Gill Infection Model for Columnaris Disease in Common Carp and Rainbow Trout


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Published online: 08 Dec 2014.


To link to this article: http://dx.doi.org/10.1080/08997659.2014.953265

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Gill Infection Model for Columnaris Disease in Common Carp and Rainbow Trout

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Abstract

Challenge models generating gill lesions typical for columnaris disease were developed for the fry of both Common Carp Cyprinus carpio and Rainbow Trout Oncorhynchus mykiss by means of an immersion challenge and Flavobacterium columnare field isolates were characterized regarding virulence. Carp inoculated with highly virulent isolates revealed diffuse, whitish discoloration of the gills affecting all arches, while in trout mostly unilateral focal lesions, which were restricted to the first two gill arches, occurred. Light microscopic examination of the gills of carp exposed to highly virulent isolates revealed a diffuse loss of branchial structures and desquamation and necrosis of gill epithelium with fusion of filaments and lamellae. In severe cases, large parts of the filaments were replaced with necrotic debris entangled with massive clusters of F. columnare bacterial cells enwrapped in an eosinophilic matrix. In trout, histopathologic lesions were similar but less extensive and much more focal, and well delineated from apparently healthy tissue. Scanning and transmission electron microscopic observations of the affected gills showed long, slender bacterial cells contained in an extracellular matrix and in close contact with the destructed gill tissue. This is the first study to reveal gill lesions typical for columnaris disease at macroscopic, light microscopic, and ultrastructural levels in both Common Carp and Rainbow Trout following a challenge with F. columnare. The results provide a basis for research opportunities to examine pathogen–gill interactions.

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Received January 10, 2014; accepted July 27, 2014
Flavobacterium columnare is a bacterial pathogen that occurs worldwide and causes columnaris disease in multiple freshwater fish species, including Common Carp Cyprinus carpio and Rainbow Trout Oncorhynchus mykiss (Tripathi et al. 2005; Suomalainen et al. 2009). The disease is notorious as it induces gill, fin, and skin lesions resulting in massive mortalities and economic losses of millions of dollars yearly (Shoemaker et al. 2011). Columnaris disease may exhibit an acute or more chronic course is which the age and immune status of the fish are major determining factors. In young fish, the disease strikes acutely and the gill is the major site of damage. In acute cases of columnaris disease in adults, yellowish-white regions of necrotic tissue may appear in the gills, causing respiratory distress and death of the fish. In more chronic cases, it takes longer for gill pathology to develop and skin and fin lesions may also occur (Pacha and Ordal 1967; Decostere 2002; Bernardet and Bowman 2006; Declercq et al. 2013b). Besides host-related parameters, the virulence of F. columnare isolates can also markedly influence the course of the disease. Highly virulent strains are able to induce acute mortality, and the vast majority of fish do not display macroscopic lesions at the time of death due to the speed of mortality (Rucker et al. 1953; Pacha and Ordal 1967).

Considering the pathogenesis of columnaris disease, especially with regard to the interaction of the pathogenic agent with the gill tissue, multiple domains remain to be explored and research questions need to be answered. This information is crucial in order to combat the disease without having to resort to antimicrobial agents. Recently, increasingly more data have become available on the reciprocal effects between F. columnare and its host. However, most of these studies focused on the interaction between F. columnare and skin tissue (Bader et al. 2003; Suomalainen et al. 2005b; Tripathi et al. 2005; Bullard et al. 2011) or linked mortality to the genomovars and genetic traits of the isolates involved (Thomas-Jinu and Goodwin 2004; Suomalainen et al. 2006a, 2006b; LaFrentz et al. 2012). This leaves the interplay between this pathogen and gill tissue a challenging wasteland to cultivate. Indeed, only a mere handful of studies have explored the interaction between F. columnare and gill tissue (Sun et al. 2012; Peatman et al. 2013) and have mainly concentrated on mucosal actors in the teleost gill.

Currently, to our knowledge, a reproducible experimental infection model eliciting gill lesions as noted in the field (DeCostere et al. 2013) does not exist, and so in-depth research on the interplay between this pathogen and the host gill is hampered. The aim of this study was to develop a reliable and reproducible experimental infection model for columnaris disease in Common Carp and Rainbow Trout, in which there would be a particular focus on eliciting gill lesions. A virulence profile of 11 F. columnare isolates was additionally assessed. Furthermore, we will provide a detailed description of the induced gill lesions, which could help in the elucidation of the pathogenesis of columnaris disease with special emphasis on the gill tissue.

**METHODS**

**Fish.**—Two-day-old Common Carp fry were obtained from a Belgian hatchery. After transportation to the experimental facilities, the fry were grown to a mean length of 5 cm before being used in the experiment. Rainbow Trout with an average length of 5 cm were kindly provided by Laboratoire de Pisciculture Huet, Leuven, Belgium, and acclimatized for 2 months. The fish were maintained in 1-m³ stocking tanks filled with 800 L of recirculating and aerated tap water. The water temperature was 22°C ± 1°C for the carp and 19°C ± 1°C for the trout. Starting from 2 weeks before the experimental challenge, the water temperature of the stocking tanks was gradually increased by 1°C every 2 d until a temperature of 25°C ± 1°C and 22°C ± 1°C was reached for the carp and trout, respectively. This water temperature was then kept constant until the onset of the challenge. Ammonia and nitrite concentrations were monitored daily and were below detectable levels at all times. A photoperiod of 12 h light : 12 h dark was provided, and the fish were fed a commercial diet (trout: Trouw Nutrition; carp: Fin Perfect Feed, Sonubaits) to satiation twice daily. Fish were deprived of food 24 h prior to the experimental challenge. Twenty-five carp and 25 trout were sacrificed with an overdose of benzocaine (ethylaminobenzoate; Sigma, Diegem, Belgium). Gill and skin were screened for parasites and for the absence of F. columnare by means examining the bacteria cultivated on modified Shieh agar (Shieh 1980; Song et al. 1988) containing 1 µg/mL tobramycin (Decostere et al. 1997) and by PCR. For the latter, DNA from the tissue samples was extracted using a DNeasy blood and tissue kit (Qiagen, Venlo, The Netherlands) according to the guidelines of the manufacturer. The PCR mixtures and cycle conditions were the same as those described by Declercq et al. (2013a).

**Bacterial propagation.**—Five Common Carp isolates (0401781, 0901393, 10009061-1, 10012573/2, and CDI-A) and six Rainbow Trout isolates (JIP 44/87, JIP P11/91, LVDJ D7461, H2, B259, and Coho 92) were used for the inoculation trials. More information concerning origin, year of isolation, and provision of the isolates, is available in Declercq et al. (2013a). The identity of all F. columnare isolates was confirmed by PCR as described by Declercq et al. (2013a). The genomovar of the koi carp (a variant of Common Carp) and Rainbow Trout isolates was determined at the Aquatic Microbiology Laboratory of Auburn University, Auburn, Alabama, using 16S-restriction fragment length polymorphism (RFLP) according to the protocol as described by Olivares-Fuster et al. (2007b). Before inclusion in the challenge trials, each isolate was passaged once in the fish species it was retrieved from to assure all isolates were first passage isolates. This was done by experimentally inoculating fish through immersion, sampling the gills 6 h after the challenge and streaking the sample onto modified Shieh agar containing 1 µg/mL tobramycin. Following incubation at 28°C for 36 h, five colonies displaying the typical F. columnare morphology were purified and the resulting
cultures stored at −70°C in freezing medium (1% casitone Difco, 0.1% MgSO₄·7H₂O, and 10% sterile glycerol resolved in distilled water, pH 6.8-7). For the experimental challenge, the isolates were defrosted and grown over 36 h at 28°C on modified Shieh agar plates. Five colonies were used to inoculate plastic, 15-mL Falcon tubes filled with 4 mL of modified Shieh broth. The inoculated broth was placed on a shaker for 24 h at 28°C at 100 revolutions per minute (rpm). The cultivated broth of two Falcon tubes was used to inoculate 392 mL of modified Shieh broth in 500-mL glass bottles. After 24 h of incubation at 28°C on a shaker at 100 rpm, bacterial titers were measured by making 10-fold dilution series in triplicate on modified Shieh agar plates. Optical densities were measured using a spectrophotometer at 600 nm (Pharmacia LKB Ultraspec III, Biotech, Gaithersburg, Maryland). The grown bacterial cultures were used in the inoculation trials as described below.

**Experimental challenge.**—For each koi isolate, a group of 20 arbitrarily chosen Common Carp and for each trout isolate, a group of 20 arbitrarily chosen Rainbow Trout were removed from the stocking tanks and placed in a 10-L tank filled with 4.6 L of aerated water at 27°C ± 1°C (carp) or 23°C ± 1°C (trout). Then 400 mL of cultivated modified Shieh broth was added to the tank water. A control group of 20 fish were immersed in a tank with 4.6 L water with 400 mL modified Shieh broth without *F. columnare*. The carp were inoculated during a period of 90-min and then a group of 20 fish were transferred to a 60-L tank filled with 48 L of recirculated aerated tap water of 25°C ± 1°C. Two liters of the challenge water from the 10-L tank were transferred as well. The trout were inoculated during a 120-min period and then a group of 20 fish were transferred to a 1-m³ tank filled with 350 L of aerated, recirculated, tap water at 22°C ± 1°C. Both the carp and the trout were monitored every 3 h and mortality was recorded. Every 12 h, 75% of the water was replaced. Following the challenge and during the remainder of the trial, the fish were checked every 1 to 2 h. As soon as the predetermined humane endpoints (no reaction to stimuli, hanging at the water surface, loss of balance) were reached, the fish were euthanized with an overdose of benzocaine and sampled. The first left gill arch was removed and cut into three parts for examination by light microscope, scanning electron microscope (SEM), and transmission electron microscope (TEM) as described below. The counterpart right gill arch served for bacterial titration of *F. columnare* as described below. The trial lasted for 7 d and at the end of the experiment, all surviving fish, including the control fish, were sacrificed and the gill tissue sampled as previously described. The gill tissue of the control fish was additionally screened for the presence of *F. columnare* using PCR as described above.

The trials as described above were executed twice at different time points. For two Rainbow Trout isolates, B259 and JIP P11/91, the challenge experiments were carried out an additional third time, as discussed below. Isolates that were able to elicit 80% mortality or higher within 72 h were assigned as highly virulent, whereas isolates causing 20% mortality or less were designated low virulent. The isolates giving a mortality rate between 20% and 80% within 72 h were assigned as moderately virulent. The experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University, under the number EC2011/012.

**Bacteriological examination.**—The right gill arch was excised using sterile scissors and crushed in a sterile plastic tray. Half of the right gill arch was placed in a plastic 15-mL Falcon tube filled with 5 mL of a 1:7 diluted (3 g/L) cation-adjusted Mueller–Hinton broth (Becton Dickinson, Erembodegem, Belgium). Tenfold dilution series were made in plastic 15-mL Falcon tubes. From each dilution, 50 µL was retrieved and inoculated on specific modified Shieh agar containing 1 µg/mL tobramycin. Following incubation at 28°C for 36 h, the bacterial titers were determined. In addition, special attention was paid to the viscosity of the cultivated broth.

**Examination by light microscope.**—In case skin lesions were noted, the skin was excised over the full length of the lesion including the transitional zone between affected and normal-looking skin. The skin tissue and one-third of the first left gill arch were placed in phosphate-buffered 4% formaldehyde at room temperature for 24 h, dehydrated in an alcohol–xylene series (Microm tissue processor STP420D, Prosan, Merelbeke, Belgium) and embedded in paraffin wax (Microm embedding station EC 350-1, Prosan). All tissues were sectioned at 8 µm thickness (Microm microtome HM 360, Prosan) and stained with hematoxylin and eosin (H&E) and Giemsa stain.

**Examination by electron microscope.**—For SEM analysis, the gill samples were preserved in a HEPES–glutaraldehyde solution. Tissue samples were postfixed in 1% buffered osmium tetroxide for 2 h and dehydrated in an increasing alcohol series followed by increasing ethanol–acetone series up to 100% acetone as described by De Spiegelaere et al. (2008). The samples were then dried to the critical point with a Balzers CPD 030 critical point drier (Sercobal bvba, Merksem, Belgium) and further mounted on metal bases and sputter coated with platinum using the JEOL JFC 1300 Auto Fine Coater (Jeol, Zaventem, Belgium). The samples were examined with a JEOL JSM 5600 LV SEM (Jeol). For TEM processing, a similar protocol as described by De Spiegelaere et al. (2008) was used. For viewing, both the JEM-1200EX II Jeol TEM (Jeol) operating at 60 kV and the JEM-1400 plus Jeol TEM (Jeol) operating at 80 kV were used. Analog micrographs were taken on the SEM and subsequently scanned with an EPSON scanner (Type V700 PHOTO). Digital images were taken on the TEM.

**Statistical analysis.**—The data for mortality were not normally distributed and therefore transformed into a binary data set with values for “moribund fish” and “fish euthanized at the end of the experiment” reclassified as “0” and “1,” respectively. Based on this classification and by means of logistic regression, the data for each isolate were compared between trials to see whether the trials were reproducible.

For each species, the mortality pattern was compared for the different isolates based on the time to death. To do so, a
RESULTS

Mortality and Bacteriologic Data

Before initiation of the experiments, all screened fish were negative for parasites and *F. columnare*. All *F. columnare* isolates included in this study displayed the typical genomovar I profile. For each trial, the optical densities and bacterial titers, as determined by a plate count of the 400-mL inoculation broth, and the percent mortality of the various adopted isolates are presented in Table 1. The optical densities (ODs) of the cultivated broth varied between 0.51 and 0.97; the lowest and highest ODs did not correspond with the lowest and highest bacterial titers, respectively, as determined by a plate count. In all control fish, no clinical signs nor mortality occurred and *F. columnare* could not be isolated from the gill samples.

For the Common Carp, the immersion challenge with four of the carp isolates (0401781, 10012573/2, 0901393, and 10009061-1) resulted in 100% mortality within 9–12 h postinoculation in both trials. Hence, these isolates were assigned as highly virulent. The bacterial broth of these isolates was assigned viscous in both trials. The challenge with isolate CDI-A caused 5% mortality within 18 h in the first trial and 10% mortality within 11 h in the second trial, designating this isolate as low virulent. The bacterial broth of this isolate was less viscous compared with the cultured broths of the highly virulent isolates. No significant differences were found in the type of mortality (spontaneous or euthanized at the end of the experiment) between the two trials for each isolate. The survival analysis showed that the mortality rate for the low-virulent isolate CDI-A differed significantly from the highly virulent isolates. The mortality rates of the highly virulent isolates did not differ significantly from each other.

The average log-transformed bacterial titers that could be retrieved from the carp gills, and the corresponding percentage of fish from which *F. columnare* retrieval from the gills of euthanized fish after the challenge experiment was possible, are presented in Table 2. No *F. columnare* colonies could be isolated from any of the fish that were sacrificed at the end of the experiment; these were the surviving fish inoculated with the low-virulent isolate CDI-A and the control fish.

In the first trial all Rainbow Trout challenged with isolate B259 died between 15 and 24 h postinoculation. However, in the second trial only 20% of the fish inoculated with B259 died starting at 30 h up until 72 h postchallenge. In the third trial, no mortality per trial for the immersion challenges in Common Carp and Rainbow Trout using *F. columnare* was observed.

### Table 1. Optical densities and bacterial titers (CFU/mL) as determined by plate count of the 400-mL cultivated Shieh broth and the associated elicited mortality per trial for the immersion challenges in Common Carp and Rainbow Trout using *F. columnare*. Values are for trials 1, 2, and 3*; note for trout isolates B259 and JIP P11/91, a third trial (3*) was performed.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Isolate</th>
<th>Optical density of cultivated broth (trial 1, 2, 3*)</th>
<th>Bacterial titers (CFU/mL) of cultivated broth (trial 1, 2, 3*)</th>
<th>Percent mortality per trial (trial 1, 2, 3*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Carp</td>
<td>0401781</td>
<td>0.58, 0.69</td>
<td>6 × 10⁸, 2 × 10⁸</td>
<td>100, 100</td>
</tr>
<tr>
<td></td>
<td>10012573/2</td>
<td>0.59, 0.65</td>
<td>2 × 10⁸, 8 × 10⁹</td>
<td>100, 100</td>
</tr>
<tr>
<td></td>
<td>0901393</td>
<td>0.56, 0.65</td>
<td>2 × 10⁸, 2 × 10⁸</td>
<td>100, 100</td>
</tr>
<tr>
<td></td>
<td>10009061-1</td>
<td>0.54, 0.66</td>
<td>8 × 10⁷, 3 × 10⁸</td>
<td>100, 100</td>
</tr>
<tr>
<td></td>
<td>CDI-A</td>
<td>0.51, 0.58</td>
<td>2 × 10⁸, 2 × 10⁹</td>
<td>5, 10</td>
</tr>
<tr>
<td>Rainbow Trout</td>
<td>B259</td>
<td>0.94, 0.97, 0.91*</td>
<td>2 × 10⁸, 6 × 10⁸, 2 × 10⁸*</td>
<td>100, 20, 100*</td>
</tr>
<tr>
<td></td>
<td>JIP P11/91</td>
<td>0.80, 0.89, 0.82*</td>
<td>1 × 10⁸, 4 × 10⁸, 4 × 10⁸*</td>
<td>0, 100, 100*</td>
</tr>
<tr>
<td></td>
<td>LVDJ D7461</td>
<td>0.63, 0.36</td>
<td>2 × 10⁸, 2 × 10⁷</td>
<td>50, 15</td>
</tr>
<tr>
<td></td>
<td>Coho 92</td>
<td>0.73, 0.80</td>
<td>4 × 10⁸, 1 × 10⁸</td>
<td>20, 5</td>
</tr>
<tr>
<td></td>
<td>H2</td>
<td>0.77, 0.89</td>
<td>2 × 10⁸, 8 × 10⁸</td>
<td>15, 0</td>
</tr>
<tr>
<td></td>
<td>JIP 44/87</td>
<td>0.61, 0.74</td>
<td>2 × 10⁸, 4 × 10⁸</td>
<td>0, 0</td>
</tr>
</tbody>
</table>

Cox regression survival analysis was performed to compare the mortality rate between the isolates for each fish species.

For the statistical analysis of the bacterial titration counts, data for positive titers, which corresponded with the ability to retrieve bacteria from the gill tissue, were log transformed and differences between the different isolates for each species were tested using one-way ANOVA. All statistical results were considered to be significant when P-values were lower than 0.05. All analyses were performed using SPSS version 21.0.

### Table 2. Mean ± SD log-transformed titers of *F. columnare* (in decreasing order) as determined by serial dilutions of the gill tissue of euthanized fish and the associated average percent retrieval from the gill tissue of Common Carp after an immersion challenge. Values followed by different letters in the same order are significantly different (P < 0.05).

<table>
<thead>
<tr>
<th>Carp isolate</th>
<th>Average log-transformed titers</th>
<th>% retrieval</th>
</tr>
</thead>
<tbody>
<tr>
<td>0901393</td>
<td>7.73 ± 0.647 x</td>
<td>100</td>
</tr>
<tr>
<td>10012573/2</td>
<td>7.70 ± 0.845 yx</td>
<td>100</td>
</tr>
<tr>
<td>10009061-1</td>
<td>7.38 ± 0.697 z</td>
<td>100</td>
</tr>
<tr>
<td>0401781</td>
<td>7.38 ± 0.912 z</td>
<td>100</td>
</tr>
<tr>
<td>CDI-A</td>
<td>7.13 ± 0.977 zy</td>
<td>100</td>
</tr>
</tbody>
</table>
mortality started 18 h postinoculation and by 54 h postchallenge, all fish had died (Table 1). The bacterial broth of this isolate was assigned nonviscous in the trial in which it produced only 20% mortality, while in the other two trials in which 100% mortality was observed, the broth was assigned viscous. Hence, the overall elicited mortality was 73%. In the group challenged with isolate JIP P11/91, no mortality occurred in the first trial. In the second and third trials on the other hand, 100% mortality occurred between 15 and 72 h in the second and within 18 h postchallenge in the third trial, resulting in an overall mortality of 67%. As was the case for B259, the bacterial broth was assigned viscous in the trials in which this isolate resulted in 100% mortality compared with nonviscous in the trial in which there was no mortality. Since isolates B259 and JIP P11/91 were able to elicit 100% mortality in two trials, they were assigned as highly virulent. Fifty percent of the fish inoculated with isolate LVDJ D7461 died, of which 80% died within 54 h and the other 20% within 138 h after inoculation. In the second trial, challenge with this isolate resulted in 15% mortality within 72 h. Challenge with isolate Coho 92 elicited 20% mortality between 9 and 99 h postinoculation in the first trial. In the second trial adopting this isolate, 5% mortality occurred 72–75 h postchallenge. Inoculating the fish with isolate H2 generated 15% mortality between 15 and 51 h postchallenge in the first trial. In the second trial, 5% mortality occurred 72–75 h postchallenge. Inoculating the fish with isolate H2 generated 15% mortality between 15 and 51 h postchallenge in the first trial but no mortality in the second trial. All fish inoculated with isolate JIP 44/87 survived. The isolate LVDJ D7461 was designated as moderately virulent, whereas isolates Coho 92, H2, and JIP 44/87 were considered as low virulent. The trials differed statistically from one another (P < 0.05) as isolates JIP P11/91 and B259 gave only 0–20% mortality in one trial, while in the other two trials they elicited 100% mortality. For the other isolates, the mortality following the challenge did not differ significantly between the two trials. Survival analysis revealed no statistically significant differences in mortality rate between the highly virulent isolates. The survival of the fish inoculated with isolates Coho 92, LVDJ D7461, and H2 was significantly longer than for the highly virulent isolates.

The average log-transformed bacterial titers that could be retrieved from the trout gills, and the corresponding percentage of fish from which F. columnare retrieval from the gills of euthanized fish after the challenge experiment was possible, are presented in Table 3. Flavobacterium columnare could not be retrieved from the gill tissue of the surviving fish at the end of the experiment; these fish were the surviving fish inoculated with the low-virulent isolates and the control fish, except for one fish inoculated with isolate H2 (average bacterial titer, 6.50E+01 CFU/100 mg gill tissue).

Necropsy Findings in Common Carp

**Macroscopic examination.**—The control fish did not reveal any abnormalities. In the Common Carp that died after inoculation with one of the highly virulent isolates, all four gill arches of both sides were affected and displayed diffusely spread yet well-delineated yellowish-white discolorations (Figure 1). No skin lesions were discerned. The fish that were challenged with the low-virulent isolate CDI-A did not display any macroscopic lesions.

**Light microscope examination.**—In the control fish, no histopathologic lesions were perceived. Upon examination of the H&E-stained gill sections of the fish that died following the challenge with one of the highly virulent isolates, the discerned gill lesions ranged from mild to severe. In one-quarter of these fish, mild to moderate tissue damage consisting of desquamation of gill epithelial cells was observed coincided with the presence of filamentous bacteria; no inflammatory cells nor fusion of the lamellae were noted. In a second quarter of the fish, lesions were moderate to severe and comprised multifocal fusion of lamellae, and lamellar loss occurred mostly at the tips and in the middle of the filaments. Numerous filamentous bacteria clustered around the disintegrated lamellae and exfoliated epithelial cells. In addition, mild hemorrhage and the occasional presence of inflammatory cells with the morphology of macrophages were recorded. No fusion of the filaments was discerned. The gill sections of one-half of the fish depicted more severe lesions with a diffuse merger of the filaments and extensive lamellar loss. The lamellae were replaced by necrotic debris and desquamated and inflammatory cells. On top of the filaments, huge clusters of long and slender bacteria embedded in an eosinophilic matrix and necrotic debris were observed (Figure 2). In the few fish that died following inoculation with the low-virulent isolate CDI-A, a minority of the lamellae showed mild fusion and filamentous

![FIGURE 1](image-url) Gill lesions in a 5-cm-long Common Carp fry 12 h postinoculation with the highly virulent F. columnare isolate 0901781. Diffuse yellowish-white discolorations of the gill arches are evident where the left operculum has been removed. [Figure available online in color.]
bacteria were noted only sporadically. No other histopathologic lesions were present. In the fish that survived the CDI-A isolate challenge, no abnormalities were observed.

Electron microscopic examination.—The SEM and TEM pictures of the gills of the control fish did not reveal any abnormalities.

The severity of gill lesions as observed with the SEM of the carp inoculated with one of the highly virulent isolates corresponded to the histopathologic traits as described above. Carp challenged with one of the highly virulent isolates revealed a diffuse fusion of filaments and lamellae with extensive clumps attaching to the full length of filaments that obscured the epithelial finger printing pattern (Figure 3a). At a higher magnification, these lumps were observed to be composed of microcolonies of long, slender, rod-shaped bacterial cells approximately 0.3–0.5 \( \mu \)m wide and 3–10 \( \mu \)m long. The bacteria were aggregated rather than spread individually throughout the epithelial surface. In between the dense twirl of bacterial cells, red blood cells and cellular debris were visible (Figure 3b). In the fish inoculated with the low-virulent isolate, no abnormalities were noted.

The TEM images of the gill tissue of the carp following the challenge with one of the highly virulent isolates all revealed severe damage in which edema and cell necrosis prevailed. Long, slender bacterial cells lined up along the lamellar epithelium and in between lamellae and were wrapped in cellular debris (Figure 4). In the gills of fish surviving a challenge with the low-virulent isolate and of fish euthanized at the end of the experiment, no abnormalities were discerned apart from some slight edema in only a minor part of the gills.

Necropsy Findings in Rainbow Trout

Macroscopic examination.—The control fish did not display any abnormal features. In the Rainbow Trout that died after the challenge with a highly virulent isolate, gill lesions mostly occurred on one side and were only visible in the first two gill arches. The lesions appeared as discolored foci of 5–10 mm scattered throughout the gill tissue (Figure 5). One-half of the fish that died following the challenge with a highly virulent isolate displayed pale discolorations of the skin typically starting around the dorsal fin and spreading laterally resembling a saddle. The lesions spread farther ventrally and caudally towards the pelvic and anal fin. Skin lesions were also noted surrounding the pectoral fin. Progressively, the delicate tissue in between the fin rays disappeared, exposing the rays, especially those of the dorsal fin (Figure 6). The fish died within 12 h following the appearance of the skin lesions.
The trout that died after inoculation with a moderately or low-virulent isolate or that were sacrificed at the end of the experiment did not reveal any macroscopic lesions.

Light microscope examination.—The gills of the control Rainbow Trout did not display any abnormalities. The gill lesions in the trout challenged with the highly virulent isolates were distributed focally, involving up to one-half of the gill filaments. In these areas, the lamellar epithelium was necrotic or had disappeared completely and was replaced by a microcolony of filamentous bacteria encased in an eosinophilic matrix (Figure 7a, b). Leukocytes with the morphology of macrophages and eosinophilic cells and red blood cells were also noted. In adjacent lamellae, mild to moderate edema was present. One-fourth of the fish that died following inoculation with a highly virulent isolate showed less severe histopathological lesions. The gill epithelia of fish that had died following challenge with the moderately and low-virulent isolates were mildly dissociated from the capillary bed. Fusion of the lamellae and the presence of inflammatory cells were also noted. The gill tissues of the fish that survived the challenge showed no abnormalities apart from mild edema.

Histopathology of the skin lesions revealed epidermal ulceration and necrosis with loss of scales accompanied by hemorrhage, massive infiltration of long, slender bacteria embedded in an eosinophilic material, and the presence of inflammatory cells. Bacterial cells even invaded the deeper skin layers resulting in interstitial infiltration of the fat and muscle tissue with myositis.

Electron microscopic examination.—The SEM and TEM images of the gills of the control fish did not reveal any abnormalities. For the trout inoculated with one of the highly virulent isolates, the affected tissue sites as observed using the SEM, showed multifocally distributed merger of filaments and lamellae. Massive bacterial clusters embedded in between mucus, red blood cells, and cellular debris were present. The bacterial cells measured 0.3–0.5 µm in width and 3–10 µm in length. The epithelial fingerprinting pattern of the gill filament was still discernible in sites adjacent to the *F. columnare* microcolony formation (Figure 8). The SEM sections of the gills of the trout challenged with the moderately and low-virulent isolates and the fish sacrificed at the end of the experiment did not reveal any abnormalities.

The TEM images of the gills of Rainbow Trout challenged with one of the highly virulent isolates revealed the lamellar epithelium to be surrounded by bacteria, cellular debris, and inflammatory cells. Loss of the normal lamellar structure with lifting of the epithelium and consequent lamellar necrosis were noted, especially in the middle and at the tips of the filaments. In one of the gills of a fish that died following the challenge with a moderately virulent isolate, the presence of a few long, slender, bacterial cells adjacent to the gill epithelium could be visualized and edema and lamellar necrosis were visible. In all other samples of trout inoculated with the moderately or
FIGURE 7. (a) H&E-stained section of the first left gill arch of a Rainbow Trout fry 27.5 h postchallenge with a highly virulent *F. columnare* isolate (JIP P11/91). The upper four gill filaments reveal lamellar necrosis and fusion of the lamellae at the middle parts and at the tips. The four gill filaments situated in the lower figure half are fused, their gill lamellae are fully necrotic and replaced by *F. columnare* bacterial cells. Bar = 200 μm. (b) Detail of inset from (a). The necrotic lamellae are nearly completely replaced by vast microcolonies of long, slender bacteria (arrows). Bar = 100 μm. [Figure available online in color.]

low-virulent isolates, no bacterial cells were discerned. Lifting of the epithelium did occur in the latter samples.

DISCUSSION

Hitherto, the virulence mechanisms of *F. columnare* are still far from being fully elucidated. In particular, the way this pathogenic agent interacts with and causes lesions to the gill tissue remains to be explored, even though a detailed description of the elicited gill lesions is currently lacking. This scarcity of data is largely rooted in the absence of a reproducible infection model that would induce gill lesions like those observed in the field. To our knowledge, this study is the first to pinpoint an experimental infection model in Common Carp and Rainbow Trout that uses immersion as a means through which severe gill lesions typical for columnaris disease are elicited. In addition, in this study the gill lesions are described in detail on a macroscopic, light microscopic, and an ultrastructural level.

In the challenge protocols for columnaris disease that have been published so far, cultivation and harvesting methods, inoculation temperatures, incubation periods, and whether tissues need to be scarified are only some of the existing variables, even with those using the same fish species. These variations have recently been reviewed by Declercq et al. (2013b) and point towards the delicate balance and complex interplay between bacterial cells, fish, and environment in the successful reproduction of this bacterial disease in the laboratory. Indeed, for the latter there are a number of obstacles to overcome. Firstly, there is the tendency of some *F. columnare* isolates to clump in broth culture and adhere strongly to the agar surface (Bernardet and Bowman 2006; Darwish et al. 2008; Kunttu et al. 2011), rendering it difficult to make a homogeneous suspension and thus adequately determine bacterial titers in challenge inocula and tissues. This phenomenon indeed poses a true challenge for developing standardized inoculation procedures. This nonhomogeneous growth was also exhibited by various isolates used in the current study, as well as in others, and is reflected by the lack of correlation between OD and bacterial titration values determined by plate count. Clumpy cultures make it difficult to take a reliable subsample for determining bacterial titers. This clump-like bacterial growth could additionally cause an underestimation of the bacterial numbers as obtained by plate culturing when more than one bacterial cell gives rise to one colony. Using quantitative PCR (qPCR) to determine bacterial titers of the inoculation broth could aid to more reliably determine bacterial inoculation titers. This technique has been described
for *F. columnare* (Panangala et al. 2007), and in merely a few hours, researchers could consult the results and decide whether to include bacterial isolates in the challenge. A disadvantage, compared with bacterial titration, is that dead bacteria are also counted, a problem that could be circumvented by combining an ethidium bromide monoazide (EMA) treatment and real-time (RT) PCR, thereby allowing differentiation between viable and dead bacteria, as recently described for quantitative detection of *Helicobacter suis* in pork (De Cooman et al. 2013). With regard to determining the number of *F. columnare* bacteria in tissues and comparing these values between isolates, besides using plate counting, it is advisable to substantiate these findings using, for example, qPCR or microscopy imaging techniques as was done in the current study. Secondly, in order to be able to develop a reliable challenge model, fish that are specifically free of *F. columnare* are needed. Various research groups have described that fish may dwell in a clinically healthy carrier status housing an isolate remaining from a previous infection with columnaris disease (Fujihara and Nakatani 1971; Suomalainen et al. 2005a). Should carrier fish be included, then the interaction patterns of the test isolates may be altered and thus would hamper a reliable outcome of the experiment. Therefore, before purchasing the fish for this study, several individuals from carp farms and retailers of varying origin were sampled for the presence of *F. columnare*, culture-based methods proved to be insufficiently reliable for diagnosing flavobacterial diseases from fish tissues. For this reason, this study used PCR to label the fish as specific *F. columnare* free. The carp that were eventually included in this study were purchased as 2-d-old larvae from a farm with no history of columnaris disease. The Rainbow Trout farm from which the trout fry were retrieved was also free from columnaris disease. All fish were negative by culture but positive by PCR (data not shown). Indeed, based on results of Suomalainen et al. (2005a), culture-based methods proved to be insufficiently reliable for diagnosing flavobacterial diseases from fish tissues. For this reason, this study used PCR to label the fish as specific *F. columnare* free. The carp that were eventually included in this study were purchased as 2-d-old larvae from a farm with no history of columnaris disease. The Rainbow Trout farm from which the trout fry were retrieved was also free from columnaris disease. All fish were housed under strict quarantine conditions. Adopting both cultivation and PCR techniques to demonstrate *F. columnare* free status of the fish included in the study has to our knowledge not been done before and thus provides a basis for future research.

A remarkable feature was that the highly virulent trout isolates JIP P11/91 and B259 induced no or only 20% mortality, respectively, in one out of the three trials in which they were adopted. This provides the rationale for carrying out the trial with both isolates three times in the course of time. Noteworthy is that in the trials where no or only low mortality was induced, the cultivated broth was much less viscous compared with the other trials in which these two isolates displayed highly viscous cultures and induced 100% mortality. Kunttu et al. (2009, 2011) described that one isolate may indeed produce different colony types with varying virulence. Those authors stated that colony morphology changes might be caused by phase variation and that different colony types isolated from infected fish might indicate different roles of the colony morphologies in the infection process of columnaris disease (Kunttu et al. 2011). Unfortunately, no correlations or speculations on the viscous character of the associated broth cultures were given, lending this topic merit for further research to determine the cause and degree of viscosity of the culture and to elucidate the possible interplay between viscosity, colony type, and virulence.

In Channel Catfish *Ictalurus punctatus*, genomovar II is considered to be more virulent than genomovar I (Olivares-Fuster et al. 2007a; Shoemaker et al. 2008). In one study the challenge to Rainbow Trout with genomovar I and II isolates of *F. columnare* induced significantly higher cumulative percent mortalities (LaFrentz et al. 2012), while in another study genomovar I demonstrated a high degree of pathogenicity against Rainbow Trout fry at 15°C (Evenhuis et al. 2014). In this study, all adopted isolates belonged to genomovar I and a correlation between virulence and genomovar could not be made.

Both macro- and microscopic examination of the affected gills revealed a different distribution pattern of the lesions as induced by the highly virulent *F. columnare* isolates in Common Carp compared with those in Rainbow Trout. Indeed, the gills of the affected carp showed a diffuse distribution of the lesions, affecting all gill arches bilaterally. At least half of the gill tissue was destroyed, and filamentous bacterial cells were spread diffusely, clustering on top of the filaments and forming vast microcolonies. In trout, the distribution pattern of the gill lesions was more focal, mostly located unilaterally and only present in the two first gill arches.

Data resulting from the bacteriologic and histopathologic examination of the gill tissue of fish with the low- and moderately virulent isolates do not seem to correlate. Indeed, the *F. columnare* gill titers following the challenge with the low-virulent carp (CDI-A) and trout (Coho 92) isolates and the moderately virulent trout (LVDJ D7461) isolate were high, whereas upon histopathologic examination of the sampled gill tissue, hardly any filamentous bacteria were discernible. The possible explanation for this phenomenon may be twofold. Firstly, one should consider that only a limited part of the gill tissue is examined by means of histological examination. If adhesion is localized or if the gills are only focally colonized, the bacteria may not be detected by histological examination but could be found by means of bacteriological examination, as was noted previously by Decostere et al. (1999). Secondly, the few bacterial cells observed within the gill tissue of the fish inoculated with the low virulent isolates were not in close contact with the host epithelium. These bacteria most likely were part of the aqueous biofilm covering the gill tissue and hence could be noted upon bacteriological examination when plating out the tissue samples. However, the processing of the gill tissue for histological examination involved several staining and washing steps and the resulting tissue sections revealed only firmly attached bacteria. The presence of the firmly attached bacteria seems to be the case only for the highly virulent isolate and to a much lesser extent for the moderately and low-virulent isolates, which explains the sparsely present bacterial cells upon histological inspection.
Indeed, upon examination of the H&E-stained gill sections of the carp and trout challenged with a highly virulent isolate, massive infiltrates of *F. columnare* cells were seen attached to the gill tissue along the gill filaments and lamellae and entangled between necrotic tissue. This feature typically resembles biofilm formation, a phenomenon that has recently been studied in vitro for *F. columnare* (Cai et al. 2013). To our knowledge, this is the first time massive clusters of microcolonies resembling biofilm formation have been visualized in the gill tissue of both Common Carp and Rainbow Trout after an experimental challenge. These in vivo findings are consistent with the demonstration of genes in *F. columnare* encoding for biofilm formation by the research group of Tekedar et al. (2012). The filamentous bacterial cells were seen encased in an eosinophilic matrix. It is tempting to speculate on the origin of this observed matrix, namely whether it is related to the bacterium or host. This embedment may afford protection for the bacterial cells against external factors, such as fish mucus containing antimicrobial substances, and even impede the penetration of antimicrobial agents that are administered via the water. Hence, this matrix formation may constitute an important step in the pathogenesis of columnaris disease and needs to be studied in greater detail.

In conclusion, the current study described an immersion inoculation model in Common Carp and Rainbow Trout that was used to elicit gill lesions typical for columnaris disease as observed in the field. In this way, an in-depth study of the interaction of this pathogen with its target tissue was facilitated. In addition, the gill lesions described in detail the progression from intact to partially or completely destroyed gill tissue as a means to clarify the pathogenesis of columnaris disease and contribute to the development of efficient methods to combat the disease without having to resort to antimicrobial agents.

**ACKNOWLEDGMENTS**

The *F. columnare* isolates were kindly provided by Jean-François Bernardet (Unité de Virologie et Immunologie Moléculaires, INRA Centre de Recherches de Jouyen-Josas, France), Laurie Caslake (Department of Biology, Lafayette College, Easton, Pennsylvania), ir. Olga L.M. Hae- nen (Fish and Shellfish Diseases Laboratory, Central Veterinary Research Institute, Wageningen, The Netherlands), and Lotta-Riina Sundberg (Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland). The Marcel Huet research platform (Louvain la Neuve, Belgium) is gratefully acknowledged for supplying the specific-pathogen-free Rainbow Trout. Professor Cova Arias and Haitham Mohammed of Auburn University, Auburn, Alabama, are thanked for the genomovar determination of the *F. columnare* isolates used in this study. We thank Professor Simoons for insightful comments on the manuscript. Furthermore, Lobke De Bels, Jurgen De Craene, Bart De Pauw, Marleen Foubert, Leen Pieters, Hanne Vereecke, and Patrick Vervaet are gratefully acknowledged for their technical assistance during the trials. The Special Research Grant (Bijzonder Onderzoeks- fonds, BOF; grant number 01Z06210) of Ghent University, Ghent, Belgium, and the Hercules foundation (grant number AU/GE/11/009) are gratefully acknowledged for financial support.

**REFERENCES**


GILL INFECTION MODEL FOR COLUMNARIS DISEASE


Exophiala angulospora Causes Systemic Mycosis in Atlantic Halibut: a Case Report

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Published online: 10 Dec 2014.

To cite this article: David P. Overy, David Groman, Jan Giles, Stephanie Duffy, Mellisa Rommens & Gerald Johnson (2015) Exophiala angulospora Causes Systemic Mycosis in Atlantic Halibut: a Case Report, Journal of Aquatic Animal Health, 27:1, 12-19, DOI: 10.1080/08997659.2014.953266

To link to this article: http://dx.doi.org/10.1080/08997659.2014.953266

PLEASE SCROLL DOWN FOR ARTICLE
Exophiala angulospora Causes Systemic Mycosis in Atlantic Halibut: a Case Report

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Abstract
Filamentous black yeasts from the genus Exophiala are ubiquitous, opportunistic pathogens causing both superficial and systemic mycoses in warm- and cold-blooded animals. Infections by black yeasts have been reported relatively frequently in a variety of captive and farmed freshwater and marine fishes. In November 2012, moribund and recently dead, farm-raised Atlantic Halibut Hippoglossus hippoglossus were necropsied to determine the cause of death. Histopathology revealed that three of seven fish were affected by a combination of an ascending trans-ductual granulomatous mycotic nephritis, necrotizing histiocytic encephalitis, and in one fish the addition of a fibrogranulomatous submucosal branchitis. Microbial cultures of kidney using selective mycotic media revealed pure growth of a black-pigmenting septated agent. Application of molecular and phenotypic taxonomy methodologies determined that all three isolates were genetically consistent with Exophiala angulospora. This is the first report of E. angulospora as the causal agent of systemic mycosis in Atlantic Halibut.

Exophiala spp. are ubiquitous fungi, historically isolated from a variety of environmental substrates such as soil and sediment, decaying wood and plant material, human hair and nails, and drinking water (Iwatsu et al. 1991; Domsch et al. 2007; de Hoog et al. 2011). These filamentous black yeasts are also opportunistic pathogens causing both superficial and systemic mycoses in warm- and cold-blooded animals (Uijthof et al. 1997; de Hoog et al. 2011; Gjessing et al. 2011). de Hoog et al. (2011) suggest that animals with moist skin are more susceptible to infection by filamentous black yeasts, where the ability of the pathogen to assimilate alkylbenzenes and accumulate melanin within the hyphae and conidia are purported to be general virulence factors for these agents. Black yeast infections in aquarium and farmed fish as well as amphibians are relatively frequent. An accurate estimation of the magnitude of the disease outbreaks has been difficult to ascertain due to the infrequent and happenstance nature of disease reports, even though outbreaks of this mycotic disease have been reported to result in severe losses (de Hoog et al. 2011).

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Received January 27, 2014; accepted July 23, 2014
Although first isolated and described from drinking water (Iwatsu et al. 1991), *Exophiala angulospora* is also an opportunistic pathogen of both freshwater and marine fishes. *Exophiala angulospora* has been reported to cause mycosis in aquaria-maintained Weedy Seadragons *Phyllopteryx taeniolatus* in the United States (Nyaoke et al. 2009). This pathogen has also been isolated from Inconnu *Stenodus leucichthys* in southern Russia (de Hoog et al. 2011) and was determined to be the cause of systemic mycosis in farmed Atlantic Cod *Gadus morhua* from Norway (Gjessing et al. 2011). Necropsy of moribund cod revealed chronic multifocal inflammation in the internal organs, consisting of dematiaceous fungal hyphae surrounded by distinct layers of inflammatory cells. Gjessing et al. (2011) noted that the fungal infection was not eliminated by this inflammatory response in the cod, resulting in a systemic infection and mortality.

In this case report we describe the diagnostic evaluation of 2-year-old juvenile Atlantic Halibut * Hippoglossus hippoglossus* that were submitted on multiple occasions from a grow-out population of approximately 8,000 fish during November 2012. These moribund fish showed reduced appetite and originated from the lowest grade (~4,000 fish) and represented less than 0.05% of the population. Affected fish were 40% smaller than the remaining cohort based on mean weights determined from batch samples of fish. Clinical observations of these fish, as reported by the onsite clinician, were limited to reduced appetite and loss of equilibrium.

Necropsies and diagnostic testing on both fresh dead and moribund fish harvested from the slowest-growing group in the population were undertaken at the Aquatic Diagnostic Services Unit of the Atlantic Veterinary College, University of Prince Edward Island, Charlottetown. Fish had been reared in 1.5 × 3.7 × 0.38-m (5 × 12 × 1.25 ft) and 1.83 × 3.7 × 0.38-m (6 × 12 × 1.25 ft) fiberglass tanks (water temperature, 12.1–12.2°C; O₂, 105–119% saturation; N₂, <96% saturation) and were being fed dry commercial fish feed (6-mm Europa 18, Skretting Feeds). Cumulative mortality during the previous 2 months was 5 to 10 times higher than normally expected, ranging from 0.8% to 1.5% per month compared with the normal rate of <0.1% per month. Although this level of mortality was still considered to be very low, fish were still submitted for routine necropsy as the producer could document incidental findings and infections. Diagnostic investigation into the cause of death revealed that the halibut were suffering from a systemic mycotic infection caused by *Exophiala angulospora*. To our knowledge this is the first report of a clinical mycosis due to *E. angulospora* in farm-reared Atlantic Halibut.

**METHODS**

**Necropsy**

Seven Atlantic Halibut—three live, three mortalities, and one fresh dead—were submitted for necropsy. The live fish were euthanized using an overdose of a saturated solution of benzocaine (PCCA, London, Ontario) dissolved in 95% ethanol. All fish were subsequently examined for gross external and internal lesions, and assessed for sex determinations and contents of the alimentary tract. Wet mounts of the gills of all live fish were examined by phase contrast microscopy.

**Histopathology**

The following tissues were selected from all seven fish at the time of necropsy, trimmed to approximately 1 cm in diameter and placed into 10% neutral buffered formalin (Fisher Scientific, Fairlawn, New Jersey): gill, brain, eye, kidney, spleen, heart, liver, nares, gonad, esophagus, stomach, intestine, pyloric ceca, pancreas, and body wall (1:10 ratio of tissue to fixative). Fixation was completed by holding all samples on a shaker for a minimum of 48 h prior to routine histological processing. For tissues containing bone and/or cartilage (cranium, eye, body wall, gill), decalcification was undertaken by immersion for a further 24 h in a commercially available decalcification fluid (CAL-EX II, Fisher Chemical). Sections from all tissues were subsequently trimmed and placed into cassettes, processed for paraffin embedding, sectioned at 5 µm, mounted and dried onto glass slides, and stained by the hematoxylin and eosin (H&E) method (Luna 1968:38–39). Selected tissues were resectioned and stained by the periodic acid-Schiff (PAS) technique (Luna 1968:158). Stained slides were examined using a Leica DM2500 light microscope and, where appropriate, digital images were obtained with a top-mounted PixelLink PL-B625 digital microscope camera using the PixelLINK µScope Standard version 3.6 software.

**Bacteriology**

A sterile swab of the kidney was provided at necropsy for routine bacteriology from each fish for phenotypic identification. Swabs were streaked onto blood agar and blood agar containing 2% NaCl, and held at both 22°C and 15°C for a minimum of 7 d. Any bacterial growth deemed significant was subcultured once onto new media and subsequently identified using routine biochemical and immunologic methodologies (AFS–FHS 2010).

**Mycology**

*Phenotypic identification.*—A sterile swab of the kidney was provided at necropsy for routine mycology from each fish. Swabs were streaked onto Sabouraud’s agar, incubated at room temperature (~22°C), and monitored daily for fungal outgrowth. Axenic fungal isolates obtained were subcultured once onto potato dextrose and Sabouraud’s agar at 22°C and held for a period of up to 2 months. Micromorphology was visualized from cellotape slide mounts prepared in lactic acid and observed under 40 × and 100 × magnification using phase contrast microscopy on a Leica DME microscope. Digital photomicrographs were obtained using a Leica EC3 camera (Leica Microsystems, Switzerland) and micromorphological measurements were made from digital photomicrographs using the Leica LAS EZ software (version 2.1.0).
**Extraction of DNA and PCR amplification.**—Genomic DNA (gDNA) was obtained from isolated strains using the fast DNA extraction kit (FastDNA SPIN Kit for Soil, MP Biomedicals) according to the manufacturer’s protocols. The ITS rRNA gene was amplified by PCR using 50 μL of reaction mixture consisting of 25 μL of Econo Taq PLUS GREEN 2 × Master Mix (Lucigen), 17 μL of sterile double-distilled H2O, 2 μL of each primer (ITS-1 and ITS-4: White et al. 1990), and 4 μL of gDNA. Reactions were run in a Biometra thermocycler using an initial denaturation step at 96°C for 3 min followed by 35 cycles of 45 s for denaturation at 96°C, primer annealing at 54.5°C for 45 s, and extension at 72°C for 1 min; the reaction was completed with a final extension step of 10 min at 72°C. The PCR amplicons were checked for correct length and concentration by electrophoresis in 1% agarose gel in 1× TAE buffer (tris base, 2.42 g; glacial acetic acid, 0.572 mL; 0.5 M EDTA, 1 mL; double-distilled H2O added to 500 mL).

**Sequencing of DNA and phylogenetic analysis.**—The ITS amplicons were sent to a commercial sequencing facility (Eurofins MWG Biotech) and sequenced on a 3730xl DNA analyzer coupled with BigDye Terminator version 3.1 cycle sequencing reagents (Applied Biosystems). The generated sequences were compared with other fungal ITS sequences from the GenBank sequence database from the National Center for Biotechnology Information using a BLASTN search algorithm. Using the software Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura et al. 2011), a data set was compiled of 42 ITS nucleotide sequences of Exophiala spp. (39 obtained from GenBank), and a sequence alignment was subsequently performed using the ClustalW algorithm with a DNA Gap Open Penalty = 15.0, DNA Gap Extension Penalty = 6.66, and a delay divergent cutoff of 30% To infer the evolutionary history of the data set, the neighbor-joining method was used to construct a bootstrap consensus tree from 2,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method and all ambiguous positions were removed for each sequence pair during analysis.

**RESULTS**

**Necropsy Findings**

A total of seven Atlantic Halibut were examined: three mortalities and four live moribund specimens. The mean weight of the three mortalities was 227 g. None of the fish showed evidence of significant external lesions to the body proper. Wet-mount evaluations of the gills from the four live fish were found to be negative for both bacteria and parasites. Lenticular opacity was noted in three of seven fish and digesta was found in the lumen of the intestine in three of seven fish. Three of seven fish showed significant internal changes as specified below, including two fish with melanized discoloration of the midbrain including regions of the cerebellum and tegmentum (Figure 1A, C). Specimen U30818_F3 was a fresh mortality that presented on gross examination with flared gills. Specimen U29736_F4 was a live female that presented on gross examination with both splenomegaly and serosanguineous ascites. And specimen U29736_F1 was a female mortality that presented on gross examination with an enlarged kidney from which a chalky white fluid oozed from a cutsection and the urinary bladder was filled with a soft mineral-like substance.

**Histopathology Findings**

**Specimen U30818_F3.**—The gills of this fish showed low frequency, random distribution of respiratory epithelial cells containing a chlamydial-like organism with no associated inflammatory infiltrate. Both kidney and spleen sections contained regions of histiocytic and granulomatous infiltration containing septate, brown-colored, dematiaceous hyphae (golden-brown in H&E staining). Brain sections revealed locally extensive multifocal fungal colonization with moderate diffuse histiocytic infiltrate and intracerebral hemorrhage (Figure 1B). No additional significant morphological changes were noted.

**Specimen U29736_F4.**—This fish presented with a unilateral liquefactive lenticular cataract. No significant morphological changes were noted in tissue sections of the body wall and nares. Brain sections revealed multifocal fungal colonization of the forebrain with evidence of necrosis and granulomatous infiltration (Figure 1B, D), especially prominent in association with the meningeal vasculature (Figure 1E). Tissue sections of the gill showed marked necrosis, granulomatous infiltrate, and hemorrhage in the gill arch that extended into an adjacent base of filaments. The lesion was colonized by a brown-colored, dematiaceous, septate fungus (Figure 2A, B). Kidney sections contained a locally extensive, necrotizing, granulomatous infiltration of the renal interstitium and urinary duct in the rostral trunk kidney, with a marked colonization by a brown-colored, dematiaceous, septate fungus. Fungal hyphae were present in high frequency in both the urinary duct lumen and adjacent peritubular interstitial tissue (Figure 2D).

**Specimen U29736_F1.**—Kidney sections from this fish showed locally extensive, necrotizing, granulomatous infiltration of the renal interstitium and urinary ducts of the rostral trunk kidney, with a marked fungal colonization of both the duct lumen and adjacent peritubular interstitial tissue (Figure 2C, D). Fungal hyphae were brown-colored, dematiaceous, and septate (Figure 2B). Application of the PAS stain highlighted the morphology of the fungal hyphae (Figure 2D). No significant morphological changes were noted in tissue sections of the ovary, liver, esophagus, stomach, intestine, spleen, liver, heart, nares, brain, or eye.

**Bacteriology Findings**

Moderate to heavy growth of *Lactobacillus* sp. was recovered on blood agar from the kidneys of two fish (U29736_F4 and U30818_F1), all other fish were found to be free of significant bacterial agents.
FIGURE 1. Gross and microscopic images of brain with encephalitis due to infection with *Exophiala angulospora* in Atlantic Halibut. (A, C) Gross midsagittal sections through formalin-fixed infected brain tissue (b) from two different fish; the circled area shows melanization of affected neuropile. (B) Inflammation of neuropile with perivascular hemorrhage (arrow). (D) Higher magnification of neuropile showing fungal hyphae (arrows). (E) PAS staining of fungal hyphae (arrow) in affected neuropile. [Figure available online in color.]
**Mycology Findings**

*Molecular identification and phylogenetic analysis.*—Three dematiaceous fungal isolates were obtained from the kidney samples in each of the three fish submitted (denoted as isolates U29736_F1, U29736_F4, and U30818_F3). BLASTN ITS sequence searches against GenBank returned a 100% sequence homology of the three isolates to various isolates of *E. angulospora*. A data set of the ITS rDNA gene was compiled and analyzed to infer the relative evolutionary history of isolates U29736_F1, U29736_F4, and U30818_F3 with other representative *Exophiala* species (Figure 3). The analysis involved 42 sequences and included 604 positions in the final data set with an overall mean distance calculated as 0.095 and an SE of 0.035. Isolates U29736_F1, U29736_F4, and U30818_F3 formed a well-supported clade (100% bootstrap support) with other isolates of *E. angulospora*, including the type strain, which was phylogenetically distinct from other representative taxa of the genus. Alignment of the ITS sequences revealed a 100% agreement between isolates U29736_F1 and U29736_F4 and the *E. angulospora* isolates VI05436 and VI03759 (isolated from Atlantic Cod) and CBS 11911 (isolated from Weedy Seadragon). Isolate U30818_F3 aligned in 100% agreement with *E. angulospora* CBS 121503 (isolated from Inconnu), and these strains differed from the aforementioned isolates by a single nucleotide base-pair substitution.

*Phenotypic taxonomy.*—Morphological phenotypes of the isolates were examined and compared with the published description of *E. angulospora* (Iwatsu et al. 1991). Colonies
were slow growing, olivaceous black, and velvety in appearance with a black reverse (Figure 4A). Conidiogenesis was annellidic with conidia accumulating in slimy masses from intercalary (Figure 4B) and branched (Figure 4D) or unbranched flask-shaped or cylindrical conidiogeneous cells that tapered to a short beak at the apex and were septate at the base (Figure 4E). Conidia were single celled and variable in shape, ranging from obovoid and oblong to angular (Figure 4C). Mature hyphae, conidiophores, and conidia were dematiaceous.
FIGURE 4. Morphology of *Exophiala angulospora* isolated from Atlantic Halibut (isolate U29736_F1). (A) Gross colony morphology on potato dextrose agar after 14 d incubation at 22°C (scale = 1 mm). Conidiogenesis was annellidic from (B) intercalary, (D) branched, or (E) unbranched flask-shaped or cylindrical conidiogenous cells (scale = 10 µm). (C) Conidia were single celled and variable in shape ranging from obovoid and oblong to angular (arrows). [Figure available online in color.]

DISCUSSION

Systemic infection caused by a dematiaceous fungus was evident in tissue samples from three of the seven Atlantic Halibut that were submitted for necropsy. From tissue outgrowths, a dematiaceous fungus was isolated and confirmed by both phenotypic examination and ITS sequence homology to be *Exophiala angulospora*. The lack of significant external lesions on the three fish, as well as the consistent occurrence of fungal hyphae within the urinary duct and renal interstitium of the kidney, suggest that the urinary tract was the site of infection ingress prior to the ultimate dissemination of the infection to the brain. In one of the three fish, evidence of fungal infection of the gills suggests a second locus of fungal ingress; however, it is more likely that the fungus was disseminated to other tissues from the kidney via the circulatory system. Systemic mycosis caused by *E. angulospora* has been previously described from two marine fishes: Weedy Seadragon and Atlantic Cod. Similar to our observations, in the case of Atlantic Cod ingress was suggested to occur via the urinary tract to the kidney, as the kidney was found to be infected in all of the diseased fish examined (Gjessing et al. 2011). Fungal ingress in Weedy Seadragons was believed to occur via the gill or skin through preexisting lesions such as abrasions (Nyaoke et al. 2009). All of these examples of infections by *E. angulospora* were incidental, chronic, and likely not highly contagious. As such, these types of fungal infection would not be considered to be a major impact to the halibut industry in Canada or worldwide.

*Exophiala angulospora* is a ubiquitous fungus, and reports of isolations have been made from water, decorticated wood, human skin and nails, soils, and diseased fish (de Hoog et al. 2011; Gjessing et al. 2011). Due to the ubiquitous nature of the fungus the origin of the *E. angulospora* inoculum found in the diseased Atlantic Halibut is unclear. In this instance, the chronic nature and low prevalence of this infection did not support a
decision to undertake treatment. In fact, there are currently no approved systemic therapeutants for fungal infections of food fish in Canada, and as such it would have been unlikely that any antifungal agent would have been prescribed to this population.

Determination of the source of the *E. angulospora* inoculum was outside the scope of this investigation; however, multiple sources are possible and *E. angulospora* could have originated from the stocked juvenile halibut, commercial feed, seawater supply, or surfaces of the rearing facility. In this case the water supply is filtered to remove fungal propagules so that the incoming water supply is likely not the source of the agent. The absence of intestinal mucosal lesions, which would be expected from infection via feed, further suggests that the feed was not the primary source. Since the stocked juveniles were not originally assessed for fungal agents, it is possible these fish were carriers of the agent and over time disseminated the fungus throughout the facility, colonizing tank walls and piping. As the kidney and urinary duct pathology supported initial infection via the urinary tract, it is likely that the benthic distribution of the fish within the rearing tanks contributed to this route of infection. Thus, it could be hypothesized the agent most likely gained access to the halibut via the urinary pore from the biofilm of the tank surfaces. To confirm or disprove this hypothesis additional epidemiological investigation and application of Koch’s postulates would be useful, as infections by *E. angulospora* clearly pose a pathological threat to onshore rearing of Atlantic Halibut.

Species-specific primers have been successfully applied in the molecular diagnosis of fungal infections from a variety of symptomatic tissues (Atkins and Clark 2004). Phylogenetic analysis of the ITS gene demonstrated that this region is suitable for the identification of *Exophiala* species and distinguishes *E. angulospora* from all other taxa of the genus. Alignment of the *Exophiala* ITS data set indicated that sufficient sequence variance between taxa is present in loci of both the ITS1 and ITS2 regions, while these regions remained conserved within species, and therefore are ideal for forward and reverse species-specific primer development. Extraction of gDNA from urinary tracts, which are routinely collected from culled fish during harvest, and PCR amplification using *E. angulospora* specific primers would allow for adequate surveillance and rapid diagnosis of disease outbreak.

**REFERENCES**


Lack of Protection following Re-Exposure of Chinook Salmon to Ceratonova shasta (Myxozoa)

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Published online: 13 Dec 2014.

To cite this article: C. N. Hurst & J. L. Bartholomew (2015) Lack of Protection following Re-Exposure of Chinook Salmon to Ceratonova shasta (Myxozoa), Journal of Aquatic Animal Health, 27:1, 20-24, DOI: 10.1080/08997659.2014.970716

To link to this article: http://dx.doi.org/10.1080/08997659.2014.970716

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Lack of Protection following Re-Exposure of Chinook Salmon to *Ceratonova shasta* (Myxozoa)

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**Abstract**

The recent identification of multiple genotypes of the salmonid parasite *Ceratonova shasta* with different virulence levels in Chinook Salmon *Oncorhynchus tshawytscha* suggests that it is possible to immunize fish against subsequent infection and disease. We hypothesized that exposure of Chinook Salmon to the less-virulent parasite genotype (II) prior to the more virulent parasite genotype (I) would decrease disease and/or result in fewer mature parasites compared with fish only infected with the more virulent genotype. To test this hypothesis, fish were challenged in a combination of field and laboratory exposures, and we measured infection prevalence, percent morbidity, and mature parasite production. Neither mortality nor mature parasite production were reduced when fish were exposed to genotype II prior to genotype I compared with fish exposed only to genotype I, suggesting that protection against *C. shasta* using a less-virulent genotype of the parasite does not occur.

In aquaculture, there are opportunities for the control of diseases through vaccination and/or treatment. Vaccines have been developed for a variety of bacterial and viral pathogens of aquatic animals, but no commercial parasite vaccines exist (Sommerset et al. 2005). However, there is evidence that fish can acquire resistance after a natural exposure to a parasite. In salmonids, resistance to parasite reinfection has been demonstrated for the microsporidians, *Kabatana takedai* (Awakura 1974) and *Loma salmonae* (Speare et al. 1998; Kent et al. 1999), the monogenean, *Discocotyle sagittata* (Rubio-Godoy and Tinsley 2004), and the myxozoan, *Tetracapsuloides bryosalmonae* (Foott and Hedrick 1987). However, one drawback with using a re-exposure strategy with only a virulent parasite is the inability to achieve high infection prevalence without causing a high incidence of disease and/or mortality after the initial exposure. For example, Foott and Hedrick (1987) demonstrated that when infection prevalence with *T. bryosalmonae* was high (82%), most fish exhibited clinical signs of disease.

Alternatively, prior exposure to attenuated parasites or naturally occurring, less-virulent parasite species and/or strains may also provide some protection from disease or reduce disease severity (Smith et al. 1999; Read and Taylor 2001). Attenuation decreases parasite virulence while simultaneously eliciting a protective immune response against future infections with an unaltered parasite. For example, prior exposure to an attenuated strain of the hemoflagellate, *Cryptobia salmositica*, protected Rainbow Trout *Oncorhynchus mykiss* against disease development (Woo and Li 1990). Protection has also been achieved for the myxozoan parasite, *Myxobolus cerebralis*, when researchers exposed fish to infective actinospores treated with ultraviolet (UV) irradiation 63 d before their exposure to fully infective parasites. Prior exposure resulted in decreased infection, parasite survival within the host, and mature parasite production after fish were exposed for a second time (Hedrick et al. 2012). Immunization of Rainbow Trout to the ciliate, *Tetrahymena thermophila*, (a less-virulent parasite species) prior to exposure to another ciliate, *Ichthyophthirius multifilis*, 6–10 weeks later increased host survival by approximately 50% (Wolf and Markiw 1982). Similarly, Sánchez et al. (2001) demonstrated that Rainbow Trout exposed to a less-virulent strain of *L. salmonae* 15 weeks prior to a more virulent strain resulted in a reduction in xenoma intensity in the gills.

The recent discovery that the myxozoan parasite, *Ceratonova shasta*, comprises four genotypes (0, I, II, and III: Atkinson and Bartholomew 2010a, 2010b) that differ in virulence presents an opportunity to immunize hatchery fish prior to their release. The life cycle of *C. shasta* is complex, in which a waterborne actinospore stage attaches to and penetrates the gills of a salmonid. Once in the host, the parasite begins proliferating and travels through the blood to the intestine (Bjork and Bartholomew 2004). Mature myxospores typically develop upon death of the fish host and are subsequently released into the water column where they encounter and infect the freshwater polychaete, *M. ayunkia speciosa* (Bartholomew et al. 1997).

Our study examined the potential for immunizing Chinook Salmon *O. tshawytscha*, which are commonly infected by both...
C. shasta genotypes I and II. In this host genotype I causes mortality from enteronecrosis, while infection with genotype II rarely results in myxospore production or mortality (Hurst and Bartholomew 2012). Thus, we hypothesized that infection of Chinook Salmon with C. shasta genotype II, followed by exposure to genotype I, will result in a decrease in disease and/or myxospore production compared with fish infected with genotype I only. The association of C. shasta with declines in adult Chinook Salmon returns (Fujisawa et al. 2011) has focused attention on strategies to reduce parasite abundance and ultimately disease. Thus, immunizing hatchery fish may provide a twofold advantage: (1) improvement in the survival of returning adults by increasing survival of out-migrating juveniles and (2) reduction in the overall number of parasites in the river by reducing myxospore transmission to the polychaete host.

METHODS

Parasite exposures.—Age-0 Chinook Salmon (5–10 g) were obtained from Iron Gate Hatchery (Hornbrook, California), transported in aerated coolers to the John L. Fryer Salmon Disease Laboratory (SDL), Oregon State University, Corvallis, Oregon, and held until exposure to the parasite. A total of 360 fish were randomly placed into four exposure treatments: (1) no parasite (control, treatment 1), (2) genotype II only (treatment 2), (3) genotype I only (treatment 3), and (4) genotype II followed by genotype I (treatment 4). Two replicates were used for treatments 1 and 2 as no mortality was expected; treatments 3 and 4 had four replicates each (Figure 1). All replicates began with 30 fish and exposures were conducted in cylindrical cages, 0.3 × 1.0 m in size.

The exposure method and timeline was constrained by the availability of myxospores and polychaetes (Hurst and Bartholomew 2012). Fish in treatments 2 and 4 were exposed to genotype II for 24 h in the Williamson River, Oregon (42°32.425′ N, 121°52.787′ W), a location where this genotype is predominant and genotype I is absent (Atkinson and Bartholomew 2010a; Hurst et al. 2012). Water temperature during exposure was 16.5°C. The remaining treatments (1 and 3) were exposed to UV-treated Willamette River water at 18°C for 24 h at the SDL.

Exposure to genotype I was conducted in the laboratory where a pure parasite culture could be obtained and occurred 53 d after the initial exposure to genotype II. This timeline allowed for activation of both the innate and adaptive immune responses (6–12 weeks; Sitjá-Bobadilla 2008) and coincided with genotype I production in the laboratory. At the SDL, each treatment replicate was placed in a separate cage within a 378-L tank that had flow-through water and contained either genotype I from cultured polychaete populations (treatments 3 and 4) or UV-treated Willamette River water (treatments 1 and 2) for 24 h (Figure 1). Water temperatures were 21°C at the time of the second exposure.

FIGURE 1. Experimental design of Chinook Salmon exposure to two genotypes of Ceratonova shasta. Both exposures were for 24 h and included four treatments; control (treatment 1), genotype II only (treatment 2), genotype I only (treatment 3), and genotype II followed by genotype I (treatment 4). For treatments 2 and 4, exposure 1 took place in both the Williamson River, Oregon, and for treatments 1 and 3 at the John L. Fryer Salmon Disease Laboratory (SDL) at Oregon State University, Corvallis, Oregon. Exposure 2 took place at the SDL for all four treatments. Five fish were euthanized immediately following both exposures to determine the extent of infection.

Parasite exposure dose.—To calculate exposure dose per fish for field and laboratory challenges, three 1-L samples of water were collected before and after each exposure. Water samples were filtered, parasite DNA was extracted, and parasite density was measured by quantitative PCR (qPCR) for each sample in duplicate (Hallett and Bartholomew 2006). Samples were considered positive if both duplicate wells fluoresced and were rerun if a difference of more than one cycle occurred between wells. One sample from the beginning and end of each exposure was tested to determine whether parasite DNA detection was inhibited by other components in the water sample filtrate (Hallett and Bartholomew 2009). If inhibition occurred, samples were diluted 1:10 and rerun. Mean parasite density for each exposure site and time was then multiplied by the velocity and exposure duration and divided by the number of fish (Ray et al. 2010). Velocity was 3 L/s in the field and 0.0083 L/s in the laboratory.

Assessment of infection.—Immediately after each 24-h exposure, five fish from each replicate were euthanized with an overdose of tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington) and the right half of the gill was excised, placed on ice, and stored at −20°C to later determine infection prevalence. The remaining fish were placed into 25-L tanks supplied with well water at 18°C. All fish were treated for external parasites and bacterial infections, fed daily, and monitored for clinical disease signs according to Ray et al. (2010). Moribund fish were removed and euthanized.
as above, and the time to morbidity was recorded. The intestine was removed and weighed, and myxospores were collected by flushing them from the intestine into a microcentrifuge tube using a pipette filled with 1 mL of tap water. Myxospores were counted using a hemocytometer at 200 × magnification. A 25-mg aliquot of the harvested myxospores was stored at −20°C to determine genotype composition (see below). Fish remaining at the end of the experiment at 113 d were euthanized using an overdose of MS-222, and a 25-mg piece of intestine was collected and stored at −20°C to later determine infection status in survivors (10 fish from each treatment were assayed).

The DNA from gills and intestines was extracted and purified as in Hurst et al. (in press). After extraction, DNA from all samples was directly tested for the presence of parasite DNA using qPCR as above. To create a standard curve for estimating the parasite DNA copy number in 0.1 g of host gill tissue, 10-fold serial dilutions of a synthetic parasite template were added to gill tissue (Hallett and Bartholomew 2006). Harvested myxospores from the intestines of 10 moribund fish in each of the treatments were sequenced to determine genotype composition (Hurst et al., in press).

Infection prevalence and copy number were determined by using gills from treatments 2 and 4 (genotype II; n = 30) and treatment 3 (genotype I; n = 20) at 1 d after exposure. The program S-PLUS version 8.2 (Tibco, Palo Alto, California) was used to compare survival between treatments 3 and 4 and among treatment replicates using a Mantel–Cox test. One replicate from treatment 4 was lost when water flow to the tank was stopped and therefore was not included in the analyses. A one-way ANOVA with Tukey’s test for highly significant differences was used to compare ln-transformed (for normality) myxospore counts among treatment replicates. If no differences were detected among replicates within a treatment, replicates were combined for analyses at the treatment level using a Student’s t-test. Differences were considered significant at P < 0.05.

RESULTS AND DISCUSSION

Previous exposure to the less-virulent genotype II of C. shasta did not prevent or limit mortality or myxospore production in Chinook Salmon after a subsequent exposure to the more virulent genotype I. There was no significant difference in survival of salmon between treatments 4 (57%) and 3 (48%) (Mantel–Cox test; t = 2.05, P = 0.15; Figure 2). There was also no difference in myxospore counts between these two treatments (t-test: t5 = 0.462, P = 0.663), which had counts of 8,173 ± 2,108 (mean ± SE) and 7,267 ± 612, respectively (Figure 3). Sequencing demonstrated that myxospores obtained from fish from both treatments were only of genotype I. Infection prevalence in surviving fish from treatments 3 and 4 was 50% and 40%, respectively. None of the fish in treatments 1 and 2 died and parasite DNA was not detected in these fish at the end of the study. These data indicate that prior exposure to C. shasta genotype II does not reduce the incidence of disease in Chinook Salmon. If genotype II was protective, we would have expected at least 74% survival (i.e., survival of the 50% infected with genotype II [45 of 90] in addition to 48% of the remaining naïve fish exposed to genotype I [22 of 45]).

Differences in exposure conditions and in genotype virulence resulted in variations in exposure dose and infection prevalence. The exposure dose of C. shasta genotype II in the field was approximately 1.1 × 104 actinospores/fish, and 50% of the fish became infected, as determined by detection of parasite DNA in fish gills. Mean parasite copy number per fish at 24 h was 4.3 (SE, 1.8) as determined by using our standard curve for gill tissue (y = −3.35x + 38.40, r2 = 0.997). The exposure dose of C. shasta genotype I in the laboratory was lower, 9.2 × 10^1

![FIGURE 2. Percent survival of Chinook Salmon after exposure to Ceratonova shasta genotype I only (black line) or after exposure to genotype II followed by exposure to genotype I (gray line). Letters in common indicate a lack of significant difference between the two groups based on a Mantel–Cox test.](Image 312x581 to 561x724)

![FIGURE 3. Mean number of Ceratonova shasta myxospores produced in 0.1 g of intestinal tissue from moribund Chinook Salmon in treatments 3 (exposure to genotype I only) and 4 (exposure to genotype II then I). Error bars indicate SE and letters in common indicate a lack of significant difference between the two groups based on a Student’s t-test.](Image 323x136 to 551x328)
actinospores/fish, but resulted in a higher infection prevalence (100%) and a higher mean parasite copy number per fish of $6.7 \times 10^2$ (SE, 48.5). Parasite DNA was not detected in the gills of control fish. The faster replication rate of genotype I (C. N. Hurst, unpublished data) combined with more optimal flow and temperature conditions for the parasite in the slower flow of the laboratory challenge tanks (Ray and Bartholomew 2013) likely contributed to the higher prevalence and intensity of genotype I infections.

Although these results suggest that previous exposure using a low-virulence genotype is not effective, modifications to exposure timing may yield different results. Although the role of the adaptive response in protection against C. shasta is not fully understood, Rainbow Trout infected with a less-virulent parasite genotype of C. shasta (genotype 0) that survived to 3 months had a 700-fold increase in immunoglobulin T (IgT) antibody levels compared with unexposed fish (Zhang et al. 2010). Work with other myxozoans indicated that specific antibodies were produced by Turbot Psetta maxima from 50 to 360 d after re-exposure in response to Entomomyxum scophthalmi infection (Sitjà-Bobadilla et al. 2007) and as early as 35 d postexposure in Rainbow Trout infected with M. cerebralis (Hedrick et al. 1998). Thus, it seems likely that the adaptive immune response to C. shasta infection would have been mounted during our experimental time frame, but a longer time between exposures may have allowed for increased production of a putative protective antibody.

The timing of the subsequent exposure to the more virulent genotype should also consider the timing of the host’s innate immune response to the parasite, which may be elicited within hours to days of infection depending on temperature and stress (Sitjà-Bobadilla 2008; Gómez et al. 2014). For example, infections with M. cerebralis resulted in upregulation of immune-relevant genes as early as 5 min after exposure (Severin and El-Matbouli 2007). Recently, Bjork et al. (2014) demonstrated that an inflammatory response to C. shasta was mounted within at least 2 weeks of exposure and was capable of resolving infection by 90 d. However, in that study sampling was not conducted between 25 and 90 d; thus, the infection may have been resolved sooner. This indicates that in our study, infection with genotype II may have been resolved before exposure to genotype I occurred. A decrease in the interval between exposures may provide some short-term protection for the fish by taking advantage of the mounted inflammatory response.

The lack of protection after initial exposure to genotype II could be attributed to a low parasite dose and could be rectified by exposing fish to a higher dose of genotype II or lengthening the exposure time. Studies with T. bryosalmonae and E. scophthalmi demonstrated fish resistance to parasite reinfection only after lengthy continuous exposures of 10 and 13 months, respectively, to the parasite (Foott and Hedrick 1987; Sitjà-Bobadilla et al. 2007). Alternatively, immunization may be specific to the parasite genotype, requiring initial exposure to the more virulent genotype to elicit an effective immune response. However, prior exposure to the more virulent genotype increases the probability of fish developing clinical disease. To minimize this risk, fish could be exposed to a low dose of genotype I associated with the resolution of infection (Bjork et al. 2014). Despite the difficulties of working with a parasite that has a complex life cycle, immunization studies with C. shasta are worth pursuing because they may improve our understanding of how to implement management actions such as dam removal and/or fish reintroduction in a manner that could provide fish, such as Chinook Salmon, with some level of natural protection against the parasite.

ACKNOWLEDGMENTS

We thank Iron Gate Hatchery for supplying Chinook Salmon. This work was funded by the Hatfield Marine Science Center William Q. Wick Marine Fisheries Award, the Flyfisher’s Club of Oregon, and NOAA’s Graduate Sciences Program.

REFERENCES


Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease I: Antibiotics in Sperm Extenders

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Published online: 12 Jan 2015.


To link to this article: http://dx.doi.org/10.1080/08997659.2014.966211

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COMMENT

Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease I: Antibiotics in Sperm Extenders

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Abstract

Bacterial coldwater disease caused by Flavobacterium psychrophilum has led to the loss of significant numbers of hatchery-reared salmonids. The bacteria can be spread from parent to progeny within contaminated sperm and ovarian fluid. Methods for disinfecting ovarian fluid and unfertilized eggs are available, but methods for disinfecting sperm have not been described. In this study we determined whether sperm extenders containing a mixture of penicillin and streptomycin can be used to eliminate F. psychrophilum. In vitro trials demonstrated that when Rainbow Trout Oncorhynchus mykiss sperm is mixed with an extender, a 15-min exposure to 0.197 mg penicillin plus 0.313 mg/mL streptomycin is effective at killing the bacteria and has no effect on sperm motility. Small-scale trials showed that egg fertilization rates were not reduced when sperm held in an extender solution containing the same antibiotic mixture for 15 min was used to fertilize eggs. Production-scale trials, however, showed a roughly 18% decrease in egg fertilization rate when sperm stored in an antibiotic containing extender was used. To determine why a reduction in fertilization capacity was observed, a small-scale experiment testing the fertilization of eggs with larger quantities of sperm was performed and showed that increasing the volume of sperm used did not increase fertilization rates. Our results demonstrate that extenders containing penicillin and streptomycin can be used to disinfect sperm, especially when small quantities of eggs are fertilized, but factors negatively affecting egg fertilization and survival on a production scale still need further investigation.

The gram-negative bacterium Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease (BCWD). Worldwide, this disease has been implicated in the loss of significant numbers of cultured salmonids (Nematollahi et al. 2003). Flavobacterium psychrophilum can be transmitted both horizontally and vertically (Brown et al. 1997; Kumagai and Nawata 2010a). In vertical transmission, one source of the bacterium is the ovarian fluid where F. psychrophilum can be found in high concentrations and enters the egg through the micropyle during fertilization (Kumagai and Nawata 2010a). Recently, Kumagai and Nawata (2010a, 2010b) demonstrated that iodophor disinfection of eggs prior to fertilization can help prevent the transmission of BCWD. Flavobacterium psychrophilum can also be found in male seminal fluid and also enters the egg during fertilization (Kumagai and Nawata 2011; R. W. Oplinger, personal observation). Few methods are available for disinfecting sperm, which can be attributed to the sensitivity of sperm to chemical treatment and the potential for chemical treatment to cause premature flagellum activation.

Sperm extenders are solutions that are specially designed to store sperm in an unactivated state, and if antibiotics are added to the extenders, these solutions can serve as vehicles for sperm disinfection. Extenders are designed to have ion concentrations and pH that mimic seminal fluid and many sperm extenders contain antibiotics (Stoss 1983). The use of extenders is common in salmonid aquaculture because the sperm of these species is known to rapidly lose motility when stored in a refrigerated state without extension (Stoss 1983). A mixture of penicillin and streptomycin has been added to many sperm extenders to reduce bacterial growth (Stoss 1983; Stoss and Refstie 1983). For example, Brown and Mims (1995) found that the addition of 5,000 IU penicillin plus 5 mg streptomycin/mL to the sperm extender for Paddlefish Polyodon spathula increased the length of time that samples could be stored. Similarly, the addition of 10,000 IU penicillin plus 10 mg streptomycin plus 25 µg amphotericin/mL increased the storage time of Channel Catfish Ictalurus punctatus semen (Christensen and Tiersch 1996). In Rainbow Trout Oncorhynchus mykiss, Stoss et al. (1978) demonstrated that the addition of penicillin and streptomycin up to concentrations of 9,000 IU penicillin plus 9.0 mg streptomycin/mL did not affect the motility of the sperm. In vitro trials have demonstrated...
that 15-min treatments with penicillin concentrations > 10,000 IU/mL or streptomycin concentrations > 5,000 mg/L are effective at killing *F. psychrophilum* (Wagner et al. 2012; Oplinger and Wagner 2013). Also, the combination of penicillin and streptomycin is lethal to *F. psychrophilum* at concentrations > 2.5 × 10^6 IU/L penicillin plus 2.5 mg/L streptomycin (Oplinger and Wagner 2013). Most work on the addition of antibiotics to sperm extenders has evaluated the effects of antibiotics over long periods of time (days to weeks). Stoss et al. (1978) noted that antibiotic concentrations > 9,000 IU/mL penicillin plus 0.9 mg/mL streptomycin reduced Rainbow Trout sperm fertility and that the lowest antibiotic concentrations tested inhibited bacterial growth (Stoss et al. 1978, lowest antibiotic concentration not specified in manuscript). The preferred antibiotic concentration for most Rainbow Trout sperm extenders is 125 IU/mL penicillin and 0.125 mg/mL streptomycin (Stoss and Refstie 1983; Negus 2008), but the effectiveness of these concentrations at killing *F. psychrophilum* in sperm has not been evaluated.

The objectives of our research were to determine whether a mixture of penicillin and streptomycin can kill *F. psychrophilum* when mixed into Rainbow Trout sperm and whether these antibiotics are detrimental to sperm when added to an extender. In our trials, sperm was held in the extenders for 15 min. We selected this duration because it is practical in a hatchery setting where several lots and thousands of fish can be spawned in a single day. Longer storage periods could slow spawning operations since the number of females that are ready to spawn and the percentage of these with good quality eggs is unknown prior. While extenders allow sperm to be stored for several days (Stoss and Refstie 1983), factors such as temperature and oxygen concentrations lead to the general maxim that the shorter the storage period, the better the quality of the sperm.

**METHODS**

We conducted several trials that evaluated sperm extenders containing penicillin and streptomycin on Rainbow Trout egg fertilization, sperm motility, and antibiotic effectiveness at killing *F. psychrophilum*. In the applicable trials (see below), a 0.5% NaCl solution was used as the diluent and water hardening occurred in well water (pH = 7.6, hardness and alkalinity = 180 mg/L). The sperm extender used consisted of 6.02 g/L NaCl, 2.98 g/L KCl, and 4.77 g/L HEPES (Stoss et al. 1978; Negus 2008) and was mixed with sperm at a 1:1 (v/v) ratio. Antibiotics were added to the extender as described below. *Flavobacterium psychrophilum* (CSF 259–93 strain: Crump et al. 2001) used in the experiments was cultured in either a maltose-infused tryptone yeast extract broth (MAT; 0.4% tryptone, 0.04% yeast extract, 0.05% CaCl₂, 0.05% MgSO₄, 1% maltose, and 0.02% C₃H₂NaO₂; Crump et al. 2001) or on a tryptone yeast extract, salt agar medium (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% CaCl₂, and 0.05% MgSO₄; Holt et al. 1993). All solutions were tempered to ensure that temperatures (9–11°C) were identical to the sperm, eggs, and ovarian fluid used in the trials. Sperm and eggs were collected from Eagle Lake and West Virginia strain Rainbow Trout housed at the Mantua State Fish Hatchery, Box Elder County, Utah, or from Kamloops strain Rainbow Trout housed at the Whiterocks State Fish Hatchery, Uintah County, Utah. Sperm was expressed by hand stripping the fish, filtered through a metallic mesh screen, and collected into a Styrofoam cup. Eggs were also expressed by hand stripping and collected on a fabric mesh screen that separated the eggs from the ovarian fluid. All data were analyzed using the R statistical software package (Hornik 2013). Q–Q plots and Shapiro–Wilks Tests (Kuehl 2000) were used to assess whether the normality and equality of variance assumptions of the analyses were met; transformations were performed as necessary. All results were considered statistically significant at *P* < 0.05. Analyses for individual experiments are described within their respective sections. Antibiotic waste produced during the trials was minimal (<10 L) and was not released into public waterways. No fish that were exposed to antibiotics were stocked, and those individuals who performed the treatments were trained on the safe handling of antibiotics and wore gloves, eye protection, and respirators.

**In vitro test of the ability of penicillin and streptomycin to kill F. psychrophilum when mixed with sperm.**—Two antibiotic concentrations were tested: (1) 0.079 mg penicillin (Russell R-Pen; 1,582 IU/mg) plus 0.125 mg/mL streptomycin (Sigma-Aldrich S6501) and (2) 2.5 times this concentration (0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin). Growth of *F. psychrophilum* was assessed from five replicate samples taken at 5-min intervals from 0 to 15 min after adding antibiotic. Our lowest antibiotic concentration was identical to what is recommended by Stoss and Refstie (1983) and Negus (2008). The higher concentration was chosen to determine whether antibiotic concentrations approached any toxicity threshold for sperm during a 15-min exposure. The objective of the experiment was to determine whether these mixtures would kill 100% of *F. psychrophilum* when treating sperm in extender solution.

Pools of sperm were formed, each containing the sperm from five fish; each pool was divided into two 10-ml aliquots, and one aliquot was extended at each antibiotic concentration. Since the prevalence of *F. psychrophilum* in infected sperm can vary widely (Kumagai and Nawata 2011) and the prevalence in the stocks used was unknown, 100 μL of a 96-h-old MAT broth culture containing *F. psychrophilum* was added to each aliquot approximately 1 min before the addition of the extender to ensure the presence of *F. psychrophilum* (~20% of similar five-fish pools lack the bacterium: R.W.O., unpublished data). The extender was sterilized by autoclaving prior to use. Antibiotics were added to the extender solutions at the start of the experiment. Tenfold serial dilutions on TYES agar indicated that the concentration of *F. psychrophilum* in the sperm solution (after MAT broth addition) was ~1 × 10⁵ cells/mL. This bacteria concentration is greater than naturally found in salmonidcs in Japan (10⁴ to 10¹⁵ CFU/mL; Kumagai and Nawata 2011), but is similar to concentrations of bacteria challenges used in other in
vitro studies ($10^4$–$10^5$: Bullock and Stuckey 1977; $10^5$: Darwish et al. 2008). Developing target treatment concentrations using higher bacteria numbers also helps to reduce the probability of developing antibiotic-resistant strains of bacteria. Furthermore, concentrations needed for in vivo treatment can be much higher than for in vitro treatment of the same bacterium (Gee and Sarles 1942).

Verification of bacterial growth or its inhibition was the only endpoint monitored in this experiment. Samples were taken 0, 5, 10, and 15 min after the addition of extender to the sperm. The 0-min sample was taken about 10 s after mixing the sperm and served as a control. This control was viewed to be preferable to a separate “untreated control” because it allowed us to assess the potential effects of transfer of antibiotic to the media plate with the inoculum as well as have a tighter relationship between control and antibiotic treatment; the number of bacteria in each particular replicate and sample was the same. To take the samples, the bacteria–antibiotic extended sperm solution was diluted by taking 100 µL of extended sperm and mixing it with 10 mL of a sterile phosphate-buffered saline (PBS) solution. Then, 100 µL of the sperm–PBS mixture was withdrawn and plated onto a TYES agar and incubated at 15°C for 10 d. After incubation, the presence or absence of *F. psychrophilum* growth was noted by assessing colony color, morphometry, Gram stains, oxidase tests, and catalase tests, and treatments with no *F. psychrophilum* growth were considered successful at controlling the bacterium. Based on the growth of bacteria at the lower concentration and an interest in testing additional replicates to verify the lethality of the higher concentration, additional replicates ($N = 4$) of the higher antibiotic concentration were tested on a second date.

**Tests of antibiotic effect on sperm motility.** —To test the effect of the antibiotics on sperm motility, antibiotics were added to the sperm extender at five different concentrations: (1) 2.05 mg/mL penicillin plus 3.25 mg/mL streptomycin, (2) 3.95 mg/mL penicillin plus 6.25 mg/mL streptomycin, (3) 7.90 mg/mL penicillin plus 12.5 mg/mL streptomycin, (4) 15.8 mg/mL penicillin plus 25.0 mg/mL streptomycin, and (5) control (no antibiotics added). Five pools (replicates) of sperm were formed and each pool contained sperm from three males. Each pool was divided into five aliquots containing 2 mL of sperm and each aliquot was extended at a different antibiotic concentration. Sperm motility checks were performed 15 min after extender addition by mixing 100 µL of sperm with 500 µL of a 0.5% NaCl diluent. From this mixture, 50 µL was pipetted onto a microscope slide and cover-slipped, and sperm was viewed at 400× magnification. A visual estimate of the percentage of motile sperm was performed to assess differences in mean percent motility and activity time among antibiotic concentrations (blocked by replicate sperm pool).

**Small-scale egg fertilization trials.** —In the paired design employed in this trial, one-half of each sperm pool was treated with the antibiotic and the other half was not. The experiment used 12 replicate pools of sperm, and each pool consisted of sperm from three fish. Each pool was divided in half, and an equal volume of extender containing 0.197 mg penicillin plus 0.313 mg/mL streptomycin was added to one-half of the sperm pool and no extender was added to the other half. Twenty-four groups of eggs were formed by transferring 50 mL of eggs (later determined to be 800–1,500 eggs per replicate) from a bucket containing eggs pooled from 20 females. After the sperm was exposed to the antibiotics for 15 min, 1.0 mL was withdrawn and used to fertilize half of the egg groups. At the same time the remaining egg groups were fertilized using 0.5 mL (equal sperm count to 1:1 diluted extender group) of nonextended sperm (nonextended sperm also used 15 min after collection). After fertilization the eggs were incubated and percent survival to the eyed stage (eye-up) and to hatch, and deformity rates were determined for each replicate by hand counting. For analysis, each performance metric (percent eye-up, hatch, and deformity) from eggs fertilized using extended sperm was compared with the eggs fertilized using nonextended sperm from the same sperm pool using paired $t$-tests.

**Production-scale egg fertilization trials.** —To determine whether eggs could be successfully fertilized at a production scale using antibiotic-treated sperm, three replicate trials were performed on separate dates. Eggs from 20 female Rainbow Trout were used in the first trial, 60 females were used for the second trial, and 30 females were used for the third trial. Eggs were fertilized in a male : female ratio of 1:2. Pools of eggs and sperm were formed and divided in half. One-half of the sperm was placed on extender containing 0.197 mg penicillin plus 0.313 mg/mL streptomycin and used to fertilize one-half of the eggs. The other half of the eggs were fertilized using nonextended sperm (control). Eggs were fertilized with extended sperm 15–34 min (depending on date; variation due to differences in time required to strip eggs from females) after the sperm was extended, whereas eggs in the control group were fertilized immediately after collection. The eggs collected on a date were pooled into a single eyeing jar for each treatment (i.e., antibiotic sperm versus control sperm). Each spawn date represented a separate replicate in the analysis. Paired $t$-tests were used to compare the eye-up rates of the eggs that were fertilized using antibiotic-treated sperm with their respective paired controls.

**Effect of increasing volume of extended sperm relative to egg volume.** —The purpose of this trial was to determine whether reductions in sperm fertility caused by extender use on a production scale (see Results) can be offset by using more sperm. Treatments were (1) sperm placed in antibiotic extender (0.197 mg penicillin plus 0.313 mg/mL streptomycin) and eggs fertilized
15 min after extender addition, (2) same as treatment 1, but double the volume of extended sperm used to fertilize the eggs, (3) sperm mixed into extender without antibiotic and eggs fertilized after 15 min with same sperm concentration as treatment 1 (extender control), and (4) control (no extender, no antibiotic, fertilized at the same sperm concentration as treatment 1, and used immediately after collection).

For each of four replicates, gametes were pooled from three males and two females. Each replicate pool of eggs was then subdivided into four beakers so that each contained 50 mL of eggs. The sperm was divided into four beakers; three of those beakers received one-fifth of the volume of pooled sperm within the replicate (used for treatments 1, 3, and 4 above) and the fourth beaker received two-fifths of the sperm volume (used for treatment 2). Extender with or without antibiotics was added to the appropriate treatments. After 15 min, the sperm–extender mixtures were added to the eggs to initiate fertilization. After fertilization, eggs were rinsed with well water and treatment replicates were incubated in separate trays, which were randomly allocated with a seven-tray stack. Survival to the eyed egg stage, percent hatch, and counts of deformed fry were performed after hatch and one-way ANOVA was used to compare these metrics among treatments.

RESULTS

In Vitro Test of the Ability of Penicillin and Streptomycin to Kill F. psychrophilum when Mixed in Sperm

We found that the ability of the penicillin–streptomycin mixture to kill F. psychrophilum varied with concentration. Controls exposed to antibiotic for just a few seconds all had bacterial growth. The lowest antibiotic concentration (0.079 mg penicillin plus 0.125 mg/mL streptomycin) did not kill the bacteria in any of the 5- or 10-min exposure replicates. After 15 min, growth was found in three of five replicates. In contrast, growth of the bacteria at the higher antibiotic concentration (0.197 mg penicillin plus 0.313 mg/mL streptomycin) was observed in five of nine of the replicates after 5 min, three of nine of the replicates after 10 min, and none of nine replicates after 15 min.

Tests of Antibiotic Effect on Sperm Motility

We found that the four antibiotic treatments, which had concentrations up to 15.8 mg/mL penicillin (= 24,996 IU/mL) plus 25 mg/mL streptomycin, had no pronounced effect on the motility of the sperm compared with controls. Across treatments, average sperm motility ranged between 32% and 68%, and no differences in the percentage of sperm that were motile were observed among antibiotic concentrations ($F_{4, 12} = 0.57, P = 0.69$; Table 1). The average motility time of the sperm ranged between 68 and 82 s and did not vary among treatments ($F_{4, 12} = 0.70, P = 0.60$).

Small-Scale Egg Fertilization Trials

Eye-up rates among eggs fertilized using extended sperm in the small-scale trials did not differ significantly from their respective paired controls ($t_{1, 11} = 0.13, P = 0.90$; Figure 1; Table 2). Similarly, hatch and deformity rates were similar and did not vary between treatments (both $P > 0.72$).

Production-Scale Egg Fertilization Trials

Between 39,000 and 140,000 eggs were fertilized on each date. On average, the eye-up rate of the eggs that were fertilized using the antibiotic-treated sperm was 18.0 ± 6.2% (mean ± SD)

<table>
<thead>
<tr>
<th>Replicate</th>
<th>Antibiotic-treated eye-up rate (%)</th>
<th>Control eye-up rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99.6</td>
<td>98.1</td>
</tr>
<tr>
<td>2</td>
<td>99.7</td>
<td>99.8</td>
</tr>
<tr>
<td>3</td>
<td>64.8</td>
<td>97.3</td>
</tr>
<tr>
<td>4</td>
<td>90.3</td>
<td>97.7</td>
</tr>
<tr>
<td>5</td>
<td>97.9</td>
<td>99.8</td>
</tr>
<tr>
<td>6</td>
<td>96.4</td>
<td>90.5</td>
</tr>
<tr>
<td>7</td>
<td>91.0</td>
<td>98.4</td>
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<tr>
<td>8</td>
<td>99.7</td>
<td>91.7</td>
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<tr>
<td>9</td>
<td>79.3</td>
<td>23.5</td>
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<tr>
<td>10</td>
<td>60.2</td>
<td>70.9</td>
</tr>
<tr>
<td>11</td>
<td>93.3</td>
<td>91.9</td>
</tr>
<tr>
<td>12</td>
<td>68.3</td>
<td>71.7</td>
</tr>
<tr>
<td>Mean</td>
<td>86.7</td>
<td>85.9</td>
</tr>
<tr>
<td>SD</td>
<td>14.7</td>
<td>22.1</td>
</tr>
</tbody>
</table>

TABLE 1. Average percentage of Rainbow Trout sperm that were motile and the time required between sperm activation and the cessation of motion (s) after 15 min of storage in a sperm extender containing various concentrations of penicillin and streptomycin. The SD for each mean is presented in parentheses.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Motile</th>
<th>Time motility ceases (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.05 mg/mL penicillin plus 3.25 mg/mL streptomycin</td>
<td>43 (43)</td>
<td>68 (17)</td>
</tr>
<tr>
<td>3.95 mg/mL penicillin plus 6.25 mg/mL streptomycin</td>
<td>46 (32)</td>
<td>70 (16)</td>
</tr>
<tr>
<td>7.90 mg/mL penicillin plus 12.5 mg/mL streptomycin</td>
<td>68 (62)</td>
<td>82 (12)</td>
</tr>
<tr>
<td>15.8 mg/mL penicillin plus 25.0 mg/mL streptomycin</td>
<td>52 (33)</td>
<td>81 (12)</td>
</tr>
<tr>
<td>Control (no antibiotics added)</td>
<td>32 (35)</td>
<td>72 (15)</td>
</tr>
</tbody>
</table>
lower than their respective paired control ($t_{1,2} = 5.03$, $P = 0.04$; Figure 1).

**Effect of Increasing Volume of Extended Sperm Relative to Egg Volume**

Eye-up of the eggs ranged between 95.0% (double sperm volume treatment) and 97.2% (control, no extender) and there were no significant differences among treatments ($F_{3,15} = 0.35$, $P = 0.79$; Table 3). Hatch rates ranged between 84.9% (double sperm volume treatment) to 91.1% (control, no extender) and also did not vary among treatments ($F_{3,15} = 0.34$, $P = 0.80$). The rate of deformities was less than 2% and did not significantly differ among treatments ($F_{3,15} = 0.28$, $P = 0.84$).

**DISCUSSION**

These results show that a mixture of penicillin and streptomycin, when added to a sperm extender, can potentially prevent the vertical transmission of *F. psychrophilum* via sperm. Our tests determined that penicillin concentrations of 0.197 mg/mL mixed with streptomycin concentrations of 0.313 mg/mL were adequate to kill *F. psychrophilum* with 15 min of exposure. Penicillin and streptomycin are often mixed together because these two antibiotics act synergistically, i.e., kill bacteria at lower concentrations of the two antibiotics than if used separately (Jawetz et al. 1950). Moellering and Weinberg (1971) showed in enteroccci that this synergism can be attributed to an increased uptake of streptomycin when bacteria are incubated in the presence of penicillin.

Despite this synergism, there is some evidence that streptomycin is more toxic to Rainbow Trout sperm than is penicillin and that motility is reduced at antibiotic concentrations greater than 9,000 IU penicillin mixed with 9,000 µg streptomycin/mL (Stoss et al. 1978). Antibiotic concentrations lower than these thresholds were tested in our study. Reduced sperm motility can be a problem when extenders are used because nonmotile sperm can clog egg micropyles and limit the ability of motile sperm to fertilize eggs (Levanduski and Cloud 1988). Poor sperm motility would be a concern in a hatchery because reduced fertility could reduce the percentage of egg hatch, which in turn would decrease the number of fish produced. In our study, simple motility tests did not detect any antibiotic-caused decrease in sperm motility. Still, reductions in fertilization were observed in production-scale tests. It is not evident why reduced fertility was observed in these tests and not detected in the small-scale trials. The longer duration of antibiotic treatment in some production-scale replicates is one possible factor, but the lack of negative effects of in vitro tests where higher antibiotic concentrations were tested suggests that the antibiotic toxicity to sperm is low. The small-scale tests suggest that extender use per se is not a factor since egg survival was not significantly reduced by extender use in these tests. Instead, it is possible that the use of the extender led to a small reduction in sperm motility and this reduction was not detected using our crude sperm motility assessment (estimated precision within ±5–10%). Small reductions in sperm motility may have been offset in our small-scale trials by the use of an excess quantity of sperm, whereas in production-scale tests, proportionally less sperm was used. Also, better mixing of sperm and eggs in the beakers used for the small-scale trials may have occurred compared with the larger buckets in which sperm and eggs were mixed for the production-scale tests. Another possible factor in the large-scale trials was air temperature, which was well below freezing during some of the trials. While efforts were made to keep sperm and extender solutions protected in coolers from the air temperatures and no freezing of solutions was noted, the cold temperature may have had some indirect effect on either eggs or sperm. Further research should isolate and evaluate the factors related to larger-scale production, such as the effect of increased volume of sperm, effects of environmental variables (air and water temperature, light, oxygen), effects of egg batch size, and diluent-to-egg ratios.

Sperm motility and fertilization ability are likely correlated with physiological condition (Levanduski and Cloud 1988), and cells that remain motile in samples that have overall low

![FIGURE 1. Percent survival to the eyed egg stage (eye-up) of Rainbow Trout eggs fertilized using sperm in an extender containing a penicillin–streptomycin mixture compared with eggs fertilized using nonextended sperm immediately after sperm collection (control). Data in the top panel are from small-scale experiments ($n = 12$), whereas data in the bottom panel are from production-scale trials ($n = 3$). Error bars represent ±1 SD of the mean.](Image 55x418 to 295x723)
percentage motility may have reduced fertilization capacity compared with sperm in samples in which a higher percentage of sperm are motile. This reduced physiological condition may exacerbate the effect that small changes in percent motility may have on egg fertilization. One option to combat reduced sperm motility associated with extender use could be to simply increase the volume of sperm used. We tested this and found that doubling the quantity of sperm used did not increase the percentage of eggs fertilized. In retrospect, these trials should be repeated at a production scale as it is likely that sperm were present in excess, even in the treatment with low sperm concentration. Regardless, there is evidence that increasing the volume of extended sperm may be counterproductive because this also increases the number of nonmotile sperm that could clog the micropyle and prevent fertilization by motile sperm (Stoss and Holtz 1981).

The results from this research demonstrate that the use of a sperm extender containing penicillin and streptomycin can be used to disinfect sperm and prevent the vertical transmission of BCWD in salmonids. Given the increase in antibiotic-resistant bacteria and concerns for both human and fish health related to aquaculture (Davies 1994; Wiklund and Dalsgaard 1998; Wang et al. 2012), antibiotics should be used responsibly. That is, antibiotic solutions should be properly disposed of (e.g., chemical or thermal degradation, not dumped down the drain) and applied in concentrations and durations that preclude development of antibiotic resistance. Antibiotics should not be used prophylactically (i.e., they should be used only to correct a particular disease problem at a given time). In addition, humans who are handling these antibiotics should be trained in the proper handling of these chemicals and wear appropriate personal protection equipment.

It appears that small numbers of eggs can be fertilized using extended sperm without compromising hatch. However, eye-up and hatch rates decreased when sperm extenders were used at a production scale. This reduced fertility could be offset by dividing large groups of eggs into smaller aliquots for fertilization and afterwards pooling the eggs for incubation. Future research should target the optimization of the use of sperm extenders at a production scale and should determine whether a reduction in *F. psychrophilum* density in the sperm leads to a reduced occurrence of BCWD after hatch.

### ACKNOWLEDGMENTS

Funding for this research was provided by the Federal Aid in Sport Fish Restoration program, project F-96-R and the Utah Division of Wildlife Resources. We thank D. Dewey and M. McCarty at Whiterocks State Fish Hatchery, Utah, for their help with the production scale test and incubating the eggs for the study. We are similarly grateful to Q. Bradwisch and K. Moulton for their assistance collecting gametes and incubating eggs at Mantua State Fish Hatchery.

### REFERENCES


### TABLE 3. Comparison of the combined effects of doubling Rainbow Trout sperm quantity (1.5:1 male : female ratio) and a 15-min antibiotic (penicillin–streptomycin) treatment of sperm with controls for each factor. Performance metrics were mean (± SD) egg survival to the eyed stage (% eye-up), percent hatch, and the deformity rate (percentage of crippled fry).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Male : female ratio</th>
<th>Eye-up rate (%)</th>
<th>Hatch rate (%)</th>
<th>Deformity rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic extender</td>
<td>1.5:1</td>
<td>95.0 (4.9)</td>
<td>84.9 (13.8)</td>
<td>8.5 (9.3)</td>
</tr>
<tr>
<td>Antibiotic extender</td>
<td>0.75:1</td>
<td>96.1 (2.9)</td>
<td>88.2 (8.2)</td>
<td>6.4 (6.3)</td>
</tr>
<tr>
<td>Extender without antibiotic</td>
<td>0.75:1</td>
<td>96.8 (2.0)</td>
<td>90.2 (6.8)</td>
<td>4.1 (3.3)</td>
</tr>
<tr>
<td>Control: no extender, no antibiotic</td>
<td>0.75:1</td>
<td>97.2 (2.7)</td>
<td>91.1 (7.0)</td>
<td>6.9 (7.6)</td>
</tr>
</tbody>
</table>


Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease II: Efficacy of Using Antibiotics in Diluents and During Water Hardening

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Published online: 12 Jan 2015.

To cite this article: Randall W. Oplinger, Eric J. Wagner & Wade Cavender (2015) Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease II: Efficacy of Using Antibiotics in Diluents and During Water Hardening, Journal of Aquatic Animal Health, 27:1, 32-37, DOI: 10.1080/08997659.2014.945049

To link to this article: http://dx.doi.org/10.1080/08997659.2014.945049

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COMMENT

Use of Penicillin and Streptomycin to Reduce Spread of Bacterial Coldwater Disease II: Efficacy of Using Antibiotics in Diluents and During Water Hardening

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Abstract
Bacterial coldwater disease, caused by Flavobacterium psychrophilum, has lead to the loss of significant numbers of hatchery-reared salmonids. The bacteria can be spread from parent to progeny within contaminated sperm and ovarian fluid and can enter the egg during fertilization. The addition of antibiotics to diluents and water-hardening solutions could prevent the spread of the disease. In separate trials, a mixture of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin was added to both a 0.5% sodium chloride fertilization diluent and hatchery well water during hardening. Tests showed that the addition of the antibiotics to the diluent and during up to 60 min of water hardening had no effect on the eye-up, hatch and deformity rates of Rainbow Trout Oncorhynchus mykiss eggs compared with the nonantibiotic-treated controls. Also, significant reductions in the prevalence of F. psychrophilum on the surface and inside eggs were observed when compared with controls. These results indicate that the addition of penicillin and streptomycin to diluents and during water hardening can prevent the vertical transmission of bacterial coldwater disease.

Worldwide, bacterial coldwater disease, caused by Flavobacterium psychrophilum, has been implicated in the loss of significant numbers of cultured salmonids (Nematollahi et al. 2003). Flavobacterium psychrophilum can be transmitted both horizontally and vertically (Brown et al. 1997; Kumagai and Nawata 2010a). During vertical transmission, bacteria from the ovarian or seminal fluid enter the micropyle during fertilization (Kumagai and Nawata 2010a). The bacterium incubates within the egg and F. psychrophilum outbreaks can occur after the eggs hatch (Kumagai and Nawata 2010a). Three possible methods for preventing the vertical transmission of F. psychrophilum are vaccination, iodine disinfection prefertilization, and antibiotic treatment. Vaccines against the bacteria are currently under development leaving the iodine (Kumagai and Nawata 2010a) and antibiotic treatments as the best available methods for limiting the spread of the disease from parent to progeny. Antibiotic treatment is a particularly appealing method for preventing the vertical spread of F. psychrophilum because it allows for the treatment of sperm, eggs, and ovarian fluid, whereas iodine treatment is only intended for eggs and ovarian fluid (Kumagai and Nawata 2010a). The bacterium is not susceptible to every antibiotic (e.g., tobramycin: Kumagai et al. 2004) but is susceptible to several including florfenicol (Bruun et al. 2000), oxytetracycline (Bruun et al. 2003), erythromycin (Brown et al. 1997; Hesami et al. 2010), penicillin (Wagner et al. 2012), doxycycline, sarafloxacin, and enrofloxacin (Rangdale et al. 1997).

Penicillin and streptomycin are inexpensive, readily available antibiotics that show promise for controlling F. psychrophilum. Previous in vitro trials have demonstrated at 15°C, that 15-min treatments with penicillin (>6.3 mg/mL: Wagner et al. 2012), streptomycin (>5,000 mg/L: Oplinger and Wagner 2013), and a mixture of these two antibiotics (1.6 mg/mL penicillin plus 2.5 mg/mL streptomycin: Oplinger and Wagner 2013) are effective at killing 100% of the bacterium. Toxicity trials have shown that Rainbow Trout Oncorhynchus mykiss eggs can tolerate 1-h exposures to high penicillin concentrations (up to 63.2 mg/mL: Wagner et al. 2012) and eggs can tolerate a mixture of penicillin and streptomycin at concentrations of 5.7 mg/mL penicillin plus 9.0 mg/mL streptomycin (Stoss et al. 1978). In contrast, most Rainbow Trout sperm extenders target antibiotic concentrations of 0.08 mg/mL penicillin mixed with 0.125 mg/mL streptomycin (Stoss and Reftstie 1983; Negus 2008). In vivo trials in which F. psychrophilum had been mixed into sperm and eggs showed that a concentration of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin is required to kill the bacterium (Oplinger and Wagner 2015, this issue).

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Received May 1, 2014; accepted July 10, 2014
One possible method for preventing the vertical transmission of *F. psychrophilum* is the application of antibiotics during fertilization and water hardening. Disinfection of salmonid eggs using Betadine or erythromycin during water hardening has been tested for multiple fish species and pathogens (e.g., Evelyn et al. 1986; Leary and Peterson 1990; Kumagai and Nawata 2010b). Research with erythromycin has shown that the antibiotic is incorporated into the perivitelline fluid during the hardening of eggs from Coho Salmon *O. kisutch* and Chinook Salmon *O. tshawytscha* (Evelyn et al. 1986). In theory, although not thoroughly tested, the vertical transmission of *F. psychrophilum* could be prevented by applying antibiotics during egg fertilization. One benefit of the application of antibiotics during fertilization is that it allows for the simultaneous disinfection of sperm, eggs, and ovarian fluid. Also, antibiotics could enter the egg through the micropyle, which would allow the treatment to continue after water hardening.

This study was undertaken to determine whether the vertical spread of *F. psychrophilum* can be prevented by the application of antibiotics during egg fertilization and water hardening. We tested the addition of a penicillin–streptomycin mixture (0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin (Olinger and Wagner 2015). The antibiotics were added to both a 0.5% sodium chloride fertilization diluent and a water-harden solution (antibiotic mixed with well water) to determine whether the antibiotics (1) had an effect on Rainbow Trout egg fertilization, hatch, and deformity rates, and (2) significantly reduced the occurrence of *F. psychrophilum* on both the surface and interior of eggs.

**METHODS**

**Egg safety trial.**—Six treatments were tested in this trial: antibiotics were added (1) to the diluent only, (2) after the post-fertilization egg rinse and during water hardening for 15 min after fertilization, (3) after the postfertilization egg rinse and during water hardening for 60 min after fertilization, (4) to both the diluent and during water hardening for 15 min after fertilization, and (5) to both the diluent and during water hardening for 60 min after fertilization, and (6) as a control (no antibiotics added) the normal diluent and water-hardening process was followed. Four replicate lots of eggs, each consisting of eggs from three female Rainbow Trout and sperm from two males were produced. Each lot was split into six equal-sized portions (prior to fertilization), each of which contained approximately 1,250–1,500 eggs and was subjected to a different treatment. The diluent solutions consisted of 0.5% NaCl with or without 0.197 mg/mL penicillin powder (Russell R-Pen, 1,582 IU/mg) and 0.313 mg/mL streptomycin powder (Sigma-Aldrich S6501). To fertilize the eggs, sperm was added and flagella were activated by adding 300 mL of the appropriate (with or without antibiotic) diluent solution. Three minutes after fertilization, the eggs were rinsed three times with hatchery water (pH = 7.6, total hardness and alkalinity = 180 mg/L). Then they were water hardened in hatchery well water for 60 min after fertilization. When applicable, antibiotics were added to the water-hardening solution at the same concentration as in the diluent. Eggs that were subjected to antibiotics for the first 15 min were also rinsed 15 min after fertilization with hatchery water and then left to sit in hatchery water (without antibiotics) for the remainder of the water-hardening process. In accordance with Utah Division of Wildlife Resources (UDWR) policy (eggs collected and reared in UDWR’s Mantua Hatchery, Box Elder County), 60 min after fertilization all eggs were disinfected for 15 min in a 100-mg/L iodine solution (Amend 1974). Eggs were incubated in Heath trays and percent eye-up, hatch, and the deformity rate among the eggs was determined by visually examining every egg in each tray. Percent eye-up was determined 5 d after eyes first appeared within the eggs, and percent hatch and the deformity rates were determined 7 d after the first eggs began to hatch. Data were analyzed as a randomized complete block design using Program R (Hornik 2013) and were considered statistically significant at *P* < 0.05. For this study, replicate groups of eggs were considered blocks.

**Egg disinfection trial.**—Gametes from six male and six female fish were collected. Approximately 20 min after the gametes were stripped, *F. psychrophilum* (CSF 259–93 strain: Crump et al. 2001) from a 96-h-old culture of a maltose-infused tryptone yeast extract (MAT) broth (incubated at 15°C; Crump et al. 2001) was added to both the sperm and eggs to reach a target concentration of 10,000 bacteria cells/mL. To reach this target concentration, the bacteria density in the 96-h culture was determined using a spectrophotometer (Thermo Electron Corporation Genesys 10 UV, wavelength set at 525 µm) and converted to an estimate of CFU/mL using the formula, cells/mL = −5,155,052.107 + 137,140,282.280 × absorbance (Olinger and Wagner 2012). The estimated density in the 96-h culture was 6.68 × 10^7 F. psychrophilum cells/mL. Then a 100-fold dilution of this 96-h culture was made by adding 1 mL of this culture to 99 mL of uninoculated MAT broth. Then to reach the target concentration of 10,000 bacteria cells/mL in the sperm and eggs, the volume of sperm and eggs (with ovarian fluid) was determined and 14.97 µL of the 100-fold dilution of the 96-h-old culture per milliliter of sperm or eggs was added. No active outbreaks of bacterial coldwater disease were observed among the broodfish and as a result the density of *F. psychrophilum* naturally occurring in the sperm and eggs was assumed to be negligible (Kumagai and Nawata 2010a).

The eggs were fertilized 60 min after the bacteria were added. Eggs mixed with *F. psychrophilum* were fertilized with sperm containing *F. psychrophilum*. Four separate treatments were tested: (1) eggs fertilized using diluent containing antibiotics and antibiotics applied during the first 20 min of water hardening, (2) eggs fertilized using diluent containing antibiotics and antibiotics applied during the first 60 min of water hardening, (3) a positive control (*F. psychrophilum* added to sperm and eggs...
and no antibiotics added to the diluent or during water hardening), and (4) a negative control (F. psychrophilum not added to sperm and eggs and no antibiotics added to the diluent or during water hardening). The antibiotic concentration for all applicable treatments was 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin. Four replicates of each treatment were tested with ~200 eggs in each treatment. The eggs were thoroughly rinsed with sterile well water 60 min after fertilization to remove the antibiotics but no external egg disinfection occurred. Twenty-five eggs from each replicate were then individually transferred using sterile forceps to sterilized 1.5-mL microcentrifuge tubes containing 0.5 mL of sterile MAT broth. Each egg was macerated with a sterile pestle and incubated at 15°C. After 24 h, the mixture was agitated briefly on a vortex mixer, 100 µL of solution was distributed with a sterile spreader on tryptone yeast extract salt agar with the antibiotic tobramycin added (TYES + T; 4.0 µg/mL tobramycin: Kumagai and Nawata 2010a). The dishes were incubated at 15°C for 10 d and counts of F. psychrophilum colonies were made at the end of this incubation period. Isolates were made from all putative F. psychrophilum colonies from each treatment replicate on TYES + T media and a PCR assay was performed to confirm that the bacteria were F. psychrophilum (Wiklund et al. 2000). Data were normalized using arcsine-square root transformations and analyzed as a one-way ANOVA using Program R (Hornik 2013). Data were considered statistically significant at P < 0.05.

RESULTS

Egg Safety Trial

Eye-up, hatch, and deformity rates among eggs treated with antibiotics during various stages of fertilization and water hardening did not significantly differ from eggs that were not treated with antibiotics (all P ≥ 0.12). Mean survival to the eyed egg stage (eye-up) was similar among treatments, ranging from 67.9% to 79.6% (Table 1). The percent hatch averaged from 60.2% to 71.7% among treatments. Deformity rates were low in all treatments (<1%). The results showed that the addition of antibiotics to both the diluent and water-hardening solution were safe for Rainbow Trout eggs.

**TABLE 1.** Eye-up, hatch, and deformity rates (%, SD in parentheses) among Rainbow Trout eggs that were treated or not treated with a mixture of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin during fertilization or during either the first 15 or 60 min of water hardening. Eggs in the control treatment were not subjected to antibiotics during either fertilization or water hardening. A. represents antibiotic treatment and N.A. represents the absence of antibiotics.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Eye-up rate (%)</th>
<th>Hatch rate (%)</th>
<th>Deformity rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Diluent only</td>
<td>70.6 (19.5)</td>
<td>63.7 (22.7)</td>
<td>0.18 (0.07)</td>
</tr>
<tr>
<td>A. Diluent + 15 min hardening</td>
<td>78.0 (14.6)</td>
<td>70.3 (19.7)</td>
<td>0.71 (0.51)</td>
</tr>
<tr>
<td>A. Diluent + 60 min hardening</td>
<td>67.9 (24.5)</td>
<td>60.2 (27.3)</td>
<td>0.84 (0.48)</td>
</tr>
<tr>
<td>N.A. Diluent + 15 min hardening</td>
<td>75.6 (16.6)</td>
<td>66.5 (22.2)</td>
<td>0.32 (0.37)</td>
</tr>
<tr>
<td>N.A. Diluent + 60 min hardening</td>
<td>79.6 (13.8)</td>
<td>71.7 (19.2)</td>
<td>0.29 (0.29)</td>
</tr>
<tr>
<td>Control</td>
<td>69.8 (21.9)</td>
<td>60.4 (31.4)</td>
<td>0.42 (0.21)</td>
</tr>
</tbody>
</table>

Egg Disinfection Trial

Significantly fewer eggs were infected with F. psychrophilum (F3,12 = 17.88, P < 0.01) and the average number of CFU on the eggs were lower (F3,12 = 21.14, P < 0.01) on eggs treated with antibiotics than on the untreated control eggs. On average, F. psychrophilum was recovered from 29 ± 24% (mean ± SD) of the eggs in the negative control treatment and 32 ± 31% of the eggs in the positive control (t1,6 = 0.36, P = 0.73; Figure 1). In contrast, no F. psychrophilum was found among the antibiotic-treated eggs and the number of colonies other than those of F. psychrophilum did not differ between the two antibiotic treatment regimes (Tukey’s honestly significantly different [HSD] test: P = 0.88).

DISCUSSION

The results from our trials showed that antibiotics can be added to both sperm diluents and during water hardening. The results from our egg safety trials demonstrate that the addition of 0.197 mg/mL penicillin plus 0.313 mg/mL streptomycin to 0.5% sodium chloride sperm diluents and to well water during the water-hardening process does not decrease the number of eggs reaching eye-up and the percent hatch or increase the deformity rate among Rainbow Trout eggs. Also, antibiotic treatment during fertilization and water hardening appears to kill 100% of the bacterium F. psychrophilum. Thus, the use of antibiotics during these egg production steps appears to prevent the vertical transmission of bacterial coldwater disease. Interestingly, the percentage of eggs infected with F. psychrophilum did not vary between the positive and negative control treatments (positive control was “spiked” with F. psychrophilum) indicating that the addition of extra bacteria did not increase the rate of detection of bacterial coldwater disease.

The use of antibiotics for control of vertical transmission of bacterial pathogens has been attempted previously by injecting broodstock with antibiotics (Brown et al. 1990; Haukenes and Moffitt 2002), but studies that have focused on egg treatment are few. Among those, Arenzon et al. (2002) used penicillin during egg incubation and Jensen et al. (1981) used a 0.002-mg/mL erythromycin phosphate solution for rinsing eggs. Erythromycin (0.002 mg/L for 1 h) was also used during water hardening.
One primary advantage to the use of antibiotics during these production stages is that this treatment limits *F. psychrophilum* production stages is that this treatment limits horizontal transmission of these antibiotics could potentially reduce the vertical transmission compared with shorter treatments. The results demonstrate that similar reductions in bacteria numbers are observed regardless of whether antibiotics are applied for 20 or 60 min during water hardening. This indicates that antibiotic use can be discontinued at the hatchery’s discretion anytime during this period without reducing the effectiveness of the treatment. Most salmonid hatcheries disinfect eggs shortly after fertilization and iodine is a common disinfectant. Many hatcheries perform this disinfection 60 min after fertilization but some hatcheries disinfected water hardening (5–60 min after fertilization). It is not known whether the mixing of iodine with antibiotics reduces egg eye-up or hatch rates or whether iodine reduces the effectiveness of the antibiotics. Our results indicate that iodine treatments performed >60 min after egg fertilization are safe to the eggs and do not inhibit the effect of the antibiotics. The development of antibiotic resistance is a growing problem in aquaculture and the rate of development of antibiotic-resistant bacteria strains may be accelerated when suboptimal antibiotic concentrations are used (Schmidt et al. 2000). Thus, the antibiotic concentration used should be adequate to kill all bacteria within the desired exposure period. Many Rainbow Trout produced in western North America are sterilized (Kozfkay et al. 2006; Budy et al. 2012) and triploid induction may be a logical time to discontinue antibiotic treatment during water hardening. In the egg safety trial we had treatments in which we tested antibiotic application for 15 min after fertilization, and we tested a 20-min application in the egg disinfection trial. This shift in time corresponds with when the UDWR induces triploidy and was made for convenience and to minimize egg handling.

We observed that the use of antibiotics did not increase the incidence of larval deformities. This deformity assessment was made 7 d after hatch and was based on the number of fry that had obvious deformities (e.g., in the spine or occurrence of two heads) or swam in circles. Research on the use of the antibiotic erythromycin during water hardening has shown that this antibiotic increases the incidence of meristic count asymmetry between the left and right side of the body (Leary and Peterson 1990); those investigators suggested that the antibiotics interrupted larval development. It is not known whether similar effects occur when a penicillin–streptomycin mixture is used.
used during water hardening, but nothing abnormal has been observed in a production group of Rainbow Trout eggs we treated with these antibiotics (R. W. Oplinger, unpublished data). In 2013, we applied the antibiotic treatment (diluent plus 20 min water hardening) on a production scale at the Mammoth Creek State Fish Hatchery (UDWR). The survival to the eyed stage was 74% for eggs from 3-year-old female Rainbow Trout (694,532 eggs) and 80% for 4-year olds (328,176 eggs). These data affirm the safety of the antibiotic treatment on a production scale. Survival of these eggs was comparable with what was observed in previous years (65–75%) at this same hatchery.

Our results have demonstrated that a penicillin–streptomycin mixture could interrupt the vertical transmission of *F. psychrophilum* in salmonids. Based on these results, we are optimistic that this antibiotic mixture, applied during fertilization and water hardening, could be a useful tool in the control of bacterial coldwater disease. We recommend that antibiotics be used sparingly, with the knowledge that bacterial resistance is inevitable. There is ample literature noting that antibiotic resistance is a growing concern for human health (Davies 1994; Waters et al. 2011; Wang et al. 2012). Antibiotic resistance in aquaculture also has been a concern, not only for fish health (Schmidt et al. 2000), but also for resistant bacteria moving up the food chain into aquaculture products (Khan et al. 2009; Bruun et al. 2000). Antibiotic resistance in bacteria is a growing concern for human health (Davies 1994; Waters et al. 2011; Wang et al. 2012). Antibiotic resistance in aquaculture also has been a concern, not only for fish health (Schmidt et al. 2000), but also for resistant bacteria moving up the food chain into aquaculture products (Khan et al. 2009; Bruun et al. 2000). Antibiotic resistance in bacteria is a growing concern for human health (Davies 1994; Waters et al. 2011; Wang et al. 2012).

ACKNOWLEDGMENTS

Funding for this research was provided by the Federal Aid in Sport Fish Restoration program, project F-96-R and the Utah Division of Wildlife Resources. This manuscript was greatly improved by the comments of three reviewers.

REFERENCES


Kumagai, A., C. Nakayasu, and N. Oseko. 2004. Effect of tobramycin supplementation to medium on isolation of *Flavobacterium psychrophilum* from *Ayu* Plecoglossus altivelis. Fish Pathology 39:75–78.


Colorimetric Method of Loop-Mediated Isothermal Amplification with the Pre-Addition of Calcein for Detecting Flavobacterium columnare and its Assessment in Tilapia Farms

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\textsuperscript{c} Aquatic Animal Biotechnology Research Center, Faculty of Science and Industrial Technology, Prince of Songkla University, Surathani Campus, Surat Thani 84100, Thailand
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Published online: 13 Jan 2015.


To link to this article: http://dx.doi.org/10.1080/08997659.2014.966212
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**Abstract**

*Flavobacterium columnare*, the causative agent of columnaris disease in fish, affects many economically important freshwater fish species. A colorimetric method of loop-mediated isothermal amplification with the pre-addition of calcein (LAMP–calcein) was developed and used to detect the presence of *F. columnare* in farmed tilapia (*Nile Tilapia* *Oreochromis niloticus* and red tilapia [ *Nile Tilapia* × Mozambique Tilapia *O. mossambicus*) and rearing water. The detection method, based on a change in color from orange to green, could be performed within 45 min at 63°C. The method was highly specific, as it had no cross-detections with 14 other bacterial species, including other fish pathogens and two *Flavobacterium* species. The method has a minimum detection limit of 2.2 × 10^2 *F. columnare* CFU; thus, it is about 10 times more sensitive than conventional PCR. With this method, *F. columnare* was detected in gonad, gill, and blood samples from apparently healthy tilapia broodstock as well as in samples of fertilized eggs, newly hatched fry, and rearing water. The bacteria isolated from the blood were further characterized biochemically and found to be phenotypically identical to *F. columnare*. The amplified products from the LAMP–calcein method had 97% homology with the DNA sequence of *F. columnare*.

*Flavobacterium columnare*, a Gram-negative bacterium commonly found in natural aquatic environments (Welker et al. 2005), can infect at least 36 different species of freshwater fish worldwide, causing columnaris disease (Austin and Austin...
Affected fishes include economically important species, such as Nile Tilapia Oreochromis niloticus (Figueiredo et al. 2005), Channel Catfish Ictalurus punctatus (Bader et al. 2003), and salmonids (Avendaño-Herrera et al. 2011). The transmission of F. columnare has been demonstrated to occur via fish-to-fish contact, by cohabitation with carrier fish that shed the bacterium, or via the water column under experimental conditions (Welker et al. 2005).

Conventional diagnosis of columnaris disease is based on its main clinical signs: yellow erosive, necrotic lesions of the skin, gills, and oral cavity; and haystack formations on skin or gills. The period between exposure to gill scrapings examined under a microscope (Durborow et al. 1998). The period between exposure to F. columnare and the outbreak of clinical disease differs greatly depending on the virulence of the bacterial strain and the ambient water temperature (Morisson et al. 1981). When the bacteriological method is used to isolate and identify F. columnare, it can take days to obtain the results (Groff and LaPatra 2000). Although PCR and real-time PCR assays are powerful tools for sensitive detection of F. columnare, they are time consuming and require sophisticated equipment and technical skill (Bader et al. 2003; Darwish et al. 2004; Welker et al. 2005; Panangala et al. 2007). A rapid detection method termed loop-mediated isothermal amplification (LAMP) has been developed for F. columnare detection based on the 16S ribosomal RNA (rRNA) gene; however, the LAMP assay still requires a second step of visualizing the amplified products on agarose gel stained with ethidium bromide, which is prone to contamination and inconvenient for field diagnosis (Yeh et al. 2006). To circumvent this problem, a single-tube, colorimetric LAMP assay with the pre-addition of calcein (hereafter, “LAMP–calcein”) has been demonstrated to simplify the assay by avoiding the use of gel electrophoresis for endpoint detection (Tomita et al. 2008; Wasling et al. 2010; Suebsing et al. 2013). This technique relies on a decrease in magnesium concentration resulting from the formation of magnesium pyrophosphate during the LAMP process (Mori et al. 2001; Tomita et al. 2008). Calcein binds to magnesium and becomes orange in color; with a decreased level of magnesium, which would occur in a positive LAMP reaction, the binding of calcein to magnesium is thus decreased, and the color of the reaction solution changes from orange to green (Tomita et al. 2008).

The present study was aimed at developing a LAMP–calcein method for F. columnare detection and using the method to detect the pathogen in apparently healthy tilapia and in rearing water. The results reveal that the method is reliable and superior to previous methods; based on prevalence and concentrations of F. columnare detected in various samples, the pathogen’s mode of transmission is discussed.

**METHODS**

*Sample collections and DNA extraction.*—For field assessment of the colorimetric LAMP–calcein assay, samples were randomly collected from Nam Sai Farms, a commercial tilapia operation in Prachinburi, Thailand. The samples included sexu-

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
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<tbody>
<tr>
<td>Flavobacterium columnare</td>
<td>ATCC 49512</td>
</tr>
<tr>
<td>F. columnare</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>F. psychrophilum</td>
<td>ATCC 49418</td>
</tr>
<tr>
<td>F. johnsoniae</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>Streptococcus agalactiae</td>
<td>DMST 17129</td>
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<tr>
<td>Streptococcus iniae</td>
<td>ATCC 29178</td>
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<tr>
<td>Staphylococcus epidermidis</td>
<td>ATCC 12228</td>
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<tr>
<td>Aeromonas salmonicida</td>
<td>ATCC 14174</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>ATCC 35654</td>
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<tr>
<td>A. sobria</td>
<td>Laboratory strain</td>
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<tr>
<td>A. veronii</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>Lactococcus garvieae</td>
<td>ATCC 49156</td>
</tr>
<tr>
<td>Edwardsiella tarda</td>
<td>ATCC 15947</td>
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<tr>
<td>Vibrio anguillarum</td>
<td>ATCC 19264</td>
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<tr>
<td>Shewanella putrefaciens</td>
<td>Laboratory strain</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>ATCC 27853</td>
</tr>
</tbody>
</table>

All tissue samples and 5 mL of each bacterial isolate (Table 1) were homogenized in TRIzol Reagent (Invitrogen) for DNA extraction according to the manufacturer’s protocols. Blood samples were subjected to extraction by a rapid boiling method using 0.005-N NaOH at a ratio of 1:2 (Kiatpathomchai et al. 2001). Extracted nucleic acids were adjusted to a final concentration of 100 ng/µL and were stored at −20°C until use. Colorimetric LAMP–calcein and PCR amplifications were carried out by using 2 µL of the DNA template.

*Colorimetric LAMP–calcein assay.*—The LAMP primers were designed according to the published sequence of the chondroitin AC lyase (cslA*) gene of F. columnare strain ATCC (American Type Culture Collection) 49512 (GenBank...
accession number CP003222; Table 2). The colorimetric LAMP assay was performed in 25 μL of total reaction mixture containing 2 μM of forward inner primer, 2 μM of backward inner primer, 0.2 μM of forward outer primer (F3), 0.2 μM of backward outer primer (B3), 2 μM of loop forward primer, 2 μM of loop backward primer, 1 × ThermoPol-supplied reaction buffer, 0.6-M betaine (USB Corporation), 25 μM of the target DNA template. A reaction mixture without template was included as a negative control. The mixtures were incubated at 65 °C for 45 min, after which the color of the reaction mixture (either orange or green) could be detected visually. For comparison, detections of LAMP-amplified products were purified with a gel purification kit (Macherey-Nagel, Dūren, Germany), and 50 ng/μL of purified product were then directly sequenced (Macrogen, Seoul, South Korea). The cslA* gene sequences were aligned with published sequences in GenBank (National Center for Biotechnology Information) by using the ClustalW program in MEGA version 4 (Tamura et al. 2007).

**RESULTS**

**Sensitivity and Specificity of the Colorimetric LAMP–Calcein Assay.**—The developed colorimetric LAMP–calcein assay was tested to determine its specificity for *F. columnare* relative to other known fish pathogens and other bacteria (Table 1). The pathogens included *F. columnare* isolated from Channel Catfish, two species of *Flavobacterium*, two species of *Streptococcus*, and three species of *Aeromonas*. All isolates were stored in 20% glycerol at −80°C. Prior to testing, the bacteria were grown in tryptic soy broth at 28–30°C for 24 h with gentle shaking.

For the sensitivity test, *F. columnare* strain ATCC 49512 was used as the reference strain to optimize LAMP conditions and was prepared in 10-fold serial dilutions containing 2.2 × 10² to 2.2 × 10⁷ CFU/mL by the standard plate count method. Using those preparations as templates, the colorimetric LAMP–calcein method and conventional PCR were compared in terms of their sensitivity in detecting *F. columnare* ATCC 49512.

**Sequence analysis.—** Samples that were positive for *F. columnare* were further amplified with the LAMP outer primers F3 and B3 and were subjected to sequencing. The amplified products were purified with a gel purification kit (Macherey-Nagel, Dūren, Germany), and 50 ng/μL of purified product were then directly sequenced (Macrogen, Seoul, South Korea). The cslA* gene sequences were aligned with published sequences available in GenBank (National Center for Biotechnology Information) by using the ClustalW program in MEGA version 4 (Tamura et al. 2007).

### Table 2. Oligonucleotide primers used in the colorimetric method of loop-mediated isothermal amplification with the pre-addition of calcein for detecting *Flavobacterium columnare*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Location (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fla-F3</td>
<td>CGTGTATACACATCGAAT</td>
<td>1911011–1911031</td>
</tr>
<tr>
<td>Fla-B3</td>
<td>CCTGTACCTAATTGGGAA</td>
<td>1911096–1911214</td>
</tr>
<tr>
<td>Fla-FIP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GCCATAACGCTATAATCATGGTTTTTTCTTGAGATTTTCTGA</td>
<td>1911032–1911055/1911088–1911112</td>
</tr>
<tr>
<td>Fla-BIP&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CATAGATCATAGCTAGCTCCATTCTTGAGATTTTCTGA</td>
<td>1911121–1911145/1911174–1911195</td>
</tr>
<tr>
<td>Fla-LF</td>
<td>CTAATGCAAGTACTAGAT</td>
<td>1911058–1911075</td>
</tr>
<tr>
<td>Fla-LB</td>
<td>GTAGTCTATGAGGAGA</td>
<td>1911151–1911164</td>
</tr>
</tbody>
</table>

<sup>a</sup> Primers are forward outer (F3), backward outer (B3), forward inner (FIP), backward inner (BIP), loop forward (LF), and loop backward (LB).

<sup>b</sup> The TTTT linker for FIP and BIP is highlighted in bold lettering within the primer sequence.
colorimetric LAMP–calcein method was highly specific to \textit{F. columnare} (Figure 1A, tubes 8–21).

Likewise, gel electrophoresis of the LAMP amplicons revealed the typical ladderlike pattern only in \textit{F. columnare} samples from $2.2 \times 10^6$ to $2.2 \times 10^7$ CFU (Figure 1B, lanes 1–5). Based on fluorescence spectrophotometry, the highest intensity of emitting wavelength was observed in the \textit{F. columnare} sample at $2.2 \times 10^6$ CFU, whereas it was almost undetectable for \textit{F. columnare} at $2.2 \times 10^1$ CFU and for other bacteria (Figure 1C). Use of conventional PCR for detection revealed an approximately 203-bp DNA fragment at \textit{F. columnare} concentrations of $2.2 \times 10^6$ to $2.2 \times 10^7$ CFU (Figure 2, lanes 1–4). At $2.2 \times 10^2$ CFU, the band was barely visible, suggesting that the sensitivity of conventional PCR was 10 times lower
(2.2 \times 10^3 \text{ CFU}) \text{ than that of the colorimetric LAMP–calcein method.}

**Detection of Flavobacterium columnare in Farmed Tilapia**

To assess the validity of the colorimetric LAMP–calcein method, the assay was employed to detect *F. columnare* in farmed tilapia and rearing water. *Flavobacterium columnare* was detected in 6 of 20 gonad samples from apparently healthy broodstock of Nile Tilapia and red tilapia; these positive samples were detected by the LAMP–calcein method but not by conventional PCR (Table 3). The minimum detection level of conventional PCR was 2.2 \times 10^3 \text{ cells} ; this indicates that the LAMP–calcein positive samples contained *F. columnare* at levels no higher than 2.2 \times 10^3 \text{ cells}/50 \text{ mg of tissue}. For the gill samples, positive detections were made in 3 of 10 Nile Tilapia and in 2 of 8 red tilapia. The proportion of positive samples was much higher for the blood samples: *F. columnare* was detected in 9 of 10 Nile Tilapia and in 7 of 8 red tilapia. For both types of tilapia, the number of positive detections of *F. columnare* in pooled samples of fertilized eggs was low. However, among newly hatched fry, the percentage of pooled samples that were positive increased dramatically to approximately 19% (25 of 131 determinations) in Nile Tilapia and 7% (10 of 136 determinations) in red tilapia. The occurrence of positive detections was lower when using conventional PCR, most likely due to the lower sensitivity of this method.

For rearing water samples, the LAMP–calcein method detected the presence of *F. columnare* in 8 of 10 samples (Table 3), whereas conventional PCR produced no positive detections. This result also suggests that the concentration of *F. columnare* in the water was no higher than 220 cells/50 mL (4–5 bacteria/mL).

Use of the F3 and B3 primers to determine the DNA sequences of the positive cases from all samples revealed 97% homology with the sequence for the *F. columnare* reference strain (data not shown). The nucleotide sequences were registered in GenBank under accession numbers KF111746–KF111753 and KJ659868–KJ659871.

Bacteria from rearing water and blood samples were cultured on tryptic soy agar supplemented with 1.5% NaCl (Brown et al. 1997) and Shieh agar (Shoemaker et al. 2005) for 24–36 h at 28°C. Homogeneously yellow colonies were observed, selected from the agar, and characterized biochemically. From the analysis, the colonies were phenotypically identical to *F. columnare* in exhibiting positive reactions for catalase, oxidase, nitrate reduction, β-galactosidase, H_2S production, and citrate utilization (data not shown). In addition, sequence analysis of the 16S rRNA and *cslA* genes from the isolates yielded results that were identical to sequences from *F. columnare* (data not shown).

### DISCUSSION

In this study, a single-tube, colorimetric LAMP–calcein assay was successfully developed for visual detection of *F. columnare* in farmed tilapia and in water samples. The integration of the LAMP technique with the pre-addition of calcein dye resulted in a clear accomplishment of the reactions without the risk of contamination caused by opening the reaction tubes. The colorimetric LAMP–calcein technique relies on detection of the magnesium concentration, which is reduced during the formation of magnesium pyrophosphate, by the pre-addition of calcein into the reaction mixtures, and this assay produced greater effects in visual detection (Tomita et al. 2008).

Validation of the LAMP–calcein method reported herein revealed high specificity for *F. columnare*, as there was no positive reaction for other fish pathogens or even for two other species of *Flavobacterium*; therefore, false-positive results are unlikely. The method’s sensitivity was about 10 times that of conventional PCR. This is not the first LAMP method for detecting *F. columnare*; Yeh et al. (2006) reported a LAMP method targeting
16S rRNA of the bacterium. However, the method described in the present study does not require detection by gel electrophoresis, and thus it is more convenient for field use. In addition, the colorimetric LAMP–calcein method was designed to amplify a 203-bp sequence of the cslA* gene of *F. columnare*; this gene and its proteases and adherence factors have been demonstrated as virulence factors of the bacterium (Newton et al. 1997). Those proteins have been found to contribute to the invasive capacity and virulence of *F. columnare* (Stringer-Roth et al. 2002; Suomalainen et al. 2006). Therefore, the specific identification of *F. columnare* at the cslA* gene will be advantageous for further assessing its pathogenicity in fish (Xie et al. 2005; Panangala et al. 2009).

Relatively low prevalence and low concentrations of *F. columnare* were found in the gonads, gills, and fertilized eggs of both Nile Tilapia and red tilapia, whereas prevalence was higher in the samples of blood and newly hatched fry. Correspondingly, the presence of *Flavobacterium* in gonads has also been reported in salmon, with *F. psychrophilum* (a close relative of *F. columnare*) being found in the ovarian fluid and eggs of mature adults (Holt 1987; Barker et al. 1989; Randegale et al. 1996; Ekman et al. 1999). Moreover, *F. columnare* had already been known to exist in rearing water, although at low levels (Welker et al. 2005).

The relatively high prevalence of *F. columnare* in the blood suggests bacteremia at low levels in most tilapia. The low prevalence in the gills, which also contain some amount of blood, was probably due to a very low volume of blood accompanying the gill tissue samples. In addition, the sensitivity of the colorimetric LAMP technique to inhibitory substances present in blood samples is lower than that of PCR when a rapid boiling method was used for DNA extraction (Kaneko et al. 2007; Jaroenram et al. 2009). The present study does not require detection by gel electrophoresis, and thus it is more convenient for field use. In addition, the colorimetric LAMP–calcein method was designed to amplify a 203-bp sequence of the cslA* gene of *F. columnare*; this gene and its proteases and adherence factors have been demonstrated as virulence factors of the bacterium (Newton et al. 1997). Those proteins have been found to contribute to the invasive capacity and virulence of *F. columnare* (Stringer-Roth et al. 2002; Suomalainen et al. 2006). Therefore, the specific identification of *F. columnare* at the cslA* gene will be advantageous for further assessing its pathogenicity in fish (Xie et al. 2005; Panangala et al. 2009).

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**ACKNOWLEDGMENTS**

This work was supported by a grant from the Cluster and Program Management Office, National Science and Technology Development Agency (Grant Number P-13–00870). We are grateful to Channarong Rodkhum (Department of Veterinary Microbiology, Faculty of Veterinary Science, Chulalongkorn University) for providing *F. columnare* isolates.

**REFERENCES**

Austin, B., and D. A. Austin. 2007. Bacterial fish pathogens: diseases of farmed and wild fish. Springer-Praxis, Chichester, UK.


Efficacy of Florfenicol for Control of Mortality Associated with Edwardsiella ictaluri in Three Species of Catfish

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Published online: 23 Feb 2015.


To link to this article: http://dx.doi.org/10.1080/08997659.2014.976672
ARTICLE

Efficacy of Florfenicol for Control of Mortality Associated with Edwardsiella ictaluri in Three Species of Catfish

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Abstract

The efficacy of florfenicol for control of mortality associated with Edwardsiella ictaluri was studied in fingerlings of Channel Catfish Ictalurus punctatus (Delta strain), Blue Catfish I. furcatus (D&B strain), and a hybrid catfish (Delta strain Channel Catfish × D&B strain Blue Catfish). On day 0, fish were immersion challenged in 65-L aquaria. For each of the three species of catfish, 10 aquaria were randomly assigned to two treatment groups, either treated with florfenicol at 0 mg/kg of body weight (unmedicated feed) or at 10 mg/kg (medicated feed). Fish were treated for 10 consecutive days, monitored for mortality during this treatment period, and observed for 14 d afterwards. Post observation, all survivors were humanely euthanized in tricaine methanesulfonate, cultured for E. ictaluri, and examined for gross pathology. The mean cumulative percent mortality from enteric septicemia of catfish (ESC) challenge among the three genotypes of catfish did not differ between Blue Catfish, hybrid, and Channel Catfish in treated or control groups. However, the florfenicol-treated fish had a significantly lower mean cumulative mortality (6%) than the controls (78%). All genotypes of catfish tested were responsive to treatment with florfenicol-medicated feed for control of mortality associated with ESC. There were no significant differences in mortality associated with hybrid catfish, blue catfish, and Channel Catfish (Delta strain).

Enteric septicemia of catfish (ESC) is caused by the bacteria Edwardsiella ictaluri, which has a high predilection for U.S. farm-reared catfish (Noga 2010:183–210). This infectious disease occurs predominately in the spring and fall when water temperatures in commercial ponds are in the range of 22–28°C. Both Channel Catfish and hybrid catfish are susceptible to E. ictaluri but hybrid catfish are reported to be more resistant to diseases than Channel Catfish (Dunham et al. 2008).

Although the cost of fry production is relatively expensive compared with Channel Catfish, hybrid catfish are a popular choice for commercial production because of rapid growth and apparent disease resistance. Diagnostic laboratory records are

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Received July 20, 2014; accepted October 10, 2014
often a biased reflection of field disease outbreaks, but com-
pared with prior years, in 2012 and 2013 the Mississippi State
University College of Veterinary Medicine Aquatic Diagnostic
Laboratory received increased accessions of hybrid and Blue
Catfish (about 23% of all accessions; Khoo et al. 2013, 2014).
Perhaps because of the popularity and increased value of hybrid
catfish, farmers are submitting them to a laboratory for diagno-
sis, including susceptibility testing of any isolated bacteria to
antimicrobials. When ESC outbreaks occur in catfish, medicated
feeds are often used to control mortality. Romet and Aquaflo-
rs are antimicrobials labeled for control of mortality associated
with ESC in the USA. Romet (ormetoprim sulfadimethoxine)
is an over-the-counter medicated feed, and Aquaflo (florfeni-
col) requires a veterinary feed directive order issued from a
licensed veterinarian. Although Aquaflo has been available as
a medicated feed to the U.S. catfish industry since 2005, no
study was previously performed to illustrate the responsive-
ess of different genotypes of cultured catfish infected with
*E. ictaluri* to the drug. To determine the relative susceptibility
of catfish to a laboratory *E. ictaluri* challenge and responsive-
to florfenicol (FFC) medicated feed, we compared the morta-
ality differences between Channel Catfish, hybrid catfish, and
Blue Catfish experimentally challenged with *E. ictaluri*, then fed
with either Aquaflo medicated feed or the nonmedicated control
feed.

**METHODS**

*Experimental design.*—Ten 65-L tanks were randomly as-
signed to each one of two treatment groups of Channel Catfish
*Ictalurus punctatus* challenged with *E. ictaluri*: (1) fed unmed-
icated (control) feed, or (2) fed 10 mg of FFC/kg body weight
in medicated feed for 10 d. There were five replicates of each
treatment group. Concurrently, 10 tanks for Blue Catfish *I. fur-
catus* and 10 tanks for hybrid catfish (Channel Catfish × Blue
Catfish) were also randomly assigned to each one of the two
treatment groups described above. There was a 2-week acclim-
atation period during which fish were fed a commercial catfish
feed excluding the day prior to bacterial challenge. Immersion
challenge with *E. ictaluri* occurred on day 0. The treatment pe-
tiod began the day after challenge on day 1 and ended on day 10.
During the observation period following treatment, fish were fed
control, unmedicated feed. Throughout the study, fish were ob-
served for mortality and adverse events. Dead fish were cultured
for bacteria according to previous methods (McGinnis 2003).
Upon termination of the study, survivors were netted from each
tank, and collectively weighed. Surviving catfish were then hu-
manely euthanized with tricaine methane sulfonate (MS-222)
and evaluated for bacterial infection and gross pathology.

*Catfish.*—Three genotypes of catfish produced at the U.S.
Department of Agriculture, Agricultural Research Service,
Warmwater Aquaculture Research Unit in Stoneville, Missis-
ippi, were used in the study: the Delta strain of Channel Catfish,
D&B strain of Blue Catfish, and a hybrid (female Delta strain
Channel Catfish × male D&B strain Blue Catfish) produced
using procedures of Chatakondi et al. (2011). Fry from several
families of each genotype were mixed and raised in common
tanks to the fingerling stage.

Catfish were laboratory reared for 90–150 d and had no
known history of exposure to *Aeromonas hydrophila*, *Flavobac-
terium columnare*, or *E. ictaluri*. For each catfish type, 100
were transferred to holding vats in the wet laboratory at Mississippi
State University, Thad Cochran National Warmwater Aquacul-
culture Center in Stoneville. Four fish from each genotype were
sacrificed, and their brain and kidney samples were cultured
for bacteria on Mueller Hinton agar plates. Brain, kidney, skin,
mouth, and gill samples were cultured for *F. columnare* on di-
luted Mueller Hinton agar with neomycin. All culture results
were negative.

Channel Catfish fingerlings (mean = 16.7 g, SE = 7.8) were
counted in groups of 10, weighed, and each group was then
placed into one of 10 tanks. Hybrid catfish (mean = 21.8 g, SE
= 8.5), and Blue Catfish (mean = 15.8 g, SE = 2.1) fingerlings
were stocked in a similar fashion.

**Fish environment, handling, and feeding.**—Fish were stocked
into tanks with single-pass, free-flowing water at the rate of 0.5
L/min aerated with an air stone for oxygen. During the study,
fish were fed at a rate of 2% body weight once a day with 32%
protein feed (Fishbelt Feeds, Inc., Moorhead, Mississippi).
Water temperature, which was measured and recorded daily, ranged
from 24.5°C to 26.7°C. Water quality, which was monitored at
the initiation and termination of the study for alkalinity, hard-
ness, chloride, pH, ammonia, and nitrite, remained within suit-
able ranges for catfish husbandry (Tucker and Hargreaves 2004).
The photoperiod was 12 h light : 12 h dark.

**Preparation of *E. ictaluri* inoculum and challenge of fish.**—
The *E. ictaluri* isolate used in the study was obtained from a
catfish submitted to the Mississippi State University College
of Veterinary Medicine Aquatic Diagnostic Laboratory during
an ESC outbreak. It was identified by biochemical identifica-
tion (BBL, Crystal, Cockeysville, Maryland), and subsequently
confirmed to be *E. ictaluri* by 16S rRNA gene sequencing us-
ing established protocols (Dorsch and Stackebrandt 1992; Soto
et al. 2012). To prepare the inoculum for challenge, four test
tubes that contained 10 mL of brain heart infusion (BHI) broth
were inoculated with 3 *E. ictaluri* colonies and incubated for
24 h at 27°C. After incubation, each tube was transferred into a
1-L BHI broth and incubated for 24 h at 27°C. Postincubation,
5 mL of inoculum was serially diluted and plated for purity
check and colony counts. Fish were immersion-challenged by
draining the tanks to a volume of 15 L and inoculating the
static tanks with 100 mL of *E. ictaluri* inoculum for 2 h. The
challenge concentration in the tanks was calculated to be 8.8
CFU/mL. After 2 h, free-flow water was restored to the tanks.

**Preparation of feed.**—Commercial catfish feed containing
32% crude protein (Fishbelt Feeds, Inc., Moorhead, Mississippi)
was used for the study. This feed was ground and repelleted via
a method described by Li et al. (1993). For FFC-medicated
feeds, Aquaflor, (50% Type A Medicated Article, Merck Animal Health, Summit, New Jersey) was added to ground catfish feed then pelleted. Feeds nominally containing 0 and 500 mg of FFC/kg of feed were manufactured in order of increasing concentration of FFC then stored in a refrigerator at 4°C. Catfish feed FFC concentrations were determined by Eurofins Scientific, Inc. (Kalamazoo, Michigan) using the method of Hayes (2005). The concentration of FFC in the medicated catfish feed was 505 mg/kg (101% of nominal), and FFC was not detected in the control catfish feed above the limit of quantitation.

Statistical analysis.—Statistical analysis was conducted using the mixed procedures of the Statistical Analysis System (SAS Institute 1999). Data on percent survival were subject to two-way ANOVA that employed genetic group and type of feed as fixed effects and aquarium within the genetic groups as random effect. Mean percent of dead fish at the end of the study period were arcsine-transformed prior the analyses. For this analysis, the tank was the experimental unit and the mean square of tank within a genetic group and type of feed fed was used as the error term to test the differences among genetic groups and type of feed groups. Fish were weighed by tank, and that weight was divided by the number of fish in the tank to yield a tank average fish weight. A one-way ANOVA was used for the analysis at $\alpha = 0.05$.

RESULTS

The first mortalities were identified in nonmedicated control tanks on day 3 in Channel Catfish tanks, on day 4 in one hybrid FFC-medicated tank, and on day 6 in nonmedicated control Blue Catfish tanks. Mortalities were noted on days 3–19 for the Channel Catfish, days 4–19 for the hybrids, and days 6–20 for the Blue Catfish (Figure 1). The heaviest mortalities occurred on day 5 for Channel Catfish (11/100 fish), day 7 for hybrids (10/100 fish), and day 7 for Blue Catfish (11/100 fish). Edwardsiella ictaluri was recovered from all dead fish except three autolyzed ones. The mean cumulative percent mortality to ESC challenge among the three genotypes of catfish did not differ ($P = 0.38$) between Blue, hybrid, and Channel Catfish (SE = 5.28; Table 1). However, post-ESC challenge, treatment with FFC-medicated feed significantly decreased mean mortality (6%) compared with mean mortality in the control treatment (78%) SE = 4.2; $P < 0.01$). The mean stocking weight of hybrids was significantly ($P < 0.01$) higher than those of Channel Catfish and Blue Catfish; however, the weights of the Channel Catfish and Blue Catfish did not differ ($P = 0.1049$).

DISCUSSION

Treatment with FFC-medicated feed significantly decreased mortality from ESC in all genotypes of catfish tested in this study, and there were no difference in survival among the three types. We also found no differences in mortality associated with ESC among the three catfish types fed control nonmedicated feed.

The hybrid catfish is generally considered superior to either parent species for many traits, including disease resistance (Wolters et al. 1996). However, many factors are known to influence the susceptibility of fish to bacterial challenge: inoculum dose and duration, water temperature and chemistry, biomass and size of fish, density and stress (Bilodeau et al. 2003), and genetics (Wolters and Johnson 1994; Silverstein et al. 2008).

<table>
<thead>
<tr>
<th>Category</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genotype</strong></td>
<td></td>
</tr>
<tr>
<td>Channel catfish</td>
<td>48.00</td>
</tr>
<tr>
<td>Blue catfish</td>
<td>38.00</td>
</tr>
<tr>
<td>Hybrid catfish</td>
<td>40.00</td>
</tr>
<tr>
<td>Pooled SE</td>
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</tr>
<tr>
<td>$P$-value</td>
<td>0.74</td>
</tr>
<tr>
<td><strong>Type of feed fed</strong></td>
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</tr>
<tr>
<td>FFC-fed</td>
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</tr>
<tr>
<td>Control-fed</td>
<td>78.00</td>
</tr>
<tr>
<td>Pooled SE</td>
<td>4.26</td>
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<tr>
<td>$P$-value</td>
<td>0.001</td>
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<tr>
<td><strong>Genotype $\times$ feed</strong></td>
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</tr>
<tr>
<td>Channel catfish $\times$ FFC</td>
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<tr>
<td>Channel catfish $\times$ CF</td>
<td>86.00</td>
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<tr>
<td>Blue Catfish $\times$ FFC</td>
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<td>Blue Catfish $\times$ CF</td>
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<tr>
<td>Pooled SE</td>
<td>7.39</td>
</tr>
<tr>
<td>$P$-value</td>
<td>0.86</td>
</tr>
</tbody>
</table>

FIGURE 1. Mean cumulative percent mortality of Channel Catfish (C), Blue Catfish (B), and hybrid catfish (H) either fed with florfenicol (FFC) or control (CF) feed for 10 d post challenge. [Figure available online in color.]
Survival rates between different catfish species exposed to bacterial epizootics were previously studied. In agreement with our findings, Dunham and Brummett (1999) found no difference in fingerling survival between hybrids (Marion × Kansas Channel Catfish females and Auburn line of Blue Catfish), and three genotypes of Channel Catfish, (one of which was a Marion × Kansas cross). It was suggested that creation of the hybrid from the same female genotype as the Channel Catfish contributed to the similar survival percentage. Likewise, Bosworth et al. (2004) demonstrated that NWAC 103 line of Channel Catfish fingerlings had a survival rate that was not significantly different than that of hybrids. However, a Gold Kist strain was used as the maternal Channel Catfish to create the hybrids resulting in a dissimilar genetic pool between the hybrid and Channel Catfish.

In a study comparing the differential susceptibility of Blue Catfish, industry pool of Channel Catfish and hybrid catfish (USDA 103 strain Channel Catfish × D&B strain Blue Catfish) to Channel Catfish virus (ictalurid herpesvirus-1; IHV-1), the industry pool of Channel Catfish actually had a higher survival rate than the hybrids. Factors that were considered to influence this outcome were differences in size of the hybrid catfish (4.1 g) versus Channel Catfish (6.3 g) and genetic disparity (Silverstein et al. 2008).

A study producing the more expected outcome showed significant differences in survival rates of catfishes exposed to E. ictaluri during immersion challenges of Blue Catfish (89.5%), hybrid catfish (female Norris strain Channel Catfish × male Blue Catfish; 73.8%), and Norris strain Channel Catfish (62.0%; Wolters et al. 1996). However, in addition to the genotypes, a higher stocking density (40 fish/120-L tank versus 10 fish/56-L tanks) and shorter challenge duration (10 min versus 2 h) were different between their study and ours.

In our experiment, we used the Delta strain Channel Catfish, which represents gene pools of from many different farms throughout the Mississippi Delta region. The vigor created by the large pooling of genes may account for the survival in the Channel Catfish in our study, which compared favorably to the hybrid and Blue Catfish genotypes.

The efficacy of FFC against ESC in Channel Catfish was reported previously (Gaunt et al. 2003; Gaunt et al. 2004; Gaunt et al. 2006). In the USA, FFC is approved for control of mortality associated with E. ictaluri and F. columnare in catfish, without reference to the species of catfish. However, it has never been documented how the other species of catfish would respond to FFC treatment either in terms of mortality or palatability. Anecdotal reports indicate that the drug is both palatable and efficacious when used in the field to control mortality associated with ESC in hybrids.

Because increasing numbers of catfish producers are raising hybrid catfish, it is probable that more farmers than before are submitting sick hybrids to fish diagnostic laboratories to protect their more costly catfish investments. Losses from ESC-related mortalities are contained by treating with FFC-medicated feed. This suggests that fish from ponds diagnosed with ESC and treated with Aquaflor limits the disease-related losses and improves profitability in catfish farming.

In conclusion all three genotypes of catfish tested were responsive to treatment with FFC-mediated feed for control of mortality associated with ESC. There were no significant differences in mortality associated with the hybrid, D&B Blue Catfish, and Delta Channel Catfish, suggesting that FFC feed and not the genotype of catfish reduces the mortality associated with ESC challenge.

REFERENCES


Khoo, L., P. Gaunt, and M. Griffin. 2014. Aquatic Research and Diagnostic Lab files: 2013 report. Mississippi State University, Delta...


Assessment of the Long-Term Viability of the Myxospores of Myxobolus cerebralis as Determined by Production of the Actinospores by Tubifex tubifex

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Published online: 23 Feb 2015.

To cite this article: R. Barry Nehring, George Schisler, Luciano Chiaramonte, Annie Horton & Barbara Poole (2015) Assessment of the Long-Term Viability of the Myxospores of Myxobolus cerebralis as Determined by Production of the Actinospores by Tubifex tubifex, Journal of Aquatic Animal Health, 27:1, 50-56, DOI: 10.1080/08997659.2014.976671

To link to this article: http://dx.doi.org/10.1080/08997659.2014.976671

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Assessment of the Long-Term Viability of the Myxospores of *Myxobolus cerebralis* as Determined by Production of the Actinospores by *Tubifex tubifex*

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Abstract

While whirling disease was first observed in Rainbow Trout *Oncorhynchus mykiss* in 1893, the complete life cycle of *Myxobolus cerebralis* (*Mc*), the causative agent of the disease, was not understood until 1984, when it was shown to involve two obligate hosts, a salmonid fish and the aquatic oligochaete *Tubifex tubifex* (*Tt*). The viability of the triactinomyxon (TAM) actinospores produced by *Tt* has been well studied, and is known to be temperature dependent and measured in days and weeks. Assertions that *Mc* myxospores produced by infected fish remain viable for years or even decades were made during the mid-20th century, decades before the *Mc* life cycle was described. Moreover, the duration of myxospore viability has not been well studied since the life cycle was elucidated. In a series of time-delay treatments, we assessed the long-term viability of *Mc* myxospores by exposure to *Mc*-susceptible *Tt* oligochaetes and quantified TAM production. As the time delay between inoculation and incubation of *Mc* myxospores in sand and water and exposure to *Mc*-susceptible *Tt* oligochaetes increased, TAM production decreased exponentially. Production among the 15-d time-delay replicates was reduced 74.7% compared with the 0-d treatment. Likewise, total TAM production was reduced 94.5, 99.4, and 99.9%, respectively, in the 90-, 120-, and 180-d time-delay treatments. Linear regression analysis of our data and the absence of TAM production among replicates of *Mc* myxospores held at 5°C for 365 d prior to exposure to *Mc*-susceptible *Tt* oligochaetes indicate that the long-term viability of *Mc* myxospores is less than 1 year under the conditions of this study.

Whirling disease (WD) was first observed in Rainbow Trout *Oncorhynchus mykiss* reared in fish culture ponds in Bavaria, Germany, in 1893 (Höfer 1903). Throughout the first 8 decades of the 20th century, myxospores of the myxozoan parasite (now known as *Myxobolus cerebralis* [*Mc*]) were thought to be the infective agent that caused WD in vulnerable salmonid fishes. It was not until the early 1980s that the true life cycle of the parasite was shown to require two obligate hosts, an aquatic oligochaete and a salmonid fish (Markiw and Wolf 1983; Wolf and Markiw 1984). Upon ingestion of *Mc* myxospores, the susceptible strain of the aquatic worm *Tubifex tubifex* (*Tt*) produces a semibuoyant, waterborne triactinomyxon (TAM) form actinospore that is infective to many species of salmonids (O’Grodnick 1979; Hedrick et al. 1998; Thompson et al. 1999; Vincent 2002). The myxospore is the final developmental stage produced by infected fish. Myxospores are dispersed into the aquatic environment when shed by infected fish (Taylor and Haber 1974; Nehring et al. 2002) or upon death and decay of the carcass (Hoffman and Putz 1971; El-Matbouli et al. 1999a, 1999b).

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Received May 14, 2014; accepted September 27, 2014
For 8 decades after WD was first described in detail (Plehn 1905), Mc was considered a pathogen capable of decimating Rainbow Trout being farm-reared in earthen ponds when the conditions were right for a severe outbreak. When there was heavy incidence of disease, mass death of fry could occur without external symptoms being evident (Bauer 1959). During this period, numerous investigators believed that the myxospores required “aging” in the sediments for 3–6 months before they became infective to the trout (Hoffman and Putz 1969; Putz 1969), while others suggested that the spores are resistant to freezing and drying (Plehn 1905, 1924; Schäperclaus 1931, 1954) and could remain viable for 12 years in a dry pond (Schäperclaus 1954). Funk (1968) asserted they could remain viable for 30 years. However, prior to the landmark study by Wolf and Markiw (1984), when the complete life cycle of M. cerebralis was first described, no reproducible test existed to determine the viability of the myxospores (Halliday 1976).

The pioneering work of Wolf and Markiw (1984), demonstrating that a tubificid worm was required for completion of the life cycle of the Mc parasite, remained controversial until other studies corroborated the results of the original research (El-Matbouli and Hoffmann 1989). Subsequent studies demonstrated that the viability of the TAMs was measured in days or weeks and was temperature-dependent (Markiw 1992; El-Matbouli et al. 1999b). However, suggestions that Mc myxospores could remain viable for 12–30 years (Schäperclaus 1954; Funk 1968) remained largely unchallenged for almost a quarter century after the elucidation the life cycle (Wolf and Markiw 1984), even though one investigation suggested they were completely nonviable after being held in a vial at 6°C for 3 years based on the disappearance of the sporoplasm (Hoffman et al. 1962).

The only irrefutable test for viability of Mc myxospores is in the production of TAMs resulting from exposure of susceptible T. tubifex worms. Hedrick et al. (2008) applied this protocol to Mc myxospores exposed to freezing, air drying, sunlight, a range of temperatures for various periods of time, chemicals and UV irradiation. We found it noteworthy that there was a 71% reduction in the average number of TAMs produced among the replicates where the myxospores suspensions were held at a temperature of 4°C for a 2-month period prior to worm introduction compared with the suspensions held at 5°C for 7 d prior to worm exposure. It is possible this large difference in total TAM production between the two time-delay treatments could have been a random anomaly or due to slight variations in the dosage between replicates or variability in the susceptibility of Tt worms in the exposed replicates. However, we wondered if it might suggest that Mc myxospores degrade much more rapidly than previously (1900–1980) thought by aquaculturists and fish pathologists (Schäperclaus 1954; Hoffman et al. 1962; Funk 1968) prior to the elucidation of the life cycle of this parasite.

Our objective was to evaluate the long-term viability of Mc myxospores held in sediment and water under environmental parameters representative of spring to fall conditions in streams and lakes supporting salmonids all year. Our working hypothesis was that if the myxospores were highly resistant to degradation, then there would be no attenuation in total TAM production with increasing time-delays prior to exposure of the worms. Alternatively, attenuation in TAM production with increasing time-delay treatments would indicate that Mc myxospores become less viable with prolonged incubation in the substrate prior to being consumed by Mc-susceptible lineage III T. tubifex. Such an outcome would suggest that Mc myxospores are not as highly resistant to degradation and are much more labile than currently believed (Hedrick et al. 2008). A period of viability for Mc myxospores measured in months instead of years or decades would have important consequences for fishery pathologists and managers and the salmonid aquaculture industry.

**METHODS**

_Study design._—Our study design called for the inoculation of Mc myxospores into 20 separate containers at the beginning of the experiment, followed by the subsequent introduction of 250 unexposed, Mc-susceptible lineage III Tt oligochaetes into each container after proscribed time-delays. The time-delay treatments were 0, 15, 30, 60, 90, 120, 180 and 365 d, with two replicates for each treatment. All replicates up to and including 180 d were seeded with 12,500 Mc myxospores at day 0, a dose rate of 50 myxospores/worm. We used this low dose exposure to maximize TAM production while concurrently minimizing the physiological costs of parasite infection among the exposed worms (Nehring et al. 2014). Two additional replicates for additional 180 d and 365 d time-delay treatments were also inoculated at day 0 with 1 million myxospores, a dose rate of 4,000 myxospores/worm. The myxospores for the 365-d time-delay treatment were refrigerated in an aqueous solution at 4–5°C for 1 year prior to inoculation. In the event that we were unable to detect any TAM production in the 180-d, low-dose treatment replicates, addition of the 180-d and 365-d, high-dose replicates would help to better approximate the point in time when the myxospores were no longer viable.

The Mc myxospores for the study were provided by the Aquatic Animal Health Research Laboratory (AAHRL) at the University of California, Davis, California. Spores harvested from freshly killed Rainbow Trout infected with the Mc parasite were purified and concentrated by centrifugation and held in dechlorinated water in a refrigerated glass vial. The density of the myxospores (number/μL) in the concentrated filtrate was estimated by AAHRL staff based on an average of three haemocytometer counts. The concentrated spores were shipped in an insulated cold storage container with a 0°C gel pack by FEDEX overnight express to our Colorado Laboratory. Upon arrival, the vial of myxospores was refrigerated at 3°C. The time delay between myxospore harvest at University of...
California–Davis and inoculation of the experimental replicates was 36 h.

At the beginning of the experiment (day 0), the aliquots of myxospores were mixed into 240 g of sterilized white sand (≤1 mm grain size) in each 1-L container, which was immediately hydrated with 750 mL of dechlorinated tap water. Each container was gently aerated with an air stone. The exposure containers were held on a shelf in an uninsulated room under ambient daylight that varied with the seasons. Diurnal fluctuations in water temperature ranged between 1°C and 2°C. Minimum and maximum temperatures ranged from 5°C to 15°C with the change of seasons over the 18-month duration of the experiment. The diurnal thermal variation and seasonal minimum and maximum temperatures in this study are well within the thermal ranges used in other laboratory experimentation assessing various aspects of the biology and epidemiology of the Mc parasite and its obligate host lineage *T. tubifex* (Arsan et al. 2007; El-Matbouli and Hoffmann 1991; El-Matbouli et al. 1999a, 1999b; Granath and Gilbert 2002; Hedrick et al. 2008; Nehring et al. 2014).

Lineage III *Tt* oligochaetes for the study were drawn from a culture that originated from East Parachute Creek, Colorado, a stream where *M. cerebralis* is not enzootic. Worms from this culture were used repeatedly as both unexposed laboratory controls and our standard laboratory treatment organism for comparative purposes in numerous exposure experiments over a 5-year period (Nehring et al. 2014), and were tested by qPCR (polymerase chain reaction) to ensure they were pure lineage III *Tt* oligochaetes (Beauchamp et al. 2001, 2002) and had not been exposed to the Mc parasite (Cavender et al. 2004). After introduction, the worms were fed weekly with a ration of 0.2 g of dehydrated spirulena discs, tetramin tropical fish granules, and ALGAMAC 2000 in a ratio of 6:3:1 by weight ground to a fine powder with a small commercial coffee bean grinder.

**Filtration and Quantification of TAMs.**—Except for a few minor modifications, we used the protocol of Thompson and Nehring (2000) for collection, filtration, and concentration of the weekly water samples to enumerate and estimate the TAM production for each replicate. The air supply to each replicate was turned off for a minimum of 1 h prior to collection of the filtrate to avoid loss of myxospores. The lack of agitation in the water column due to the water currents set up by aeration allowed any myxospores that might have been suspended to settle onto the sand substrate. Approximately 95% of the water from each replicate was gently decanted off and passively filtered through a form-fitted, cone-shaped, 20-µm mesh screen inserted into a perforated plastic funnel with a top width of 100 mm. After filtration was complete, the cone-shaped screen was removed, inverted and the material retained by the screen was back-washed into a funnel and rinsed into the sample collection jar. Filtrate volumes generally ranged from 15 to 50 mL. Five-2 mL aliquots were drawn by micro-pipette and placed into 10-mL scintillation tubes and stained with 60 µL of saturated aqueous crystal violet stain. A single 160-µL subsample was drawn from each of the five scintillation tubes, placed onto a gridded petri dish, covered with a disposable coverslip, and scanned by stereo-zoom microscopy for TAMs.

When extremely large numbers of TAMs were being produced in some replicates, the number of TAMs in the 160-µL subsample were far too numerous to count correctly under the microscope in a reasonable amount of time. In our previous studies (Thompson and Nehring 2000; Nehring et al. 2003, 2014) high densities of TAMs in a filtrate often lead to clumping and entanglement of the TAMs, which could lead to overestimation of the true density and total number of TAMs produced. To avoid this, the concentrated filtrate was diluted with distilled water to a volume that would result in less than 100 TAMs being enumerated in a single 160-µL subsample. This modification of the filtration protocol eliminated this potential source of error in the TAM enumeration and quantification process. In previous studies (Nehring et al. 2014) it was sometimes necessary to dilute a single filtrate with 20 L of distilled water to keep the number of TAMs observed in a single subsample ≤100.

**Statistical analyses.**—Our statistical analyses were performed with Proc GLM in SAS system software (SAS Institute 2010). Total TAM production estimates (dependent variable) for all replicates inoculated with 12,500 myxospores were regressed against the respective time-delay in days (independent variable). Estimates of total TAM production were subjected to natural log transformation to reduce the variance and linearize the relationship between the variables.

**Quantitative PCR testing.**—Upon termination of exposure for each time-delay treatment, all worms were removed from the substrate in each replicate and separated into two size categories. Large worms were classified as adults and small worms (produced during the 210-d exposure periods) were classified as juveniles. Our objective was to test a minimum of two aliquots of 50 adult worms per replicate by qPCR (Cavender et al. 2004) for DNA of the *Mc* parasite to qualitatively assess the differences in the level of infection between the time-delay treatments. Each aliquot of *Tt* oligochaetes was preserved in 70% ETOH, and submitted for qPCR testing.

**RESULTS**

The TAM production among the two replicates for the 15-d time-delay treatment was reduced by 74.7% compared with the day-0 time-delay treatment (Table 1), indicating that significant degradation processes affect *Mc* myxospores soon after liberation from fish tissues and exposure to sand, water, and the elements. For the 120-d and 180-d time-delay treatments, TAM production was reduced by more than 99% compared with the 0-day treatments. Linear regression of total TAM production estimates (log transformed, dependent variable) for all replicates inoculated with 12,500 myxospores regressed against the respective time-delay treatments in days (independent variable) was highly significant (*P* < 0.0001), suggesting a negative
relationship between total TAM production and the length of time that the Mc myxospores were incubating in the sand and water prior to the introduction of the Mc-susceptible Tt worms (Figure 1). Extension of the regression line to the x-axis suggests that all of the myxospores subjected to the conditions in this experiment would have been rendered nonviable in slightly less than 400 d, assuming the degradation process of the transformed data remains linear. The plot represents a negatively exponential decreasing function of the untransformed TAM data.

Among those 180-d time-delay treatment replicates, where the myxospore inoculation rate was increased 80-fold, total TAM production was increased compared with the low-dose replicates, but was still 43.8% less than the production among the 0-day time-delay replicates dosed at a rate of 50 myxospores/worm (Table 1). No TAM production was observed among the replicates inoculated with 1 million myxospores re-frigerated in an aqueous suspension for 365 d prior to the introduction of Tt oligochaetes, suggesting that 100% of the Mc myxospores were nonviable prior to inoculation and concordant with a duration of viability of ≤1 year.

The test results for the adult Tt worms screened by qPCR (Cavender et al. 2004) for evidence of Mc infection are summarized in Table 1. Among those time-delay treatments where total TAM production ranged from 750,000 to more than four million, all qPCR aliquots of 50 adult worms tested positive for Mc DNA, indicative of high levels of infection. Among the 120-d and 180-d time-delay treatments that were exposed at a rate of 50 myxospores/worm, TAM production was reduced by more than 99% compared with the 0-day time-delay treatments, and all aliquots of 50 adult worms tested negative for DNA of the Mc parasite, indicative of a much lower level of infection. These results are congruent with the very low TAM production in those replicates (Table 1). Five aliquots of 50 adult lineage III Tt worms from each replicate for the 365-d time-delay treatment were tested by qPCR (Cavender et al. 2004) and found to be negative for any presence of Mc DNA, congruent with the lack of TAM production over the 210-d exposure period.

**DISCUSSION**

Our results strongly support the possibility that the viability of Mc myxospores rapidly degrades upon release into the aquatic environment. Our findings are congruent with those of Hedrick et al. (2008), suggesting that Mc myxospores decrease in viability at an exponential rate and do not remain viable for long periods, i.e., up to years or even decades, as suggested by studies from the mid-20th century (Schäperclaus 1954; Bauer 1959; Hoffman et al. 1962; Funk 1968). Our experimental results suggest the long-term viability of Mc myxospores is more than 6 months but less than 1 year. Among the four replicates where the myxospores were incubated in sand and water for 120 or 180 d prior to introduction of unexposed lineage III Tt worms, TAM production was less than 1% compared with the average TAM production among the worm replicates exposed to the myxospores at day 0 (Table 1). The lack of TAM production by the Tt oligochaetes introduced to the replicates inoculated with 1 million myxospores indicates that those

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**TABLE 1. Estimated total triactinomyxon (TAM) production among time-delay treatments of *Myxobolus cerebralis* myxospores held in sterilized sand and continuously aerated, dechlorinated tap water prior to introduction of 250 unexposed, lineage III *Tubifex tubifex* oligochaetes and held in the same container for an additional 210 d or longer. Filtration and enumeration of TAMs was terminated in individual replicates when weekly TAM production dropped below 0.5% of the total TAM production elaborated during the entire exposure period. Initiation of TAM release among the replicates varied from 75 to 90 d post introduction of the *T. tubifex* worms. Total degree-days of exposure refers to the total accumulated thermal units of exposure for the worms from the time of introduction until the termination of the treatment.**

<table>
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<tr>
<th>Time-delay treatment (d)</th>
<th>Estimated total TAM production</th>
<th>Total TAMs versus number on day 0% change</th>
<th>Terminal PCR test results (number/total number)</th>
<th>Degree-days (°C) total exposure per treatment</th>
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</tr>
</tbody>
</table>

aNot applicable; no TAMs were ever detected in the negative control replicates.
bThe replicates were inoculated with 1 million Mc myxospores at day 0, an 80-fold increase in the dose rate.

*Myxospores for these replicates were refrigerated in an aqueous suspension for 365 d at temperatures 4–5°C prior to inoculation into sand and water and introduction of 250 lineage III *T. tubifex* into each replicate. Each replicate was screened once each week for 210 d, beginning at 74 d postexposure.
myxospores were nonviable 1 year after liberation from the infected fish carcass. Our nondetection of Mc DNA among any of Tt worms from these replicates screened by qPCR (Cavender et al. 2004) at the termination of the 210-d exposure period (Table 1) is congruent with the hypothesis that the viability of the Mc myxospores was <1 year, at least under the exposure conditions and variables in this experiment.

The only unexpected results in the study were observed in the 60-d time-delay treatment, where the average TAM production was only reduced 17.3% compared with the 0-day treatment (Table 1). Moreover, total TAM production among both 60-d replicates was higher than those observed among the 30-d and 90-d replicates. There could be any number of possible explanations for this outcome. However, the same questions could be asked as to why the great variations between the replicates within the 90-d replicates seeded with 12,500 myxospores and the 180-d replicates inoculated with 1 million myxospores. We have observed high variations in TAM production among similarly exposed replicates of Tt oligochaetes on a few occasions in previous experiments (Nehring et al. 2014). Despite the great variations in TAM production between replicates within and among the different time-delay treatments, there was an exponential decrease in total TAM production with increasing time-delay treatments that was highly significant (Table 1; Figure 1).

Assessment of the long-term viability of Mc myxospores has been investigated in the past (Hoffman et al. 1962; El-Matbouli and Hoffmann 1991; Hedrick et al. 2008). Hoffman et al. (1962) held Mc myxospores in two separate vials for 3 years. One vial was held at room temperature, the other at 6°C. While the spores appeared “normal” at 22 months under microscopic examination, at 3 years the sporoplasms had either completely disappeared or appeared degraded and were believed to be 100% nonviable. However, this study was completed two decades prior to the discovery that the life cycle of the Mc parasite involved two obligate hosts producing two distinct sporogonic phases: the triactinomyxon (TAM) and the myxospore (Markiw and Wolf 1983; Wolf and Markiw 1984). Subsequent to the elucidation of the life cycle, El-Matbouli and Hoffman (1991) observed TAM production among 100 g of specific-pathogen-free tubificids (90% T. tubifex and 10% Limnodrilus hoffmeisteri) after exposure to Mc myxospores aged for 5 months in sterilized sand. These worms began producing TAMs 80 d post exposure, but TAM production was not quantified. Hedrick et al. (2008) observed a 71% reduction in TAM production among lineage III Tt oligochaetes exposed to Mc myxospores aged in sterilized sand 60 d prior to initial exposure compared with TAMs produced in a second trial where the myxospores were aged in sand for 7 d.

Many investigators have evaluated various treatments for controlling WD in the aquaculture industry (Bauer 1959; Hoffman et al. 1962; Hoffman and Putz 1969; Hoffman and Hoffman 1972; Wagner 2002; Hedrick et al. 2008). Exposure of Mc myxospores (in vitro) to measured concentrations of calcium oxide (≥0.5%), potassium hydroxide (1%), and calcium hypochlorite (400 mg/L) killed 100% of the spores (Hoffman and Hoffman 1972) in 2 d. Irradiation of the hatchery water supply with ultra violet light can be effective in deactivation of TAMs and myxospores (Hedrick et al. 2008). Treatment of earthen ponds with calcium oxide at a rate of 380 g/m² for 14 d eliminated
all life stages of the $Mc$ parasite (Hoffman and Hoffman 1972). However, that equates to 3,800 kg/ha. While these various treatments can be efficacious for remediation of this parasite in an aquaculture setting, none of them are applicable or practical for control in natural streams or lakes.

We are unaware of any case where it has been shown that the $Mc$ parasite has been eliminated from a natural lake or stream once it has become enzootic. However, we hypothesize that it might be feasible in some select circumstances if the viability of $Mc$ myxospores is less than 1 year in the natural environment. First, any incoming water must be free of the $Mc$ parasite, whether the water source is from snowmelt or rainwater flowing into a natural lake, such as a cirque-basin lake in mountainous terrain, or the headwaters of a stream. Second, the salmonid fish population must be completely removed from a natural lake or stream where the parasite is enzootic, either through chemical reclamation using piscicides such as rotenone or antymycin, or mechanical removal by electrofishing or gill netting. This would remove the source of myxospore production, as long as the parasite was not reintroduced through upstream migration of infected fish from enzootic areas. Third, the lake or the stream would have to remain fishless for a minimum of 3 years to ensure the elimination of the $Mc$ parasite, allowing all $Mc$-infected lineage III $Tt$ oligochaetes to die of old age before reintroduction of any salmonids. Granath and Gilbert (2002) kept $Mc$-infected $Tt$ alive in a laboratory setting for more than 3 years. They found that $Mc$-infected $Tt$ oligochaetes periodically released TAMs at intervals of 6–9 months and tested positive for $Mc$ DNA at the time of death at more than 600 d after a single, initial exposure. The life expectancy of $Tt$ oligochaetes is variable, and population density can be related to the organic richness of the aquatic habitat (Finogenova and Lobasheva 1987; Granath and Gilbert 2002; Granath et al. 2007). However, once all infected worms had expired, the source of $Mc$ TAMs would be eliminated, and the life cycle of the parasite would have been broken.

The final step in any effort to eradicate the $Mc$ parasite from a lake or a stream would include extensive sentinel cage exposures of susceptible salmonid fry to test for evidence of infection with qPCR screening (Cavender et al. 2004) to ensure that salmonids are not reintroduced prematurely. Studies are underway in Colorado to test the validity of this hypothesis.

ACKNOWLEDGMENTS

This study was conducted in 2009–2010 as part of the Whirling Disease Investigations research project F-237R, through the Aquatic Research section of the Wildlife Programs Branch of the Colorado Division of Wildlife. Funding for this study was provided in part by the Federal Aid in Sport Fish Restoration Act. Myxospores of the $M. cerebralis$ parasite were provided by Dolly Baxa and Kavery Mukatira at the Aquatic Animal Health Research Laboratory at the University of California–Davis. Kurt Fausch and Kevin Rogers provided valuable advice on the design of the study.

REFERENCES


Journal of Aquatic Animal Health
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uahh20

The Impact of Egg Ozonation on Hatching Success, Larval Growth, and Survival of Atlantic Cod, Atlantic Salmon, and Rainbow Trout
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Published online: 25 Feb 2015.


To link to this article: http://dx.doi.org/10.1080/08997659.2014.983278
The direct exposure of fish eggs to ozonated water has generated interest as a means of ensuring pathogen-free eggs without the use of harsh chemicals. However, there are numerous knowledge gaps, including safe contact times, exposure levels, and potential long-term effects on aquaculture species in both freshwater and seawater. The effect of different ozone (O₃) doses (0.5–1.0, 1.5–2.0, and 2.5–3.0 mg of O₃/L for 90 s) on recently fertilized eggs of Atlantic Cod Gadus morhua and eyed eggs of Atlantic Salmon Salmo salar and Rainbow Trout Oncorhynchus mykiss was evaluated in comparison with the effects of two commercial disinfectants: Perosan (0.004 mg/L) and Ovadine (100 mg/L). The impact of ozone application was evaluated based on hatching success, larval nucleic acid concentration, larval growth, and survival. Overall, results indicated that ozonation of Atlantic Cod eggs at a dose less than 3.0 mg/L for 90 s produced no negative effect on the larvae up to 30 d posthatch. Furthermore, ozonation of Atlantic Salmon and Rainbow Trout eggs generated no negative effect on the larvae, based on monitoring until 85% yolk sac re-absorption (16 d posthatch).

The production of high-quality, viable eggs—and subsequently the production of larvae with high survival and growth rates—is one of the most important aspects of juvenile fish aquaculture (Abi-ayad et al. 1997). Effective egg disinfection allows hatcheries to be stocked with disease-free eggs and to produce healthy offspring. Current chemical disinfection methods used in hatcheries include iodophors, chloramines, hypochlorites, glutaraldehydes, hydrogen peroxide, and common antibiotics (Grotmol et al. 2003; Peck et al. 2004). However, recent studies have shown that some viral pathogens are more resistant to
these disinfection methods than are bacterial pathogens (Grotmol et al. 2003), thus highlighting the importance of successful laboratory-scale trials using ozone (O$_3$) to eradicate viruses in fish eggs (Grotmol et al. 2003; Buchan et al. 2006). For fungal infections, chemicals such as hydrogen peroxide and formaldehyde are often used. Application of hydrogen peroxide requires a precise dosing that is temperature dependent, and this chemical cannot be used on food fish at any life stage (Burka et al. 1997). Although formaldehyde is quite efficient for controlling a number of pathogens, its use prompts concerns for the health of personnel conducting the application, as it is a recognized carcinogen (Marking et al. 1994); formaldehyde might also have an environmental impact if released into effluents. Therefore, there is an increasing need for safe and environmentally sustainable disinfection alternatives that can eradicate both bacterial and viral pathogens in freshwater and saltwater systems. Previous studies have documented ozone toxicity to marine organisms (e.g., Mimura et al. 1999; Grotmol and Totland 2000; Battaglene and Morehead 2006) and freshwater organisms (e.g., Wedemeyer et al. 1979; Gagné et al. 2007). In particular, the presence of high concentrations of bromide ions in seawater changes the chemistry of ozone decomposition in comparison with that in freshwater (Jones et al. 2006), thus requiring caution in dealing with marine hatchery effluents after ozonation. However, due to its fairly short half-life, ozone remains a promising candidate for egg disinfection (Forneris et al. 2003). Ozone can oxidize amino acids, fatty acids, and proteins containing sulphhydril groups and pyrimidine nucleotides (Carmichael et al. 1982), and thus ozone has the potential to serve as a potent disinfectant. However, as suggested by Asbury and Coler (1980), the membrane of the fish egg could provide a barrier that protects the developing embryo against the oxidative action of ozone, allowing for the effective application of ozone as a surface disinfectant. Ozone is used to sterilize water supplies and discharge for both flow-through and recirculating systems. It has been implemented as a means of improving water quality by removing particles and protein, reducing microflora (Summerfelt and Hochheimer 1997), and eliminating fish pathogens (Liltved et al. 2006). However, the direct application of ozonated water to fish eggs as a means of disinfection against pathogens is in the early stages of development.

Ozone has been used successfully to disinfect the eggs of saltwater species, including Atlantic Halibut Hippoglossus hippoglossus, Turbot Scophthalmus maximus, Haddock Melanogrammus aeglefinus, and Atlantic Cod Gadus morhua, on a non-industrial scale (Grotmol et al. 2003; Buchan et al. 2006). However, ozone toxicity appears to be an issue, as it has resulted in delayed, asynchronous, or reduced hatching in several marine fish species (Mimura et al. 1999; Grotmol and Totland 2000; Battaglene and Morehead 2006). Oxidants that are formed during seawater ozonation may negatively impact egg chorion functionality (Grotmol and Totland 2000; Grotmol et al. 2003). Ozone concentration (C) and contact time (T) both determine the impact of the disinfection process on eggs. The strength of the disinfection is scored as a combined value of $C \times T$ (hereafter, CT). In Atlantic Cod eggs, an optimum level to ensure the destruction of fish pathogens without affecting hatch was a T of 2 min or less at 2 mg of O$_3$/L of water (i.e., CT $\leq$ 4; Grotmol et al. 2003). Operationally, ozonated seawater applied at 1.5–2.0 mg/L for 90 s (CT = 2.25–3.00) has been used on Atlantic Cod eggs at the Joe Brown Aquatic and Research Building (JBARB; Department of Ocean Sciences, Memorial University of Newfoundland, St. John’s, Newfoundland and Labrador) since 2004 in response to the use of nodavirus-positive broodstock in initial production cycles. However, this use of ozonated water as a surface disinfectant has never been validated scientifically, and knowledge gaps are numerous, including exposure levels, duration, and potential toxicity to the gametes. In particular, questions remain on the effects of ozone exposure timing during egg development stages. Application of ozone treatments at the introduction of embryos (after fertilization) into a production cycle presents a significant advantage for achieving initial stocking of disease-free eggs in hatcheries.

Ozone has been the focus of considerable research into the treatment of freshwater for rearing salmonids (Øye and Rimstad 2001); however, very little work exists on the direct application of ozone as a disinfectant for eggs in freshwater species. At the egg and larval stages of Sockeye Salmon Oncorhynchus nerka, acute exposure to low levels of ozone resulted in high mortality up to the eyed-egg stage (Grischkowsky et al. 1983), although the authors of the study acknowledged that ozone levels were inconsistent and significantly higher than originally intended. When used in repeated applications of 0.01, 0.03, or 0.20 mg/L at 2-d intervals for 46–60 d, ozonated water effectively controlled saprolegniosis in Brown Trout Salmo trutta eggs that were incubated in California trays (Forneris et al. 2003).

The intent of this research was to examine the application of ozonated water to fish eggs in seawater and freshwater at a semi-commercial hatchery. We scaled up our ozone disinfection trials from laboratory-scale models (e.g., Grotmol and Totland 2000; Forneris et al. 2003; Grotmol et al. 2003), which are too small for large-scale disinfection of egg batches (Buchan et al. 2006); this allowed us to evaluate ozone’s effects on a high number of eggs subjected to different hatching methods, such as vertical trays (salmonids). The higher organic load of large egg biomasses will significantly shorten the half-life of ozone, so sufficient water volume and ozone flow are necessary to maintain appropriate disinfection doses (Buchan et al. 2006). In addition, the timing of ozone application to fish embryos was selected to mimic the initial stocking of a hatchery shortly after fertilization for Atlantic Cod (i.e., broodstock held in the same facility) as opposed to application before hatch (as in Grotmol et al. 2003) or at the eyed stage for salmonids (after transport).

One marine species (Atlantic Cod) and two freshwater species (Atlantic Salmon Salmo salar and Rainbow Trout Oncorhynchus mykiss) were exposed to ozone at 0.5–3.0 mg/L (CT $= 3.75–4.50$) to determine any measurable effect on egg quality, survival, and larval performance. For Atlantic Cod, the
effect of ozonation was measured using metrics of egg and larval quality, such as blastomere normality, egg diameter (Kjorsvik et al. 1990), hatch rate, larval survival up to 30 d posthatch (dph), and larval RNA : DNA ratio (Caldarone 2005). The physiological basis of the RNA : DNA ratio is the constant amount of DNA in somatic cells (genetic material) relative to the varying RNA concentration due to protein synthesis (Buckley 1984; Caldarone et al. 2003). Hence, organisms in good condition will tend to have higher RNA : DNA ratios than organisms in poor condition (Chícharo and Chícharo 2008). The ozonation procedure was also compared with the usage of Perosan (active ingredients = peroxyacetic acid and H₂O₂), a disinfectant that is commonly employed in marine finfish hatcheries. In freshwater, ozone application on eyed eggs of Atlantic Salmon and Rainbow Trout was examined in comparison with use of the commercial disinfectant Ovadine (active ingredient = iodine). The effect of disinfection treatments was evaluated using measures of egg and larval success (percent survival), growth (larval weight and length), and growth rate (yolk sac conversion efficiency [YSC]).

**METHODS**

**Atlantic Cod egg collection and treatment.**—Communally spawned Atlantic Cod eggs were collected in 2011 at the JBARB. The broodstock were comprised of captive wild fish that were initially transferred to the facility in 2005. An external egg collector was emptied at 1800 hours on the night prior to a collection and was reset with a clean, empty Nitex bag; eggs were then retrieved from the collector the next day at 0800 hours. Therefore, only fertilized eggs (fertilization rates > 50%) that had been spawned within the previous 14 h in the broodstock tanks were used. Floating eggs (viable eggs) from within the tank into a Nitex bag that was suspended in seawater. The egg collector was emptied at 1800 hours on the night prior to a collection and was reset with a clean, empty Nitex bag; eggs were then retrieved from the collector the next day at 0800 hours. Therefore, only fertilized eggs (fertilization rates > 50%) that had been spawned within the previous 14 h in the broodstock tanks were used. Floating eggs (viable eggs) from a single batch were divided into five volumes of 300 mL (100,000–150,000 eggs). Each beaker containing 300 mL of eggs was randomly assigned to one of five treatments as follows: untreated (control); Perosan at 0.004 mg/L; or a 90-s disinfection treatment with ozone at 0.5–1.0 (low ozone), 1.5–2.0 (standard ozone), or 2.5–3.0 mg/L (high ozone). The protocol for Perosan treatment consisted of a static bath with 40 mL of Perosan in 10 L of filtered, ultraviolet (UV)-treated seawater for 1 min at 6–8 °C. The ozone system.—Ozone for egg disinfection was generated using a corona-discharge ozone generator (Pacific Ozone Technologies, California) previously installed in the JBARB hatchery. Ozonated seawater was pumped into an egg contact basket and remained there for 90 s until removal of the basket from the system.

**Atlantic Cod egg quality and survival.**—Egg quality for Atlantic Cod was assessed on 100 eggs/batch prior to disinfection. Variables examined included fertilization success (number of eggs showing cell division out of the 100 eggs), egg development stage (number of cells), and six criteria for blastomere normality (as revised for Atlantic Cod; Shields et al. 1997; Penny et al. 2006): (1) uniformity of cell size and shape, (2) cell adhesion, (3) cell margins, (4) clarity of cell cytoplasm, (5) cell symmetry, and (6) cell numbers. Blastomere normality variables were expressed as percentages. Finally, the diameters (mm) of 10 eggs from each batch were measured and recorded. Egg quality in all of the treatment groups was reassessed at 5 h post-treatment. This period allowed for cell division to occur while ensuring that the number of cells could still be counted visually under the microscope. Eggs were incubated in 50-L, conical flow-through incubators with a water flow rate of 25 L/h and gentle aeration. Temperature in the incubators was monitored daily (mean = 5.9 °C) and was used to calculate degree-days for individual batches. Dead eggs were collected through the bottom valve of the incubator, and the total volume was measured to assess egg mortality (for calculation of egg survival).

**Atlantic Cod larval rearing and sampling.**—After all eggs within a treatment had hatched, three 50-mL samples were removed, and the number of hatched larvae was determined by extrapolating to the total number of larvae in the incubator based on the mean of three counts. Due to tank limitations and restrictions on live feed, only four batches of eggs (i.e., batches 1–4) were reared, and therefore the 0.5–1.0-mg/L ozone treatment group was terminated. From the remaining four batches, 30,000 larvae/treatment were transferred to 500-L tanks. Larvae were maintained under 24-h light and 95% oxygen saturation at 10°C (SE = 1) and were fed a diet of rotifers enriched with Ori-green (Skretting, Bayside, New Brunswick) three times daily. Larvae were sampled at 0, 10, 20, and 30 dph for nucleic acid analysis (n = 10 larvae) and to determine SL (mm) and myotome height (body height [mm] at the anus; n = 10 larvae). Larval condition factor (K) was calculated by dividing the myotome height by the SL as per Koslow et al. (1985) and Puvanendran and Brown (2002). Single larvae were sampled and placed in RNase/DNase-free microcentrifuge tubes and were stored at −70°C. At 30 dph, all larvae were siphorned from each tank, and the final numbers of larvae were determined in the same manner as at hatch to determine survival.

**Nucleic acid analysis of Atlantic Cod larvae.**—The RNA and DNA concentrations in Atlantic Cod larvae were determined by
the method of Caldarone et al. (2001). Briefly, a single larva was submerged in 1% N-lauro sarcosine and was centrifuged. The supernatant was loaded into a 96-well microplate along with ethidium bromide. The resulting fluorescence was measured (in triplicate) using a microplate reader (BioTek KC4 Synergy HT; BioTek Instruments, Winooski, Vermont) with excitation and emission levels set at 530 and 590 nm, respectively, and quantified relative to a standard curve of molecular-grade 18S and 28S ribosomal RNA purified from calf liver (BioWorld, Dublin, Ohio). After enzymatic digestion of the RNA by using RNase (Sigma-Aldrich), the process was repeated to measure the DNA of the same sample (in triplicate), this time relative to a DNA standard curve created using genomic ultra-pure calf thymus DNA (Sigma-Aldrich). Four samples per treatment and time point (0, 10, 20, and 30 dph) were processed for each batch. After the DNA-attributed fluorescence reading was completed, DNase was added to all wells on the plate, and a third and final reading was taken to ensure the purity of the reagents and the standard curve solutions.

Atlantic Salmon and Rainbow Trout eggs.—Thirty-thousand eyed eggs of Atlantic Salmon (age = 381.7 degree-days) were transferred by ground transportation from Northern Harvest Smolt Ltd. (Stephenville, Newfoundland and Labrador) to the Fisheries and Marine Institute at Memorial University of Newfoundland. Prior to the experiment, Atlantic Salmon eggs were held at a density of 5,000 eggs/tray in vertical incubation units supplied with 8°C, UV-treated freshwater. Forty-thousand eyed eggs of Rainbow Trout (245 degree-days) were transported by air from AquaSearch Ova ApS (Jelling, Denmark) to the Fisheries and Marine Institute. Prior to experimental trials, the Rainbow Trout eggs were held at a density of 5,000 eggs/tray in vertical incubation units supplied with 9.5°C, UV-treated freshwater.

Salmonid egg incubation.—Throughout the study, salmonid eggs were incubated in four vertical incubation units, with 8 trays/unit. Each tray was divided into two chambers using Lexan dividers; thus, 64 chambers were used. The units were to be used as an individual water supply to each tray. The freshwater ozonation system was a prototype system designed for plug-and-play use, similar to that described for the Atlantic Cod study. For each group of eggs, the total egg volume was subdivided into four treatment groups of equal volume. The four volumes were randomly assigned to one of the following treatment groups: (1) control (similar handling but no chemical disinfection), (2) Ovadine exposure, (3) ozone applied at 0.5–1.0 mg/L (low ozone), or (4) ozone applied at 2.5–3.0 mg/L (high ozone). The Ovadine treatment was completed in accordance with the manufacturer’s recommendation (1:100 dilution) by mixing a 10-mL/L solution of Ovadine and submerging the eggs for 10 min. Exposure to ozone was completed as described for Atlantic Cod. After treatment, eggs were transferred to the vertical incubation trays and maintained with a supply of UV-treated freshwater at a mean temperature of 8.7°C (SE = 1.4) for Atlantic Salmon eggs and 9.5°C (SE = 1.4) for Rainbow Trout eggs.

Salmonid larva morphometrics.—Atlantic Salmon and Rainbow Trout egg mortalities were counted and removed daily. At hatch, TLs of larvae (both species) were measured in each of the trays. Rainbow Trout larvae were also measured (n = 8 larvae/tray) for mass (g) and yolk sac volume (YSV) at three time points: (1) hatch (0 dph); (2) midway through the endogenous feeding period (8 dph); and (3) at 85% (SE = 5) yolk sac re-absorption (16 dph). Mass and YSV were not investigated for Atlantic Salmon larvae. For Rainbow Trout larvae, YSV was calculated as per Kosiken et al. (2002), and specific growth rates for both TL (mm) and mass (g) were derived by the method of Jobling (1994). The YSC was estimated as described by Fraser et al. (2008),

\[ YSC = \frac{\text{Size}_{YS,\text{re-absorption}} - \text{Size}_{\text{hatch}}}{\text{YSV}} \]

where \( \text{Size}_{YS,\text{re-absorption}} \) is larval TL (mm) at yolk sac re-absorption and \( \text{Size}_{\text{hatch}} \) is larval TL (mm) at hatch.

Statistical analysis.—Prior to parametric analysis, all data were tested for normality of the residuals and for homogeneity of variances. When necessary, percentage data were square root transformed. If the data set failed to meet the assumption of equal variances (\( P \leq 0.05 \)), a nonparametric Kruskal–Wallis ANOVA on ranks was performed.

For the Atlantic Cod trials, one-way ANOVA was used to evaluate the age of the eggs within a batch, which was determined based on cell number. The percentages of eggs with different numbers of cells (2, 4, 8, 16, 32, 64, or >64 cells) within one batch were compared by this method. The effect of the treatments (Perosan; and low, standard, and high ozone applied for 90 s) on blastomere normality (six criteria) was also assessed by using a series of one-way ANOVAs. The effects of treatment on egg survival, larval hatch rates, and larval survival were evaluated using a randomized-block general linear model (GLM) with two categorical variables: treatment (fixed term) and batch (random term). The effect of treatment on specific growth rate was evaluated by the same GLM method; however, the effect was measured at each time point (10, 20, and 30 dph). The effects of treatment on \( K \) and RNA : DNA ratios were assessed by using a series of one-way ANOVAs.

For the Atlantic Salmon and Rainbow Trout trials, the effects of treatment on larval weight and TL were measured with ANCOVA. For both salmonid species, mean initial larval mass or TL was used as the continuous variable. The YSC of Rainbow Trout and the cumulative survival of both salmonid species were analyzed using a GLM with two categorical variables: treatment and status (both fixed terms).

For all data, when significant differences were detected, Tukey’s honestly significant difference test for pairwise comparisons was used to determine differences between treatments. In all analyses, the significance level \( \alpha \) was set at 0.05. Statistical analyses were conducted in Statistica version 10 (StatSoft,
TABLE 1. Initial fertilization success (%) and cell stage (%) of Atlantic Cod eggs used for the ozonation study.

<table>
<thead>
<tr>
<th>Batch</th>
<th>Fertilization (%)</th>
<th>2–16 cells</th>
<th>16–64 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>82.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>49.0</td>
<td>63.0</td>
<td>37.0</td>
</tr>
<tr>
<td>4</td>
<td>94.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>77.0</td>
<td>74.0</td>
<td>26.0</td>
</tr>
<tr>
<td>6</td>
<td>100.0</td>
<td>88.0</td>
<td>12.0</td>
</tr>
<tr>
<td>7</td>
<td>98.0</td>
<td>99.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>99.0</td>
<td>94.0</td>
<td>6.0</td>
</tr>
<tr>
<td>9</td>
<td>99.0</td>
<td>94.0</td>
<td>6.0</td>
</tr>
<tr>
<td>10</td>
<td>99.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Tulsa, Oklahoma) and SigmaPlot version 12.0 (Systat Software, Chicago).

RESULTS

Atlantic Cod Trials

The development stages of the eggs within each of the 10 batches were statistically similar ($P = 0.418–0.996$; Table 1; cell stages were pooled into two groups for clarity). At 5 h posttreatment, blastomere normality criteria did not vary among treatment groups ($P = 0.181–0.943$). Likewise, egg survival up to the time of hatch did not differ among treatments ($F_4 = 0.806, P = 0.421$; Figure 1). All egg batches hatched at between 92.4 and 108.8 degree-days. Further, hatch was synchronous for all batches except batch 1, in which the control group hatched 11.9 degree-days prior to the other four treatments. The results showed no significant difference in hatch rates ($P = 0.280$; Figure 2). Similarly, no significant differences were observed in specific growth rates among treatments at 10, 20, or 30 dph ($P = 0.334–0.996$). Larval survival ranged from 21.7% to 43.3% at 30 dph and was not significantly different ($F_3 = 1.0726, P = 0.408$) among the treatments.

Mean $K$ showed some significant differences due to treatment in select batches, but there was no specific trend in the manner in which treatments differed (Table 2). Overall RNA concentrations ranged from 3.18 µg/larva (SD = 1.02) to 23.12 µg/larva (SD = 10.23) between 0 and 30 dph, while DNA concentrations ranged from 1.07 µg/larva (SD = 0.36) to 8.50 µg/larva (SD = 3.70). The RNA : DNA ratio showed statistically significant effects of treatment at 0, 10, 20, and 30 dph and within several batches (1, 2, 3, and 10), with each age showing a unique effect of treatment. Furthermore, RNA : DNA ratio values ranged between 1.30 and 3.73 throughout the experiment.

Atlantic Salmon and Rainbow Trout Trials

There was no significant difference ($P > 0.05$) in length among treatments for Atlantic Salmon or Rainbow Trout at any life stage (Atlantic Salmon: $P = 0.842$; Rainbow Trout: $P = 0.951$; Table 3). Rainbow Trout did not exhibit among-treatment differences at any life stage in terms of weight ($P = 0.316$), yolk sac absorption ($P = 0.783$), specific growth rate in weight ($P = 0.334–0.996$), or specific growth rate in length ($P = 0.334–0.996$; results not shown). There was also no among-treatment difference in percent survival of Atlantic Salmon (ANOVA: $F_3 = 0.33, P = 0.800$). Survival of Rainbow Trout larvae differed among treatments ($P < 0.001$; Table 3); the control larvae had...
a significantly higher percent survival (81.8%) than the larvae treated with Ovadine (74.1%), low ozone (74.5%), or high ozone (73.6%). However, Rainbow Trout larval survival was equivalent within 1% among the disinfection treatments.

**DISCUSSION**

**Atlantic Cod Trials**

In this study, initial egg quality and cell stage were checked in every batch to confirm consistent quality between batches, thereby ensuring that any observed effect was the result of treatment and not due to the use of poor-quality eggs. Delayed hatch or reduced hatching success has been reported as a result of saltwater ozone disinfection for eggs at different stages of development in Atlantic Halibut, Striped Trumpeter *Latris lineata*, and Japanese Flounder *Paralichthys olivaceus* (Mimura et al. 1999; Grotmol and Totland 2000; Battaglene and Morehead 2006). However, Grotmol et al. (2003) found that when ozone was applied to Atlantic Cod eggs 2 d prior to expected hatch at O₃ levels no greater than 2.2 mg/L for 30 s (*CT* ≤ 1.1), most of the ozone-treated groups hatched within the same period as the control. Our results showed that Atlantic Cod embryos treated with ozone at 2.5–3.0 mg/L for 90 s within 18 h of fertilization (2–32-cell stage) had egg survival equivalent to that of both the control group and the Perosan-treated group. Similarly, no detrimental effect of ozone treatment on egg survival was observed in Haddock, another gadid species, when eggs in different periods of time (*CT* = 4–16-cell stage) were treated with ozone at 3 mg/L for 10 b. Other batches were treated with Ovadine (74.1%), low ozone (74.5%), or high ozone (73.6%). However, Rainbow Trout larval survival was equivalent within 1% among the disinfection treatments.

**TABLE 2. Differences (P-values) in mean condition factor (K) and RNA : DNA content among Atlantic Cod larvae hatched from eggs that were exposed to different disinfection treatments (dph = days posthatch; ND = not determined). Within each batch and age-group, significant differences (P < 0.05) due to treatment are indicated by bold italic P-values.**

<table>
<thead>
<tr>
<th>Age (dph)</th>
<th>Batch</th>
<th>K</th>
<th>Differences</th>
<th>RNA : DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>0.080</td>
<td>0.409</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>0.149</td>
<td><strong>0.030</strong></td>
<td>C &gt; L, S</td>
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</tr>
<tr>
<td>4</td>
<td>0.812</td>
<td>0.258</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><strong>0.038</strong></td>
<td>C &gt; L</td>
<td>0.913</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.227</td>
<td>0.679</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0.877</td>
<td>0.625</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.950</td>
<td>0.073</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td><strong>0.047</strong></td>
<td>P &gt; C</td>
<td>&lt;0.001</td>
<td>C, P &gt; S &gt; L</td>
</tr>
<tr>
<td>10b</td>
<td>1</td>
<td>0.054</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.084</td>
<td>ND</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>0.273</td>
<td><strong>0.015</strong></td>
<td>H &gt; C</td>
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<tr>
<td>4</td>
<td>0.555</td>
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<td></td>
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<tr>
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<td>0.620</td>
<td><strong>0.023</strong></td>
<td>C &gt; P</td>
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<td>P &gt; S, H</td>
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<tr>
<td>4</td>
<td>&lt;0.001</td>
<td>P, C, S &gt; H</td>
<td>0.060</td>
<td></td>
</tr>
</tbody>
</table>

---

a Differences between the different treatments tested (C = control; P = Perosan; L = low ozone [0.5–1.0 mg of O₃/L]; S = standard ozone [1.5–2.0 mg/L]; H = high ozone [2.5–3.0 mg/L]).

b After 10 dph, only four batches were considered, and the low-ozone treatment was no longer evaluated.

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content (Chicharo and Chicharo 2008). There was considerable variability in RNA : DNA ratio among egg batches, particularly at 0 dph, with a predominance of RNA : DNA range of 1.30–3.73 (maximum value = 6.54). Larval variability has been observed to be high during the yolk sac stage, which in Atlantic Cod extends up to 5 dph (Kjorsvik et al. 1991), and some authors have cautioned against extrapolating from this time period for RNA : DNA growth models (Westerman and Holt 1994; Folkvord et al. 1996; Caldarone et al. 2003). Individual larval growth is known to vary greatly, even within a single treatment, leading to difficulties in establishing mean growth patterns (Caldarone et al. 2003), as was evident in this study. The variability in RNA : DNA ratio among treatments within a single batch was lower than the variability observed when comparing across batches. Therefore, each batch was examined as a single unit, exploring the effect of disinfection treatment relative to the control. Significant differences were found in 25% of the batch/age periods considered, but no particular pattern from treatment application was discernible. The RNA : DNA ratios and RNA content are
better predictors of recent length- and weight-specific growth rates than they are of absolute length and weight (Bulow 1987; Ferguson and Danzmann 1990). Despite the interindividual and interbatch variability in the RNA : DNA ratio and the significant differences observed in certain batches, there was no evidence that any of the disinfection methods negatively impacted growth up to 30 dph. This conclusion is supported by the absence of a disinfection treatment effect on $K$, specific growth rate, and survival. Survival levels ranged from 21.7% to 43.3%, with the highest survival occurring in the two ozone-treated larval groups. Puvanendran and Brown (1999) observed larval survival of approximately 24% for Atlantic Cod reared under similar conditions at 14 dph and later, and in a separate study, they (Puvanendran and Brown 2002) observed 40% survival at 42 dph when examining optimal photoperiod and light intensity.

**Atlantic Salmon and Rainbow Trout Trials**

For salmonid eggs, disinfection was applied at the eyed stage of development, as this stage is past a critical developmental point allowing for egg transport. For both Atlantic Salmon and Rainbow Trout eggs, hatching success was comparable among treatments. There was no significant effect of low ozone, high ozone, or Ovadine application relative to the untreated control. Hatching success of both salmonids ranged between 73% and 85%. Ozonated freshwater was applied at the eyed-egg stage with no delay or decrease in hatch, which has been a reported side effect of ozone toxicity in some marine fish species (Mimura et al. 1999; Grotopl and Totland 2000; Battaglene and Morehead 2006). Overall, the results of this study are supported by the limited data available on ozone disinfection of salmonid eggs. Grischkowsky et al. (1983) observed that Sockeye Salmon hatched with a hatching success of 76.4% when treated with continuous low levels of ozone throughout the egg development stages. Similarly, freshwater application of ozone at 0.15 mg/L to eggs of Persian Sturgeon Acipenser persicus yielded hatching success of 76.4% (Ghomi et al. 2007).

There was no impact of disinfection on TLs of Atlantic Salmon or Rainbow Trout larvae. For both species, all three disinfection treatments yielded growth results (length, weight, and YSC) that were equivalent to the results from their respective control groups. Atlantic Salmon survival was above 81% in all treatments. However, Rainbow Trout survival in the ozone treatments (73.5–74.5%) was significantly lower than that of the control group (81.8%). However, a reduction of less than 10% in Rainbow Trout survival at hatch due to disinfection treatment would not outweigh the long-term benefits of halting the transmission of disease if present within a population.

In conclusion, the intent of this research was to investigate the safety of ozone for use with three species of interest to aquaculture. Ozone applications were focused on specific stages of egg development corresponding to hatchery handling or transport points during production. In addition, this investigation provided an opportunity to increase the scale of ozone trials from a laboratory-scale model to a semi-commercial facility, thereby optimizing the amount of disinfectant required for a larger quantity of eggs. The treatment of recently fertilized Atlantic Cod eggs with ozone concentrations ranging from 0.5 to 3.0 mg/L caused no negative impact on the eggs or resultant larvae up to 30 dph in comparison with the untreated control or Perosan-treated groups. Similarly, our results for Atlantic Salmon and Rainbow Trout indicated no negative impact on the growth or survival of eyed eggs from treatment with ozonated freshwater at 3.0 mg/L or less for 90 s ($CT \leq 4.5$) relative to Ovadine-treated eggs. Rainbow Trout eggs and larvae appeared to be more sensitive (i.e., lower survival) to both of the disinfection methods and handling. Further research examining the efficacy of ozone ($CT \leq 4.5$) as a disinfectant against specific aquatic diseases is necessary.

**ACKNOWLEDGMENTS**

This project was funded by the Aquaculture Collaborative Research and Development Program. We are grateful to the staff of JBARB and the Fisheries and Marine Institute, Memorial University of Newfoundland, for assistance throughout this project.

**REFERENCES**


Carmichael, N. G., C. Winder, S. H. Borges, B. L. Blackhouse, and P. D. Lewis. 2001. Proteinase K activity and nucleic acid content as indicators of developmental stage of larval 


JOURNAL OF AQUATIC ANIMAL HEALTH

Guide for Authors

Editorial Policy

The Journal of Aquatic Animal Health serves the North American and international communities of scientists concerned with the health of aquatic organisms. We encourage the submission of papers dealing with the causes, effects, treatments, and prevention of diseases of marine and freshwater organisms, particularly fish and shellfish. We also welcome manuscripts describing biochemical and physiological investigations into fish health that relate to assessing the impacts of both environmental and pathogenic factors.

Manuscript Submission and Review

Manuscript Categories

Manuscripts may be submitted in any of the following categories: (1) Articles are reports of substantial, controlled research that will be judged on their scientific merit. Critical reviews of timely topics will also be considered in this category. Articles should ordinarily not exceed 5,000 words, excluding references and tables (about 20 double-spaced manuscript pages), but longer ones will be considered. (2) Communications are shorter papers based on more restricted study objectives, sometimes without extensive statistical data, but with sound biological observations; promising work that may lead to additional in-depth studies; thorough testing of a technique; or case histories. Such papers will be evaluated as much for their practical utility as for their scientific quality. Communications should generally not exceed 3,000 words (about 12 double-spaced manuscript pages). (3) Comments are critiques of papers published by this journal, responses to which will be invited from the original authors; brief presentations of experiences or additional data related to previously published papers; or short discussions of technical issues pertinent to the aquatic animal health community.

Submission Procedures

Manuscripts and associated correspondence should be submitted at the journal’s online submission and tracking site, http://mc.manuscriptcentral.com/jaah (this site may also be accessed through the Publications section at the American Fisheries Society’s Web site, www.fisheries.org). Detailed instructions, including acceptable file formats, are available at the site.

Although the submission site permits authors to include a cover letter, such letters are generally not necessary; they should be included only when they contain information that cannot easily be incorporated into the standard submission form.

The site also permits authors to recommend certain reviewers and/or to request that certain reviewers not be used. Recommendations are encouraged but are not required.

Review Process

Submitted papers will be critically reviewed by at least two experts in the relevant discipline(s) and evaluated by one of the journal’s editors. A manuscript may be returned to its author without review if it is judged to be of poor quality or inappropriate for this journal.

All submissions are electronically screened for the inappropriate use of material from previously published sources. In submitting a paper, you are stipulating that, except where explicitly indicated otherwise, all of the statements, data, and other elements reflect your own work and not that of others. All allusions to the work of others should be properly cited; exact quotations from other sources should be in quotation marks. Authors are also cautioned not to repeat long passages from their own publications. Failure to follow these requirements may result in rejection of the paper and, in extreme cases, restrictions on publishing in this journal.

Authors have the option of not having their names revealed to the reviewers (to facilitate the selection of reviewers, however, the editor and associate will always be aware of the authors’ identities). If authors wish to exercise this option, they should: (1) check the appropriate block on the submission page, (2) put the title page in a separate file that can be excluded from the manuscript file that the reviewers’ receive, and (3) remove from their manuscript any other information that may reveal their identities.

Review of manuscripts relies on volunteers and can be a fairly lengthy process. However, we strive to get decisions to authors in 9–12 weeks. If revisions are requested, authors should make them promptly, normally within 30 days of receiving the editor’s decision (short extensions will be allowed if there are justifiable delays). If a revision is not received within the allowed time, the paper will be considered withdrawn; late revisions will be treated as new submissions and may have to go through the review process again.
Publication Charges

Publication charges are US$100 per printed page and will be billed when the paper is in proof. Full and partial subsidies are available to voting members of the American Fisheries Society who certify that grant or agency funds are not available. Manuscript reviews are not affected by requests for subsidies; however, at least one author must be (or become) an AFS member by the time that a paper is published. Every paper published in the journal is subject to a $30 fee to offset handling costs. Authors will receive an “e-print” of the published article (i.e., a special link that grants complimentary access to the online version that may be shared with up to 49 other people); they may also purchase reprints of their papers from the printer when they receive their proofs.

Manuscript Preparation

Components

A typical manuscript will have the following components:

Title page.—The title page should give the title of the paper and the name(s) and complete mailing address(es) of the author(s). In addition to accurately reflecting the content of the paper, the title should be short (preferably no more than 12 words) and to the point. See a recent issue of the journal for the format to use for authors’ names and addresses. A suggested running head (shortened version of the title) may also be included on the title page. Keywords are not used in this journal, however, and so should not be included.

Abstract.—Articles and communications require abstracts; comments do not. The abstract should consist of one paragraph (up to 300 words for an article and up to 200 words for a communication) that concisely states why and (generally) how the study was done as well as what the results were and what they mean. It should not simply outline the contents (e.g., avoid statements to the effect that such-and-such is presented) or present the methods in detail. Citations and footnotes are not allowed in abstracts, and abbreviations should be used sparingly. De-
nomenclature (e.g., ages of anadromous fish and structures of fatty acids).

Tables.—In general, tables should be designed to present related information as simply and directly as possible. A good rule of thumb is to establish the point(s) that the table is intended to make, then to select the information required to do that and determine the most logical order in which to present it. Detailed guidelines for the preparation of tables may be found in chapter 12 of the AFS style guide, but a few of the more important ones may be mentioned here:

1. We prefer to print tables in “portrait” orientation but will allow ones in “landscape” orientation as long as they take up no more than one page.
2. Tables that are too long or too wide to fit on one page can be carried over to a facing page, but authors should try to avoid creating tables that span more than two pages. In general, very large tables should appear as supplements in the online version of the article only.
3. Tables should contain only three horizontal rules (lines)—one before the column headings, one after those headings, and one at the bottom of the table—and no vertical rules.
4. As a rule, captions should be detailed enough that the table can be understood apart from the text (if there is more than one table with the same general structure, only the first needs to have a detailed caption). Captions should be written so as to stress the purpose of the table and not merely list its contents in a mechanical way.
5. There should be only one set of column headings. If the information to be presented seems to require more than that, the table should be redesigned (e.g., by switching the rows and columns) or split into two or more tables.
6. Bold, centered headings may be used within the body of the table to distinguish different types of data as long as they do not conflict with the column headings.
7. Only the first letter of a row or column heading should be capitalized (along with words or symbols that would be capitalized in ordinary text).
8. The data within the body of the table should not be crowded; if need be, blank rows can be inserted to separate data into logical groups or provide guides for the eye.
9. Significant differences should be indicated by lowercase letters, beginning with the letter “z” (“z” may mark either the highest or the lowest value[s], but subsequent letters have to follow suit); in most cases, there should be no omissions in the sequence of the letters. The letters should be set on the same lines as the values to which they pertain (not as superscripts) and be separated from those values by single spaces.
10. Values less than 1.00 should be preceded by zeroes (e.g., 0.78).
11. Values need not be reported to all significant digits if a lesser number of digits conveys the information in a meaningful way.
12. Footnotes should be indicated by superscripted lowercase letters, beginning with the letter “a”; the letters may appear in the row and column headings as well as the body of the table but not in the caption. The footnotes per se should be listed on separate lines at the bottom of the table.

Figure captions.—Figure captions should appear with the figures themselves rather than (as formerly) in a separate list; however, the name of the corresponding author should still be given outside the image area of each figure for purposes of identification. Like table captions, figure captions should generally be detailed enough that the figure can be understood apart from the text. To the extent possible, however, panel descriptions, (full) variable names, units of measure, legends, and so forth should be included in the figure itself rather than in the caption; in no case should they be given in both places. Different panels may be designated “A,” “B,” and so forth, but it is preferable to give them substantive labels (e.g., “Treatment” and “Control”).

Figures.—Figures include visual materials such as graphs, maps, diagrams, and photographs. Figures have proved to be one of the most troublesome aspects of the publishing process. As the Journals Department has only limited ability to modify figures, they frequently have to be sent back to the authors for correction.

At the most fundamental level, figure design should follow certain commonsense principles: figures should be as simple and straightforward as possible; have a high enough resolution to be easily readable (300 dpi or more); and be consistent in the use of lettering, line widths, and other graphic elements. In addition, they need to conform to AFS style. It is particularly important to remember that most figures will be reduced by up to 50% when printed and thus need to be designed with this in mind. We recommend that authors use a copier to reduce each figure to the width of one or two printed columns (3.50 and 7.25 inches, respectively), depending on the dimensions of the particular figure, and verify that all elements are still legible. The following are particularly problematical: bold type (which tends to blur), italic type (which tends to become less visible), dashed lines (which tend to appear continuous) and dotted lines (which tend to disappear entirely). Additional guidelines for the preparation of figures may be found in the AFS style guide.

In the print version of the journal, all figures will be reproduced in black and white unless authors have made specific arrangements with the Journals Department to cover the extra costs of color printing. (In the online version, color figures will be reproduced in color at no additional charge.) Because color printing is expensive, authors are advised not to use color to distinguish phenomena when other means (different shading, symbols, and so forth) are adequate. If you have to use color in a figure, avoid using similar colors or shades that may be difficult for readers to distinguish. Also, in deference to readers with color blindness, avoid using red and green in the same figure.
Digital files in EPS, TIFF, and PSD formats are preferred; figures should be submitted as separate files rather than being imbedded in text files.

Mathematical and statistical expressions.—Chapter 4 of the AFS style guide covers the treatment of these expressions in detail, but a few general points may be mentioned here:

1. Symbols representing variables and parameters should be italicized if they consist of single letters in the Latin alphabet (e.g., \( K \) and \( F \)). All other symbols except Greek letters may be italicized or not, provided that the treatment is consistent (e.g., \( CPUE \) or \( CPUE \)). Greek letters should never be italicized.

2. Natural logarithms may be expressed as \( \log_e \) or \( \ln \); logarithms with other bases should identify the base (e.g., \( \log_{10} \)).

3. Long equations should be “broken” at logical points, normally after an operator such as a plus or minus sign.

4. Definitions of variables and parameters may be run into the text if only a few such terms are involved. If there are a number of them or they are used in more than one equation, a list is preferable (see section 4.8 of the style guide).

5. Avoid the expressions “the mean length was 45.2 \( \pm \) 3.84 mm” and “the mean (\( \pm \) SD) length was 45.2 \( \pm \) 3.84 mm” because they are at best awkward and at worst inaccurate. Use the expressions “the mean \( \pm \) SD length was 45.2 \( \pm \) 3.84 mm” or “the mean length was 45.2 mm (SD, 3.84)” instead.

Appendices and supplements.—In addition to the standard elements of a paper, authors may submit certain supplemental material, such as additional data or results, the derivations of equations, computer code, and so forth. For publication purposes, such material will be treated either as an appendix (which will appear with the article in both the print and online versions) or as a supplement (which will appear only in the online version). Of course, all material that is essential to understanding an article should be included in the article itself. Closely related material that will be of interest to a large number of readers may be placed in an appendix. Other material may be made available through a supplement if the editors deem it important enough for readers to have ready access to. In terms of format, appendices should be regarded as extensions of articles and thus follow AFS style strictly. Supplements, by contrast, may be in any format that is suitable for their contents; however, (1) there should be consistency between the symbols, abbreviations, and so forth used in the supplement and those used in the article and (2) either the title of the supplement or the first paragraph should make clear how it relates to the article.

Style and Format

Published articles represent the culmination of research efforts, often lengthy and highly sophisticated ones. To do those efforts justice, however, the articles must be well written; poorly written articles not only place an unnecessary burden on readers, they also cast doubt on the quality of the research itself.

Although some people naturally write better than others, most can develop the ability to write well through practice and attention to detail. The introduction to the AFS style guide should be a particularly valuable resource in this regard; in a few pages, it identifies the errors in composition mostly commonly encountered in the papers submitted to AFS journals and shows how to correct them. We also encourage authors to have other fisheries professionals critique their initial drafts with respect to presentation as well as substance. Authors whose native language is not English should make a point of having English speakers review their manuscripts before submission.

In writing for AFS journals, authors are also expected to follow certain style conventions pertaining to capitalization, spelling, punctuation, mathematical expressions, technical terms, and so forth. For instance, we require that the letter \( P \) (indicating the degree of statistical significance) be capitalized as well as italicized, whereas some journals require that it be lowercased. Although some of the more important style conventions are noted below, all of them are discussed in detail in the AFS style guide. Authors would be well advised to become familiar with the main elements of AFS style and to consult the guide frequently in preparing their manuscripts.

Resources for authors.—As suggested above, the principal resource on matters of style is the AFS style guide. Authors may also find it helpful to consult the Chicago Manual of Style (University of Chicago Press, Chicago) and Scientific Style and Format (Council of Science Editors, Chicago), though the AFS style guide always takes precedence.

The standard resource for word usage and spelling is Webster’s Third New International Dictionary, as updated by the latest edition of Merriam-Webster’s Collegiate Dictionary. Appendix A of the AFS style guide shows the proper way to spell many of the terms used in fisheries writing (some of which are not in the dictionary), including terms for which our preferred spelling differs from that in the dictionary.

The standard resource for the common and scientific names of North American fish species is the current edition of Common and Scientific Names of Fishes from the United States, Canada, and Mexico (American Fisheries Society, Bethesda, Maryland). For other aquatic species, authors should follow the companion publications World Fishes Important to North Americans and Common and Scientific Names of Aquatic Invertebrates from the United States and Canada (the volumes Mollusks, Decapod Crustaceans, and Cnidaria and Ctenophora are currently available in the latter series).

In most cases, scientific names should be included only at first mention in the abstract and text; full common names (e.g., “Coho Salmon” rather than simply “Coho”) should be used elsewhere. The format for the first mention is

Coho Salmon \textit{Oncorhynchus kisutch},
in which all parts of the common name are capitalized and the scientific name follows the common name but is not given in
2. All numerical values should be expressed in metric units.

In papers about population dynamics, we prefer the notation used by W. E. Ricker in *Computation and Interpretation of Biological Statistics of Fish Populations* (Fisheries Research Board of Canada Bulletin 191, 1975). However, all symbols should be defined anew in every paper. Our standard sources for chemical and enzyme names are the current editions of the *Merck Index* (Merck & Co., Rahway, New Jersey) and *Enzyme Nomenclature* (Academic Press, San Diego, California), respectively. The preferred treatment of allozymes is noted in the article “Gene Nomenclature for Protein-Coding Loci in Fish” by J. B. Shaklee et al. (*Transactions of the American Fisheries Society* 119:2–15, 1990). Additional information on the treatment of these and other technical matters may be found in chapter 11 of the AFS style guide.

**Manuscript format.**—As an aid to reviewers and editors, authors should

1. use a line spacing of at least space and a half for all components of the paper, including the title page, footnotes, and tables;
2. number all pages sequentially and provide continuous line numbering beginning with the title page;
3. use a 12-point font throughout;
4. use three levels of headings, as follows: for the major sections of the paper (Methods, Results, Discussion, Acknowledgments, and References), type them flush left with initial letters capitalized (except for prepositions and conjunctions) in ordinary type, preceded by “<A>” (e.g., <A>Methods); for subsections in Results and Discussion, type them flush left with initial letters capitalized in ordinary type preceded by “<B>” (e.g., <B>Treatment 1); and for subsections in Methods and sub-subsections in Results and Discussion, run them into the text with only the initial letter of the first word capitalized, all words italicized, and followed by a period and a long dash (e.g., Sampling design.—); and
5. turn off automatic hyphenation and justification.

**General style conventions.**—A detailed presentation of AFS style is beyond the scope of these guidelines. The following conventions, however, are so general as to apply to virtually every paper:

1. Only symbols and abbreviations included in Webster’s dictionaries or listed at the end of these guidelines (as well as at the back of each printed issue of the journal) may be used without definition. All others should be defined at first use (e.g., index of biotic integrity [IBI]). Abbreviations should not be introduced unless they are used at least two more times.
2. All numerical values should be expressed in metric units. The only exceptions are a few quantities that are typically expressed only one way (e.g., g [of medication]/lb [of feed]).
3. Single-digit numbers should be spelled out unless they are used with units of measure or in conjunction with larger values (e.g., 8 Walleyes and 16 Saugers). Numbers with four or more digits should contain commas; those less than 1.00 should be preceded by zeroes.
4. Ratios involving two values or units of measure should be indicated by forward slashes (e.g., 0.30 g/d); ratios involving three such terms should be indicated by negative exponents (e.g., 0.01 g·g⁻¹·d⁻¹).
5. Ages of fish should be expressed by Arabic numerals and not contain plus signs (e.g., a fish is age 1 [not age 1+] from the January 1 after it hatches to the following December 31).
6. Dates should be expressed as month–day–year (e.g., January 11, 2011). Note that the term “Julian day” does not mean day of the year and should not be used in that context.
7. Time should be expressed in terms of the 24-hour clock followed by the word “hours” (e.g., 1435 hours rather than 2:35 p.m.).

**Reference formats.**—Text citations should conform to the author–year system. Examples of common types are as follows:

- (Johnson 1995)
- (Johnson and Smith 1996)
- (Johnson et al. 1997, 1998) [three or more authors]
- (Johnson and Smith 1999, 2001; Smith 2000)
- (Johnson 2000a, 2000b)
- (Johnson, in press)
- (E. M. Johnson, National Marine Fisheries Service, personal communication)

Note that with one exception citations should be listed in chronological order; the exception is that all citations to the same author(s) should be grouped together (see the fourth example above).

In reference lists, references should be in strict alphabetical order by authors’ last names; if there are two or more references with the same authors, those references should then be listed chronologically. All authors must be named in references.

Detailed information on reference formats may be found in chapter 8 of the AFS style guide. The more common types are as follows:

**Articles in journals**


Note that (1) except for the first author, authors’ initials come before their last names; (2) only the first word of the title of the article is capitalized (along with any other words that would be capitalized in ordinary text); and (3) the name of the journal is given in full.

**Books**

Chapters in books


Government reports

Reports that are issued on a regular basis are treated much like articles in journals (the principal difference being that page numbers should not be given); other reports are treated like books:


Electronic publications

References to books and reports should be formatted in the usual way even if they are only available online (or are available in print form but were accessed online):


Universal resource locator (URL) addresses may be given in the text for established databases, computer code, and other items that should remain accessible over the long term and in the reference list for sources that would otherwise be difficult to locate.

If a journal is available in print form, authors should use the standard reference format even if they accessed the article online. If a journal is only available electronically, the format depends on the way(s) in which articles are designated. Two possible formats are as follows:


Note that digital object identifiers (DOIs) should only be included for articles still in press.