremia of carp virus: lateral (upper panel) and ventral (lower panel) ecchymosis, multifocal to coalescing, is apparent in the skin and fins. [Figure available in color online.]

A Qiagen One-Step RT-PCR Kit was used for amplification. Published primers (SVCV F1: 5′-TCTTGGAGCCAAATAGCCTCARRTC-3′; SVCV R2: 5′-AGATGGTATGGACCCCAATACATHACNCA-CAY-3′) were used to amplify a sequence of the SVCV glycoprotein gene (Stone et al. 2003). The RT-PCR thermal cycling protocol consisted of 30 min at 50°C; 15 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C; and 10 min at 72°C. The PCR product was visualized with ethidium bromide after 1% agarose gel electrophoresis.

The amplified PCR product was purified by using the Qiagen PCR Purification Kit and was sequenced at the Advanced Genomic Analysis Center, University of Minnesota. The sequencing was performed in both directions with the same forward and reverse primers used in the RT-PCR. The sequences obtained were aligned using Sequencher software (www.msi.umn.edu) followed by BLAST analysis (www.ncbi.nlm.nih.gov). The nucleotide sequence was compared with published sequences in GenBank by alignment with ClustalW in MEGA version 4.0 (Warg et al. 2007). The evolutionary distances were computed using the maximum composite likelihood model. A phylogenetic tree of aligned sequences was constructed by the neighbor-joining method using 1,000 bootstrap replicate values.

RESULTS

Bacteriology

On aerobic bacterial culture, *Chryseobacterium* (98.8%) and *Aeromonas hydrophila* group 2 (98.8%) were isolated from the kidney and *Actinobacter baumannii/calcoaceticus* (94.8%) was isolated from the eye. The bacterial isolates were moderate in abundance and were pure. Although these bacteria may have contributed to the mortality, they are not significant for this report.

Virology

Cytopathic effects were observed on the EPC cell line at 48 h postinoculation, with widespread cell aggregation, degeneration, and vacuolization. The cytopathic effects were observed
again 48 h after inoculation of a 1:100 dilution of the infected cell culture fluid into fresh cells.

**Electron Microscopy**

Numerous enveloped, bullet-shaped particles (≈180 × 70 nm in size) were detected (Figure 2b, inset) by negative contrast electron microscopy. The helical nucleocapsids were surrounded by an envelope with prominent spikes on the surface. Ultrastructurally, changes consisted of individualization of cells, with condensation of mitochondria, mitophagy, vacuolation, replication, and dilation of secular organelles, such as endoplasmic reticulum. Depolymerization and peripheral aggregation of the chromatin, chromatin fragmentation, cytoplasmic blebbing, and formation of numerous apoptotic bodies were also observed (Figure 2a). Numerous virions were attached to the plasma membrane, and nucleocapsids were present within vesicles and tubular organelles (Figure 2b).

**Reverse Transcription PCR and Sequencing**

Consistent with SVCV (Dixon 2009), a 714-bp band was observed by gel electrophoresis. Since this isolation of SVCV was the first for this location, sequencing was performed to further confirm the identity of the isolate. The sequence was submitted to GenBank with Accession Number JQ247697 (MN/27923/11). Phylogenetically, the virus isolate was grouped with SVCV subtype Ia, with 96.8–99.0% similarity (Figure 3). Maximum homology was observed with the Canadian SVCV isolate (99%; EF194065, from common carp in Lake Ontario), followed by isolates from Illinois and Ohio (98.6%) and from Wisconsin (98%). The Minnesota isolate showed comparatively low homology with isolates from koi in Missouri (divergence = 2.4%), North Carolina (2.6%), and Washington (2.8%), thus indicating geographical divergence, host divergence, or both.

**DISCUSSION**

This is the first report of SVCV within the state of Minnesota. A previous common carp mortality event caused by SVCV was observed during 2007 in Mississippi River Pool 8 (near Genoa, Wisconsin), approximately 280 km south of this outbreak on the Minnesota border. The virus has not subsequently been isolated from fish in that location or any other location on the Mississippi River. These outbreaks are of particular concern given the long duration of subclinical persistence in wild populations and the distance traveled. Causes for the outbreak in Minnesota are unknown, but poor overwintering conditions and water temperatures are often critical factors in determining host susceptibility to SVCV (Ahne et al. 2002). Two weeks prior to the outbreak (June 1–7, 2011), record-high temperatures (average daily temperature = 24.9°C) and only a trace of precipitation were experienced at Minnehaha Creek. During the week prior to the outbreak (June 8–14, 2011), the weather cooled rapidly at this location and temperatures dropped to an average of 17.7°C, accompanied by 4.2 cm of precipitation. These temperatures are within the range that is considered favorable for disease progression. Thus, environmental conditions (rapid fluctuations in temperature, pH, dissolved oxygen, etc.) likely depressed the immune systems of the hosts, resulting in their increased susceptibility to SVCV.
FIGURE 3. Phylogenetic analysis performed on the basis of 515-nucleotide sequences of the spring viremia of carp virus (SVCV) glycoprotein gene by using MEGA version 4.0. Phylogenetic tree showing SVCV genogroups 1a–1d was constructed by the neighbor-joining method using the p-distance substitution model with 1,000 bootstrap replicates and a cutoff value of 70%. The sequence marked with a black triangle (JQ247697/common carp/MN/USA) is from the present case report; all other sequences (presented as GenBank accession number/species/location) are from previously published isolates (NC002803/common carp/Yugoslavia is the reference sequence). Other species from which previous isolates were obtained include the goldfish *Carassius auratus*, bighead carp *Hypophthalmichthys nobilis*, and rainbow trout *Oncorhynchus mykiss*.

It is unknown how or when SVCV was introduced into this area. The most logical scenario is that the virus was transported via natural movement of fish from the Mississippi River Pool 8 outbreak in 2007. This is supported by the high degree of genetic similarity between the two isolates; however, the Minnesota isolate is most closely related to the virus isolated from common carp in Lake Ontario during 2006. Additional molecular analysis is needed to further evaluate the differences. It is also plausible that SVCV was introduced with the movement of recreational or commercial fishing boats. These boats frequently travel long distances in the Mississippi River with water (in the live well, bait bucket, bilge, etc.) or fomites (nets, tackle, trailer, etc.) that may be carrying viable virus. In either event, the long duration of subclinical infections in the wild and the potential for anthropogenic movement of the virus deserve investigation.

Active surveillance in high-risk SVCV waters during the spring and early summer months is highly recommended. These activities would better inform fish health managers in devising strategies to prevent the spread of SVCV into new areas. Furthermore, investigating the subsequent spread and persistence of SVCV in surviving fish after an outbreak would help fish health managers to better understand the epidemiology of the virus. For example, early identification of SVCV could advise
managers to limit the movement of infected fish (e.g., by use of carp barriers) so as to prevent the virus from spreading to the high-value fisheries connected to the Mississippi River.

ACKNOWLEDGMENTS
We thank Wendy Wiese, Becca Wheeldon, and Don Ariyakumar for their exceptional technical assistance at the MVDL. We also thank Don Pereira, Paula Phelps, and Ling Shen from MDNR for critical review of this case report.

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The Use of a One-Step Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for the Surveillance of Viral Hemorrhagic Septicemia Virus (VHSV) in Minnesota

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ARTICLE

The Use of a One-Step Real-Time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for the Surveillance of Viral Hemorrhagic Septicemia Virus (VHSV) in Minnesota

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Abstract
Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus of fish. The virus infects more than 70 fish species worldwide, in both fresh and salt water. A new viral strain (VHSV-IVb) has proven both virulent and persistent, spreading throughout the Great Lakes of North America and to inland water bodies in the region. To better understand the geographic distribution of the virus, we used a modified real-time reverse transcription polymerase chain reaction (rRT-PCR) assay for high-throughput testing of fish for VHSV. The assay was shown to be twice as sensitive as the gold standard, virus isolation, and did not cross react with other viruses found in fish. In addition, the diagnostic turnaround time was reduced from 28 to 30 d for virus isolation to 2–4 d for rRT-PCR. To demonstrate the usefulness of the rRT-PCR assay, 115 high-priority water bodies in Minnesota were tested by both methods from April 2010 to June 2011. All survey sites tested negative for VHSV by both methods. The survey results have informed fisheries managers on the absence of VHSV in Minnesota and have better prepared them for the eventual arrival of the disease. In addition, the results demonstrate the value of this rRT-PCR as a surveillance tool to rapidly identify an outbreak so that it can be controlled in a timely manner.

Viral hemorrhagic septicemia virus (VHSV) is a highly contagious and pathogenic virus of fish. The virus has a broad host range including more than 70 fish species and is capable of causing significant biological and economic losses (OIE 2009; Kim and Faisal 2011). The presence of this virus was first suspected as early as 1938, but was not confirmed until 1963 when VHSV was isolated from freshwater rainbow trout *Oncorhynchus mykiss* from farms in Denmark (Schaeperclaus 1938; Jensen 1963). Currently, there are four genotypes of VHSV (VHSV-I to VHSV-IV). Genetic analysis of viral isolates through the 1990s from Europe (VHSV-I, II, III), East Asia (VHSV-I, II, IV), and North America (VHSV-IV) concluded that VHSV is of marine origin but has also mutated to cause disease in freshwater fish hosts (Einer-Jensen et al. 2004; Pierce and Stepien 2012). The routes of transmission between regions and environments are not fully known, but the transfer of infected water, baitfish, and fish for stock enhancement are likely candidates (Herve-Claude et al. 2008; Bain et al. 2010; VHSV Expert Panel and Working Group 2010).

In 2003, VHSV was isolated in the Laurentian Great Lakes (Lake St. Clair) basin from a muskellunge *Esox masquinongy*. This was a first event in several regards: (1) presence of VHSV in freshwater of North America, (2) susceptibility of this particular host species, and (3) presence of fish rhabdovirus in the Great Lakes basin (Kim and Faisal 2011). Unfortunately, this isolate was not confirmed to be VHSV until 2005 during a second muskellunge mortality event (Elsayed et al. 2006). Since then the virus has proven to be highly pathogenic in this new environment, naturally infecting 28 different fish species and causing significant losses throughout much of the Great Lakes basin (USDA-APHIS 2010; Kim and Faisal 2011; Thompson et al. 2011). Although confirmed positive for VHSV by surveys of apparently healthy fish, Lake Superior has not experienced mass mortality events similar to those in

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the eastern Great Lakes (Bain et al. 2010; Thompson et al. 2011).

Within the North American VHSV genetic group IV, the marine and freshwater isolates are grouped into VHSV-IVa and VHSV–IVb, respectively (Meyers and Winton 1995). The genetic divergence within VHSV–IVb is very low (maximum of 1.05% in 669 nucleotides of the G-gene), which is consistent with a recent introduction of the virus in a naive population (Thompson et al. 2011; Pierce and Stepien 2012). Although the differences were minor, Thompson et al. (2011) did identify multiple unique isolates in single outbreak locations, including inland lakes. This has supported the covert nature of the virus and its ability to repeatedly move or be introduced undetected. Therefore, continued virus surveillance is critically important to reduce further spread of VHSV within, and out of, the Great Lakes basin.

Several methods are currently utilized for regulatory, clinical, and surveillance testing of VHSV. Virus isolation is the gold standard diagnostic assay for all fish viruses as recommended in the U.S. Fish and Wildlife Service (USFWS) and American Fisheries Society, Fish Health Section (AFS-FHS) Blue Book (USFWS and AFS-FHS 2010) and the World Organization for Animal Health Manual of Diagnostic Tests for Aquatic Animals (OIE 2009). As such, virus isolation is widely used and accepted for regulatory testing of fish to certify virus-free status (Purcell et al. 2011). However, for surveillance or other voluntary testing without movement or legal implications, the selection of diagnostic tests can be much more flexible. While an assay for surveillance purposes must be validated and proven robust to high standards, it should be best fit for the purpose. One method that has gained popularity in recent years for surveillance testing is real-time polymerase chain reaction (rt-PCR) (Mackay et al. 2002; Walker 2002; Espy et al. 2006). The rt-PCR can improve sensitivity, speed, and accuracy, while lowering cost, compared with traditional methods of virus isolation (Leland and Ginocchio 2007; Hope et al. 2010). These advantages allow managers to have a better understanding of virus distribution and respond more rapidly to an outbreak. Limitations, such as poor validation and risk of cross-contamination in molecular assays, have been significantly reduced with increases in laboratory experience and technological advancements (OIE 2009; Purcell et al. 2011).

Real-time PCR has been widely used and federally recognized as a primary detection and surveillance assay for a variety of terrestrial animal diseases including avian influenza, exotic Newcastle disease, classical swine fever, swine influenza, foot and mouth disease, and many others (OIE 2009; USDA-APHIS 2010; USFDA 2011). While the same cannot be said for rt-PCR for aquatic pathogens, there has been widespread development and validation for their use in research and clinical cases (Purcell et al. 2011). Both conventional reverse transcription PCR (RT-PCR) and real-time RT-PCR (rRT-PCR) methods exist for the detection of VHSV in fish (Winton and Einer-Jensen 2002; Hope et al. 2010; Garver et al. 2011; Jonstrup et al., in press). These assays have been validated to varying standards and have been designed for specific purposes. For example, conventional RT-PCR is more commonly used for confirmatory testing following virus isolation (OIE 2009; USFWS and AFS-FHS 2010). Two of the previously published rRT-PCR assays were designed to detect all known strains of VHSV (Garver et al. 2011; Jonstrup et al., in press), while another assay was designed to detect only the VHSV–IVb strain in the Great Lakes (Hope et al. 2010). All of these assays have demonstrated their value as a surveillance tool by increasing sensitivity and significantly reducing turnaround time compared with virus isolation (Knuesel et al. 2007; Bain et al. 2010; Hope et al. 2010; Garver et al. 2011; Jonstrup et al., in press).

Until the regulatory framework is established, the voluntary use of rRT-PCR for the detection of VHSV can significantly improve management of this virus. For example, prior to 2010, the majority of Minnesota water bodies used for aquaculture or recreational purposes were never tested for VHSV. As a result, the VHSV status for much of the state was unknown, despite routine movements of fish between water bodies for bait and stock enhancement. In the present study, a one-step version of the Garver et al. (2011) assay was used for high-throughput testing of fish for VHSV surveillance. This initial survey has informed epidemiologists and managers on the extent of the virus presence in Minnesota, which has better prepared them for the eventual arrival of VHSV. In addition, the survey has satisfied the necessary regulatory testing requirements for aquaculture producers to move fish within the state (Minnesota statutes 2011, Section 17.4991, subdivision 3).

METHODS

Source of samples.—A survey of Minnesota fish populations was performed for the presence of VHSV from April 2010 through June 2011. Registered license holders for aquaculture facilities (n = 145) and baitfish harvesters (n = 289) were contacted by mail to participate in the surveillance program. The program offered a free “regulatory inspection,” which consisted of collecting fish and testing them simultaneously by virus isolation (the gold standard test) and modified rRT-PCR. Fish species included in the survey were limited to those on the U.S. Department of Agriculture, Animal and Plant Health Inspection Service VHSV susceptible species list (USDA-APHIS 2008) at the time of the survey. Sample locations were selected based on (1) water body use by the aquaculture industry, (2) presence of VHSV susceptible species, (3) distribution throughout Minnesota, and (4) high risk or high priority as identified by the Minnesota Department of Natural Resources (MNDNR).

On-site fish collection was overseen by an accredited veterinarian or MNDNR field biologist with specialized fish health training, or by an American Fisheries Society certified Fish Health Inspector. A minimum of 60 fish per site were collected, targeting species on the aquaculture permit, such as the spottail shiner *Notropis hudsonius* and walleye *Sander vitreus*. If the target species was unattainable owing to seasonal
availability, 150 fish of other VHSV susceptible species were collected. For large populations (>1,000 fish), sampling of 60 fish provided 95% confidence in detecting VHSV, assuming the virus was present in 5% of the population. The sample of 150 susceptible fish maintained 95% confidence, but accounted for a potentially lower prevalence of 2%. Fish collections primarily occurred during the spring and fall when water temperatures were suitable for VHSV.

Freshly dead fish were submitted on ice to the Minnesota Veterinary Diagnostic Laboratory (MVDL) within 24 h of collection. Immediately upon arrival, each fish was visually inspected for clinical signs of disease and a necropsy was performed to obtain the appropriate samples e.g., kidney and spleen from fish >6.0 cm, entire viscera from fish 4.0 to 6.0 cm, and the entire fish when it was <4.0 cm in length. Tissues from five fish were pooled together and stored at 4°C for no more than 24 h. The pools were tested for VHSV by both virus isolation and rRT-PCR. Results were made immediately available online for the owner and the regulatory agency for review.

Sample preparation.—Because of regulatory implications of the survey results, all samples from this study were tested by both virus isolation and rRT-PCR. Tissue processing was identical for both methods and followed the protocols recommended by USFWS and AFS-FHS Blue Book (2010). Briefly, a 10% suspension of the tissue was prepared in Hanks’ balanced salt solution (HBSS). The suspension was processed in a stomacher for 30 s followed by centrifugation at 4°C for 15 min at 2,900 x g. An equal amount of antibiotic incubation medium was added to the supernatant. After mixing, the suspension was incubated for 2 h at 15°C and finally recentrifuged for 15 min at 2,900 x g. The resulting supernatant was immediately used for virus isolation and rRT-PCR.

Virus isolation.—Virus isolation was performed according to the USFWS and AFS-FHS Blue Book (2010). Monolayers of epithelioma papulosum cyprini (EPC) cells (Fijan et al. 1983) were prepared in 48-well microtiter plates. When monolayers were 80% confluent, the cell culture media was decanted and the sample was inoculated (100 µL/well). The inoculated cells were incubated at 15°C for 60 min to allow for viral adsorption. Maintenance medium was then added and the plates incubated at 15°C for 14 d. The plates were examined twice weekly for the appearance of cytopathic effects (CPE). Unless CPE was observed, all samples were blind passaged at day 14 and determined negative if no CPE appeared after 14 d of incubation. A VHSV-IVb isolate (Great Lakes reference strain: MI03) was used as a positive control to monitor the sensitivity of the cell line to the virus. The CPE from the positive control was occasionally confirmed by rRT-PCR, sequencing, and negative contrast electron microscopy. Cell culture supernatant was stored at 4°C for no more than 24 h prior to extraction.

Total nucleic acid extraction.—MagMAX Express 96 Viral RNA Isolate kit (AM1836; Life Technologies, Grand Island, New York) was used for total nucleic acid extraction from sample suspension and from infected cell culture supernatants. The extraction was performed according to a custom protocol developed by the MVDL in association with Life Technologies. The primary difference from the standard protocol is a starting volume of 100 µL and final elution volumes of 75 µL, compared with 50 µL for each in the standard protocol. This method was originally optimized for high-throughput extraction of a swine virus, the porcine reproductive and respiratory syndrome virus (PRRSV), and has been found to be effective for a wide variety of other animal pathogens (data not shown). Along with unknown samples, a VHSV-IVb positive isolate grown in EPC cells was extracted for each run to serve as a positive extraction control. The extraction procedure was also performed on 100 µL of 1 x PBS to serve as a negative extraction control. The resulting RNA was stored on ice for immediate testing or frozen at −80°C for later testing.

Real-time reverse transcription polymerase chain reaction.—Real-time RT-PCR for VHSV was performed on an ABI 7500 Sequence Detection System (Life Technologies). The master mix was prepared in a 20-µL volume, with 12.5 µL of 2× Path-ID Multiplex RT-PCR Buffer (Life Technologies), 1.25 µL of 10× Path-ID Multiplex enzyme mix (Life Technologies), 1.0 µL of 5 µM FAM dye-labeled MGB Probe (5′-Fam-TAC GCC ATC ATG AGT AGT-3′), 0.375 µL of each 40-µM forward (5′-ATG AGG CAG GTG TCG GAG G-3′) and reverse (5′-TGT AGT AGG ACT CTC CCA GCA GCC-3′) primers, and 4.5 µL of nuclease-free water. The primer-probe set was designed to detect the N-gene of all known strains of VHSV (Garver et al. 2011). Five microliters of extracted RNA (unknown samples), nuclease-free water (negative control), VHSV-known-positive RNA (positive PCR control), VHSV-known-positive cell culture supernatant (positive extraction control), or negative extraction control were added to each well. Each sample was run in duplicate. The thermal cycling protocol consisted of 10 min at 45°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

Analytical specificity and sensitivity.—The specificity of the modified assay was confirmed with spring viremia of carp virus, infectious salmon anemia virus, channel catfish virus, koi herpes virus, golden shiner virus, two fathead minnow picornaviruses, and an unknown paramyxovirus of white suckers Catostomus commersonii. For sensitivity determination, serial dilutions of VHSV-positive cell culture supernatant were tested in duplicate by virus isolation and rRT-PCR on four separate days. The lowest dilution that yielded a positive result was determined to be the limit of detection. The threshold cycle (Ct) for the rRT-PCR was considered as the cycle in which the amplification curve crossed the automatic threshold (Life Technologies) during the exponential growth phase of the curve. The rRT-PCR reaction had a cut-off at 40 cycles, but all samples with Ct values 37–40 were automatically retested. If they remained below 40 cycles, they were called positive.
RESULTS

VHSV Survey

Of 434 contacted, 30 aquaculture producers and wild baitfish harvesters participated in the VHSV survey. The industry impact was probably much higher given that once a water body was certified VHSV-free, anyone with the appropriate license could harvest bait. From April 2010 to June 2011, 115 water bodies were tested for VHSV as part of this survey (Figure 1). The sampling locations with high aquaculture and wild baitfish harvest were well distributed across Minnesota. A total of 7,098 individual fish were collected, comprising 1,420 pooled samples. Approximately 56% \( (n = 65) \) of the surveyed sites consisted of sport fish, while 44% \( (n = 50) \) consisted of baitfish. Species collected included walleye \( (n = 3,375) \), spottail shiner \( (n = 3,000) \), black crappie \( Pomoxis nigromaculatus \) \( (n = 285) \), muskellunge \( (n = 265) \), northern pike \( Esox lucius \) \( (n = 80) \), bluegill \( Lepomis macrochirus \) \( (n = 60) \), yellow perch \( Perca flavescens \) \( (n = 18) \),

FIGURE 1. Locations of 115 VHSV survey sites in Minnesota collected from April 2010 to June 2011. Circles indicate sport fish \( (n = 65) \) and squares indicate baitfish \( (n = 50) \). All locations tested negative for VHSV by virus isolation and rRT-PCR. Image generated with Google Earth. [Figure available online in color.]
and smallmouth bass Micropterus dolomieu (n = 15). All sample pools were found negative for VHSV by both virus isolation and rRT-PCR. Throughout the survey, the virus isolation and rRT-PCR assays were 100% accurate in identifying the positive and negative controls. The turnaround time for virus isolation and rRT-PCR was 28–30 d and 2–4 d, respectively.

**VHSV rRT-PCR**

*Analytical specificity.*—The rRT-PCR was specific to VHSV and did not cross react with other viruses tested. Although the VHSV-IVb isolate was the only VHSV tested in this validation, sequence alignment and previous data show the primers used can detect all known VHSV strains (Garver et al. 2011).

*Analytical sensitivity.*—The analytical sensitivity of this assay was comparable with other rRT-PCR assays for VHSV. The standard curve was linear over six logs of virus dilution and had a starting concentration of 4.6 × 10⁶ TCID50 (tissue culture infectious dose with a 50% endpoint) (Figure 2). The correlation coefficient (r) and PCR efficiency were 0.9942 and 104.5%, respectively. The minimum detection limit of the rRT-PCR assay was determined to be a 1.44 TCID50 with a corresponding Ct of 37.2. Compared with this rRT-PCR assay, virus isolation was half as sensitive and had a detection limit of 2.88 TCID50 and a corresponding Ct of 34.8.

**DISCUSSION**

A newly modified one-step rRT-PCR for the detection of VHSV was used in this study. The assay was shown to be twice as sensitive as the gold standard, virus isolation. Since VHSV can persist in wild fish populations at low levels without producing clinical signs of disease, this improved sensitivity is critically important to prevent the spread of VHSV. In addition, rRT-PCR can significantly decrease the turnaround time and lower labor and laboratory costs. The analytical data generated from this study showed this assay to be an excellent candidate for further evaluation and fit for the purpose of surveillance testing as demonstrated in a 2-year survey of Minnesota waters for VHSV. With nearly 1,500 sample pools tested, no false-positive results were obtained, while both tests accurately identified the negative and positive controls. The results from this survey have certified 115 locations to be VHSV negative, allowing in-state fish movement (for 1 year post inspection).

Aquaculture industry support for this study was very strong in Minnesota, owing to a desire to move fish within days of sample submission rather than having to wait a month. This is essential for any industry that survives on quick turnaround and accurate test results. The survey data have informed management agencies and researchers of the distribution of VHSV in Minnesota. Applications for these data are wide-reaching and will improve selection criteria for future testing, support risk-assessment studies, and ease public concern for this emerging fish disease.

Despite the aforementioned advantages, there are certain limitations of rRT-PCR. One significant limitation is the appropriate interpretation of results. If rRT-PCR is used for surveillance testing, positive rRT-PCR results should remain suspect until confirmed by virus isolation. If virus isolation does not support the suspect-positive result, it should be considered “a population of interest, in need of further testing” or “negative” as it would have been without the use of rRT-PCR. A suspect-positive by rRT-PCR should not result in immediate depopulation or other regulatory action. In the event of conflicting results, other factors should be considered to warrant additional action, such as clinical signs of disease, previous testing history, disease risk factors, or the number of suspect-positive results. Additional action may include, retesting the original material, resampling the population, sequencing the rRT-PCR product, and other confirmatory tests.

One other limitation is proper validation and quality control in the laboratory. Validation standards are outlined by the OIE (2009) and Purcell et al. (2011). While the degree to which an assay is validated depends on its proposed use, the assays used for widespread surveillance testing should be thoroughly scrutinized. The assay used in this study, for example, should undergo an interlaboratory validation to evaluate various platforms, technicians, and diagnostic sensitivity and specificity. Furthermore, proper controls should be used throughout the rRT-PCR assay, including positive and negative controls for RNA extraction and rRT-PCR. These controls were used in this study and increased confidence in the interpretation of results. Endogenous controls were not used in this study, but are recommended to confirm the quality and quantity of nucleic acid (Bland et al. 2012). Other concerns such as laboratory conditions, experience, and capacity are rapidly improving in the United States as technology and demand increases.

Given the threat of aquatic animal pathogens, such as VHSV, new and improved diagnostic tests must be developed, validated, and recognized by agencies for use in surveillance testing. Rapid
detection and quick turnaround time are paramount to identify and effectively control an outbreak. This need has been well demonstrated with the emergence of VHSV-IVb in the Great Lakes. As technology continues to improve and appropriate evaluations are performed, rRT-PCR will become the assay of choice for the detection of viral hemorrhagic septicemia and other emerging diseases of aquatic animals.

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The Impact of Mitochondrial and Thermal Stress on the Bioenergetics and Reserve Respiratory Capacity of Fish Cell Lines

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COMMUNICATION

The Impact of Mitochondrial and Thermal Stress on the Bioenergetics and Reserve Respiratory Capacity of Fish Cell Lines

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Abstract
Various stressors affect the health of wild and cultured fish and can cause metabolic disturbances that first manifest at the cellular level. Here, we sought to further our understanding of cellular metabolism in fish by examining the metabolic responses of cell lines derived from channel catfish *Ictalurus punctatus* (CCO), white bass *Morone chrysops* (WBE), and fathead minnow *Pimephales promelas* (EPC) to both mitochondrial and thermal stressors. Using extracellular flux (EF) technology, we simultaneously measured the oxygen consumption rate (OCR; a measure of mitochondrial function) and extracellular acidification rate (ECAR; a surrogate of glycolysis) in each cell type. We performed a mitochondrial function protocol whereby compounds modulating different components of mitochondrial respiration were sequentially exposed to cells. This provided us with basal and maximal OCR, OCR linked to ATP production, OCR from ion movement across the mitochondrial inner membrane, the reserve capacity, and OCR independent of the electron transport chain. After heat shock, EPC and CCO significantly decreased OCR and all three cell lines modestly increased ECAR. After heat shock, the reserve capacity, the mitochondrial energetic reserve used to cope with stress and increased bioenergetic demand, was unaffected in EPC and CCO and completely abrogated in WBE. These findings provide proof-of-concept experimental data that further highlight the utility of fish cell lines as tools for modeling bioenergetics.

As organisms intimately associated with their environment, fish are sensitive to numerous environmental insults that can alter their physiology on a cellular level. Both wild fish and fish subject to intensive farming practices can experience a host of acute and chronic stressors such as changes in dissolved oxygen, temperature, and water quality, as well as physical affronts including high density, confinement and handling. Indeed, such stressors can disrupt homeostasis and result in metabolic perturbations that are central to the pathophysiology of disease in both wild and cultured fish.

The process of cellular metabolism hinges upon the uptake of substrates, namely oxygen, glucose, and fatty acids, which are subsequently converted to energy via a series of enzymatic redox (oxidation–reduction) reactions. These reactions ultimately result in the production of ATP and byproducts such as lactate and CO₂, which are released into the extracellular milieu (Ferrick et al. 2008; Nicholls et al. 2010). Importantly, aberrations in cellular metabolism are commonplace in numerous diseases including age-related diseases, cardiovascular disease, diabetes, and obesity (Nicholls 2002; Ferrick et al. 2008; Hill et al. 2009; Dranka et al. 2010). Likewise, the concept of metabolic stress and altered bioenergetics in fish is not new, as a preponderance of literature is committed to characterizing the responses of live fish to numerous stressors such as heat shock, hypoxia, toxicants, confinement, and handling (Pickering et al. 1987; Pickering and Pottinger 1989; Pottinger et al. 1992; Iwama et al. 1998, 2004; Barton 2002; Falciani et al. 2008; Pottinger 2008). Interestingly, the overwhelming majority of studies in this regard have focused on whole-animal models, which are useful as they account for the physiological organization of the whole animal (Iwama et al. 1998). Curiously though, few studies have focused on the cellular bioenergetics of fish-derived cells. Clearly, cell lines have numerous advantages including their ease of maintenance, low cost, reproducibility, uniformity, commercial availability (affords standardization among laboratories), and as an alternative to increasingly scrutinized live animal experimentation (Iwama et al. 1998; Lakra et al. 2011).
Thus, in the present study, we sought to further our understanding of cellular metabolism in fish by examining the comparative stress response of cells derived from fish species that are of both ecological and commercial importance: channel catfish *Ictalurus punctatus*, white bass *Morone chrysops*, and fathead minnow *Pimephales promelas*.

To this end, we employed an instrument that noninvasively detects changes in oxygen (O₂) levels and pH within the media directly surrounding cells. By measuring the changes in oxygen and proton concentrations immediate to cells (termed extracellular flux; EF), the respective contributions of aerobic respiration and glycolysis—the energy yielding pathways of the cell—can be assayed simultaneously (Ferrick et al. 2008). We characterized the aerobic and glycolytic configurations of cells at rest, after a heat-shock stimulus, and after treatment with several compounds that target distinct complexes within the respiratory chain of the mitochondrion.

From these informative bioenergetic experiments we generated distinct metabolic signatures for each cell type. These findings provide key proof-of-principle experimental data that will further highlight the utility of fish cells and cell lines as tools for modeling bioenergetics. Ultimately, we hope to translate the metabolic phenotypes shown here into biomarkers predictive of how fish will cope with cellular insults encountered in the wild and in food fish production settings.

**METHODS**

**Cells.**—White bass embryo (WBE) cells were obtained from the American Type Culture Collection. Channel catfish ovary (CCO) cells were provided by the Auburn University Department of Fisheries and Allied Aquacultures, Auburn University, Alabama. *Epithelioma papulosum cyprini* (EPC) cells (derived from fathead minnow, Winton et al. 2010) were provided by the Fish Disease Diagnostic Laboratory at the University of Arkansas at Pine Bluff, Pine Bluff, Arkansas. All chemicals came from Sigma-Aldrich (St. Louis, Missouri) unless otherwise noted. Cells were maintained with Leibovitz’s L-15 medium or unbuffered Dulbecco’s Modified Eagle Medium (DMEM; Seahorse Bioscience, Billerica, Massachusetts) supplemented with 10% fetal bovine serum containing 100 U/mL penicillin, 100 U/mL streptomycin, and 25 µg/mL amphotericin B. Cells were cultured at 27°C, a temperature within the acceptable ranges of each cell line (Bowser and Plumb 1980; Fijan et al. 1983; Shimizu et al. 2003). Cells were passaged when 90% confluency was reached with 0.25% Trypsin (Mediatech, Manassas, Virginia). Cells were counted with a hemacytometer, and cell viability after each experiment was determined by routine Trypan Blue (Invitrogen, Carlsbad, California) exclusion methods. Micrographs of cells were captured at 200× magnification with a Zeiss Axiosvert 40 using PlasDIC contrast and AxioVision software (Zeiss, Thornwood, New York).

**Compounds.**—Oligomycin, carbonyl cyanide 4-(trifluoro methoxy)phenylhydrazone (FCCP), and rotenone were used to systematically target different aspects of mitochondrial respiration. Oligomycin inhibits ATP synthase, FCCP is a proton ionophore that uncouples mitochondrial respiration and stimulates oxygen consumption, while rotenone inhibits Complex I (NADH dehydrogenase) of the mitochondrial respiratory chain. Working concentrations of these compounds were carefully determined following the suggestions of Diers et al. (2010), so that maximum effects for each cell type could be reached without causing overt toxicity to the cells. The concentrations used for all experiments were: oligomycin (1 µg/mL), FCCP (3 µM), and rotenone (1 µM).

**Profiling cellular bioenergetics.**—A Seahorse Bioscience XF24 Extracellular Flux Analyzer (Seahorse Bioscience) was used to measure cellular respiration and glycolysis in adherent cell lines. The XF24 instrument detects small changes in oxygen levels and pH within the media immediately surrounding cultured cells. This is achieved by creating a transient <10-µL microchamber in which changes in oxygen and pH can be measured noninvasively, sensitively, and repeatedly over time (Ferrick et al. 2008). The oxygen consumption rate (OCR) is a measure of mitochondrial function and the extracellular acidification rate (ECAR), which is measured simultaneously, is a marker of glycolytic flux; glycolysis results in lactate formation and excretion and acidifies the unbuffered media surrounding the cells.

First, the optimal seeding density of WBE, CCO, and EPC cells in assay plates was determined. The optimum density is reflected by uniformly distributed semiconfluent monolayers of cells that are not clumped in appearance after an overnight incubation. Concentrations of 15,000, 30,000 and 60,000 cells per well were tested. Measurements were carried out in a manner similar to that described previously (Qian and Van Houten 2010) but with modifications. Briefly, prior to measurements, medium was aspirated and cells were washed with unbuffered DMEM leaving approximately 50 µL of residual media to protect cells from drying. A final volume of 650 µL of unbuffered DMEM containing no fetal bovine serum, 10 mM glucose, and 1 mM sodium pyruvate was added to the cells. All measurements were performed at 27°C. The instrument was programmed to gently mix the medium for 3 min, pause for 2 min, and measure for 3 min. For basal metabolic pathway determination this program was repeated five consecutive times.

To profile cellular bioenergetics, a previously described mitochondrial function protocol used in the study of mammalian cells (Jekabsons and Nicholls 2004; Diers et al. 2010) was employed. A baseline OCR is first established (first 2–3 measurements), followed by the sequential injection of oligomycin, FCCP, and rotenone. After injection of each compound two measurements were collected before injection of a subsequent compound. From this single assay, several important mitochondrial parameters were determined: (1) basal OCR, (2) maximal OCR, (3) oxygen consumption linked to ATP production, (4) oxygen consumption due to ion movement across the mitochondrial inner membrane, referred to as proton leak, (5) the reserve respiratory capacity,
referred to as reserve capacity, and (6) oxygen consumption that occurs by other means such as the reduction of oxygen to yield reactive oxygen species (Diers et al. 2010).

Because growth kinetics differ by cell type, all measurements were normalized to total protein levels in each well assayed at the completion of the XF24 protocol. Cells were lysed with M-PER mammalian protein extraction reagent (Pierce/Thermo Scientific, Rockford, Illinois), and total protein concentrations were determined with the Coomassie Plus (Bradford) assay kit (Pierce/Thermo Scientific) (Bradford 1976).

Heat-shock experiment.—We next assayed the metabolic responses of all three cell types to heat shock, a frequent stressor that fish encounter. Cell cultures were subjected to a heat-shock stimulus for 14 h at 32°C, a water temperature and duration that can regularly occur during the summer months at our research facility and throughout the United States (Green and Rawles 2010). A mitochondrial profiling experiment (as described above) was performed with these cells and compared with the identical passage of cells cultured overnight at 27°C for the same duration.

Statistics.—Oxygen consumption rate (pmol O2·min⁻¹·µg protein⁻¹) and ECAR (mP·min⁻¹·µg protein⁻¹) are reported as mean ± SE. Statistical differences between the mean OCR or ECAR for all cell lines and treatments were evaluated using a Student’s t-test in SigmaPlot 11 (Systat Software, San Jose, California). Differences were considered significant if \( P \leq 0.05 \).

RESULTS

Basal Metabolic Phenotype

The optimum cell-seeding density was determined to be 60,000 cells per well for each cell type, as a density of 30,000 cells per well was too sparse after overnight culture for reliable measurements (not shown). This density was also selected for each cell type because it yielded measurements within the linear range of the instrument. Next, we determined the basal metabolic phenotype, which is derived from the simultaneous measurement of aerobic respiration (as indicated by OCR) and the rates of glycolytic flux (as indicated by ECAR) in WBE, CCO, and EPC cells. All three cell lines exhibited distinct basal metabolic phenotypes (Figure 1). The CCO cells showed the greatest aerobic respiration (highest OCR) and EPC cells were the most glycolytic (highest ECAR). Basal oxygen consumption rates by cell type were: WBE = 14.9 ± 0.28 pmols O2·min⁻¹·µg protein⁻¹, CCO = 29.9 ± 0.29 pmols O2·min⁻¹·µg protein⁻¹, and EPC = 19.3 ± 0.9 pmols O₂·min⁻¹·µg protein⁻¹ (Figure 1A). The CCO cells were significantly more aerobic than both WBE and EPC, while EPC cells were significantly more aerobic than WBE. Basal ECARs were: WBE = 0.369 ± 0.029 mP·min⁻¹·µg protein⁻¹, CCO = 0.678 ± 0.038 mP·min⁻¹·µg protein⁻¹, and EPC = 0.95 ± 0.110 mP·min⁻¹·µg protein⁻¹ (Figure 1B). Basal ECAR was significantly lower in the WBE cells compared to CCO and EPC cells.

Mitochondrial Function Assay

Using a mitochondrial function assay, we examined the effects of various compounds targeting the electron transport chain on the OCR of WBE, CCO, and EPC cells (Figure 2). The oligomycin sensitive fraction of respiration, which differentiates oxygen consumption linked to ATP production, was greatest in CCO cells (22.8 ± 1.01 pmols O2·min⁻¹·µg protein⁻¹), followed by EPC cells (13.1 ± 0.53 O2·min⁻¹·µg protein⁻¹) (Figure 2C). There were no significant differences between the cells in proton leak or rotenone-insensitive OCR. The reserve respiratory capacity was significantly greater in WBE cells (13.1 ± 0.52 mP·min⁻¹·µg protein⁻¹) when compared with the either CCO (6.7 ± 0.83 mP·min⁻¹·µg protein⁻¹) or EPC cells (6.2 ± 0.39 mP·min⁻¹·µg protein⁻¹) (Figure 2D).

Heat-Shock Experiment

After heat shock, both EPC and CCO cells exhibited a significant decrease in OCR, while WBE cells were relatively unaffected (Figure 3). However, all three cell lines showed increased ECAR after heat shock yet the differences between each cell line were not statistically different (Figure 3). After heat shock, the reserve respiratory capacity was markedly decreased in WBE cells, slightly decreased in CCO cells, and significantly increased in EPC cells (Figure 3). Cell viability was greater than 95% for each cell line after heat-shock treatment, and viability was not different from cells cultured overnight at 27°C.

DISCUSSION

Stress is regarded as a state of threatened homeostasis that is re-established by a complex suite of adaptive responses...
FIGURE 2. (A) Schematic showing the use of specific compounds to delineate how oxygen consumption was dedicated within the cell. After three baseline oxygen consumption rate (OCR) measurements, oligomycin, FCCP, and rotenone, were injected sequentially, and OCR was measured at two timepoints after each injection. (B) Bioenergetics of WBE, EPC, and CCO cells generated after the sequential injection of oligomycin (1 µg/mL), FCCP (3 µM), and rotenone (1 µM). (C) From the basal and the oligomycin-sensitive rates the ATP-linked oxygen consumption and the OCR resulting from proton leak (oligomycin-insensitive respiration) were calculated. Injection of rotenone allows for the measurement of oxygen consumption by other means (designated as “Other” shown in panel A). (D) Injection of FCCP serves to determine the approximate maximal OCR and allows for the calculation of the reserve capacity (FCCP stimulated respiration minus basal respiration). Different lowercase lettering above bars denotes a statistically significant ($P < 0.05$) difference between groups.

There is a large body of literature documenting whole-animal-level responses to stress ranging from insults encountered in aquaculture production to anthropogenic disturbances. However, the precise molecular mechanisms mediating the stress response in fish remains incredibly complex as it varies widely among species, geography, and cell and tissue type (Iwama et al. 2004). Nevertheless, linking cellular mechanisms to the generalized stress response at higher levels of biological organization is crucial in furthering our understanding of how fish respond to exogenous stressors.

The differential bioenergetics demonstrated by the cells in this study could be a result of their differences in lineage or tissue of origin (i.e., embryonic versus epithelial). Moreover, variation may also be explained by differing degrees of transformation or acclimatization to culture as CCO and EPC cells were isolated approximately two decades before WBE cells. Interestingly, some of the variation shown here may also be a consequence of the species that our cells were derived from. Marked differences in oxygen consumption by isolated liver mitochondria have been documented between rainbow trout *Oncorhynchus mykiss* and channel catfish, and rainbow trout mitochondria have greater basal oxygen consumption (Smith et al. 1975). Moreover, mitochondrial respiration in rainbow trout was more sensitive to inhibition by antimycin (Smith et al. 1975), a drug that inhibits mitochondrial respiration similar to rotenone (albeit by a different mechanism) used in the present...
study. Respiration in isolated mitochondria can be measured, yet we opted to examine the metabolic phenotypes in the whole cell as all of the regulatory elements would be intact. Using the important proof-of-concept experimental findings shown here, ongoing studies in our laboratory are examining bioenergetic phenotypes in primary cells and tissues, such as liver and muscle, as well as in isolated mitochondria.

A growing number of studies are using a mitochondrial-profiling study similar to the present study. Indeed, this assay is emerging as a viable means by which to assess baseline cellular metabolism, identify mitochondrial dysfunction, and predict the capacity of a cell population to cope with stress (Twig et al. 2008; Choi et al. 2009; Liu et al. 2009; Wu et al. 2009). One of the most important parameters derived from this assay is the reserve capacity (also termed reserve or spare respiratory capacity), which is an index of the mitochondrial energy reserves in place to increase energy production in the face of increased bioenergetic demand. The reserve capacity has emerged as a critical component in maintaining cellular function during acute and chronic stress (Hill et al. 2009; Sansbury et al. 2010). Indeed, in numerous studies published in the field of biomedical sciences, a loss of the reserve capacity has been predictive of cell death or organ dysfunction (Gong et al. 2003; Yadava and Nicholls 2007; Hill et al. 2009; Dranka et al. 2010). For example, under basal conditions neonatal rat ventricular myocytes can possess a substantial reserve capacity to respond to increasing energy demand if needed (Hill et al. 2009). However, upon exposure to increasing concentrations of the reactive lipid species 4-hydroxynonenal, a product formed during acute and chronic cardiac dysfunction, the bioenergetic reserve capacity was depleted and cell death followed (Hill et al. 2009).

The marked decrease in the reserve capacity of WBE cells (compared with the reserve capacity measured at 27°C) after heat shock may be explained by the differences in tolerance to thermal extremes by the different species the cell lines were derived from. Both channel catfish (origin of CCO) and fathead minnow (origin of EPC) are exceedingly more tolerant to thermal extremes than the white bass, the species of origin for the WBE line. The reported critical thermal maxima (CTM) for channel catfish and fathead minnow are 42.1°C and 40.4°C, respectively (Beitinger et al. 2000). In contrast, CTM for white bass was found to be 35.3°C (Spotila et al. 1979). Importantly, we contend that the loss of the reserve capacity may be permissive for, or exacerbate, the development of pathophysiological conditions in fish. Consequently, we anticipate that the differential reserve capacities shown here may be extended to a practical application, such as a novel biomarker that may be extrapolative to the theoretical performance of a fish in settings of stress.

Since the isolation of the first fish cell line (Wolf and Quimby 1962), fish cell lines have been employed in the study of physiology, toxicology, carcinogenesis, and gene expression, yet the majority of studies use fish cell lines as tools for diagnostic and mechanistic virology (Lakra et al. 2011). Despite the relative importance of fish cell lines, their usage as models currently lags behind that of mammalian cell lines (Lakra et al. 2011). Bioenergetics have been studied in fish cells, particularly in freshly isolated primary cells such as hepatocytes and mitochondria (Krumschnabel and Nawaz 2004; Bains and Kennedy 2004). One of the most important parameters derived from this assay is the reserve capacity (also termed reserve or spare respiratory capacity), which is an index of the mitochondrial energy reserves in place to increase energy production in the face of increased bioenergetic demand. The reserve capacity has emerged as a critical component in maintaining cellular function during acute and chronic stress (Hill et al. 2009; Sansbury et al. 2010). Indeed, in numerous studies published in the field of biomedical sciences, a loss of the reserve capacity has been predictive of cell death or organ dysfunction (Gong et al. 2003; Yadava and Nicholls 2007; Hill et al. 2009; Dranka et al. 2010). For example, under basal conditions neonatal rat ventricular myocytes can possess a substantial reserve capacity to respond to increasing energy demand if needed (Hill et al. 2009). However, upon exposure to increasing concentrations of the reactive lipid species 4-hydroxynonenal, a product formed during acute and chronic cardiac dysfunction, the bioenergetic reserve capacity was depleted and cell death followed (Hill et al. 2009).

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2005). These studies have commonly used oxygen electrodes (e.g., Clark electrodes, or oxygraphs) and are different in approach and technology to the methodologies employed in the present study. The Clark electrode–oxygraph approach provides valuable kinetic information but introduces artifacts by its continuous consumption of oxygen, which presents a decreasing oxygen pressure to the cells or isolated mitochondria in the measurement chamber (Ferrick et al. 2008). Although oxygen consumption is a good indicator of mitochondrial function, it only measures one component of cellular bioenergetics and therefore an investigator may be unaware of other pathways that contribute to bioenergetic equilibrium, namely glycolysis, which was simultaneously measured in the present study. In addition to bioenergetics, fish cell lines have proven invaluable in the study of the heat-shock response with a principal focus on the characterization of heat-shock proteins (Mosser et al. 1986; Hightower and Renfro 1988; Misra et al. 1989; Iwama et al. 1998). The increase in glycolytic flux (i.e., ECAR) after the heat-shock treatment recapitulates in vivo data derived from fish where surrogates of glycolysis increase after heat shock (Viant et al. 2003). Similar to our results, heat shock can also increase lactate production in rainbow trout erythrocytes (Currie et al. 1999).

It is important to note that the cell types used in the present study were carefully selected to represent species of economic and ecological importance. However, we do recognize the limitations of extrapolating these observations to the whole animal. Accordingly, the principle fish species studied at our research facility is the hybrid striped bass (female white bass × male striped bass Morone saxatilis). The hybrid striped bass food fish industry is considered one of the fastest growing segments of aquaculture in the United States. A notable limitation to the production of hybrid striped bass exists in the form of stress as some of the highest levels of the stress hormone cortisol have been reported in fish within the genus Morone (Mazik et al. 1991). Compared with the hybrid, such hormonal differences have also been associated with both lower survival and resistance to infection in striped bass after confinement stress (Noga et al. 1994). As a model of the genus Morone, we used white bass embryo (WBE) cells, which were obtained from the dissociation of white bass embryos 6–48 h postfertilization (Shimizu et al. 2003). The cells exhibit epithelial characteristics by expressing cytokeratin and possessing extracellular junctions (Shimizu et al. 2003). Ultimately, we expect to use this cell line and other cell types derived from Morone species to characterize the concomitant genes and gene products that are associated with various bioenergetic phenotypes. This approach is made more feasible by the notion that fish cell lines have been transfected to stably express heterologous genes (Bearzotti et al. 1992; Falco et al. 2009). Clearly, gene expression methodologies would enhance mechanistic studies.

In summary, the applicability of extracellular flux technology towards examining bioenergetic phenotypes and measuring cellular stress in fish-derived cells is promising. More importantly, our application of new bioindices to piscine-derived cells, notably the reserve capacity, is a basis for future studies to link cellular metabolism to whole-animal performance in various settings of stress.

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Clinical and Pathological Effects of the Polyopisthocotylean Monogenean, *Gamacallum macroura* in White Bass

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Abstract

An aquaculture research facility experienced high mortality rates in white bass *Morone chrysops* associated with a monogenean infestation of the gills, but not in striped bass *Morone saxatilis* in the same facility. All mortalities had pale gills. Monogeneans, identified as *Gamacallum macroura* (MacCallum and MacCallum 1913) Unnithan 1971, were found on the gills. Pale-gilled and healthy white bass were selected with no particular attention to condition for venipuncture and euthanasia for postmortem examination, including parasite counts from gills. The median packed cell volume (PCV) of fish with gill pallor was 12.5% (range 9–37%) while PVC of fish with more normal color was 30% (27–33%). Association between the PCV and gill pallor score was statistically significant, as was the association between PCV and the number of monogeneans found on the gills of each fish. Median estimated white blood cell count of fish with gill pallor, at 12.05 × 10³/µL (range 3.8–24.7), was significantly lower than of apparently healthy fish: 24.7 × 10³/µL (17.3–31.5). Histopathology of the gill arches of pale-gilled fish revealed multifocal moderate to severe branchitis, focal areas of dilated hyperplastic lamellae occluded by fibrin, and monogeneans attached to the lamellae. Fish that were apparently healthy had grossly similar histologic lesions, but at lower frequency and severity.

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Hybrid moronids, the sunshine bass (female white bass *Morone chrysops* × striped bass *M. saxatilis*) and palmetto bass (female striped bass × white bass), became established in the USA because of the hybrid’s favorable growth rate compared with either parent species, increased disease resistance, and tolerance of a wide range of salinities and temperatures (Gempesaw et al. 1992; Ludwig 2004).

Hybrid striped bass production requires the management of both white bass and striped bass brood stocks as well as the hybrid fish themselves. Knowledge of differences in susceptibility to environmental and disease conditions among these species can be very important to the success of the industry. This is particularly an issue in parasitic infestations, where specific identification of parasites is essential to inform choice of therapeutic agents and to evaluate methods for treatment, control, and prevention of future outbreaks (Stoskopf 1993; Hayward et al. 2007). Knowing individual pathogens can also play a role in monitoring biosecurity alerting staff when possible infectious agents have been translocated to a facility (Hayward et al. 2007). We identified the presence of, pathological changes associated with, and possible options for treatment of the monogenean, *Gamacallum macroura* (MacCallum and MacCallum 1913) Unnithan 1971, affecting white bass in an aquacultural setting.

**METHODS**

*Case history and husbandry.*—The Pamlico Aquaculture Field Laboratory of North Carolina State University (NCSU), located in Aurora, North Carolina, is a national provider of white bass and striped bass broodstock for the hybrid striped bass industry and conducts reproductive research. The facility’s water is mixed from two sources, a brackishwater creek (South Creek), and a 40-m-deep freshwater well. The salinity in holding tanks is generally maintained at 5–10 g/L during the winter months and at 2–5 g/L during the summer months, and general husbandry practices in the facility have been previously reported (Hodson and Sullivan 1993).

In 2007, staff biologists observed moderately high mortality of white bass after handling or spawning during the spring months. Almost all fish that died had severe gill pallor. Standard survival gill biopsies (i.e., removal of the tips of three to four gill filaments and scalp blade scrapings of the skin from the pectoral fin along the dorsum of the animal) were performed on survivors for microscopic examination of wet mount preparations. No ectoparasites were noted on microscopic examination. All animals at the facility were treated every 4–6 weeks, as deemed necessary by the staff biologists, presumptively with single low-dose formalin applications of 0.150 mL/L using 37% formaldehyde. Despite treatment, mortalities continued to occur. Mortalities were not observed in the striped bass broodstock. Both species of fish were kept on the same facility grounds and in the same parallel water flow-through system, although individual tanks were segregated by species. In April 2010, during the spawning season, affected white bass with pale gills were isolated into separate tanks from the general white bass population, where more concerted efforts were made to characterize the cause of mortalities.

Environmental water variables at the facility were managed to maintain a temperature of approximately 20°C, 90–100% dissolved oxygen saturation, and salinity of 2.9–5.0 g/L. Other water chemistry metrics were alkalinity = 120 mg/L, hardness = 684 mg/L, nitrite = 0.03 mg/L, ammonia = 0.2 mg/L, and pH = of 8.

**Sample collection and analysis.**—One day after spawning, six presumably affected white bass were selected from the tank, and six unaffected presumptively healthy individuals were selected from tanks on a different water system. Each individual was placed in a separate tub and immobilized with an overdose of eugenol, dosed to effect. Once immobile, a brief physical examination was performed and total length, weight, and sex were recorded. A subjective ordinal scale of gill pallor was used to assign a score for each fish. The scale assigned a 0 for normal red to pink gills and 1–4 for linearly escalated degrees of visually perceived pallor, 4 being pale tan to almost white gills. Gill pallor was scored by a single observer (author, EOC) to eliminate interobserver subjectivity in scaling the gill coloration. The animals were placed in dorsal recumbency for venipuncture of the caudal vein. Approximately 3 mL of blood was collected in heparinized syringes. Blood transferred to a heparinized hematocrit tube was centrifuged for 5 min at 7,000 rpm to determine packed cell volume (PCV). Thin blood smears were fixed and stained with a rapid hematology stain (Harleco Hemacolor, Gibbstown, New Jersey) and evaluated by a single observer (author, EOC) to establish differential and estimated total white blood cell (WBC) counts. Estimates of total WBC counts were determined by taking the average number of white blood cells in 10-high-power dry fields (400× magnification), and multiplying by 2,000 (Latimer and Bienzle 2000; Anderson et al. 2010). Absolute differential WBC counts were calculated by multiplying the WBC count times the percentage of each white blood cell type categorized by counting 100 cells under oil immersion (1,000×). A wet mount preparation from a skin scraping taken behind the pectoral fin and extending along the dorsum of the animal, was obtained for microscopic evaluation for ectoparasites to rule out any other factors contributing to mortalities.

The anesthetized fish were euthanized by cranial concussion. A full gross necropsy was performed, evaluating the GI tract, swim bladder, liver, gallbladder, spleen, gonads, muscle tissue, skin, dorsal fin, and anterior and posterior kidney. All gill arches of each fish were removed bilaterally, examined, and evaluated under a dissecting microscope. Each individual gill arch was examined, and the number of monogeneans observed on the lamellae and filaments of the gills was recorded for each fish. Multiple monogeneans found on the gills were collected and preserved in neutral buffered formalin for later identification.

Subsamples of gill, liver, anterior kidney, and spleen from each fish were placed in 10% neutral buffered formalin,
processed routinely, embedded in paraffin, sectioned at 5-μm, and stained with haematoxylin and eosin at the College of Veterinary Medicine Histology Laboratory, NCSU. Histologic slides were examined by a board-certified veterinary pathologist experienced with fish pathology (JML), blinded to fish group.

To provide comparative information, 12 clinically unaffected adult striped bass housed in the same facility and water flow-through system as the clinically unaffected white bass, were euthanized using cranial concussion. All gill arches of each individual fish were removed bilaterally, examined, and evaluated under a dissecting microscope for ectoparasites.

Monogenean identification.—Worms were stained in Harris hematoxylin (Ricca Chemical Co., Arlington, Texas), cleared in xylene and mounted with Permount (Thermo Fisher Scientific, Inc., Waltham, Massachusetts). Identifications were based on published descriptions and keys (MacCallum and MacCallum 1913; MacCallum and MacCallum 1913; Tripathi 1954; Unnithan 1971; Hendrix 1994). Also, voucher specimens of nominal species of *Microcotyle* from the U. S. National Parasite Collection (USNPC) (*Microcotyle eueides* – 36517; *Microcotyle macroura* – 36524, 36525, 36529; *Microcotyle lineatus* – 36526, 36530) and the H.W. Manter Laboratory of Parasitology (*Microcotyle australiensis* – 1434; *Microcotyle pomatomi* – 1435; *Microcotyle spinicirrus* – 20495; *Neomicrocotyle pacifica* – 39538; *Polymicrocotyle manteri* – 39542; *Protomicricotyle nayaritensis* – 39548) were examined for comparison. Specimens from current study have been submitted to USNPC, accession number 105810.00, storage number SH231:6-87/97.

Monogenean classification is that of Hoffman (1999, page 95), while morphological terminology and species designation and spelling is that of Hendrix (1994). Fish host names follow that of Nelson et al. (2004), and Burgess (1980a, 1980b, 1980c).

Statistical analysis.—Continuous data were tested for normal distribution by the Shapiro–Wilk *W* test. Because some continuous data were not normally distributed, and other data were ordinal, nonparametric methods were employed throughout. Estimated WBC count, PCV, and monogenean parasite load were compared between pale-gilled and apparently healthy fish by the Wilcoxon rank-sums test (Hollander and Wolfe 1973). Association of PCV with gill pallor scores was assessed using the Jonkhoeve test of ordered alternatives (Hollander and Wolfe 1973). Association of PCV with monogenean parasite load was tested with the Spearman rho test (Hollander and Wolfe 1973). Statistical analyses were performed with a commercial software package (JMP, SAS Institute Inc., Cary, North Carolina), except for the Jonkheere test, which was performed manually (Hollander and Wolfe 1973). Statistically significance was established at $\alpha = 0.05$.

RESULTS

Clinical Pathology and Gill Pallor

Gill pallor scores, PCV, estimated WBC counts, and monogenean numbers are presented in Table 1. The PCV between fish with gill pallor and apparently healthy fish did not differ significantly ($P = 0.07$), but degree of gill pallor was significantly associated with decreased PCV ($P = 0.007$). Packed cell volume was negatively associated with parasite load ($P = 0.04$), and fish with pale gills had a greater parasite load than apparently healthy fish ($P = 0.001$). Fish with pale colored gills had a significantly lower estimated WBC values ($P = 0.02$). There were no significant differences in white blood cell differential counts between the two groups of fish (neutrophils, $P = 0.6$; lymphocytes, $P = 0.2$; monocytes, $P = 0.2$). Clinical signs and mortalities were noted only in white bass, never in striped bass. Monogeneans were detected on all of the 12 white bass. No other ectoparasites were observed on either gill clippings or skin scrapings and wet mount preparations from any white bass sampled. No monogeneans or other ectoparasites were observed on the 12 striped bass examined.

Monogeneans

The monogeneans collected during our investigation were determined to be *G. macroura*, but at $\leq 4.5$ mm, the specimens were shorter than previously described specimens (12 to 15 mm; MacCallum and MacCallum 1913; Figures 1–3).

Our specimens possessed characteristics diagnostic for *G. macroura* (MacCallum and MacCallum 1913; Unnithan 1971: (1) a distinct bilaterally symmetric haptor with 20–25 clamps in a single row on each side of the haptor, (2) the middle clamps are larger than either the anterior or posterior clamps, (3) vaginal opening is dorsomedial, unarmed and genital atrium is ventromedial, unarmed, (4) male copulatory organ is armed with few chitinous spicules, and (5) intestine diverticulae extends into the haptor (MacCallum 1913; MacCallum and MacCallum 1913; Tripathi 1954; Unnithan 1971; Hendrix 1994).

Members of the genus *Pauiciconibula* Dillon and Hargis 1965 most closely resemble *G. macroura* by having unarmed vaginal
and genital opening and middle haptoral clamps larger than the posterior or anterior clamps (Tripathi 1954). However, members of *Pauciconfibula* lack an armed copulatory organ and have a clamp arrangement dissimilar to *G. macroura*. For example, *Pauciconfibula subsolana* (Chisholm et al. 1991) found on white perch *Morone americana* has 2 rows of clamps on each side of the haptor with 9–13 clamps on each side (Chisholm et al. 1991); *Pauciconfibula pogoniae* (MacCallum 1913; Chisholm et al. 1991) from black drum *Pogonias cromis* has 2 rows of clamps on each side of the haptor but with approximately 54 clamps each side (MacCallum 1913); and *Pauciconfibula trachini* (Parona and Perugia 1890; Dillon and Hargis 1965) from the starry weever *Trachinus radiatus* has only 8–10 clamps in a single row on each side of the haptor (Parona and Perugia 1890).

This fifth primary report on *G. macroura* (others being MacCallum and MacCallum 1913; MacCallum 1913; Zwerner and Lawler 1972; Paperna and Zwerner 1976) adds new host (white bass) and locality (Aurora, North Carolina) records. However, it seems that white bass is a susceptible but dead-end host for *G. macroura* because no mature specimens were observed. Of 76 mounted specimens, only 2 were over 4 mm long, had 20 pairs of clamps, fully developed organs (but with only a few productive vitelline follicles), and a minor amount of yolk in the vitelline reservoir. Of the 76 specimens 27 (36%) were from 1 mm to 3.3 mm long with 8–18 pairs of clamps. The smallest specimens (34%) were less than 0.5 mm long, and had only 2–3 pairs of clamps. Furthermore, the larval haptoral anchors were retained by these smallest specimens but were shed by specimens that had developed 4 pairs of clamps.

**Pathology and Histopathology**

At necropsy, no remarkable macroscopic internal abnormalities were noted in any of the examined white bass or striped bass. Microscopic lesions were limited to the gills (Figures 4–6). In histologic sections from the pale-gilled fish, multifocal areas of the lamellae were thickened by moderate to marked epithelial hyperplasia, often with lamellar atrophy and (or) fusion, along with variable infiltrates of mixed mononuclear inflammatory cells and edema (branchitis, Figures 4, 5). In some, lamellar capillaries were occluded by fibrin and nuclear debris, indicating thrombus formation. We found *G. macroura* were found firmly attached to the gills, apparently by grasping lamellae with the parasite’s many haptoral clamps (Figure 4). Tissue injury at the attachment sites was evident, with loss of scattered lamellae and erosion and thickening of the underlying epithelium by similar inflammatory changes (described above) and variable amounts of reactive epithelial proliferation and limited granulation tissue formation. The entire group of pale-gilled fish had subjectively increased numbers of lamellar aneurysms and thrombi,
FIGURE 4. Hematoxylin- and eosin-stained section of a white bass gill with *Gamacallum macroura* shown attached to a gill filament by grasping a focal area of the lamellae via haptoral claspers. Damage around the attachment site is evident because this filament is focally denuded of most lamellae. The underlying epithelium is multifocally eroded and is moderately thickened by edema and scattered mononuclear inflammatory infiltrates, along with mild proliferation of new blood vessels and connective tissue matrix (granulation tissue). Several adjacent lamellae show mild to moderate epithelial hyperplasia and inflammatory infiltrates. Note, however, that most lamellae away from the attachment site as well as on other filaments are essentially normal.

indicating focal capillary damage with rupture of supporting pillar cells (Figure 6). There were also many gill filaments that were essentially normal; these filaments presumably had either not experienced injury due to parasite attachment or had undergone complete restitution and healing by the time of examination. Clinically unaffected fish had only minimal to mild pathologic changes of the gill lamellae, observation of *G. macroura*,

FIGURE 5. Hematoxylin- and eosin-stained section of a gill from a pale-gilled white bass. Lamellae are focally thickened and fused together due to reactive epithelial hyperplasia and inflammatory infiltrates, presumably due to parasite-induced injury.

FIGURE 6. Hematoxylin- and eosin-stained section of gill with a focal lamellar aneurysm from a pale-gilled white bass.

lamellar aneurysms, and thrombi being rarely seen or seen in less frequency on histology.

In all fish, no parasites were observed in any internal organs. Histologic sections of liver, spleen, and anterior kidneys were considered to be within normal limits. The livers of all fish had moderate hepatocellular macroversicular vacuolation, consistent with lipid storage and considered a normal finding in fish on production feeds (Noga 2010). Splenic pigmented macrophage aggregates were found in all of the animals and were also considered to be within normal limits. No other remarkable pathologic changes were observed.

DISCUSSION

Pathology

Previously, *G. macroura* has been reported only from striped bass (MacCallum and MacCallum 1913; Paperna and Zwerner 1976; Chisholm et al. 1991; Hendrix 1994), no studies reporting parasite-associated pathology. This is not unexpected because many polyopisthocotyleans have been regarded as non-pathogenic, host-specific parasites (Rubio-Godoy 2007; Noga 2010). Interestingly, while white bass in our study demonstrated gill injury from monogeneans, no *G. macroura* were observed from the 12 striped bass that shared the same flow-through water with infected white bass. Also, no striped bass within the facility demonstrated suspicious clinical signs. Being valuable broodstock, we were allowed to necropsy only a few striped bass, which did not assure detection of infections at a low prevalence or intensity. Ultimately, the original source of infection of *G. macroura* at the facility is unknown.

In our investigation, *G. macroura* were associated with anemia and gill pathology in white bass. Although many polyopisthocotyleans have been regarded as nonpathogenic (Rubio-Godoy 2007; Noga 2010), some have been reported to cause pathology and even mortality in fish (Kim et al. 2000; Kim
Microcotyle pomatomi can cause a hypochromic microcytic anemia and decreased gill function in gilthead seabream Sparus aurata within 5–10 weeks of infection (Sitjà-Bobadilla and Alvarez-Pellitero 2009). Affected fish have been documented with mortalities occurring in high-stress situations. Microcotyle sebastis is a major cause of mortalities in juvenile Korean rockfish Sebastes schlegeli in the Korean aquaculture industry (Kim et al. 2000). Microcotyle pomatomi has been reported to cause mortality in bluefish Pomatomus saltatrix and striped bass (Buckel and McKown 2002). Microcotyle mulgilis causes increased mortalities during the summer in Greek mullet aquaculture facilities (Ragias et al. 2005), which agrees with our investigation, where mortalities in white bass were more pronounced in the spring and summer months.

The white bass we examined with gill pallor exhibited a lower estimated WBC count than the nonclinically affected fish. This may be in part due to hematopoietic exhaustion from chronic infection and parasite infestation, although other species of fish have been documented to have an increased WBC count in the face of monogenean infections (Sitjà-Bobadilla and Alvarez-Pellitero 2009). Gilthead seabream infected with Sparicotyle chrysophrii displayed increases in neutrophils, eosinophils, and plasma cells, suggesting infected fish increased production of specific antibodies to the parasite (Sitjà-Bobadilla and Alvarez-Pellitero 2009).

Similar microscopic lesions have been described with other microcotylid species infections (Rubio-Godoy 2007; Sitjà-Bobadilla and Alvarez-Pellitero 2009). Gill pathology was described as secondary lamellar fusion, lamellar hemorrhage, epithelial hyperplasia, and increased mucus production. (Dezfuli et al. 2007; Rubio-Godoy 2007; Sitjà-Bobadilla and Alvarez-Pellitero 2009). Concussive force used as the method for euthanasia of fish may also have created telangiectasia, due to transient spikes in blood pressure from the concussive blow (Crespo et al. 1988). However, in the pale-gilled fish, we observed remnants of nuclear debris and fibrin in the affected lamellae, indicating thrombus formation. These findings indicate ante-mortem and not peri-mortem lesions from concussive force. The possibility of some telangiectasia resulting from concussive force cannot be ruled out in our study, however.

Polyplastoschotyle and monoplastoschotyle monogeneans have been documented to have certain attachment site predilections on the host (Rubio-Godoy 2007; Sitjà-Bobadilla and Alvarez-Pellitero 2009). We observed G. macroura at the intersection of the filament and gill arch. This location was probably the reason why the monogeneans were not diagnosed on routine shallow gill clippings and wet-mount preparations performed by staff biologists. A deeper more aggressive and lethal collection of gill clippings facilitated detection of G. macroura on the gills.

Treatment

Although treatment of G. macroura has not been described in the literature, treatment of other monogeneans species in the Microcotylidae family has been documented. Praziquantel immersion baths at 100 mg/L for 4 min and praziquantel-adsorbed food at 0.003 mg/kg of fish q 48 h for 7 d (20 g/kg of feed q 48 h at 1% body weight) were both effective in reducing the numbers of Microcotyle sebastis on rockfish in Korean net-pen aquaculture (Kim et al. 2000). This method may prove effective in large aquaculture settings such as hybrid striped bass operations.

Monogeneans

Gamacallum macroura is a polyplastoschotyle monogenean belonging to Microcotylidae. Members of this group are oviparous with direct life cycles and feed on the blood of their fish hosts (Hoffman 1999). There are approximately 74 microcotylid species, many of the species infecting marine hosts (Hoffman 1999).

The salinity preferences of G. macroura have not been fully described, but its primary host species, striped bass, can tolerate a wide range of salinities (Chisholm et al. 1991). G. macroura have been reported on striped bass in the lower Chesapeake Bay and along the Atlantic coastline (MacCallum and MacCallum 1913; MacCallum 1913; Zwerner and Lawler 1972; Paperna and Zwerner 1976). Based on observations in the current investigation, it appears that G. macroura may be an euryhaline species because the facility’s water was kept at a salinity ranging from 2 to 5 g/L, but salinity tolerances of the parasite were not tested.

In conclusion, increased G. macroura parasites located at the cartilaginous base of the gills of white bass were associated with gill pallor, anemia, severe gill pathology, and lower estimated WBC values than fish with lower numbers of parasites. No mature parasites were observed on the white bass. If in fact, the parasite is unable to mature on white bass then the source for pathogenic levels of the parasite population is still unidentified. However, we suspect that low-grade infections in the natural but pathologically resistant striped bass may be the source.

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Impact of Thiamine Deficiency on T-cell Dependent and T-cell Independent Antibody Production in Lake Trout


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Impact of Thiamine Deficiency on T-cell Dependent and T-cell Independent Antibody Production in Lake Trout

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Abstract
Lake trout Salvelinus namaycush on thiamine-replete and thiamine-depleted diets were evaluated for the effects of thiamine status on in vivo responses to the T-dependent antigen trinitophenol (TNP)-keyhole limpet hemocyanin (TNP-KLH), the T-independent antigen trinitrophenol-lipopolysaccharide (TNP-LPS), or Dulbecco’s phosphate-buffered saline (DPBS; negative control fish). Plasma antibody concentrations were evaluated for possible differences in total anti-TNP activity as well as differences in response kinetics. Associations between anti-TNP activity and muscle and liver thiamine concentrations as well as ratios of muscle-to-liver thiamine to anti-TNP activity were also examined.

Thiamine-depleted lake trout that were injected with TNP-LPS exhibited significantly more anti-TNP activity than thiamine-replete fish. The depleted fish injected with TNP-LPS also exhibited significantly different response kinetics relative to thiamine-replete lake trout. No differences in activity or kinetics were observed between the thiamine-replete and -depleted fish injected with TNP-KLH or in the DPBS negative controls. Anti-TNP activity in thiamine-depleted lake trout injected with TNP-KLH was positively associated with muscle thiamine pyrophosphate (thiamine diphosphate; TPP) concentration. A negative association was observed between the ratio of muscle-to-liver TPP and T-independent responses. No significant associations between anti-TNP activity and tissue thiamine concentration were observed in the thiamine-replete fish. We demonstrated that thiamine deficiency leads to alterations in both T-dependent and T-independent immune responses in lake trout.

Diet and nutritional status are known to affect immune function and disease resistance in fish (Lall 2000; Balfry and Higgs 2001). The immunomodulatory role of specific vitamins and their effects on disease resistance in fishes has been examined in some detail. Ascorbic acid (vitamin C; Waagbø et al. 1992; Dunier et al. 1995; Sahoo and Mukherjee 2003; Tewary and Patra 2008) and the tocopherols (vitamin E; McCoy et al. 1994; Puangkaew et al. 2004) have received considerable attention. Although the effect of thiamine (vitamin B1) deficiency has been investigated in respect to salmonid embryo survival (McDonald et al. 1998; Hill and Nellbring 1999; Honeyfield et al. 2005), disease resistance and immune function have not been studied.

Early mortality syndrome (EMS) results in embryonic mortality in salmonids (coho salmon Oncorhynchus kisutch, Chinook salmon O. tshawytscha, steelhead O. mykiss, brown trout Salmo trutta, and lake trout Salvelinus namaycush).

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Clinical symptoms of EMS include loss of equilibrium, swimming in a spiral pattern, lethargy, hyperexcitability, and hemorrhage and death occurring between hatch and first feeding (Marquenksi and Brown 1997; McDonald et al. 1998). Stocks of Atlantic salmon *Salmo salar* from the Finger Lakes, New York, and the Baltic Sea exhibit a similar early life stage mortality, called Cayuga syndrome (Fisher et al. 1996) and M74 syndrome (Børjeson and Norrgren 1997; Brown et al. 2000), respectively. Low egg thiamine levels and enhanced survival following thiamine treatments are common characteristics of EMS, Cayuga Syndrome, and M74 syndrome. Incidence of Baltic M74 syndrome continues to be problematic, as does EMS in the Laurentian Great Lakes basin.

Despite this low apparent mortality from EMS, virtually no recruitment of wild or unmarked lake trout in Lake Michigan has been observed (Czesny et al. 2009). One possible cause for this continued lack of recruitment might be physiological impairments due to thiamine deficiency resulting in significantly higher indirect latent mortalities. Brain lesions have been found in some thiamine-deficient lake trout fry that survived EMS (Brown and Honeyfield 2001) but not in others (Jaroszewiska et al. 2009; Lee et al. 2009). Carvalho et al. (2009) reported that visual detail discrimination and motion detection in lake trout fed a low thiamine diet were significantly lower than those in thiamine-replete lake trout fry. In addition, the ability of lake trout fry to avoid a piscine predator and their ability to forage on *Daphnia* are negatively affected by low thiamine status (Fitzsimons et al. 2009). Combined, these reports indicate that thiamine deficiency in lake trout fry can adversely impact physiological function, which could lead to an increase in mortality.

The physiological impacts of thiamine deficiency are likely to be far reaching and it is not known if later life stages are negatively affected (Brown et al. 2005). Early mortality syndrome is a result of thiamine deficiency in the sexually mature component of the lake trout populations. Thiamine pyrophosphate (thiamine diphosphate; TPP) is a required cofactor for enzymes involved in multiple physiologic pathways including those leading to ATP synthesis (Agyei-Owusu and Leeper 2009). Thiamine depletion can significantly reduce the activity of the enzymes transketolase, alpha-ketoglutarate dehydrogenase, glucose-6-phosphate dehydrogenase, and cytochrome p450 1A in Baltic Atlantic salmon sac fry (Amcoff et al. 2000). Given that adult lake trout experience thiamine deficiency, as evidenced by the occurrence of EMS, two central questions need to be asked. Which (if any) physiologic pathways, functionally altered by thiamine depletion in a laboratory setting, could contribute to reductions in survival probabilities? Do wild thiamine-depleted adult lake trout experience reduced survival probabilities as a result of one or more of the identified thiamine-dependent physiologic impairments? In this study we begin to address the first of these two questions.

Pathogen-appropriate immune responses that lead to disease resistance are critical to vertebrate survival. Antibody production (humoral immunity) is one of several functional components within the teleost immune-response repertoire known to contribute to bacterial (Swain et al. 2008), viral (Workenhe et al. 2010), and parasitic (Alvarez-Pellitero 2008; Sijá-Bobadilla et al. 2008) disease resistance. Here we evaluated antibody production to T-cell dependent and T-cell independent antigens and examined possible interactions between these responses and concentrations of specific forms of thiamine quantified from skeletal muscle and liver.

The examinations of antibody responses to T-dependent and T-independent antigens were used during the characterization of mammalian humoral immunity (Rittenberg and Amkraut 1966; Jacobs and Morrison 1975) and subsequently for similar characterizations in fish (Etlinger et al. 1979; Arkoosh and Kaattari 1991; Vallejo et al. 1991, 1992; van Ginkel et al. 1992; Killie and Jøgenson 1994; Lam et al. 2004). In mammals, the major difference in the response to T-dependent and T-independent antigen is the participation of T helper (Th) cells. In the former, Th participation is a hallmark of this type of response but is unnecessary in the latter. Response to T-independent antigens may include the participation of other T-cell types as well as other immune cells such as natural killer cells (Mond et al. 1995). Both T-dependent and T-independent responses are well characterized in mammalian models and major differences in response kinetics, modes of isotype switching, and development of immunologic memory as well as in the participating cell types and cytokines have been demonstrated (Parker 1993; Mond et al. 1995; Constant and Bottomly 1997). Responses in fish are similar to those in mammals when allowances are made for ectothermic physiology (Le Morvan et al. 1998) and a less diverse immunologic repertoire (Bird et al. 2006; Whyte 2007; Flajnik and Kasahara 2010; Hirano et al. 2011). Analysis of thiamine-depletion effects on lake trout antibody production using both T-dependent and a T-independent antigen allowed us to address our primary question regarding effects on antibody production and potentially provides indications toward more refined questions on the role of thiamine depletion in immune function. The latter use of our data was made most robust by contrasting the observed responses to both antigen types.

**METHODS**

*Fish origin and maintenance.*—Seneca strain lake trout were spawned, hatched, and reared at the U.S. Geological Survey (USGS), Leetown Science Center, Northern Appalachian Research Laboratory, Wellsboro, Pennsylvania, using thiamine-replete broodstock and egg handling methods that have been previously described (Honeyfield et al. 2005). Adult lake trout were maintained in either 182-cm circular tanks or 58 × 305-cm rectangular tanks supplied with 17–19 L/min of oxygenated well water. Tank illumination was provided by ambient light supplemented by fluorescent fixtures. Fish maintenance and spawning were performed following standard hatchery practices (Piper et al. 1982). Adult fish were fed...
commercial feed. Fertilized eggs were incubated in Heath-style trays provided with 9°C oxygenated well water. Feeding fry and juvenile fish were reared using the same housing conditions and maintenance methods described for the adults. A minimum fish size of 100 g was selected for use in this study to provide ample blood plasma for antibody analysis and to shift our focus from fry to a later life stage. Fish were reared to approximately 100 g on commercial feed and then randomly divided into two groups. Thiamine deficiency was achieved in the thiamine-deficient group via delivery of a casein bacterial thiaminase diet (CBT; Table 1; Honeyfield et al. 2005). Thiamine-deficient status was confirmed by skeletal muscle levels in treatment subsamples (Brown et al. 1998). Fish were on the CBT diet for 12 weeks at which time mortalities began to be observed and muscle total thiamine concentrations were at or below 500 pmol/g. Once a thiamine-depleted state was achieved, the fish were fed a similar diet (ACBT 0.4; Table 1) without thiaminase and containing 0.4 mg/kg dietary thiamine, which was sufficient to prevent death while retaining the thiamine-depleted state. Thiamine-replete fish were fed the ACBT diet with 2.0 mg/kg thiamine (ACBT 2.0; Table 1) through the entire study. The thiamine-deficient diet was administered on a twice weekly basis to prevent death while retaining the thiamine-depleted state. Thiamine-replete fish were fed the ACBT diet with 2.0 mg/kg thiamine (ACBT 2.0; Table 1) through the entire study. The

### TABLE 1. Composition of casein bacterial thiaminase (CBT) diet, thiamine-replete (ACBT 2), and marginal thiamine (ACBT 0.4) diets fed to lake trout.

<table>
<thead>
<tr>
<th>Diet ingredients (g/kg unless otherwise noted)</th>
<th>CBT</th>
<th>ACBT 2</th>
<th>ACBT 0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>410</td>
<td>410</td>
<td>410</td>
</tr>
<tr>
<td>Gelatin</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Herring meal (70%)</td>
<td>86</td>
<td>86</td>
<td>86</td>
</tr>
<tr>
<td>Menhaden fish oil</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Starcha</td>
<td>150</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>Dextrin</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin premixa</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral premixc</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ascorbyl-2-polyphosphate</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Betaine HCl</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Sand</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Thiamine HCl (mg/kg)</td>
<td>0.0</td>
<td>2.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Bacterial thiaminasee</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

aMira-gel 463, Skidmore Sales, West Chester, Ohio.
bVitamin premix supplied units per kilogram of diet: vitamin A, 6,250 IU; vitamin D, 6,000 IU; vitamin E, 125 IU; vitamin K, 13.75 mg; biotin, 0.375 mg; vitamin B12, 0.025 mg; folic acid, 5.0 mg; niacin, 25 mg; pantothenate, 50 mg; pyridoxine, 15.0 mg; riboflavin, 17.5 mg.
cMineral premix (Bernhart-Tomarelli, Dyets Inc.) supplied mg/kg of diet: calcium carbonate, 630; calcium phosphate, 22,050; citric acid, 68.1; cupric citrate 2H2O, 13.8; ferric citrate 3H2O, 1,674; magnesium oxide, 780; manganese citrate, 250.5; potassium iodide, 0.3; potassium phosphate dibasic, 2,430; potassium sulfate, 2,040; sodium chloride, 918; sodium phosphate, 64.2; zinc citrate 2H2O, 39.9.
dStay-C, 1% l-ascorbyl-2-polyphosphate.

Immune function studies were conducted at the USGS, Leetown Science Center, Fish Health Branch, Kearneysville, West Virginia. During these studies the trout were maintained in an indoor flow-through system consisting of 900-L circular tanks supplied with 12°C oxygenated spring water. Water flow into the tanks was maintained at rate of 10 total volume replacements per day. Tanks were illuminated by fluorescent lighting with photoperiod adjusted to match local seasonal change. Trout were fed daily to satiation with either ACBT 2 or ACBT 0.4 diets. Prior to handling and depending on experimental requirements, trout were either anesthetized or euthanized with tricaine methanesulfonate (Finquel, Argent Chemical Laboratories, Redmond, Washington) immediately after removal from the tanks. The lake trout were acclimated to tank conditions for at least 2 months prior to initiation of experimental protocols. No morbidity or mortality was observed during the acclimation or experimental periods.

**In vivo antibody production.—**Unless otherwise stated, all reagents were obtained from Sigma Chemical (St. Louis, Missouri). Trinitrophenol (TNP) was conjugated to keyhole limpet hemocyanin (KLH; Rittenberg and Amkraut 1966) and lipopolysaccharide (LPS; E. coli O111:B4; Jacobs and Morrisson 1975). Final dialysis of both the TNP-KLH and TNP-LPS antigens was performed against Dulbecco’s phosphate buffered saline (DPBS). The replete and depleted lake trout were injected with 100 µg of TNP-KLH, 100 µg TNP-LPS, or with a volume of DPBS (negative control) equivalent to that of the TNP-KLH and TNP-LPS as described by Arkoosh and Kaattari (1987). The antigens and DPBS were mixed with an equal volume of Freund’s complete adjuvant and were administered as intracoelomic injections at a final volume of 200 µL. After injection, the lake trout were returned to the 900-L tanks; fish were segregated into six separate tanks according to thiamine status and antigen (one tank per thiamine status and antigen group). Fish were stocked into the final tanks at a stocking density of 40 fish per tank, which was about one-third the stocking density used during the tank acclimation period. Fish were maintained on thiamine-specific diets and fed as described above.

Blood samples were obtained from thiamine-replete and -depleted lake trout at 5 and 12 weeks after injection. Twenty fish were sampled from each of the six thiamine status and antigen groups at each time point. On each sample date fish were euthanized as described above and bled with heparinized syringes. Liver and muscle samples were obtained aseptically and were immediately frozen at −80°C for later tissue thiamine determinations as described below. Plasma was separated from whole blood by centrifugation at 1,000 × g at 4°C for 20 min and stored at −80°C. Processing during both weeks 5 and 12 was performed over a 2- to 3-d period with thiamine-replete and thiamine-depleted fish that had received the same antigens processed on the same day. The time period for sample processing varied with the injection date.
collection was used to accommodate staff scheduling and was not a part of the intrinsic experimental design.

The anti-TNP enzyme-linked immunosorbant assay (ELISA) was performed following a modification of the methods described by Kaattari and Yui (1987). Plates were processed under low light condition throughout the assay and all plate washing was performed using a Skan Washer 400 (Molecular Devices Corporation, Sunnyvale, California). Trinitrophenol was conjugated to bovine serum albumin (BSA) following the methods cited above for the conjugation to KLH. Ninety-six well plates were loaded with 50 µL/well of a 15-µg/mL solution of TNP-BSA in coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6). Plates were incubated for 2 h at 37°C in a sealed container and then washed three times with 300 µL/well of tris-buffered saline (50 mM trizma-base, 1.5 mM ethylenediaminetetraacetic acid, 154 mM sodium chloride, pH 8.0) containing 0.1% Tween 20 (TBST). Plates were blocked for 2 h at 37°C in a sealed container with 300 µL/well TBST containing 1% fetal bovine serum (blocking buffer). Blocking buffer was removed from plates without additional washing, and 50 µL/well of plasma samples from weeks 5 and 12, serially diluted pooled lake trout plasma from fish previously injected with TNP-KLH (standard), and blocking buffer only (negative control) were loaded into the plates. The primary plasma dilutions were 1:30 and subsequent serial dilutions were 1:3. All dilutions were made in blocking buffer. Plates with plasma dilutions were incubated overnight at 15°C in a sealed container. After the overnight incubation, plates were washed three times as above and then loaded with 50 µL/well of a 1:70 dilution of anti-trout immunoglobulin monoclonal antibody (catalog F11, Microtek International, Saanichton, British Columbia) in blocking buffer. Plates were covered and incubated for 2 h at room temperature. Plates were washed three times as above and then loaded with 50 µL/well of a 1:1,000 dilution in blocking buffer of goat antimouse immunoglobulin G conjugated with horseradish peroxidase (code 115-035-146, Jackson Immuno Research Laboratories, West Grove, Pennsylvania), covered, and incubated for 1 h at room temperature. Plates were washed five times with 300 µL/well TBST and then 50 µL/well of ABTS substrate solution (4.8 mL of 10 mM citrate buffer, pH 4, with 200 µL of 18.2 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) was added. Plates were read using a 405-nm wavelength after 45 min incubation at room temperature using a V-max kinetic microplate reader (Molecular Devices Corporation). The same standard and negative control were included in each plate as a quality control measure. All samples, the standard, and the negative control were replicated in duplicate. The standard x-intercept was defined as the point on the standard titration curve at which the optical density half-maximum occurred and was assigned a unit value of 1,000. Sample antibody activity was expressed as the x-intercept derived from the intersect point between the sample titration curve and the standard curve optical density half-maximum value. Sample antibody activity was expressed relative to the standard unit value of 1,000.

 RESULTS

Total thiamine concentrations from each treatment group are reported to provide comparisons with previously published values (Table 2). Average weights of fish in each treatment group at each sample time are also provided to facilitate comparisons with other our future studies (Table 3). Plasma samples from four fish were not evaluated for antibody activity due to problems with postthaw quality or quantity. Since only complete data sets were considered, sample sizes for the week-5 DPBS thiamine-depleted group, week-5 TNP-LPS thiamine-depleted group, week-12 DPBS thiamine-replete group, and the week-12 TNP-KLH thiamine-replete group were reduced from 20 to 19.

At both weeks 5 and 12 postinjection, thiamine-depleted fish exhibited significantly lower concentrations of both muscle and liver TPP and significantly lower muscle-to-liver TPP ratios in all injection groups (Figure 1). At week 5 postinjection, thiamine-replete fish injected with TNP-KLH and TNP-LPS exhibited significantly lower muscle TPP than the DPBS-injected controls (Figure 2). In this same group at week 5, fish injected with TNP-LPS exhibited significantly lower liver TPP than the TNP-KLH- or DPBS-injected groups and both the TNP-KLH- and TNP-LPS-injected groups exhibited significantly lower muscle-to-liver TPP ratios than the fish injected with DPBS (Figure 2). At week 12 postinjection, the muscle...
TABLE 2. Concentrations of total thiamine in lake trout skeletal muscle and liver expressed by thiamine status, antigen treatment group, and time after antigen injection.

<table>
<thead>
<tr>
<th>Week postinjection</th>
<th>Thiamine Status</th>
<th>Treatment or control a,b</th>
<th>Muscle median; maximum, minimum (pmol/g)</th>
<th>Liver median; maximum, minimum (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Replete</td>
<td>DPBS</td>
<td>2,166; 3,820,1,569</td>
<td>13,085; 16,456, 9,564</td>
</tr>
<tr>
<td>5</td>
<td>Depleted</td>
<td>DPBS</td>
<td>490; 858, 379</td>
<td>5,804; 8,668, 4,601</td>
</tr>
<tr>
<td>5</td>
<td>Replete</td>
<td>TNP-KLH</td>
<td>1,425; 2,296, 876</td>
<td>12,460; 13,617, 6,424</td>
</tr>
<tr>
<td>5</td>
<td>Depleted</td>
<td>TNP-KLH</td>
<td>516; 1,167, 307</td>
<td>5,237; 7,438, 3,984</td>
</tr>
<tr>
<td>5</td>
<td>Replete</td>
<td>TNP-LPS</td>
<td>1,459; 2,024, 1,140</td>
<td>11,167; 14,689, 8,664</td>
</tr>
<tr>
<td>5</td>
<td>Depleted</td>
<td>TNP-LPS</td>
<td>453; 722, 319</td>
<td>5,270; 7,506, 3,640</td>
</tr>
<tr>
<td>12</td>
<td>Replete</td>
<td>DPBS</td>
<td>1,047; 1,670, 752</td>
<td>8,750; 12,004, 4,040</td>
</tr>
<tr>
<td>12</td>
<td>Depleted</td>
<td>DPBS</td>
<td>441; 569, 318</td>
<td>4,939; 7,274, 3,758</td>
</tr>
<tr>
<td>12</td>
<td>Replete</td>
<td>TNP-KLH</td>
<td>1,288; 1,997, 866</td>
<td>11,643; 14,229, 5,891</td>
</tr>
<tr>
<td>12</td>
<td>Depleted</td>
<td>TNP-KLH</td>
<td>369; 640, 202</td>
<td>4,091; 6,803, 3,894</td>
</tr>
<tr>
<td>12</td>
<td>Replete</td>
<td>TNP-LPS</td>
<td>1,180; 1,901, 830</td>
<td>8,895; 12,564, 5,436</td>
</tr>
<tr>
<td>12</td>
<td>Depleted</td>
<td>TNP-LPS</td>
<td>420; 606, 275</td>
<td>4,459; 5,907, 3,786</td>
</tr>
</tbody>
</table>

aDPBS: Dulbecco’s phosphate-buffered saline; TNP-KLH: trinitrophenol conjugated to keyhole limpet hemocyanin; TNP-LPS: trinitrophenol conjugated to lipopolysaccharide.

bSamples sizes (n) by sample week were as follows:
Week 5: replete DPBS, n = 20; depleted DPBS, n = 19; replete TNP-KLH, n = 20; depleted TNP-KLH, n = 20; replete TNP-LPS, n = 20; depleted TNP-LPS, n = 19.
Week 12: replete DPBS, n = 19; depleted DPBS, n = 20; replete TNP-KLH, n = 19; depleted TNP-KLH, n = 20; replete TNP-LPS, n = 20; depleted TNP-LPS, n = 20.

TTP and liver TTP concentrations observed in the DPBS- and TNP-LPS-injected replete fish were significantly lower than that of the TNP-KLH-injected fish (Figure 2). Muscles to liver TTP ratios in the thiamine-replete fish at week 12 were not significantly different in the DPBS, TNP-KLH, and TNP-LPS groups (Figure 2). Thiamine-depleted fish exhibited no significant differences between the DPBS, TNP-KLH, and TNP-LPS groups at week 5 postinjection (Figure 3). At week 12, the depleted fish did exhibit significantly higher muscle TTP in the TNP-LPS group relative to both the DPBS and TNP-KLH groups, significantly higher liver TTP in the DPBS group relative to the TNP-KLH group, and significantly lower muscle-to-liver TTP ratios in the DPBS group relative to both the TNP-KLH and TNP-LPS groups (Figure 3).

TABLE 3. Weight of thiamine-replete and thiamine-depleted lake trout 5 and 12 weeks after injection with Freund’s complete adjuvant mixed with trinitrophenol (TNP)-conjugated keyhole limpet hemocyanin (KLH), TNP-conjugated lipopolysaccharide (LPS), or with Dulbecco’s phosphate-buffered saline (DPBS). Values are expressed as mean ± SE. See Table 2 for sample sizes for treatment and week.

<table>
<thead>
<tr>
<th>Thiamine Status</th>
<th>Injection group</th>
<th>Week 5 weight (g)</th>
<th>Week 12 weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Replete</td>
<td>DPBS</td>
<td>299.5 ± 18.0</td>
<td>303.0 ± 15.9</td>
</tr>
<tr>
<td>Depleted</td>
<td>DPBS</td>
<td>356.1 ± 25.6</td>
<td>396.4 ± 24.3</td>
</tr>
<tr>
<td>Replete</td>
<td>TNP-KLH</td>
<td>362.9 ± 17.4</td>
<td>366.0 ± 15.0</td>
</tr>
<tr>
<td>Depleted</td>
<td>TNP-KLH</td>
<td>345.9 ± 15.2</td>
<td>364.6 ± 25.6</td>
</tr>
<tr>
<td>Replete</td>
<td>TNP-LPS</td>
<td>337.4 ± 10.5</td>
<td>349.4 ± 13.8</td>
</tr>
<tr>
<td>Depleted</td>
<td>TNP-LPS</td>
<td>356.2 ± 15.4</td>
<td>368.8 ± 17.2</td>
</tr>
</tbody>
</table>

Between weeks 5 and 12 after injection, replete fish that were injected with DPBS and TNP-LPS exhibited significant decreases in both muscle and liver TTP (Figure 3). Thiamine-depleted lake trout injected with DPBS also exhibited decreased muscle and liver TTP at week 5 relative to week 12 (Figure 4). The thiamine-depleted trout injected with TNP-LPS, however, did not exhibit a similar decrease (Figure 4). Replete lake trout injected with TNP-KLH did not exhibit a significant change in either muscle or liver TTP concentrations between weeks 5 and 12 postinjection (Figure 3). The thiamine-depleted fish injected with TNP-KLH exhibited a significant increase in muscle TTP and a significant decrease in liver TTP over this same time period (Figure 4). Muscle-to-liver ratios in both thiamine-replete (Figure 3) and thiamine-depleted (Figure 4) trout injected with DPBS significantly declined between weeks 5 and 12 postinjection. The muscle-to-liver ratios in the replete and depleted fish injected with TNP-KLH and TNP-LPS exhibited no significant change over this time period.

At week 5 postinjection, lake trout injected with TNP-KLH and TNP-LPS exhibited significantly higher levels of anti-TNP activity than those thiamine-status-specific fish injected with DPBS (Figure 5). In both the thiamine-replete and thiamine-depleted trout, anti-TNP activity in the TNP-LPS-injected groups was significantly higher than that determined for TNP-KLH-injected fish at this same time point (Figure 5). Anti-TNP activity in TNP-LPS-injected thiamine-depleted trout was significantly higher than comparable injected thiamine-replete fish at week 5 postinjection, but no thiamine-status-associated significant differences were exhibited by fish injected with TNP-KLH or DPBS (see Figure 7). At week 12 postinjection, anti-TNP activities of TNP-KLH-injected fish remained significantly higher than activity observed for DPBS-injected controls in both
FIGURE 1. Comparisons of (A, B) muscle thiamine pyrophosphate (MTPP), (C, D) liver thiamine pyrophosphate (LTPP), and (E, F) the muscle-to-liver ratio of thiamine pyrophosphate (M:L TPP) in thiamine-replete (REP) and thiamine-depleted (DEP) lake trout injected with Dulbecco’s phosphate-buffered saline (DPBS), keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or lipopolysaccharide-trinitrophenol (TNP-LPS) as measured from tissues obtained (A, C, E) 5 weeks and (B, D, F) 12 weeks postinjection. Note differences in axis scaling. The upper and lower boundaries of the boxes represent the 75th and 25th percentiles, respectively, for each data set. Whisker caps indicate the 90th and 10th percentile and dots show outliers. Lines within boxes indicate the data set medians. Parenthetically listed above each box are the data set sample size (n) and the results from statistical comparisons using Kruskal–Wallis one-way ANOVA by ranks with a Mann–Whitney U-test as an evaluation of treatment effect. Significant differences for sample week (5 or 12) and injection group (DPBS, TNP-KLH, or TNP-LPS) between observed values in REP and DEP fish are indicated by different letters. Test results were considered significant when \( P \leq 0.05 \).
FIGURE 2. (A, D, G, J) Muscle thiamine pyrophosphate, (B, E, H, K) liver thiamine pyrophosphate, and (C, F, I, L) the muscle-to-liver ratio of thiamine pyrophosphate in (A–F) thiamine-replete and (G–L) thiamine-depleted lake trout injected with Dulbecco’s phosphate-buffered saline (DPBS), keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or lipopolysaccharide-trinitrophenol (TNP-LPS) as measured from tissues obtained (A–C, G–I) 5 weeks and (D–F, J–L) 12 weeks postinjection. Note differences in axis scaling. Box plot properties are as described in Figure 1. Parenthetically listed above each box are the data set sample size (n) and the results from statistical comparisons (see Figure 1 for details). Significant differences between injection groups (PBS, TNP-KLH, and TNP-LPS) within thiamine status group, tissue source, and sample week are indicated by different letters. Test results were considered significant when \( P \leq 0.05 \).
FIGURE 3. (A, B, C) Muscle thiamine pyrophosphate, (D, E, F) liver thiamine pyrophosphate, and (G, H, I) the muscle-to-liver ratio of thiamine pyrophosphate in thiamine-replete lake trout injected with Dulbecco’s phosphate-buffered saline (DPBS), keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or lipopolysaccharide-trinitrophenol (TNP-LPS) as measured from tissues obtained 5 and 12 weeks postinjection. Note differences in axis scaling. Box plot properties are as described in Figure 1. Parenthetically listed above each box are the data set sample size (n) and the results from statistical comparisons (see Figure 1 for details). Significant differences between sample weeks are indicated by different letters. Test results were considered significant when P ≤ 0.05.

the thiamine-replete and thiamine-depleted groups (Figure 5). The levels of anti-TNP activity in both thiamine-replete and thiamine-depleted trout injected with TNP-KLH were significantly higher than that observed in the thiamine-status-specific TNP-KLH-injected groups sampled during week 5 (Figure 6). As was the case at week 5, the week 12 postinjection anti-TNP activity did not differ significantly between thiamine-replete and thiamine-depleted trout injected with either TNP-KLH or DPBS (Figure 7). The thiamine-replete TNP-LPS-injected lake trout sampled at week 12 exhibited a significant decrease in anti-TNP activity relative to replete TNP-LPS-injected fish sampled at week 5 (Figure 6). This level of anti-TNP activity did not differ significantly from the activity observed in the week 12 thiamine-replete DPBS-injected controls and was significantly lower than exhibited by TNP-KLH-injected replete fish at this same time point (Figure 5). At week 12 postinjection, the anti-TNP activity of TNP-LPS-injected thiamine-depleted fish continued to be significantly higher than that exhibited by TNP-LPS-injected thiamine-replete fish (Figure 7). Also at week 12, TNP-LPS-injected thiamine-depleted lake trout exhibited significantly higher anti-TNP activity than their DPBS-injected controls (Figure 5). As was the case with thiamine-replete lake trout, however, TNP-LPS-injected thiamine-depleted fish did exhibit a significant decrease in anti-TNP activity at week 12 relative to week 5 (Figure 6). This level of TNP-LPS-associated anti-TNP activity did not significantly differ from that observed...
at week 12 in the thiamine-depleted fish injected with TNP-KLH (Figure 5).

Relationships between anti-TNP activities and TPP were found in thiamine-depleted lake trout only (Figure 8). At week 5 postinjection, depleted lake trout injected with DPBS exhibited a significant negative relationship between anti-TNP activity and TPP concentrations in both muscle ($P = 0.011$) and liver ($P = 0.041$). Depleted fish injected with TNP-KLH exhibited a significant positive relationship with muscle TPP concentrations at week 12 postinjection ($P < 0.001$). At week 12 postinjection, the thiamine-depleted lake trout injected with TNP-LPS exhibited a significant negative relationship ($P = 0.014$) between anti-TNP activity and the ratio of muscle-to-liver TPP concentrations.

**DISCUSSION**

Central to our observations on the response of thiamine-replete and thiamine-depleted lake trout to TNP-KLH and TNP-LPS are the differences in both magnitude and kinetics of the anti-TNP response. When differences were observed they included both anti-TNP activity as well as differences in the relative tissue distribution of TPP. The kinetics of the anti-TNP response (Figures 5, 6, 7) generated by the thiamine-replete lake trout were similar to those previously reported by Arkoosh and Kaattari (1987). Muscle and liver total thiamine concentrations in both the thiamine-replete and thiamine-depleted lake trout (Table 2) included in this study were comparable with those previously reported in wild (Brown et al. 2005) and
FIGURE 5. Plasma anti-trinitrophenol (anti-TNP) activity in (A, C) thiamine-replete and (B, D) thiamine-depleted lake trout injected with Dulbecco’s phosphate-buffered saline (DPBS), keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or lipopolysaccharide-trinitrophenol (TNP-LPS) as measured from plasma obtained (A, B) 5 weeks and (C, D) 12 weeks postinjection. Box plot properties are as described in Figure 1. Parenthetically listed above each box are the data set sample size \((n)\) and the results from statistical comparisons (see Figure 1 for details). Significant differences in anti-TNP activity between injection group (DPBS, TNP-KLH, or TNP-LPS) in fish with the same thiamine status determined from plasma collected at week 5 or 12 postinjection are indicated by different letters. Test results were considered significant when \(P \leq 0.05\).

Anti-TNP activity was observed in DPBS-injected thiamine-replete and thiamine-depleted lake trout. These fish were not exposed to the TNP hapten (Figures 5, 6, 7). Production of anti-TNP activity by these fish, in the absence of antigen-specific stimulation, presumably occurred via a generalized activation of the resident B-cell or plasma-cell (transformed, antibody-producing B cells) populations. Freund’s complete adjuvant was used in the delivery of all inoculants including the DPBS negative control. The adjuvant was used to enhance antibody production through generalized immune cell activation (e.g., macrophages, B- and T-lymphocytes) as well TNP-specific immune-cell clonotypes (Billiau and Matthys 2001). Given that thiamine-depleted lake trout injected with either experimental (Honeyfield et al. 2005) fish. When considering potential relationships between anti-TNP activity and thiamine concentrations we chose to examine tissue concentration ratios as well as the absolute tissue concentrations. Both muscle and liver thiamine concentrations were used as surrogates for the thiamine concentrations that could be available to the immune cells involved in the generation of anti-TNP activity. The use of muscle-to-liver thiamine ratios was based on the assumptions that the relative concentration of thiamine in muscle compared with liver may better reflect the unknown thiamine concentration in immune cells than absolute tissue values. This assumption proved to be partially correct, but we cannot currently offer a definitive explanation as to why.
FIGURE 6. Plasma anti-trinitrophenol (anti-TNP) activity in (A, C, E) thiamine-replete and (B, D, F) thiamine-depleted lake trout injected with (A, B) Dulbecco’s phosphate buffered saline (DPBS), (C, D) keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or (E, F) lipopolysaccharide-trinitrophenol (TNP-LPS) as measured from plasma obtained 5 and 12 weeks postinjection. Box plot properties are as described in Figure 1. Parenthetically listed above each box are the data set sample size (n) and the results from statistical comparisons (see Figure 1 for details). Significant changes in anti-TNP activity between sample weeks 5 and 12 within thiamine status and injection group are indicated by different letters. Test results were considered significant when $P \leq 0.05$.
FIGURE 7. Plasma anti-trinitrophenol (anti-TNP) activity in thiamine-replete (REP) and thiamine-depleted (DEP) lake trout injected with Dulbecco’s phosphate-buffered saline (DPBS), keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or lipopolysaccharide-trinitrophenol (TNP-LPS) as measured from plasma obtained (A) 5 and (B) 12 weeks postinjection. Box plot properties are as described in Figure 1. Parenthetically listed above each box are the data set sample size (n) and the results from statistical comparisons (see Figure 1 for details). Significant differences in plasma anti-TNP activity between REP or DEP fish determined within injection group at weeks 5 and 12 are indicated by different letters. Test results were considered significant when $P \leq 0.05$.

DPBS or TNP-LPS exhibited a negative relationship between anti-TNP activity and tissue TPP concentration and that similar associations were not exhibited in thiamine-replete fish (Figure 8), there does appear to be a possible association between nonantigen-specific (non-clonal-type specific) immune cell activity, antibody production, and available TPP. The positive association between available TPP and anti-TNP activity in thiamine-depleted lake trout inoculated with TNP-KLH (Figure 8), when contrasted with the negative relationships observed between available TPP and anti-TNP activity in the thiamine-depleted DPBS and TNP-LPS-inoculated fish, suggests that the mechanisms involved with a T-dependent versus a T-independent antibody response can significantly alter the relationship between available TPP and antibody production. The decrease in tissue TPP concentrations observed between weeks 5 and 12 postinjection in thiamine-replete fish injected with DPBS and TNP-LPS, but not in those injected with TNP-KLH (Figure 3), seems to bolster the hypothesis that the T-independent and T-dependent responses could have a differential effect on TPP concentrations. The thiamine-depleted lake trout, however, exhibited a different response with TPP tissue concentrations decreasing from week 5 to week 12 in TNP-KLH-injected fish (Figure 4). The differences in TPP concentration change associated with the T-dependent and T-independent antigens in the thiamine-replete and thiamine-depleted lake trout contradict a simple T-dependent versus T-independent response explanation for changes in TPP concentration. The possibility that thiamine-replete and thiamine-depleted lake trout may differ in how they use available TPP needs be considered. A more focused comparison of the cellular and soluble factors involved in the T-dependent and T-independent process placed in the context of thiamine depletion may improve our understanding of this relationship between available thiamine and antibody production. There are, however, some fundamental questions regarding the physiologic processes associated with thiamine depletion that need be addressed before a general understanding of the relationships between thiamine, immunity, and disease resistance can be developed.

The observed difference in antibody production associated with the T-dependent and T-independent responses in the thiamine-replete and thiamine-depleted trout could be a direct result of differences in energy requirements for responses and thiamine-status-dependent variation in available energy. The TPP requirements of cell types directly or indirectly involved in immune responses are not known. Ames (2006) proposed a triage hypothesis whereby vital functions such as ATP synthesis would receive preferential allocation of scarce micronutrients. It is not known where immune responses would rank in a triage-based allocation of TPP. In general, little is known regarding the specific regulatory mechanisms influencing TPP tissue distribution. A possibility that needs be considered is that our observed TPP tissue distributions and anti-TNP
FIGURE 8. Plasma anti-trinitrophenol (anti-TNP) activity in thiamine-replete (REP; closed circle) and thiamine-depleted (DEP; open circle) lake trout injected with (A, B) Dulbecco’s phosphate-buffered saline (DPBS), (C) keyhole limpet hemocyanin-trinitrophenol (TNP-KLH), or (D) lipopolysaccharide-trinitrophenol (TNP-LPS) expressed relative to (A, C) muscle, (B) liver, or (D) the muscle-to-liver ratio of thiamine pyrophosphate measured at (A, B) week 5 or (C, D) week 12 postinjection. Note differences in axis scaling. Associations between anti-TNP activity and thiamine pyrophosphate concentration or ratios for each sample date and injection type were evaluated by linear mixed regression modeling followed by hypothesis testing. The relationships between antibody production and thiamine-pyrophosphate were significant in each of the displayed graphs for thiamine-deficient lake trout. Corresponding plots for thiamine-replete trout were not significant. Test results were considered significant when $P \leq 0.05$.

A repercussion of thiamine depletion is oxidative stress. This effect has been widely reported in mammals, particularly in association with brain pathology (Hazell and Butterworth 2009; Araki et al. 2010). The association between oxidative stress and thiamine depletion has also been reported in fish (Lundström et al. 1999a; Amcoff et al. 2000; Vuori and Nikinmaa 2007). Similarly, thiamine depletion is associated with tissue pathology (Lundström et al. 1999b, 2002; Fitzsimons et al. 2001) and altered brain function (Amcoff et al. 2002). Hypoxia-inducible transcription factor (HIF-1 alpha) is directly associated with pathology observed in thiamine-depleted Baltic Atlantic salmon (Vuori et al. 2004). Brain pathology (Brown and Honeyfield 2001) and altered brain function (Carvalho et al. 2009; Fitzsimons et al. 2009) observed in thiamine-depleted fish may provide additional clues to the cause of immune dysfunction in thiamine-depleted lake trout. The interaction between the neuroendocrine axis and immune function is well established in mammals and lower vertebrates including fish (Chiappelli et al.
Acknowledgments

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Predicted Redistribution of Ceratomyxa shasta Genotypes with Salmonid Passage in the Deschutes River, Oregon

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Predicted Redistribution of Ceratomyxa shasta Genotypes with Salmonid Passage in the Deschutes River, Oregon

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Abstract

A series of dams on the Deschutes River, Oregon, act as migration barriers that segregate the river system into upper and lower basins. Proposed fish passage between basins would reunite populations of native potamodromous fish and allow anadromous fish of Deschutes River origin access to the upper basin. We assessed the potential redistribution of host-species-specific genotypes (O, I, II, III) of the myxozoan parasite Ceratomyxa shasta that could occur with fish passage and examined the influence of nonnative fish on genotype composition. To determine the present distribution of the parasite genotypes, we exposed eight salmonid species—three native and five stocked for sport fishing—in present and predicted anadromous salmonid habitats. We monitored fish for infection by C. shasta and sequenced a section of the parasite ribosomal DNA gene from fish and water samples to determine parasite genotype. Genotype O was present in both upper and lower basins and detected only in steelhead Oncorhynchus mykiss. Genotype I was spatially limited to the lower basin, isolated predominately from Chinook salmon O. tshawytscha, and lethal for this species only. Genotype II was detected in both basins and in multiple species, but only as a minor component of the infection. Genotype III was also present in both basins, had a wide host range, and caused mortality in native steelhead and multiple nonnative species. Atlantic salmon Salmo salar and kokanee O. nerka were the least susceptible to infection by any genotype of C. shasta. Our findings confirmed the host-specific patterns of C. shasta infections and indicated that passage of Chinook salmon would probably spread genotype I into the upper Deschutes River basin, but with little risk to native salmonid populations.

Noble (1950) first described Ceratomyxa shasta from infected hatchery trout from northern California. This myxozoan parasite, commonly found throughout the Pacific Northwest (PNW; Bartholomew 1998), causes the disease ceratomyxosis. Notable clinical signs of the disease include inflammation, necrosis, and hemorrhaging of the intestine; in severe cases the development of asciases causes a grossly distended abdomen (Wales and Wolf 1955). Fish become infected when they encounter the actinospore stage of the parasite, which is released into the water column from the invertebrate host Manayunkia speciosa. The myxospore stage, which develops in the salmonid fish, infects the invertebrate host (Bartholomew et al. 1997). The pathogenicity of the parasite depends upon the host species and origin, and native salmonids from endemic waters are generally less susceptible and able to survive exposure to higher parasite densities than salmonids from nonendemic waters (Bartholomew 1998). Recent studies in the Klamath River, California–Oregon, identified multiple host-specific genotypes of the parasite (Atkinson and Bartholomew 2010a, 2010b). In that river system, genotype O infects native steelhead Oncorhynchus mykiss (anadromous rainbow trout) and redband trout (rainbow trout subspecies), genotype I causes mortality in native Chinook salmon O. tshawytscha, genotype II exists as two biotypes that cause mortality in either native coho salmon O. kisutch or nonnative rainbow trout (Hurst and Bartholomew 2012), and genotype III was only detected at low levels, but in multiple species (Atkinson and Bartholomew 2010b). These host–parasite relationships have not been evaluated outside the Klamath River system and have implications for stocking and fish passage plans throughout the parasite’s range.

Strategies for stocking salmonids in waters in which C. shasta is endemic have been guided by results from in-river sentinel

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fish exposures. These natural challenges tested the susceptibility of particular fish strains but were often conducted in watersheds other than where stocking was proposed, with the fundamental assumption that the parasite is equivalent throughout its range (Schafer 1968; Sanders et al. 1970; Zinn et al. 1977; Ching and Parker 1989). But outcomes of stocking programs have varied, and in retrospect, the perceived resilience of some species or strains may have resulted from the absence of a pathogenic genotype (Atkinson and Bartholomew 2010a). In some instances, the stocking of nonnative salmonids has increased parasite levels with little or no consequence to native populations because the genotype that increased was specific to the introduced species. For example, stocking of susceptible populations because the genotype that increased was specific to a naive host, yet is not infectious for native salmonids (Hurst and Bartholomew 2012). However, introducing susceptible species may also result in amplification of parasite strains that can affect native fish, as has been the case for another myxozoan Myxobolus cerebralis (Nehring and Walker 1996). Thus, data on the parasite genotypes present in a river system and the host specificity of these genotypes should be synthesized to develop informed stocking decisions that minimize disease risks.

Dam removal and expanded fish passage are expected to benefit salmonid populations by increasing habitat. However, an unintended consequence may be the exposure of presently isolated fish populations to pathogens associated with introduced populations and species. In the state of Washington, increased numbers of anadromous salmonids passed above the Cowlitz Salmon Hatchery, coincided with increases in ceratomyxosis at that facility (E. Ray, Washington Department of Fish and Wildlife, personal communication). Pathogens can also exist solely above migration barriers and thus present a risk to reintroduced anadromous fishes. A survey conducted in the Elwha River, Washington, identified a myxozoan in kokanee O. nerka that occurred only in the upper portion of the system and could potentially affect lower-basin fish populations after removal of the migration barrier (Jones et al. 2011). To minimize risks of introducing pathogens, sentinel studies can be used to inform fisheries managers of the parasite populations present above and below migration barriers. For example, Zielinski et al. (2010) determined M. cerebralis is established in the lower Deschutes River basin and recommended only marked salmonids of upper Deschutes River basin origin be passed beyond barriers. Similarly, in the Klamath River, testing for C. shasta-associated risks has been conducted prior to anadromous fish passage. Sentinel exposures of extirpated Chinook salmon and coho salmon in the upper Klamath River basin resulted in minimal mortality because host-specific genotypes were absent (Atkinson and Bartholomew 2010a). However, the movement of fishes beyond migration barriers could expose trout and salmon to different C. shasta genotypes, creating new host–parasite interactions with unknown effects.

Similar to many watersheds of the PNW, the Deschutes River, Oregon, is partitioned by several dams that restrict fish migration, and recreational angling is supplemented by stocking native and nonnative salmonids. Improvements have been made to permit downstream migration, and upstream passage has recently begun (Northwest Power and Conservation Council 2004). Ceratomyxa shasta is endemic in the Deschutes River basin, being present in the lower basin in the river main stem and above the dams in the reservoir, several upper basin lakes, and two of the three major tributaries (Sanders et al. 1970; Ratliff 1983). To determine C. shasta genotype distribution and infection risks to native and nonnative salmonid populations, we collected and analyzed water samples for parasite density and genotype composition from known positive locations in the lower and upper basin. Additionally, we conducted sentinel fish exposures at these locations using salmonid species currently stocked in the upper Deschutes River and those that will gain passage beyond the barrier. We determined genotype host range by sequencing parasite DNA from infected fish and host suitability (the ability of the parasite to sporulate within a particular host) by examining for mature myxospores. Our data revealed differences in genotype composition across the basin, confirmed the host-specific patterns of genotypes observed previously in the Klamath River (Atkinson and Bartholomew 2010a, 2010b), and further defined the host range of genotype III.

METHODS

Study location.—The Deschutes River in central Oregon flows north 278 river kilometers (rkm) into the Columbia River. A series of dams, collectively referred to as the Pelton–Round Butte Hydroelectric Project (PRB), were built between 1956 and 1964, with Round Butte Dam (rkm 177) being the ultimate migration barrier that divides the Deschutes River into an upper and lower basin. Round Butte Dam impounds Lake Billy Chinook, which is fed by the Metolius, Crooked, and upper Deschutes rivers. The former two rivers were historically inhabited by anadromous fishes, but the latter has a natural migration barrier, Big Falls. Ceratomyxa shasta is endemic to both the upper and lower basins (Sanders et al. 1970; Ratliff 1983). Two sentinel fish exposure locations were chosen based on accessibility and were within the present and projected anadromous salmonid range: lower basin—Deschutes River at rkm 76 (45°27′30″N, 121°04′30″W), upstream of the Oak Springs State Hatchery effluent; upper basin—Crooked River at rkm 12 (44°29′45″N, 121°17′15″W) below the Opal Springs Dam (Figure 1).

Analysis of river water samples.—Triplicate 1-L river water samples were taken at the beginning and end of the exposure period (7 and 11 June 2010) from each exposure location. Water filtration, DNA extraction, and C. shasta quantitative PCR (qPCR) assay were done according to Hallett and Bartholomew (2006) with an additional step of using acetone to digest the nitrocellulose filter (Hallett et al. 2012). To estimate parasite density at exposure localities, we evaluated the river water quantitative
cycle (Cq) results using values from previously assessed standards of 1 and 10 actinospores (Hallett and Bartholomew 2006). Parasite DNA from water samples was amplified for sequencing using C. shasta genotyping primers according to the protocol of Atkinson and Bartholomew (2010b). Resulting PCR products were purified using ExoSAP-IT (USB, Cleveland, Ohio) at a ratio of 5-µL PCR product to 2-µL ExoSAP-IT and sequenced at the Oregon State University Center for Genome Research and Biocomputing using an ABI Prism 3730 DNA analyzer. Sequence chromatograms were analyzed with the program 4Peaks (version 1.7.2) to determine the number of ATC repeats that define each genotype. For mixed infections, the percentage of each genotype was estimated from the average height ratios of coincident peaks at five different sequence positions (Atkinson and Bartholomew 2010a).

Fish groups and exposure.—We obtained eight salmonid species, all age 0, from Oregon Department of Fish and Wildlife (ODFW) state hatcheries (Table 1). Sizes varied between species (average weight per fish, 2–10 g) because of differences in spawning times and growth rates; however, previous studies demonstrated that salmonids of a wide range in size and age are susceptible to C. shasta (Zinn et al. 1977; Bjork and Bartholomew 2009). Fifty fish of each species were transported to each sentinel location in separate coolers with aeration. Species were exposed in separate cages for 5 d, 7–11 June 2010, similar to Stocking et al. (2006) but without a formalin bath treatment postexposure, then transported to the John L. Fryer Salmon Disease Laboratory (SDL), Corvallis, Oregon. A submersible temperature logger (Optic StowAway, Onset Computer Corporation, Pocasset, Massachusetts) was used at each exposure location (triangles), migration barriers (black bars) and major tributaries.

Exposed and control fish were held for 60 d in separate, nonreplicated, 25-L tanks. Fish were supplied with specific-pathogen-free 18°C water and fed daily an Oregon Moist Pellet diet with 2–4% oxytetracycline (~3.5 g per 45 kg of feed; TM200, Pfizer, Atlanta, Georgia). The antibiotic reduces the chance of bacterial infection, but presumably does not affect C. shasta infections. We monitored the fish daily, and any moribund fish were removed and euthanized by overdose with the anesthetic tricaine methanesulfonate (MS-222; Argent Laboratories, Redmond, Washington) and counted as “mortalities.” After 60 d, all remaining fish were euthanized and counted as “survivors.”

Infection assessment.—For all moribund fish, clinical signs of disease were noted and fresh wet mounts from intestinal scrapes were examined microscopically at 100–250 × magnification for up to 3 min for the presence of characteristic arcuate myxospores of C. shasta (AFS-FHS 2010). All moribund fish were necropsied and their intestines removed and stored at −20°C. Those without myxospores were subjected to a diagnostic PCR assay. Following the methods of Stocking et al. (2006), DNA was extracted from intestines and primers specific for C. shasta, Cs1 and Cs3 were used to determine whether parasite DNA was present in moribund fish (Palenzuela et al. 1999). Individual fish were considered C. shasta positive (Cs+) if myxospores were identified by microscopic evaluation or if C. shasta DNA was amplified by diagnostic PCR; C. shasta negative (Cs−) samples were negative by both tests. Subsamples of 10 survivors per exposed and control groups were examined microscopically and molecularly similar to mortalities to determine the extent of infection. The level of susceptibility (low, moderate, or high) for each group was based on clinical signs (absent to severe) and the extent of Cs+ mortality.

To determine if the infecting C. shasta genotypes were able to persist and cause mortality, we randomly sampled five fish from each group at 7 and 21 d postexposure (dpe) and froze portions of their intestines. Chinook salmon exposed in the lower basin were sampled only at 7 dpe as insufficient fish remained at 21 dpe.

<table>
<thead>
<tr>
<th>Species</th>
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<tbody>
<tr>
<td>Atlantic salmon Salmo salar</td>
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<td>Kokanee O. nerka</td>
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<tr>
<td>Chinook salmon O. tshawytscha</td>
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</tr>
<tr>
<td>Steelhead (anadromous rainbow trout)</td>
<td>Native</td>
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FIGURE 1. The Deschutes River basin in the Pacific Northwest showing exposure locations (triangles), migration barriers (black bars) and major tributaries.
(26 fish escaped from their tank early in the monitoring period). Ten visually Cs+ fish mortalities per group were selected for genotyping; if groups had fewer than 10 Cs+ fish mortalities, 7 and 21 dpe samples were genotyped. As described above DNA was extracted for fish intestines and samples were genotyped similar to water samples.

Percent total mortality and C. shasta cumulative mortality (visual and PCR Cs+) were calculated as the number of mortalities divided by the starting count (minus fish sampled at 7 and 21 dpe) and multiplied by 100. To determine the mean days to death (MDD) we averaged the number of days postexposure for Cs+ cumulative mortalities for each group. Kaplan–Meier survivorship curves show the probability of survival at a given time and were drawn using R (version 2.9.2 for Macintosh), censoring visually and PCR Cs− mortalities and survivors. Survival of a species between basins was not compared because of differences in dose and genotype, or between species because of the lack of replication.

RESULTS

Water Samples

Water was ~13°C throughout the exposure at both exposure localities. At the beginning of the exposure, parasite density was higher in water samples from the lower basin (1–10 spores/L) than the upper basin (<1 spore/L). When fish were collected 5 d later, parasite densities at both exposure localities were ~1 spore/L. Genotypes O (33%) and III (67%) were identified from three upper basin water samples at the end of the exposure period. The quantity of parasite DNA in earlier samples from the upper basin was insufficient for performing the genotyping assay. Five out of six lower basin water samples were genotyped with an average composition of 89% genotype I and 11% genotype III.

Sentinel Fish: Upper Basin

Genotype III was isolated from all eight fish species and was the dominant genotype detected in all mortalities (Table 2). Genotype II was detected as a minor component (<29%) in multiple species, and genotype 0 was detected only from steelhead sampled at 21 dpe. Survivorship curves reflected the susceptibility of each species to genotypes O and III and identified categories with varying degrees of susceptibility (Figure 2).

High susceptibility.—Nonnative rainbow trout and cutthroat trout had 100% Cs+ cumulative mortality. Clinical signs were severe and often systemic with lesions and myxospores observed in the liver, kidney, spleen, and epidermis, and abdominal swelling was common. Brook trout also had 100% mortality, but 15 mortalities occurred prior to the onset of ceratomyxosis and were presumably caused by Flavobacterium columnare (based on 18°C water temperatures and clinical signs). When these fish were censored from the analysis, Cs+ cumulative mortality was 58%. Positive brook trout mortalities had typical clinical signs of ceratomyxosis (myxospores, swollen intestines).

<table>
<thead>
<tr>
<th>Species</th>
<th>Morts (%)</th>
<th>Cs+ (%)</th>
<th>MDD</th>
<th>Genotype (morts)</th>
<th>Genotype (7 and 21 d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Genotype (I)</td>
<td>Genotype (II)</td>
</tr>
<tr>
<td>Upper basin</td>
<td></td>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Rainbow trout</td>
<td>41 (100)</td>
<td>41 (100)</td>
<td>25</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Cutthroat trout</td>
<td>38 (100)</td>
<td>38 (100)</td>
<td>24</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Brook trout</td>
<td>39 (100)</td>
<td>14 (58)</td>
<td>25</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Brown trout</td>
<td>21 (57)</td>
<td>18 (49)</td>
<td>41</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>Steelhead</td>
<td>7 (18)</td>
<td>4 (11)</td>
<td>33</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Kokanee</td>
<td>3 (8)</td>
<td>2 (6)</td>
<td>33</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Atlantic salmon</td>
<td>7 (18)</td>
<td>1 (3)</td>
<td>36</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Chinook salmon</td>
<td>1 (3)</td>
<td>0 (0)</td>
<td></td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

| Lower basin       |           |         |     | Genotype (I) | Genotype (II) | Genotype (III) | Genotype (O) |
|-------------------|-----------|---------|-----| I               | II                  | III            | O             |
| Rainbow trout     | 31 (100)  | 31 (100)| 26  | 2               | 10                  |                |               |
| Cutthroat trout   | 38 (100)  | 35 (92) | 24  |                 | 12                  |                |               |
| Brook trout       | 39 (100)  | 22 (92) | 24  | 4               | 11                  | 10             |               |
| Brown trout       | 12 (29)   | 6 (15)  | 41  | 4               | 8                   |                |               |
| Steelhead         | 0 (0)     | 0 (0)   |     |                 | 1                   | 6              |               |
| Kokanee           | 4 (9)     | 1 (2)   | 48  | 1               | 2                   | 4              |               |
| Atlantic salmon   | 8 (21)    | 0 (0)   |     |                 | 1                   | 10             |               |
| Chinook salmon    | 19 (100)  | 15 (79) | 19  | 10              | 1                   | 4              | 4              |

aData were censored to exclude mortalities that occurred prior to the detection of C. shasta by PCR.
Moderate susceptibility.—Brown trout had 49% \( Cs^+ \) cumulative mortality. Mortalities had slightly swollen intestines with mostly underdeveloped or deformed myxospores visible in intestinal scrapes. Of the upper-basin survivors, only brown trout tested positive for \( C. shasta \) by PCR assay (20%), but myxospores were not observed.

Low susceptibility.—Steelhead had 11% \( Cs^+ \) cumulative mortality and clinical signs were less apparent than in other species, but included myxospores and slightly swollen intestines. None of the exposed kokanee, Atlantic salmon, or Chinook salmon developed clinical signs of disease and \( Cs^+ \) cumulative mortality was 6%, 3% and, 0%, respectively. The two visually \( Cs^+ \) kokanee also had a heavy infection with another myxozoan in the intestines and kidneys that resembled \( Myxidium minteri \). Kokanee were the only species that tested negative after 21 dpe.

Sentinel Fish: Lower Basin

Similar to the upper basin, the dominant genotype isolated from all mortalities was genotype III, except for Chinook salmon. Genotype I was predominant in Chinook salmon mortalities and was also present as a minor component in brook trout mortalities and in 7- and 21-dpe samples. Genotype O was detected only in steelhead at 7 and 21 dpe. As in the upper basin, mixed infections with <22% genotype II were detected in several species. Survivorship curves reflected the susceptibility of each species to genotypes I and III (Figure 3).

High susceptibility.—Rainbow trout and cutthroat trout were highly susceptible, with clinical disease similar to their cohorts exposed in the upper basin. Brook trout had 100% mortality, with 92% of those \( Cs^+ \); again, there was a complicating infection with \( Flavobacterium columnare \) (15 mortalities prior to the first \( Cs^+ \) detection). The \( C. shasta \)-infected brook trout had clinical signs of ceratomyxosis and visible myxospores. In contrast to their low susceptibility in the upper basin, Chinook salmon suffered high \( Cs^+ \) cumulative mortality (79%) and had myxospores and swollen intestines.

Low susceptibility.—Brown trout mortality was 15% with mild clinical signs, and again, the majority of myxospores were underdeveloped or deformed. No mortality occurred in steelhead and no \( Cs^+ \) mortality was recorded for Atlantic salmon. One kokanee mortality tested PCR positive. Again, kokanee were the only species that tested negative at 21 dpe, and brown trout were the only surviving species in the lower basin that were \( Cs^+ \) by PCR assay (20%), with myxospores observed in 10% of fish.

Mean Days to Death

Chinook salmon exposed in the lower basin had the most rapid \( Cs^+ \) mortality rate (19 MDD) among all groups. Brown trout from both exposure locations had the slowest rate of death (41 MDD). Only a single kokanee exposed in the lower basin died at 48 dpe. For all other species, the MDD was 24–33 (Table 2).

Controls

All control mortalities (13% of Atlantic salmon, 28.6% of brown trout, 32.4% of cutthroat trout, and 2.4% of steelhead) and the 10-fish subsets of each species were visually and PCR \( Cs^- \).
DISCUSSION

This Deschutes River study confirmed the host-specificity patterns of C. shasta genotypes observed in the Klamath River (Atkinson and Bartholomew 2010b). In both rivers, genotype I had a narrow host range, predominately infecting Chinook salmon. During this short study period, genotype I was not detected from the upper basin of either system, presumably as a result of the extirpation of Chinook salmon after construction of the dams. Similar to the Klamath River, genotypes O, II, and III were able to persist above and below migration barriers in current populations of suitable hosts. We examined the host range of genotype III, and while this genotype infected all eight salmonid species, steelhead were the only native species to develop clinical signs of disease. These results suggest native salmonids in the upper basin, such as kokanee and redband trout, would probably have low susceptibility to genotype I if the parasite was reintroduced by Chinook salmon.

Genotype I was the only parasite strain with a restricted distribution. Historically, spring Chinook salmon migrated into the Metolius and Crooked rivers of the upper basin and would have carried genotype I to their spawning grounds. Our inability to detect genotype I in the Crooked River suggests that without a suitable host, this genotype was unable to persist after fish migration was blocked by construction of the PRB and the failure of fish passage. We predict that passing spring Chinook salmon over the dam into the upper basin will eventually reestablish genotype I in the Crooked River, but this genotype presents a low disease risk for native upper-basin salmonids due to its strict host specificity. In this study, the only detection of genotype I in a species other than Chinook salmon was as a mixed infection in brook trout (a nonnative species) exposed in the lower basin. The inability to detect genotype I in fish or water from the Crooked River, despite brook trout being present in this river since their introduction in 1918, suggests this species is not a suitable host for genotype I. Previous studies presented conflicting results on brook trout susceptibility (Schaefer 1968; Zinn et al. 1977), and this may reflect that this species can become infected by multiple C. shasta genotypes.

The presence of genotype O in the Crooked River indicated that a host other than steelhead is proliferating this genotype. Atkinson and Bartholomew (2010b) documented native Great Basin redband trout O. mykiss newberrii infected with genotype O in the Klamath River basin; hence, we presume that in the upper Deschutes River basin, ubiquitous, native Columbia River basin redband trout O. mykiss gairdneri perpetuate genotype O. In this study we exposed two O. mykiss forms (native steelhead and nonnative rainbow trout) and only steelhead became infected with genotype O; we did not test native redband trout. We did not observe pathological changes associated with genotype O in native O. mykiss, which was consistent with other studies (Atkinson and Bartholomew 2010b; Stinson 2012), although water samples had only low amounts of genotype O and this low exposure dose would be expected to have reduced pathogenic effects.

The only parasite strain not detected in water samples was genotype II. However, low levels of genotype II were detected in many salmonid species and in both basins. No mortality was associated with this genotype despite using the same nonnative rainbow trout stock used in the Klamath River exposures where high mortalities resulted from genotype II infection (Atkinson and Bartholomew 2010a). This difference in genotype composition between the Deschutes and Klamath rivers may reflect differences in the salmonid species native to each basin. Coho salmon, the presumed natural host for genotype II (Atkinson and Bartholomew 2010b), are native and extant in the Klamath River but are extinct in the Deschutes River (Williams et al. 1991). Genotype II was documented in a wide range of hosts surveyed throughout the PNW (Stinson 2012) and could be proliferating at low levels in other salmonids residing in the Deschutes River.

Genotype III had a wide host range, infecting all native and nonnative salmonids tested, resulting in varying levels of disease. All Cs+ mortalities, except Chinook salmon, were predominately infected with genotype III. Many of these were nonnative salmonids, representing new hosts for C. shasta, thus highlighting the nonspecific nature of genotype III. The only native host that died from an infection by genotype III was steelhead. The only species in which genotype III myxospores did not develop were Atlantic salmon and Chinook salmon, but even these species were positive for genotype III at 7 and 21 dpe.

Although we could not statistically compare the susceptibilities between species because of the lack of replication for the both exposure in the river and subsequent holding at the SDL, these experimental design constraints probably had minimal influence on study conclusions. Closely positioned sentinel cages have been demonstrated to receive a similar exposure dose (Ray et al. 2010) and postexposure holding conditions in this study were identical for all species. Additionally, intertank variation in disease severity should not have been a factor with C. shasta, which has a two-host life cycle and no fish-to-fish transmission, in contrast to directly transmissible agents that spread rapidly within aquaria, such as bacteria and viruses (Bricknell 1995).

The ability of some myxozoans to infect a wide range of hosts was recognized by Shul’man (1966), who hypothesized that the level of development within the host is a measure of the compatibility of the host–parasite relationship. Considering the development of myxospores as evidence that the parasite can complete its fish host phase, we can make some observations on the suitability of each species as hosts for C. shasta genotype III. Kokanee, Chinook salmon, and Atlantic salmon were unsuitable hosts as they were either able to clear the infection or no myxospores developed. Under the current study conditions, the high MDD and incomplete development of myxospores in brown trout suggest this species is also not a suitable host for genotype III. Nonnative brook, rainbow trout, and cutthroat trout were highly susceptible and suitable hosts, but they probably represent incidental hosts for this genotype. The only native species in which genotype III matured was steelhead. Thus, although there
is only evidence for one native host, genotype III has adapted to a suite of hosts.

In addition to supporting the host–parasite relationships first observed in the Klamath River, results of this study can inform stocking and fish passage plans in the Deschutes River and suggest strategies for similar river systems. The likely reintroduction of genotype I into the upper basin would present a low risk to native salmonids due to its high host specificity; a similar conclusion was made by Hurst and Bartholomew (2012) in the Klamath River. In contrast to the Klamath River, genotype III is widely distributed in the Deschutes River and infects a wide range of salmonids; thus, it could be amplified through stocking of susceptible species. We predict that passage of steelhead would not introduce novel genotypes, as genotypes O and III are already established in the Crooked River and are potentially present in other endemic areas of the upper Deschutes River basin. Ultimately, reintroduced anadromous salmonids will overlap with resident salmonids (mountain whitefish *Prosopium williamsoni*, bull trout *Salvelinus confluentus*, and redband trout) in major tributaries of the Deschutes River, but for *C. shasta*, the disease risk associated with introduction will be limited by the host specificity of each genotype.

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**REFERENCES**


