Pathogenicity of *Diplostomum* cercariae in rainbow trout, and alternative measures to prevent diplostomosis in fish farms

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Abstract
The pathogenicity of *Diplostomum* cercariae shed from two different snail species (*Lymnaea stagnalis* and *Radix auricularia*) to rainbow trout fry (5-6 cm body length) was investigated. Thus, 1000 cercariae per fish elicited 100 % mortality within 24 h. Cercariae shed from *L. stagnalis* (*Diplostomum pseudospathaceum*) elicited higher mortality in rainbow trout compared to cercariae (*Diplostomum paracaudum*) from *R. auricularia*. Furthermore, cercariae from *R. auricularia* were more successful in terms of migrating and establishing as metacercariae in the lenses of rainbow trout. Alternative measures to prevent diplostomosis in fish farms were subsequently studied.

*Diplostomum* cercariae could be eliminated by mechanical filtration methods. Filters with 32 micrometer mesh size eliminated 99 % while 160 and 200 micrometer meshes removed approximately 50 % of the cercariae. Treatment of water with sodium percarbonate (20 mg/L or higher) killed all infective larvae. Therefore, filtration and sodium percarbonate treatment of fishpond water are suggested as sustainable methods for control of eye-fluke populations in fish farms.

Introduction
Diplostomid metacercariae (eye-flukes) are frequently found in the eye lenses of fish in fresh and brackish waters (Chappell et al., 1994). These parasites use fish as their second intermediate host, and have been reported from more than 125 species of freshwater fish world-wide (Skrijabin, 1964; Sweeting, 1974). *Diplostomum* spp. cercariae are released from many different snail species (the first intermediate host), but usually host specificity is high and a particular diplostomid limited to one or two related snail species. *D. spathaceum* exploits *Radix balthica* (syn. *Lymnaea ovata* and *L. peregra* (Glöer, 2002)), and *R. auricularia* (syn. *Lymnaea auricularia* (Glöer, 2002)) as their first intermediate hosts (Niewiadomska and Kiseline, 1994), whereas *D. pseudospathaceum* develops in *L. stagnalis* and *S. palustris*. Finally, *D. paracaudum* use *R. balthica*, *R. auricularia*, and in rare cases *S. palustris*, as their first intermediate host (Niewiadomska and Kiseline, 1994). Fish become infected when cercariae invade through the skin and migrate to the eyes where they often elicit parasitic cataract (Betterson, 1974; Shariff et al., 1980). Heavily infected fish become easy prey for birds, and growth is reduced even when food is abundant (Crowden and Broom, 1980; Owen et al., 1993; Buchmann and Uldal, 1994). Thus, chronic infections with

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diplostomids are of wide economic importance to freshwater fish producers (Whyte et al., 1988). However, information on the pathogenic effect of cercarial penetration into fish has been limited. Therefore, the present experiments have been conducted in order to elucidate the pathogenicity of the penetrating cercariae. In addition, possible alternative ways for the fish farmers to prevent outbreaks of diplostomosis were evaluated. Previous studies have shown that treatment with praziquantel supplemented feed or in water baths affects the metacercariae (Bylund and Sumari, 1981; Székely and Molnár, 1991). In addition, increase of water current velocity will reduce infections (Field and Irwin, 1994). The present study deals with the mechanical removal (filtration) of Diplostomum cercariae, and treatment of cercaria contaminated water with formalin and sodium percarbonate.

Methods and materials
Snails and cercariae
Two infection experiments were carried out with Diplostomum cercariae shed from two different snail species L. stagnalis and R. auricularia, respectively. Due to limitation of infected snails the Diplostomum cercariae used in the filtration experiment were taken from L. stagnalis whereas studies on the effect of sodium percarbonate and formalin on Diplostomum cercariae were conducted with cercariae shed from R. auricularia. The L. stagnalis snails were collected at a fish farm in the Northwestern part of Denmark in August 2003, and the R. auricularia snails were collected at Lake Furesø on Zealand, Denmark, in September 2003. The snails were brought to the laboratory, placed in separate beakers with de-chlorinated water and monitored for cercarial shedding at room temperature as described by Lyholt and Buchmann (1996). For identification purposes, a sample of the cercariae was fixed in buffered neutral formalin (4%) or glutar-aldehyde (2.5 %). The cercariae fixed in formaldehyde were later stained using Alcian Blue and Meyer’s haematoxylin, and mounted on slides with glycerol gelatine. The cercariae were characterised by using light microscopy and SEM (Scanning Electron Microscopy). Measurements in μm of five cercariae were taken from each snail species using a compound microscope (Olympus CH-2), and means were calculated. For SEM cercariae were post-fixed in tannic acid, critical point dried, sputtered with gold/palladium and examined in a JEOL JSM840 SEM.

Fish
Rainbow trout Oncorhynchus mykiss (Walbaum, 1792) were obtained from a pathogen free re-circulated fish culture system. The mean body length of the rainbow trout exposed to Diplostomum cercariae from L. stagnalis was 52.83 mm (SD 4.86 mm), and the mean body weight was 1.434 g (SD 0.513 g). The mean body length of the rainbow trout exposed to Diplostomum cercariae from R. auricularia was 57.92 mm (SD 5.94 mm), and the mean body weight was 1.981 g (SD 0.760 g).

Filters
Cylindrical filters (diameter 100 mm, height 40 mm) were delivered from Retsch GmbH and Co. KG (Haan, Germany) with the following mesh sizes (in micrometers): 32, 80, 112, 160, 200, 300 and 500.
Infection procedure

Two infection experiments comprising a total of 50 fish were performed. Four groups of rainbow trout with five fish in each group were exposed to cercariae from *L. stagnalis* and correspondingly four groups were exposed to cercariae from *R. auricularia*. Individual fish in each of the four groups were either placed in a suspension of 100, 300, 600, and 1000 cercariae in 250 ml water (in a 500 ml glass beaker), respectively. Control groups were treated likewise except for exposure to cercariae. The mortality of the fish in each group was then recorded every six hours for 24 hours. After this period the surviving fish from each group were transferred to 10 litre glass aquaria containing 3 litres of water, with daily water exchange. After 30 days the fish were killed with an overdose of MS 222 (3-aminobenzoic acid ethyl ester) (Sigma A-5040). The fish were dissected and the numbers of metacercariae in their eyes were recorded. All experiments were conducted in a thermostat controlled room at 11-12 °C, and a light/darkness interval of 12 hours.

Filtration procedure

The *Diplostomum* cercariae were shed naturally from the snail into a 200 ml glass beaker with water. Then, 100 cercariae were randomly taken out using a pipette. They were released into 250 ml de-chlorinated tap water and poured through the filters. However, before the cercariae were poured through the filter, it was checked (using Olympus stereomicroscope, 7–40x magnifications) that they were intact. Subsequently, the filter was rinsed with 300 ml de-chlorinated water and examined counting the number of cercariae using a stereomicroscope.

Formalin and sodium percarbonate exposure

Solutions of formalin and sodium percarbonate (\(\text{Na}_2\text{CO}_3\cdot3\text{H}_2\text{O}_2\)) in seven different concentrations each (10, 20, 40, 60, 80, and 100 mg/l) were tested. The cercariae in the control group were placed in de-chlorinated tap water. A total of 130 cercariae were used. Each concentration (including the control group) was tested on 10 cercariae.

<table>
<thead>
<tr>
<th><strong>Diplostomum cercariae from</strong></th>
<th><strong>Length (min. - max.)</strong></th>
<th><strong>Mean</strong></th>
<th><strong>Width (min. - max.)</strong></th>
<th><strong>Mean</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body of cercariae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. auricularia</em></td>
<td>133 - 155</td>
<td>146.0</td>
<td>60 - 63</td>
<td>61.5</td>
</tr>
<tr>
<td><em>L. stagnalis</em></td>
<td>163 - 178</td>
<td>170.5</td>
<td>50 - 63</td>
<td>57.5</td>
</tr>
<tr>
<td><strong>Tail stem</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. auricularia</em></td>
<td>253 - 260</td>
<td>256.0</td>
<td>28 - 35</td>
<td>32.5</td>
</tr>
<tr>
<td><em>L. stagnalis</em></td>
<td>228 - 238</td>
<td>233.0</td>
<td>28 - 40</td>
<td>35.5</td>
</tr>
<tr>
<td><strong>Furcae</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td><em>R. auricularia</em></td>
<td>275 - 288</td>
<td>281.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. stagnalis</em></td>
<td>255 - 263</td>
<td>259.5</td>
<td></td>
<td></td>
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</tbody>
</table>

Table 1. Morphometrics of *Diplostomum* cercariae from the two species of snails used in the experiments. All measurements in micrometer. (N=5 in each group).
Newly released cercariae were randomly taken out using a pipette and placed in separate wells on multiwell plates (NUNC Brand Products, cat. no. 143982) in a small drop of water. Then the solutions of formalin or sodium percarbonate were added (2.5 ml in each well). The cercariae were examined after 2, 5, 10, 16, 22, 28, and 34 hours. Death was defined by lack of response to touch, or when the tail of the cercaria was shed.

Data processing
Kaplan-Meier Survival Analyses (Log Rank Survival Test) were conducted using Sigma Stat 3.0 to elucidate the effect of the exposure of cercariae on the mortality of rainbow trout compared to the control group. The same test was used in order to determine the effect of the different concentrations of sodium percarbonate and formalin on survival time of cercariae compared to the control group. In all experiments differences were considered significant when p<0.05.

Results
Cercarial morphology
The morphometrics of Diplostomum cercariae from R. auricularia (Figures 1 and 2) and Diplostomum cercariae from L. stagnalis (Figures 3 and 4) are listed in Table 1. Neither of the two cercariae possesses fin-folds, and their acetabulum is situated at mid-length of body. Furthermore, they possess pre-oral spines and sensillae as well as post-oral spines distributed in ten concentric rows reaching

Figure 1. SEM of Diplostomum paracaudum cercaria (whole larva) from Radix auricularia. Scale bar 100 micrometer.

Figure 2. SEM of Diplostomum paracaudum (forebody). Scale bar 10 micrometer.
the acetabulum, which also possess spines, apparently arranged in two rows. The posterior body is covered with smaller dispersed spines. When resting in water the cercariae exhibited their distended furcae at an angle of about 180°, and tail stem bent at an angle of about 90°.

Infection experiment
All rainbow trout in the control groups and fish exposed to 100 cercariae survived for 24 hours. The mortality in the two groups with 300 cercariae differed. In the first experiment (Diplostomum cercariae from L. stagnalis) 3 out of 5 fish died during the first 24 hours, whereas only 1 out of 5 fish died in the second experiment (Diplostomum cercariae from R. auricularia). However, neither of the two groups had a significantly higher mortality than the control group. In the experiment carried out with 600 cercariae from L. stagnalis per fish all fish died (p<0.05 compared to the control group), whereas only 1 fish died (after 18 hours) in the group with the same number of cercariae from R. auricularia per fish (statistically insignificant compared to the control group). Finally, all the fish exposed to 1000 Diplostomum cercariae from L. stagnalis died within 6 hours, whereas the fish exposed to 1000 Diplostomum cercariae from R. auricularia died within 18 hours. Both groups had a significantly higher mortality compared to the fish in the control group. The dissection of the surviving fish showed that

Figure 3. SEM of Diplostomum pseudospathaceum (whole larva) from Lymnaea stagnalis. Scale bar 100 micrometer.

Figure 4. SEM of Diplostomum pseudospathaceum (forebody). Scale bar 100 micrometer.
46% of the *Diplostomum* cercariae from *L. stagnalis* migrated to the fish eye and transformed to the metacercarial stage, and 79% of the *Diplostomum* cercariae from *R. auricularia* were recovered as metacercariae in the host lenses.

**Filtration experiment**
Approximately 50% of the cercariae were filtered off when using a filter with a mesh size of either 160 or 200 micrometer. None of the filters were able to completely remove all cercariae. The best result was achieved with the 32 micrometer mesh size filter in which only one cercaria out of 100 passed through.

**Effects of sodium percarbonate and formalin**
The cercariae in the formalin solutions generally died faster compared to the cercariae in the sodium percarbonate solutions. Three of the cercariae in the control group were still alive, when the experiment was ended after 34 hours. Within 16 hours all the cercariae in the formalin solutions were dead, whereas 34 hours were needed to kill all cercariae in sodium percarbonate (20 mg/L). All concentrations (except sodium percarbonate 10 mg/L) of the two compounds tested caused a significantly shorter survival time of the cercariae compared to the cercariae in the control group.

**Discussion**

**Infection experiment**
*Diplostomum* cercariae from *L. stagnalis* elicited higher mortality than cercariae from *R. auricularia*. It has been suggested by Niewiadomska and Kiseliene (1994) that different *Diplostomum* species are specifically associated with corresponding snail species. According to the morphology, morphometrics, cercariae resting position, and snail host species of the cercariae used in this study they could represent the two closely related species, *Diplostomum pseudospathaceum* Niewiadomska, 1984 and *Diplostomum paracaudum* (Iles, 1959), respectively. Chaetotaxy, a technique showing characteristic distribution of tegumental sensillae, in order to classify the cercariae was omitted because there are no differences between *D. pseudospathaceum* and *D. paracaudum* (Niewiadomska and Kiseliene, 1994). Furthermore, the results indicate that *Diplostomum* cercariae from *R. auricularia* are more successful in terms of migrating and establishing as metacercariae in the lenses of rainbow trout. It may be speculated that penetrating *Diplostomum* cercariae from *L. stagnalis* are more pathogenic to rainbow trout due to erratic migration in the host compared to *Diplostomum* cercariae from *R. auricularia*.

**Filtration experiment and the effect of sodium percarbonate and formalin**
Diplostomosis is normally prevented by keeping fish-eating birds away from the pond area, or by reducing snail populations in ponds, increasing the water flow (Field and Irwin, 1994), or treating infected fish with praziquantel (Drontit) (Bylund and Sumari, 1981; Székely and Molnár, 1991). In this study two alternative measures were evaluated. We found a clear correlation between the filter mesh size and the number of cercariae passed through. At the smallest mesh size (32 micrometer) almost all cercariae are removed (99%) but even filters with 200 micrometer mesh removed 50 % of the cercariae.
Microscreening with various mesh sizes is commonly used in fish farms removing particles from the water, and their use for prevention of diplostomosis should be further tested. In addition, the effect of an alternative and environmentally friendly compound (sodium percarbonate) was tested on the Diplostomum cercariae. The environmental impact of sodium percarbonate is low. When dissolved in water, hydrogen peroxide is released and the end products are oxygen, sodium, carbonate, and water. Furthermore, sodium percarbonate has previously been shown effective in eradicating ectoparasites. In vitro experiments with sodium percarbonate showed that the compound was effective in eradicating theronts of Ichthyophthirius multifilis Fouquet, 1876 (Buchmann et al., 2003), and in vivo experiments showed that sodium percarbonate in a concentration of 80 mg/L (bath treatments) eradicated Gyrodactylus derjavini Mikailov, 1975, on rainbow trout (Buchmann and Kristensson, 2003). The present results show that even small concentrations of formalin and sodium percarbonate significantly shorten the life span of Diplostomum cercariae. Within 16 hours all cercariae in the solution with 10 mg formalin per litre (or higher) died. The concentration of sodium percarbonate needs to be higher (minimum 40 mg/L) in order to eradicate the parasites within the same period. However, sodium percarbonate has some advantages compared to formalin. The compound is granulate, it is easy to handle, and when dissolved in water it releases hydrogen peroxide over a longer period (Buchmann and Kristensson, 2003). The results suggest that sodium percarbonate (20 mg/L or higher) could be a suitable replacement for formalin treatment of Diplostomum cercariae in fish farms. In conclusion our results indicate that filtration and sodium percarbonate treatment of fishpond water may be used as sustainable method to control eye-fluke infections in fish farms.

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