RED SPOT DISEASE OF SEA MULLET *(Mugil cephalus)*

FINAL REPORT TO FRDC

Project No. 89/81

RICHARD B. CALLINAN

NSW FISHERIES

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BACKGROUND

Results of a previous FIRTA-funded project (86/53) indicated that red spot disease (RSD), a cutaneous ulcerative disease of estuarine fish, is caused by a number of interacting factors. The study showed that lower catchment rainfall is an important determinant of RSD outbreak occurrence. The study also showed that dissolved oxygen concentrations at representative estuarine sites fell to very low levels within 7-10 days of such rain events. Concentrations remained low for a further 8-10 days, as flood waters receded. These findings indicate that rapid and severe changes in one or more water quality parameters, including dissolved oxygen concentrations, pH, salinity and temperature, may be important in initiating RSD outbreaks.

Another key finding of the study was that RSD ulcers are a consequence of massive invasion of skin and underlying muscle by morphologically similar, non-septate fungal hyphae. These invasive hyphae cause extensive tissue destruction and provoke a characteristic granulomatous inflammatory response.

There was no evidence that bacteria, cutaneous ectoparasites or viruses were essential in induction of lesions.

The present project was designed to identify water quality parameters important in induction of RSD lesions, and to identify invasive fungi associated with ulcers .

OBJECTIVES

Project objectives were:

- Carry out field and laboratory trials designed to reproduce RSD in sea mullet.
- Isolate and identify pathogenic fungi associated with ulcers in naturally infected fish.

RESULTS

Disease reproduction trials

Field trials

Field trials were conducted in two stages:

Low rainfall period

In March 1989, juvenile mullet were placed in cages in the Richmond River at 6 representative mainstream and tributary sites; two of the latter were drains receiving groundwater and/or run-off from acid sulphate soil areas. Water quality parameters were measured daily (dissolved oxygen, pH, temperature, conductivity) or twice weekly (turbidity, non-filterable residue, volatile suspended solids, total phosphorus, filterable reactive phosphorous, total Kjeldahl nitrogen, ammonia-nitrogen, nitrates/nitrites, chlorophyll a and selected ions). Fish were examined daily for evidence of disease.

All fish at the 2 acid sulphate soil drainage sites died within 2 days of the commencement of the experiment; death was attributed to low pH (< 4.6) and low dissolved oxygen concentrations (< 3.8 ppm) at the sites. The experiment was discontinued after 3 weeks. Water quality at the other sites remained within acceptable limits and there was no evidence of RSD in fish held at these sites.

High rainfall period

A major rainfall event on the lower Richmond River catchment occurred in the first week of April 1989. Within 7 days of this event, juvenile mullet were placed in cages at 8 representative sites (including the 6 sites used above). Water quality and RSD prevalence were monitored as above. All fish caged at the 2 acid sulphate soil drains, a non-acid sulphate soil drain, and a mainstream site downstream of these, died within 5 days of placement of the cages. Deaths were attributed to low dissolved oxygen concentrations (< 1.5 ppm). Fish at one mainstream site were exposed to low dissolved oxygen concentrations (2.1-2.5 ppm), but showed no evidence of RSD. Water quality paramaters at all other sites remained within acceptable limits and fish at these sites also failed to show evidence of RSD. The experiment was discontinued after 5 weeks.

Laboratory trial

An experiment was conducted to determine whether sub-lethal exposure to low concentrations of dissolved oxygen (1.5-2.5 mg/l), together with exposure to RSD-related Aphanomyces spores (100 spores/ml) was sufficient to induce RSD lesions.

Juvenile mullet were held in aquaria and subjected to the following treatments:

- low dissolved oxygen concentrations
- low dissolved oxygen concentrations, fungal spores
- fungal spores
- untreated control

Spores were added from days 1-10; dissolved oxygen concentrations were maintained at low levels on days 3-6.

No fish developed lesions consistent with RSD.

Isolation and identification of pathogenic fungi

Broad, non-septate, fungal hyphae are a constant finding in the necrotising dermatitis lesions and dermal ulcers characteristic of RSD. During a one year period, fungi with hyphae morphologically consistent with those seen in histological sections of lesions were recovered from 27 out of 28 RSD lesions on sea mullet, *Mugil cephalus*, yellowfin bream, *Acanthopagrus australis*, and sand whiting, *Sillago ciliata*, collected from the Clarence and Richmond Rivers in NSW and from Saltwater Creek in north Queensland. Cultural and morphological characteristics, including spore formation, of 24 of these isolates were examined. In addition, tolerances to different salinities, growth on different media and growth at different temperatures were studied for 3 representative isolates, one for each species of fish. These studies suggested that all isolates belonged to a single species within the genus *Aphanomyces*. The presence of the *Aphanomyces* isolates in the 3 species of fish, the high isolation rate, the absence of other oomycete fungi, and their recovery from widely separated localities at different times, strongly suggests they are responsible for the fungal granulomas seen in RSD. Results are presented in full in Fraser, Callinan and Calder (1992); see Appendix.

CONCLUSIONS

The above study was highly successful in that it identified the putative causative infectious agent of RSD, an *Aphanomyces* sp. This finding has proven to be a major breakthrough in understanding the aetiology and pathogenesis of RSD in Australia and of the identical condition, epizootic ulcerative syndrome (EUS), in Asia (see below).

Attempts to induce RSD in field and laboratory trials were unsuccessful. In both situations, a possible reason was failure of sufficient infectious propagules to contact the exposed fish. In the field trials, cages were positioned at the water surface, to minimise deaths due to hypoxia. Subsequent studies have suggested that motile zoospores, the probable infectious stage, are concentrated at the bottom of the water column, at the sediment/water interface. Consequently, the caged fish probably encountered few zoospores. In the laboratory trials zoospores added to aquaria may have become encysted, ie. inactivated, by agitation during the addition procedure. No effective count technique was then available to detect this change.

The findings of FIRTA Projects 86/53 and 89/81 have provided a critical springboard for further studies of RSD and EUS.

- A 3-year study of possible relationships between RSD outbreaks, run-off from acid sulphate soils and aphanomycetes has been funded by Land and Water Resources Research and Development Corporation (Project UNS12: Avoidance of soil and estuary degradation by better drainage management).
- A major 3-year collaborative project, funded by Australian Centre for International Research and involving scientists from Australia, Indonesia and the Philippines, was begun in 1993 (Project 9130: Improving fish production in freshwater aquaculture and in estuaries by reducing losses due to epizootic ulcerative syndrome). One of the objectives of this project is to more clearly identify the causes of RSD in estuarine fish in Australia. Comparative mycological and pathological studies, based on data obtained under the FRDC-funded studies, have already been conducted (see Appendix), and further studies are in progress. A successful outcome should allow identification of management strategies to minimise the impact of outbreaks on the fisheries.

These projects, and the potential benefits arising from them, would not have been possible had the above FRDC-funded studies not been conducted.

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Aphanomyces species associated with red spot disease: an ulcerative disease of estuarine fish from eastern Australia

G. C. FRASER, R. B. CALLINAN & L. M. CALDER *New South Wales Agriculture & Fisheries, Regional Veterinary Laboratory, Wollongbar Agricultural Institute, Wollongbar, Australia*

Abstract. Broad, non-septate, fungal hyphae are a constant finding in the necrotising dermatitis lesions and dermal ulcers characteristic of red spot disease. During a one-year period, fungi with hyphae morphologically consistent with those seen in histological sections of lesions were recovered from 27 out of 28 lesions on sea mullet, *Mugil cephalus* L., yellowfin bream, *Acanthopagrus australis* (Owen), and sand whiting, *Sillago ciliata* Cuvier, collected from three widely separated river systems in eastern Australia. Cultural and morphological characteristics, including spore formation, of 24 of these isolates were examined. In addition, tolerances to different salinities, growth on different media and growth at different temperatures were studied for three representative isolates, one from each species of fish. These studies suggest that all isolates belonged to a single species within the genus *Aphanomyces*. Their role in the pathogenesis of red spot disease, and their relationship with other *Aphanomyces* spp. affecting fish, is discussed.

Introduction

Red spot disease (RSD) is an ulcerative skin disease affecting estuarine fish in New South Wales (Callinan, Fraser & Virgona 1989) and Queensland (McKenzie & Hall 1976; Rodgers & Burke 1981). A similar, probably identical, disease affects estuarine fish in the Northern Territory (Humphrey & Langdon 1986; Pearce 1990) and Western Australia (D. A. Pass, unpublished data).

Although the cause of RSD remains unresolved, outbreaks typically occur after prolonged heavy rainfall in lower river catchments, and large numbers of fungal hyphae are present in early lesions on fish captured in the second week after these rain events (Callinan *et al.* 1989).

In this paper, the authors report the isolation of oomycete fungi, consistent with *Aphanomyces* spp., from early RSD lesions. Morphology and some growth characteristics of these isolates are described.

Materials and methods

Sea mullet, *Mugil cephalus* L., yellowfin bream, *Acanthopagrus australis* (Owen), and sand whiting, *Sillago ciliata* Cuvier, with skin lesions consistent with RSD were captured during May and June 1989 at three sites in the lower Richmond River, north-eastern New South Wales. Water conductivity measured during four of these six collections indicated salinities varied between 2 and 9‰. RSD-affected bream were collected at a single site in the lower Clarence River, north eastern New South Wales in June 1989; in May 1990, affected mullet

Correspondence: G. C. Fraser, Regional Veterinary Laboratory, Wollongbar Agricultural Institute, Wollongbar, NSW 2477, Australia.

were collected at a single site in Saltwater Creek, northern Queensland. Fish were transported alive to the laboratory in river water.

Isolation and maintenance of fungi

Fish were killed by decapitation, and those with necrotising dermatitis lesions (Callinan *et al.* 1989) with minimal ulceration were selected for further examination. The fish were pinned to a dissecting board and scales around the lesion were carefully removed. The skin around the lesion was then seared with a spatula and the fish and board removed to a Class II microbiological safety cabinet containing filtered air free of fungal elements. The necrotic skin was then reflected by incising through the seared area so that the underlying muscle was exposed. Fungal isolation was attempted only when the muscle appeared yellow and soft. Using aseptic technique, up to four pieces of damaged muscle, approximately $2 \times 2 \times 2$ mm, were removed and transferred to a single Petri dish containing Czapek Dox agar (Gibco Ltd, Paisley, Scotland) with penicillin G (100 units ml^{-1}) and streptomycin sulphate (100 $\mu g\,ml^{-1}$). The medium was inoculated within 24 h of preparation and the surface was not dried before use.

Inoculated plates were sealed with adhesive tape and incubated at room temperature (approximately 15–24°C) under normal laboratory lighting. Plates were examined daily for 10 days. Emerging hyphal tips were repeatedly transferred to fresh plates of Czapek Dox agar (CDA) or Czapek Yeast Autolysate agar (CYAA) (Smith & Onions 1983) until cultures were free of bacterial contamination. For each of these primary cultures, small blocks of agar, which had been invaded by fungus, were removed after 7–10 days incubation and placed in 20 ml tubes of sporulating medium (SM) consisting of 0·25 mM $CaCl_2$ and 0·25 mM KCl in distilled water (Griffin 1978, cited by Dykstra, Noga, Levine, Moye & Hawkins 1986). Two sterilized half hemp seeds were added to each tube. Tubes were maintained under natural light at room temperature and colonies were examined for sporulation using phase contrast microscopy with an inverted microscope after one and two days incubation. Approximately 0·5 ml of SM containing secondary zoospores was spread on CYAA medium and, after 1–2 days incubation, a representative colony of each isolate was subcultured onto another plate of the same medium. Such cultures were considered axenic. Primary cultures and axenic cultures were maintained on slopes of CYAA medium under oil (Smith & Onions 1983).

Three axenic cultures, one from a bream from the Clarence River and one each from a whiting and a mullet from the Richmond River were selected for studies of growth on different media, growth and sporulation at different salt concentrations, and growth at different temperatures.

Growth studies

In order to standardize the size of inoculum, mycelial mats were produced using a modification of the method described by Dykstra *et al.* (1986). A small inoculum from a 7–10-day-old colony of each of the three axenic isolates on CYAA was transferred into a 250 ml flask containing 30 ml of GY broth, consisting of 5 g glucose, 2·5 g yeast extract in 1 l distilled water (Griffin 1978, cited by Dykstra *et al.* 1986). The flask was shaken gently at one oscillation per second for 6–7 days at room temperature under normal laboratory lighting. The resultant mycelial mats were individually washed three times in sterile distilled water before use.

A single washed mat was applied to the centre of each plate of medium, the plate sealed with tape and then incubated in the dark at 22°C, except as indicated below. For each isolate,

each test treatment was replicated five times. None of the media contained antibiotics, and plates that became contaminated with fungi or bacteria were discarded.

The six media tested for ability to support growth were CDA, CYAA, agarized GY medium (GYA) described by Dykstra *et al.* (1986), cornmeal agar (CMA) supplemented with 2% glucose and 1% yeast extract (Dykstra *et al.* 1986), glucose yeast extract agar (GYEA) described by Willoughby & Pickering (1977) and fish meat extract agar (FMEA) (Hatai, Egusa, Takahashi & Ooe 1977), modified by the substitution of sea mullet flesh. After the final measurement, 7 days after inoculation, portions of each isolate were scraped from the edge of one colony on each medium, resuspended in water and coverslipped. The mean hyphal diameter of 10 randomly selected hyphae was calculated from measurements made with the aid of a calibrated graticule at ×400 magnification.

Ability to grow at different NaCl concentrations was tested on GYA at NaCl concentrations of 0, 2, 4, 8, 12, 16 and 20‰ over 7 days. The effect of temperature on growth was tested on GYA at 3, 13, 22, 25, 31 and 37°C over 6 days.

Colonies were measured immediately after they had been placed on test media and at daily intervals thereafter. The arithmetic mean diameter of each colony was calculated from its largest diameter and a diameter bisecting it at right angles. Growth, determined by change in mean colony diameter, was tested for significance by analysis of variance. For salt and temperature studies, growth over the initial 24-h period and subsequent growth were analysed separately for significant differences from zero. Mean growth for each treatment was weighted inversely according to the variance of that treatment. Differences were considered significant when $P < 0{\cdot}05$.

Effect of salinity on sporulation

Single washed mycelial mats of each of the three axenic isolates were placed in two tubes containing 20 ml of either water, SM or SM supplemented with NaCl at 2, 4, 8, 12, 16 and 20‰. Two sterilized hemp seed halves were added to each tube. Tubes were incubated at room temperature under natural light and examined for sporulation and motile secondary zoospores twice daily for 4 days.

Results

Twenty-eight lesions on 27 fish, comprising 19 sea mullet (17 from the Richmond River and two from Saltwater Creek), five yellowfin bream (three from the Richmond River and two from the Clarence River) and three sand whiting (Richmond River), were selected for fungal culture. One mullet from the Richmond River had two lesions, both of which were cultured.

Fungal isolation

Fungi with hyphae morphologically consistent with those seen in histological sections of RSD lesions were recovered from 27 of 28 lesions cultured; the single exception being one lesion on a sea mullet from the Richmond River. At ×70 magnification, broad, thick-walled hyphae were first noticed 15 h after inoculation, emerging from putative granulomas in the muscle. Developing colonies were flat and transparent with individual fungal hyphae clearly visible to the naked eye, particularly at the edge of the colony. After several days incubation, colonies became slightly opaque and had an unevenly white velvety surface. At no stage were aerial hyphae apparent. All these primary cultures were similar in morphology and growth rate.

Sporulation

Two primary cultures from New South Wales fish were lost through bacterial contamination before spore production was induced, and one of the two primary cultures from Queensland fish was not examined further. Each of the 24 remaining primary cultures, and the 24 axenic cultures derived from them, produced both terminal and intercalary sporangia within 2 days of incubation in SM. Impending sporulation was indicated by increased density of refractile granules in hyphal segments separated from remaining vegetative hyphae by thin septa. Subsequently, a single row of primary spores developed in these sporangia. In intercalary sporangia, primary spores discharged through lateral evacuation tubes (Fig. 1), whereas in terminal sporangia, they discharged through lateral evacuation tubes, the hyphal tip or both. A thin strand of cytoplasm connected the primary spores (Fig. 2) as they moved through the sporangium to an orifice, where typically, 30–50 spores encysted in a roughly spherical cluster. Motile secondary zoospores were seen within 12 h of sporangial development. The secondary zoospores were subspherical and biflagellate. No sexual reproductive structures were seen.

Sporulation occurred only in water, SM and SM with 2‰ NaCl. Few motile secondary zoospores were observed at 2‰ NaCl.

Growth on various media

All media supported growth of the three axenic isolates tested and there were significant interactions between isolates and media. However, maximum growth for all isolates occurred on GYA or GYEA (Table 1). Colonies on GYA and GYEA were more opaque and slightly more velvety than those on other media. For each medium tested, the mean hyphal diameter of the three isolates did not differ significantly. Mean hyphal diameters ranged between 9 μm on GYEA and 16 μm on FMEA.

Effect of temperature on growth

At 24 h, small increases in mean colony diameter were observed at all temperatures. Subsequently, no growth occurred at 3 or 37°C. There were significant differences between isolates

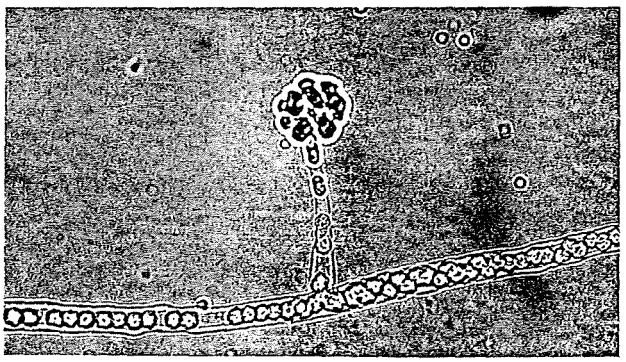

Figure 1. Primary spores discharging through a lateral evacuation tube of a sporangium. Discharged primary spores have encysted in a spherical cluster (×225).

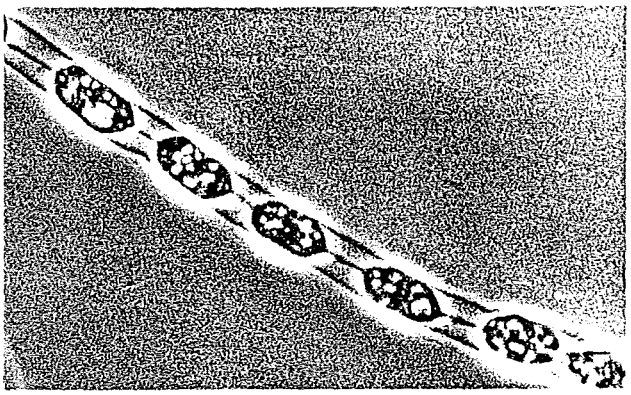

Figure 2. A single row of primary spores, linked by thin strands of cytoplasm, within a sporangium (×350).

Table 1. Influence of culture medium on growth of three representative fungal isolates

	Mean colony growth after 7 days (mm)*		
Medium	Mullet isolate	Whiting isolate	Bream isolate
GYEA	48(1)	47(1)	44(1)
GYA	45(1)	47(1)	43(1)
CMA	27(1)	24(1)	29(1)
CDA	21(3)	34(3)	27(3)
FMEA	25(2)	20(1)	23(1)
CYAA	12(3)	21(3)	23(3)

* Standard error of mean in brackets.

in their subsequent growth at the other temperatures; maximum growth for the bream and mullet isolates occurred at 31°C, and for the whiting isolate at both 22°C and 25°C (Fig. 3).

Effect of salt concentration on growth

At all salt concentrations, small changes in mean colony diameter of the three isolates occurred after 24 h. Further growth declined progressively as salt concentration increased, and no isolate made further growth on media containing 12, 16 or 20‰ NaCl. The mullet isolate grew significantly better than both the other isolates at 8‰ NaCl and better than the bream isolate at 4‰ NaCl (Fig. 4).

Discussion

In previous studies, *Saprolegnia* spp., *Pythium* spp., and various imperfect fungi were recovered from ulcerative lesions of fish with RSD (G. C. Fraser, R. B. Callinan & L. M. Calder, unpublished data). These isolates were subsequently dismissed as contaminants or secondary invaders because of their low recovery rate or because they did not resemble the

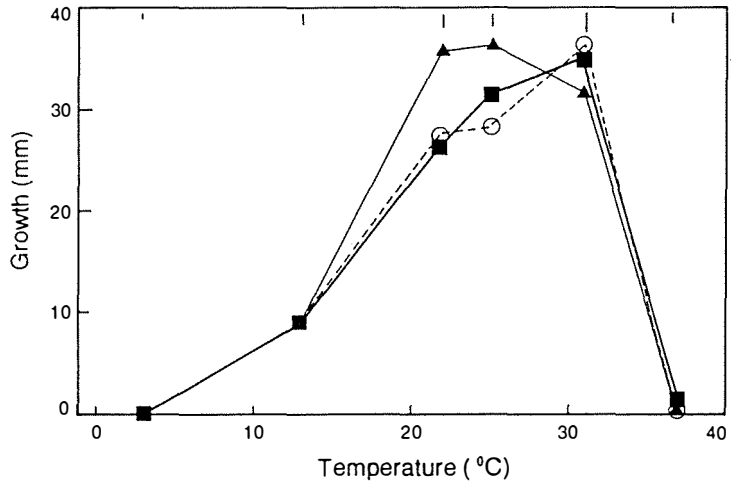

Figure 3. The effect of temperature on growth of *Aphanomyces* isolates from yellowfin bream (■), sand whiting (▲) and sea mullet (○). Growth occurring between 1 and 6 days of incubation was measured. Vertical bars represent the largest standard error of the mean at each temperature.

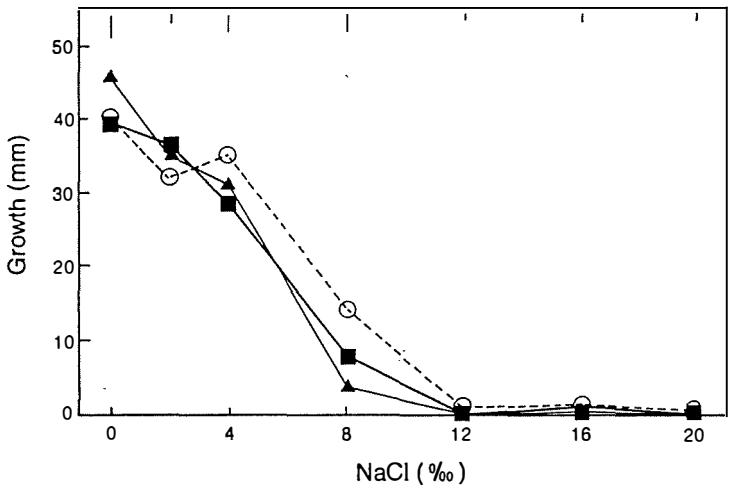

Figure 4. The effect of salinity on growth of three *Aphanomyces* isolates. Growth occurring between 1 and 7 days of incubation at 22°C was measured. Symbols as in Fig. 3.

apparently viable fungi seen in crush preparations of these lesions. In this study, the authors recovered fungi typical of *Aphanomyces* spp. from 27 out of 28 lesions examined. No other oomycete fungi were recovered.

The present authors attributed the high isolation rate of *Aphanomyces* spp. in this study to several factors. The authors cultured only damaged muscle underlying early necrotising dermatitis lesions, since only at this stage of lesion development were apparently viable hyphae containing motile organelles consistently present in crush preparations of muscle. In addition, granuloma formation which might inhibit fungal growth was minimal at this stage. Bacteria

are known to reduce isolation rates and growth of *Aphanomyces* spp. (Dykstra, Levine, Noga, Hawkins, Gerdes, Hargis, Grier & Te Strake 1989). The present authors reduced the risk of bacterial contamination by selecting early lesions on freshly killed fish, by aseptically removing the skin overlying the damaged muscle, and by including antibiotics in the culture medium. Finally, although the present studies show that the three axenic isolates grew more slowly on CDA than on some other media used in previous isolation attempts (G. C. Fraser, R. B. Callinan & L. M. Calder, unpublished data), CDA appears to be the most suitable medium for isolation of fungi associated with RSD. The present authors frequently observed water of syneresis around pieces of inoculum on the plates and this might allow the relatively slow growing hyphae to reach the medium before desiccation of the inoculum occurs.

Most *Aphanomyces* spp. are parasites or saprophytes on plants or freshwater crustaceans; there are few reports of infections of fish (Scott 1961). Shanar & Saslow (1944) described *Aphanomyces* sp. infection of the skin and muscle of several freshwater fish species maintained in an aquarium. In Japan, *A. piscicida* was isolated from mycotic granulomatosis affecting wild and cultured freshwater fishes (Hatai *et al.* 1977; Hatai, Takahashi & Egusa 1984). In the south-eastern United States, Dykstra *et al.* (1989) associated one or more *Aphanomyces* sp. with an ulcerative mycosis affecting several estuarine fish species, including the commercially important Atlantic menhaden, *Brevoortia tyrannus* (Latrobe).

Ulcerative mycosis and mycotic granulomatosis have many pathological similarities to RSD (Noga, Levine, Dykstra & Hawkins 1988; Miyazaki & Egusa 1973). The present authors' isolates appear distinct from an isolate described from ulcerative mycosis by Dykstra *et al.* (1986). Compared with their isolate, the three axenic isolates the present authors studied, while showing similar temperature-growth relationships, have slower growth rates, fail to grow at salinities greater than 8‰, and lack salt requirements for optimum growth. In addition, Dykstra *et al.* (1989) suggested this menhaden isolate resembled *A. laevis*, which has a simple, filamentous sporangium (Scott 1961); all the present authors' isolates have a complex sporangium. Further, in contrast to the menhaden isolate, these isolates fail to sporulate at salinities greater than 2‰. However, the temperature-growth and salinity-growth relationships of the present authors' isolates are similar to those of an *A. piscicida* isolate recovered from a lesion of mycotic granulomatosis on an ayu, *Plecoglossus altivelis* Temminck & Schlegel, (Hatai & Egusa 1978).

Environmental studies in the Richmond and Clarence rivers have shown that salinities, even in the lower reaches, approach zero following prolonged heavy rainfall (R. B. Callinan, G. C. Fraser, J. L. Virgona, E. A. Scribner & R. Lea, unpublished data). At these times, salinities in much of the river would be suitable for growth and sporulation of the *Aphanomyces* isolates described in the present study. In susceptible fish species, rapid changes in water quality following these rain events may initiate changes to the skin which allow attachment of fungal spores and subsequent invasion of underlying tissue (Callinan *et al.* 1989). In the current study, fungi were recovered from ulcerated fish collected in water with salinities of 2–9‰. The three isolates the present authors studied, however, sporulated poorly in water of salinity 2‰ and not at all in water of salinity 4‰ or higher. Some fish may be infected in fresh water and then move to brackish water where the fungus would be expected to continue to grow in the damaged tissue. However, Hearth & Padgett (1990) have demonstrated that spores of an *Aphanomyces* isolate from ulcerated menhaden will germinate in sea water in the presence of exogenous nutrients. If the present isolates share this property, then the fish studied here may have been infected at the capture site by spores originating in fresh water. This mechanism could explain some infections of fish such as yellowfin bream and sand whiting. These species

are rarely found in fresh water under normal conditions (R. West, personal communication).

The identification of *Aphanomyces* spp. relies on the morphology of oogonia and antheridia (Scott 1961); until the present isolates can be induced to undergo sexual reproduction it is difficult to determine their relationships with established species, or whether more than one species is involved. However, the consistent hyphal and zoosporangial morphology, and the generally consistent growth and sporulation characteristics, suggest these isolates constitute a single species within the genus *Aphanomyces*. Further, the presence of the *Aphanomyces* isolates in three species of fish, the high isolation rate, the absence of other oomycete fungi, and their recovery from widely separated localities at different times, strongly suggest they are responsible for the fungal granulomas seen in RSD of fish from eastern Australia. Further studies are in progress to define relationships between these isolates, to determine their geographic distribution, and to identify the conditions under which they are pathogenic.

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Aphanomyces species associated with epizootic ulcerative syndrome (EUS) in the Philippines and red spot disease (RSD) in Australia: preliminary comparative studies

R.B. Callinan[1], J.O. Paclibare[2], M.G.Bondad-Reantaso[2], J.C. Chin[3] and R.P. Gogolewski[3]

[1] NSW Fisheries, Regional Veterinary Laboratory, Wollongbar, NSW Australia

[2] Fish Health Section, Bureau of Fisheries and Aquatic Resources, Quezon City, Metro Manila, Philippines.

[3] NSW Agriculture, Elizabeth Macarthur Agricultural Institute, Camden, NSW, Australia.

ABSTRACT: Fungi morphologically consistent with class Oomycetes were recovered on primary culture from 20 of 22 ulcers on 21 fish with epizootic ulcerative syndrome (EUS) collected from 5 sites in the Philippines. Eleven primary isolates, and the unifungal cultures derived from them, were identified as Aphanomyces spp.; the remaining 9 primary isolates were lost through contaminant overgrowth. The Aphanomyces isolates were morphologically indistinguishable from those recovered from red spot disease (RSD) in Australia. Comparison of 4 representative Australian Aphanomyces isolates from RSD lesions with 3 representative Philippines Aphanomyces isolates from EUS lesions, using SDS-PAGE, revealed very similar polypeptide banding profiles, indicative of a single Aphanomyces species. These findings, combined with epidemiological and pathological similarities between EUS and RSD, suggest the two conditions are identical, and that a single Aphanomyces sp. may be the primary infectious cause.

Since 1980, severe periodic outbreaks of epizootic ulcerative syndrome (EUS) have affected wild and cultured freshwater fishes, as well as wild estuarine fishes, in many countries of South and Southeast Asia (ABD/NACA, 1991). Typically affected fish have one or more large dermal ulcers with varying degrees of destruction of underlying tissues; mortality rates are often high. In the Philippines, periodic outbreaks of EUS have occurred since 1985; many fish genera, including Mugil, Arius and Scatophagus, are affected (Llobrera and Gacutan 1987, Reantaso 1991).

In eastern and northern Australia, periodic outbreaks of a similar syndrome, known colloquially as 'red spot disease' (RSD), have occurred since 1972 in wild freshwater and estuarine fish (McKenzie and Hall 1976, Rodgers and Burke 1981, Pearce 1990). Affected fish genera include those listed above for the Philippines.

The patterns of spread of both EUS and RSD suggest involvement of one or more primary infectious agents (Rodgers and Burke 1981, Roberts et al. 1990). Although a number of viruses or bacteria have been recovered inconsistently from fish with EUS (Frerichs et al. 1986, Hedrick et al. 1986, Roberts et al. 1986, Llobrera and Gacutan 1987, Roberts et al. 1990, Torres et al. 1990) and RSD (Burke and Rodgers 1981, Callinan and Keep 1989, Pearce 1990), no aetiological agent has been conclusively identified for either condition.

Numerous invasive fungal hyphae, morphologically consistent with fungi of the classes Oomycetes or Zygomycetes, are present in histological sections of advanced ulcers from EUS-affected fish (Tonguthai 1985, Bondad-Reantaso et al. 1990, Roberts et al. 1990) and in early and advanced ulcers on RSD-affected fish (McKenzie and Hall 1972, Callinan et al. 1989, Pearce 1990). Severe necrotising granulomatous dermatitis and myositis are associated with the invasive hyphae. While it is possible that these fungi are opportunists which have invaded EUS ulcers initiated by other infectious agents, the similar fungal morphology and host tissue responses in numerous species of fish with EUS and RSD suggests the fungi may be primary infectious agents in both conditions. Several studies have attempted to identify them. Achlya spp. have been recovered form EUS-affected fish in Thailand (Pichyankura and Bodhalimik 1983) and Sri Lanka (Subasinghe et al. 1990). However, they may have been contaminants; Achlya spp. occur commonly in freshwater in Southeast Asia (I. Dogma, personal

communication) and, in our experience, the culture methods used in these studies are likely to result in a high rate of recovery of contaminant fungi. By contrast, Fraser et al. (1992), using methods which minimise contamination of inocula, isolated an apparently single Aphanomyces sp., an oomycete fungus, from 27 of 28 RSD lesions on 3 species of estuarine fish from widely separated river systems in eastern Australia. These isolates were morphologically and culturally very similar, and the authors suggested the putative Aphanomyces sp. was the cause of the typical RSD granulomas.

In November and December 1991, EUS-affected fish were collected from 4 widely separated freshwater sites and one coastal, fresh-to-brackish water site on Luzon Island, the Philippines; they were then transported live, in local water, to a laboratory. Twenty one fish (14 striped snakehead Ophicephalus striatus, 3 mullet Mugil sp., 2 bar-eyed goby Glossogobius giurus, 1 walking catfish Clarias batrachus and 1 three spot gourami Trichogaster trichopterus) with typical early to advanced dermal ulcers were killed, and muscle tissue from 22 lesions on these fish was cultured for fungi. The methods of Fraser et al. (1992) were used, with the following modifications: media were inoculated in a Class I microbiological safety cabinet with the airflow system disconnected, and inoculated plates were incubated at 28-30°C. Cultures were examined daily and the methods of Fraser et al. (1992) used to obtain contaminant-free primary fungal cultures and to derive unifungal cultures from them.

Broad, non-septate, sparsely branching fungal hyphae, indistinguishable from Aphanomyces spp., grew from muscle tissue inocula from 20 of the 22 lesions cultured (Table 1). Nine of these primary cultures were lost due to overgrowth of bacteria or of fungi morphologically distinct from those present in lesions. The remaining 11 primary cultures, and the unifungal cultures derived from them, were identified as Aphanomyces spp. (Scott 1961); all had filamentous sporangia indistinguishable from hyphae, and an "achlyoid" manner of primary spore discharge and encystment. Furthermore, colonial morphology, growth rates and microscopic morphology of the isolates were consistent with descriptions given by Fraser et al. (1992) for the Aphanomyces isolates from RSD-affected fish; most notably, sporangia were both terminal and intercalary, and 30-50 encysted spores were usually present in each spore cluster. No other fungi, morphologically consistent with those present in lesions, were recovered in cultures.

Table 1

Histopathological examination of ulcer sections from all 21 fish cultured showed lesions typical of EUS and indistinguishable from those of RSD (Figure 1).

Figure 1

Total protein electrophoresis provides useful, but not definitive, information concerning relationships within and between species of oomycete fungi (Bielenin et al. 1988, Chen et al. 1991). Extracts from 4 representative Australian Aphanomyces isolates from RSD lesions, 3 representative Philippines Aphanomyces isolates from EUS lesions, as well as an Aphanomyces laevis type isolate, an Aphanomyces cochloides type isolate and an Aphanomyces euteiches type isolate were compared electrophoretically (Figure n). Fungal mats were derived from GY broth (Griffin 1978, cited by Dykstra et al. 1986) cultures incubated at 30°C in the dark for15 days. Mats were rinsed in distilled water, dried and stored at -80°C. Prior to extraction, frozen fungal mats were placed in liquid nitrogen and ground to a powder with mortar and pestle. 100 mg aliquots of fungi were then extracted by boiling for 5 min in 500 ul of reducing mixture (2% sodium dodecyl sulphate, 5% 2-mercaptoethanol, 10% glycerol in 62.5 mM Tris-HCl at pH 6.8). The mixture was clarified at 5000 x *g* and 20 ul of the supernatant was loaded into 12% acrylamide and electrophoresced under reducing conditions according to Laemmli (1970). Peptides resolved by SDS-PAGE were visualised by silver staining (Tsai and Frasch 1982).

Band similarities or differences between isolates were assessed on the basis of band clusters over specific molecular weight class ranges. On this basis, all isolates from RSD and EUS-affected fish were very similar over a range of band clusters from 94 to 14 kDa. The particular similarities between the snakehead (Bautista), snakehead (Buguey) and bream (Clarence) isolates indicate a close degree of relatedness between these Philippine and Australian strains. Aphanomyces cochloides, A. euteiches and A. laevis clearly differ from the ulcerative disease isolates in several band clusters, notably in the range between 35 to 50 kDa. The overall similarity in banding profiles between isolates supports the concept that Aphanomyces isolates recovered from RSD in Australia and EUS in the Philippines belong to a single species, but this putative species cannot definitely be differentiated from A. cochloides, A. euteiches and A. laevis by the method used. Such definitive differentiation must be backed up by DNA and PCR analyses as well as other taxonomic parameters.

Figure 2

The high rate of recovery, in the current study, of Aphanomyces spp. from EUS-affected fish in the Philippines, combined with the epidemiological and pathological similarities between EUS and RSD, indicates these conditions are identical; we therefore propose the term EUS be used to designate the condition in Australia. The study results also suggest that, as in Australia, a single Aphanomyces sp. is associated with EUS in the Philippines and elsewhere in Asia, and that it may be the primary infectious cause. In recent decades, this agent could have spread throughout the region, possibly through movements of fish or water, in a manner similar to the spread through Europe of Aphanomyces astaci, the cause of crayfish plague (Alderman et al. 1987).

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Table 1: Recovery of Aphanomyces spp. from EUS lesions on fish from the Philippines

Collection site	Fish species	Aphanomyces-like growth on primary culture	Aphanomyces on primary and axenic cultures
A	Mugil sp.	+	+
	Mugil sp.	+	+
	Mugil sp.	NFG	.
	Glossogobius giurus	+	+
	G. giurus	NFG	.
B	Ophicephalus striatus		
	Lesion 1	+	+
	Lesion 2	+	+
	O. striatus	+	+
	O. striatus	+; lost	.
C	O. striatus	+	+
	O. striatus	+; lost	.
	O. striatus	+; lost	.
	O. striatus	+; lost	.
D	O. striatus	+; lost	.
E	O. striatus	+	+
	O. striatus	+	+
	O. striatus	+	+
	O. striatus	+; lost	.
	O. striatus	+; lost	.
	O. striatus	+; lost	.
	Clarias batrachus	+	+
	Trichogaster trichopterus	+; lost	.

A Coastal lagoon, Buguey, Cagayan Province, Northern Luzon.

B Ricefield, Buguey, Cagayan Province, Northern Luzon.

C Pond, Bautista, Pangasinan Province, Central Luzon.

D Swamp, Pulilan, Bulacan Province, Central Luzon.

E Laguna Lake, Laguna Province, Southern Luzon.

NFG No fungal growth

Fig 1 Necrotising granulomatous myositis associated with invading fungal hyphae (arrows) in EUS-affected fish from the Philippines (haematoxylin and eosin; x 330) (a) Striped snakehead, Ophicephalus striatus, from a ricefield (b) Mullet, Mugil sp., from a coastal lagoon.