

***Kudoa neurophila* in striped trumpeter: identification,
diagnostic development and histopathology**

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Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by this university or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

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The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

G.W. Gossel, **I. Dyková¹**, **J. Handler²** and **B.L. Munday³**. *Pentacapsula neurophila* sp.n. (Multivalvulida) from the central nervous system of striped trumpeter, *Latris lineata* (Forster). Journal of Fish Diseases 2003, 26, 315-320.

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Abstract

Striped trumpeter, *Latris lineata* (Forster), are being experimentally cultured by the Tasmanian Aquaculture and Fisheries Institute (TAFI) at Taroona, Hobart, Tasmania, Australia. Fish that survive beyond 30 days develop nervous aberrations associated with a severe granulomatous meningoencephalomyelitis. The myxozoan parasite *Pentacapsula neurophila* was described as the parasite causing the disease in the striped trumpeter juveniles. Molecular Bayesian phylogenetic analysis using small subunit ribosomal DNA (ssu rDNA) gene sequence and the covariotide evolutionary model, has shown *P. neurophila* to reside firmly within the clade comprised of *Kudoa* species, histozoic parasites of fish from the order Multivalvulida with 4 or more shell valves containing polar capsules. This has provided molecular evidence resulting in the proposed new combination of the Kudoidae to include this *Pentacapsula* species.

A polymerase chain reaction (PCR) diagnostic assay was developed from the ssu rDNA gene sequence to detect *Kudoa neurophila* (formerly known as *Pentacapsula neurophila*). The assay is sufficiently species specific and sensitive enough to detect a small fragment of the parasite ssu rDNA gene (0.1 spore or 60 fg DNA or 4 spores g⁻¹ / 25 µl PCR reaction). Specifically, the test is capable of detecting early stages of the life cycle within the fish host and consequently diagnosing an infection not normally detected using histology. The PCR test can also be used to screen water supplies and prey cultures throughout the hatchery system to determine bio-security efficacy, assist in epizootiology studies, identify infected alternative or other primary hosts indicating the location of the disease reservoir, and enable a targeted approach to disease prevention in an aquaculture situation.

Histology and *in situ* hybridisation techniques were incorporated into the study of the histopathology of the disease caused by this parasite to elucidate its entry point on the fish host and migratory pathway to terminal stage sporulation. *Kudoa neurophila* enters the fish via epithelial cells as early as 25 days post hatch (dph). Parasite cells then appear within plasmodia in skeletal muscle tissue between 50 – 80 dph. This appears to be the first key proliferation stage which is followed by another plasmodial stage in the peripheral nerve pathways near the spinal cord (70 – 115 dph). It is during this stage that clinical signs of the disease, such as whirling, become apparent. The presporogonic cells then enter the spinal cord where terminal stage sporulation occurs throughout the brain and spinal cord (105 – 130 dph) causing acute pathology, eventually resulting in the death of the animal.

The information gained from the research conducted in this thesis, along with incorporated epizootiology studies, form the foundation of understanding that has assisted the hatchery management team to make informed health management decisions with an outlook to produce healthy juvenile striped trumpeter for on-going research into the development of this species for marine sea cage aquaculture.

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Chapter 1

General Introduction

1.1. Introduction

As developers of new species for intensive aquaculture production, we do not have the benefit of many years husbandry experience held by traditional primary producers. New species for aquaculture require an initial research and development phase to assess factors such as the viability, feasibility and marketability of the product and industry sector in which it will compete for market share. Other factors that influence sustainable development are social and environmental, where local and state governing bodies initiate research and development in the interest of progressing communities in a sustainable, environmentally aware and socially responsible manner. Many new species not only bring new managerial problems, they also bring with them new and unidentified disease problems. Some of these diseases are common with wide host specificity, but in an intensive aquaculture production situation, the opportunity of increased pathogenicity is highly probable. During the developmental phase, the fundamental objectives relating to disease are to identify disease threats, develop effective diagnostic capabilities, understand both the biology of the organism causing the disease and the pathology of the disease, and finally develop efficient management strategies as a means of prevention or control.

1.2. Marine finfish aquaculture in Tasmania

Intensive marine finfish aquaculture has been rapidly expanding world wide and has more than doubled since 1992 (FAO Yearbook, 2001). Many countries are commercially

producing a diverse range of species to meet the market and resource demands of the 21st century. In Tasmania, Australia, the State Government has initiated programs to capitalize on improved marine aquaculture technology and the pristine marine environment of Tasmania in order to foster a competitive and diverse marine aquaculture industry. The Tasmanian Aquaculture & Fisheries Institute (TAFI) has identified marine finfish species with aquaculture potential and is currently developing the premium table fish, striped trumpeter, *Latris lineata*, as a marine aquaculture species suitable for sea cage rearing (Battaglene et al., 2003). The choice to develop striped trumpeter was founded on the basis of suitability for restaurants where it is used for both traditional western meals and sashimi. In addition, it is mainly caught by hand line and the supply is limited, thus further encouraging artificial propagation of this species. The sea cage culture of striped trumpeter, it is hoped, will supplement the existing Atlantic salmon industry and diversify the finfish species grown in the marine environment, thereby decreasing exposure to risk and adding value and market strength to the local aquaculture industry.

1.3. Myxozoan disease in aquaculture caused by *Kudoa* spp.

Disease caused by myxozoan parasites to wild and farmed fish is becoming increasingly important as marine aquaculture expands globally (Kent et al., 2001). Of particular economic importance to aquaculture and commercial fisheries species are the histozoic marine myxozoans from the genus *Kudoa* (Moran et al., 1999a & b; Kent et al., 2001; Dyková et al., 2002; Whipps et al., 2004). Members of the Kudoidae are not generally associated with mass mortalities in aquaculture and fisheries (Lom & Dyková 1995).

However, they are associated with reduced product quality, as infections are mainly found in muscle tissue, causing unsightly cysts in commercial species such as Atlantic herring (Lom & Dyková 1992) or post-mortem myoliquefaction in farmed Atlantic salmon (Whitaker & Kent 1991). Most commercially important species of *Kudoa* infect muscle tissue (Moran et al., 1999a). However, 7 species have been reported as infecting the central nervous system (CNS) of marine finfish. The 4 valved CNS *Kudoa* include; *K. cerebralis* (Paperna & Zwerner 1974), *K. tetraspora* (Narasimhamurti & Kalavati 1979), *Kudoa* sp. (Langdon 1990) and *K. paralichthys* (Cho & Kim 2003). The 3 reported >4 valve *Kudoa* include; the 7 valve *K. yasunagai* (Yasunaga et al., 1981), the 6 valve *Kudoa* sp. (Egusa 1986a & b) and the 5 valve *K. neurophila* (Grossel et al., 2003; Chapter 2).

Generally, Myxozoa are metazoan parasites of freshwater, brackish water and marine fish with a number of exceptions such as a species found in the brain of a mole *Talpa europaea* (Friedrich et al., 2000), in the testicles of the Australian dwarf green tree frog *Litoria fallax* (Browne et al., 2002), another in African hyperoliid frogs (Mutschmann 2004) and a report involving *Myxobolus* spp. spores found in immunocompromised human stool samples (Hessen & Zamzame 2004). The complex myxozoan life cycle involves myxospore and actinospore life stages within a fish host and an invertebrate host respectively (Wolf & Markiw 1984) and over 25 complete life cycles have been described (Kent et al., 2001), including the life cycle of *Ellipsomyxa gobii*, the only marine myxozoan life cycle elucidated to date (Køie et al., 2004). In Tasmania, Australia, there have been several reports of myxozoan parasites of wild marine fish (Willis 1949; Su & White 1994; Su & White 1995) and a single case of

morphologically determined *Kudoa thyrsites* (Gilchrist) infecting the somatic muscle of farmed Atlantic salmon (Munday et al., 1998) with no other species reported in marine aquaculture.

1.4. Identifying myxozoan parasites morphologically

Shortly after research on striped trumpeter commenced, a number of managerial and disease problems were identified and eventually overcome. However, the juvenile cultured striped trumpeter persistently suffered from a severe meningoencephalomyelitis, extensive throughout the brain and spinal cord and in all neural cellular layers except peripheral nerves (Grossel et al., 2002). The CNS disease thought to be caused by this pathogen was recognised as one of the bottlenecks to ongoing research and the production of sufficient commercial quantities of viable juvenile fish for culture assessment. Prior to the commencement of the work described in this thesis, initial diagnostic analysis of infected fish CNS tissue by simple wet squash preparations, diff-quick stained smears and hematoxylin and eosin stained sections suggested the pathogen may be a myxozoan parasite in the pentacapsulid form. The family Pentacapsulidae (Naidjenova & Zaika 1970) was established for myxozoan marine multivalvulidan histozoic parasites that have 5 shell valves each containing a single polar capsule. Since the first report of *Pentacapsula schulmani* (Naidjenova & Zaika 1970) there have only been 2 other *Pentacapsula* species reported from anywhere in the world, namely *Pentacapsula muscularis* (Cheung, Nigrelli & Ruggieri 1983) and *Pentacapsula cutanea* (Kovaleva & Gaevskaya 1984). All species are multivalvulidan, histozoic parasites of marine finfish (Lom & Dyková 1992). To date, no other 5 polar capsule species of

Myxozoa have been identified from Australian waters or are known to cause significant disease problems in a species being examined for aquaculture. The first research task undertaken in this thesis reports on the identification, morphological description, taxonomic classification and the naming of this unidentified pathogen with reference to the International Code of Zoological Nomenclature.

1.5. Identifying myxozoan parasites using molecular techniques

Within members of the phylum Myxozoa, morphological characterisation of spores was the primary means of classification (Lom & Arthur 1989; Lom & Dyková 1992; Moran et al., 1999a). From the mid 1990's onward, molecular data has been employed to determine the relationship of the Myxozoa to other metazoan taxa (Smothers et al., 1994; Siddall et al., 1995) as well as to clarify relationships within the Myxozoa (Hervio et al., 1997; Andree et al., 1999; Xiao & Dessler 2000; Kent et al., 2001; Okamura & Canning 2004; Whipps et al., 2004). Although phylogenetic trees constructed from analysis of the small subunit ribosomal DNA (ssu rDNA) gene sequence are consistent at the family level with early myxozoan phylogenetic hypotheses by Shulman (1966), Kent et al. (2001) recommended that further collection of molecular and other data are required to resolve existing controversies. Myxozoa from the order Multivalvulida are generally marine histozoic parasites with 3 or more shell valves and have traditionally been divided into families based on shell valve and polar capsule numbers. Of particular interest are the *Kudoa* multivalvulidan species, which are a current topic of discussion, as recombination of the genus has been suggested by Whipps et al. (2003b) and Whipps et al. (2004) based on comparative molecular phylogenetic analyses. These studies also

propose that Myxozoa from the *Pentacapsula*, *Hexacapsula* and *Septemcapsula* be synonymized in favour of *Kudoa* (Whipps et al., 2003b; Whipps et al., 2004). This thesis reports on the molecular phylogeny of the pentacapsulid myxozoan pathogen infecting striped trumpeter through phylogenetic analysis of the molecular sequence from the ssu rDNA gene to gain a more complete understanding of its taxonomic classification and its relationship to other histozoic myxozoan species within the order Multivalvulida. The molecular phylogeny presented in this thesis has employed the Bayesian method of phylogenetic analysis and the covariotide evolutionary model, not yet used within myxozoan phylogenetic analysis, to assess relationships within the Multivalvulida.

1.6. Molecular diagnostic assay development

The ability to detect a parasite as early as possible and to identify its source is critical to long term effective health management. A very sensitive and specific DNA based diagnostic test using standard polymerase chain reaction (PCR) molecular technology is a management tool capable of detecting early developmental stages of the parasite or very light but mature infections in the fish host. The ability of PCR to perform this function is particularly useful as it can detect sub-clinical myxozoan infections much earlier than routine histology (Saulnier & de Kinkelin 1997; Andree et al., 1998; Kent et al., 1998; Palenzeula et al., 1999; Yokoyama et al., 2000; Morris et al., 2002). In the case of the myxozoan parasite in striped trumpeter, traditional techniques such as light microscope examination of stained sections and wet squash preparations are only effective in detecting the terminal spore forming stage and in rare instances, presporogonic plasmodial stages of the disease. This thesis reports on the development of an efficient,

sensitive and specific PCR diagnostic assay for detecting a fragment of the ssu rDNA gene of the parasite and its use in the hatchery as an effective aquaculture health management tool.

1.7. Histopathology

Long term routine health monitoring of striped trumpeter has been conducted since 1994 when the first larval fish were reared in the purpose built marine finfish hatchery located at TAFI (Taroom, Tasmania). It was noted in the daily recording sheets that fish surviving through the larval development stage began to show signs of a disease associated with the nervous system. Retrospective health records extending back to 1994 were examined to determine the significance of these observations. Additionally, histological results prior to the commencement of research for this thesis consistently showed that all surviving cultured striped trumpeter juveniles (100% prevalence) from each spawned cohort (in a phase shifted and extended spawning season) eventually developed advanced CNS pathology with heavy infections of terminal stage myxozoan sporogonic plasmodia. Although a few surviving fish seem to function adequately with some 4 to 5 year old fish now reaching sexual maturation, they are all heavily infected with the myxozoan parasite, do not function to their full biological potential and are not considered to be of the quality required for grow-out in sea cages or further research. This thesis describes the pathology of the brain disease caused to striped trumpeter by the parasite, together with aspects of the migratory and developmental pathway taken by the parasite from its entrance point into the fish host, to sporulation in the brain and spinal cord. The development of the parasite life stages within its fish host was traced using a

combination of modern molecular and traditional histological techniques. To elucidate the entry locus of the parasite into the fish host, an *in situ* hybridisation technique was developed from labelled oligonucleotide primers that were initially developed for the PCR diagnostic assay from the ssu rDNA sequence. This was followed by traditional histology such as hematoxylin and eosin stained sections to further trace the parasite to its terminal stage within the CNS target tissue.

1.8. Epizootiology

As part of the overall health management of striped trumpeter culture, aspects of the epizootiology of the disease caused by the parasite was examined and is presented in this thesis. The epizootiology of the disease was studied in and around the hatchery system, including the adjacent marine aquatic reserve, and was used to help build a more complete understanding of the biology of this parasite. The PCR diagnostic assay was used extensively and in conjunction with traditional diagnostics to assist in the identification of the parasite infecting other potential primary fish hosts and potential alternative hosts such as polychaete worm species. It is hoped that the frequency and distribution of the disease caused by this parasite will help establish the disease reservoirs so that a targeted approach to health management can commence.

1.9. Disease prevention and control

Techniques for controlling myxozoan parasites are still in their infancy. The antibiotic chemotherapeutant, fumagillin and its analogue, TNP-470, have been extensively trialed, particularly on cyprinid and salmonid fishes (Molnar et al., 1987; Hedrick et al., 1988;

Higgins & Kent 1998; Yokoyama et al., 1999; Wang et al., 2001; Higgins & Kent 2002; Morris et al., 2003), but the efficacy of these treatments vary greatly between species (Yokoyama et al., 1999; Wang et al., 2001; Whipple et al., 2002) and concern exists over the toxicity of these drugs (Rigos et al., 2000; Morris et al., 2003). The human anti malarial drug quinine and the antibiotic salinomycin have had some success when used against *Henneguya* sp. infecting tapir fish, *Gnathonemus petersii* (Dohle et al., 2002). Control of disease caused by myxozoans to date has been mostly based on environmental management such as quarantine and restricting the movement of live fish (Hoffman 1990), environmental control such as vector control (El-Matbouli et al., 1999; Wagner et al., 2003; Liyanage et al., 2003), habitat and water improvement through water re-direction, ozonation (Higgins & Kent 2002), UV sterilization (Hedrick et al., 2000) and filtration (Arndt & Wagner 2003; Nehring et al., 2003). Other control measures such as vaccination (Martínez de Velasco & Cuéllar 2003; Kelley et al., 2004) and biological control (El-Matbouli et al., 1999) using benthic grazers have been explored and suggested and restocking management (Bartholomew 1998; Whipple et al., 2002) and particularly selective breeding for disease resistance (Bartholomew 1998, Bartholomew et al., 2003; Hedrick et al., 2003; Nichols et al., 2003; Sollid et al., 2003) are also being actively researched. At the present stage of research into the myxozoan parasite infecting striped trumpeter, there are large gaps in our understanding of its biology, particularly the closing of the parasites life cycle through the discovery and identification of an alternative host. However, the research conducted in this thesis to: identify the parasite, develop diagnostics, elucidate aspects of epizootiology in and around the hatchery, and ascertain the histopathology of the disease caused by the parasite, has provided essential

biological information that is sufficient for the development of effective disease prevention methods based on environmental management, health management strategies and quarantine. The disease prevention and control initiative, based on the research in this thesis, resulted in modifications to the hatchery system and is presented and discussed in Chapter 4 and the general discussion. The fundamental aim of the disease prevention modifications is to deliver pathogen free water to the hatchery system and prevent infection by the parasite. The 2004 spawning season will represent the first cohorts of striped trumpeter grown under the new water treatment regime and the practical outcomes of the research into disease control will be the focus of ongoing research.

1.10. Aims of this thesis

The aims of this thesis are outlined as follows:

1. The identification, morphological description and taxonomic classification of this myxozoan parasite causing disease in striped trumpeter (Chapter 2).
2. To further clarify the taxonomy of this parasite by comparative molecular phylogeny using the small subunit ribosomal DNA gene sequence (Chapter 3).
3. To develop a diagnostic assay based on polymerase chain reaction for use as a flexible health management tool with applications extending to further research in the field and laboratory (Chapter 4).
4. Describe the epizootiology of the disease in and around the hatchery with the desired outcome of using all the information gathered to assist in making effective

- and efficient management decisions that will result in the prevention and control of myxozoan caused brain disease in cultured striped trumpeter (Chapter 4).
5. To clarify the pathology of the disease caused by the parasite to its fish host using a combination of molecular techniques and traditional histology (Chapter 5).

The research component of this thesis comprises of 4 chapters (Chapters 2–5) intended for publication as journal articles. Each chapter is an individual study in logical sequence according to the aims of the thesis. The results are discussed in detail at the conclusion of each topic of research. The general discussion at the end of this thesis will attempt to briefly review all research chapters and draw conclusions relating to the desired outcomes from the research conducted.

Chapter 2

***Pentacapsula neurophila* sp.n. (Multivalvulida) from the central nervous system of striped trumpeter, *Latris lineata* (Forster).**

2.1. Abstract

Striped trumpeter, *Latris lineata* are being experimentally cultured by the Tasmanian Aquaculture and Fisheries Institute at Taroona, Hobart, Tasmania, Australia. Fish surviving over 30 days post-hatching have frequently developed nervous aberrations associated with a severe granulomatous meningoencephalomyelitis. The myxozoan parasite *Pentacapsula neurophila* sp.n. was revealed as the parasite causing the disease in the striped trumpeter juveniles. Measurements made of isolated spores indicated that the organism was distinct from all previously described *Pentacapsula* species. This is the first report of a marine myxozoan parasite from the genus *Pentacapsula* in Australian waters.

2.2. Introduction

To meet the demand for a variety of premium table fish, the government of Tasmania and private industry initiated a Finfish Development Program to propagate striped trumpeter, *Latris lineata*. The choice of this fish was made on the basis of suitability for high class restaurants where it is used for both traditional western meals and sashimi. Also, as it is mainly caught by hand line the supply is limited, thus further encouraging artificial propagation of this species. To date in Tasmania, Australia, there have been several reports of myxozoan parasites of wild marine fish (Willis 1949, Su & White 1994, Su & White 1995). However, except for a single case of morphologically determined *Kudoa thyrsites* (Gilchrist) infecting the somatic muscle of farmed Atlantic salmon, *Salmo salar* L., (Munday et al., 1998) no other species have been reported in marine aquaculture from this region. During routine health surveillance of juvenile striped trumpeter mortalities a pentagonal shaped myxozoan with 5 shell valves was found in the central nervous system associated with a severe granulomatous meningoencephalomyelitis.

The family Pentacapsulidae (Naidjenova & Zaika 1970) was established for myxozoan parasites that have 5 shell valves each containing a single polar capsule. Since the first report of *Pentacapsula schulmani* (Naidjenova & Zaika 1970) there have only been 2 other *Pentacapsula* species reported from anywhere in the world, namely, *Pentacapsula muscularis* (Cheung et al., 1983) and *Pentacapsula cutanea* (Kovaleva & Gaevskaya 1984). All species are multivalvulidan, histozoic parasites of marine fish (Lom & Dyková 1992).

The myxozoan described here is the first species with 5 shell valves each containing a polar capsule identified from Australian waters and it has the potential to severely affect the embryonic striped trumpeter aquaculture industry.

2.3. Materials and methods

2.3.1. *Animals*

Striped trumpeter collected for this study displayed clinical signs of disease such as loss of spatial control. The fish were spawned and cultured at the Tasmanian Aquaculture and Fisheries Institute, Marine Research Laboratories, Taroona, Hobart, Tasmania, Australia, as a part of the Finfish Development Program. The several infected fish from which mature spores were extracted ranged in age and size from 643 days old, 88 g to 783 days old, 273 g.

2.3.2. *Spore Extraction*

Brains and spinal cords were removed and spores were extracted using a combination of digestion and sedimentation. Neural tissue was digested by allowing the tissue to autolyse in 0.9% saline solution at 20 °C for 4 days. The liquefied tissue was then subjected to a sedimentation/floatation technique to extract mature intact spores as follows. One ml of liquefied tissue was overlaid onto 10 ml of NaCl solution with a specific gravity of 1.2 and centrifuged at 2100 *g* for 30 min. The NaCl solution had a higher specific gravity than the neural tissue debris and lower specific gravity than the spores allowing clean separation of the spores into a pellet. The pellet containing the spores was washed by re-suspending in fresh 0.9% saline solution prior to collection.

2.3.3. *Spore Measurements*

An Olympus® BX41 microscope (Olympus America Inc. Melville, NY, USA) was used to examine 1410 fresh mature spores.

Photographs of fresh specimens and a micrometer were taken using an Olympus® DP11 microscope digital camera (Olympus America Inc. Melville, New York, USA). Images were transferred to Analytical Imaging Station™ software (Version 3.0 Rev 1.1, St Catherines, Ontario, Canada) and all means and standard deviations of each characteristic and descriptions of the myxozoan were recorded following the guidelines set out by Lom & Arthur (1989) and Lom & Dykova (1992). However, as a measurement protocol for recording the width of *Pentacapsula* was not set out in the above guidelines, in this study width measurements were determined from fresh spores in apical view by measuring the distance of a straight line drawn in any direction through the centre of the spore (Fig. 2.3). Polar filaments were extruded by placing fresh specimens into 1M potassium hydroxide. Phase contrast was used to determine the orientation of the filament coil and calculate the number of times the polar filament was coiled inside the polar capsule. Line drawings were made with the assistance of a light viewing table.

2.3.4. *Scanning electron microscopy*

To prepare specimens for scanning electron microscopy, the purified spores were collected and fixed in 10% neutral buffered formalin. The spores were then thoroughly rinsed in water to remove the maximum amount of remaining foreign particles from their surface followed by concentration in a small amount of water. Both operations were made using a depression slide. Small cover-slips were then coated with 0.1% aqueous solution of poly-lysine hydrobromide (Polysciences Europe, Eppelheim, Germany). A drop of spore suspension was taken from the depression slide and placed on the surface of the cover-slip. Excess fluid was drawn off and the attached spores were fixed in 1%

osmium tetroxide for 45 min, washed in distilled water, dehydrated and critical point dried using CO₂, then sputter-coated with gold and examined with a JEOL JSM-6300 scanning electron microscope at an accelerating voltage of 15kV.

2.4. Results

2.4.1. *Pentacapsula neurophila* sp.n.

2.4.2. *Type host*: *Latris lineata* (Forster, 1801), Latridae (trumpeters), Perciformes.

2.4.3. *Site of infection*: Central nervous system.

2.4.4. *Type locality*: Crayfish Point, Derwent River, Tasmania, Australia. Latitude 42.52S. Longitude 147.19E.

2.4.5. *Type material*: Hapantotype number G463712 deposited in the Queensland Museum, Brisbane, Australia (Appendix 1.1).

2.4.6. *Etymology*: The species name was derived from tissue tropism, “neurophila” meaning that the species has an affinity for nervous tissue.

2.4.7. Description

Scanning electron microscopy reveals the mature spores resembling a 5-petal rose in apical view and having a conical shape in frontal view with prominent channels for polar filaments (Fig. 2.1). The bottom of the spore is smooth without sculpturing. The pentaradiate form of the spore is uniform. Suture lines unifying 5 shell valves are indistinct under light microscopy (Fig. 2.2). Physical dimensions (Table 2.1; Appendix 1.2) in $\mu\text{m} \pm \text{SD}$ are width and length 7.73 ± 0.56 and 6.13 ± 0.45 respectively. Each of the 5 shell valves contains an equal-sized pyriform-shaped polar capsule 3.78 ± 0.28 long by 2.27 ± 0.19 wide with long axes at equal angle (Fig. 2.3). The polar filament is coiled loosely within the pyriform polar capsule with an average of 1.5 coils parallel to the long axis of the polar capsule (Fig. 2.3). Polar filaments are 8.99 ± 0.77 when extruded (Fig. 2.4; Appendix 1.3).



Figure 2.1. Scanning electron micrograph of *Pentacapsula neurophila* sp.n.. Top three images are in side view. Middle three images are in apical view and the lower three images are in bottom view (bar = 2 μ m).

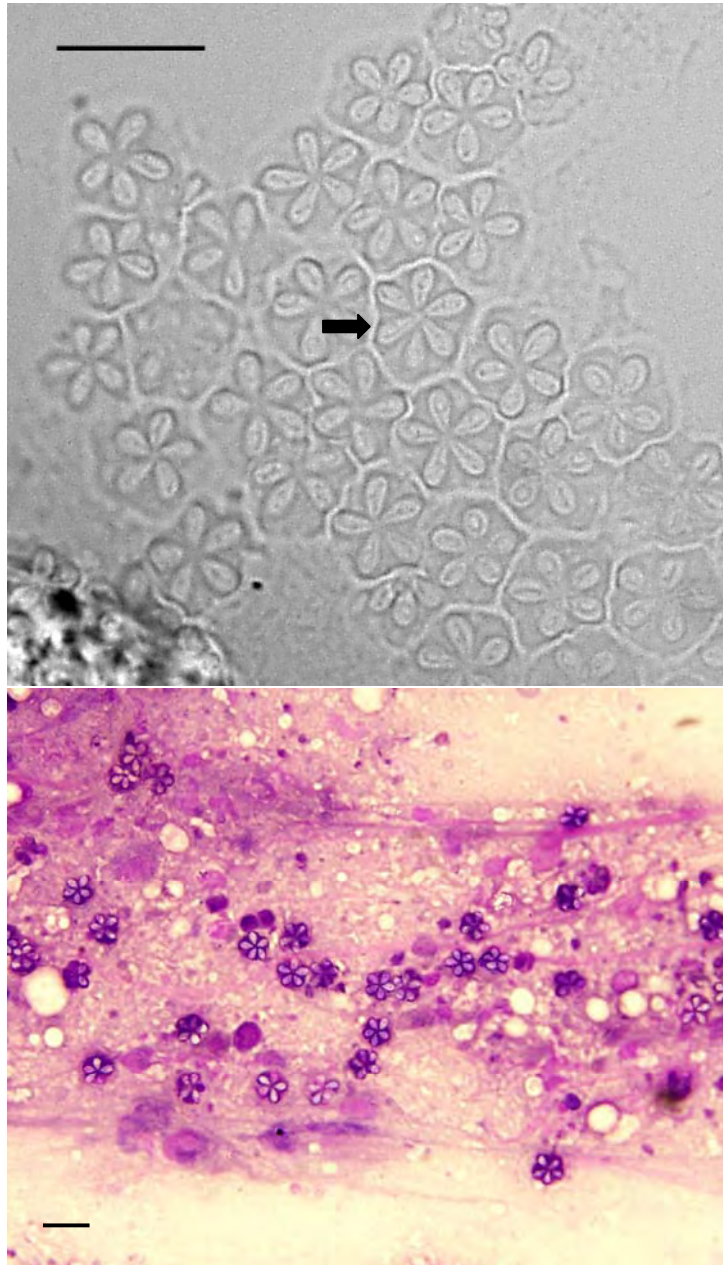
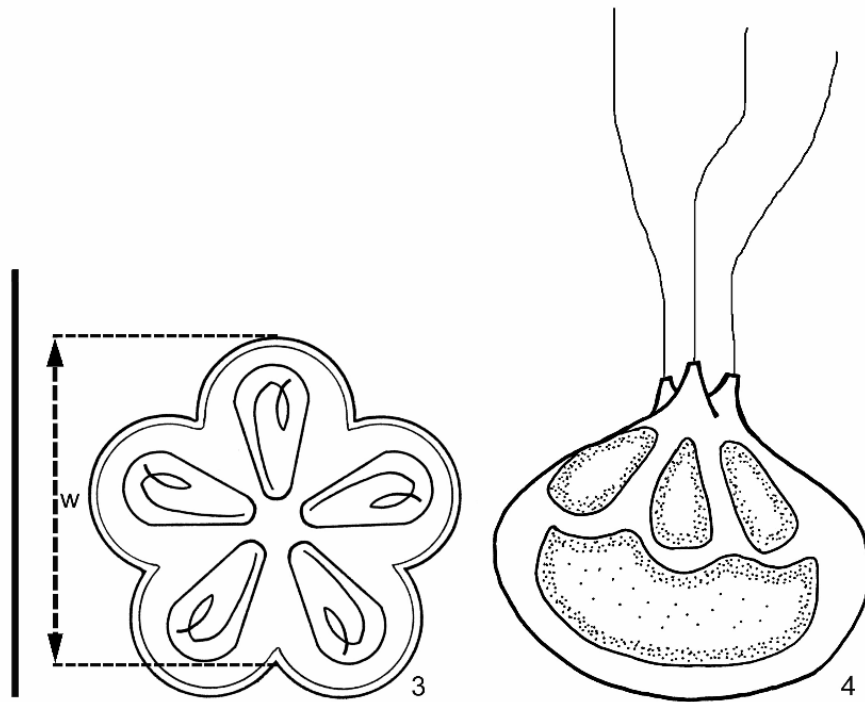


Figure 2.2. Top panel, photomicrograph of spores of *Pentacapsula neurophila* sp.n. in apical view as seen in a wet preparation of spinal cord tissue. Note the spore in the centre of the image containing six polar capsules (arrow). This was the only morphological anomaly found. Bottom panel, diff-quick stained spinal cord smear highlighting the unique blossom shape of the spores (bars = 10 μ m).



Figures 2.3 – 2.4. Diagram of a mature *Pentacapsula neurophila* sp.n. spore. (3) Apical view. Perforated lines represent method of width measurement (w = width). (4) In side view with polar filaments extruded (bar = 10 μ m).

	<i>P. neurophila</i>	<i>P. muscularis</i>	<i>P. schulmani</i>	<i>P. cutanea</i>
Width	7.73 ± 0.56	7.5 ± 0.2	7-8	12-13.3
Length	6.13 ± 0.45	5.5 ± 0.3		
Polar capsule length	3.78 ± 0.28	3.5 ± 0.1	2-2.5	6.65
Polar capsule width	2.27 ± 0.19	1.5 ± 0.1		3.3-4
Filament length	8.99 ± 0.77	15 ± 0.1	5-6	15
Target tissue	Central nervous system	Dorsal muscle	Dorsal muscle	Subepidermal
Host	<i>L. lineata</i>	<i>C. collare</i>	<i>N. japonicus</i>	<i>Echiodon</i> sp.
Locality	Tasman Sea Pacific Basin	Phillipine Is. Indo-Pacific	Indian Ocean	South Atlantic Ocean

Table 2.1. Morphological characteristics of mature spores of all reported *Pentacapsula* species. All measurements are in μm (mean \pm SD).

2.5. Discussion

Morphologically, the discriminating features of Pentacapsulidae (Naidenova & Zaika 1970; Lom & Dyková 1992) are all present in *P. neurophila*. Obvious differences in the physical measurements and overall shape set *P. neurophila* apart from other *Pentacapsula* species and identify it as a distinctly different species (Table 2.1). Foremost is the difference in shape when viewing the mature spore from an apical perspective. The 5 shell valves of *P. neurophila* pentaradiate equally from the centre and are rounded on their peripheral edge. They resemble individual flower petals which together give the appearance of a blossom (Figs 2.1, 2.2 & 2.3). Line drawings of all 3 other *Pentacapsula* species described thus far have a standard, straight sided pentagonal geometry in apical view. *Pentacapsula neurophila* also differs markedly from these when viewed from the side. It has a shape resembling a bulb of garlic (Figs 2.1 & 2.4) whereas the other 3 *Pentacapsula* species are cone shaped at the top in side view. Also, significant differences are noted with the polar filaments. As distinguished from the line drawings of *P. muscularis* from Cheung et al. (1983) and *P. cuteana* from Kovaleva & Gaevskaya (1984), the polar filament when coiled within the polar capsule has 5 – 6 turns for *P. muscularis* and 3 – 4 turns in *P. cuteana* with the orientation of the coils perpendicular to the length of the polar capsule in both species. *Pentacapsula neurophila* differ markedly from these 2 species in that its polar filament has only 1.5 turns and its orientation is parallel to the length of the polar capsule (Fig. 2.3).

Whilst *P. cutanea* is the largest of the 4 *Pentacapsula* species, *P. neurophila*, *P. muscularis* and *P. schulmani* (Naidenova & Zaika 1970) are of similar width in apical view, even though the method for taking this measurement has not been documented by

authors. Because apical width measurement only differs by a small amount, the best distinction between the 3 species, when in apical view, is the length and width of the polar capsule. *Pentacapsula neurophila* polar capsules are almost twice the length of those of *P. schulmani* yet only about half the length of those of *P. cutanea* and slightly larger than *P. muscularis*.

It is important to note that guidelines for apical view width and thickness in *Kudoa* species with 4 shell valves as set out in Lom & Arthur (1989) is appropriate for a 4 sided figure or any equilateral polygon with an even number of sides as a straight line drawn through the centre in any direction will not be the same length. This standard measure for width and thickness does not apply to equilateral polygons with an uneven number of sides as they are the same length in any direction if a straight line is drawn through the centre. Alternatively, Cheung et al. (1983) took an apical view width measurement for *P. muscularis* which they termed the polar view diameter. This is a circle drawn around the outside of an equilateral pentagon that will also give width in side view equal to the diameter of the circle or the circumradius of a regular equilateral pentagon. However, in apical view, the size of the equilateral pentagonal spore is best represented by a straight line drawn in any direction through the centre of the spore. This will give a measure of equal distance in any direction. Since all *Pentacapsula* species discovered so far have been described prior to the publication of the guidelines and have equilateral pentagonal geometries, future use of this method to determine spore width in apical view will be unambiguous and provide a more standard procedure to satisfactorily describe these species avoiding descriptions that may contain misleading variance.

Pentacapsula neurophila also differs from the other described *Pentacapsula* species by virtue of host species, site of infection and geographic location. The sites of infection of the various *Pentacapsula* species differ in that *P. neurophila* targets the tissues of the central nervous system whereas *P. muscularis* and *P. schulmani* develop into mature spores in the dorsal muscle and subepidermis respectively. Type host and locality also differ for each species described. These differences provide further support for *P. neurophila* being a new species. Although Lom & Authur (1989) state these differences do not provide adequate justification alone for creating a new species, morphological and morphometrical differences are sufficient to distinguish the Tasmanian organism from previously described *Pentacapsula* species. Research effort concerning the 18S rDNA sequence for *P. neurophila* is underway and is the subject of Chapter 3 regarding the molecular phylogenetic relationships of this species with other myxozoans.

Myxozoan parasites are increasingly becoming important in net pen mariculture throughout the world as the diseases they cause often have economic impacts concerning fish marketability (Munday et al., 1998). This also coincides with the fact that global mariculture is expanding at a great rate (Kent et al., 2001; Kent 2002). The marine aquaculture industry in Tasmania has not previously been exposed to the potential negative impacts of myxozoan parasites and the presence of *P. neurophila* represents the first myxozoan problem as the state government and industry explore the potential to expand net pen mariculture with striped trumpeter. The potential of *P. neurophila* to cause problems in the embryonic striped trumpeter industry is significant as the disease caused by this parasite is already appearing as a disease bottleneck to producing

commercial quantities of viable juvenile fish. The source of infection, prevalence of infection in wild stock and the ability of the parasite to infect and cause disease in adult fish are presently unknown, therefore, any negative impacts *P. neurophila* may have on any future striped trumpeter industry will be the ongoing focus of research.

In this chapter, a new myxozoan pathogen using morphological techniques was described. In Chapter 3, further research into the taxonomic classification of this unusual myxozoan species was performed using comparative ssu rDNA phylogenetic analysis.

Chapter 3

Small subunit rDNA phylogeny places *Pentacapsula neurophila* (Myxosporea: Multivalvulida) within Kudoidae.

3.1. Abstract

Striped trumpeter, *Latris lineata* is a premium food fish much sought after for the restaurant trade. To meet the demand for this fish, a propagation program to develop striped trumpeter as an aquaculture species was initiated at the Tasmanian Aquaculture and Fisheries Institute (TAFI). Fish surviving longer than 30 days old develop nervous aberrations associated with meningoencephalomyelitis caused by the myxozoan parasite, *Pentacapsula neurophila*. Morphological evidence shows *P. neurophila* to be the first reported species from the genus *Pentacapsula* in Australia. Molecular phylogenetic analysis shows *P. neurophila* to reside firmly within the clade comprised of *Kudoa* species, histozoic parasites of fish from the order Multivalvulida with 4 or more shell valves containing polar capsules. The multivalvulidans are characterized as having 3 or more shell valves with an equal number of polar capsules. Recent molecular studies on the phylogeny of the Multivalvulida show a number of myxozoans with >4 valves residing within the 4 valve *Kudoa* clade. Bayesian phylogenetic analysis of the Kudoidae, using small subunit ribosomal DNA (ssu rDNA) gene sequence and the covariotide evolutionary model, was used to compare *P. neurophila* with other closely related species. Hence molecular evidence is provided which supports the redescription of Kudoidae to include this *Pentacapsula* species.

3.2. Introduction

Disease caused by myxozoan parasites in wild and farmed fish is becoming increasingly important as marine aquaculture expands to meet the resource demands of the 21st century (Kent et al., 2001). In the state of Tasmania, Australia, the State Government together with industry seek to diversify the finfish species grown in the marine environment. The Tasmanian Aquaculture and Fisheries Institute (TAFI) has identified marine species with aquaculture potential and is currently developing striped trumpeter, *Latris lineata* as a marine aquaculture species (Battaglione et al., 2003). Juvenile fish have been lost to conditions such as failure of swim bladder inflation, bacterial enteropathy and nodavirus, but these problems have been overcome. Fish surviving over 30 days post hatch (dph) have frequently developed meningoencephalomyelitis caused by *Pentacapsula neurophila* (Grossel et al., 2003; Chapter 2), a histozoic marine myxozoan with 5 shell valves and polar capsules that sporulates in all neural tissues. This disease was identified as one of the bottlenecks to ongoing research and the production of large commercial quantities of viable juvenile fish for grow out in sea cages.

Within the phylum Myxozoa, morphological characterization of spores is the primary means of classification (Lom & Arthur 1989; Lom & Dyková 1992; Moran et al., 1999a). However, from the mid 1990's onward, molecular data has been employed to determine the relationship of the Myxozoa to other metazoan taxa (Smothers et al., 1994; Siddall et al., 1995) as well as to clarify relationships within the Myxozoa (Hervio et al., 1997; Andree et al., 1999; Xiao & Dessler 2000; Kent et al., 2001; Okamura & Canning 2004; Whipps et al., 2004). Although phylogenetic trees constructed from analysis of the ssu rDNA gene sequence are consistent at the family level with early myxozoan

phylogenetic hypotheses by Shulman (1966), Kent et al. (2001) recommended that further collection of molecular and other data are required to resolve existing controversies.

Myxozoa from the order Multivalvulida are generally marine histozoic parasites with 3 or more shell valves and have traditionally been divided into families based on shell valve and polar capsule numbers. Of particular interest are the *Kudoa* multivalvulidan species, which are a current topic of discussion, as redescription of the genus has been suggested by Whipps et al. (2003b) and Whipps et al. (2004) based on comparative molecular phylogenetic analyses.

This study reports on the molecular phylogeny of *P. neurophila* through phylogenetic analysis of the molecular sequence from the ssu rDNA gene to gain a more complete understanding of its taxonomic classification and its relationship to other histozoic myxozoan spp. within the order Multivalvulida. We have employed the Bayesian method of phylogenetic analysis and the covariotide evolutionary model, not yet used within myxozoan phylogenetic analysis, to assess relationships within the Multivalvulida.

3.3. Materials and methods

3.3.1. *Animals and spore isolation*

Striped trumpeter collected for this study displayed clinical signs of disease such as loss of spatial control. Fish were spawned and cultured at TAFI, Marine Research Laboratories, Taroona, Hobart, Tasmania, Australia (Battaglione et al., 2003). The infected fish from which mature spores were extracted ranged in age and size from 643 dph, 88 g to 783 dph, 273 g.

Brains and spinal cords were removed and neural tissue was digested by autolysis in 0.9% saline solution at 20 °C for 4 days. The liquefied tissue was then subjected to a sedimentation/floatation technique to extract intact mature spores by overlaying 1 ml of liquefied tissue onto 10 ml of NaCl solution with a specific gravity of 1.2 and centrifuging at 2100 *g* for 30 min. The resulting spore pellet was washed by re-suspending it twice in phosphate buffered saline (PBS), pH 7.4, prior to final collection.

3.3.2. *DNA extraction*

The pellet containing the spores was re-suspended in 500 µl PBS and subjected to 2 liquid nitrogen freeze-thaw cycles to ensure the spores break open and sufficient DNA is released prior to being placed in the lysis solution (Proteinase K 200 µg/ml Tris 1 M pH 8.0, NaCl 4 M, EDTA 500 mM, SDS 10%) and incubated overnight at 37 °C. DNA was isolated by 2 phenol/chloroform extractions followed by 2 chloroform extractions and precipitated with 5 M NaCl and ice cold 100% ethanol then incubated overnight at –20 °C. Genomic DNA was collected by centrifugation and re-suspended in 100 µl TE buffer

(Tris 10mM, EDTA 1 mM pH 8.0) and quantified using an Eppendorf BioPhotometer (Brinkmann Instruments, Inc., NY, USA).

3.3.3. *PCR and sequencing*

A variety of general and *Kudoa* specific myxozoan primers conserved among members of the Multivalvulida were used to amplify overlapping regions of the entire ssu rDNA from the 5'-end in association with the 18e primer (Hillis & Dixon 1991; Appendix 2.1). PCR reactions in 50 µl volumes included 5 µl of 10 x PCR buffer, MgCl₂ 2.5 mM, dNTP 2 mM, 0.5 µl of each primer, 0.4 µl Platinum[®] Taq DNA Polymerase (Invitrogen Australia, Mount Waverley, Victoria) and 2 µl of template DNA. Amplifications were performed using an MJ Research PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts, USA). DNA was denatured for 3 min at 95 °C and 29 amplification cycles were performed as follows: 30 sec at 94 °C for denaturation, 45 sec for primer annealing at 53 °C, and 1 min for Taq extension at 72 °C. Five µl of the reaction product was electrophoresed on a 1% agarose gel then stained in 4 µl ethidium bromide stock solution (10 mg ml⁻¹) per 100 ml distilled water for 30 min and visualized on a UV transilluminator box. PCR products were purified using a QIAquick PCR purification kit (QIAGEN Inc, Valencia, California) following the manufacturers protocol. Sequencing was carried out by Supamac laboratories, Sydney, Australia on an ABI 3700 automated sequencer. The entire ssu rDNA sequence (Appendix 2.2) was produced by aligning overlapping regions using ClustalX (Thompson et al., 1997).

3.3.4. Phylogenetic analysis

The data set chosen for analysis consisted of ssu rDNA sequences from closely related Myxozoa available from DDBJ/EMBL/GenBank (Fig. 3.1; Appendix 2.4).

1552 base pairs (bp) of the *Pentacapsula neurophila* ssu rDNA sequence (GenBank accession number AY172511) was aligned with other myxozoan sequences using ClustalX and its default parameters. Three sequence datasets were analysed. The first sequence alignment (dataset A) contained unedited ssu rDNA sequence data for each species and the alignment extended to 1992 nucleotide positions. The second alignment (dataset B) was produced by manually editing dataset A by truncating the extended ends of some sequences and removing single additional insertions not found in any of the other sequences (Appendix 2.5). This alignment contained 1630 nucleotide positions. The third alignment (dataset C) considered the rRNA molecule secondary structure. Only helical regions, defined by the RNAfold program (Hofacker 2003) were included. All alignments underwent a final manual check to ensure consistency and overall alignment quality.

The choice of outgroup species included in this study were representatives of basically marine genera (Kent et al., 2001) and is obviously limited by the number of sequences available from such taxa. The 5 species chosen (*Ceratomyxa shasta*, *Enteromyxum scopthalmi*, *Enteromyxum leei*, *Zschokkella mugilis* & *Parvicapsula minibicornis*) were deemed sufficient as changes such as additions or subtractions of other closely related outgroup species did not seemingly affect the analyses. The likelihood-ratio test (Huelsenbeck & Crandall 1997) was employed to test for the most suitable and robust model of nucleotide evolution using neighbour-joining analysis with PAUP version 4.0b10 (Swofford 1998). Overall phylogeny was tested with the

Huelsenbeck test (Huelsenbeck 2002). The data was analysed with the most robust evolutionary model using likelihood and Bayesian inference of phylogeny with MrBayes version 3.0b4 (Ronquist & Huelsenbeck 2003; Appendix 2.6). Clade credibility values (CCV's) were calculated by MrBayes using the covarion evolutionary model as the proportion of the trees that contain each of the branches. This approach was previously described as a more accurate measure of branch support than the commonly used bootstrap method (Douady et al., 2003). The final maximum posterior probability tree, a majority-rule consensus tree, was drawn using TreeView version 1.6.6 (Page 1996).

3.4. Results

PCR amplification and DNA sequencing generated 1552 bp of unambiguous sequence for the ssu rDNA of *P. neurophila*. The rDNA sequence obtained was aligned with rDNA from other multivalvulidans and the phylogeny investigated. Initially the likelihood-ratio test was used to assess different models of evolution with datasets derived from the rDNA described here. The likelihood-ratio test resulted in the simpler evolutionary likelihood models to be rejected (unequal base frequencies, transitions and transversions $\delta=1319.1$, $P<0.001$) in favour of the most complex evolutionary model, the general time reversible (GTR) substitution model. This model, also known as the GTR+I+ Γ model, as summarised by Morrison et al. (2004), allows base frequencies to vary, all 6 possible substitution rates to vary, a proportion of the sites to be invariant, and the variable sites to vary with a discrete gamma distribution. This result infers the additional rate parameters of the GTR+I+ Γ model, when used with the multivalvulidan rDNA dataset, are more biologically meaningful than other likelihood models tested. Additionally, the GTR+I+ Γ model (proportion of invariable sites) was compared to the covariotide model of DNA substitution that allows rates to vary over time. The null hypothesis of equal evolutionary rates among lineages was rejected ($\delta=157.4$, $P<0.001$) in favour of the covariotide model (Lockhart et al., 1998; Huelsenbeck 2002; Morrison et al., 2004). Bayesian analysis was therefore used to execute this task using MrBayes version 3.0b4.

Consistently similar results were derived from datasets A, B and C that were additionally analysed with distance, maximum likelihood and parsimony approaches (not shown). However, results yielded low bootstrap support values compared to the more confident and higher CCV's from Bayesian analysis using the covariotide model. The

similarity of results from dataset A (unedited) and dataset C (helical regions from secondary structure) suggest that both these datasets contain phylogenetically important information and only the strength of association is weakened by either leaving in long sequence insertions or removing non-helical regions from ssu rDNA secondary structure. The sequence alignment yielding the highest CCV's was dataset B and the results from these analyses are summarised here (Figs. 3.1 – 3.2). Approximately 10 000 generations were required for the likelihood parameters to converge resulting in 10 000 as the setting for the burn-in period, followed by another 15×10^6 generations sampled every 100 generations giving 5101 trees.

A majority rule consensus tree was produced from the 5101 trees sampled (Fig. 3.1). CCV's (Fig. 3.2) were 10% higher overall than bootstrap values generated for the same data set analysed using distance, maximum likelihood and parsimony analyses with default parameters in PAUP (not shown). Furthermore, at 5 nodes with the lowest bootstrap values of 60 or less, CCV's were 33% greater. Bayesian phylogenetic inference from ssu rDNA clearly supports the multivalvulidans as a monophyletic group with CCV's higher compared to bootstrap values. The group of *K. hypoepicardialis*, *K. shiomitsui*, *K. crumena*, *K. amamiensis* and *K. amamiensis* AUS form a sister clade to the rest of the multivalvulidans with *K. hypoepicardialis* and *K. shiomitsui* forming sister species with high CCV's and similar tissue tropism (Fig. 3.2). Not only *P. neurophila* but all of the >4 valve species are firmly rooted within the Kudoidae with very high CCV's. The 6x >4 valve species grouped without any 4 valve species and were separated into 2 groups. One group consists of *P. neurophila*, *Septemcapsula yasunagai* and *Hexacapsula* sp. AY302738 and the other group contains *K. permulticapsula*, *Hexacapsula* sp.

AY302737 and *Hexacapsula* sp. AY302739. The other groups comprise species with 4 valves and very high CCV's.

Major *Kudoa* clades are distinctively separated into 5 well supported groups (Figs. 3.1 – 3.2) with the exception of *K. ovivora* and *K. quadricornis*. Although well supported, their separation and basal placement to 4 groups excluding the *K. crumena* group renders the relationship between these 2 species and the rest of the *Kudoa* unclear given the limited data set.

The sequence most similar to that of *P. neurophila* is that of *S. yasunagai*. These 2 multivalvulidans with 5 and 7 valves respectively are consistently represented through various methods of ssu rDNA analysis as closely related sister species from the western Pacific Ocean with very high CCV's (Fig. 3.1). Although the data set is deficient in other 5 and 7 valve species, these 2 group together and display the same tissue tropism (Fig. 3.2).

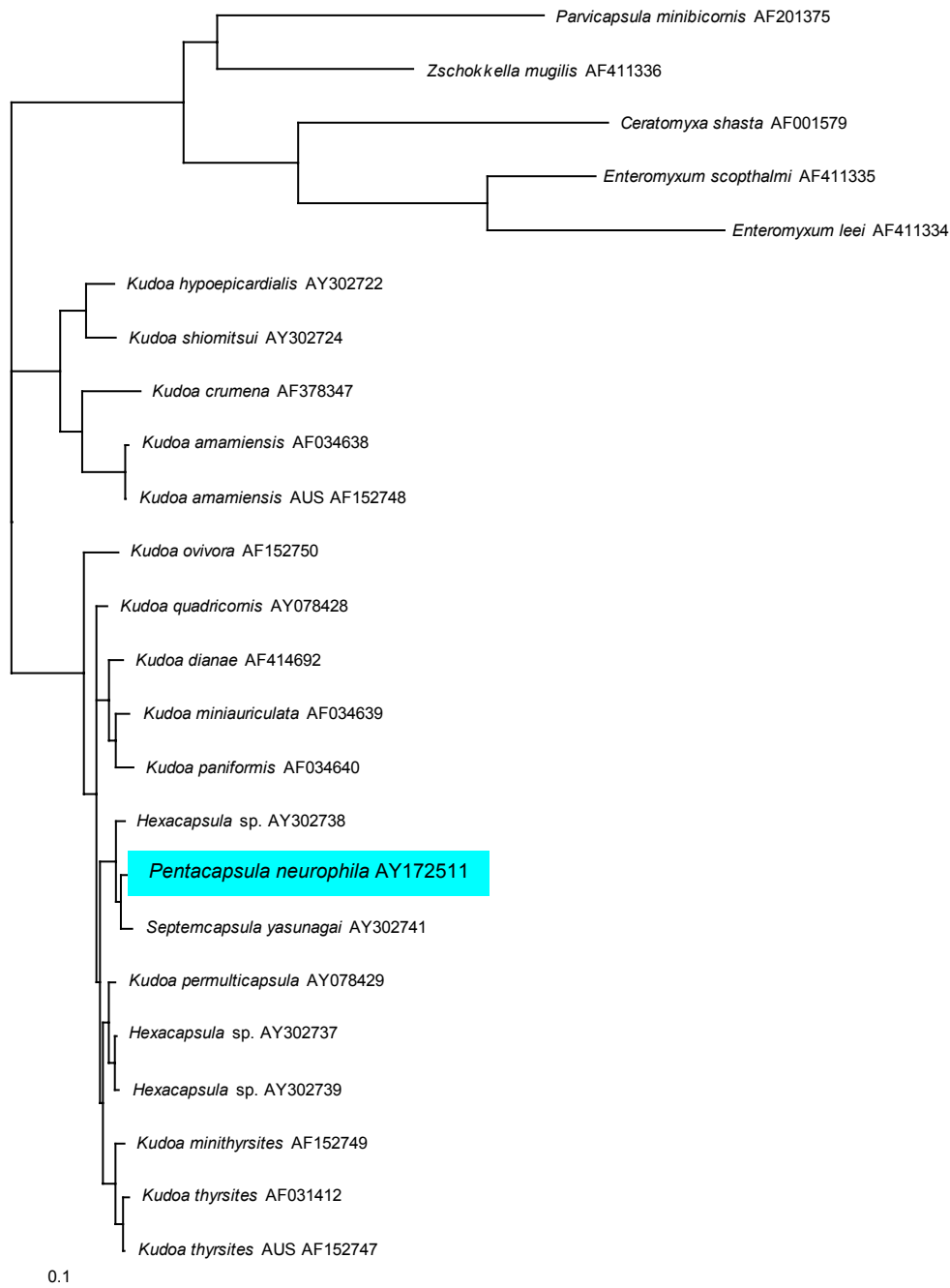


Figure 3.1. Phylogenetic tree produced for the multivalvulidans by Bayesian maximum posterior probability analysis. The DDBJ/EMBL/GenBank accession number is provided for each species. Scale bar = amount of inferred evolutionary change along the branch lengths.

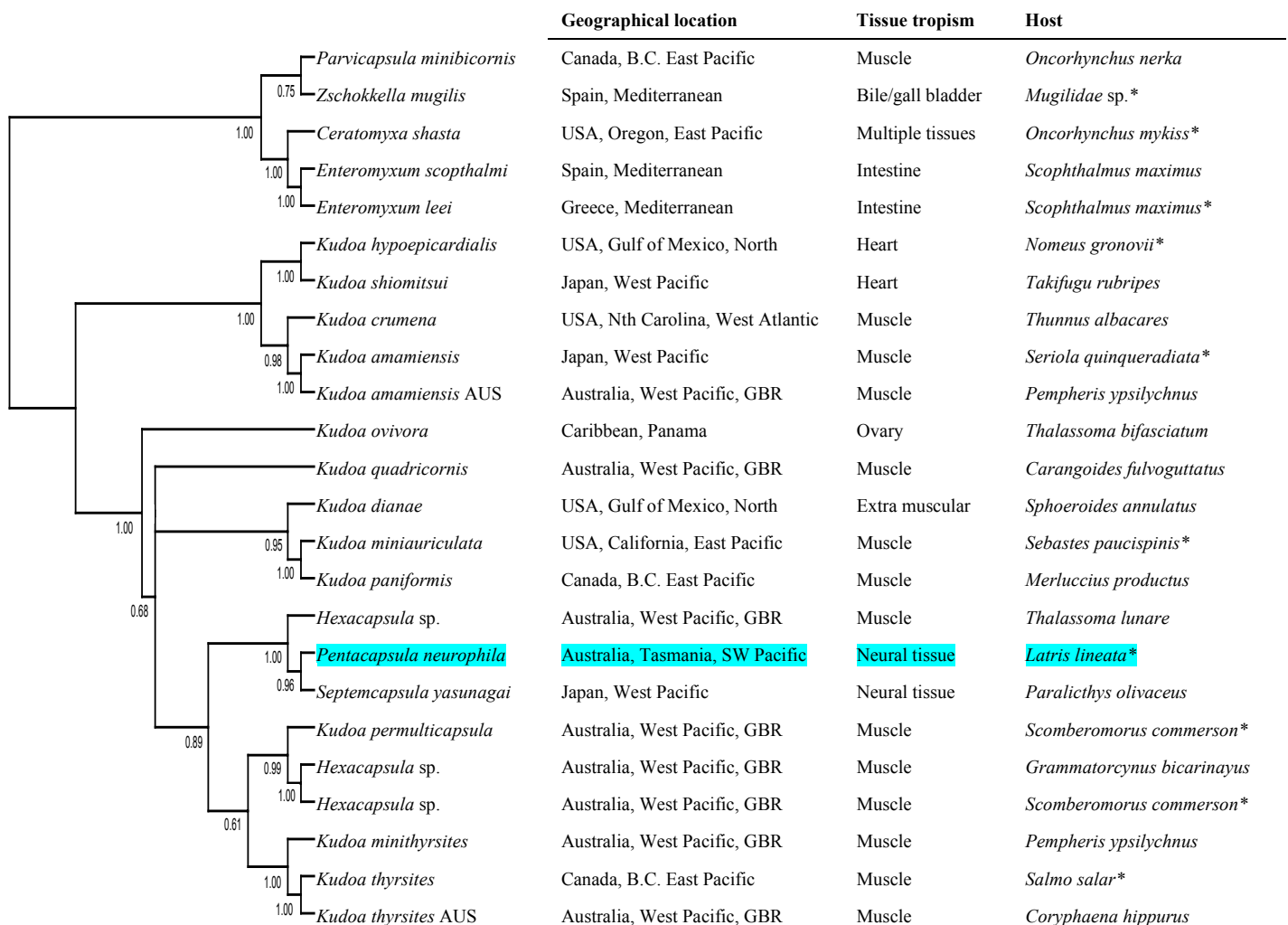


Figure 3.2.

Bayesian maximum posterior probability cladogram of the Multivalvulidae showing; the clade credibility values at branches; multivalvulidan species; geographical location; tissue tropism and host. AUS following the species name refers to the species being found in Australian waters for species with the same name. GBR Great Barrier Reef.* denotes the parasite infects more fish species than the single host listed.

3.5. Discussion

Traditional morphological classification has resulted in assignation of the members of the multivalvulidans containing 5 shell valves and 5 polar capsules to the genus *Pentacapsula* (Naidjenova & Zaika 1970; Cheung et al., 1983; Kovaleva & Gaevskaya 1984; Lom & Dyková 1992; Grossel et al., 2003). In the phylogenetic analysis of the ssu rDNA gene sequence, this *Pentacapsula* species was found to cluster firmly within the group comprised of *Kudoa* species with very high CCV's. Historically, Meglistch (1960) described the family Kudoidae as histozoic Myxozoa with 4 or more shell valves and polar capsules. However, previous classification schemes divide the multivalvulidans into separate families based on valve numbers (Lom & Dyková 1992). In a recent study by Whipps et al. (2003b), an unusual myxozoan with 13 valves and polar capsules was shown to be closely related to the *Kudoa* by ssu rDNA sequence analysis and described as *Kudoa permulticapsula*. Further ssu rDNA analyses by Whipps et al. (2004) showed other >4 valve myxozoans were also closely related to *Kudoa* species. This work has resulted in the redescription of *Kudoa* to include myxozoans with 4 to 13 valves and polar capsules. The current analyses of *P. neurophila* using 3 different ssu rDNA sequence alignments and a robust phylogenetic analytical model shows this 5 valved multivalvulidan to reside deeply within a monophyletic clade comprising the *Kudoa* species. *Pentacapsula neurophila* and the rest of the >4 valve species separate into 2 distinct groups without any other 4 valve species; however, this fact should not render the Kudoidae as paraphyletic. The analyses provides, through high levels of probability and support for the clades, an argument for the maintenance of the monophyletic status of the group; reinstatement and amendment of the description of Kudoidae by Meglitsch

(1960); the redescription of *Pentacapsula*, *Hexacapsula* and *Septemcapsula* to *Kudoa* and overall taxonomic consistency for the multivalvulidans, as proposed by Whipps et al. (2004). Although the *Kudoa* ssu rDNA data set does not include up to 65% of the limited number of *Kudoa* described (Moran et al., 1999a; Pampoulie 2002; Dyková et al., 2002; Cho & Kim 2003; Grossel et al., 2003; Reimschuessel et al., 2003; Whipps et al., 2003a; Whipps et al., 2003b; Blaylock et al., 2004), the stronger results delivered from Bayesian tree building analysis and the covariotide model of evolution, compared to analyses based on less appropriate mathematical models, provide the most credible summary of the multivalvulidans to date.

The very close relationship demonstrated between *P. neurophila* and *S. yasunagai* with 5 and 7 valves respectively, is difficult to address given the data set is deficient in representatives of other 5 and 7 valve species. Host-parasite relationships, geographical location, spore morphology and tissue tropism at the terminal stage within the fish host are far more complex parameters to consider concerning relationships amongst the multivalvulidans. This complexity is especially due to members of this group displaying great variability for each parameter and in some cases within each parameter, such as the spore morphology described for *S. yasunagai* (Egusa 1986a) and *K. permulticapsula* (Whipps et al., 2003b). Morphometric spore variation has also been shown among the same species of triactinomyxon, requiring a molecular approach to clarify relationships (Hallet et al., 2004). However, *P. neurophila* and *S. yasunagai* share a close relationship more due to tissue tropism than spore morphology. The importance of tissue tropism in the biology of these taxa, and its role in clarifying relationships among the group can be further considered through evidence provided by the 3x 6 valve *Hexacapsula* species,

also from the western Pacific Ocean. These species share closer simple spore morphology to *P. neurophila* but are found in muscle tissue and not as closely related to *P. neurophila* in the ssu rDNA analysis. The exact tissue tropism of plasmodial development is increasingly being considered to gain a better understanding of the *Kudoa* and other genera (Dyková et al., 2002; Blaylock et al., 2004; Eszterbauer 2004) but it is too early to draw significant conclusions from any discrete patterns with the limited data available. General tissue tropism, together with sequence similarity as seen in this analysis by the sister grouping of the heart tissue myxozoans and the neural tissue myxozoans, rather than simple spore morphology, geographic location or host-parasite relationships, combine as the most distinctive evidence to date for determining the closeness of the relationships amongst this limited group of marine histozoic multivalvulidans. Tissue specificity and its importance in classification has also recently been reported among closely related *Myxobolus* species (Bahri et al., 2003; Eszterbauer 2004). Similarity in geographic location of *Kudoa* species (Hervio et al., 1997) is not consistent in this study and host-parasite relationships are complex as the *Kudoa* appear to have wide host specificity and are often found not only to infect multiple species, but also species from a variety of fish families (Moran et al., 1999a).

Another *Kudoa* gene sequence, the large subunit ribosomal RNA gene sequence, has been examined via comparative sequence analysis to elucidate relationships among the multivalvulidans (Whipps et al., 2004; Appendix 2.3), but the data set is by no means extensive and as long as researchers focus on the ssu rDNA sequence, it will remain the most extensive and reliable data set available. Other authors have also used a number of different models to analyse *Kudoa* ssu rDNA data but have applied the analysis to very

limited sets of data and have often concluded that low bootstrap values do not comprehensively support their findings and perhaps other genes should be included in the analysis. This study employs Bayesian maximum posterior probability using the covariotide model to infer the phylogeny of *P. neurophila* with ssu rDNA data. The covariotide evolutionary model was chosen to perform the analysis based on likelihood-ratio tests (Huelsenbeck & Crandall 1997), which compared the suitability of the covariotide model against simpler models. Most models of phylogenetic inference are stationary and do not allow constraints to change over time. The covariotide mathematical model of evolution on the other hand, allowed nucleotide substitution to vary over the evolutionary history of the Kudoidae and should therefore be considered the most appropriate model for analysis of this group. Including the majority of complete or near complete *Kudoa* ssu rDNA sequences in the analysis, together with the strong phylogenetic signal, suggests an increased accuracy of phylogenetic reconstruction of evolutionary history. Although this analysis is by no means comprehensive, it delivers a more accurate analysis of classification for *P. neurophila* and its position firmly within the Kudoidae with higher and more credible support values than previously used phylogenetic methods. Bayesian analysis of the Kudoidae supports recombination of *P. neurophila* to *Kudoa neurophila* and may also provide an alternative and more comprehensive method for reviewing and resolving relationships using the myxozoan ssu rDNA dataset available.

Following this research, the Kudoidae has been recombined to include this *Pentacapsula* species. *Pentacapsula neurophila* is now known as *Kudoa neurophila*.

Chapter 4

Diagnostic polymerase chain reaction assay to detect *Kudoa neurophila* (Myxozoa: Multivalvulida) in a striped trumpeter, *Latris lineata*, hatchery and the epizootiology of the disease caused by the parasite.

4.1. Abstract

A polymerase chain reaction (PCR) diagnostic assay was developed from a small subunit ribosomal DNA (ssu rDNA) gene sequence to detect the myxozoan parasite *Kudoa neurophila*, the causative agent of myxozoan disease in the hatchery reared marine finfish, striped trumpeter, *Latris lineata* (Forster). The assay was developed for use as a disease control management tool in a hatchery system specifically designed to research and produce marine finfish such as striped trumpeter juveniles for aquaculture. The assay is sufficiently species specific and sensitive to detect a small fragment of the parasite's ssu rDNA. At the lower limits of detection, the test is consistently positive to an estimated 0.1 spore or 60 fg of parasite DNA per 25 µl PCR reaction in serial dilution and positive to an estimated 0.1 spore in 25 mg of infected fish central nervous system (CNS) tissue (4 spores g⁻¹). Specifically, the test is capable of detecting early stages of the life cycle within the fish host and consequently diagnosing an infection not normally detected using traditional histological techniques.

The epizootiology of the disease caused by the parasite was studied in and around the hatchery system. The PCR test was extensively utilised in epizootiology studies as it proved effective for screening filtrates from water supplies, prey species cultures and assisted in the identification of alternate or other primary fish hosts and potential

alternative host species. This information was used to indicate the location of the pathogen reservoirs and to enable a targeted approach to disease prevention. The test was also used throughout the hatchery system to determine bio-security efficacy of the water treatment system designed to deliver pathogen free water.

4.2. Introduction

Global marine finfish aquaculture has more than doubled since 1992 (FAO Yearbook 2001). Many countries are commercially producing a diverse range of species to meet the market and resource demands of the 21st century. In Tasmania, Australia, the State Government has initiated programs to capitalise on improved marine aquaculture technology and the pristine marine environment in order to foster a competitive and diverse marine aquaculture industry. The Tasmanian Aquaculture & Fisheries Institute (TAFI) has identified marine finfish species with aquaculture potential. It is currently developing striped trumpeter, *Latris lineata* (Forster) as a marine aquaculture species suitable for sea cage rearing (Battaglione et al., 2003) to supplement the existing Atlantic salmon industry.

Shortly after research on this species commenced, the myxozoan *Kudoa neurophila* (Grossel et al., 2003; Chapter 2; Chapter 3), a marine multivalvulidan histozoic parasite with pentavalvid spores infecting the tissue of the central nervous system (CNS), was discovered during routine histological analysis. The disease caused by the parasite is a severe meningoencephalomyelitis, extensive throughout the brain and spinal cord and in all neural tissue types except peripheral nerves (Grossel et al., 2002). Although the mode of transmission of this parasite is unknown, it is generally accepted that the complex myxozoan life cycle involves myxosporean and actinosporean life stages within a fish and invertebrate host respectively (Wolf & Markiw 1984). An alternate invertebrate host for *K. neurophila*, responsible for releasing the infective actinosporean stage of the parasite has not yet been discovered, although juvenile fish become infected during larval stages and develop nervous aberrations suggestive of a

disease of the CNS around 70 days post hatch (dph). Mature spores of *K. neurophila* representing the terminal life cycle stage within the fish host can first be detected with a high prevalence of infection (100% in surviving fish) from approximately 100 dph (Grossel et al., 2002). This developmental pattern is similar to other closely related marine Myxozoa such as *Kudoa thyrsites* in netpen-reared Atlantic salmon (Moran et al., 1999b). *Kudoa neurophila* has been recognised as a significant disease restricting striped trumpeter culture assessment. The disease directly inhibits the production of sufficient quantities of viable juvenile fish required to test large-scale marine aquaculture.

Pentavalvid forms of *Kudoa* were previously morphologically distinguishable as species belonging to the genus *Pentacapsula* by having 5 shell valves and 5 polar capsules (Naidenova & Zaika 1970; Lom & Dykova 1992) with only 4 species discovered to date (Naidenova & Zaika 1970; Cheung et al., 1983; Kovaleva & Gaevskaya 1984; Grossel et al., 2003). However, comparative small subunit ribosomal DNA (ssu rDNA) gene sequence analysis shows *K. neurophila* to be very closely related to other histozoic marine multivalvulidans with 4 or more shell valves and polar capsules residing within the family Kudoidae. Recent molecular phylogeny studies propose the *Pentacapsula*, *Hexacapsula* and *Septemcapsula* genera be synonymised in favour of *Kudoa* (Whipps et al., 2003b, Whipps et al., 2004). This is the first 5 polar capsule species of the *Kudoa* genus to be discovered in Australia and the first to cause significant disease problems in a species being examined for aquaculture.

At the present stage of research into *K. neurophila* there are large gaps in our understanding of its biology, hence effective control has initially been based on environmental management, health management strategies and quarantine to prevent

infection by the parasite. The ability to detect the parasite as early as possible and to identify its source is critical to long term effective health management. A very sensitive and specific DNA-based diagnostic test using standard polymerase chain reaction (PCR) molecular technology is a management tool capable of detecting early developmental stages of the parasite or very light but mature infections in the fish host. The ability of PCR to perform this function is particularly useful as it can detect sub-clinical myxozoan infections much earlier than routine histology (Saulnier & de Kinkelin 1997; Andree et al., 1998; Kent et al., 1998; Palenzeula et al., 1999; Yokoyama et al., 2000; Morris et al., 2002). In the case of *K. neurophila*, traditional techniques such as light microscope examination of stained sections and wet squash preparations are only effective in detecting the terminal spore forming stage, and in rare instances, presporogonic plasmodial stages of the disease. This study reports on the development of an efficient, sensitive and specific PCR diagnostic assay for detecting a fragment of the ssu rDNA gene of *K. neurophila* and its use in the hatchery as an effective aquaculture health management tool.

To gain a more detailed understanding of the biology of *K. neurophila* so that a targeted approach to disease prevention can be taken, epizootiological studies incorporating the PCR diagnostic assay were undertaken in and around the hatchery system, including the adjoining Crayfish Point Marine Aquatic Reserve from which the sea water for the hatchery is drawn. Members of the Kudoidae have wide host specificity (Moran et al., 1999a). This may also be true for *K. neurophila* and therefore would be of particular concern regarding the reservoirs of the disease, the perpetuation of the parasite life cycle and the subsequent health management decisions required.

4.3. Materials and methods

4.3.1. *Animals and spore isolation*

Striped trumpeter collected for this study displayed clinical signs of disease such as scoliosis and loss of spatial control. The fish were spawned and cultured at TAFI, Marine Research Laboratories, Taroona, Hobart, Tasmania, Australia (Battaglene et al., 2003). The infected fish from which mature spores were extracted ranged in age and size from 643 dph, 88 g to 783 dph, 273 g. Brains and spinal cords were removed and neural tissue was digested by autolysis in 0.9% saline solution at 20 °C for 4 days. The liquefied tissue was then subjected to a sedimentation/floatation technique to extract mature intact spores by overlaying 1 ml of liquefied tissue onto 10 ml of NaCl solution with a specific gravity of 1.2 and centrifuged at 2100 *g* for 30 min. The resulting spore pellet was washed by re-suspending it twice in phosphate buffered saline (PBS), pH 7.4, prior to final collection.

4.3.2. *DNA extraction and primers*

DNA was extracted using the DNeasy Tissue Kit (QIAGEN Inc., Valencia, California) according to the manufacturer's protocol. DNA was routinely extracted from 25 mg of tissue and suspended in 100 µl of the buffer provided in the kit unless otherwise stated. All DNA was quantified using an Eppendorf BioPhotometer (Brinkmann Instruments, Inc., NY, USA). The ssu rDNA sequence (GenBank accession number AY172511) of 1552 base pairs from the 5' end and 18e primer (Hillis & Dixon 1991) was subject to National Centre for Biotechnology Information (NCBI) BLAST searches (Altschul et al., 1997). The sequences from closely related species were then aligned using ClustalX (Thompson et al., 1997) and used to design primers from regions that displayed sufficient

variation from the alignment of sequences. Twelve 20–24mer oligonucleotide primers were subsequently designed (Appendix 3.1) giving 20 possible primer combinations (Appendix 3.2). The physical characteristics for each primer were tested for suitability using Net Primer (available at www.premierbiosoft.com). Each primer was subject to NCBI BLAST searches to determine the individuality of each primer sequence. Only primers specific to *K. neurophila* that did not match any other known GenBank sequence were selected for testing.

4.3.3. Specificity

To further determine specificity, the primers were tested against the target species, a panel of related myxozoan species and a panel of non-target environmental isolates. The target species consisted of template DNA extracted from *K. neurophila*. The related species panel consisted of template DNA extracted from closely related species including: *Kudoa* sp. (Langdon 1990) from cysts of infected southern bluefin tuna *Thunnus maccoyii* muscle tissue collected from farmed fish in Port Lincoln, South Australia; *Kudoa thyrsites* from Atlantic mackerel *Scomber scombrus* muscle, France; *Kudoa ovivora* from the ovaries of bluehead wrasse *Thalassoma bifasciatum*, Panama; and *Hexacapsula* sp. from the muscle of moon wrasse *Thalassoma lunare*, Great Barrier Reef, Australia. A local myxozoan, *Myxobolus aldrichetti* from the gill of mullet *Aldrichetti forsteri* was also included in the panel. The non-target environmental isolate panel consisted of template DNA from wild caught adult striped trumpeter, *Latris lineata* (primary host); rainbow trout *Oncorhynchus mykiss*; greenback flounder *Rhombosolea tapirina*; damsel fish *Acanthochromis polyacanthus*; and blue throat wrasse *Notolabrus*

tetricus (primary host). All fish except for wild striped trumpeter and wild blue throat wrasse were spawned and cultured at the School of Aquaculture, University of Tasmania, Launceston, Tasmania, Australia. Template DNA from the polychaete worm *Galeolaria caespitose* was included as a potential alternative host representative.

4.3.4. Sensitivity

Sensitivity of the PCR was initially determined in the laboratory using a serial dilution of *K. neurophila* DNA to find the lowest limit of detection of the target organism. Spores were counted using a haemocytometer prior to DNA extraction. DNA was extracted from 1000 spores and resuspended in 100 μl of buffer provided ($10 \text{ spores } \mu\text{l}^{-1}$) followed by 10-fold dilutions in sterile distilled water (1 and 0.1 spore μl^{-1} respectively). Serial dilutions were used to determine the lowest number of spores able to be detected by producing a response from a 25 μl PCR that could be easily visualised on a gel.

Sensitivity dilutions were also seeded with striped trumpeter DNA extracted from 25 mg of uninfected brain tissue to simulate infected fish and determine any inhibitory effect of the fish DNA in the PCR reaction. To further test the sensitivity of the PCR for use in the hatchery, 3 samples of uninfected striped trumpeter brain weighing 25 mg were seeded with 1000, 100 and 10 spores respectively prior to DNA extraction. DNA was resuspended in the standard 100 μl of buffer provided to produce 10, 1 and 0.1 spore μl^{-1} respectively for use in the PCR reaction. Positive controls for PCR reactions included template DNA from infected striped trumpeter brain and spinal cord samples. These fish were histologically determined to be heavily infected with the parasite in the terminal stage of infection by hematoxylin & eosin (H & E) stained brain sections and wet squash

preparations using a light microscope. Negative controls in PCR reactions included template DNA from the brain tissue of wild caught adult striped trumpeter, determined to be uninfected by histology.

4.3.5. PCR reaction and thermo cycling

All PCR reactions were carried out in 25 µl volumes consisting of 18.6 µl sterile distilled H₂O, 2.5 µl 10 x PCR buffer (1x), 1.75 µl 50 mM MgCl₂ (3.5 mM), 0.5 µl 10 mM dNTP (0.2 mM), 0.25 µl of each primer (50 pmol µl⁻¹, 12.5 pmol), 0.2 µl (1 unit) Platinum[®] *Taq* DNA Polymerase (Invitrogen Australia, Mount Waverley, Victoria) and 1 µl of template DNA. Amplifications were performed using an MJ Research PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, Massachusetts, USA). DNA was denatured for 3 min at 95 °C. Twenty nine amplification cycles were performed as follows: 30 sec at 94 °C for denaturation, 45 sec for primer annealing at 63.2 °C, and 1 min for Taq extension at 72 °C. Four µl of the PCR product were electrophoresed on a 1% agarose gel then stained in a water bath containing 0.4 µg ml⁻¹ ethidium bromide stock solution in distilled water for 30 min and visualised over a UV transilluminator box. PCR products were purified using a QIAquick PCR purification kit (QIAGEN Inc., Valencia, California) following the manufacturer's protocol. Sequencing was carried out by Supamac Laboratories, Sydney, Australia on an ABI 3700 automated sequencer for positive identification by comparison with the *K. neurophila* ssu rDNA gene sequence.

4.3.6. PCR efficiency

The efficiency of the PCR could not be tested against striped trumpeter owing to 100% infection rates which were easily detected using all diagnostic methods. A local wild fish species, blue throat wrasse, *Notolabrus tetricus* inhabits the area surrounding the hatchery water intake. This species was found to be infected by *K. neurophila* at a low prevalence compared to cultured striped trumpeter juveniles (see Results section 4.4.5). Due to the low prevalence of infection and comparatively low susceptibility to the disease, the blue throat wrasse was chosen as an appropriate model for testing PCR efficiency compared to traditional histological techniques. Fourteen sample fish were caught by trap and handline from around the sea water intake. Following euthanasia, brains and spinal cords were collected. One 25 mg brain sample was placed in 95% ethanol for PCR, another sample placed in 10% neutral buffered formalin (NBF) for histological examination (H & E stained 5 µm section) and a final wet preparation sample was squashed in a drop of filtered, sterilised saline solution and viewed under a light microscope. A positive or negative result was recorded for wet preparation and H & E stained sections by the presence or absence of the mature spore stage in CNS tissue. DNA taken from a fin clipping of a disease free blue throat wrasse was used as a negative control for PCR. Positive PCR samples were sequenced and compared to the *K. neurophila* ssu rDNA gene sequence.

4.3.7. Detection of earlier life stages

To detect early life stages of the parasite within the fish host, PCR was used on a cohort of winter spawned cultured juvenile fish. Unsampled fish from this group subsequently

developed 100% infection, displaying mature spores in wet squash preparations and in the terminal stage of the parasite life cycle at 136 dph. Sample fish were stored in 95% ethanol refreshed weekly prior to use. Template DNA was extracted from the fish at 23, 44, 77 and 136 dph (n = 20 at each time point). Remains of whole fish from 23 and 44 dph following wet-squash preparation and sampling for H & E were used to extract DNA and ≤ 25 mg brain samples were used for the fish at 77 and 136 dph. DNA was re-suspended in 100 μ l and stored at -20°C prior to use.

4.3.8. *Epizootiology incorporating PCR use in and around the hatchery*

Following development, the PCR test was used to determine the presence and infection potential of the parasite within and around the hatchery. This information was used to facilitate health management decisions to prevent the pathogen from entering the hatchery such as the installation of an effective water treatment system.

Incoming untreated sea water, thought to be the source of the infectious actinosporean stage of the disease, was filtered to 1 μm using a microfibre filter bag (Filter Specialists Inc. Michigan, IN, USA). DNA was extracted from 25 mg filtrate samples and subjected to PCR. Incoming sea water treated with sand and cartridge filters, foam fractionation, ozonation, UV sterilisation and charcoal filtration was also filtered to 1 μm and DNA was extracted using the same technique described above. Samples (n = 56) were collected and analysed by PCR every week throughout the spawning season extending from June to December 2003 to determine any possible source of the infectious stage of the parasite and to gauge the bio-security efficacy of the installed water treatment system. Filtrate samples from both the untreated sea water 1 μm filters and the treated sea

water 1 μm filters were examined daily from June to December under light microscope for the presence of actinospore-like organisms entering the hatchery from the aquatic reserve.

Rotifer and artemia cultures, prepared with treated sea water (described above) further filtered to 0.2 μm , and used as prey species for juvenile fish, were routinely sampled, examined under light microscope and tested using PCR once every week.

Ten species of wild fish thought to act as other fish hosts for the parasite were collected using trap and handline within the boundary of aquatic reserve and around the sea water intake from August 2003 to February 2004. Fish species collected during the sampling period included:

<i>Meuschenia freycineti</i> , 6 spined leather jacket	n = 5
<i>Notolabrus tetricus</i> , blue throated wrasse	n = 14
<i>Pictilabrus laticlavius</i> , senator fish	n = 7
<i>Latridopsis forsteri</i> , bastard trumpeter	n = 1
<i>Nemadactylus macropterus</i> , jackass morwong	n = 4
<i>Meuschenia australis</i> , brown striped leather jacket	n = 2
<i>Notolabrus fucicola</i> , purple wrasse	n = 2
<i>Acanthaluteres vittiger</i> , toothbrush leather jacket	n = 1
<i>Neosebastes scorpaenoides</i> , ruddy gurnard perch	n = 1
<i>Platycephalus bassensis</i> , sand flathead	n = 16

Organ and skeletal musculature samples were taken and subject to histological examination (H & E stained sections), wet squash preparation and brain samples (25 mg)

were collected from every fish, stored in 95% ethanol prior to DNA extraction and subjected to the PCR diagnostic assay.

Invertebrates such as oligochaete and polychaete worms, thought to represent potential alternative hosts for the parasite life cycle and responsible for releasing the infectious actinosporean stage, were collected from June 2003 to February 2004 from the hatchery system, from the tidal zone of the neighbouring aquatic reserve and from immediately around the sea water intake situated within the aquatic reserve 150 m from the shore and in 5 m deep water. Worms within the hatchery system were collected from the sea water inlet pipes, 10 000 litre header tanks, inlet pipes throughout the hatchery, fish holding tanks within the hatchery, drainage systems and the hatchery drainage outlet pipe located on the shore at the edge of the aquatic reserve approximately 500 m from the sea water intake. Two types of oligochaete and 4 types of polychaete worms were sampled. Only 1 polychaete, *Galeolaria caespitose*, was identified to species level. Approximately 1500 worms were sampled from each species. Each worm was sectioned into 3 equal pieces comprising the head, middle and tail. A small sample was removed from each section and prepared as a wet squash preparation in a drop of saline and examined for potential myxozoan infection. Invertebrates suspected to be infected were photographed and subject to PCR testing. Invertebrates infected with other parasitic species were also photographed and subject to PCR. Four main colonies of worms consisting mainly of single species were examined. Colony 1 consisted of an unidentified polychaete forming a dense colony carpeting the drainage system beneath the broodstock conditioning and juvenile rearing tanks within the hatchery. Colony 2 consisted of an unidentified marine oligochaete worm found only in the 2x 10 000 litre header tanks

feeding sea water to the hatchery. Colony 3 consisted of the polychaete worm *G. caespitose* found in dense colonies along the tidal zone of the aquatic reserve. Colony 4 consisted of an unidentified marine polychaete found in colonies in very close proximity to the sea water intake. Large numbers (n = 334) of another free living oligochaete worm was found living with colony 2. A smaller number (n = 92) of an unidentified free living marine polychaete worm was also found living amongst the 4 main colonies examined. These miscellaneous, non-colony-forming worms were also examined as described above.

To determine the infection status of adult striped trumpeter used for broodstock, a total of 56 large wild adult striped trumpeter caught by professional fishermen from various locations off the east coast of Tasmania were subject to PCR testing, histological examination and CNS tissue wet squash preparations following removal of the fillet for public consumption. A further 23 specially selected broodstock fish previously sourced from Tasmanian waters and conditioned within the hatchery for at least 18 months were also examined as described above.

4.4. Results

All 20 primer combinations from the 12 primers designed and tested for this assay produced PCR fragments of the predicted size that were 100% identical to *K. neurophila* ssu rDNA gene sequence. There were no non-specific products visualised on 1% agarose gel (Appendix 3.2). Subsequent validation of each primer pair resulted in the elimination of unsuitable combinations. These combinations failed specificity testing by amplifying PCR products from closely and further phylogenetically removed myxozoan template DNA which, when visualised appeared identical in size to the *K. neurophila* target fragment. Some combinations produced many non-specific amplicons when tested against the template DNA of the closely related species panel and non-target environmental isolates panel. The non-specific amplification could not be satisfactorily removed through optimising thermocycling or PCR reaction conditions. The PEN3F-PEN4R primer pair (sense primer PEN3F 5'–GA CCC ATC AAA GAC TCA CTA–3' and antisense primer PEN4R 5'–CC AAA GCC GAAACA CTA GGT–3') proved to be the most successful and displayed a specific single DNA fragment, consistently able to produce a 188 base pair PCR product fragment 100% identical to *K. neurophila* sequence from infected host CNS tissue (Appendix 3.3).

4.4.1. Specificity

The PEN3F-PEN4R primer combination, when further tested against template DNA from closely related and a local myxozoan species showed positive single DNA fragment specificity to *K. neurophila* under the optimized PCR reaction and thermocycling conditions. No positive results or non-specific products were visualised for any of the

closely related myxozoan species tested (Appendix 3.3). Specificity of the PEN3F-PEN4R primer combination was further validated when tested against the non-target environmental isolates panel by displaying no amplification against any of the fish or polychaete worm template DNA (Fig. 4.1; Appendix 3.3). This result suggests no interference to the PCR reaction by host or other DNA's tested when using a standard 25 mg sample.

4.4.2. Sensitivity

The limit of sensitivity of the PCR assay from serially diluted preparations, also containing host template DNA and from spiked 25 mg CNS tissue samples prior to DNA extraction, was consistently achieved at an estimated 0.1 spore per 25 μ l reaction (Fig. 4.2). Quantification of *K. neurophila* DNA extracted from known spore numbers and dilutions was shown to be as low as 60 fg per positive PCR reaction. Positive PCR signals for DNA equivalent to 0.1 spore using a standard 25 mg of CNS tissue in each DNA extraction extrapolates to a sensitivity measure approximately equivalent to 4 spores g^{-1} . Although consistency was also achieved at an estimated 0.01 spore per PCR reaction, PCR product intensity visualised under UV illumination varied from very faint to barely detectable following the standard 30 min ethidium bromide staining procedure. Specific positive DNA fragments were 100% identical to *K. neurophila* following sequencing (Appendix 3.4) and no other PCR products or false negative results were observed from the inclusion of uninfected host CNS tissue prior to DNA extraction.

4.4.3. PCR efficiency

Of the 14 blue throat wrasse collected from around the sea water intake, 3 (21%) were shown to harbour mature *K. neurophila* spores in CNS tissue using wet squash preparations. The same 3 fish (21%) were diagnosed as infected following sectioning and H & E staining. The PCR test also showed a positive result for the fish and a positive result for 2 additional fish (36%) previously shown to be disease free by the 2 other diagnostic methods employed (Table 4.1). Overall, the PCR test detected a further 15% of infections from the population sampled and proved 66% more efficient than traditional diagnostic techniques when used on the blue throat wrasse model. PCR products following sequencing were 100% identical to the *K. neurophila* ssu rDNA gene sequence. DNA from disease free blue throat wrasse did not result in false positive signals (Fig. 4.1).

4.4.4. Detection of early life stages

PCR analysis was tested on a 2002 winter spawning cohort of larval and juvenile striped trumpeter (23, 44, 77 and 136 dph) exposed to untreated sea water. The terminal stage of infection with mature parasite spores in the CNS was confirmed in all 136 dph fish by microscopic examination of H & E stained 5 µm sections and wet squash preparations. The few surviving fish were noted to be very heavily infected with the parasite and some fish showed displacement of up to 80% of brain and spinal cord tissue by parasite pseudocysts. All preserved fish with infections of earlier parasite presporogonic life stages undetected in H & E stained 5 µm sections, wet squash preparations or diff-quick

stained brain smears were positive to the *K. neurophila* ssu rDNA 188 base pair fragment when tested against negative and positive controls (Table 4.1, Fig. 4.1).

4.4.5. *Epizootiology incorporating PCR use in and around the hatchery*

Untreated sea water filtrates drawn from the adjacent aquatic reserve were consistently positive using the PCR test whereas sea water filtrates following purification treatments were negative (Table 4.2; Appendix 3.3). Daily examination of untreated water filtrates under light microscope did not reveal the consistent presence of an organism potentially resembling an actinospore.

Prey species cultures of rotifer and artemia sampled throughout the spawning season were consistently negative by the PCR test (Table 4.2) and light microscope examination.

Of the 53 wild adult fish from 10 species sampled from within the aquatic reserve and around the sea water intake, 12 fish from 7 species representing 45 fish in total (27% from the species infected with *K. neurophila*) were PCR positive for *K. neurophila* (Table 4.2). Eight of the 11 species sampled (73% of species), including striped trumpeter, had mature *K. neurophila* infections of the CNS (Table 4.3).

No invertebrates were observed to be infected with a myxozoan life cycle stage by wet squash preparation and examination under light microscope and all suspected infections subject to PCR were found to be negative (Table 4.2). In colony 2, 35% of the marine oligochaete worms sourced from the hatchery inlet header tanks had gut infections of an unidentified nematode worm and 20% were infected with an unidentified new species of gregarine (Protista: Apicomplexa) (Appendix 3.5). Colony 3, *G.*

caespitose from the tidal zone of the aquatic reserve also had nematode gut infections (12%). No other infections were found in any other worm species examined.

Wild collected adult striped trumpeter were negative by PCR and of the 23 broodstock fish tested, 1 fish was found to have a very mild infection with a single cyst visualised in wet squash and positive in PCR (Table 4.2). This adult male fish was collected from the wild and had been held in captivity for 18 months over the 2002 and 2003 spawning seasons. The fish displayed unusual behaviour, fast uncontrolled swimming and suffered acute stress necessitating euthanasia.

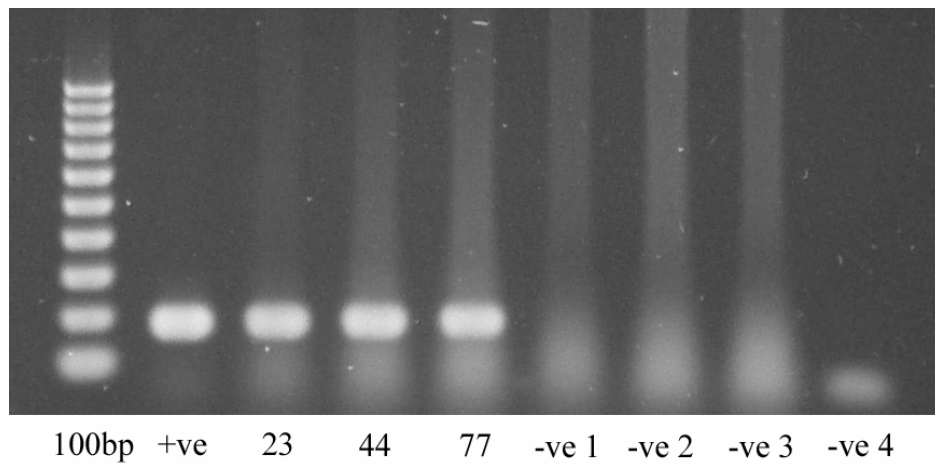


Figure 4.1. Detection of early stages of parasite infection in juvenile cultured striped trumpeter with positive (+ve) and negative (–ve n) controls. The positive control contains DNA from heavily infected fish brains at 136 dph. 23, 44 and 77 refer to the age of the fish in days post hatch. All juvenile fish were from the same cohort. –ve 1 contains uninfected wild striped trumpeter DNA. –ve 2 contains uninfected blue throat wrasse DNA. –ve 3 contains polychaete worm (*Galeolaria caespitose*) DNA. –ve 4 contains no DNA in the PCR reaction.

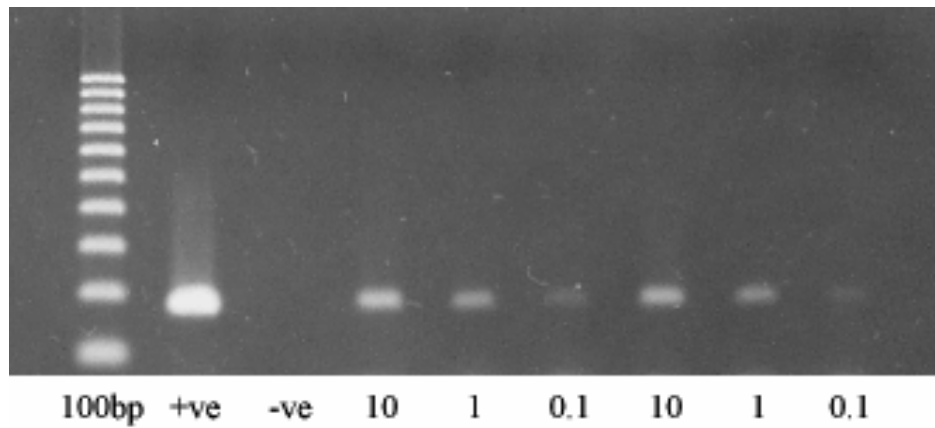


Figure 4.2. Sensitivity with positive (+ve) and negative (–ve) controls. 10 fold serial dilution containing striped trumpeter DNA equivalent to 10 spores to 0.1 spore followed by 25 mg striped trumpeter CNS tissue spiked with mature spores prior to DNA extraction to give 10, 1 and 0.1 spore per 25 µl PCR reaction.

Fish #	Wet	H & E	PCR
1	+	+	+
2	–	–	–
3	–	–	–
4	–	–	–
5	–	–	+
6	+	+	+
7	–	–	–
8	–	–	–
9	+	+	+
10	–	–	–
11	–	–	–
12	–	–	–
13	–	–	–
14	–	–	+
%	21%	21%	36%
23dph	–	–	+
44dph	–	–	+
77dph	–	–	+
136dph	+	+	+

Table 4. 1. PCR efficiency when tested on the blue throat wrasse model and striped trumpeter early life stages. Fourteen adult blue throat wrasse and 20 striped trumpeter from each life stage at 23, 44, 77 and 136 dph were tested for parasites using wet squash preparations (Wet), H & E stained 5 µm sections (H & E) and PCR (PCR). Positive and negative infections are shown as + and – respectively and the percentage of positive infections are calculated at the bottom of each column for the blue throat wrasse model.

PCR SUBJECT	# +VE PCR	# –VE PCR
Pre treated water filtrates	28	0
Post treated water filtrates	0	28
Wild fish species	12	45
Rotifer (early stage prey)	0	28
Artemia (later stage prey)	0	28
Suspected invertebrates	0	15
Wild striped trumpeter	0	56
Adult broodstock fish	1	22

Table 4. 2. The number of PCR positive (# +VE PCR) or negative (# –VE PCR) results from tests conducted in the hatchery and from the adjacent aquatic reserve and sea water intake. Pre and post treated water filtrates and prey species were sampled once per week for 28 weeks over the spawning period from June to December 2003.

Species	Common name	# infected	% infection
<i>Meuschenia freycineti</i>	6 spined leather jacket	1/5	20%
<i>Notolabrus tetricus</i>	blue throated wrasse	5/14	36%
<i>Pictilabrus laticlavius</i>	senator fish	0/7	0%
<i>Latridopsis forsteri</i>	bastard trumpeter	0/1	0%
<i>Nemadactylus macropterus</i>	jackass morwong	1/4	25%
<i>Meuschenia australis</i>	brown striped leather jacket	2/2	100%
<i>Notolabrus fucicola</i>	purple wrasse	1/2	50%
<i>Acanthaluteres vittiger</i>	toothbrush leather jacket	0/1	0%
<i>Neosebastes scorpaenoides</i>	ruddy gurnard perch	1/1	100%
<i>Platycephalus bassensis</i>	sand flathead	1/16	6%
Overall =		12/53	23%

Table 4. 3. Prevalence of *K. neurophila* infections in the 10 species (species and common name) sampled within the boundary of the aquatic reserve, not including striped trumpeter. Including the overall number and percentage of fish determined to be positively infected by using either the PCR test, H & E histology or wet squash preparation.

4.5. Discussion

Traditionally, diagnosis of *K. neurophila* in striped trumpeter depended on microscope examination of H & E stained sections through the CNS, wet squash preparations and giemsa or diff-quick stained smears from brain or spinal cord tissue. All 3 traditional histological techniques rely upon the ability to visualise the distinctive pentagonal features of the parasite in its terminal mature spore stage within the definitive fish host (Appendix 3.6). These traditional diagnostic tools are unable to detect early or presporogonic stages of the parasite, the migratory route it takes through the fish to the CNS or its morphological characteristics as the parasite develops to terminal stage sporulation. Diagnosis of the parasite at the mature spore stage is too late for the hatchery manager to take preventative measures against infection, because by this stage, any surviving fish are 100% infected and will not survive or be of the quality required for research or an intensive aquaculture situation such as sea cage rearing. Traditional methods are also labour intensive, cost inefficient and limited in their overall use as an effective diagnostic and health management tool. The highly specific and sensitive molecular test described utilises PCR to detect ssu rDNA from any parasite developmental stage, particularly in the early stage. It provides a flexible, multiple use tool with diagnostic and health management capabilities which is more cost effective and quicker than traditional methods. The test is also designed to utilise commercially available molecular biology kits that have been shown here to be effective, efficient and reliable when used in the field situation. Myxozoan detection research has determined similar outcomes using the PCR approach to diagnosis and health management for both wild fishery management and aquaculture worldwide (Saulnier & de Kinkelin 1997;

Andree et al., 1998; Kent et al., 1998; Palenzuela et al., 1999; Morris et al., 2002; Yokoyama et al., 2000).

The PCR test was able to specifically amplify a fragment of the ssu rDNA from *K. neurophila* and strong amplicons were always visualised from fish known to harbour the parasite infection at the terminal stage, as determined by traditional histology. The short 188 base pair fragment from the PEN3F-PEN4R primer combination performed with better amplification efficiency to target the ssu rDNA than any other primer combination tested. A similar result was reported by Docker et al. (1997), demonstrating greater efficiency for a shorter fragment to detect the microsporean *Loma salmonae* parasitising chinook salmon, *Oncorhynchus tshawytscha*. The improved sensitivity of 2 primers producing a 272 bp PCR fragment was 3 orders of magnitude more sensitive than another primer set producing a 627 bp PCR fragment.

Following establishment of an acceptable level of specificity to *K. neurophila* ssu rDNA, further testing confirmed the lower limits of detection with the robust PEN3F–PEN4R primer combination. This was shown to be consistent with an estimated 0.1 spore per 25 µl PCR reaction (60 fg DNA, 4 spores g⁻¹ tissue) both in laboratory controlled serial dilutions of spore template DNA and in brain tissues that had been spiked with spores prior to DNA extraction. Although very faint yet consistent products were attained at an estimated 0.01 spore, human error caused by the inability to visualise such a weak signal is likely to result in a false negative outcome. Sensitivity for this diagnostic assay is similar to previous myxozoan PCR based tests developed to date (Kent et al., 1998; Palenzuela et al., 1999; Morris et al., 2002) and is more sensitive than current diagnostic methods. Accuracy in diagnosis is also improved by the ability to process whole fish,

whole brains and spinal cords, whereas techniques such as microscopic examination of 5 µm sections only examines a portion of the targeted tissue introducing greater probability of error. Sensitivity was not compromised by the addition of host DNA or by seeding uninfected host tissue with known concentrations of spores prior to the DNA extraction process. This signifies that the presence of host DNA does not interfere with the PCR process and hence does not produce non-specific amplification or false positive results in controlled seeded matrix combinations of parasite and host DNA (Fig. 4.2).

By using the blue throat wrasse as a useful test model, we were able to demonstrate the PCR method to be 66% more efficient when compared to routine traditional histological techniques. Thus, future diagnostics for health management purposes will have increased accuracy and no longer need to rely entirely on traditional histological confirmation.

Twenty three, 44 and 77 dph fish that displayed no sign of infection when microscopically examined using H & E stained sections, diff-quick stained spinal cord smears or wet squash preparations were found to be infected using the PCR test. The ability of this test to detect previously undetectable presporogonic life stages or possible light infections of the mature spore stage of the disease in an efficient and cost effective manner is of invaluable benefit and provides the aquaculture hatchery manager with a powerful diagnostic and decision making tool. Disease prevalence data collected will be used in ongoing epizootiological studies to determine periods of highest infection risk. A controlled spawning strategy, such as phase shifting spawning events to times outside a window of highest infection risk, may prove to be a contributing and effective health management strategy against this disease.

As part of the ongoing health management process, the PCR test has been used to screen wild adult fish and mortalities among wild brood stock fish caught and conditioned in the same water believed to be the possible source of the actinosporean or infectious stage of the disease. So far, with the exception of a single broodstock fish, adult wild fish and brood stock fish exposed to untreated sea water and possible infectious stages have been found to be free of the parasite when examined microscopically and with the PCR diagnostic assay. It is unknown if the brood fish contracted the infection in the hatchery during its 18 months stay or in the wild. If the parasite was contracted within the hatchery, susceptibility to disease was very low in comparison to heavily infected juvenile fish held under the same conditions during this time. This may suggest that striped trumpeter are only susceptible to heavy infection in the larval or juvenile stages or can be infected at low levels of intensity as adults. Alternatively, juvenile fish with very low levels of infection survive in the wild to adulthood as seen in the local adult wild fish species. The PCR result indicating very low prevalence of the infection in adult wild striped trumpeter used for broodstock does not point to these fish as being the source of the disease. The developmental point at which hatchery fish are no longer susceptible will require determination through attempted transmission of infection to naive age classes so that a strategic reduction in the costly anti-parasitic health management measures can occur and fish can be conditioned to natural sea water prior to sea cage rearing.

To gain a more complete biological and epizootiological understanding of the parasite and the disease it causes, the PCR was field tested within and outside the hatchery system. The results confirm another 7 species of fish resident within the aquatic

reserve and inhabiting the area around the sea water intake for the hatchery were infected with *K. neurophila*, albeit at lower prevalence and infection intensity than cultured juvenile striped trumpeter. Further positive PCR results from the incoming water filtrates and negative PCR results from within the hatchery suggest that the parasite occurs naturally in some species of wild fish within the reserve, and the infectious actinosporean stage of the parasite released from an alternative invertebrate host is being drawn into the hatchery system from this area. In effect, the aquatic reserve and some species of fish and invertebrates resident therein act as the reservoir for the parasite and the hatchery is providing the conditions necessary for the disease to flourish amongst the juvenile cultured fish. As a result, the health management decision to treat water before entering the hatchery system was implemented and the PCR is applied to pre- and post-water treatment filtrate samples to gauge the efficacy and bio-security of the water treatment system. However, it is possible that the use of PCR may detect DNA from presumptive actinosporean stages in the incoming water as well as non-living life stages of *K. neurophila* in the stomach contents of prey species or pre-actinosporean parasite stages in infected alternative hosts. Application of the tool in this form also raises the question of false positives, which are likely due to limitation of PCR specificity against every possible environmental isolate entering the hatchery (Hiney & Smith 1998). Positive results from filtrates would require sequencing to further validate the result and this approach may be time consuming and cost prohibitive. However, testing water filtrates pre- and post-treatment provides an effective broad screening technique. The negative PCR results in post-treatment filtrates indicates that the water treatment system eliminates the possibility of a potential infectious actinosporean stage of the parasite

entering the hatchery during the spawning and larval rearing period when it is believed that the fish are most at risk of infection. Negative results from post-treatment filtrate samples have provided the information required for implementing effective disease management. They have also shown that the infectious stage may be present in the incoming water throughout the entire current spawning season, warranting continued surveillance and maintenance of the water treatment system.

To further understand the biology of this parasite, the PCR assay will continue to be used to extensively screen invertebrate populations in and around the hatchery and water intake to test for the presence of an infected alternative host. PCR results have previously been successfully used to link myxozoan life stages on a molecular level to initially determine possible alternative host candidates and also used to add evidential weight to transmission and life cycle studies (Andree et al., 1997; Bartholomew et al., 1997; Anderson et al., 1999; Lin et al., 1999; Anderson et al., 2000; Pote et al., 2000; Negredo et al., 2003). By elucidating the life cycle of *K. neurophila* through incorporating ssu rDNA sequence comparison we hope to increase knowledge on the biology of marine myxozoans, as only a single marine life cycle has been discovered so far (Køie et al., 2004). Additionally, we also hope to provide further evidence of the exact location of the disease reservoirs.

Presporogonic morphological development of this histozoic parasite once it enters the fish and up to the terminal mature spore stage encapsulated within plasmodia in the CNS is still not clear. As part of pathology studies on this disease, the oligonucleotide primers developed for this assay are currently being incorporated into an *in situ*

hybridisation technique to determine the route of entry and migratory development in the definitive fish host.

Myxozoan parasites of fish have the potential to cause problems among wild fish stocks and aquaculture. Molecular detection of the pathogen as a routine diagnostic tool has proved to be the most efficient and effective means of diagnosis available (Kent et al., 2001). Not only can this molecular technique be used for efficient and effective diagnosis, it has wider application extending beyond basic diagnostics as a flexible multiple use tool. It also has the added ability to perform biological and epizootiological research and ultimately facilitate correct implementation of health management decisions for the hatchery manager and researcher in an aquaculture situation.

This chapter described the development of a molecular diagnostic assay and the epizootiology of the disease. Some of the data has been submitted for review and accepted for publication (see Appendix 6.3). The oligonucleotide primers developed here were used for *in situ* hybridisation studies to investigate entry locus pathology and contribute to the last research chapter of this thesis.

Chapter 5

Histopathology and *in situ* hybridisation techniques to describe the myxozoan central nervous system disease of striped trumpeter, *Latris lineata*, and trace the migratory pathway of the agent *Kudoa neurophila* (Myxosporea: Multivalvulida).

5.1. Abstract

The myxozoan parasite *Kudoa neurophila* causes brain disease in the cultured marine fish striped trumpeter, *Latris lineata* in Tasmania, Australia. Histopathology and *in situ* hybridisation (ISH) were used to study the pathology caused by this parasite, from its entry point on the fish host and, via the migratory pathway, to terminal stage sporulation. *Kudoa neurophila* entered the fish host via epithelial cells as early as 25 days post hatch (dph). Parasite cells then appeared in the skeletal muscle between 50 – 80 dph. At this point, the parasite cells undergo presporogonic proliferation within a multicellular plasmodium. The fish start to display clinical signs such as whirling as the multicellular plasmodia develop along peripheral nerve pathways, continuing to proliferate on a migratory route toward the final site of infection (70 – 115 dph). The presporogonic stages enter the spinal cord via the connecting peripheral nerves where terminal stage sporogonic plasmodia develop throughout the brain and spinal cord causing a severe granulomatous pan-meningoencephalomyelitis between 105 – 130 dph. Host response in the central nervous system (CNS) is limited and plasmodial development in the major brain divisions of the CNS is most prolific in the spinal cord, medulla oblongata and optic lobes.

5.2. Introduction

Histozoic marine myxozoans from the genus *Kudoa* are increasingly becoming economically important parasites of aquaculture and commercial fisheries species (Moran et al., 1999a & b; Kent et al., 2001; Dyková et al., 2002; Grossel et al., 2003; Whipps et al., 2004). Although members of the *Kudoa* family are not generally associated with mass mortalities in aquaculture and fisheries (Lom & Dyková 1995), their detrimental effect is associated with product quality, as infections are more often associated with muscle tissue, causing unsightly cysts in commercial species such as Atlantic herring (Lom & Dyková 1992) or post-mortem myoliquefaction in farmed Atlantic salmon (Whitaker & Kent 1991). *Kudoa* infections are not limited to muscle tissue and 7 species have been reported to infect the CNS of marine finfish. The 4 valved CNS *Kudoa* include; *K. cerebralis* (Paperna & Zwerner 1974), *K. tetraspora* (Narasimhamurti & Kalavati 1979), *Kudoa* sp. (Langdon 1990) and *K. paralichthys* (Cho & Kim 2003). The 3 reported >4 valve CNS *Kudoa* include; the 7 valve *K. yasunagai* (Yasunaga et al., 1981), the 6 valve *Kudoa* sp. (Egusa 1986a) and the 5 valve *K. neurophila* (Grossel et al., 2003). The latter 3x >4 valve *Kudoa* species were formerly classified as *Pentacapsula*, *Hexacapsula* and *Septemcapsula* largely based on morphological criteria (Lom & Arthur 1989; Lom & Dyková 1992). However, they have recently been found to be very closely related to other *Kudoa* species by comparative ssu rDNA sequence analysis (Whipps et al., 2004) resulting in the redescription of the multivalvulidans.

Striped trumpeter is a marine finfish currently undergoing development for large scale commercial sea cage aquaculture in Tasmania, Australia (Battaglione et al., 2003). The histozoic multivalvulidan marine myxozoan *Kudoa neurophila* (Grossel et al., 2003;

Chapter 2; Chapter 3) causes a significant CNS disease threatening the production of large quantities of juveniles for on-growing and further research by causing losses of close to 100%. Spawning events are phase shifted and can occur from May through to December within a purpose built marine finfish hatchery. Records of every spawning season from 1994 to 2003 show that fish surviving through the larval development stage display signs of a disease associated with the nervous system from around 70 dph, previous records also indicate older fish display granulomatous myxozoan pathology. Generally, by 130 dph all surviving fish from each spawned cohort show advanced CNS pathology with heavy infections of terminal stage myxozoan sporogonic plasmodia. In some cases, brain and spinal cord histology shows pseudocysts and associated host reactions displace up to 90% of neural tissue, and although the few surviving fish seem to function adequately in culture with some 4 to 5 year old fish now reaching sexual maturation, they are all heavily infected with the parasite, do not function to their full biological potential and are not considered to be of the quality required for grow-out in sea cages or further research.

This report describes the pathology of the brain disease caused to striped trumpeter by *K. neurophila*, together with aspects of the migratory and developmental pathway taken by the parasite. From the entry point into the fish host, to presporogonic development in the skeletal musculature and peripheral nerve pathways, to sporulation in the brain and spinal cord.

5.3. Materials and methods

5.3.1. *Animals and histology*

As well as collation of the pathology from archived materials, histopathology and entry point pathology was also assessed under controlled conditions. Animals collected for parasite entrance point determination using *in situ* hybridisation (ISH) were spawned in November 2003. 2500 fish were naturally challenged with the pathogen by exposure to untreated sea water drawn from the neighbouring aquatic reserve. Negative control fish were reared in the same facility and held under a strict bio-secure water treatment system utilising filtration, ozonation and UV sterilisation to deliver pathogen free water. Twenty fish from each group were sampled daily for 29 days at which point no fish remained due to the difficulties encountered in breeding this species for longer periods. Small fish, up to 150 dph, used for presporogonic pathology were sourced from hatchery stock and fixed whole in 10% neutral buffered formalin (NBF) at 4 °C for 2 days then stored in 95% alcohol prior to wax embedding for histopathology or PCR testing (Grossel et al., 2005; Chapter 4). Brains and spinal cords used for terminal stage sporulation pathology were removed from 1, 2 and 5 year old fish (n = 12) and fixed as described above.

Following sampling for PCR and wax embedding, whole fish for presporogonic pathology were sectioned along the dorsal plane at 3 µm, stained with hematoxylin and eosin (H & E) and mounted for microscopic examination. A 6 µm section was also taken and mounted on a 3-(aminopropyl)triethoxysilane (APES) coated slide for ISH (described below). Brains used for terminal stage sporulation pathology were separated into 5 major anatomical divisions; telencephalon (forebrain); mesencephalon (optic lobes); diencephalon (mid brain); metencephalon (cerebellum) and myelencephalon

(medulla oblongata). Spinal cords were sampled in 3 sections; a 1 cm long section from immediately behind the medulla oblongata (head); a 1 cm section from the middle (mid); a 1 cm section from the tail end (tail). The 5 brain divisions were cut in half along the transverse plane and spinal cord sections were cut in half along the dorsal plane. From these sections a single 3 μ m section was cut and stained with H & E and mounted for observation.

To determine CNS tissue tropism and host response at the terminal stage of infection throughout each section, individual plasmodia and pseudocyst numbers were recorded for each section taken from the 5 brain divisions and an average recorded from the 3 spinal cord divisions previously described. An immunological response from the host was characterised by the formation of a granuloma or host cell encysted plasmodia containing electron-dense material. The number of positive responses was recorded to measure the relationship between pseudocyst numbers and host response from each brain division. Archival records were retrieved for all cohorts of cultured juvenile fish extending from 1994 to 2003 and clinical signs of disease relating to nervous aberrations were recorded from cohorts of fish surviving for periods of time over 30 dph and up to 100 dph. The cohorts were spawned at various times throughout the phase shifted 8 month season extending from May to December each year and encompass 11 cohorts of fish surviving to 100 dph. Fish were noted as suffering a nervous disorder if they were found to swim in a whirling pattern or displayed general loss of spatial control. Over this period, wild caught adult broodstock fish had been collected from waters around Tasmania and held in the hatchery for domestication and conditioning for spawning over several years. The adult fish were exposed to the same untreated sea water and conditions

as all infected juvenile cultured offspring. Twenty-three broodstock at the end of their productive life were examined for *K. neurophila* caused infection by a combination of wet squash preparation, H & E stained brain sections and PCR to determine the susceptibility of adult fish to the pathogen. Tissue tropism in the anatomical CNS divisions for fish in all age and size categories was compared using chi-square analysis ($P < 0.01$).

All light microscopy was performed using an Olympus® IX71 inverted research microscope (Oakleigh, Vic, Australia) and all images were taken with an attached Optronics Magnifire 2.0 camera (Goleta, CA, USA).

5.3.2. *In situ* hybridisation technique

The 20 samples per day collected from both the natural infection challenge and negative control group were fixed and stored in 95% ethanol prior to wax embedding for ISH or diagnostic analysis using PCR. Six µm sections were cut and mounted on APES coated slides. Sections were deparaffinised and rehydrated in graded ethanol solutions followed by equilibration in tris-buffered saline (TBS, pH 8). Sections were permeabilised with 50 µg ml⁻¹ of proteinase K (Ambion, TX, USA) in TBS, pH 8 for 15 min at 37 °C, washed for 5 min in phosphate-buffered saline (PBS, pH 7.2) and post-fixed for 15 min at 4 °C with 0.4% paraformaldehyde in PBS. Sections were then rinsed in sterile distilled water (sdH₂O) and immersed in 10% H₂O₂ in methanol for 10 min to stop non-specific peroxidase binding. The H₂O₂ was washed off in sdH₂O and sections were dried at 45 °C. Two oligonucleotide primers specific to *K. neurophila* small subunit ribosomal DNA, PEN3F and PEN4R (Grossel et al. 2005), were 5' end labelled with biotin (Geneworks,

Hindmarsh, S.A., Australia). The sections were encircled with a liquid repellent slide marker (Daido Sangyo, Tokyo, Japan) followed by addition of the hybridisation buffer (4x saline-sodium citrate buffer (SSC) in TBS containing 0.5% Ficoll, 0.5% polyvinylpyrrolidone, 0.5% bovine serum albumin, 100 $\mu\text{g ml}^{-1}$ calf thymus DNA, and 1.5 $\text{ng } \mu\text{l}^{-1}$ of each oligonucleotide probe). Covered sections were denatured for 5 min at 95 °C followed by hybridisation to parasite DNA for 2 hours at 45 °C. Hybridisation buffer and cover slip were removed by rinsing in 2x SSC followed by a high-stringent wash of 0.1% SSC containing 0.1% TWEEN20 at 45 °C for 15 min to ensure specificity of probe binding. The hybridisation signals were detected using the mRNAlocator™-Biotin kit (Ambion, TX, USA). Briefly, streptavidin incubation for 30 min at 37 °C followed by 3 washes in Tris for 4 min each. Followed by incubation in NBT/BCIP for 2 hours at 37 °C resulting in a purple signal. Sections were then counterstained with 0.5% methyl green for 10 min and dehydrated in a graded alcohol series, transferred to xylene and mounted for light microscopy using an Olympus® IX71 inverted research microscope (Oakleigh, Vic, Australia) (Appendix 4.1).

5.4. Results

All cohorts of fish surviving more than 100 dph from 1994 to 2003 became heavily infected with *K. neurophila* at 100% prevalence, regardless of the time at which they were spawned during the extended 8 month breeding season. Development of the parasite within the fish host occurred in 4 distinctive stages: Stage 1, entry point into the epithelial cells of the skin, gut and gill. Stage 2, presporogonic proliferation in muscle tissue. Stage 3, presporogonic proliferation along peripheral nerve pathways in the skeletal musculature. Stage 4, terminal stage sporogonic development in the brain and spinal cord.

5.4.1. Stage 1: Entry point. Following exposure to the infectious stage of the parasite, positive PCR signals were detected at 23 dph (Appendix 4.2). PCR was negative for *K. neurophila* at 23 dph in the negative control group (Appendix 4.2). The first purple coloured signals from ISH results were detected in the positive control group at 25 dph from the epithelial cells of the skin (Fig. 5.1 A–F), the epithelial cells of the gill (Fig. 5.1 G) and the epithelial cells of the intestinal tract (Fig. 5.2 A–D). Positive signals were detected up to 29 dph in the small larval fish, 5.7 mm \pm 0.7 in size (mean \pm SD), at which point the fish were sampled to extinction.

5.4.2. Stage 2: Presporogonic proliferation in muscle tissue. Large presporogonic plasmodia were then detected with ISH for the first time developing inside skeletal muscle fibres starting at 50 dph (Fig. 5.3 A & B) from fish fixed in cold 10% NBF for 2 days and stored in 95% alcohol prior to wax embedding. Muscle fibre cysts ranged in

diameter from 10 – 50 μm (mean = 20 μm). H & E stained 3 μm sections taken from fish at 50 – 80 dph and from serial sections taken from the same fish used for ISH were shown to have eosinophilic staining, presporogonic proliferative parasite stages within the muscle fibres which were finely granular in appearance (Fig. 5.3 C & D).

5.4.3. Stage 3: Presporogonic proliferation along peripheral nerve pathways in the skeletal musculature. By 70 dph infected fish started to display eosinophilic staining, granular presporogonic plasmodia developing in the peripheral nerve pathways of the skeletal muscle from 15 – 48 μm (mean = 26 μm). As fish advanced in age to 80 dph, plasmodial development within muscle fibres was no longer evident and development was now focussed in peripheral nerve pathways and in closer proximity to the spinal cord (Fig. 5.4 A & B). The onset of clinical signs of disease, such as whirling and loss of spatial control (as recorded from archived records between 1994 and 2002 and including observations from this study in 2003), commenced during this stage at day 72 ± 2.6 (week 10, mean \pm SD) for the 11 groups of fish surviving up to 100 dph since 1994.

5.4.4. Stage 4: Terminal stage sporogony in the brain and spinal cord. Most fish at 115 dph but as early as 105 dph start to display spinal cord pathology with early developmental plasmodial sporogonic stages $12.8 \mu\text{m} \pm 0.3$ in size (Fig. 5.5 A–D). Some fish had a small remnant number of presporogonic cysts in very close proximity to the spinal cord at the same time as very early stage 4 sporogonic development within spinal cord neural tissue until as late as 115 dph. Once the parasite gained entry to the spinal cord and brain it could be found developing throughout all major anatomical brain

divisions, meninges and in all neural cellular layers and cell types within each division (Fig. 5.6 A–D), and in one rare case, in the inner and outer nuclear cells layers of the retina (Fig. 5.7 A). Single plasmodia continue to develop and contain approximately 100 fully mature spores by 130 dph, $48.6 \mu\text{m} \pm 0.6 \mu\text{m}$ in size (Fig. 5.6 A–D). Plasmodia continually increase in size until they eventually join together to form large pseudocysts (Fig. 5.7 B–D). Some cysts attained sizes up to 1 mm or more by continuing to join resulting in the compression and displacement of large areas of CNS tissue and marked vacuolation of the remaining surrounding CNS tissue (Fig. 5.7 C & D). The overall developmental time frame throughout the 4 stages of development from initial infection as larvae to terminal stage sporogony in the definitive fish host is approximately 130 days (18 weeks), with transition from one stage to the next lasting over a short period of approximately 2 weeks.

An immunological response by the host was not detected during the first 3 stages of development. It was not until stage 4 that a response was detected for the first time within the brain and spinal cord at terminal stage sporulation. The host response occurs within all anatomical brain divisions and is predominantly evidenced by host epithelial cells encapsulating parasite pseudocysts which stain deeply basophilic with an electron dense centre of parasite spores (Appendix 4.3). Some plasmodia initiate a granulomatous immunological response comprised of mononuclear epithelioid cells (Fig. 5.6 A & B) with no evidence of granulocyte involvement. Presporogonic stages developed synchronously and were not detected in any other tissues or organs apart from the epithelial cells of the skin, gut and gill, the muscle fibres and the peripheral nerve pathways. No asynchronous developmental stages were found.

The mature parasite displayed a clear preference for anatomical divisions within the CNS regardless of fish age or size ($P < 0.01$, $n = 12$). The centrally located spinal cord, medulla oblongata and optic lobes contained 84% of all parasite cysts recorded from the single 3 μm section cut from the centre of each division (Fig. 5.8). The 3x 1 cm spinal cord segments examined from each fish showed uniform rates of infection throughout the length of the spinal cord. Clearly, the spinal cord contained most of the CNS tissue and if sectioned along its entire length would contain the vast majority of parasite cysts. An average of the 3x 1 cm segments from each fish were used for similar size comparison with the smaller brain divisions. The more peripheral brain divisions, including the cerebellum, mid-brain and fore-brain contained 13.6%, 2%, 0.4% of the total parasite cysts respectively. There was no clear preference for cell type within each major CNS anatomical division described and pseudocysts were found scattered or clumped throughout all parts of the brain and spinal cord. An immunological host response recorded as the formation of an epithelioid cell granuloma or host cell encapsulated pseudocyst was limited in each section within the CNS. Host response counts through each central section examined appeared to reach a level of saturation, with an overall CNS average of 6.1 host responses/central section (Fig. 5.8, no more than 19% overall). At no time did histopathology show breaching of the CNS by parasite cysts, resulting in an intact blood brain barrier and hence no leukocyte infiltration and accompanying inflammatory response within the brain tissue or spinal cord.

A small single cyst (110 μm) was found in one of the 23 wild caught and domesticated adult broodstock fish in a post-mortem wet squash examination and a 30 mg brain tissue sample from this fish was positive to the PCR diagnostic assay.

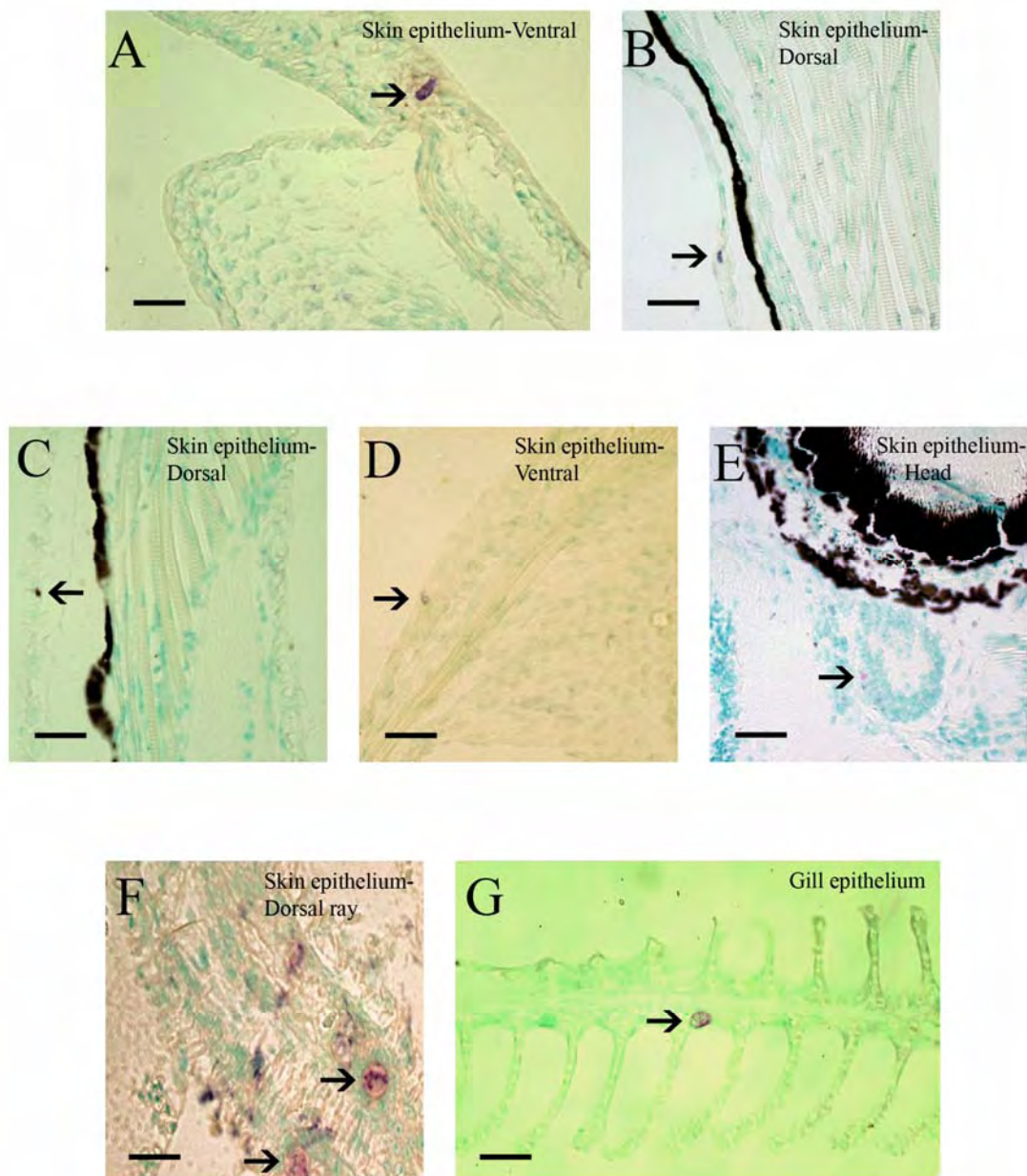


Figure 5.1. Panels A–D, stage 1 entry point ISH signal (arrows) detected at 25 dph from the epithelial cells of the skin on the dorsal and ventral surfaces of the fish (magnification 400X, bar = 10 μ m). Panel E, ISH signal in the skin epithelium of the head, close to the eye (magnification 400X, bar = 20 μ m). Panel F, higher magnification (1000X) of an ISH signal in the epithelial cells of the dorsal ray (bar = 5 μ m). Panel G, ISH signal in the gill epithelium (magnification 640X, bar = 10 μ m).

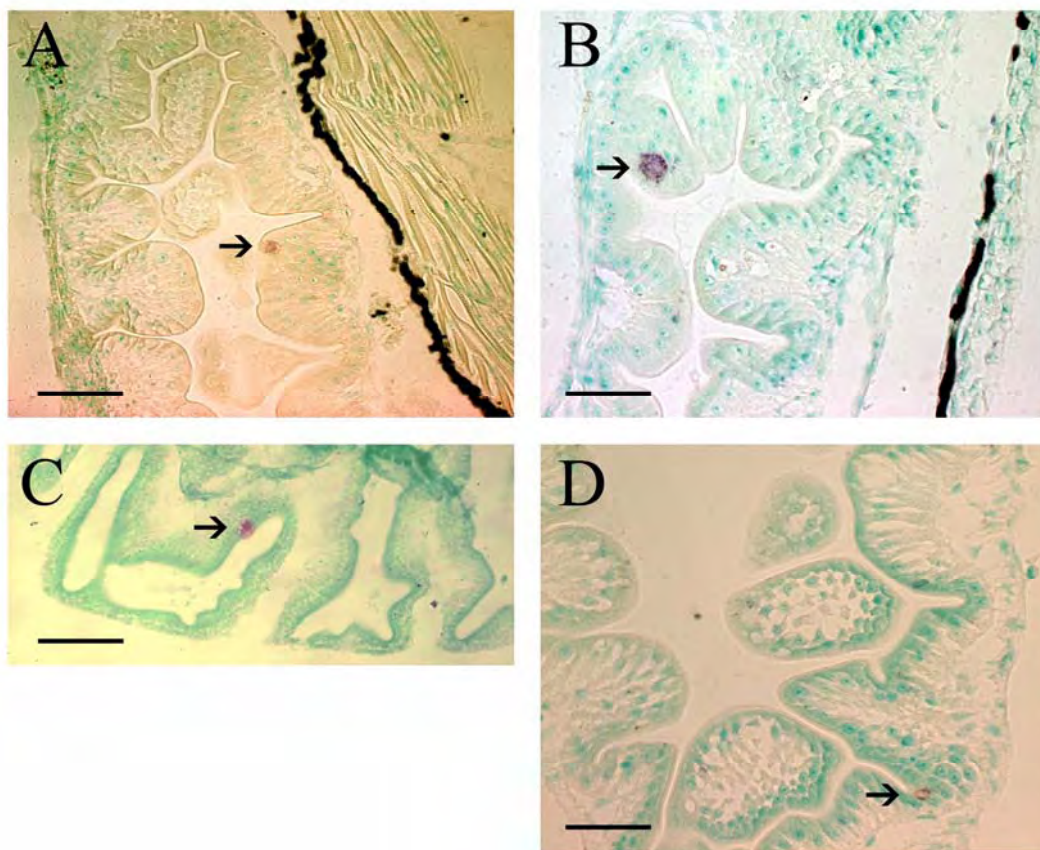


Figure 5.2. Stage 1 entry point presporogonic plasmodia detected with ISH (arrows, purple signal) developing in the epithelium of the intestinal tract in striped trumpeter juveniles at 25 dph. Magnification, 400X for panels A, C & D (bar = 20 μ m) and 640X for panel B (bar = 10 μ m).

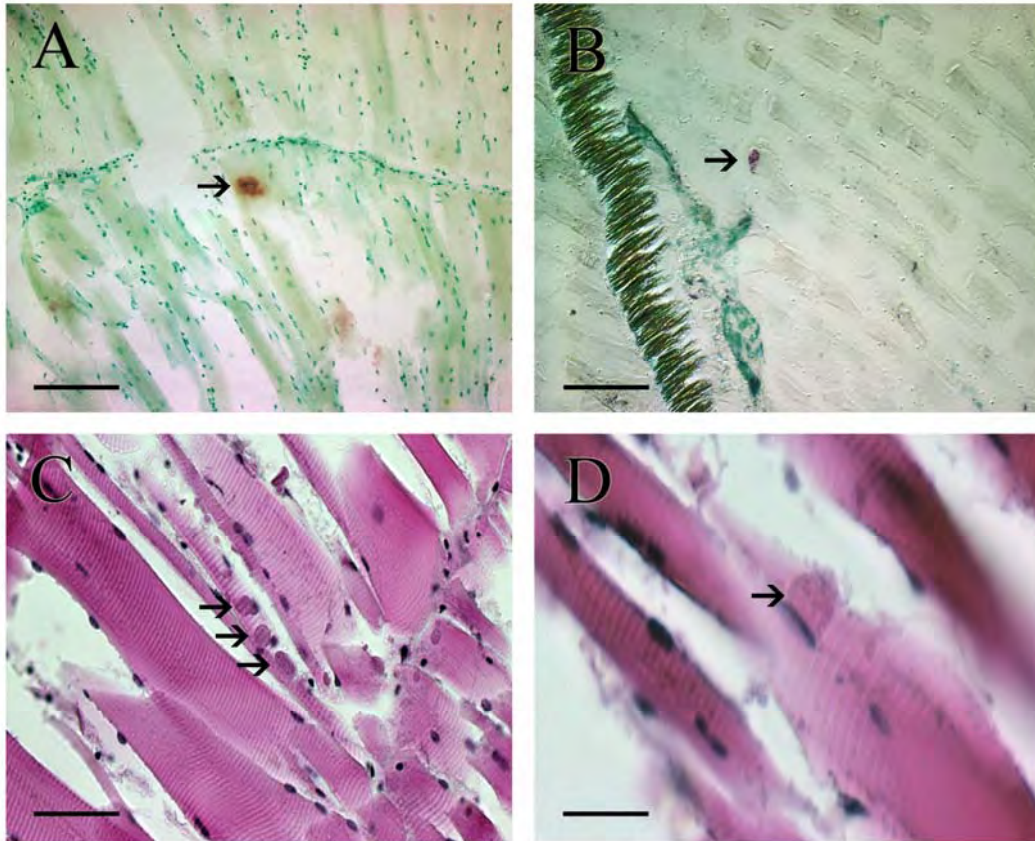


Figure 5.3. Panels A & B, stage 2 presporogonic plasmodia detected with ISH (arrows, purple signal) within the skeletal muscle fibres of juvenile striped trumpeter at 50 dph (magnification 400X, bar = 20 μ m). Panels C & D, stage 2 presporogonic plasmodia seen in skeletal muscle fibres of striped trumpeter at 50 dph (arrows) from 3 μ m sections stained with H & E. Magnification 400X for panel C (bar = 20 μ m) and 640X for panel D (bar = 10 μ m).

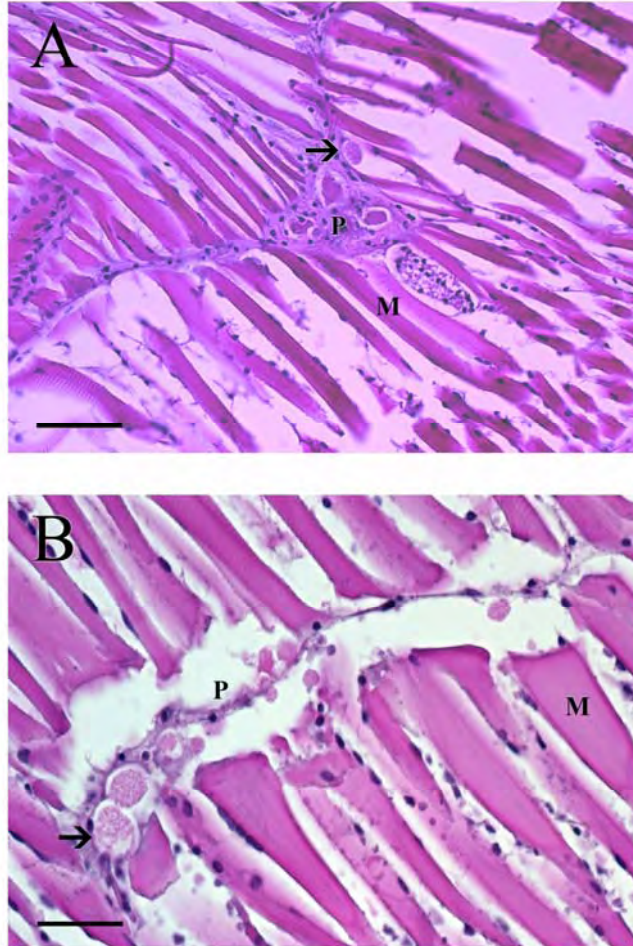


Figure 5.4. Stage 3 H & E stained section of an infected juvenile fish at 84 dph showing granular presporogonic plasmodia (arrows) developing at the peripheral nerve pathways (**P**) of the skeletal muscle (**M**) and in close proximity to the spinal cord. Magnification for panel A, 200X (bar = 40 μ m) and 400X for panel B (bar = 20 μ m).

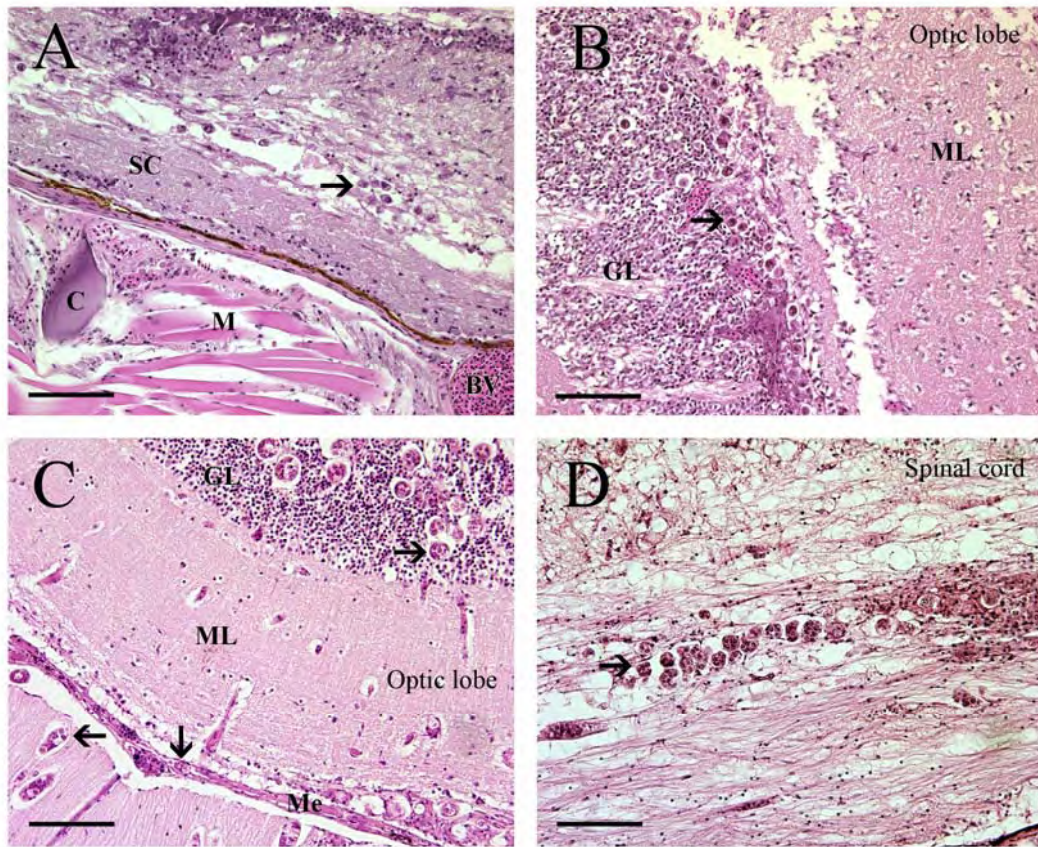


Figure 5.5. H & E stained section of early stage 4 sporogonic development. Panel A, an infected juvenile fish at 102 dph showing very early sporogonic development (arrow, single example) in the spinal cord (SC). Cartilage (C), muscle (M), blood vessel (BV) (magnification 200X, bar = 20 μ m). Panel B, very early sporogonic development (arrow, single example) at 115 dph in the granular layer (GL) of the optic lobe. Molecular layer (ML) (magnification 200X, bar = 20 μ m). Panel C, slightly more advanced sporogonic development (arrows) in all tissue types of the optic lobe at 115 dph. Granular layer (GL), molecular layer (ML) & meninges (Me) (magnification 400X, bar = 10 μ m). Panel D, sporogonic development (arrow) in the spinal cord at 115 dph (magnification 400X, bar = 10 μ m).

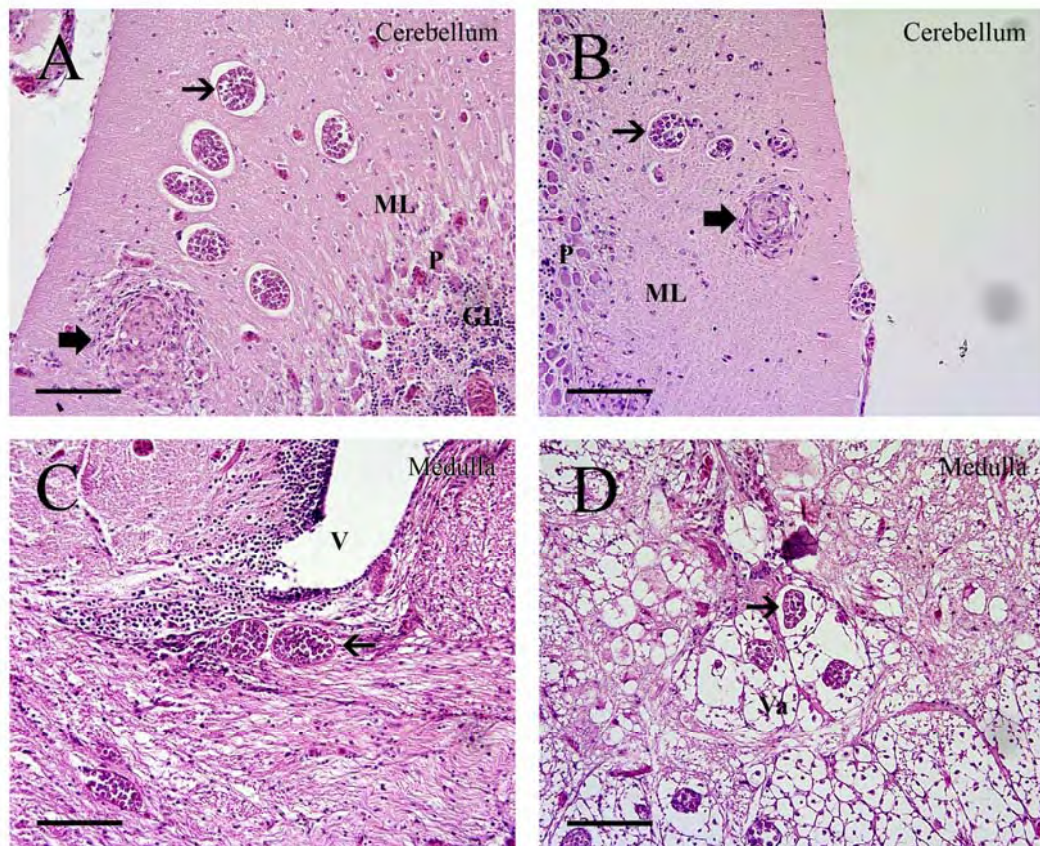


Figure 5.6. Stage 4 development, H & E stained section showing mature spores within individual polysporic plasmodia (small arrows) developing in different sections of the brain (Panels A & B cerebellum, panels C & D medulla oblongata) at approximately 130 dph. A granulomatous immunological response is present (large arrows) in A & B, comprised of mononuclear epithelioid cells. Purkinje cell layer (P), granular layer (GL), molecular layer (ML) & meninges (Me). Panel C shows plasmodia with mature spores in the medulla in close proximity to the ventricle (V) and causing marked vacuolation (Va) in D. Magnification 200X (bar = 50 μ m).

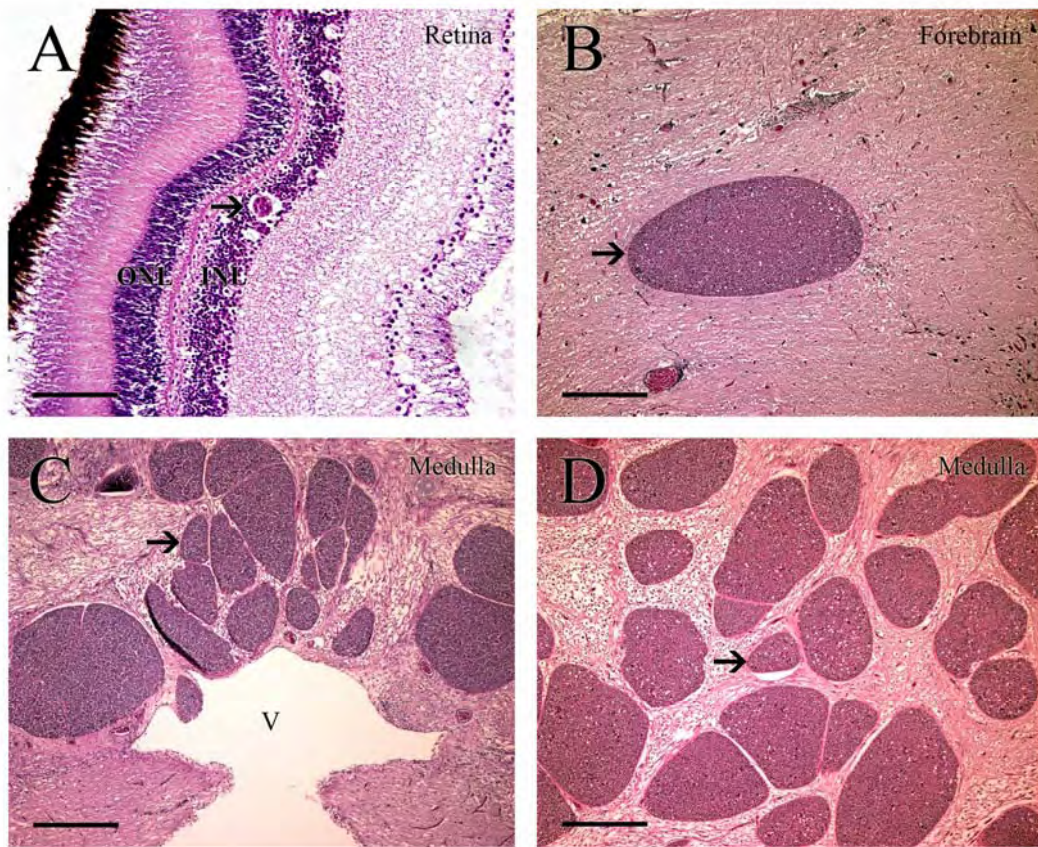


Figure 5.7. Late stage 4 development, H & E stained section showing mature spores within polysporic plasmodia. Panel A, a rare development in the inner nuclear layer (INL) of the retina (arrow) in a 2 year old striper trumpeter. Outer nuclear layer (ONL) (magnification 200X, bar = 50 μ m). Panel B, large joined polysporic plasmodia forming into a single plasmodia containing many mature spores in the forebrain (magnification 100X, bar = 0.1 mm). Panels C & D, plasmodial development (arrows, single example) in the medulla oblongata after 130 dph resulting in the compression and displacement of large areas of CNS tissue and marked vacuolation of the remaining surrounding tissue. Ventricle (V) (magnification 100X, bar = 0.1 mm).

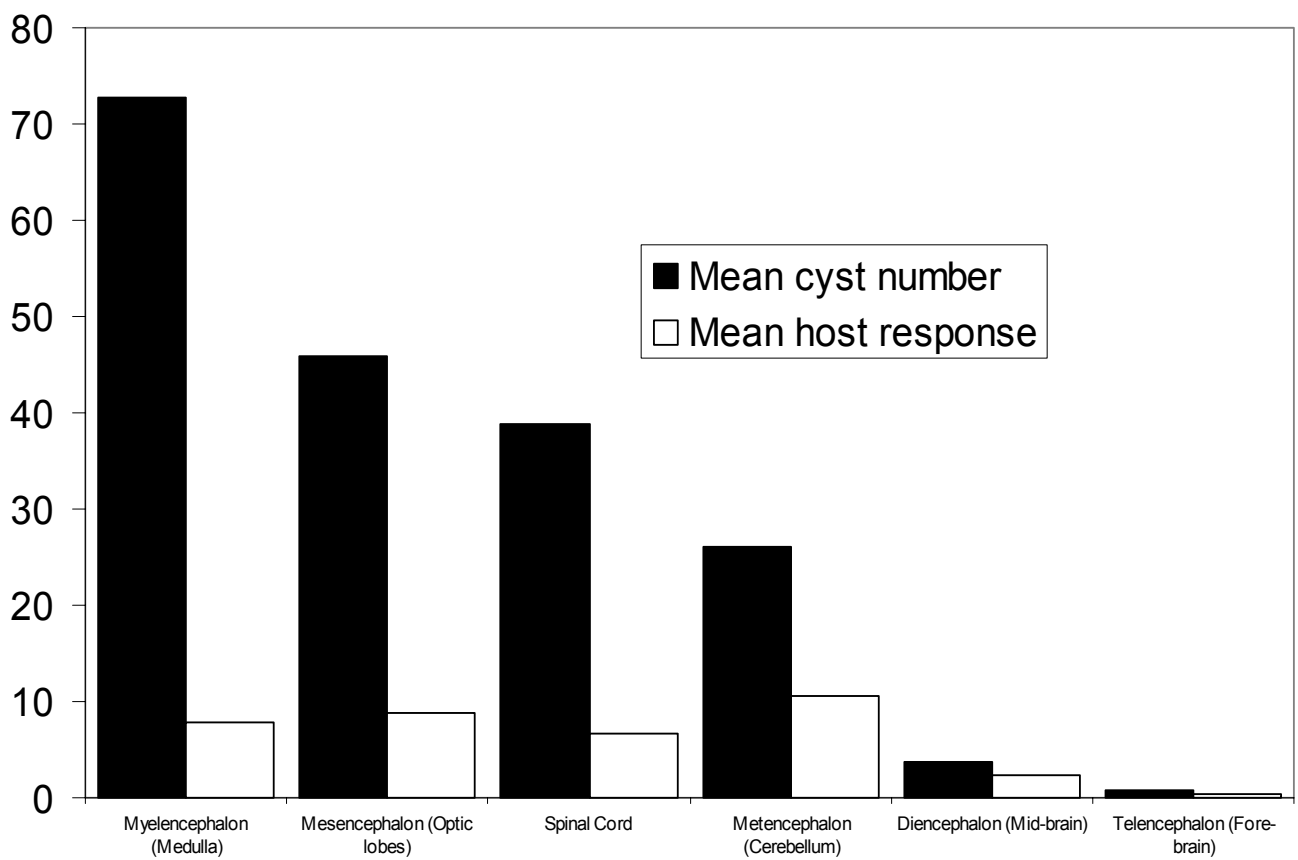


Figure 5. 1. Mean distribution of parasite pseudocysts from the 5 major anatomical brain divisions and spinal cord recorded from each central 3 μ m section including the mean distribution of cysts eliciting a host response in each brain division from 12 heavily infected 1, 2 & 5 year old fish.

5.5. Discussion

An invertebrate host for the marine myxozoan *K. neurophila* is unknown. However, an infectious actinosporean stage is thought to be released by an alternative host and enter the hatchery via the intake water which is sourced from an adjacent aquatic reserve. Epizootiological studies have identified 8 species of fish infected with *K. neurophila* from the environment immediately surrounding the water intake for the hatchery (Grossel et al., 2005; Chapter 4). This evidence suggests that within the aquatic reserve, the parasite life cycle is perpetuated between a number of resident fish species and an alternative host and therefore, the aquatic reserve acts as the infection reservoir. The amount of infectious waterborne actinospores entering the hatchery and the subsequent rate of exposure is unknown. All surviving cultured juvenile fish from phase shifted spawning seasons, extending back to 1994 and exposed to untreated sea water from the aquatic reserve, become heavily infected with the parasite at a prevalence of 100%. This indicates consistent exposure to the infectious stage is occurring within the hatchery system, which in turn is providing the conditions required for increased pathogenicity of the parasite, irrespective of any seasonal variations that may occur within the infection reservoir.

Using the PCR diagnostic assay, larval fish cultured in the hatchery were first noticed to be infected with the parasite at 23 dph. With ISH, infection was first detected between 25 dph and 29 dph. However, earlier infection cannot be ruled out as the fish are still very small (3 – 6 mm in length) and only minute portions of tissue are available for ISH testing. A positive ISH signal at the entry point into the fish was only detected in the epithelial cells of the skin, gut and gill and in relatively small numbers in the very small

fish. The apparent low numbers of actinospores in the hatchery system and subsequent low initial infection in the very small fish larvae suggest the parasite undergoes a highly proliferative stage given the number of spores that finally develop within the brain and spinal cord.

Following entry point of infection, there is a gap in our knowledge of the migratory pathway from approximately 30 – 50 dph. This is largely due to striped trumpeter having an extended larval phase accompanied by very low survival during this time frame (Bransden et al., 2003). Further difficulties are encountered in the ongoing culture of this species and hence there is a lack of samples available from this age class. A blood stage of development in the transition from skin epithelium to terminal stage sporogony within the skeletal muscle fibres has been reported in *K. thyrsites* infections in Atlantic salmon (Moran et al., 1999a). The possibility of a blood stage of *K. neurophila* from 30 – 50 dph, as shown for *K. thyrsites*, was not demonstrated using the ISH technique employed here, but cannot be discounted. Although *K. neurophila* is closely related to *K. thyrsites* (Chapter 3), the migratory pathway of *Myxobolus cerebralis* from outer epithelial layers to deeper migration into the subcutis and onto peripheral nerves (El-Matbouli et al., 1995) may more closely represent the migratory pathway of this neurotropic myxozoan.

At stage 2, plasmodia are seen developing at varying sizes in the skeletal muscle fibres located under the subcutis from 50 – 80 dph with no detectable immunological response from the fish host. This plasmodial stage appears to be the first key presporogonic proliferative phase of development and can be detected in very heavily infected PCR positive fish with traditional diagnostics and ISH. Stage 3 occurs from 70 –

115 dph in the peripheral nerve pathways as stage 2 is seen to diminish to the point where it is no longer detectable with ISH or histology. This stage appears to be the second proliferative developmental phase providing the means of trophozoite recruitment into the spinal cord via the connecting nerves. The presporogonic cysts in stage 2, and particularly in stage 3, appear similar to xenoma formations seen in microsporidial infections. This type of presporogonic plasmodial development has also been reported for myxozoan species such as *Myxidium lieberkuehni* (Lom et al., 1989) and a single morphological determination from a member of the Kudiodae, *Kudoa clupeiidae*, infecting Atlantic menhaden with little or no host response (Reimschuessel et al., 2003). Although no ultrastructural examination was undertaken, the presporogonic cysts at stage 3 are clearly seen to be multicellular and granular in appearance and are always found close to the spinal cord in the peripheral nerve pathways leading onto the skeletal musculature. All fish sampled from this age class are also positive by PCR and all surviving fish go on to become heavily infected with the parasite. Additionally, no other infections were detected in any other tissues or organs.

The onset of clinical signs such as loss of spatial control and swimming in a whirling pattern coincides with the appearance of prespore cysts in peripheral nerve pathways in close proximity to the spinal cord. The generally large cysts may be responsible for the onset of whirling by disrupting or displacing and compressing the nerves controlling muscle contraction and normal swimming behaviour. Abnormal swimming behaviour proved to be the most distinctive and reliable clinical sign of *K. neurophila* caused CNS disease. While some fish were observed with acute scoliosis, it is more likely to be a common phenomenon associated with a nutrition deficiency in

cultured marine finfish larvae. Recent disease free cohorts of juveniles from the 2004 winter and spring spawnings, cultured in pathogen free water, still display scoliosis in similar numbers to previous infected cohorts over 10 years. Although it has been well documented that myxozoan brain disease causes scoliosis, particularly among *Myxobolus* spp. targeting the CNS (Lom & Dyková 1992) or surrounding cartilage (Hedrick et al., 1998), scoliosis is not a widely reported phenomenon in captive cultured marine finfish under constant observation and suffering myxozoan related brain disease. So far, 7 *Kudoa* spp. have been described as infecting the CNS of cultured and wild marine and brackish water fish, but scoliosis has not been reported to be associated with myxozoan brain disease in any of these fish species. Reports of *Kudoa* spp. infecting the CNS in wild collected fish (Paperna & Zwerner 1974; Narasimhamurti & Kalavati 1979) do not describe details concerning clinical signs such as whirling or abnormal swimming behaviour, nevertheless, this is the most consistently reported symptom of *Kudoa* caused CNS disease in cultured and observed research or aquarium fish (Yasunaga et al., 1981; Egusa 1986a; Cheung & Nigrelli 1990; Langdon 1990; Cho & Kim 2003). A more mild form of scoliosis eventually develops in surviving, heavily infected cultured striped trumpeter from the few remaining fish in the 3 – 5 year old age class. The fish have a single bend at a point immediately behind the head at the terminal end of their life-span. When swimming motion was observed next to uninfected wild caught broodstock fish, the infected fish swim by waving their head left to right rather than using swimming muscles. This unusual swimming motion appears to be a form of paraplegia associated with dark colouration, emaciation and severe spinal cord atrophy. Light microscopy of heavily infected fish confirms acute myxozoan pathology within all sectors of the spinal

cord from head to tail and compression and displacement of almost all remaining spinal cord tissue with mature pseudocysts. Bent fish often straighten out when anaesthetised suggesting involuntarily contracted muscles caused by damaged and disrupted nerve pathways are relaxed during anaesthesia.

The final proliferative prespore stages are sometimes seen in very close proximity to the spinal cord at the same time as very early sporogonic plasmodium within the spinal cord and may provide further and final recruitment of the parasite into the CNS resulting in the acute infections seen in terminal stage sporulation. While parasite development is synchronised in the fish host there is a lag phase of approximately 2 weeks between initial and final transitional stages as shown by the presence of 2 stages at once from stage 2 onwards. By the time terminal stage sporulation in the CNS is reached, usually by 130 dph (18 weeks), no other clearly asynchronous stages can be detected. This is a similar developmental time frame as reported for the histozoic multivalvulidan *Kudoa thyrsites* in Atlantic salmon (Moran et al., 1999a). By this time, non-random distribution was evident in the major anatomical brain divisions. *Kudoa neurophila* is similar to many other myxozoans infecting the CNS of their fish host in that the parasite appears to display non-random distribution with a clear preference for certain anatomical sections of the brain (Paperna & Zwerner 1974; Narasimhamurti & Kalavati 1979; Langdon 1987; Dzulinsky et al., 1994; Frasca et al., 1998; Longshaw et al., 2003). No evidence was found for preference of cell type within each anatomical division described and parasites were frequently seen in all organised cellular layers, including the meninges. Entry into the CNS via peripheral nerves to the spinal cord is most likely the initial and only route of entry into the CNS based on sequential histopathology presented in this study. The

parasite is always seen firstly in the spinal cord during the very early stages of sporogony prior to further trophozoite movement and migration toward the brain via the spinal cord. The medulla oblongata is the first part of the brain encountered on route from the spinal cord and is the most heavily infected major anatomical brain division. Pseudocysts are always recorded in higher numbers in the centrally located brain divisions of the medulla oblongata and optic lobes via a direct route from the spinal cord. The more peripherally situated brain divisions including the cerebellum (above the medulla oblongata), the mid-brain (below the optic lobes) and the fore-brain (front of the brain) displayed consistently lower rates of parasite infiltration and development. The non-random distribution of sporogonic cyst development in the CNS has previously been proposed as a factor of the migratory route of the parasite (Frasca et al., 1998) and seems to be the most credible explanation for the pattern of distribution of *K. neurophila* in cultured striped trumpeter.

The host was found to mount a limited immunological response to the parasite during the final stage of parasite sporogonic development within the CNS. It was noted that the CNS immunological response was indicative of the brain's own immune system due to the formation of an epithelioid granuloma or an electron dense parasite pseudocyst surrounded by a fibrous capsule, no breaching of the blood brain barrier and no infiltration or proliferation of inflammatory leukocyte cells. The introduction of inflammatory leukocyte cells and the displacement or replacement of functional CNS tissue is probably limited by the brain's own specific immune response in order to maintain enough CNS tissue required for the preservation of organ function. Although some fish have up to 80% compression and displacement of CNS tissue, they still function in the hatchery environment, whereas an acute inflammatory response may

severely impact on the fish hosts function and potential life span and its ability to carry the parasite through to the next stage of its development. This is representative of an effective host – parasite relationship whereby the target tissue has a limited host response to maintain function and preservation of the host and at the same time deliver enough viable infective spores into the environment at the end of the definitive hosts life.

Adult wild caught broodstock fish are maintained and conditioned for extended periods of time in the same raw sea water thought to contain the infectious actinosporean stage of the parasite and only a single fish held for 18 months was found to be infected with the parasite. It is possible this fish was infected prior to entering the hatchery system as it had a very minor infection which may be representative of a naturally occurring *K. neurophila* infection and prevalence similar to 7 other wild species caught from around the hatchery intake (Chapter 4). The noticeable resistance to *K. neurophila* in adult broodstock fish, held in the same untreated sea water, suggests striped trumpeter are more susceptible when in the larval or juvenile stage of their life. The age at which cultured fish become resistant to the disease is unknown and is the subject of ongoing research so that expensive anti parasitic treatments can cease and fish can be conditioned to raw sea water in preparation for on-growing in sea cages. Juvenile wild and cultured fish have often been reported to be more susceptible to myxozoan CNS infection than adult fish (Langdon 1990; Markiw 1992; Hedrick et al., 2001b; Bartholomew et al., 2003; Longshaw et al., 2003; Sollid et al., 2003). A number of possibilities have been proposed concerning age susceptibility, especially for *Myxobolus cerebralis* infections (Markiw 1992; Ryce et al., 2004). These include increased skeletal ossification, CNS maturity (Rose et al., 2000), immune system maturity and parasite penetrability (Markiw 1989;

Hedrick et al., 2001a) and life history patterns (Bartholomew et al., 2003) as the fish ages. The synchronised developmental pattern for *K. neurophila* is probably a product of initial infection and parasite recruitment during the first few weeks of larval growth before an effective skin/scale barrier and/or immunity has developed sufficiently to prevent further penetration and infection by actinosporean stages.

Not only does this *Kudoa* spp. have wide host specificity (Chapter 4), it has been a devastating pathogen limiting the culture of striped trumpeter juveniles for on-growing. These factors will necessitate Tasmanian hatcheries that are intending to produce marine finfish for on-growing and sale to consider the bio-security status of their water treatment systems and understand the biology and impact of *Kudoa* spp. myxozoan parasites on production figures and the quality of their product. Future prospective marine finfish aquaculture candidates in Tasmania will need to be hatched and reared in untreated sea water known to contain the infectious stage of this disease to test the species susceptibility and potential requirement to deliver actinosporean-free water. Furthermore, existing and potential aquaculture sites located around the coastal regions of Tasmania would benefit from routinely screening wild fish in the vicinity of their water intake for the presence of myxozoan parasites so that infection potential and preventative strategies can be assessed.

Chapter 6

General Discussion

The process of developing striped trumpeter in Tasmania as a species for aquaculture has focused on broodstock management, egg quality, improved growth and survival of larvae, enhanced hatchery production with improved nutrition and disease identification, prevention and control (Battaglione et al., 2003). The research undertaken in this thesis relates to a specific myxozoan disease that is limiting striped trumpeter culture.

6.1. Chapter 2

Routine disease surveillance of striped trumpeter, since the initiation of the project, ascertained few disease problems associated with the intensive rearing of this species. Problems such as an incident of nodavirus were overcome and were not considered a threat to production. However, the brain disease occurring in all surviving fish, caused by a marine myxozoan parasite, was persistent and severely impacted on the ability of the research team to produce healthy juveniles for further research. The research undertaken in this thesis has identified the parasite responsible for causing the brain disease using morphological techniques and named it *Pentacapsula neurophila* due to the location of the mature form of the parasite in the target tissue of the CNS of its fish host (Chapter 2).

6.2. Chapter 3

Initially, the physical features of this myxozoan parasite were recognised as a new species identified in the pentacapsulid form containing 5 shell valves and 5 polar capsules as determined by Naidenova & Zaika (1970) and Lom & Dyková (1992). The marine

species of myxozoans are histozoic parasites belonging to the order Multivalvulida which were traditionally classified into separate families based on valve numbers (Lom & Dyková 1992). Families making up the multivalvulidans include the Trilosporidae, Kudoidae, Hexacapsulidae, Pentacapsulidae and Septemcapsulidae, classified by having 1, 4, 5, 6 and 7 valves respectively. During a research trip to the Great Barrier Reef, Australia, fish were caught and examined for myxozoan infections. The popular pelagic recreational fish, Spanish mackerel *Scomberomorus commerson* was found to have a muscle infection caused by a marine histozoic myxozoan with 13 polar capsules (Whipps et al., 2003b). Further molecular phylogenetic analysis using ssu rDNA revealed the multi-capsule myxozoan to cluster firmly within the 4 valved monophyletic Kudoidae (Whipps et al., 2003b). The study raised questions concerning the accuracy of traditional classification of the multivalvulidans based on morphological and morphometric information. Applying molecular phylogenetic analysis together with more traditional morphological phylogeny was recommended by Kent et al. (2001) to solve such discrepancies within the Myxozoa and hence prompted the re-examination of the Multivalvulida using comparative ssu rDNA analysis by Whipps et al. (2004). In contribution to the re-examination of the multivalvulidans, the ssu rDNA sequence from *P. neurophila* was provided for molecular phylogenetic analysis. In conjunction with this analysis, the research undertaken in this thesis employed Bayesian maximum posterior probability using the covariotide model of evolution to infer the phylogeny of the multivalvulidans. It has been shown, using molecular phylogenetic analysis, the classification for *P. neurophila* and its position firmly within the Kudoidae with higher and more credible values than previously used phylogenetic methods that employ

bootstrap values. The Bayesian tree building analysis not only supports the analysis by Whipps et al. (2004) but is more comprehensive and delivers stronger results when compared to less appropriate mathematical models and has provided the most credible summary of the Kudoidae multivalvulidans to date. Bayesian analysis of the Kudoidae supports redescription of *P. neurophila* to *Kudoa neurophila* and that Pentacapsulidae, Hexacapsulidae and Septemcapsulidae be synonymised with Kudoidae. Additionally, Bayesian analysis using the covariotide model of evolution will provide a more comprehensive and robust method for reviewing and resolving future relationships using the myxozoan ssu rDNA dataset as it continues to expand.

6.3. Chapter 4 PCR development and use

Having identified the parasite responsible for the primary disease threatening the continued successful production of juvenile fish and resolving taxonomic issues within the multivalvulidans, the TAFI research team required a diagnostic tool with greater flexibility than traditional routine diagnostics. Because the myxozoan life cycle is complex and involves many morphological changes, including development in an alternative host (Wolf & Markiw 1984), the early presporogonic stages or alternative host stages were unable to be detected or identified as being myxozoan using traditional histology, which is capable of detecting only the distinctive terminal spore stage in the definitive fish host. Molecular genetic sequence information from the ssu rDNA (Chapter 3) was used to develop a sensitive and specific PCR diagnostic assay (Chapter 4). The PCR test has the additional capacity to determine a result from any stage of the parasite during its 2 host life cycle. This flexibility makes it possible to further elucidate

biological and epizootiological aspects of the parasite. Thus, the test can be utilised to facilitate important managerial decisions associated with understanding, controlling or preventing infection by the parasite and subsequent development of the fatal brain disease it causes.

6.4. Chapter 4 biology, epizootiology and disease prevention

Following the sexual stage of development of the myxozoan parasite, usually in an invertebrate host (El-Matbouli & Hoffman 1998), a stage is released into the water that is infectious to the fish host. This waterborne infectious stage, also known as the actinosporean stage of the same parasite, can infect the host either by direct attachment to the skin epithelium or ingestion and subsequent attachment to the host digestive tract epithelial cells. The most thoroughly researched example of such a life cycle is the triactinomyxon stage of *Myxobolus cerebralis*, released from the annelid worm *Tubifex tubifex* (Wolf & Markiw 1984; El-Matbouli et al., 1995). The actinospores attach to the host cell by discharging a sticky coiled filament from the polar capsules in response to mechanical or chemical stimuli (Cannon & Wagner 2003). Once attached, the parasite sporoplasm is passed into the host cell to develop further (El-Matbouli et al., 1995). Although the actinosporean stage is unknown for *K. neurophila*, *in situ* hybridisation showed the skin, gut and gill epithelium as the entry locus, suggesting a waterborne actinospore stage with an attachment process similar to other actinospores is possible. Additionally, the myxospore stage of this parasite has been shown to have 5 polar capsules containing filaments which can be extruded artificially (Chapter 2) and may function as a means of attachment to an alternative invertebrate host.

Armed with this further knowledge concerning the biology of the parasite, epizootiology studies incorporating the PCR test commenced in and around the hatchery system (Chapter 4) with the aim of discovering an alternative host and/or locating the reservoirs of the disease. An invertebrate infected with the parasite was not located within the hatchery or the adjacent aquatic reserve from which the hatchery sources its water. Conversely, the epizootiology study showed 8 species of wild marine finfish were infected with the parasite and living in very close proximity to the water intake. We consider the aquatic reserve as the disease reservoir from which we are drawing the infectious actinosporean stage of the parasite. This information, based on the research herein, has enabled the development a water treatment system with a targeted approach to deliver pathogen free water to the marine finfish facility. The health management strategy employed is based on environmental management and is designed to mechanically and chemically prevent infectious stages of the parasite from entering the hatchery and prevent myxozoan brain disease. By treating the incoming water in this fashion, we hope we have been able to provide a non discriminating approach that can also prevent other parasitic, bacterial and viral pathogens entering from the nearby marine environment and causing disease in striped trumpeter juveniles. Hatchery managers can now focus on producing healthy fish that are suitable for further aquaculture assessment.

6.5. Chapter 5

Research was then conducted to ascertain the pathology of the disease caused by the parasite to its fish host during its life cycle. Prior to the installation of an effective water treatment system, the parasite was prevalent in 100% of juvenile cultured fish resulting in

the loss of all fish, apart from a few heavily infected survivors with diminished potential as an aquaculture candidate. The pathology of the brain disease caused by the parasite was studied to gain an understanding of how the disease contributes to the eventual death of the fish host. Following entry into the fish host, the parasite migrates and proliferates in the muscle, followed by the peripheral nervous system on its way toward its destination in the target tissue of the CNS. Once in the brain and spinal cord the host response is limited and the parasite matures to final stage sporulation. Plasmodia, containing maturing and mature spores, continue to join and expand, compressing and displacing large areas of neural tissue which severely compromises normal function and eventually leads to the death of the host. Myxospores are then released into the environment to continue their life cycle in another host. We speculate that the spore is not released until the death of its host as histology of fish up to 5 years old, displaying advanced CNS pathology, show no sign of the parasite pseudocysts ever breaching the blood brain barrier in a possible route out of the live fish host.

6.6. Novel targets for disease control

Our understanding of how histozoic species such as *K. neurophila* develops in its host is the first step in understanding the disease it causes and will contribute to the future development of chemotherapeutics or vaccines that target a particular life stage of the parasite. Recent studies have identified important enzymes responsible for *M. cerebralis* developmental physiology during certain periods of its life stage in rainbow trout (Kelly et al., 2004). This discovery will hopefully lead to the identification of the genes responsible for encoding parasite enzymes and is one such targeted approach which may

lead to the control of the disease with chemotherapeutics or vaccines. While a similar mechanism of enzyme mediated parasite migration may also be operative during the development of *K. neurophila*, we have not elucidated the life cycle nor do we have the means to infect naïve classes of juveniles for further study in this area. However, the developmental complexity of *K. neurophila*, as evidenced by a series of morphological transformations (Chapter 5), offers a number of possibilities for targets to disease control. A recent study by Belli et al. (2004) has shown cross-linking of tyrosine-rich residues in proteins in the formation of oocyst walls of apicomplexan parasites. UV fluorescence is one of the detection methods used to indicate the presence of the dityrosine cross-linking proteins implicated in the wall forming process (Belli et al., 2004). Study of the spores of *K. neurophila* show the parasite forms walls at terminal stage sporulation that may act to protect the parasite from exposure in the transition stage from host to host. Myxozoan spore viability, due to its outer protective shell, following exposure, has previously been shown to be true for *M. cerebralis* (El-Matbouli & Hoffman 1991). A pilot study on the spore walls of *K. neurophila* (data not shown) has shown the spore walls of this multivalvulidan myxozoan to autofluoresce when examined under UV light (Appendix 5.1). This preliminary result suggests that the parasite forms the walls of its spore by utilising a mechanism to develop protective extracellular matrices in a similar fashion to many other outer wall forming parasites (Waite 1987; Chen et al., 1992; Belli et al., 2004). Identifying the encoding genes responsible for spore wall formation is a novel target for the control of myxozoan caused diseases that have serious economic and environmental impacts on commercial and recreation fisheries.

6.7. Myxozoa and future marine farming in Tasmania

The Kudoidea have wide host specificity (Moran et al., 1999a) and many species can commonly be found in several fish hosts. In the case of *K. neurophila*, it has been found in 8 species of marine finfish from a variety of fish families (Chapter 4). This pattern of host specificity suggests that the parasite is capable of indiscriminate infection of fish provided that the host is exposed to a sufficient quantity of the infectious actinosporean stage within the area of the parasite's geographical distribution. Hence, this *Kudoa* species alone may have the pathogenic potential to severely affect the viability of marine finfish aquaculture in Tasmania. The ramifications of this possibility extend to considerations toward the development of new and existing species for the culture of marine finfish in Tasmanian coastal waters. Future aquaculture development and subsequent intensive production of fish in the marine environment would benefit from thorough risk assessment in relation to the biological and financial impacts of myxozoan pathogens.

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Guide to Electronic Appendix

Appendix 1

1.1 Museum submission form.

MS Word document.

Museum specimen submission form sent with samples to;

Dr Robert D. Adlard

Curator of Protozoa, Queensland Museum

PO Box 3300, South Brisbane, Queensland 4101.

18 September 2002.

1.2. Morphometric data.

MS Excel document.

MS Excel file contains the raw data used to calculate the morphometric information for *Kudoa neurophila* that appears in Table 1.1. This table also contains additional information regarding the amount of shrinkage occurring for mature spores following fixation in either 10% formalin or 95% alcohol when compared to fresh spores.

1.3. Photos of filament length.

MS Word document.

Photomicrograph of a fresh spore placed into a solution containing 1M potassium hydroxide to extrude the polar filament. Photos were then taken using an Olympus[®] DP11 digital camera attached to an Olympus[®] BX41 microscope (Olympus America Inc., Melville, NY, USA). Images of spores and a measurement graticule were transferred to

Analytical Imaging StationTM software (Version 3.0 Rev 1.1, St Catherines, Ontario, Canada) to measure filament length.

Appendix 2

2.1. Oligonucleotide primers used.

MS Word document.

The document contains sequence information of the primers used to extract the entire small subunit rDNA (ssu rDNA) gene from *Kudoa neurophila*, the primer combinations, the start point on the sequence from the 5'-end and 18e primer (Hillis & Dixon 1991), fragment size in base pairs, reference to first use and photos of the agarose gels containing the PCR fragment sent for sequencing.

2.2. *Kudoa neurophila* AY172511 ssu rDNA sequence and GenBank information.

Text document.

Information and ssu rDNA sequence submitted electronically to GenBank by Geoffrey Grossel including the GenBank accession number.

2.3. *Kudoa neurophila* AY302735 long subunit (lsu rDNA) sequence and GenBank information.

Text document.

Information and lsu rDNA sequence submitted electronically to GenBank by Christopher Whipps including the GenBank accession number.

2.4. Sequence data for all Myxozoan ssu rDNA used.

Text document.

Folder containing 24 text documents of all the Myxozoan ssu rDNA sequences used in Bayesian phylogenetic analysis of the multivalvulidans.

2.5. ClustalX alignment.

Text document.

ClustalX alignment in text format of the 24 Myxozoan sequences used for the Bayesian analysis results that appear in the main body of the thesis. This alignment was edited by truncating extended ends and removing single additional insertions not found in any of the other sequences prior to the addition of Bayesian analysis code.

2.6. Bayesian analysis code.

Text document.

ClustalX alignment in text format of the 24 Myxozoan sequences including the Bayesian analysis code used to set the conditions of the phylogenetic analysis.

Appendix 3

3.1. PCR primers. Design.

MS Word document.

Specific oligonucleotide primers designed from the ssu rDNA sequence of *Kudoa neurophila*.

3.2. PCR primers. Combinations.

MS Word document.

Table 3.2.1. Combinations of sense and anti-sense PCR primers tested on *Kudoa neurophila* genomic DNA.

Figure 3.2.1. PCR products from *Kudoa neurophila* genomic DNA using the primer combinations 1–9 targeting the small subunit rDNA gene.

Figure 3.2.2. PCR products from *Kudoa neurophila* genomic DNA using the primer combinations 10–18 targeting the small subunit rDNA gene.

Figure 3.2.3. PCR products from *Kudoa neurophila* genomic DNA using the primer combinations 17 & 19–21 targeting the small subunit rDNA gene.

3.3. PCR primers chosen and testing.

MS Word document.

The following figures (Figures 1–5) show 1% agarose gels from the development of primer combination PEN3F–PEN4R. The successful combination was chosen from the 21 primer combinations tested due to specificity and sensitivity to the target species.

Figure 3.3.1. This gel represents the most suitable annealing temperature (63.2°C) at the most suitable MgCl^{++} concentration of 3.5 mM in the 25 μl PCR reaction. The annealing temperature and MgCl^{++} concentrations were tested using *Kudoa neurophila* gDNA from the equivalent of 1 spore per 25 μl PCR reaction.

Figure 3.3.2. Primer combination PEN3F–PEN4R tested for specificity on gDNA from a very closely related species panel consisting of: 1. *Myxobolus aldrechetti* 2. *Kudoa* n.sp. from southern bluefin tuna 3. *Kudoa thyrsites* 4. *Kudoa ovivora* 5. *Hexacapsula* species

(GenBank #AY302739) from Spanish mackerel 6. *Hexacapsula* species (GenBank #AY302738) from moon wrasse 7. *Septemcapsula yasunagi*. +ve = gDNA taken from purified isolated *Kudoa neurophila* spores.

Figure 3.3.3. Primer combination PEN3F–PEN4R tested for specificity on gDNA from a non-target species panel consisting of uninfected: 1. Rainbow trout *Oncorhynchus mykiss* 2. Greenback flounder *Rhombosolea tapirina* 3. Damsel fish *Acanthochromis polyacanthus* and 4. Blue throat wrasse *Notolabrus tetricus* (alternate primary host).

Figure 3.3.4. Further primer combination PEN3F–PEN4R testing for specificity on gDNA from non-target species consisting of: Lane 5 Polychaete worm *Galeolaria caespitose* (potential actinosporean stage host). Lanes 1 & 6 Histologically determined uninfected striped trumpeter. Lane 2–4 = some +ve test results from pre-spore infected striped trumpeter juveniles 77, 44 & 23 days old respectively. Lane 7 –ve no DNA.

Figure 3.3.5. Example gel of the results from PCR testing of primer combination PEN3F–PEN4R on seawater filtrates before and after treatment for the month of June in the 2003 spawning season. Lane 1, first week pre-treatment filtrates. Lane 2, first week post-treatment filtrates. Lane 3, second week pre-treatment filtrates. Lane 4, second week post-treatment filtrates.

3.4. ClustalX alignment check.

Text document.

ClustalX sequence alignment to check PCR fragment produced is from the targeted *Kudoa neurophila* ssu rDNA gene.

3.5. Invertebrate screening

MS Word document.

The following figures (A, B, C & D) show an unidentified gregarine (Protista: Apicomplexa) found parasitizing an unidentified marine oligochaete worm found living in the silt of header tanks that provide fresh sea water to the hatchery from the aquatic reserve.

Figure 3.5.1. Photograph A. An unidentified marine oligochaete worm found living in the silted bottom of the 10 000 liter header tanks supplying the marine finfish hatchery. The whole worm in this photograph has been prepared as a simple wet squash for viewing under a light microscope.

Figure 3.5.2. Photograph B. An unidentified species of gregarine (Protista: Apicomplexa) found parasitizing the marine oligochaete worm from the header tanks supplying the hatchery. This photograph shows the simultaneous development of trophozoite cells within an unruptured oocyst and within the body cavity of its invertebrate host.

Figure 3.5.3. Photograph C. Trophozoites from a ruptured oocyst following wet squash preparation.

Figure 3.5.4. Photograph D. Higher magnification of a trophozoite.

3.6. Traditional diagnostics.

MS Word document.

Photomicrographs of the various methods of traditional diagnostics used to detect mature *Kudoa neurophila* spores in an infected cultured striped trumpeter central nervous system.

Figure 3.6.1. Light microscope photograph (400X) of a wet squash preparation from the brain of a juvenile striped trumpeter infected with *Kudoa neurophila*. The picture clearly shows mature spores within intact plasmodia. Scale bar = 5 μm .

Figure 3.6.2. Light microscope photograph (400X) of a diff-quick stained spinal cord smear from the head end of the spinal cord of a juvenile striped trumpeter infected with *Kudoa neurophila*. The picture shows the distinctive pentagonal or blossom shaped mature spores. Scale bar = 5 μm .

Figure 3.6.3. Light microscope photograph (100X) of **A.** 3 μm hematoxylin and eosin stained section & **B.** 3 μm giemsa stained section, from the cerebellum of a juvenile striped trumpeter infected with *Kudoa neurophila*. Scale bar = 20 μm .

Appendix 4

4.1. ISH positive control.

MS Word document.

In situ hybridisation (ISH) positive controls.

Figure 4.1. A. *In situ* hybridisation positive control performed on a 6 μm section from the brain of a heavily infected striped trumpeter juvenile. The shape of the plasmodia can be seen (arrow) filled with spores stained deeply purple from the ISH procedure. Some

freely scattered spores can also be seen. Methyl green counter staining was not used in the initial development using positive controls; hence CNS morphology is not clearly distinguishable. Scale bar = 50 μm .

Figure 4.1. B. *In situ* hybridisation positive control performed on a 6 μm spinal cord section of a striped trumpeter juvenile at 120 dph and representing the first stage of parasite development in the target tissue of the CNS. The shape of the plasmodia can be seen (arrow) stained purple from the ISH procedure. No mature spores are noticeable as this stage is still in a prespore developmental phase. Methyl green counter staining was used in this positive control to highlight the morphology of the CNS. Scale bar = 20 μm .

4.2. PCR test on positive control fish.

MS Word document.

Example gels of PCR tests performed on positive (Figure 4.2.1.) and negative (Figure 4.2.2.) control fish as part of the natural infection trial conducted to provide samples for *in situ* hybridisation.

Figure 4.2.4. PCR result on positive control fish exposed to untreated seawater at 23 days post hatch. Lanes marked 1–5 contain gDNA from 25 mg whole fish samples in the PCR reaction. Also included on this gel are a positive control (+ve) and a 100 base pair ladder (100bp).

Figure 4.2.2. PCR result on negative control fish exposed to treated seawater at 23 days post hatch. Lanes marked 1–6 contain gDNA from 25 mg whole fish samples in the PCR reaction. Also included on this gel are a positive control (+ve) and a 100 base pair ladder (100bp).

4.3. Host response.

MS Word document.

Host response. Host epithelial cells encapsulating parasite pseudocysts within a fibrous capsule. The electron dense centre of parasite spores stain deeply basophilic.

Figure 4.3.1. Light microscope photograph (200X) of a 3 µm H & E stained section from the optic lobe in the brain of a juvenile striped trumpeter infected with *Kudoa neurophila*. The picture shows mature spores within large plasmodia with no host response (**N**) and host response with spores forming basophilic staining, electron dense centres (**E**) surrounded by a fibrous epithelioid capsule of host cells (**F**). Scale bar = 0.1 mm.

Figure 4.3.2. Light microscope photograph (100X) of a 3 µm giemsa stained section from the optic lobe in the brain of a juvenile striped trumpeter with an acute *Kudoa neurophila* infection causing the granular cell layer to be completely displaced by large mature plasmodia. The picture shows mature spores within large plasmodia with no host response (**N arrow**) and host response with spores forming basophilic staining, electron dense centers (**H arrow**) surrounded by a fibrous epithelioid capsule of host cells (**H arrow**). Scale bar = 0.2 mm.

Figure 4.3.3. Higher magnification of a light microscope photograph (200X) of a 3 µm giemsa stained section from the optic lobe in the brain of a juvenile striped trumpeter infected with *Kudoa neurophila*. The picture shows mature spores within large plasmodia with no host response (**arrow**) and host response with spores forming basophilic staining, electron dense centres (**E**) surrounded by a fibrous epithelioid capsule of host cells (**F**). Scale bar = 0.1 mm.

Appendix 5

5.1. Fluorescence photos.

MS Word document.

UV fluorescence detection of *Kudoa neurophila* spores.

Figure 5.1.1. A, B & C. Positive control showing UV fluorescence in yeast cells using an Olympus® IX71 inverted research microscope (Oakleigh, Vic, Australia) and an Optronics Magnifire 2.0 camera (Goleta, CA, USA). Spores were visualized under 330–385 nm UV light. 200X magnification. Scale bar = 20 µm.

Figure 5.1.2. Mature spores of *Kudoa neurophila* isolated from the brain of an infected striped trumpeter at 150 dph. An Olympus® IX71 inverted research microscope (Oakleigh, Vic, Australia) and an Optronics Magnifire 2.0 camera (Goleta, CA, USA) was used to show UV fluorescence in *K. neurophila*. Spores were visualized under 330–385 nm UV light. 200X magnification. Scale bar = 20 µm.

Appendix 6

A1-Journal articles in PDF format.

The electronic folder titled Appendix 6 contains the A1-Journal articles arising from the research performed in this thesis and are organized as follows;

6.1. G.W. Grossel, I. Dyková, J. Handler and B.L. Munday. *Pentacapsula neurophila* sp.n. (Multivalvulida) from the central nervous system of striped trumpeter, *Latris lineata* (Forster). Journal of Fish Diseases 2003, 26, 315-320.

6.2. C.M. Whipps, **G.W. Grossel**, R.D. Adlard, H. Yokoyama, M.S. Bryant, B.L. Munday and M.L. Kent. The phylogeny of the Multivalvulidae (Myxozoa: Myxosporea) based upon comparative rDNA sequence analysis. *Journal of Parasitology* 2004, 90, 618-622.

6.3. G.W. Grossel, J. Handler, S. Battaglione and B.L. Munday. Diagnostic polymerase chain reaction assay to detect *Kudoa neurophila* (Myxozoa: Multivalvulida) in a marine finfish hatchery. *Diseases of Aquatic Organisms*. In Press for 2005, DAO reference D1432.

6.4. G.W. Grossel, J.T. Ellis, J. Handler and B.L. Munday. Small subunit rDNA phylogeny places *Pentacapsula neurophila* (Myxosporea: Multivalvulida) within Kudoidae. Submitted to *Systematic Parasitology* and under review.