Elevated Water Temperature Treatment of Ayu Infected with *Glugea plecoglossi*: Apparent Lack of Involvement of Antibody in the Effect of the Treatment

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The possibility of antibody-mediated effect of elevated water temperature treatment of *Glugea*-infected ayu was investigated by challenge test and ELISA. The treatment effectively reduced the prevalence and intensity of infection. In naturally infected fish, the prevalence of infection was significantly decreased by elevated water temperature treatment. The treatment was more effective in naturally infected fish treated thrice than those treated twice, as demonstrated by the smaller number of cysts in the former group of fish than in the latter. The antibody levels were also significantly increased by the treatment in naturally infected fish. However, in the experiment using uninfected fish, no significant increase in the antibody levels by the treatment was seen, though the prevalence of infection was significantly decreased by the treatment. Through the challenge test, it was clear that the effect was temporary, and protective immunity was not confirmed by the treatment. These results suggest that the effect may not be antibody-mediated. Thus it is recommended that the elevated water temperature treatment be repeated and applied with continuous monitoring of reinfection.

**Key words:** Microsporidia, *Glugea plecoglossi*, ayu, *Plecoglossus altivelis*, treatment, ELISA, antibody

Microsporidia are obligate intracellular parasites which infect a wide range of animals and their development, in certain hosts, depends on the environmental temperature (Lom and Dyková, 1992). *Microsporidium takedai* stopped development when infected fish were kept at 8.2–8.4°C (Awakura, 1974), and *Glugea stephani* required a temperature of above 15°C for its development (Olson, 1981). Development of *Glugea plecoglossi* also seemed to be temperature-dependent, cysts failing to appear below 16°C (Takahashi and Egusa, 1977). However, this temperature effect was only to arrest or delay the development of the parasites, but not to kill the parasites. So, when the infected fish was returned to its optimal maintenance temperature, the parasite resumed normal growth (Olson, 1981; Antonio and Hedrick, 1995).

Infection with *Glugea plecoglossi* is thought to be endemic in ayu from Lake Biwa (Takahashi, 1980). However, the prevalence of infection falls to around zero during summer, as the surface water temperature becomes elevated above 25°C. Takahashi and Ogawa (1997) suggested the possibility of treating *Glugea*-infected fish by elevating the water temperature, and reported that most cysts disappeared by raising the water temperature to 29°C for 5 days twice with an interval of 1 week. However, the mechanism of this treatment remains unknown. The present study was conducted to examine whether this treatment is effective against reinfection and whether the effect of this treatment owes to antibody production using challenge test and ELISA.

**Materials and methods**

*Fish*

A stock of cultured ayu (n = 277; average body weight: 10.1 g) raised at Shiga Prefectural Fisheries Experimental Station were maintained at a temperature of about 20°C for Experiments 1 and 3. For Experiment 1, *Glugea* cysts were seen with the naked eye on 5 of 25 randomly selected fish and thus the initial prevalence of infection was judged to be 20%. For Experiment 3, 30 fish were examined as above and the prevalence of infection was considered to be 30%.

For Experiment 2, *Glugea*-free ayu weighing about 3.0 g (n = 375) were obtained from Yamanashi Prefectural Fisheries Technology Center and subsequently
maintained at the Fisheries Laboratory of the University of Tokyo, Shizuoka Prefecture, at a temperature of about 20°C.

Parasites

Glugea plecoglossi spores were obtained from naturally infected ayu and purified as described in a previous paper (Kim et al., 1996). For Experiments 2 and 3, spores were used immediately after purification. For ELISA, spores were purified and stored at -20°C until required.

Experimental design and serum collection

In Experiment 1, a group of fish were treated by gradually raising the water temperature to 29°C. Then after 5 days treatment, the water temperature was returned to 20°C. After 7 days, the same treatment was repeated (duplicated treatment group; T2). In another group of fish, the same treatment was performed a total of 3 times (triplicated treatment group; T3). The control group did not receive any treatment. Forty days after the last treatment, all fish were anesthetized by 2-phenoxyethanol and blood was collected for ELISA. They were dissected to investigate the prevalence of infection.

In Experiment 2, after confirming the absence of cysts, three groups of fish were given live G. plecoglossi spores orally with food (9.2×10⁶ spores/fish) for 3 consecutive days. After 7 days, the same treatment as in Experiment 1 was conducted once (single treatment group; t1) or twice (t2). The control group did not receive any treatment. Fish were anesthetized and blood was collected. They were dissected 40 days after the last treatment.

In Experiment 3, after conducting the elevated temperature treatment twice on two groups of fish, one group was challenged with live G. plecoglossi spores given perorally (1.8×10⁷ spores/fish) with food for 3 days as described above (elevated temperature-treated and challenged group; TC), while the other group was reared until the end of the experiment without a subsequent challenge (TX). Forty days after the challenge, all fish were anesthetized and dissected to investigate the prevalence of infection and blood was collected for ELISA. Control groups (untreated and unchallenged group; XX) did not receive any treatment.

ELISA

ELISA was conducted and antibody units were calculated from the means of the optical density as described previously (Kim et al., 1996).

Statistics

Data from Experiments 1–3 were analysed by the chi-square test and Student’s t-test. Tests were considered significant at probability values of P < 0.05.

Results

Experiment 1

The prevalence of infection in elevated temperature treated groups was significantly lower than that in the control group (P < 0.05, chi-square test). The prevalence in the latter group increased when compared with that observed before the commencement of the experiment. The difference in prevalence of infection between T2 and T3 was not significant (Table 1). However, the intensity of infection did appear different between groups T2 and T3; all fish having 0–1 cyst in T3, while some fish had more than 2 cysts in T2 (Fig. 1).

The antibody level was increased significantly in the treated groups, and the difference between T2 and T3 was also significant (P < 0.05, Student’s t-test) (Fig. 2).

Table 1. Effect of multiple elevated water temperature treatments on ayu naturally infected with Glugea plecoglossi

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of fish*¹</th>
<th>BW*² (g)</th>
<th>PI*² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>21.5</td>
<td>76.0</td>
</tr>
<tr>
<td>T2</td>
<td>29</td>
<td>26.0</td>
<td>27.6*</td>
</tr>
<tr>
<td>T3</td>
<td>28</td>
<td>29.9</td>
<td>10.7*</td>
</tr>
</tbody>
</table>

*¹: Number of fish examined at the end of experiment
*²: Body weight at the end of the experiment
*²: The prevalence of infection before dividing into each group was 25.0%.
**: Significantly different from control (P < 0.05, chi-square test)

Abbreviations: PI (prevalence of infection); BW (body weight); T2 (duplicated treatment group); T3 (triplicated treatment group)
Antibody uninvolvement in Glugea treatment

Experiment 3

The prevalence of infection was decreased significantly by the elevated temperature treatment (TX in comparison with XX, \( P < 0.05 \), chi-square test). The prevalence of infection in TC was not increased significantly compared to TX (Table 3). On the other hand, heavily infected fish with more than 10 cysts appeared after the challenge test (Fig. 3). The antibody level was not significantly different between any groups; 10.09 ± 6.00 for XX, 13.86 ± 2.17 for TX and 8.56 ± 3.55 for TC.

Discussion

Although the lower limit of environmental temperature for microsporidian growth has been investigated by some authors as mentioned in the introduction, no information is available on the upper limit. In the case of Glugea...
hertwigi infecting freshwater smelts *Osmerus eperlanus mordax*, the prevalence of infection was highest during late summer to early winter when water temperatures were around 20°C (Delisle, 1972). *Glugea stephani* showed a similar seasonal prevalence of infection to the above in winter flounder *Pseudopleuronectes americanus* (Takvorian and Cali, 1984). Kalavati et al. (1985) suggested that the optimal temperature for microsporidians to infect fish might be around 20°C, based on Glugea plecoglossi infections have also been seen to exhibit seasonal fluctuations, with heavily infected fish appearing most frequently when the water temperature is higher than 20°C (Takahashi, 1981).

The development of piscine microsporidians is influenced by temperature, and the accelerated growth was observed when water temperatures were high (Olson, 1976; Takahashi and Egusa, 1977). However, if a microsporidian is subjected to a higher than physiologically relevant temperature for its survival, detrimental effects may occur. Nakajima and Egusa (1975) reported that the rate of polar tube discharge of *G. plecoglossi* was decreased by heating the spore suspension and that all the spores died at 52°C. *Glugea malabaricii* infection of *Carangoides malabaricus* was not observed in summer, when the surface water temperature was around 30°C (Kalavati et al., 1985). This coincides well with the water temperature effects on *G. plecoglossi* infection in ayu recorded in the current study. Some authors have reported a detrimental effect of high temperature on insect microsporidians as well (Wilson, 1979; Jouvenaz and Lofgren, 1984). However, the direct effects of high temperature on fish microsporidians are still not well-characterized.

Mature spores within the xenoma in fish tissues are finally phagocytized by macrophages (Dykova and Lom, 1980), and it was suggested that spores might be presented as antigens to antigen-presenting cells (Kim et al., 1987).

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. of fish</th>
<th>BW (g)</th>
<th>PI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XX</td>
<td>59</td>
<td>11.8</td>
<td>72.9</td>
</tr>
<tr>
<td>TX</td>
<td>35</td>
<td>21.7</td>
<td>25.7+</td>
</tr>
<tr>
<td>TC</td>
<td>50</td>
<td>18.0</td>
<td>48.0</td>
</tr>
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</table>

*a*: Number of fish examined at the end of experiment  
*b*: Body weight at the end of the experiment  
*c*: The prevalence of infection before the start of experiment was 30.0%.  
+ : Significantly different from XX (*P* < 0.05, chi-square test), all other comparisons of PI were not statistically significant.

Abbreviations: XX (untreated and unchallenged group); TX (treated and unchallenged group); TC (treated and challenged group)

Other abbreviations are as in Table 1.

**Fig. 3.** Frequency distribution of the number of *Glugea* cysts in naturally infected ayu after elevated water temperature treatment and subsequent challenge test. Abbreviations are given in Table 3.
Plecoglossi was almost the same among artificially infected fish, while in Experiment 2, there was no significant increase in the antibody level of the treated fish. In the case of Experiment 1, using naturally infected fish, the time of invasion of the parasite was not clear, nor was the development of the parasites synchronous in individual fish. Moreover, naturally infected fish had possibly been exposed to spores before initiation of the experiment. In Experiment 2, the developmental stage of G. plecoglossi was almost the same among artificially infected fish, but the time interval between spore administration and elevated temperature treatment was different from that of Experiment 1. These differences probably produced the different antibody levels between the two groups.

Unfortunately, it was not possible to conduct the challenge test after treatment of initially Glugea-free fish because of an accidental loss of experimental fish. Consequently, Experiment 3 was carried out using naturally infected fish. Although no statistically significant increase both in the antibody level and in the prevalence of infection was seen in response to the challenge, heavily infected fish with more than 10 cysts appeared again in the challenged fish. These results suggest that the effect of elevated temperature treatment is temporary and that the parasite-specific antibodies are not protective against reinfection. Miyakawa (1980) conducted elevated water temperature treatments and continuous monitoring. In Experiment 3, using naturally infected fish, the development of the parasites synchronous in individual fish. Although the time of invasion of the parasite was not clear, the presence of morphologically abnormal spores in the infected muscle tissue following treatment. However, he also recovered live spores from the treated fish and suggested the possibility of a relapse of infection. Antibody-mediated protective immunity to teleost microsporidians has yet to be reported.

Hence it is thought that ayu can be subjected to reinfection when they come into contact with G. plecoglossi again after the treatment. Although efficacy of the treatment depended largely on the developmental stage of the parasite present at the time of its usage (Takahashi and Ogawa, 1997), the treatment of cultured ayu with visible Glugea-cysts was quite effective (Table 1). Thus, it is suggested that the elevated water temperature treatment against Glugea infection may be successfully utilised at ayu farms which incorporate repeated treatments and continuous monitoring.

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