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Aerosol dispersal of the fish pathogen, *Amyloodinium ocellatum*

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Abstract

Amyloodinium ocellatum, a frequently encountered parasite in marine aquaculture, was investigated to determine if infective dinospore stages could be transported in aerosol droplets. We used an in vivo model incorporating static and dynamic airflow systems and found dinospores of *A. ocellatum* could travel in aerosol droplets (up to 440 mm in a static system and up to 3 m in a dynamic one). This is the first record of this transmission pathway for a marine protozoan parasite. It is possible that other marine protozoans can transfer via the aerobiological pathway. Management of *A. ocellatum* infections in aquaculture facilities could be affected, particularly where tanks and ponds are situated in close proximity.

Keywords: *Amyloodinium ocellatum*; Aerosol; Protozoa; Parasite

1. Introduction

Amyloodinium ocellatum Brown, 1931, Oodiniaceae, is considered an ubiquitous protozoan parasite in tropical and subtropical marine waters. It is currently the only known representative of this genus. The life stages of *A. ocellatum* include a trophont that attaches to and feeds on fish gill and epithelial tissue, a tomont that undergoes division on the substrate and a free-swimming, infective dinospore (Brown and Hovasse, 1946).

A. ocellatum infections in wild fish populations rarely cause mortality or even present signs of acute amyloodiniosis because the dilution factor in the wild prevents repeated infections of the same host individual (Kuperman and Matey, 1999; Lawler, 1980). Fish maintained at high densities, such as those in inshore cage or pond aquaculture systems, are constrained to a finite area, providing ideal conditions for fulminating infections once *A. ocellatum* has been introduced into the system. Land based culture facilities can also be significantly affected by *A. ocellatum* (Fielder and Bardsley, 1999). Inadequate quarantine and biosecurity procedures are typically blamed for parasites gaining access to land based facilities. Entry pathways probably include introduced broodstock, food or inefficiently filtered influent water.

Of the possible methods of entry into aquaculture systems, one pathway that has generally been

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overlooked is via aerosol. Common fish pathogens such as the ciliate *Icthyopthirius multifiliis* Fouquet, 1876 and the bacterium *Aeromonas salmonicida* Emmerich and Weibel, 1894 have been found to disperse in aerosols generated from infected water (Bishop et al., 2003; Wooster and Bowser, 1996).

The aerobiological pathway consists of launch, aerial transport and deposition (Bishop et al., 2003). Natural aerosol droplets can range in size from 0.5 μm up to 100 μm in diameter (Bishop et al., 2003). Droplets larger than 100 μm require strong air currents to carry them any distance (Bishop et al., 2003). Persistence of natural aerosol particles is strongly dependent on ambient temperature and relative humidity at the time of aerial transport. These factors allow aerosol dispersal of parasites over small distances in static systems, as they significantly affect the internal environment of the aerosol, and render the pathogen non-viable through either desiccation or hyper-salinity (Bishop et al., 2003; Wooster and Bowser, 1996). For *I. multifiliis* this distance has been found to be up to 91.4 cm in a static airflow experiment (Bishop et al., 2003). The infective stage (theront) of *I. multifiliis* measures approximately 30×50 μm (Dickerson and Dawe, 1995), while the diameter of dinospores of *A. ocellatum* is significantly smaller (range from 11.6–15.4×10.4–14.5 μm (Lom and Dykova, 1992)), suggesting that on this parameter alone, *A. ocellatum* is a prime candidate for aerosol transport.

Here we examine the ability of *A. ocellatum* dinospores to disperse via aerosols and determine the effective range in static and dynamic systems using an in vivo model.

2. Materials and methods

2.1. Assessment of infection

Trophonts of *A. ocellatum* detach from host marine fish within minutes when exposed to freshwater (Montgomery-Brock et al., 2001). Infection of experiment fish with *A. ocellatum* was assessed by bathing fish in freshwater for 5 min and passing the bath contents through a 47 μm screen. Captured tomonts, approximately 90 μm diameter, were placed into a Petri dish in clean seawater and counted. Tomonts were then monitored over a 24 h period to determine whether division occurred, as a measure of viability (Paperna, 1984a).

2.2. Fish

Fish used in aerosol experiments were juvenile barramundi, *Lates calcarifer* Bloch, 1790 of 5–11 cm in total length obtained from a commercial barramundi growout facility (Barramundi Australia, Brisbane). Barramundi were used because they are euryhaline and thus could be sourced from freshwater farms (naïve to marine pathogens), able to be fed a commercial pellet diet, and easy to maintain in captivity. This system provided a ready supply of fish, which were not infested with *A. ocellatum*. Confirmation that experiment fish were not infested with *A. ocellatum* prior to experimentation was achieved by bathing fish in freshwater for 5 min and then observing the fish behaviour for 30 min. At no time did barramundi in the experiment tanks exhibit typical flashing behaviour indicative of infection (Montgomery-Brock et al., 2001) nor did freshwater bathing yield tomonts of *A. ocellatum* before the commencement of any trial. Fish were acclimatised to filtered (10 μm) seawater (35 ppt) in experiment tanks for 3 days before commencement of each trial. At the end of each trial experiment tanks were washed with freshwater and allowed to dry completely prior starting the next trial.

2.3. Pathogen

A. ocellatum tomonts were originally collected from juvenile snapper *Pagrus auratus* Forster, 1801 at the New South Wales Department of Primary Industries, Port Stephens Fisheries Centre (PSFC) then transported in bags containing oxygen and seawater to the experiment aquarium system at the University of Queensland Veterinary Science Farm, Pinjarra Hills. An in vivo infection was then maintained in two 100 L, aerated, plastic tanks, which were each stocked with three teleost fish of either *Acanthopagrus australis* Gunther, 1859 (Sparidae), *Sillago ciliata* Cuvier, 1829 (Sillaginidae) or *Tetractenos hamitoni* Richardson, 1846 (Tetraodontidae). Approximately 25% of the tanks' volume was exchanged each week with filtered (10 μm) seawater. Infected fish were maintained in an infection room which was discrete from the experiment room and strict quarantine procedures, including foot baths, no equipment transfer and hand washing, were put in place to minimise possible cross contamination of the experiment room. The experiment and infection room were both maintained at 19–20 °C with a relative humidity of 75–80%. *A. ocellatum* tomonts were obtained for experimental use by removing infected fish from the 100 L tanks and placed into 10 L tanks filled with freshwater for 5 min to detach parasites from body surfaces. Collected bath water was then passed through a series of 150 and 47 μm nylon filter screens to remove organic debris and collect tomonts, respectively. Tomonts were transferred to a Petri dish containing

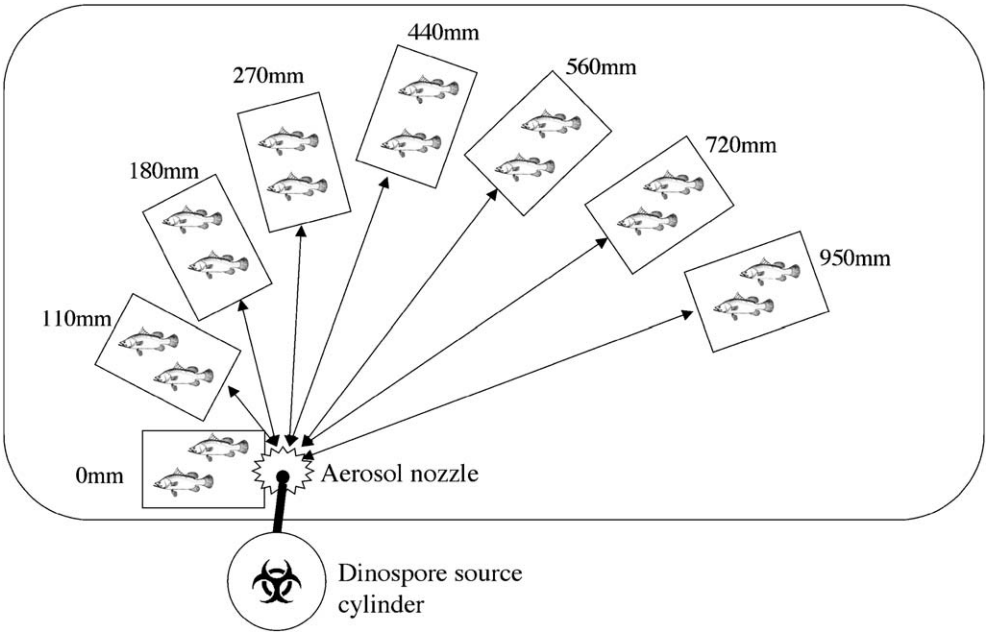

Fig. 1. Overhead view of static airflow experimental design. Arrows indicate the closest distances of tanks to the aerosol source. Two fish were present in each tank.

seawater at room temperature and held for 3 days until sporulation and dinospore emergence.

2.4. Static airflow trials

Six replicated trials were undertaken in 10 L aquaria filled with filtered seawater to 200 mm from the top to determine the effect of aerosol on transfer of *A. ocellatum* under windless (static) conditions (Fig. 1). Tanks were placed in a spiral arrangement increasing in distance from the aerosol source. Experiment tanks were not aerated in order to stop secondary aerosol generation. Two *A. ocellatum* naïve *L. calcarifer* were placed into each of the eight experiment tanks (0, 110, 180, 270, 440, 560, 720 and 950 mm from aerosol source). To minimise direct tank-to-tank contamination from fish splashing, tanks were half filled with seawater. The tank system was completely enclosed within a level, rectangular box ($2 \times 1 \times 0.5$ m) made from opaque fibreglass sheeting to minimise the influence of extraneous air movement within the system.

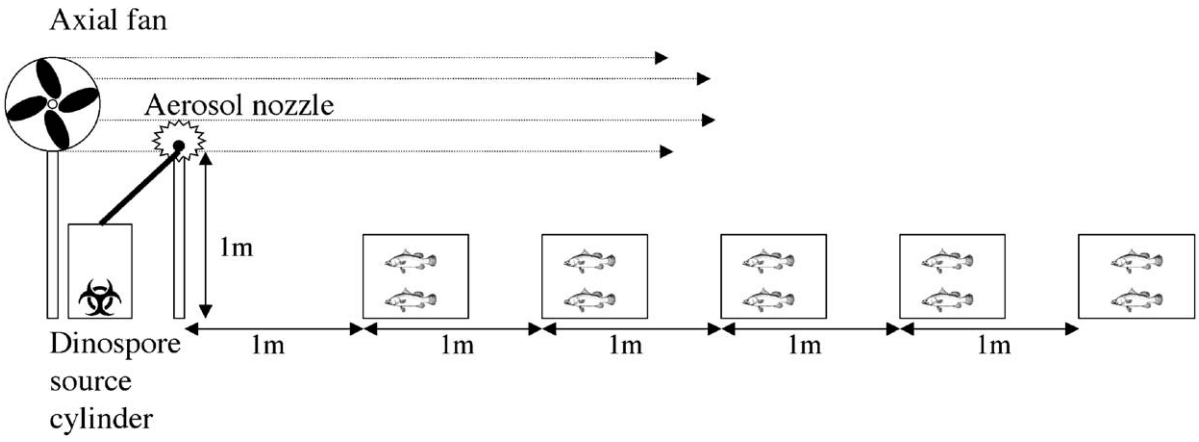

Fig. 2. Dynamic airflow experimental design. Dashed lines indicate air currents created by the pedestal fan. Two fish were present in each tank.

Table 1
Aerosol dispersal of *Amyloodinium ocellatum* to infect barramundi in a static airflow system

Distance (mm)	Tomonts recovered		
	Trial 4	Trial 5	Trial 6
0	89	110	107
110	80	62	74
180	14	35	28
270	22	8	8
440	5	3	0
560	0	0	0
720	0	0	0
950	0	0	0

Data indicates the number of tomonts recovered by freshwater bathing both fish at each distance.

In each trial approximately 100,000 dinospores were placed into a dispersing tank (Fig. 1) filled with seawater. The number of dinospores was estimated by allowing 500 tomonts selected at random from the in vivo infection to sporulate, then assuming that a maximum of 256 dinospores (Brown and Hovasse, 1946) emerged from the majority of tomonts. In the first three trials the aerosol was created by diffusing air through an air stone with an air pump (SPP 40GJ-L, Techno Takatsuki Co. Ltd.) at 5 L/min, which was placed on the bottom of the dispersal container. The diffuser ran continually for the first three days of each trial and was then turned off. The static system was then maintained for a further three days before the status of infection of each fish was assessed, allowing for any infection present to build within the individual experiment tanks. After a further 3 days another infection assessment was undertaken to confirm that any low intensity infections were not overlooked.

Trials four to six used a modified aerosol dispersing apparatus. A misting nozzle was fitted to a pressurised garden sprayer (Garden pressure sprayer 6 L, Toro Australia Pty Ltd). The canister was filled with 2 L of seawater and 100,000 dinospores added. The canister was pressurized using the hand pump to deliver the whole volume through the spray nozzle in 2 min (i.e. 1 L/min flow rate at nozzle). All other parameters were identical to those used in trials 1–3.

2.5. Dynamic airflow trials

A dynamic airflow model was used in 3 replicated trials (Fig. 2) to determine the effect of aerosol on transfer of *A. ocellatum* under dynamic airflow conditions. Five experiment tanks (described for trials 1–6) were placed at 1 m intervals, in a single line (1, 2, 3, 4 and 5 m) from a pressurised canister (as described for trials 4–6) filled with 100,000 dinospores. A pedestal fan (M1248HVS, Linda Australia Pty. Ltd.) of 1 m elevation was placed 60 cm behind the aerosol source, which was also elevated to 1 m. This configuration minimised downward air currents while allowing upward and horizontal airflow past the aerosol nozzle thus maximising the distance that droplets were broadcast. The fan was run until all liquid was sprayed from the pressurised canister containing the source of dinospores, after which the system was returned to stasis. Tanks were half filled to reduce the possibility of splash contamination and spaced such that the distance was greater than the maximum travelled distance of infection in the static trials. Assessment of infection occurred after 6 days followed by a second screening at 9 days.

3. Results

3.1. Static airflow

Trials one to three, in which a diffuser was used as the aerosol source, showed no transfer of infection. Tanks were allowed to stand for up to 12 days to allow potential infections to propagate to detectable levels. However, no *A. ocellatum* infection was detected using freshwater baths and none of the fish displayed any behavioural signs consistent with infection.

In trials four to six, fish in tanks up to 440 mm from the aerosol source were infected with *A. ocellatum*. Tomont numbers collected from the varying tank distances are shown in Table 1. The intensity of infection decreased as distance from the aerosol source was increased.

3.2. Dynamic airflow

All trials demonstrated infection transferring into all tanks up to and including 2 m from the aerosol source, albeit at a low intensity (Table 2). However, trial

Table 2
Aerosol dispersal of *Amyloodinium ocellatum* to infect barramundi in a dynamic airflow system

Distance (m)	Tomonts recovered		
	Trial 7	Trial 8	Trial 9
1	9	17	15
2	8	7	8
3	0	1	0
4	0	0	0
5	0	0	0

Data indicates the number of tomonts recovered after freshwater bathing both fish at each distance.

8 yielded a single trophont from one barramundi in a tank 3 m from the source of infection. Intensity of infection decreased as distance increased.

4. Discussion

Our results demonstrate that *A. ocellatum* dinospores are capable of travelling in aerosol droplets and infecting naïve fish to a distance of at least 4 m from their origin under our experimental conditions. This is only the second protozoan fish pathogen found capable of transferring infection via this pathway (Bishop et al., 2003). More importantly it is the first marine species found capable of such an infection pathway. Aerosols derived from seawater can be considered a hostile environment due to a rapidly increasing salt concentration as evaporation and droplet volume reduction occur (Song and Carmichael, 1999). However, the broad environmental tolerance of *A. ocellatum* is well recognised, in particular salinity tolerances from 1 to 60 ppt in which motile dinospores have been observed (Paperna, 1984b), and this would contribute significantly to maintaining viability of this pathogen and allowing it to exploit such a transmission pathway. It should be noted that little information exists on aerosol transmission of other aquatic pathogens, but those with broad halotolerance, or those that possess environmentally resistant stages, should be considered potential candidates for such a pathway.

Identification of this transmission pathway may explain why *A. ocellatum* and other infections are able to bypass even the strictest biosecurity protocols, which focus typically on transmission through either contaminated seawater, equipment or staff. The need for marine fish hatcheries to maximise fish production often results in increased culture intensity, and it is common to place ponds or tanks close together in order to fit more in to a given space. The demonstrated distances in our experiments are certainly enough for infection transfer to occur in closely placed tanks or ponds.

No transfer of infection occurred using air diffusers and this may be due to air bubble size or aerosol particle size. Air bubble size may have been too large for sufficient aerosol generation. If aerosol particles generated from these bubbles were in the larger part of the range then they may not have been able to carry far enough from the aerosol source. The pressurized canister was able to deliver all the dinospores into roughly uniform particles and dispersed them over a short period of time.

For obvious reasons, many aquaculture facilities are located close to a body of seawater. Often they consist of a number of outdoor ponds, roofed tanks, polyhouses and hatchery sheds. Most of these structures do not provide barriers to airborne infection and are thus at risk of within-facility parasite distribution.

At the Port Stephens facility from which the *A. ocellatum* was originally sourced, broodstock are maintained in an outdoor pond in an unfiltered flow-through system using estuarine water. Examination of fish from this pond revealed no physical signs of disease. However, it is possible that a latent infection below detectable levels exists within this pond. Ponds are only 1–2 m apart, well within distances that aerosols of *A. ocellatum* dinospores were found to travel under the experimental conditions reported in our study. Zooplankton blooms are often present and can lead to a foamy build-up on pond surfaces. All ponds are constantly aerated and also exposed to prevailing winds, which regularly reach up to 30 knots. These circumstances provide ideal conditions for infection transfer via aerobiological pathways. Ponds are used for juvenile growout and experimental work. After use they are drained and the liners are allowed to dry in attempts to kill any infection present previously. While biosecurity in ponds is unable to be as strict as in the hatchery, all equipment are chlorine sterilised after each use and transfer of animals between ponds rarely occurs. Despite this, recurrent infections of *A. ocellatum* still occur, lending support to our hypothesis that *A. ocellatum* is transferred by aerosol. The likelihood that infections are being transferred from pond to pond is high. Unfortunately, ponds are particularly hard to treat due to their large volumes and high associated treatment costs.

5. Conclusion

This study has shown *A. ocellatum* dinospores dispersing via aerosols and is the first record of this transmission pathway for a marine protozoan pathogen. This result shows that traditional biosecurity measures may be inadequate to exclude this pathogen from aquaculture facilities and certainly inadequate to control spread within a facility. Control measures for *A. ocellatum* should include minimisation of airflow between tanks in hatcheries and broodstock facilities where possible.

Growout ponds are likely to be susceptible to contamination via aerosols. High levels of aeration, zooplankton blooms that form foamy build-ups on pond surfaces combined with strong winds across ponds make transfer between ponds likely. Airflow in these situations is uncontrollable. Similar parasites should be subjected to this form of testing to identify the extent to which aerosol contamination occurs.

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