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High Mortality in a Large-scale Zebrafish Colony (*Brachydanio rerio* Hamilton & Buchanan, 1822) Associated with *Lecythophora mutabilis* (van Beyma) W. Gams & McGinnis

Michael J. Dykstra, PhD,[1] Keith M. Astrofsky, DVM,[2] Mark D. Schrenzel, DVM, PhD,[2] James G. Fox, DVM,[2] Robert A. Bullis, DVM,[3] Sarah Farrington, PhD,[4] Lynne Sigler,[5] Michael G. Rinaldi, PhD,[6] and Michael R. McGinnis, PhD[7]

Zebrafish (*Brachydanio rerio*) have become an important model system for studying vertebrate embryonic development and gene function through manipulation of genotype and characterization of resultant phenotypes. An established research zebrafish colony without substantial disease problems for more than 7 years of operation began experiencing appreciable mortalities in November of 1997. Young fish (fry), from five to 24 days after hatching, spontaneously developed elongate strands of organic material protruding from the mouth, operculum, and anal pore, leading workers in the laboratory to describe the infected fish as "bearded." Unlike typical freshwater fish fungal infections, the skin surface did not have evidence of fungal colonization. The disease was associated with progressive lethargy, reduced feeding, and subsequent mortality. From 10 to 100% of the fry in a given tank were affected. Initial examination indicated that the biofilm around the head of affected fry consisted of bundles of septate fungal hyphae, large numbers of mixed bacterial populations, and protozoans. Environmental samples of air and water in the laboratory were obtained to ascertain the source of the infective agent and to isolate and identify the fungus. A fungus identified as *Lecythophora mutabilis* was isolated repeatedly from infected fish and water samples from infected fish tanks, and from the main laboratory water supply tanks, but not from laboratory air. Some biofilm beards on fish were found to consist of relatively pure bacterial populations, and beards on occasional fish examined in the later part of the study consisted of hyphae and spores of the oomycete genus *Aphanomyces*. *Lecythophora mutabilis* did not invade tissues; however, elimination of the epizootic correlated with reduction in the number of *L. mutabilis* conidia in the water following modification of the laboratory water system by use of new filtration and sterilization systems. We conclude that the dense hyphal strands of *L. mutabilis* composing the predominant biofilm type, along with mixed bacteria and protozoa, contributed to the die-off in young fry by occluding the oral cavity and/or gills, leading to starvation and/or asphyxiation.

The zebrafish, *Brachydanio rerio*, is a freshwater, sexually dimorphic, oviparous species of the family Cyprinidae that is native to the region surrounding the Ganges River of India (1). Introduced originally as a popular aquarium fish, the zebrafish (also known as the zebra danio or *Danio rerio*) has emerged as an increasingly popular biomedical model for early vertebrate embryonic development, gene function analysis, and mutagenesis studies (2-4).

In 1992, a zebrafish colony was established at the Massachusetts Institute of Technology for the purpose of conducting mutagenesis studies intended to produce clones of genetically modified zebrafish with which to identify important developmental genes in vertebrates (5). The fish brought into the facility were obtained from clinically disease-free stock. After an initial quarantine period, further stock was not introduced from outside sources to decrease the possibility of pathogen introduction. The colony was expanded by breeding until approximately 27,000 fish were being continuously maintained in the laboratory by 1997.

In November of 1997, laboratory staff began observing young fish (fry) housed in individual standing water containers that were exhibiting unusual masses of white material extending from the oral cavity and the opercula covering their gills. Most fry infected during the initial outbreak in the zebrafish laboratory were between five and 24 days old. The affected fish became lethargic, had decreased feeding behavior, and died. Morbidity among affected fish ranged from 10 to 100%. The clinical outbreak appeared to coincide with a major renovation of the water system for the building, which supplied reverse-osmosis-purified water (RO) to all the research laboratories within the building. After mortality was observed, water quality parameters were evaluated and found to be within the normal range for tropical fish culture, with the exception of low calcium values (0.1 ppm). Bacteria from several genera (*Flavobacterium*, *Pseudomonas*, and *Acinetobacter*) were successfully isolated from the water, but initial attempts to isolate fungi

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[1]Microbiology, Pathology and Parasitology Department, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606, [2]Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, [3]Center for Applied Aquaculture, The Oceanic Institute, Waimanalo, Hawaii 96795, [4]Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, [5]University of Alberta Microfungus Collection and Herbarium, Devonian Botanic Garden, University of Alberta, Edmonton AB T6G 2E1, Alberta, Canada, [6]Fungus Testing Laboratory, Pathology Department, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284, [7]Medical Mycology Research Center, School of Medicine, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555.

**Corresponding author: Michael J. Dykstra, Microbiology, Pathology and Parasitology Department, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606.*

were unsuccessful. A new water polishing system consisting of an ultraviolet unit, carbon filter, and a 0.2-µm filter (Fluid Solutions, Inc., Lowell, Mass.) was installed on the main water line feeding the life support system. Mortality continued over the ensuing three months without identification of the causative agent, suggesting the need for environmental sampling for fungi.

The purpose of the study reported here was to isolate and characterize a fungus, subsequently identified as *Lecythophora mutabilis*, as an opportunistic pathogen associated with the mortality in laboratory-maintained zebrafish. We also wanted to identify the source of this fungus, which to our knowledge was not previously described in fish. In addition, characterization of the fish pathologic features associated with this fungus and antifungal susceptibility testing of *L. mutabilis* were undertaken, along with examination of the water-handling system in the laboratory to help determine how to eliminate the fungus.

Materials and Methods

Zebrafish facility. The main fish-housing laboratory contained approximately 680 tanks, each tank holding between 15 and 75 fish in two to 55 L of water re-circulated from five life support systems with a common RO supply. The water flow rate to the fish holding tanks was approximately 3.5 L/h, and the fish tank water continually overflowed from an outflow tube positioned several centimeters from the top edge of the containers. This overflow water was filtered and re-circulated to the tanks.

Air sampling for fungi. An Andersen N-6 viable air sampler (Graseby-Andersen, Smyrna, Ga.) was used to impinge particulates from the air onto supplemented cornmeal agar (CM^+) containing 17 g of corn meal agar, 2 g of glucose, 1 g of yeast extract, 0.1 g of streptomycin sulfate, and 0.1 g of penicillin G in 1 L of distilled water, as described by Dykstra and co-workers (6). Briefly, Petri dishes containing the growth medium were placed inside the N-6 sampler, and three replicate samples were collected at an air-flow rate of 28.3 L/min for 5 min at each sampling site. The samples were transported to North Carolina State University (NCSU) and were incubated at room temperature under normal laboratory lighting conditions for four days, at which time the number of colony-forming-units (CFU) was scored and mathematically converted to CFU/m^3 of air sampled. Fungal colonies were identified to genus where possible.

Water sampling for fungi. Water samples from tanks containing fish and water supply tanks for the recirculating system in the laboratory, and from the main water purification system providing water to the entire building housing the fish were collected periodically over a 14-month period. Water samples were placed in sterile 50-ml centrifuge tubes and transported to NCSU. Approximately 45 to 50 ml of water from the collection tubes was pushed through sterile 0.45-µm pore size filters by use of 50-ml syringes. The filters were then appressed sample-side down to the surface of CM^+ media in Petri dishes in three or four locations, depending on the experiment, and were incubated at room temperature under normal laboratory lighting conditions. The Petri dish cultures were monitored daily for fungal growth, and colony numbers were counted at three days. Observed fungi were identified to genus where possible.

Swab specimen cultures were made from the biofilm in the overflow trays below fish holding tanks. The swabs were streaked across the surface of CM^+ media in Petri dishes and incubated as described previously.

Resin biofilter beads from the recirculating water system filter tanks in the laboratory were lightly pressed against the surface of CM^+ media in Petri dishes and incubated as described previously for five days, at which time fungi were counted and identified as described previously.

Water sampling for bacteria. During the outbreak, water sampling was conducted at five points within the facility. Two samples were collected from direct purified water lines used for two fish rooms (samples A and B). Two additional samples were collected directly from the reservoir tank (sample C) and the tap water line in one of these fish rooms (sample D). One sample was taken directly from one of the tanks inhabited by the fish (sample E) within the same fish room. All samples were submitted for bacterial and fungal culture to Aqua Analysis, Inc. (Watertown, Mass.) and were evaluated for basic water quality parameters.

Bacterial cultures. Water samples were streaked onto MacConkey, blood, and chocolate agars, and into tryptone soy broth (TSB), then were incubated for a minimum of seven days at 37°C and 5% CO_2. Bacterial species were then identified biochemically, using standard assays.

Measurement of water quality parameters. Standard techniques (7) were used to measure overall water hardness, the concentration of $CaCO_3$, ammonia, nitrite, nitrate, halogens, phosphates, iron, copper, and zinc, and pH and water conductivity.

Microscopic examination of zebrafish. Live fish were examined by light microscopy to check for fungal infection and to observe the extent of external tissue involvement. All fish submitted for necropsy and histologic examination in this study had died as a result of natural mortality attributable to disease or were euthanized by use of tricaine methanesulfonate (MS-222, Finquel, Argent Chemical Laboratories, Redmond, Wash.). Overdosing with MS-222 is an approved method of euthanasia for fish species as outlined by the 2000 Report of the AVMA Panel on Euthanasia (8). The Massachusetts Institute of Technology Animal Care and Use Committee approved the euthanasia methods applied to this study. Selected infected fish were fixed in neutral-buffered 4% formaldehyde and were embedded in paraffin or JB-4 glycolmethacrylate resin for subsequent microtomy prior to staining with hematoxylin and eosin (H&E), Gomori-methenamine-silver (GMS), or toluidine blue O. Wet mounts of some infected fish were made by putting the fish directly into a drop of the fungal staining medium, lactophenol-cotton blue, on a glass slide, then flattening them by use of a coverslip.

Isolation, culture, and characterization of *L. mutabilis*. Individual infected fish with "beards" were examined by use of light microscopy to verify the presence of fungal hyphae. Fish with fungal beards were either placed directly on CM^+ agar in Petri dishes or blotted with Whatman No. 1 filter paper to decrease adherent water containing bacteria, then placed onto the agar. The dishes were incubated at room temperature under normal laboratory lighting conditions and examined daily for fungal outgrowth. Once outgrowth was noted, a subculture was made and the resulting isolate was maintained by monthly subculture on CM^+ incubated at room temperature. One isolate, MIT-1, originating from oral hyphae on a live zebrafish fry from MIT, was sent to the University of Alberta Microfungus Collection and Herbarium where identification as *L. mutabilis* was confirmed by microscopic and colonial features. The isolate was deposited as UAMH 9186.

The MIT-1 isolate was used to evaluate the effects of temperature on growth rate and susceptibility to antifungal agents. To as-

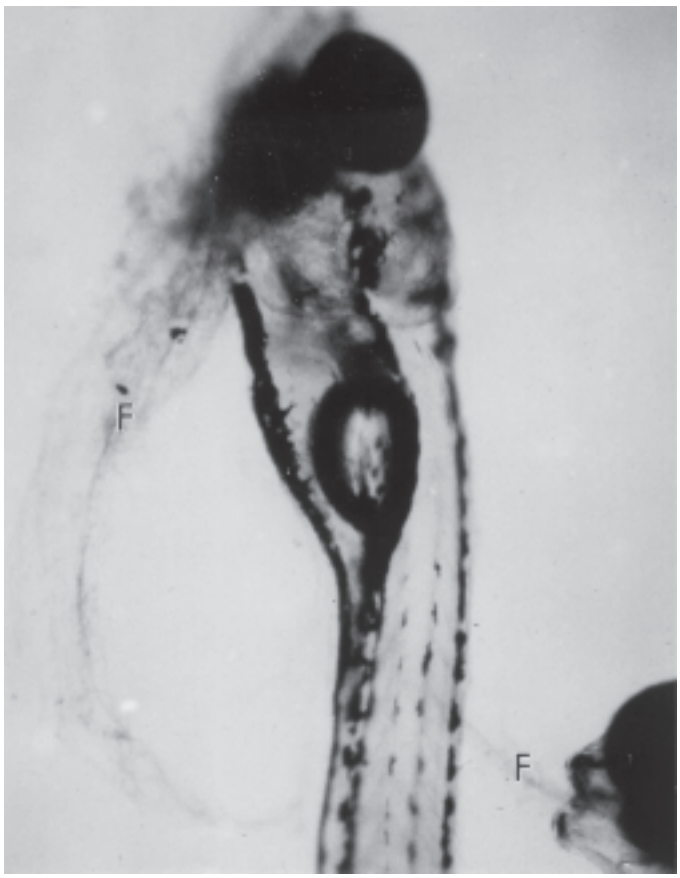

Figure 1. Unstained whole-mount of two zebra fish fry with fungal hyphae (F) protruding from the opercular region (large fish) and from the mouth of the fish in the lower right.

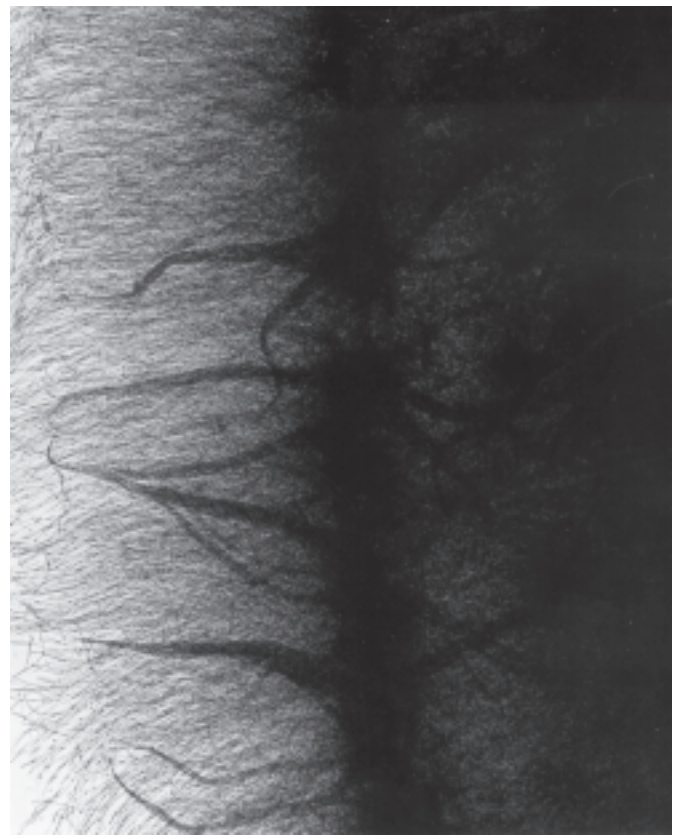

Figure 2. Photomicrograph of a section of an inoculation plug in Petri dish culture of *Lecythophora mutabilis*. Notice the arrangement of hyphae in compact fascicles. Lactophenol-cotton blue stain; Magnification, 75×.

sess temperature tolerance, 6-mm-diameter plugs from the actively growing periphery of a colony on CM^+ agar were transferred to the center of three CM^+ agar plates. Each plate was then sealed with Parafilm, covered with aluminum foil, inverted, and incubated at 4°C, room temperature (approx. 22°C), or 36 to 38°C for 14 days. Three replicates were prepared for each temperature condition. At the end of 14 days, colony diameter was measured and mean diameter of colonies grown at each temperature was determined.

Isolate MIT-1 of *L. mutabilis* was sent to the Fungal Testing Laboratory (The Department of Pathology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Tex. 78284-7750), and assessed for in vitro susceptibility to amphotericin B, 5-fluorocytosine, fluconazole, and itraconazole according to methods outlined by Sutton and co-workers (9) to determine whether chemotherapeutic methods could be used for remediation of the disease outbreak.

Results

Air sampling for fungi. The main fish-housing laboratory (room 327) contained a mean of 9.78 CFU/m^3 of air, and the hallway outside the laboratory had a mean of 11.67 CFU/m^3 of air. *Cladosporium* sp. and *Penicillium* sp. were found in the air of both locations, and that of room 327 also produced one yeast colony. *Lecythophora mutabilis* was not found.

Water sampling for fungi. Multiple sets of samples were evaluated for fungi over a 14-month period between January 1998 and March 1999. Samples were collected from various locations (reservoirs, individual fish tanks, sumps, water lines, and biofilter beds) within the main zebrafish facility (Room 327), the nursery facility (Room 326a), and the main building RO supply system.

In January and February of 1998, sampling indicated that the highest *L. mutabilis* colony counts were obtained from room 327's main reservoir tank (January, five colonies; February, 77 colonies) and room 327 fry tanks with water from the main reservoir (January, 30 colonies). Fry tanks containing presterilized water, room tap water lines, room distilled water lines, and the building's RO supply did not yield *L. mutabilis*. Low numbers of *Cladosporium* sp., *Penicillium* sp., *Aureobasidium* sp., and *Mucor* sp. were found in various samples, with the exception of tanks containing fry reared in presterilized water and the water from the building's main RO system.

In March 1998, following installation of a new main reservoir tank in room 327, *L. mutabilis* colonies were not identified from the new tank. Water obtained from the old reservoir tank had the highest *L. mutabilis* colony counts recorded (100 colonies) anywhere during the sampling period. Only three of 21 water samples from fry tanks in room 327 yielded *L. mutabilis* colonies. Further analysis of water samples obtained in April 1998 continued to reveal *L. mutabilis* colonies in low numbers in room 327 water samples.

Between May and October of 1998, four sets of water samples (May, June, August, and October) were evaluated and were cul-

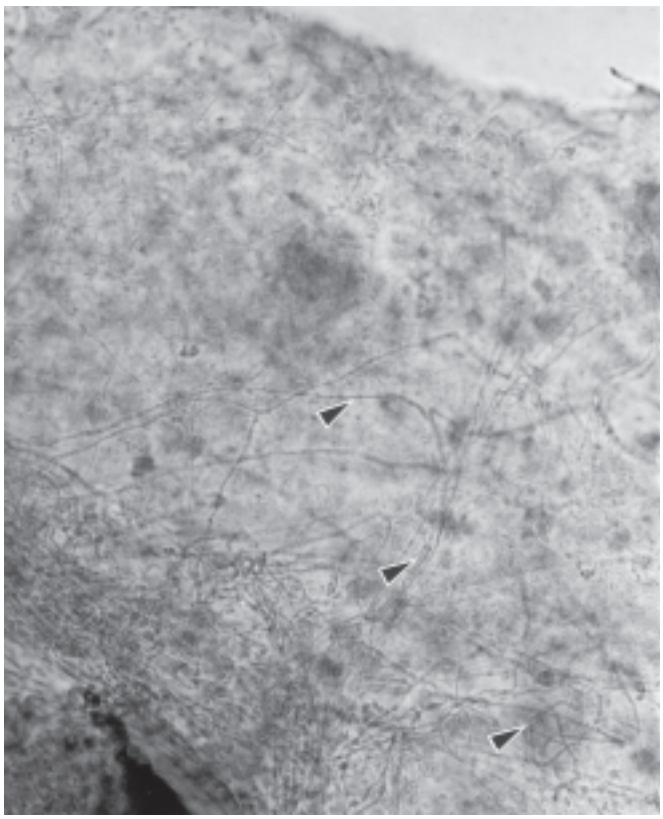

Figure 3. Photomicrograph of a section of the biofilm associated with the head of a fish. Notice numerous hyphae (arrowheads). Lactophenol-cotton blue. Magnification, 270×.

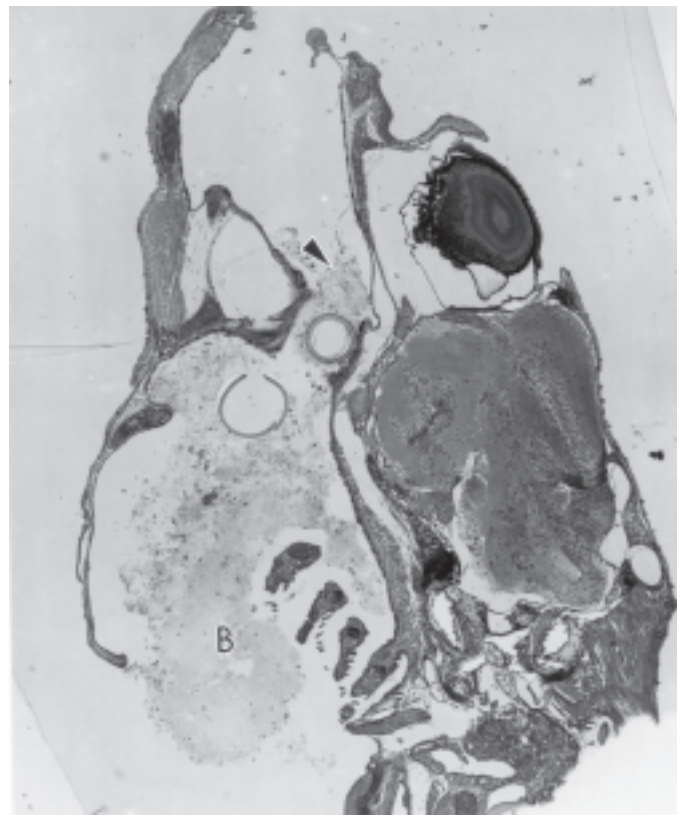

Figure 4. Photomicrograph of a glycolmethacrylate (JB-4)-embedded section of infected fish head stained with H&E. Notice biofilm (B) material in the gill region under the operculum as well as in the rear of the oral cavity (arrowhead). Magnification, 45×.

ture negative for *L. mutabilis* except for one sample obtained from a water source within room 327. Samples collected from a beaker identified as containing infected fish and another from room 327 yielded, for the first time, several oomycete colonies.

A final set of samples was evaluated in March of 1999. Samples were taken from water tanks in two rooms. One sample contained fish that had filamentous growth on their surfaces; however, *L. mutabilis* was not isolated. Oomycetes were grown from the filter impression of this sample. One fish had oomycete hyphae along with copious quantities of bacteria extending from the opercula, and three dead fish were colonized with bacteria and oomycete hyphae as well as unidentified amoebae. The oomycete hyphae were identified as *Aphanomyces* sp. Swab specimens taken from the trays beneath fish culture vessels into which the vessel water dripped for recovery by the re-circulation system also yielded *Aphanomyces* sp.

Water sampling for bacteria. Water samples A and B (collected from direct purified water lines within two fish rooms, rooms 326a and 327) yielded high total bacterial counts of 7,500 and 5,800 cells/ml, respectively. Samples C (collected from the room 327 reservoir tank) and D (collected from a room 327 tap water line) had bacterial counts of 1,700 and 1,200 cells/ml, respectively. Sample E, taken directly from one of the tanks containing fish in room 327, had the highest count of 46,000 cells/ml. *Pseudomonas* sp. and *Micrococcus* sp. were the primary bacterial species isolated while coliforms and *Aeromonas* sp. were noted, but in lower numbers. Several affected fish that were cultured yielded *Flavobacterium* sp.

Water quality parameters. The most important finding was that total hardness and calcium (i.e., $CaCO_3$) concentrations were extremely low (undetectable) in all water samples obtained from the RO system (< 0.1 ppm). The facility management attempted to remedy the low hardness values by increasing the amount of mineral salts added to the water, but attempting to raise the hardness values of very soft water through addition of mineral supplements cannot be easily accomplished without influencing other water chemical parameters. Addition of mineral compounds, such as calcium sulfate and sodium bicarbonate, may adversely influence the buffering capacity, pH, and salinity values of the water. Although the hardness has been increased to approximately 3 to 5 ppm, the water still would be considered soft water. All other water chemical parameters (pH, ammonia, nitrite, nitrate, halogens, phosphates, and conductivity) measured were within normal limits. Water samples also were negative for the presence of iron, copper, and zinc.

Microscopic examination of zebrafish and cultured *L. mutabilis*. Whole mounts of fish fry without staining (Fig.1) revealed elongate strands of material extending up to two thirds the length of the body from beneath the opercula and/or mouth. The strands consisted of fungal hyphae organized into fascicles similar to those produced by the fungus grown on artificial media (Fig. 2). An enlarged view showing the hyphal nature of the material extending from the opercula of a fish is shown in (Fig. 3).

Fish embedded in JB-4 resin had a complex assemblage of microorganisms consisting of bacterial aggregations, fungal hyphae, and protozoans forming a biofilm beneath the operculum

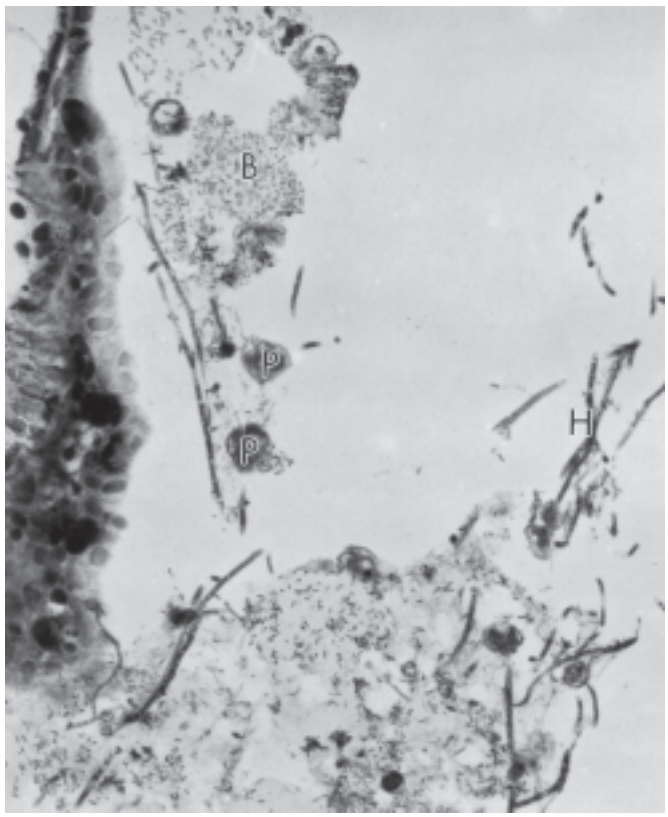

Figure 5. Enlarged view of material within the oral cavity shown in Fig. 4. Narrow hyphae (H), bacterial aggregations (B), and protozoans (P) are evident. H&E stain; Magnification, 405×.

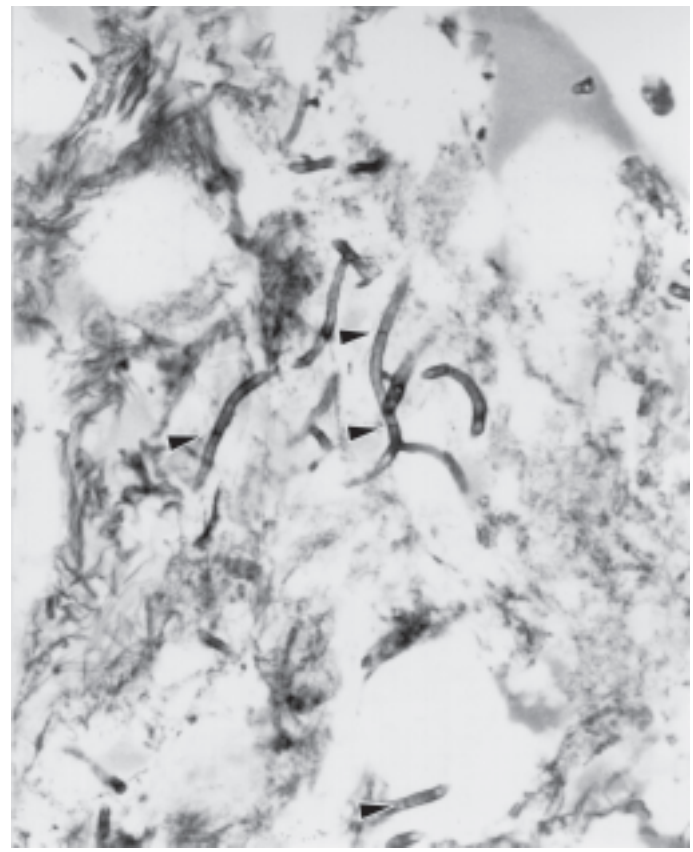

Figure 6. Photomicrograph of glycolmethacrylate-embedded section of biofilm material from beneath the operculum of an infected fish stained with Gomori methenamine silver (GMS) to demonstrate hyphal elements. Notice prominent septa (arrowheads). Magnification, 810×.

and extending into the oral cavity (Fig. 4 and 5). Material from beneath the operculum of another fish stained with GMS showed branching hyphae with septa (Fig. 6).

One set of infected fry examined near the end of the study had an anomalous infection pattern. Hyphae were associated with various areas on the skin (Fig. 7) rather than on the opercula and/or mouth; they tended to branch at right angles and had rare septa and the prominent cytoplasmic streaming characteristic of Oomycetes (Fig. 8). Subsequent cultural evaluation allowed identification of this organism as an *Aphanomyces* species.

Haematoxylin and eosin- or PAS-stained sections of more than 20 affected fry predominantly < 10 days old were examined by light microscopy. A consistent pattern of pathologic changes was not observed in the population studied. Hyphae were found in the opercular cavity of only one fish. Hyphae were not found on any external skin surfaces or in any internal organs. A minor amount of hyperplasia and evidence of slight inflammation was associated with gill tissue in several fish.

The fungus repeatedly isolated from the oral and opercular cavities of fry and from a number of the water samples, particularly from the room 327 reservoir was identified as *L. mutabilis* on the basis of microscopic and colony characteristics. It produced cylindrical to slightly curved narrow conidia (Fig. 9) formed in wet clusters at the ends of short peg-like or needle-shaped phialides along the hyphae (Fig. 10). Some hyphae aggregated into fascicles or strands. The species is distinguished from other members of the genus *Lecythophora* by development of oval or cylindrical brown chlamydospores that form after extended growth on sporulation media such as CM. Isolate MIT-1 developed chlamydospores on CM after two weeks' growth at room temperature and after 30 days at 36 to 38°C on CM^+ (Fig. 11). Colonies are moderately fast growing, and are initially pale yellow to orange color, but darken to gray with the development of the brown chlamydospores. Isolate MIT-1 had the most growth at 22°C, reduced growth at 36 to 38°C, and almost no growth at 4°C.

Antifungal susceptibility testing. *Lecythophora mutabilis* isolate MIT-1 was found to be susceptible to three of four antifungal drugs evaluated, with minimal inhibitory concentration (MIC) values of 0.03 µg/ml to itraconazole, 8 µg/ml to fluconazole, and 0.5 µg/ml to amphotericin B. The isolate was resistant to 5- fluorocytosine (> 64 µg/ml).

Discussion

During the initial outbreak resulting in "bearded" fry, the septate hyphae associated with the oral and opercular areas were organized into fascicles. When these fish were placed onto nutrient agar, the fungus recovered was septate, formed fascicles of hyphae, and was identified as *L. mutabilis*. Preliminary studies with fry exposed to up to 1,000 conidia/ml did not produce infected fish, and water from vessels containing naturally infected fish rarely yielded > 100 spores/ml (M. Dykstra, unpublished observations). Despite our inability to fulfill Koch's postulates, isolation of *L. mutabilis* repeatedly from naturally infected fish, observation of fasciculate organization of hyphae on affected fry, and subsequent isolation of

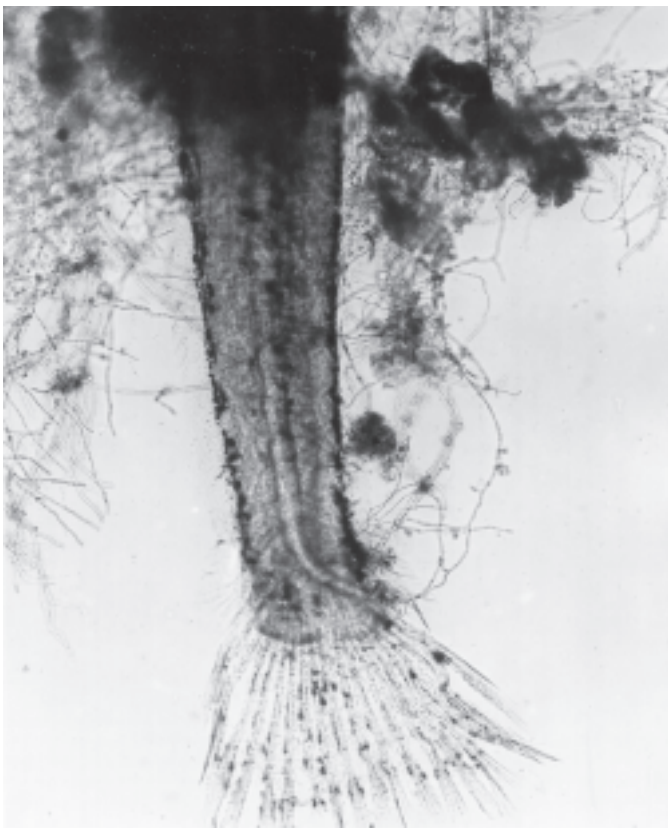

Figure 7. Whole mount of infected fish with superficial fungal infection caused by, and characteristic of *Aphanomyces* sp., which was subsequently isolated. The fungus was not restricted to the head region, but was attached to points over the entire surface of the fish. Magnification, 45×.

the fungus from fish and from water samples strongly implicates this agent in the disease process involving these zebrafish.

Lecythophora mutabilis (van Beyma) W. Gams & McGinnis, previously undescribed as a fish pathogen, was isolated from the system water and from diseased fish, but not from laboratory air. This fungus, reclassified from the genus *Phialophora* by McGinnis and Gams (10), has been found in soil and on decaying vegetation (9), in air and river water, and from wood of preservative treated utility poles (11, 12). *Lecythophora mutabilis* has been implicated, sometimes under the former name *Phialophora mutabilis,* as a rare human pathogen causing peritonitis (13), eye infection (14, 15) and endocarditis (16, 17). Wild fruit bats (*Eidolon helvum*), with mycotic infections of the liver and lung from which *L. mutabilis* could be isolated, have been described. When mice were injected intravenously, subcutaneously, or intraperitoneally with an isolate from the bats, lesions were induced (18). These reports suggest that *L. mutabilis* is a ubiquitous fungus in moist environments and a potential opportunistic pathogen in various compromised host species.

Aquatic fungi and fungus-like organisms (Oomycetes) typically produce a cottony growth on freshwater fish skin and can be associated with body orfices, such as the anus, eyes, olfactory pits, and oral cavity (19). The epithelium that covers the surface of a fish is metabolically active and can quickly respond to stress (20). Common environmentally induced stressors are excess nitrogen, heavy metals, fluctuating pH and temperature, salinity, nutrition, fish density, low alkalinity, and water hardness (21).

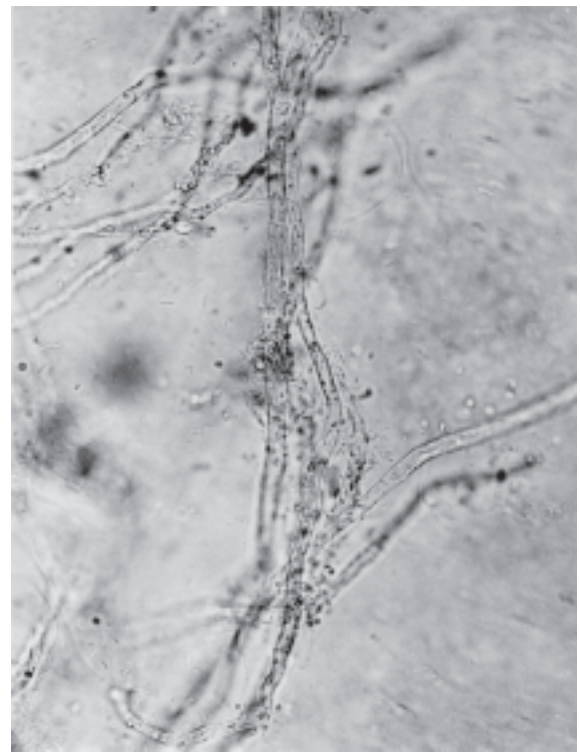

Figure 8. Enlarged view of hyphae from the fish shown in Fig. 7. Notice the hyphae essentially devoid of septa. Magnification, 324×.

Figure 9. Spores produced in culture by *L. mutabilis* isolate MIT-1 (isolated from an infected fish). Lactophenol-cotton blue. Magnification, 840×.

Fish utilize inorganic ions for normal physiologic processes and rely on the uptake of essential minerals, such as calcium, by diffusion across the gill. Adequate concentrations of dissolved

Figure 10. Photomicrograph of characteristic clusters of spores (arrowheads) along hyphae of *L. mutabilis*, isolate MIT-1. 475×.

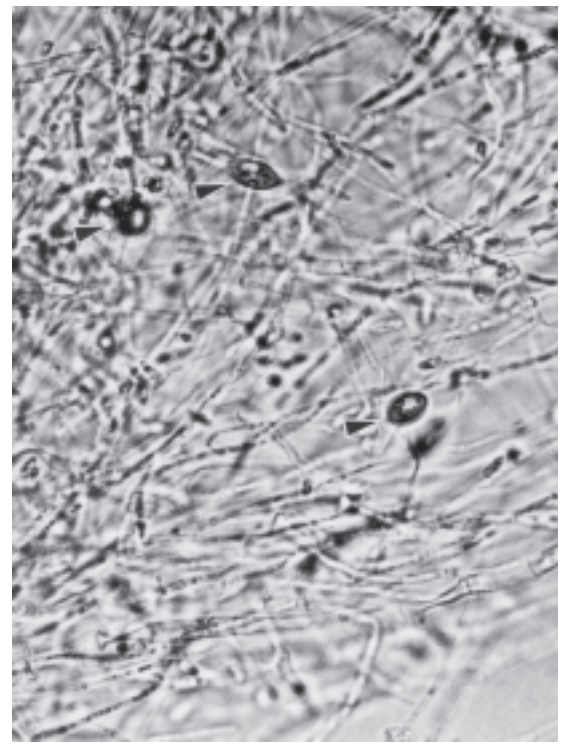

Figure 11. *Lecythophora mutabilis* isolate MIT-1 grown 30 days on CM^+ at 36 to 38°C, resulting in the production of oval or cylindrical, brown, thick-walled chlamydospores (arrowheads). Lactophenol-cotton blue. Magnification, 840×.

minerals in the water are essential and contribute substantially to dietary requirements of fish. The ability of the fish to utilize these aqueous minerals is based on the concentration and biological activity of these minerals present in the water system (22). Zebrafish are considered to be a 'hard water' species preferring total hardness and calcium values of approximately 80 to 200 ppm (1). Most fish remain healthy in water over a wide range of hardness values. However, with low hardness values, fish appear to be more susceptible to other adverse water quality conditions (7). Adequate hardness has been reported to be important in preventing low survival rates, decreased growth, and reduced disease resistance in developing fry (23). Specifically, decreased survivability and growth of catfish fry were noted in fish maintained in water with < 5 mg of calcium/L (24). The fungus identified in our study colonized only young fry between 5 and 24 days of age. Since the dietary requirement during this period is dynamic and the fry have increased metabolic demands, high mortality is commonly observed.

Pseudomonas sp., *Micrococcus* sp., *Aeromonas* sp., *Flavobacterium* sp., and other secondary coliform inhabitants were isolated from the water systems and are considered normal flora for freshwater aquaria. However, these species can cause secondary or opportunistic disease in compromised fish due to trauma, environmental imbalance, or concurrent disease (7, 22, 25). Disease associated with these various opportunistic bacterial species in laboratory zebrafish colonies has been documented (26, 27). Although the total bacterial counts obtained were high for purified water systems, these counts are within normal limits of tolerance for an optimal recirculating water system housing aquatic species. Established recirculating systems frequently have 10^4 to 10^6 total bacteria/ml due to nutrient sources, such as fish excretions and decaying food. The clinical significance of these 'high' bacterial counts and the species identified in this study were, therefore, not considered the primary cause of the epizootic.

Water filtration and sterilization systems added to the water recirculation system markedly decreased the number of *L. mutabilis* colonies isolated from water samples. More importantly, this reduction in culturable fungus coincided with the elimination of bearded fish with septate hyphae. In the later months of this study, bearded fish appeared rarely, and when examined had biofilms comprised principally of bacterial mats with associated protozoans. On the one occasion when hyphae were associated with moribund fish in several fish-rearing vessels during this period, the organism isolated was a non-septate Oomycete, *Aphanomyces* sp. These infected fish differed from those associated with the *L. mutabilis* epizootic because they had hyphae all over the surface of the body, rather than hyphae just being restricted to the head region. These infected fish most likely resulted from overfeeding in the affected tanks. Thus, the presentation of beards on fry was the result of bacterial and protozoal biofilms, *L. mutabilis* infections, *Aphanomyces* sp. infections, or various combinations of these organisms.

When the water systems were initially evaluated, there were a number of dead-end pipes containing food debris, nematodes, protozoa, bacteria, and fungal spores. Sanitizing these microbial reservoirs also may have contributed to the decrease in *L. mutabilis* conidial numbers and interfered with probable synergistic microbial relationships that allowed expression of clinical disease.

In summary, pathologic examination of the affected fish did not reveal a consistent pattern of tissue involvement. The only consistent feature associated with this syndrome was that the oral and the gill cavities beneath the opercula were filled with hyphae, bacteria, and protozoa organized into a dense biofilm. Apparently, the fish died either of starvation due to simple me-

chanical blockage of the oral cavity by this biofilm, or asphyxiation caused by the biofilm inhibiting oxygen uptake by the gills. This study highlights the importance of maintaining, operating, and closely monitoring water and life support systems to minimize the adverse consequences of opportunistic diseases in large zebrafish colonies.

Acknowledgments

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