Artificial Infection by Water Molds Following Net-shake Treatment in the Platyfish (Xiphophorus maculates)

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Abstract: Artificial infection with the fungi Saprolegnia diclina BKKU 0506 and Achlya bisexualis BKKU 0616 was induced in the platyfish (Xiphophorus maculates) via exposure to zoospores following artificial injury by scale removal and net-shake or “ami-momi” treatment. The results suggested that ami-momi treatment, but not scale removal, could be used to establish severe fungal infection in small freshwater tropical fish as well as salmonids.

Key words: Platyfish; Achlya bisexualis; Saprolegnia diclina; Pathogenicity

Ever since the work of Tiffney in 1939, the platyfish (Xiphophorus maculates) has been used in artificial infection tests with water molds. In these experiments, the fish are exposed to fungal zoospores following artificial injury by scale removal (SR) at a temperature of 15°C, which inhibits bacterial growth in the injured regions (Vishniac and Nigrelli 1957; Scott and O’Bier 1962). The platyfish is an ideal experimental animal for these tests as it can tolerate a sudden drop of water temperature, and its health status is relatively easy to maintain in the laboratory. The present study tested the pathogenicity of the fungi Saprolegnia diclina and Achlya bisexualis, which were isolated from cultured Nile tilapia (Oreochromis niloticus) eggs, in platyfish after either SR (Tiffney 1939) or net-shake (“ami-momi” or AM) treatment (Hatai and Hoshiai 1994; Hussein et al. 2007). The fungi were cultured on glucose yeast extract (GY) agar (Hatai and Egusa 1979) at 25°C and were sub-cultured every 2 – 4 weeks.

All of the S. diclina and A. bisexualis strains used in the present study were isolated from cultured Nile tilapia eggs from northeastern Thailand. The S. diclina BKKU 0506 strain was isolated from the Khon Kaen Inland Fisheries Station, Khon Kaen Province, in June 2005, and was identified using the classification of Seymour (1970) (Panchai et al. personal communication)**. The following three strains of A. bisexualis were used: BKKU 0504 isolated from the Khon Kaen Inland Fisheries Station, Khon Kaen Province, in June 2005; BKKU 0612 isolated from a private fish farm, Kalasin Province, in March 2006; and BKKU 0616 isolated from Sakon Nakhon Inland Fisheries Research and Development Centre, Sakon Nakhon Province, in March 2006. The classification of Johnson (1956) was used to identify all of the A. bisexualis strains (Panchai et al. 2007). The fungi were cultured on glucose yeast extract (GY) agar (Hatai and Egusa 1979) at 15°C and were sub-cultured every 2 – 4 weeks.

The actively growing edge of the fungal colony of each strain on the GY agar was cut with a number 2 cork borer (5.5 mm in diameter) and cultured in GY broth (Hatai and Egusa 1979) at 25°C for 24 – 48 h. The growing hyphae were then cut with a pair of sterile scissors and washed three times with sterile tap water (STW) before being transferred to 20 ml STW and incubated at 25°C for 12 – 20 h to induce zoospore production. Zoospore sporulation of each strain was observed under an inverted microscope (Nikon Phase Contrast-2 ELWD 0.3; Nikon, Japan). The number of zoospores was counted using a hemacytometer (improved Neubauer; Erma, Tokyo), and diluted with 1 ml STW to obtain the following three concentrations: 1 × 10^7 spores/ml (low), 1 × 10^8 spores/ml (medium) and 1 × 10^9 spores/ml (high).

Healthy platyfish were purchased from a tropical fish distributor in Tokyo, Japan. They were transported to the Laboratory of Fish Diseases at the Nippon Veterinary and Life Science University in Tokyo, where they were acclimatized to laboratory conditions over 3 – 4 days. The fish had a standard body length of 3 – 4 cm. Approximately 100 fish were kept in an...
aerated aquarium filled with dechlorinated tap water at 25°C. The fish were fed ad libitum commercial pellets (Tetramin; Tetra, German) once a day. Feeding was stopped 1 day before the artificial infection test began.

For experiment I (transmission by SR), several scales posterior to the left pectoral fin were removed in order to induce fungal infection. This experiment consisted of four groups: a control group (five intact fish and five SR fish) without exposure to zoospores; test group 1 (three intact fish and three SR fish) exposed to a low concentration; test group 2 (five intact fish and five SR fish) exposed to a medium concentration; and test group 3 (three intact fish and three SR fish) exposed to a high concentration. The fungal strains used were S. diclina BKKU 0506, and A. bisexualis BKKU 0504, BKKU 0612 and BKKU 0616.

In test groups 1–3, the intact and SR fish were separately immersed in spore suspensions in small glass tanks that were aerated and maintained at 15°C for 24 h. After challenge with the fungal strains for 24 h (1 day after inoculation), the fish were moved to separate, larger glass rearing tanks with 10 l STW that were aerated and maintained at the same temperature, and were reared for 7 days. The fish in the control group were initially immersed in 1 l STW without zoospores in small glass tanks that were aerated and maintained at 15°C for 24 h, and then moved to larger glass tanks with 10 l STW and reared at 15°C for 7 days. A 2-l sample of the rearing water from each large tank was removed every 2 days, and an equal amount of STW was added. The experiment was performed in a room equipped with an air conditioner to maintain the water temperature at 15°C. This was intended to inhibit bacterial contamination of the region injured by SR, based on temperature at 15°C equipped with an air conditioner to maintain the water temperature at 15°C for 24 h.

The test groups were immersed in small glass tanks with the respective spore suspensions, and were aerated and maintained at 15°C for 24 h. The control group was immersed in a small glass tank with 1 l STW that was aerated and maintained at 15°C for 24 h. All of the fish were then moved to separate, larger glass tanks with 10 l STW that were aerated and maintained at 15°C for 7 days. All groups were maintained under the conditions described for experiment I, and the moribund and dead fish were sampled as described above.

Fungal re-isolation from the moribund and freshly dead fish with fungal lesions in experiments I and II was performed by inoculating a small piece of the trunk muscle of each fish onto GY agar. To inhibit bacterial growth, 500 μg/ml penicillin G and 500 μg/ml streptomycin sulphate (both from Meiji Seika Kaisha, Co. Ltd., Tokyo, Japan) were added to the medium. Fungal colonies were subcultured on GY agar to obtain pure cultures. The identification of the isolate was made on hemp-seed cultures in STW at 25°C (Johnson 1956). Using light microscopy, the hemp-seed cultures were examined to detect the zoosporangia morphology, secondary zoospore cyst ornamentation, mode of cyst germination and oogonia, according to the classifications of Johnson (1956) and Seymour (1970).

After re-isolation, the moribund and freshly dead fish, with or without fungal lesions, were fixed whole by immersion in 10% phosphate buffered formalin for 24 h. For the fish with fungal lesions, the areas of skin covered by fungal mycelia were cut into small pieces. Sections of skin without fungal infection were also collected from the dorso-lateral regions around the dorsal fins and cut into small pieces. All of the skin samples were decalcified with ethylenediaminetetraacetic acid for 24 h, embedded in paraffin wax and sectioned at 3–5 μm intervals. These sections were stained with methenamine silver-nitrate Grocott’s variation, and counter stained with haematoxylin and eosin (Grocott–H&E), in order to detect fungal elements.

Table 1 shows the numbers of moribund and dead fish with fungal lesions, as well as the survivors, in each group of experiment I. There was no evidence of infection or mortality during the 7-day experimental period in test group 1 upon challenge with S. diclina and the three strains of A. bisexualis. By contrast, one of the five intact fish in test group 2 died 1 day after exposure to A. bisexualis BKKU 0616, without showing any gross symptoms of fungal infection. One of the three injured fish in test group 3 challenged with A. bisexualis BKKU 0616 died 3 days after exposure, and bore hard, rough, puffy and whitish-coloured mycelia on the left side of its body where the scales had been removed (Fig. 1). The
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The fungus re-isolated from the dead fish was identified as *A. bisexualis* according to the classification of Johnson (1956).

The numbers of moribund and dead fish with fungal lesions, as well as the survivors, in experiment II are summarized in Table 2. No mortality, macroscopic evidence of fungal infection or apparent external lesion by physical damage of AM treatment was observed in the control group during the 7-day experimental period. At 2 days after exposure, one of the three fish from each of the test groups challenged with *S. diclina* BKKU 0506 and *A. bisexualis* BKKU 0504, BKKU 0612, and BKKU 0616 became moribund and exhibited external mycelial growth that was visible to the naked eye. In the test group exposed to *S. diclina* BKKU 0506, one of remaining two fish died 3 days after exposure, without gross symptoms of fungal infection, whereas the remaining fish in the same group died 5 days after exposure and presented with mycelial growth on the body surface. The mycelial growth observed in the test groups that were exposed to *A. bisexualis* BKKU 0504 and BKKU 0612 tended to be minor in comparison with those in the other groups, and disappeared over time. In these two test groups, one of the two remaining fish died without exhibiting the characteristic external signs of mycelial growth on their bodies, 3 and 7 days after exposure, respectively. In the test group exposed to *A. bisexualis* BKKU 0616, both of the remaining fish died 4 days after being challenged. Their symptoms included white, cotton-like mycelia on various parts of the body surface, especially on the flanks, dorsal fin, pelvic fins and caudal fins (Fig. 2). The fungus re-isolated from the dead fish in the test group exposed to *S. diclina* BKKU 0506 was identified as *S. diclina* according to the classification of Seymour (1970). The re-isolated fungus from the dead fish in the test group exposed to *A. bisexualis* BKKU 0616 was identified as *A. bisexualis* according to the classification of Johnson (1956). Insufficient isolate for species identification was obtained from the dead fish in the other groups.

<table>
<thead>
<tr>
<th>Table 1. Mortality following SR treatment</th>
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<tr>
<td><strong>Zoospore concentration (spores/ml)</strong></td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>intact</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>Challenged with</td>
</tr>
<tr>
<td><em>S. diclina</em> BKKU 0506</td>
</tr>
<tr>
<td><em>A. bisexualis</em> BKKU 0504</td>
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<tr>
<td><em>A. bisexualis</em> BKKU 0612</td>
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<tr>
<td><em>A. bisexualis</em> BKKU 0616</td>
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</table>

These fish challenged with *S. diclina* and three strains of *A. bisexualis* zoospores at various concentrations after SR treatment.

Data represent the number of dead and moribund fish/ total number of fish.

* This fish died 1 day after inoculation without fungal growth.

** These moribund and dead fish exhibited fungal growth on the body surface.

<table>
<thead>
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<th>Table 2. Mortality and fungal growth following ami-momi treatment</th>
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<tr>
<td>Total No. of fish</td>
</tr>
<tr>
<td>No. of dead and moribund fish</td>
</tr>
<tr>
<td>No. of surviving fish</td>
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These fish were challenged with *S. diclina* and three strains of *A. bisexualis* at a high zoospore concentration (1 x 10^6 spores/ml) after "ami-momi" treatment.
Histopathological examination of the cutaneous lesions of the moribund and freshly dead fish infected with *S. diclina* and *A. bisexualis* in experiments I and II revealed well developed hyphal growth on the body surfaces. The epidermis and stratum spongiosum had sloughed off, leaving the degenerated stratum compactum. Fungal hyphae had penetrated the deeper underlying tissues, such as the subcutaneous tissue and trunk muscle layer. Numerous hyphae penetrating into the muscle layer had caused severe floccular degeneration of the skeletal muscle fibres, associated with abundant cellular debris and secondary bacterial infection (Fig. 3).

The fish that had died without gross symptoms of fungal infection in experiment II (two of which died 3 and 7 days after exposure to *A. bisexualis* BKKU 0504 and *A. bisexualis* BKKU 0612) did not exhibit fungal elements in the cutaneous tissues; however, the epidermis had partly sloughed off and a few inflammatory cells, mainly lymphocytes, were observed in the dermal layer. The skin region of fish from experiment I that died 1 day after exposure to *A. bisexualis* BKKU 0616 (at $1 \times 10^5$ spores/ml) presented with normal appearance without any fungal elements.

The gross and histopathological features of the moribund and freshly dead fish with fungal infection observed in the present study were similar to those reported previously in other fish species infected with *Saprolegnia* and/or *Achlya* (Hatai and Hoshiai 1994; Kitancharoen et al. 1995; Grandes et al. 2001; Hussein and Hatai 2002). The histopathological features indicated

**Fig. 2.** This fish died 4 days after exposure to *A. bisexualis* BKKU 0616 following challenged by AM treatment in experiment II. The symptoms included white, cotton-like mycelia on various parts of the body surface.

**Fig. 3.** Histopathological features of a cutaneous lesions in a fish exposed to *A. bisexualis* BKKU 0616 at $1 \times 10^5$ spores/ml in experiment II. Numerous hyphae penetrating into the muscle layer caused severe floccular degeneration of the skeletal muscle fibres (arrows) associated with abundant cellular debris and secondary bacterial infection (*`). Grocott-H&E, Bar=30 μm.
that the fungal infection could destroy the integument rapidly, inducing a marked acid-base imbalance and a deficiency of osmoregulation (Pickering and Willoughby 1982) that resulted in mortality. However, the results of experiment I indicated that SR treatment did not strongly predispose the fish to develop severe fungal infection with the isolates tested. By contrast, the AM treatment was shown to induce severe fungal infection in small freshwater tropical fish species, as well as in salmonids (Hatai and Hoshiai 1994; Hussein and Hatai 2002), although another detailed study with more platyfish should be required. The findings suggested that the fungal isolates tested in the present study were pathogenic to platyfish, and that AM treatment would perform better than SR treatment in determining the pathogenicity of water molds isolated from the eggs of tropical freshwater fish. We were unable to confirm why two of the fish died without fungal infection in experiment II; however, it is possible that additional factors in the artificial infection test, such as the inclusion of novel pathogenic elements in the spore suspensions, might have been responsible for mortality in the absence of fungal infection. The death of a fish in experiment I at 1 day after exposure to *A. bisexualis* BKKU 0616 (1 × 10^4 spores/ml) was thought likely to be due to the physical stress associated with handling and SR treatment.

**References**


