POTENTIAL BIOLOGICAL CONTROL AGENTS FOR THE SALMON LOUSE

Lepeophtheirus salmonis (KRØYER, 1837).

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By

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DECLARATION

I declare that this thesis has been compiled by myself and is the result of my own investigations. It has not been submitted for any other degree and all sources of information have been duly acknowledged.

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ABSTRACT

The salmon louse, *Lepeophtheirus salmonis* (Krøyer), is an obligate ectoparasitic copepod that lives on the external surface of salmonid fish. It is the most common parasite on cage reared Atlantic salmon (*Salmo salar* L.), costing the aquaculture industry in Scotland millions of pounds each year to control. Traditional methods used to control sea lice have centred on the use of chemical pesticide treatments, which are expensive, hazardous to handle, potentially deleterious to the marine environment, and are sometimes ineffective. Furthermore, the misuse use of two previously efficacious chemotherapeutants has led to a build up of resistance in sea lice populations.

The aims of this study were to investigate a potential alternative control strategy, concentrating on the isolation of the naturally occurring enemies of sea lice, and evaluating their potential for use as biological control agents. A screening protocol was undertaken to examine sea lice removed from harvest size farmed fish and wild Atlantic salmon, to look for the presence of epibiotic and hyperparasitic organisms on the external surfaces of the sea lice. A screening protocol was also undertaken to look for invasive microorganisms, such as fungal pathogens, internal symbionts, and other internal hyperparasites.

Sea lice were examined microscopically for the presence of external epibionts and obvious signs of internal invasion by microorganisms and parasites. Surface sterilised sea lice were incubated on growth media to screen for the presence of fungal pathogens. Fresh tissue squashes were performed on lice showing clinical signs of infection and screening / diagnostic PCRs were used to detect and identify endosymbionts and invasive

pathogens and parasites. Wax histology, TEM and SEM were used to further investigate host parasite interactions in order to evaluate pathogenicity where appropriate. *In vitro* and *in vivo* challenge trials were performed with an isolated hyperparasite to effect transmission and to determine pathogenicity.

The stalked suctorian ciliates *Ephelota gemmipara* and *Ephelota gigantea*, and the monogenean worm *Udonella* sp. were frequently found utilising *L. salmonis* as a substrate. High densities of *Udonella* were observed on the cephalothoracic shield and genital segment of adult lice, and very high densities of *Ephelota* spp. were found on the genital segment, abdomen, and egg strings. The prevalence and seasonal pattern of occurrence of these epibionts were assessed.

Fourteen fungal isolates were obtained, but failed to sporulate *in vitro* culture and were hence not identifiable. No endosymbionts were detected in either the body cavity or the egg strings by using the screening and diagnostic PCRs.

An hyperparasitic microsporidian was found heavily parasitising adult sea lice. The microsporidian infection arose in the epidermal cells lying beneath the cuticle, infection was not observed in other tissues. Horizontal transmission was assumed as developing eggs and egg strings were not seen to be infected. The ultrastructure and complete developmental cycle of this hyperparasite in the salmon louse were described. Specific PCR primers were designed for use as a diagnostic tool and a molecular phylogeny was constructed using rRNA gene sequences. Taking into account its taxonomic positioning, its morphology and its unique characteristics, it appears that this parasite represents both a new genus and a new species. The microsporidian was not found at all farm sites visited and was not detected in sea lice from wild caught Atlantic salmon. The microsporidian

infected up to 10% of lice sampled when most prevalent, but was sometimes absent, and showed no clear seasonal pattern in its occurrence. Transmission of the microsporidian hyperparasite to uninfected sea lice was not achieved and evidence for an alternative / intermediate host for the microsporidian is presented. This is the first report of a microsporidian from sea lice, and indeed from marine copepods; it is also the first report of a hyperparasitic microsporidian in crustacea.

The potential for the epibionts and endobionts found in association with sea lice to be used as biological control agents are presented.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION TO THE SALMON LOUSE

Lepeophtheirus salmonis (Krøyer 1837), a copepod from the family Caligidae, is a common marine ectoparasite of the Atlantic salmon *Salmo salar* L., and also parasitises other species of marine salmonid fish. It shares the common name 'sea louse' with other caligid copepods such as *Caligus elongatus* (Nordmann 1832) but is also known as the 'salmon louse'. *L. salmonis* has the potential to cause severe damage both in wild (Tully. O. *et al.*, 1993; Whelan, 1993; Todd *et al.*, 2000) and farmed (Wootten *et al.*, 1982; Jonsdottir *et al.*, 1992; Costello, 1993; Pike, 1989) salmonids, and is regarded as the most significant ectoparasite affecting these fish in the Northeast Atlantic (Wootten *et al.*, 1982; McVicar, 1997).

Lepeophtheirus salmonis is taxonomically placed as follows:

Kingdom:	Eukaryota
Phylum:	Arthropoda
Class:	Crustacea
Subclass:	Copepoda, Milne Edwards, 1840
Infraclass:	Neocopepoda, nov Huys and Boxshall, 1991
Superorder:	Podoplea, Giesbrecht, 1882
Order:	Siphonostomatoida, Thorell, 1859
Family:	Caligidae, Burmeister, 1835
Genus:	*Lepeophtheirus*, von Nordmann, 1832
Species:	*salmonis*, (Krøyer 1837)

Figure 1.1 Diagram of the basic body plan of *Lepeophtheirus salmonis*, showing both male and female lice. Modified from Kabata 1979.
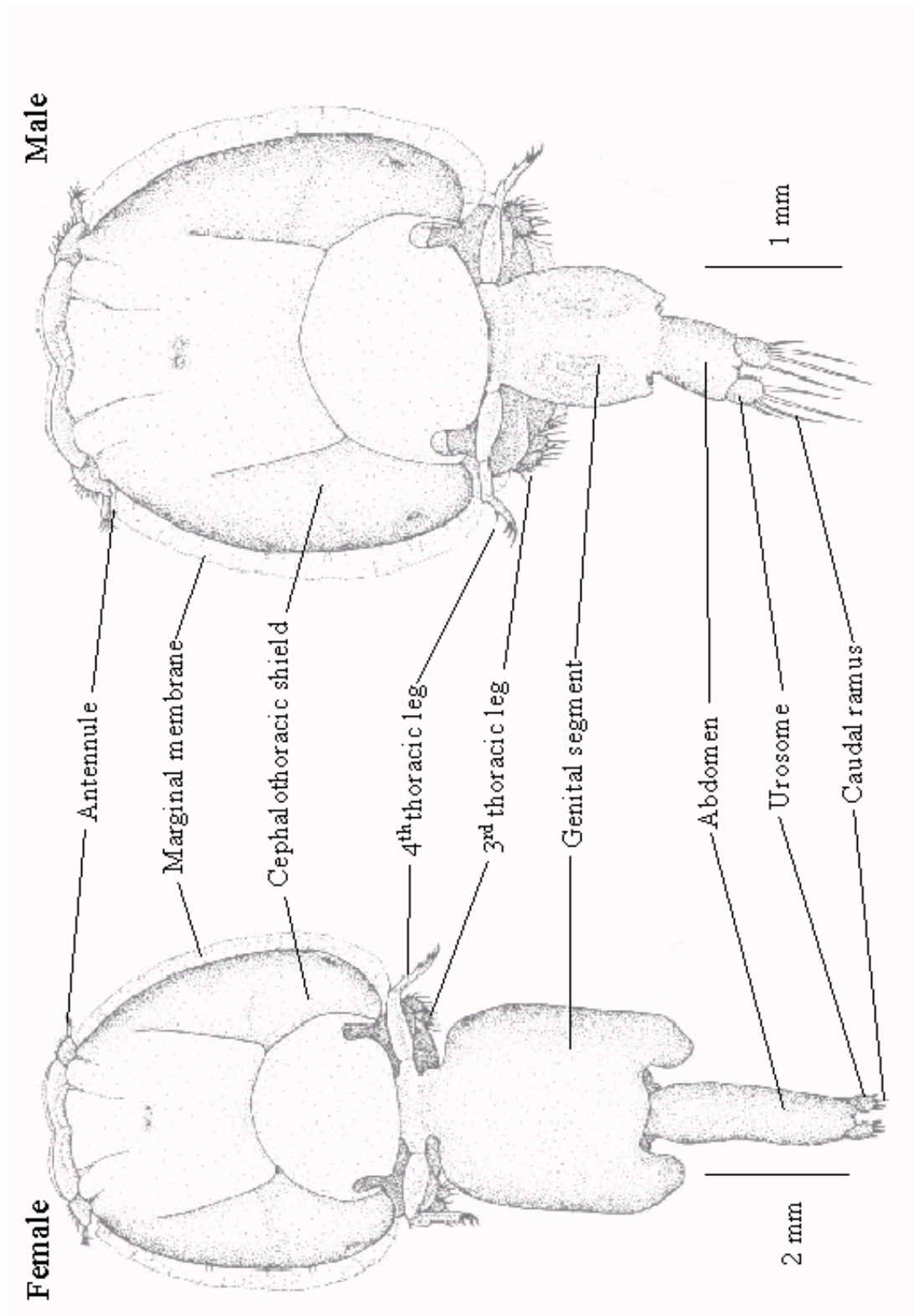
1.1.1 Life cycle and feeding

Lepeophtheirus salmonis has ten stages to its life cycle, two free-living planktonic naupliar stages, one free-swimming infectious copepodid stage, four attached chalimus stages, two pre-adult stages and an adult stage (Johnson *et al.*, 1993). Both nauplius stages and the copepodid whilst free-living do not feed. The infective copepodid finds and attaches to the host. The following four chalimus stages attach to the host by their frontal filament, whereas the pre-adults and adults lose this anchor and are freely mobile upon their hosts. After attachment to the host, the copepodids, chalimus larvae, pre-adults and adults feed on the hosts mucus, skin and blood (Kabata, 1974; Brandal *et al.*, 1976a). Feeding is achieved by pressing the tubular mouth onto the host skin and grazing with a sawing action of the mouthparts (Kabata, 1974; Kabata, 1979). This action results in the removal of mucus, leading to broken skin and ultimately the exposure of muscle and other tissues. Most damage is caused by the larger mobile adults, but the sessile chalimus stages can also damage the host (Jones *et al.*, 1990; Johnson & Albright, 1992).

1.1.2 Reproduction

During reproduction the male copepod locates a pre-adult female, then guards her until her final moult to an adult, when he deposits a pair of spermatophores over the female's genital apertures (Boxshall, 1990). These spermatophores can supply sufficient sperm for the fertilization of several clutches of eggs, which are extruded in long, paired egg sacs by the female. The egg string length and number of eggs per string vary seasonally (Tully, 1989; Tully. O. & Whelan, 1993; Ritchie *el al.*, 1993) and strings may contain differing numbers of eggs.

Figure 1.2 Life cycle of the salmon louse *Lepeophtheirus salmonis*. Modified from parasitology group, Institute of Aquaculture

Life-cycle of the salmon louse *Lepeophtheirus salmonis*
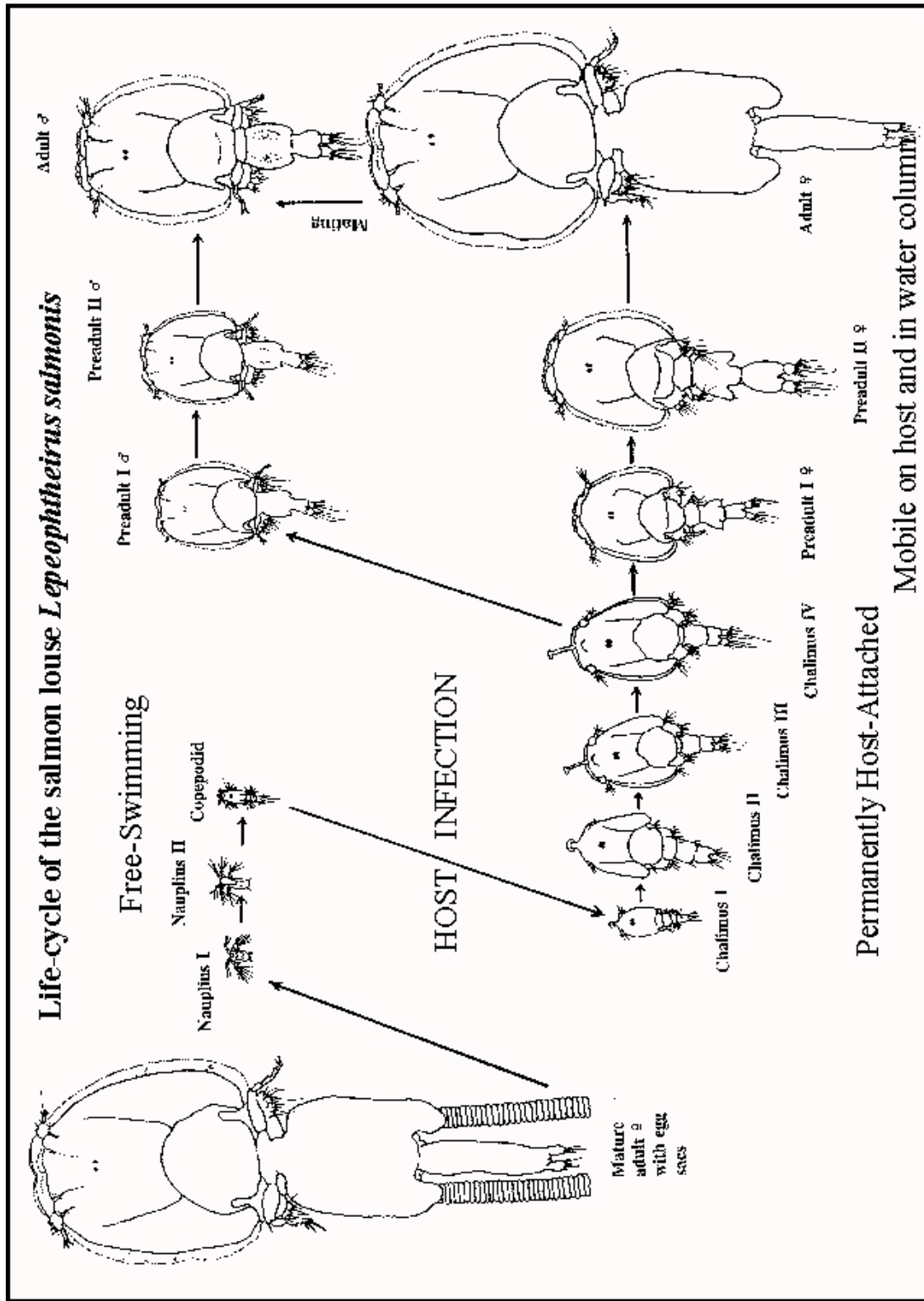
In *L. salmonis* at 10 ˚C, eggs hatch after about 8 days, the nauplius stages last 4 days, copepodid 10 days, chalimus stages 25 days, pre-adults 17 days for male and 22 days for female (Johnson & Albright, 1991). The longevity of the adults is not fully known

and generation time depends on water temperature and can range from 48 to 92 days (Tully, 1989).

1.1.3 Pathology

Damage to the host occurs as a result of the feeding activity of the attached stages of *L. salmonis* (Jones *et al.*, 1990). The occurrence of lice on farmed salmon and their level of impact are variable. Fish deaths due to lice damage have been recorded (Brandal & Egidius, 1979; Wootten *et al.*, 1982), with numbers of 2000 lice per fish recorded in Norway (Brandal & Egidius, 1977). In less extreme cases, lice damage results in poorer growth, increased susceptibility to disease and morphological damage resulting in a decrease in the market value of the fish. However, secondary infections may result from wounds on fish created by sea lice (Jonsdottir *et al.*, 1992). It has been claimed that farm infestations may also be responsible for impact upon wild salmonids (Bristow & Berland, 1991).

These problems have resulted in the need for novel, safe and effective treatment to control *L. salmonis* on farmed salmon.

1.2 CURRENT METHODS FOR CONTROL OF SEA LICE

Salmon production from farms around the world had reached over 800,000 million tonnes in 1997, an increase of approximately 800% over the previous ten years (Roth, 1998). It is therefore not surprising that a great deal of research effort has been put towards the control of sea lice. The choice of control method depends upon its efficacy, stress to the fish, environmental effects, cost, potential hazard to staff, availability, marketing applications and ease of application. Useful reviews articles on various sea lice control measures have been compiled by (Costello, 1993; Treasurer, 2002; Grant, 2002).

1.2.1 Chemical control

Current chemical control measures rely on expensive toxic chemicals such as organophosphates, pyrethroids, avermectins, chitinase inhibitors and hydrogen peroxide (Roth *et al.*, 1993). These chemotherapeutants may be administered either as in-feed treatments such as emamectin benzoate (Slice®) or as bath treatments such as the organophosphate azamethiphos (Salmosan®), or hydrogen peroxide, and the synthetic pyrethroid, cypermethrin (Excis®).

Bath treatments are labour intensive, costly and cause considerable stress to the fish. They do not permit the treatment of all cages simultaneously at a farm site, allowing potential reinfection between cages to occur, and may contribute towards the development of chemical resistance in the adjacent untreated cages that only receive flushed chemicals. Many chemicals used in bath treatments are not efficacious against all stages of sea lice, allowing chalimus stages to survive and continue the cycle of infestation. Furthermore, such treatments may not be feasible on exposed farm sites during severe weather conditions (Stone *et al.*, 1999). In-feed treatments are preferred

to bath treatments, however these chemicals have been shown to accumulate in the sediment below cages and may be toxic to some non-target benthic species (Collier & Pinn, 1998; Roth *el al.*, 1993; Davies & Rodger, 2000).

The large-scale reliance on very few effective chemotherapeutants, apart from the environmental impact to non-target species, runs the inevitable risk of selecting for genetically determined resistance in target organisms (Denholm *et al.*, 2002). A phenomenon previously observed with misuse of certain sea lice chemotherapeutants (Jones *et al.*, 1992; Tully & Mcfadden, 2000; Treasurer *et al.*, 2000).

1.2.2 Biological control in salmon farming

The search for alternative ways to reduce lice numbers on farmed salmon, coupled with increasing concern about chemical use and the environment, have led to the consideration of various forms of biological control to be used. These include the release of sterile males and finding a pathogen or organism that would parasitise the lice (Treasurer, 1993; Treasurer, 2002). Although these methods have not yet been employed, the use of wrasse (Labridae) as cleaner-fish to remove lice from salmon has been used in Norway (Bjordal, 1991), Ireland (Darwall *et al.*, 1991) and Scotland (Treasurer, 1993). Predominately the goldsinny *wrasse Ctenolabrus rupestris* L., is used but other species such as the corkwing wrasse *Crenilabrus melops* L., and the rock cook wrasse *Centolabrus exoletus* L., have also been utilised (Sayer *el al.*, 1996). The use of wrasse as an alternative control method has obvious advantages over the use of chemotherapeutants, both environmentally and with respect to reduced stress to the fish. However, there are fears in the salmon industry that wild caught wrasse may transfer pathogens to farmed salmon; although findings suggest that the parasitic fauna of wrasse should not pose a threat to salmon (Treasurer, 1997). However vibriosis and *Aeromonas salmonicida* have been recorded in wrasse (Gibson, 1995;

Gravningen *el al.*, 1996). The lack of suitably sized fish, non over-wintering of wrasse in salmon cages, possible disease transfer, over exploitation of local wild populations and the lack of suitable species in sufficient numbers, are all problems associated with the use of wrasse in the control of sea lice. It would appear that a solution to these problem may be found in the commercial culture of wrasse, and pilot projects indicate that wrasse hatchery technology is indeed possible (Stone, 1996; Skiftesvik *el al.*, 1996). At present wrasse are no longer used in salmon cages in Scotland and have a limited future as biological control agents.

1.2.3 Farm management and other strategies

Physical methods of lice control may be possible on land-based farms using filtration and other water treatments, but such techniques are impractical on sea-cages (Costello, 1993). Unsuccessful attempts have been made to reduce the infection rate by the photopositive copepodid by shading salmon pens and using light traps beside cages (Huse *et al.*, 1990).

Fallowing of farm sites in the culture of Atlantic salmon is now widespread, for the control of disease and to reduce a build up of sediment beneath the cages (Bron *et al.*, 1993). This allows the cycle of the sea lice to be broken, hence delaying reinfection once restocking begins. Fallowing of all farms in a given area at the same time and stocking of single year classes with an all fish in and all fish out policy, coupled with intercompany management agreements to synchronise treatments may also help to prevent reinfection (Costello, 1993).

Integrated pest management (IPM) which is a strategy designed to optimise productivity by combining optimum mixes of pest control techniques, was developed to avoid the problems of pest-resistance build-up (leading to pest resurgence), secondary pest outbreaks, human health problems, the high cost of pesticide control

and environmental degradation caused by excessive and inappropriate chemical pesticide use (Kogan, 1998). Farm management techniques employing IPM strategies have been shown to increase production whilst reducing costs (Kogan, 1998), and may also be applicable to the salmon industry (Grant, 2002).

Other more experimental strategies such as the use of semiochemicals and vaccines are also being researched (Ingvarsdottir *et al.*, 2002; Raynard *et al.*, 2002).

As sea lice continue to be problematic in the salmon farming industry today, alternative possibilities for their successful management, one of which may be the use of natural enemies as microbial and other biological control agents need to be investigated.

1.3 BIOLOGICAL CONTROL

The majority of organisms produce more progeny than is required to maintain a constant population density. Therefore there is, in any given generation, an initial surplus of progeny over parents, giving rise to a theoretical exponential population growth for each species. Yet, this situation clearly does not arise, quite conversely, populations are usually relatively stable and maintain characteristic densities which, although fluctuating between certain upper and lower limits do not change much in a given ecosystem over long periods (Woodworth, 1908). This state of equilibrium is produced by the interaction of a potential for unlimited population growth combined with factors of control exerted by the environment. These environmental factors are know as 'natural control' and include:

Natural enemies (parasites, predators and pathogens), weather and other physical factors, food quality and availability, interspecific and intraspecific competition, spatial or territorial requirements.

Biological control can be broadly defined as the use of one species for the suppression of another (Molloy, 1998), or more precisely as the action of parasites, predators or pathogens in maintaining another organism's population density at a lower average than would occur in their absence (Debach, 1964).

As sea lice can occur at very high densities on their farmed salmonid hosts throughout most of the year, it would appear that, in the farmed environment at least, the environmental 'natural control' factors exert little pressure to reduce population density. However, the introduction of a natural enemy, or biological control, to act as an environmental 'natural control' factor could potentially exert more environmental pressure on the sea lice and decrease louse numbers.

In recent years biological control as a viable practice in modern agriculture and horticulture has increased dramatically. There are several reasons for this heightened interest in biological control, one of these is the ever increasing demands on food production and the intensification of farming to cope with this increase in demand, coupled with a general increase in public awareness of the potential ecological hazards posed by the use of pesticides and potential danger to human health (Holt & Hochberg, 1997). Of equal importance is the fact that many of these chemical pesticides have had their effectiveness greatly reduced as a result of the emergence of resistance in pest populations (Holt & Hochberg, 1997; Jutsum *et al.*, 1998). The problem arises from the fact that many pesticides have such specific sites of attack, that a single mutation in a pest genome has been sufficient to alter or remove a sensitive site (Jutsum *et al.*, 1998). It is much less likely that pests would develop resistance to biological control agents because the genetic changes in the pest necessary to achieve resistance would be more substantial and the control agent is just as likely to co-evolve along with the pest (Debach & Rosen, 1991).

All major pests are assumed to have natural enemies such as predators, parasites and competitive antagonists (Huffaker & Messenger, 1976). Successful biological control depends on the manipulation of these natural enemies or introduced / novel enemies to reduce the pest population below an economically damaging threshold or one of environmental damage (Legner & Bellows, 1999). The two basic methods of biological control are the 'classical' and the 'inundative'. In the former an exotic beneficial organism is introduced into an area where an exotic pest has become established in the absence of the natural enemies that existed in its original environment. 'Inundative' methods involve, single or multiple applications of an agent, indigenous or introduced, in much higher concentrations of propagules than normally encountered by the pest.

There are many categories of biological control agent operating against each major type of pest. Predatory, phytophagous and parasitic insects were the pioneers for traditional biological control agents (Huffaker & Messenger, 1976), but now increased use of parasitic and pathogenic nematodes, protozoa, bacteria, viruses and fungi are being utilised as weapons of biological control, as reviewed by DeBach & Rosen (1991), and more recently by Legner & Bellows (1999). The involvement of microorganisms in biological control has been the major focus of attention of late. Fungi and bacteria have distinct advantages, not least of which is the ease with which they can be cultured to produce the necessary inundative concentrations of inoculum. The propagules are usually small enough to be applied using standard spraying techniques to hit the target pest, or to be brought into the pest's ecosphere in the manner of a conventional chemical pesticide. Pathogenic microorganisms have the added potential advantage of the capacity for rapid reproduction in or on the host pest, and further dissemination in the habitat.

The use of biological control agents has a long and successful record in the reduction of pest numbers in terrestrial systems. However, the use of biological control agents in the aquatic environment, especially the marine environment, is in its infancy. This proven track record in terrestrial, biological control systems may be an invaluable source for information, however, the criteria required for a terrestrial, biological control agent may well differ from one to be used in the aquatic environment. Caution should be taken before introducing either exotic, or inundative amounts of indigenous organisms that are not host specific to the target pest species.

1.4 STUDY OBJECTIVES

This study aims to investigate the possibility of using natural pathogens of sea lice from farmed and wild Atlantic salmon as biological control agents. The study involves the examination of large numbers of sea lice, which will be examined both externally and internally for the presence of naturally occurring hyperparasites and microorganisms. The organisms encountered will be evaluated for their potential use as biological control agents against sea lice on farmed Atlantic salmon.

CHAPTER 2

GENERAL METHODS

2.1 GENERAL ASPECTS

2.1.1 Louse collection

Sea Lice *L. salmonis* (and when present *C. elongatus*) were collected from marine salmon farms on the West Coast of Scotland. Motile stages (preadult and adult) were carefully removed using forceps from recently culled fish during routine harvests. Specimens were transferred from the farm site to the laboratory in 10 L bags of ambient temperature and salinity seawater; the temperature regulated using insulated boxes and ice. Once in the laboratory, specimens were maintained in seawater collected from the farm site, aerated at 10°C in 10 L aquaria. Daily removal of senescent and dead lice with 80% water change allowed the lice to survive without their hosts for several days.

Larval stages were obtained by maintaining gravid females in separate containers until their egg strings hatched. Naupliar stages showed varied survival rates in the laboratory (possibly due to incorrect temperature regulation on transportation from the farm site to the laboratory). The first naupliar stage hatched immediately from dark egg strings and generally moulted to the second stage within 24 hrs at 10°C. The copepodid stage was reached approximately 48 hrs after the second naupliar stage. The copepodid were sampled using a 150μm sieve; this stage was used to infect naïve fish for various experimental procedures.

2.2 MICROSCOPY

2.2.1 Light microscopy

Small organisms, juvenile stages or dissected material were examined using a cavity slide and a cover slip. Observations were made using an Olympus BH-2 compound microscope. Photographs were taken using an Olympus C-35AD-2 camera mounted on the microscope. Larger organisms and adult stages were examined whole, using an Olympus SZ40 dissecting microscope with the same photographic system.

2.2.1.1 Wax histology

Paraffin wax embedding was utilised for routine histology of tissues. Paraffin wax (Tissue Tek N°2, Bayer) allowed adequate retention of tissue morphology for general staining techniques.

Tissue samples for wax histology were first fixed in 10% neutral buffered formalin for 24 hr before processing in a Shandon Citadel automatic tissue processor. After embedding in wax, blocks were trimmed and soaked in water for one hour prior to cutting. Sections of 5μm were cut on a Reichert-Jung Biocut microtome before being stretched on a water-bath at 45 ˚C and floated on to slides. Slides were dried overnight in an oven at 60 ˚C prior to staining. See appendix 1 for staining solutions and procedures.

All sections were de-waxed or 'taken to water' before staining could be carried out, this process involved:

Xylene (de-wax)   5 min

Ethanol   2 min

Methylated spirits   1.5 min

Running tap water prior to staining protocol (according to individual staining procedure)

2.2.2 Transmission Electron Microscopy (TEM)

Dissected specimens were fixed in 2.5% gluteraldehyde for 2 hours, washed in cacodylate rinse buffer (0.1M, pH 7.2) overnight. Samples were then post-fixed in 1% osmium tetroxide (in 1% borax solution) for one hour, before dehydration through a graded acetone series. After dehydration to 100% acetone the samples were transferred to a 1:1 mix of Spurr resin (Spurr, 1963) and acetone for 1hr on a rotator followed by a further 2 hrs in a mix of 3:1 Spurr: acetone. Finally, the specimens were rotated for 24 hrs in 100% Spurr resin before being embedded in Beem capsules and polymerised at 60 ˚C for 48 hrs.

All sections were cut using a glass knife on a Reichert Ultracut E ultramicrotome. Semi-thin sections of 1μm were first cut and visualised by staining with 1% alcian blue for 5 mins and examined under light microscopy. Thin sections (80nm) were mounted on 200 mesh Formar coated copper grids and stained with uranyl acetate and lead citrate. Thin sections were examined with a Philips 301 TEM operating at 80kV. Photographs were taken with a flat plate camera using black and white Kodak 4489 EM film.

2.2.3 Scanning Electron Microscopy (SEM)

Specimens were fixed at 4 ˚C for one hour in 1% gluteraldehyde in 0.1M cacodylate buffer. This initial fixation was followed by a 2-3 day immersion in 3% gluteraldehyde in 0.1M cacodylate buffer at 4 ˚C, followed by a rinse in 0.1M cacodylate buffer only. Samples were then post-fixed in 1% osmium tetroxide (in 1% borax solution) for two hours, before dehydration through a graded ethanol series. Once in 100% ethanol, samples were transferred to a 50:50 mix of ethanol and

hexamethyldisilazane (HMDS) for 30 min before being transfered to 100% HMDS for a further 30 min. Samples were air-dried at room temperature over night, mounted on aluminium stubs and coated with gold at 40 mA for 90s using an Edwards S150B Sputter Coater.

Samples were examined using a Philips 500 scanning electron microscope operating at 15kV. Photographs were taken using an integral camera with Ilford FP4-125 black and white roll film.

2.3 DNA EXTRACTION

DNA extractions were performed on whole sea lice and other small invertebrates and on dissected internal organs of fish (approx. 20 mg of tissue). The fish tissues were carefully dissected with sterile scalpel blades whereas whole invertebrates were first washed in sterile seawater to minimise surface contamination. The sterile samples were then homogenised in 0.5 ml high concentration urea buffer (TNES-Urea: 10 mM Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 0.5% SDS; 4 M urea - modified from Asahida, Kobayashi, Saitoh & Nakayama, (1996) using a sterile micro-pestle. Proteinase K was added to a concentration of 100 µg/ml and digestion allowed to occur overnight in a 37 °C waterbath. DNA was subsequently extracted with Tris-saturated phenol and phenol: chloroform: isoamylalcohol (25:24:1), treated with RNase A, further extracted with phenol: chloroform: isoamylalcohol (25:24:1) and diethyl ether, and then precipitated at −80 °C with 2.5 x volume of 95% cold ethanol and 0.1 x volume 4M sodium acetate, and finally re-suspended in MilliQ water.

2.3.1 PCR amplification and band visualisation

PCR reactions were performed in 25 µl volumes, each reaction tube contained ~50ng of genomic DNA, 25pmol of each primer and utilised Ready-To-Go™ PCR Beads

(Amersham Pharmacia Biotech) which comprise ~1.5 units of *Taq* DNA polymerase, 10mM Tris-HCl, (pH 9.0 at room temperature), 50mM KCl, 1.5 mM $MgCl_2$, 200μM of each dNTP and stabilisers including BSA. All amplifications were performed on a Perkin-Elmer GeneAmp PCR System 2400 thermocycler.

Amplified products were separated on a 1% agarose gel (0.5x TAE see appendix 1) and visualised by staining with ethidium bromide (0.5μg/ml) and photographed on an ultraviolet transilluminator.

2.4 INFECTION OF ATLANTIC SALMON WITH L. SALMONIS

Gravid adult female *L. salmonis* were incubated as described in section 2.1.1 until hatched larvae had reached the copepodid stage. Fish for infection were maintained in 1 m round tanks, at a constant temperature of 10 ^{o}C and 34 ppt salinity. Approximately 150 copepodids per fish were introduced into each tank for infection. The water supply to each tank was turned off during exposure for approximately 3 hours and the tank covered with black polythene to allow maximum attachment of the copepodids to the fish. After attachment of the copepodids was complete, the water supply was returned and the fish left for 3 days. After which, 5 random fish were anaesthetised to facilitate an approximate count of successfully settled copepodids per fish.

CHAPTER 3

EPIBIONTS ASSOCIATED WITH SEA LICE

3.1 INTRODUCTION

Freshwater and marine crustacea have frequently been reported in the literature as being substrates for a large and diverse range of epibiont fauna (Cook *et al.*, 1998; Xu & Burns, 1991; Turner *et al.*, 1979). Epibiosis is the facultative association between two organisms: the epibiont and the basibiont (Wahl, 1989). The term 'epibiont' describes organisms that, during the sessile phase of their life cycle, are attached to the surface of a living substratum, the basibiont (Threlkeld *et al.*, 1993).

Copepods have frequently been reported as acting as carriers of protozoan ciliates, this relationship is described as phoresis (Kinne, 1980). This phoretic relationship has been described as non-parasitic or ectocommensal, where the epibiont benefits without damaging the host (Lom, 1973).

Among the most frequently reported epibionts associated with crustacea are the peritrich and suctorian ciliates (Fernandez-Leborans & Tato-Porto, 2000a; Fernandez-Leborans & Tato-Porto, 2000b). The stalked peritrich, *Zoothamnium* sp. has been reported from the copepods *Centropages abdominalis* (Sato) in a saline lake connected to Tokyo bay (Nagasawa, 1988) and *Acartia tonsa* (Dana) in the Patuxent River, Maryland (Herman & Mihursky, 1964). Another widely reported stalked peritrich, *Epistylis* sp. has been reported from the calanoid copepod *Leptodiaptomus minutus* (Lilljeborg) in Crystal lake, Wisconsin (Xie *et al.*, 2001), from the parasitic copepod

Lepeophtheirus salmonis in Scotland (Gresty & Warren, 1993) and from *Daphnia* sp in New Zealand (Xu & Burns, 1991) amongst many others. Whereas the mobiline peritrich *Trichodina* sp. has been reported from many different crustacean species such as calanoid copepods from billabongs in Australia (Green & Shiel, 2000). The stalked suctorian ciliates *Ephelota* spp. have been reported from the euphausiacean *Meganyctiphanes norvegica* (Villosus), (Nicol, 1984), on cultures of larval European lobster *Homarus gammarus* L. and penaeid shrimp *Penaeus monodon* (Fabricus) (Dale & Blom, 1987; Gacutan *et al.*, 1977) and on the parasitic copepod *Lepeophtheirus salmonis* (Stone & Bruno, 1989). A detailed review on peritrich and suctorian ciliates associated with crustacea was publishd by Fernandez-Leborans (2000a, 2000b).

Other organisms associated with copepods are worms from the genus *Udonella*, Monogenean: Platyhelminth. *Udonella* spp. are unlike other Monogenea in that they have neither a ciliated larval stage nor hooks at any point in their life cycle. There are four described species, which have been reported exclusively as hyperparasites of caligid copepods parasiting marine fish (Aken'Ova & Lester, 1996). The type species, *Udonella caligorum* (Johnston) has been reported from *Lepeophtheirus salmonis* and *Caligus elongatus* on farmed Atlantic salmon in Ireland (Minchin & Jackson, 1993). *Udonella caligorum* produces clusters of elongate, pyriform eggs of about 130 μm, each fixed to the copepod surface by a long spiral stalk with a discoidal attachment plate. From these eggs hatch juvenile worms, which attach to the copepod by means of a single posterior sucker, the caudal haptor (Minchin & Jackson, 1993).

Udonella myliobati (Guberlet) has been reported from *Caligus epidemicus* (Hewitt) on sea-caged yellow-fin bream *Acanthopagrus australis* (Owen) in Australia (Aken'Ova & Lester, 1996) and from *Lepeophtheirus natalensis* (Kensley & Grindley) on the spotted

ragged-tooth shark *Carcharias taurus* (Rafinesque) in South Africa (Olivier *et al.*, 2000).

Some parasitic dinoflagellates and related genera are also epibiotic on copepods, but are generally internally located, and hence will be reviewed in chapter four.

Although epibiosis is described as the facultative and opportunistic association of two organisms (Wahl, 1989), their direct and indirect interactions during this association may have major effects on the species involved. When the effects are predominately beneficial for epibiont and basibiont, co evolution can be expected to lead to associational or obligate specificity (Lust, 1950; Wahl & Mark, 1999). However, as many epibionts are non-specific substratum generalists (Wahl & Mark, 1999), they could be potentially detrimental or parasitic to their basibiont hosts.

High infestations rates of epibionts have previously been reported as being potentially detrimental to their hosts. Herman & Mihursky (1964), suggested that the high infestation rates of *Zoothamnium* sp. on *Acartia tonsa* were responsible for it being replaced as the dominant copepod species by *Acartia clausi* (Giesbrecht), a species that was not susceptible to infestation with the ciliate. Nagasawa (1988) also reports that infestation of the copepod *Centropages abdominalis* with the ciliate *Zoothamnium* sp. may be responsible for copepod population changes. An *Ephelota* sp. has been reported as parasitic on tank-reared *Penaeus monodon* larvae producing violent kicking movements by the host, reduced feeding rates and heavy mortalities (Gacutan *et al.*, 1977). Turner (1979), suggested that the copepod *Acartia tonsa* suffered loss of body fluids through lesions caused by the attachment of the ciliate *Epistylis* sp., and this was responsible for secondary bacterial colonisation.

Experimental studies with the copepod *Boeckella triarticulata* (Thomson) have demonstrated that when food is limited, copepods infested with the ciliate *Epistylis*

daphniae (Faure-Fremiet) survived for shorter periods of time, suggesting the epibiont might contribute to the decline of the copepod population when they are in a food-limited environment (Xu & Burns, 1991). Laboratory studies on *Daphnia* sp. have shown two clear effects of epibiont abundance on their cladoceran hosts: they significantly reduce fecundity and they lower resistance to oxygen deficiency (Green, 1974).

3.2 OBJECTIVES

The aim of the present study was to determine what natural epibiont fauna was associated with the salmon louse *Lepeophtheirus salmonis* on farmed Atlantic salmon in Scotland. The study concentrated on adult female sea lice, i.e. mature sea lice that have undergone their final moult, increasing the chances of finding bio-fouling organisms that otherwise may be lost during the moulting process. It was intended to develop and apply a protocol to screen many lice, generating an overall perspective as to what organisms are associated with salmon lice, and to assess their status as harmless ectocommensals or hyperparasitic natural enemies, and hence potential biological control agents. Thus, specific identifications, precise location, and number of each epibiont species were not recorded. Particular attention was given to species that developed high population densities and those that were found to be fouling egg strings. Comparisons in the occurrence of epibiont species between farm sites, seasonality in epibiont occurrence, and an assessment of the impact of their colonisations were made. An egg string hatching study was undertaken to establish whether fecundity and egg hatching was affected by the presence of epibiont infestation on the egg strings.

Sea lice were also examined from wild caught salmon to determine if similar epibiont fauna were associated with non-farmed fish and to maximise the potential for isolating pathogens from a wider range of environments.

3.3 METHODS

3.3.1 Louse collection and examination

Sea lice were collected from farm sites as detailed in chapter 2.1.1.

Fifteen farms sites were visited over a period of three years (see Figure 3.1), with a total of 46 farm visits in total. One site was revisited frequently and resampled, yielding seasonal data. Wild salmon were caught using seine nets in Loch Duich and beach nets at the mouth of the North Esk, Montrose bay.

A random sample of 100 adult female *Lepeophtheirus salmonis* were examined from each site within 24 hr for the presence of epibionts, using a stereo dissecting microscope. If epibionts were present the number of lice infested with each epibiont were counted. When epibionts were found to be present on adult female lice, adult male lice were also examined to establish if any preference existed between female and male lice by the epibionts.

3.3.2 Hatching studies on egg strings

Adult female sea lice with egg strings were placed individually in 500 ml beakers containing seawater from the site of collection, adjusted to 34 ppt with either distilled water or instant sea water® and maintained at 10 ºC with gentle aeration, water was changed daily. Ten beakers each contained one sea louse with a suctorian ciliate infestation over the egg strings, and ten beakers each contained a female with a greatly reduced number, or no suctorian ciliates on the egg strings. Each sea louse had been previously examined, to ascertain the total number of eggs per mm of egg string and the

length of each egg string, and grouped as having < 10 ciliates per egg string or > 100 ciliates per egg string. Lice were examined daily, after hatching was complete, the number of unhatched eggs were counted.

3.3.3 Egg string extrusion

Sixty adult female sea lice without extruded egg strings were examined and placed individually in 500 ml beakers containing seawater from the site of collection, adjusted to 34 ppt with either distilled water or instant sea water® and maintained at 10 ºC with gentle aeration, water was changed daily. Thirty beakers each contained one adult female infested with *Udonella* sp. and thirty contained lice with no udonellid infestation. The lice were examined periodically for 20 days for the continued presence of *Udonella* sp., any moribund or dead lice were removed and females that had produced egg strings were counted and removed.

3.3.4 SEM of sea lice infested with epibionts

Sea lice were fixed and prepared for SEM as described in chapter 2.2.3. Specimens were examined for cuticle damage and the presence of bacterial colonies at the point of epibiont attachment.

Figure 3.1 Map of Scotland showing the sea lice sampling sites. Red dots indicate farm sites and red asterisk indicates wild fish sites. Numbers along the map edges denote latitude and longitude.
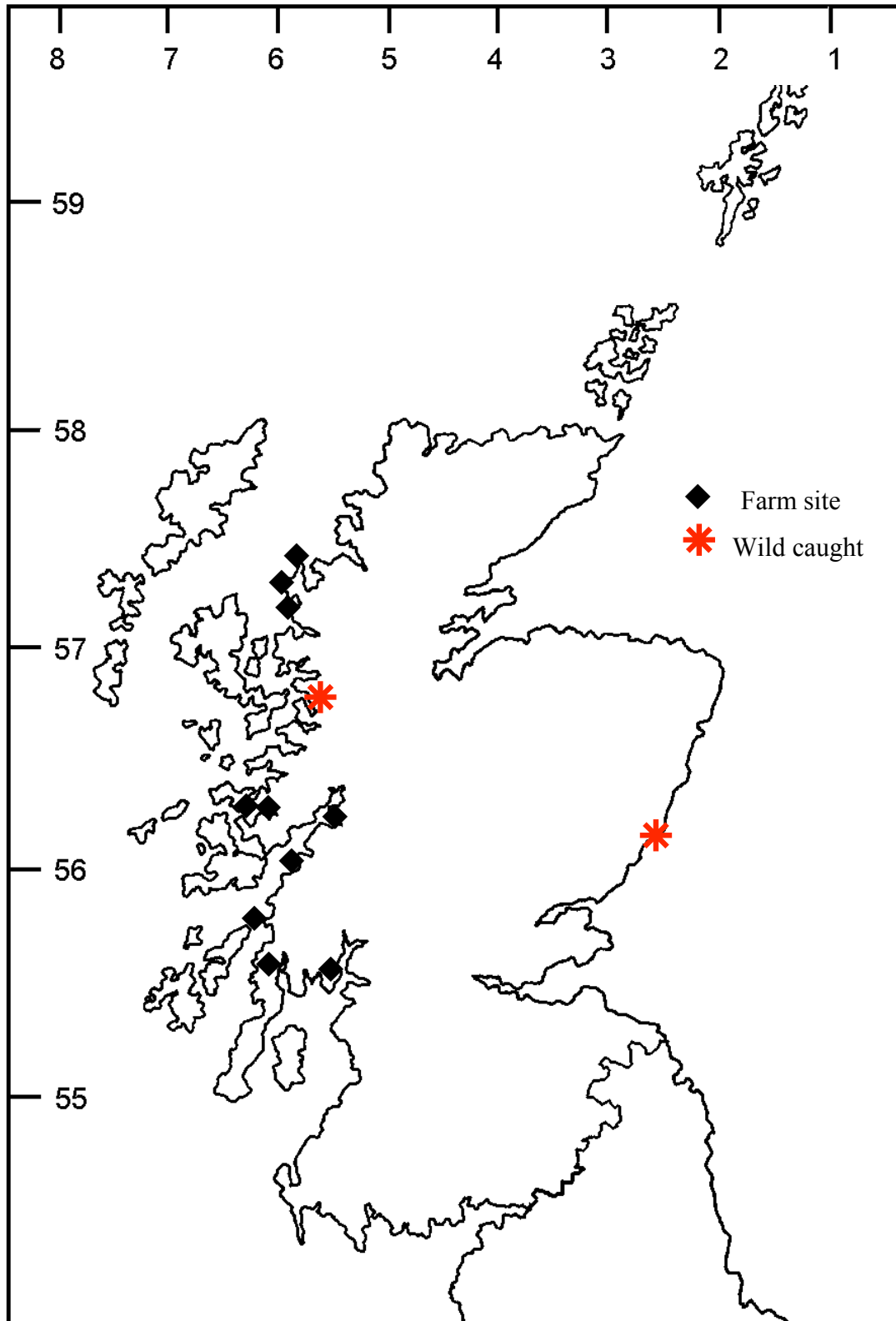
3.4 RESULTS

3.4.1 Epibionts recorded from adult female *Lepeophtheirus salmonis* on farmed salmon

The epibionts which were found in association with *L. salmonis* can be broadly classified into three groups:

> i. Algae and cyanobacteria
>
> ii. Ciliated protozoa
>
> iii. Monogenean: Platyhelminth.

See Appendix 2 for a list of epibionts present at each site.

3.4.1.1 Algae and cyanobacteria

Filamentous cyanobacteria were present on sea lice from all farm sites visited. The number of sea lice per site with cyanobacteria present varied between 4% and 98%, with an average of 48% of lice having filamentous cyanobacteria per site. The filamentous growth was found mainly on the marginal membrane of the cephalothorax (Figure 3.5). The colonies of these filamentous bacteria were limited to a few filaments on the egg strings, with larger colonies present on the marginal membrane. Filamentous cyanobacteria were also observed growing on other epibionts attached to sea lice.

Filamentous brown and green algae were found attached to the dorsal surfaces of the sea lice as shown in Figure 3.6, on the cephalothorax, the genital segment and the abdomen. Figure 3.7 shows a high power magnification (x400) of a brown filamentous algae showing discoid chloroplasts, similar to those found in *Pilayella* spp. and *Giffordia* spp.. Filamentous algae was present at ten of the fifteen sites visited and the number of sea lice sampled with filamentous algae per site varied from 1% to 100%, with an average of 17% of sea lice having filamentous brown or green algae per site when

present. The extent of the algal growth on each sea louse varied dramatically, from a few minute filaments to large trailing filaments up to four times the length of the sea louse's body.

Other algal species were less frequently recorded. Red algae were found in seven of the fifteen sites, the number of sea lice per site colonised by red algae varying from 1% to 10%, with an average of 3.8% of sea lice per site having red algae when present. Red algae were most often seen attached to the posterior of the abdomen as shown in Figure 3.8. Stalked diatoms were also sampled from seven of the fifteen sites, the number of sea lice sampled with stalked diatoms per site varied from 4% to 86%, with an average of 25% of sea lice having stalked diatoms per site when present. Stalked diatoms were always associated with high numbers of other epibionts, usually cyanobacteria or filamentous brown or green algae, and were infrequently found attached directly to the sea lice (Figure 3.9).

3.4.1.2 Ciliated protozoa

Stalked suctorian ciliates were found infesting sea lice; two species of *Ephelota* were recorded, and were identified as *E. gemmipara* (Hertwig) and *E. gigantea* (Fernandez-Leborans, pers. com.), the former being sampled more regularly. *Ephelota* spp. was present on sea lice from ten of the fifteen sites sampled. The number of sea lice per site infested with *Ephelota* spp. varied between 2% and 100%, with an average of 46% of sea lice having *Ephelota* spp. per site when present. *Ephelota* spp. was located mainly on the genital segment, abdomen and egg strings. Infestations varied from solitary individuals to dense colonies of greater than a hundred individuals per louse. Figures 3.10 and 3.11 show dense infestations of *Ephelota* spp. on adult female and male lice respectively. Figures 3.12 and 3.13 show dense colonies of *Ephelota* spp. on unhatched

and hatched egg strings, Figure 3.14 is a high power magnification (x200) of *E. gemmipara* showing a striated stalk and characteristic suctorian tentacles on the body.

A cyrtophorine ciliate, closely resembling *Trochilioides* sp. was found in only three of the fifteen sites. Infestation rates varied from between 20% to 90% of sea lice infected per site, with an average of 55% of sea lice having *Trochilioides* sp. per site when present. *Trochilioides* sp. was mainly found on the marginal membrane of the cephalothorax and on the ventral surface of the sea lice on the appendages (Figure 3.15).

3.4.1.3 Monogenea

The monogenean worm *Udonella* sp. was present on sea lice from seven of the fifteen sites. The number of sea lice per site infested with *Udonella* sp. varied between 2% and 100%, with an average of 54% of sea lice having *Udonella* sp. per site when present. *Udonella* sp. was observed as immature and mature worms and as clusters of eggs. Adult worms were located in similar positions to the eggs but were often found more centrally or dorsally positioned on the cephalothoracic shield, as shown in Figures 3.16 and 3.17. *Udonella* eggs were grouped in clusters ranging from one to twenty plus, and located on the posterior margins of the cephalothoracic shield, the genital segment and abdomen, as shown in Figures 3.18 and 3.19. Juvenile worms were found in similar regions, but often more distally positioned on the marginal membrane of the cephalothoracic shield (Figure 3.20). Rates of infestation varied from a few eggs and worms per sea louse to greater than 500 eggs per sea louse (Figure 3.21) with many adult worms present (Figure 3.17). The egg strings were rarely colonised with *Udonella*, with only an occasional juvenile worm present. *Udonella* sp. was observed in association with *Ephelota* spp. when both species were prevalent, as shown in Figure 3.22.

3.4.1.4 Other epibionts encountered

Occasionally other organisms were found as epibionts of *L. salmonis*. A stoloniferous hydroid was seen on two individuals (Figures 3.23 and 3.24) and recently settled bivalves were sometimes encountered in association with large amounts of filamentous algae.

3.4.2 Epibionts on male and juvenile *Lepeophtheirus salmonis*

All of the epibionts described above infesting adult female *L. salmonis* were also present on adult male *L. salmonis* sampled from the same locations. The epibionts showed similar site preferences on the body in both sexes, and although epibiont loads on males did reach similar densities to that of females, as shown in Figures 3.11 and 3.20, more often the epibiont load on males was far lower than that on females. Epibionts were occasionally seen on pre-adult males and females and on chalimus stages, albeit in greatly reduced numbers.

3.4.3 Epibionts on *Caligus elongatus*

Occasionally *Caligus elongatus* was sampled during the routine collection of *L. salmonis* from salmon farms. *Caligus elongatus* was rarely found in great numbers, but was examined for the presence of epibionts when present.

All the epibionts described for *L. salmonis* were found on *Caligus elongatus* specimens from the same locations. In a population of *L. salmonis* with high infestations of *Ephelota* spp. or *Udonella* sp., *C. elongatus*, when present, had the same epibionts, but at much lower densities.

3.4.4 Epibionts on sea lice from wild caught Atlantic salmon

In July 1999 seven wild salmon were caught using seine nets in Loch Duich, the fish had a total of 45 adult *L. salmonis* but no epibionts were present.

In July 2000 wild salmon were sampled from beach nets near the mouth of the North Esk, north of Montrose. Forty-four adult female *Lepeophtheirus salmonis* were found, 17% had filamentous cyanobacteria and 7% had *Udonella* sp. present. Six adult male *L. salmonis* were examined and were found to be free of epibionts. Sixteen adult female *Caligus elongatus* were also collected from the same fish in the July 2000 sample, one of which had *Udonella* sp. present. The *Udonella* infestations on *L. salmonis* and *C. elongatus* were very low, with only a few eggs or one or two worms present. *Lepeophtheirus pectoralis* (Muller) was also found on dab and flounder caught in the same nets used in the July 2000 sample; from 16 adult lice examined no epibionts were present.

A further sample of wild salmon were caught in July 2001 from the mouth of the North Esk. 58% of adult female *L. salmonis* collected were infested with *Udonella* sp., 4% had stalked diatoms and 5% had filamentous green algae. *Caligus elongatus* was also found, with 32% of adult female *Caligus* infested with *Udonella* sp.. The *Udonella* infestations were much denser than the previous year, with some sea lice heavily infested with more than 100 eggs and 50 worms per louse. The *Udonella* infestation was less dense on *C. elongatus* than on *L. salmonis*.

A repeat sample of wild salmon was taken three weeks later in July 2001 from the same site. A similar number of sea lice were collected but no epibionts were present.

3.4.5 Farm site specificity in epibiont occurrence

See Appendix 2 for a full list of epibionts sampled per site.

3.4.5.1.Cyanobacteria and filamentous algae

Filamentous cyanobacteria were found on all the sites sampled as epibionts of sea lice in Scotland. All sites sampled had sea lice with filamentous cyanobacteria present.

Brown and green filamentous algae showed no site specificity and were sampled from most sites, especially when other epibiont numbers were high.

3.4.5.2 Ciliated protozoa

Although the stalked suctorian ciliates *Ephelota* spp. were sampled in ten of the fifteen sited visited, some site specificity was apparent. Three sites in close proximity to each other in Loch Linnhe had very low percentage infestation rates of *Ephelota* sp., at a time of the year that other sites were heavily infested. Sixteen samples were taken from one site over an 18-month period and *Ephelota* spp. were always present, albeit at different levels of infestation.

From 45 farm visits, the cyrtophorine ciliate *Trochilioides* sp. was observed only five times from three different locations. The ciliate was always present when these sites were sampled, but was not observed elsewhere.

3.4.5.3 *Udonella* sp.

Udonella sp. was present form seven of the fifteen sites sampled and was always present at one site sampled 17 times over an 22-month period, albeit at differing infestation levels. In contrast, three farm sites in close proximity to each other in Loch Sunart were sampled nine times over a one-year period and *Udonella* sp. was never observed.

3.4.6 Seasonal trends of epibiont occurrence

One site that exhibited high epibiont numbers was chosen for seasonal data collection.

The site was visited 17 times over a 22-month period to estimate the infestation levels

of certain epibionts (see Figure 3.2).

Figure 3.2 shows how the percentage of lice infested with either *Ephelota* spp. or *Udonella* sp. varied from one site over a period of twenty-two months. The red arrow indicates when treatments were administered for sea lice control and the yellow line indicates sea temperature. When the site was visited more than once in any given month the data was averaged.
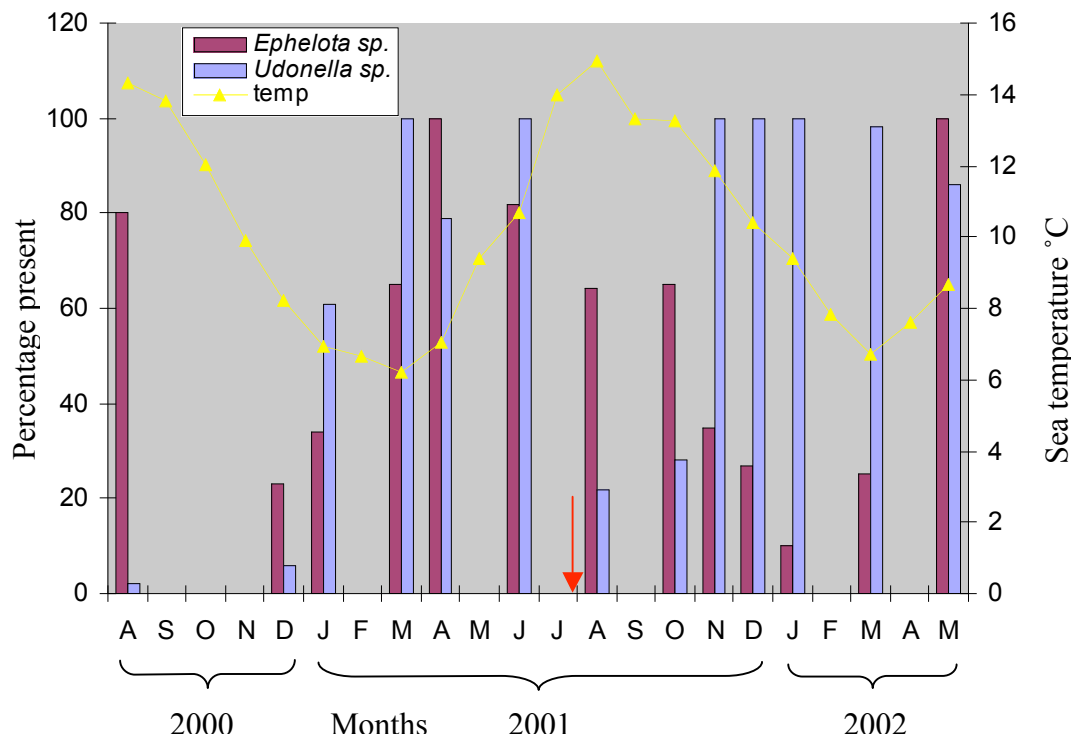
Ephelota spp. were most prevalent between March and October in 2001, peaking at

100% prevalence in April, becoming less prevalent from November through to January

2002, with numbers starting to rise again by March 2002 and increasing to 100%

prevalence in May 2002. All fish on this site were treated with the organophosphate

azamethiphos to control sea lice during the end of July and the beginning of August

2001 (indicated by red arrow on Figure 3.7), at which time the infestation rate of

Ephelota spp. on sea lice dropped from 82% prior to treatment of the fish to 64% after

treatment was given.

Udonella sp. showed very high rates of infestation throughout the year 2001, from 61% in January, rising to 100% by March and June. A rapid decline was observed in August when infestation rates dropped to just 22%, but rose steeply again to 100% by October, and remained high till May 2002. This dramatic drop exactly coincided with the administering of the sea lice control chemotherapeutant given to the fish in July and August 2001.

3.4.7 Egg string hatching study

Egg strings that were infested with greater than 100 *Ephelota* spp. per egg string averaged 19.43 eggs per mm of egg string, after hatching was complete an average of 3.29% of the eggs remained unhatched. Egg strings that were infested with less than 10 *Ephelota* sp. per egg string averaged 19.13 eggs per mm of egg string and after hatching was complete an average of 2.38% of the eggs remained unhatched, the results are shown in table 3.1, and the raw data set in appendix 2.

Table 3.1 Table of the average number of eggs per mm of egg string and average number of unhatched eggs from sea lice with high and low infestations of *Ephelota* sp. on egg strings, table also shows standard deviation.

Ephelota sp. per egg string	Mean No eggs / string ± SD	Mean egg string length (mm) ± SD	Mean eggs / mm of egg string ± SD	Mean percentage eggs unhatched (%)± SD
Greater than 100	425 ± 54	22 ± 2.9	19.43 ± 2.3	3.29 ± 1.9
Less than 10	416 ± 62	21.7 ± 2.4	19.13 ± 1.3	2.38 ± 2.8

The data collected for the percentage of unhatched eggs between the two populations (appendix 2) was tested for normality using Kolmogorov-Smirnov test, and were both found not to be normally distributed (greater than 100, K-S stastic 0.203, p= 0.2; less than 10, K-S statistic 0.318 p=0.005). The data set did however show homogeneity of variance, shown by using the Levene test for homogeneity of variance (Levene stastic 0.003, p= 0.956). A subsequent non-parametric Mann-Whitney test found there was no

significant difference in the number of unhatched eggs between the two populations (Z=-1.209 p=0.257).

The data collected for the number of eggs per mm of egg string for the two populations (appendix 2) was also tested for normality using Kolmogorov-Smirnov test, and were both found not to be normally distributed (greater than 100, K-S stastic 0.264, p= 0.047; less than 10, K-S statistic 0.209 p=0.200). The data set did however show homogeneity of variance, shown by using the Levene test for homogeneity of variance (Levene stastic 0.019, p= 0.893). A subsequent non-parametric Mann-Whitney test found there was also no significant difference in the number of unhatched eggs between the two populations (Z=-0.308 p=0.796).

3.4.8 Egg string extrusion study

The number of egg strings extruded from uninfected females and females infected with *Udonella* sp. is shown in table 3.2. Upon termination (day 20) of this experiment, there had been 16 mortalities and 12 pairs off egg string produced from the 30 sea lice that were not infested with *Udonella* sp.. This compared to 17 mortalities and 10 pairs of egg strings extruded in the 30 sea lice infested with *Udonella* sp.. At the end of the experiment two lice remained alive in the control population and three lice remained alive in the *Udonella* infested population.

Day	Control (non-infected)		Udonella sp. infected	
	Numbers of paired egg strings produced	Mortalities	Numbers of paired egg strings produced	Mortalities
0	0	0	0	0
6	5	3	4	5
9	3	3	3	2
13	2	4	0	4
15	2	3	3	2
20	0	3	0	4
Total	12	16	10	17

Table 3.2 Table showing the number of egg strings produced and the number of mortalities over a twenty-day period, from sea lice either not infested or infested with *Udonella* sp.

Figures 3.3 and 3.4 show Kaplan-Meier survival analyses for egg string extrusion and mortality rates, these show that there was no significant difference between the two populations in the rate that egg strings were produced or the rate of mortalities, Kaplan-Meier statistic 0.28; p= 0.596 and Kaplan-Meier statistic 0.01; p= 0.919 respectively. Although most sea lice survived for many days away from their salmonid hosts, adult *Udonella* sp. became moribund and detached from the sea lice within 2 to 5 days. *Udonella* eggs continued to hatch as long as the sea lice remained healthy, and the juvenile worms were active on the cephalothoracic shield for 3-7 days before coming moribund and detached. During the experiment, no juvenile worms matured to adult worms, no further eggs were produced by existing adult worms and no adult or juvenile worms remained on the sea lice at the end of the experiment.

Figure 3.3 The rate of extrusion of egg strings from *Udonella* infected and uninfected *L. salmonis* over 20 days, censored individuals represent mortalities.
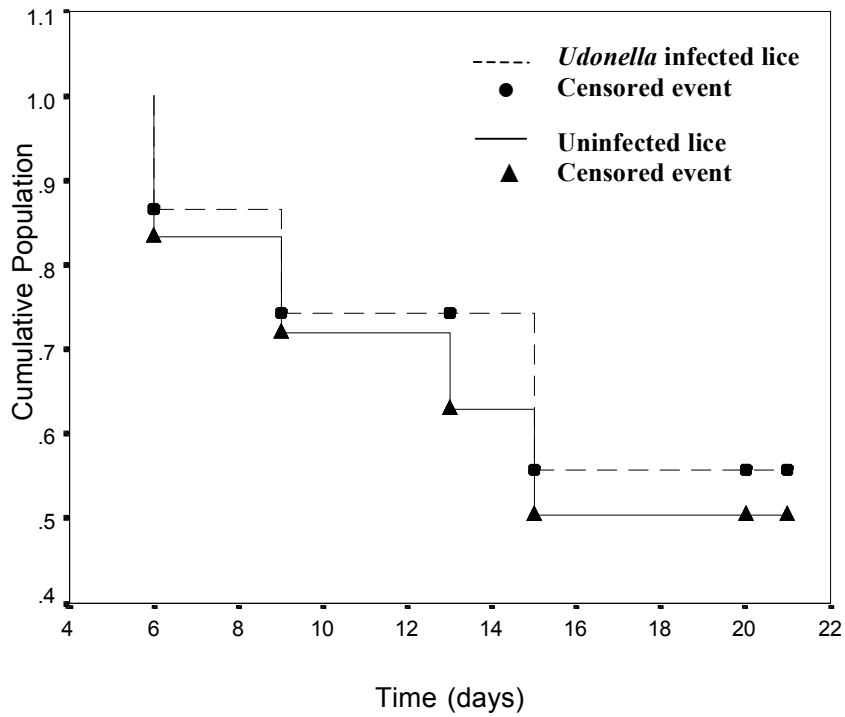
Figure 3.4 The rate of mortality from *Udonella* infected and uninfected *L. salmonis* over 20 days; censored individuals represent removed sea lice due to egg string production.
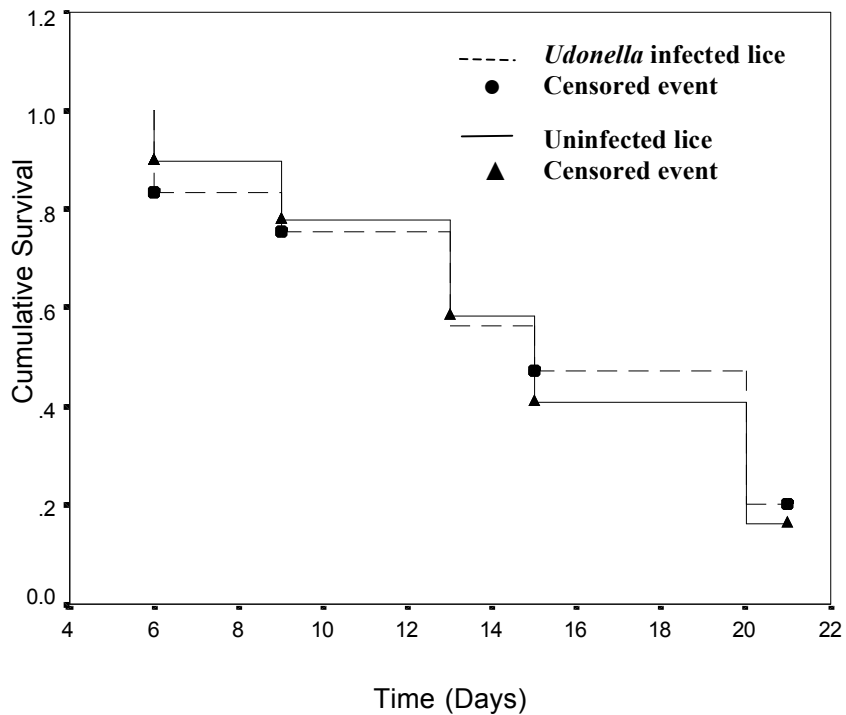
3.4.9 SEM of louse cuticle and epibionts

Figure 3.25 shows that no damage to the external surface of the sea lice cuticle was observed when udonellid worms were present on *L. salmonis*. High numbers of bacteria were found on the surface of the udonellid worms, but only very few of these bacteria were actually observed on the sea lice cuticle near the attachment sites of the worm, as shown in Figure 3.26. The attachment of udonellid egg filaments to the cuticle appeared to create a circular depression at the cuticular surface with a few bacteria present on the disc, the filament was comprised of a least five intertwined smaller filaments as shown in Figure 3.27.

The cuticle surface showed a large increase in the bacterial numbers surrounding the areas of attachment of the stalked ciliate *Ephelota* sp., although no actual cuticle damage was apparent (Figures 3.28 to 3.30). Egg strings infested with *Ephelota* sp. showed no external damage, but also had increased numbers of bacteria associated with their presence.

3.5 DISCUSSION

The epibionts described from this study infesting the salmon louse *L. salmonis* have all been previously recorded in the literature as epibionts species associated with crustacea, although there remains a certain ambiguity as to the type of relationships they share with their hosts. An attempt was made to evaluate the nature of these relationships between the epibionts and the sea lice; the terms defining such relationships are used according to Lom (2002).

3.5.1 Algae and cyanobacteria

Cyanobacteria are aquatic and photosynthetic; because of this they are often called "blue-green algae". Cyanobacteria are relatives of the bacteria, not eukaryotes, and it is only the chloroplast in eukaryotic algae to which the cyanobacteria are related.

The filamentous algae and cyanobacteria found in association with the salmon louse were never associated with cuticle damage and did not appear to be detrimental to the lice. These organisms are found growing on many different substrates and their ubiquitous nature indicates a facultative epibiosis with the salmon louse. However, when filamentous green and brown algae are present in very large dense clumps as shown in Figure 3.6, it could be argued that swimming ability could be potentially impaired, affecting the ease in which adult male copepods actively swim between fish to look for prospective mates. Mated adult females that change fish hosts less regularly (Hull *et al.*, 1998) may be less affected, but might inevitably experience more hydrodynamic drag from the fish's forward movement, which could potentially result in their premature removal from the fish host.

3.5.2 Ciliates as epibionts

Ciliates have frequently been reported in the literature as living in close association with crustacea, both as facultative and phoretic epibionts and as pathogens.

The most frequently observed ciliate in this study was *Ephelota*. This stalked suctorian ciliate was frequently observed as dense colonies on the external surfaces of the salmon louse, with infestation rates of greater than one hundred individuals per egg string. However, the hatching success rate was not significantly impaired and no signs of cuticular damage were observed at the attachment sites of these stalked ciliates. This is in contrast to an SEM study conducted by Turner *et al* (1979), in which lesions were observed on the copepod cuticle at the sites of attachment of the stalked ciliate *Epistylis*

sp. in the free-living copepod *Acartia tonsa*. These lesions were suggested to be responsible for the loss of body contents and for the increased number of bacteria observed around the lesion. Hence it was suggested that they were a potential reason for the seasonal succession of *Acartia tonsa* as the dominant copepod species (Turner *et al.*, 1979). Comparisons were made to lesions previously reported from freshwater fish suffering from an infestation of *Epistylis* sp. in which secondary bacterial infections were also observed (Esch *et al.*, 1976). However, the ciliate *Epistylis* has been previously reported living on the surface of freshwater fish with no detectable damage occurring to the host cell membrane, and Lom (1973) considered it to be an ectocommensal.

Ephelota sp. has been reported to be highly pathogenic to cultured juvenile penaeid shrimp causing high mortalities, with infestation rates as low as one ciliate per shrimp. The damage was suggested as being caused by the penetration of the cuticle by the piercing tentacles of this ciliate (Gacutan *et al.*, 1977). The present study found no evidence of this cuticular piercing, even with infestation rates of greater than 100 *Ephelota* sp. per host. This finding is in agreement with Stone & Bruno (1979) who also reported an *Ephelota* sp. from the salmon louse *L. salmonis*, which was not found to impair feeding behaviour even with infestation rates as high as 80 to 90 ciliates per louse. Thus, it seems unlikely that the high mortalities reported in tank reared penaeid shrimp were caused by *Ephelota* sp. particularly with infestation rates so low, and, as other pathogens were noted as being present at the time, this suggests that other organisms may have been responsible for the high mortalities observed.

The stalked ciliate *Ephelota* sp. observed in this study exhibited site preferences on its host, preferring the genital segment, abdomen and egg strings. Other ciliated epibionts demonstrate a much higher degree of site specificity on their hosts. A study of the

epibionts associated with the fresh water isopod *Asellus aquaticus* L., found that the highest ciliate loads occurred on the mouthparts and gills (Cook *et al.*, 1998). The distribution of eleven species of ciliates on a portunid crab revealed the chonotrich ciliate *Chilodochona quennerstedti* (Wallengren) to be the most abundant species present, colonising only specific sites on the crab's mouthparts. Whereas other ciliates including *Ephelota gemmipara* were much less site specific on the crab (Fernandez-Leborans & Cordoba, 1997).

In the present study *Ephelota* sp. was present on *Caligus elongatus* as well as on *L. salmonis*, but large differences in prevalence and epibiont density were noted, with fewer *C. elongatus* being infected with fewer ciliates than *L. salmonis* sampled from the same population. Similar findings have been reported from three different species of *Daphnia*, where the epibionts were not host specific but showed large differences in prevalence between the three host species (Stirnadel & Ebert, 1997). However, some epibiont species have been shown to exhibit a high degree of host specificity, Herman & Mihursky (1964) reported the ciliate *Zoothamnium* sp. infesting the copepod *Acartia tonsa* when no other members of the zooplankton community were affected. The stalked ciliate *Epistylis lacustris* (Imhoff) was reported to be highly specific to the calanoid copepod *Leptodiaptomus minutus*, with infestation rates as high as 250 ciliates per copepod when no other members of the zooplankton community were affected (Xie *et al.*, 2001).

Ciliated epibionts have been reported as being detrimental to their crustacean hosts, Herman & Mihursky (1964) suggested that the infestation of the copepod *Acartia tonsa* with *Zoothamnium* sp. lead to an increase in the sinking rates of infested copepods, which was detrimental and resulted in a population decline. In an experimental study by Xu & Burns (1991) the copepod *Boeckella triarticulata* infected with the ciliate

Epistylis daphniae survived for shorter periods of time than non-infected copepods when food was absent, suggesting that colonial peritrichs may contribute to the decline of copepod populations when they are in a food-limited environment (Xu & Burns, 1991). Ciliated epibiont infestations have also been shown to significantly reduce clutch size in *Daphnia* spp. (Green, 1974; Stirnadel & Ebert, 1997).

An increase in bacterial numbers was often observed using the SEM at the attachment points of *Ephelota* sp., filamentous bacteria and algae on the cuticle of sea lice. Bacteria were also seen by Gresty & Warren (1993) who observed bacterial colonies at the attachment areas of the stalked peritrichous ciliate *Epistylis* sp. to the cuticle of *L. salmonis* from Japan. This may be due to the creation of a protected area at the attachment sites of these epibionts creating a microenvironment in which bacteria can exist. Bacteria have previously been reported as being found most frequently in protected regions of the body surface of copepods such as the joints of the cuticle, the limbs and swimming appendages (Dumontet *et al.*, 1996; Nagasawa *et al.*, 1985). Copepods have a complex exocrine system consisting of various gland structures with associated secretions, one group of which are the tegumental glands. The tegumental glands lie close beneath or in direct contact with the body cuticle and one of the functions attributed to these glands is anti-fouling (Bell *et al.*, 2000; Brunet *et al.*, 1991; Brunet *et al.*, 1991; Bannister, 1993). It has also been suggested that exocrine secretions may have anti-bacterial properties (Boxshall, 1982; Bannister, 1993), evidence for which exists in terrestrial insects (Kovac & Maschwitz, 1991). As some species of stalked ciliates are able attach to the cuticle of copepods, the area of attachment or 'terminal plate' of the ciliate (Lom, 1973) may not receive the secretion from the copepods tegumental glands and hence any anti-bacterial properties attributed to the secretion would be lost in these regions, leading to a potential build up of bacterial

numbers. This might explain the increase in bacterial numbers observed in these regions, or simply that the terminal plate in ciliates is a preferred substrate for bacterial growth.

The stalked ciliate *Ephelota* sp. described from *L. salmonis* in this study was not found to inhibit the production or hatching of eggs or to compromise the integrity of the cuticle. It could be argued that, in very dense colonies, swimming ability could be affected, but sea lice appeared unaffected by their epibiont load both on and off their fish hosts. Stalked ciliates have been shown to compete for food with their host in a food-limited environment (Xu & Burns, 1991) and to foul essential areas such as the mouthparts and gills (Cook *et al.*, 1998). As the feeding and respiratory apparatus of *L. salmonis* is located ventrally in close contact with the fish host, it is an unsuitable location for stalked ciliate attachment. *L. salmonis* is a parasitic copepod and does not compete for food with the ciliates, indicating that the detrimental issues associated with ciliates and free-living copepods will nor arise in sea lice. The fact that *Ephelota* sp. appears to prefer to live on the external surfaces of crustacea suggests that they may benefit by doing so, possibly by gaining easier access to waterborne particles (Lom, 2002). It is therefore suggested that the stalked ciliate *Ephelota* sp. described in this study has a phoretic relationship with its sea lice host, being nutritionally independent, but benefiting by being transported to potentially food rich environments.

The mobile peritrichous ciliate observed in this study, *Trochilioides* sp., was not observed to cause pathogenicity to the salmon louse. This is in agreement with the previous study of Gresty & Warren (1993) in which *Trochilioides* sp. was observed attached to the salmon louse, with no apparent damage occurring. *Trochilioides* sp. is not widely reported in association with crustacea but is associated with poor water

quality where it feeds on the abundant filamentous microorganisms (Inamori *et al.*, 1991).

3.5.3 Monogenean worms

The monogenean worm *Udonella* sp. was frequently found in association with the salmon louse *L. salmonis*. Adult sea lice with heavy infestations of *Udonella* did not appear to be disadvantaged in any way. No cuticle damage was observed in the SEM study and no increase in bacterial numbers were observed at the sites of attachment of the worms despite high numbers of bacteria being present on the worms themselves. However, the discoidal attachment area of the egg filament appeared to produce a depression in the cuticle surface. This depression is possibly caused by the attachment of the egg filament to the procuticle and not the outermost layer of the cuticle, the epicuticle layer. The procuticle is described as a more laminate layer than the epicuticle (Bresciani, 1986), and hence may be a more suitable substrate for egg filament attachment. No previous SEM study of *Udonella* spp. *in situ* has been reported in the literature, but a recent histological study by Carvajal *et al* (2001) on adult *Udonella* sp. attached to the egg strings of the parasitic copepod *Caligus rogercresseyi* (Boxshall & Bravo) infesting the Chilean rock cod *Eleginops maclovinus* (Valenciiennes) revealed that *Udonella* sp. did not damage the egg string cuticle.

The presence of *Udonella* on adult female sea lice did not significantly affect the production of egg strings or increase the mortality rate when the sea lice were maintained in the laboratory away from their fish host. This is in agreement with a recent study in which *Udonella* sp. were found to have no effect on fecundity or on survival rates in the parasitic copepod *C. rogercresseyi* infesting the Chilean rock cod *Eleginops maclovinus* (Marin *et al.*, 2002).

The Udonellids are enigmatic monogeneans, not having hooks at any point in their life cycle. This lack of hooks could be an evolutionary result of its copepod association, whose hard exoskeleton is in all likelihood unsuitable for hook penetration (Littlewood *et al.*, 1998). Udonellids like most of their monopisthocotylean monogenean relatives have a close association with fish and have only been reported from copepods that parasitise fish and not from free-living copepods or other crustacea. Indeed, it has been suggested that *Udonella* sp. are more host specific to the fish than the copepod on which they live (Byrnes & Rohde, 1992). *Udonella* is frequently found on the parasitic copepod *C. rogercresseyi* infesting the Chilean rock cod, but is absent from the same species of copepod infesting cultured salmonids, in spite of the fact that the rock cod feed in close proximity to the caged salmonids (Marin *et al.*, 2002). In contrast to earlier suggestions that *Udonella* were hyperparasitic worms on their copepod hosts (Kabata, 1973), it is now suggested that *Udonella* in fact feed off the fish mucus and not from the copepod (Carvajal *et al.*, 2001; Marin *et al.*, 2002). In this study, the survival of adult *Udonella* on sea lice removed from their fish hosts was only 2-5 days during which time no eggs were deposited and no juvenile worms matured to adults before becoming moribund and detached. However, it was possible to maintain a healthy population of *Udonella* on copepods that were maintained on experimental salmonid fish in 1m seawater tanks (personal observation). This suggests that *Udonella* derive some if not all of their nutrition from the fish and not directly from the copepod.

The reason that *Udonella* spp. use caligid copepods as host and do not live directly on fish as other monogenea is unexplained but, by living on copepods, it is possible that they may be avoiding the fish's immune responses to their presence.

The *Udonella* sp. described from this study was not found to be detrimental to its copepod host and is thought to feed mainly from fish mucus that becomes available due

to the copepods' feeding activity over the fish. *Udonella* is undoubtedly dependent on the copepod for survival, and is therefore suggested to have an obligate ectocommensal relationship with its copepod host.

3.5.4 Other epibionts of the salmon louse

Occasionally other organisms were found as epibionts of *L. salmonis*. A stoloniferous hydroid and recently settled bivalves were sometimes encountered in association with large amounts of filamentous algae on the copepod cuticle. These organisms have been previously reported in association with *L. salmonis* on farmed salmonids in Ireland (Minchin & Jackson, 1993) and, despite completely smothering the genital segment of the infected female sea lice did not appear to be detrimental or affect the production or hatching of egg strings, and are suggested to be facultative epibionts.

3.5.5 Epibionts on male and juvenile *Lepeophtheirus salmonis*

Although epibiont numbers in this study did occasionally reach similar densities on male sea lice to that of female sea lice, males were usually less heavily infected with any given epibiont than the females in the same population. This is in agreement with Marin *et al* (2002) who state that *Udonella* show a marked preference for female copepods. However, other researchers reporting epibiont loads on parasitic copepods do not report this phenomenon (Gresty & Warren, 1993), indeed, in a study on free-living copepods, Nagasawa (1988) reports a higher incidence of infestation of *Zoothamnium* sp. on male copepods to females (Minchin & Jackson, 1993). The differences observed in epibiont load between adult male and female *L. salmonis* in this study could be attributed to the fact that females are generally regarded to have a greater life span than males (Jacobsen & Gaard, 1997) and hence would simply be available for colonisation for a greater period of time. Male *L. salmonis* are reported to spend more time in the water column swimming between fish (Hull *et al.*, 1998) and, although no diffenences

exist in the number of exocrine glands between male and female *L. salmonis* (Bell *et al.*, 2000), males may potentially secrete more from their exocrine glands to facilitate this swimming behaviour. This secretion is reported to have anti bio-fouling properties in copepods (Bannister, 1993), which may inhibit the ease in which epibionts can colonise the male cuticle. The low epibiont load associated with juvenile sea lice is most probably due to the loss of existing epibionts during the moulting process.

3.5.6 Epibionts on *Caligus elongatus*

In the present study the epibionts observed attached to *L. salmonis* were occasionally observed in association with *Caligus elongatus*, but usually at much lower densities. *C. elongatus* was not regularly sampled in large numbers, so the data available from this study on their associated epibionts is limited.

L. salmonis is essentially a parasite of salmonids, predominately infesting Atlantic salmon *Salmo salar* and sea trout *Salmo trutta* in Scottish coastal waters, whereas *C. elongatus* has a very wide host range of over 80 species of both elasmobranch and teleost fishes belonging to 43 families (Kabata, 1979).

Some epibionts have demonstrated a greater degree of host specificity to the fish parasitised by the copepod than to the copepod itself, Byrnes & Rohde (1992) and Marin & Sepulveda (2002) suggest that *Udonella* can only survive on their copepod host if the copepod is parasitising their fish of choice.

Certain environmental conditions may favour the successful settlement and development of a particular epibiont species on a copepod, if that copepod was to change fish host species, those environment conditions may significantly change and become unfavourable for the epibiont to maintain its population. Therefore it seems possible that a copepod that changed fish host species could potentially reduce numbers

of certain species of epibionts by altering the environment or fish host from that in which the epibiont can flourish.

High degrees of host specificity have been reported for many ciliated epibionts of copepods (Herman & Mihursky, 1964; Turner *et al.*, 1979) and varying rates of infestation have been reported in three coexisting copepods by the ciliate *Zoothamnium* sp. (Nagasawa, 1988). Therefore, it would seem acceptable to suggest that *L. salmonis* may simply be more susceptible to infestation by the epibionts described from this study than *C. elongatus*.

3.5.7 Epibionts on sea lice from wild caught salmonids

Epibionts have been previously reported from parasitic copepods infesting fish in their natural habitat. *Udonella* sp. is widely reported from caligid copepods parasitising wild teleost fish and sharks (Aken'Ova & Lester, 1996; Kabata, 1973). The single published study that looked for epibionts on sea lice from wild caught salmonids by Minchin & Jackson (1993) stated that *Udonella* sp. had not been observed on copepods from captured wild salmonids.

The epibionts reported from this study found in associated with sea lice from wild caught salmonids are either ubiquitous in the marine environment and have a facultative epibiosis with wild sea lice, or are obligate ectocommensal which have had a long evolutionary association with sea lice. The ubiquitous filamentous cyanobacteria and filamentous green and brown algae were occasionally observed in low densities on copepods from wild salmonids. The obligate ectocommensal, *Udonella* sp. however, was sometimes present in very high numbers with 58% of adult female *L. salmonis* infested with up to 100 eggs and 50 worms per individual and 32% of adult female *C. elongatus* being infested. This observation was not consistent and, during a repeat sample from the same location just three weeks later, *Udonella* was completely absent

when a similar number of sea lice were screened. Other epibionts regularly associated with sea lice on farmed salmonids, e.g. *Ephelota* sp. were never observed from copepods on wild caught salmonids in this study.

Although the data collected from wild salmonids in this study is somewhat limited, the associated epibiont fauna is similar to that from copepods on farmed salmonids; the notable exception is one of the most frequently observed epibionts in farmed salmon sea lice, *Ephelota* sp. This possibly indicates that the ciliate is not suited to the environment of the wild salmonid and is potentially a recently acquired non-specific epibiont to parasitic copepods on farmed salmon, being usually found on free-living copepods and other crustacea. The presence of *Udonella* sp. on wild caught salmonids was predictable; *Udonella* has an obligate association with parasitic copepods and must therefore be capable of sustaining a population on parasitic copepods from wild fish. *Udonella* was observed on both *C. elongatus* and *L. salmonis*, although the frequency and intensity of the infestation on *L. salmonis* was higher. The presence of high numbers of *Udonella* followed by its sudden disappearance could reflect the recent history of the fish. If the salmon have been in close contact with other species of fish parasitised by *C. elongatus* that are in turn infested with *Udonella*, the changing of host by *C. elongatus* could easily spread *Udonella* from one fish species to another, as suggested by Minchin & Jackson (1993). Hence, *Udonella* infected *C. elongatus* could act as vectors, transmitting *Udonella* to previously uninfected sea lice on different fish species. *Udonella* infested free-swimming adult *C. elongatus* have been sampled from the plankton, indicating that the transfer between fish species is indeed possible (Minchin, 1991).

It is suggested that sea lice infestation rates reach higher densities in farmed salmonids due to the intensification of farming practices (Tully, 1989). Therefore, one could

assume that higher numbers of copepods per fish combined with high numbers of fish per unit area, may lead to an increase in epibiont load per sea louse. Higher copepod densities have been reported to lead to increased ciliate numbers for a free-living copepod infested with the peritrichous ciliate *Epistylis* sp. (Xie *et al.*, 2001). However, *Udonella* population size is reported to be constant irrespective of copepod and fish numbers in both farmed (Minchin & Jackson, 1993) and wild fish (Marin *et al.*, 2002). As wild Atlantic salmon are not shoaling fish in the oceans, the presence of *Udonella* and the absence of stalked ciliate species as epibionts could simply be due to the lack of contact and hence transmission possibilities between wild oceanic fish.

There are many factors, which could lead to confusing results when studying sea lice and their associated epibionts from wild caught salmon. A salmon returning from the open ocean may encounter caged farmed salmon or the coastal dwelling sea trout, and may remain coastal for some days before either being caught by fishermen or entering the river, thereby allowing time for sea lice to potentially change hosts. Hence, the sea lice collected from wild, returning salmon, caught in coastal waters or estuaries may not be a true representation of oceanic salmon lice. The sea lice collected in this study, however, were all collected from Atlantic salmon, with the majority of sea lice being collected from the East coat of Scotland in a location distant from farmed salmon sites.

3.5.8 Farm site specificity

The facultative epibionts such as filamentous cyanobacteria and filamentous green and brown algae were ubiquitous in occurrence, and showed no farm site specificity. *Ephelota* sp. was observed at ten of the fifteen farm sites visited. Some of these sites only maintained a sparse population of *Ephelota* with low infestation rates, whilst other farm sites at similar times of the year had very high infestation rates. The presence of ciliates has been correlated to water quality (Nagasawa, 1988), which could be the case

for the cyrtophorine ciliate, *Trochilioides* sp., which was only observed at three out of the fifteen sites, where it was always present in samples. This ciliate is not widely reported in association with crustacea, but is has been previously associated with poor water quality feeding on filamentous microorganisms (Inamori *et al.*, 1991).

As previously stated the maintenance of a population of *Udonella* is not dependent on sea lice numbers (Minchin & Jackson, 1993), therefore, if a farm site contained sea lice infested with *Udonella*, unless the entire population of copepods were eradicated, one could assume that the population of *Udonella* would persist. This hypothesis is in agreement with the findings of this study. One site was sampled seventeen times over a period of twenty-two months and *Udonella* was always found, albeit at differing infestation levels. This farm site does not practise any fallowing strategy between production cycles and in fact is in constant production with mixed year classes present at all times. Other farm sites that utilise differing farm management strategies, for example fallowing between production cycles, could in effect eradicate the lice population with its associated epibionts.

It is hard to conclude convincingly from this study that site specificity occurs in these epibionts species. It is perhaps more likely that the large differences seen in epibiont presence and numbers between sites is due to differing farm management strategies and chemical treatment regimes used for sea lice control.

3.5.9 Seasonality in epibiont occurrence

This study yielded seasonal data from one site which was sampled 17 times in 22-months. *Ephelota* sp. was most prevalent in the spring months, in both 2001 and 2002, when the water temperature was between 7-9 °C. The percentage prevalence steadily fell from 100% in April 2001 to 25% by December 2001, then increased back to 100% by May 2002; this observation is in agreement with Stone & Bruno (1989), who

reported a decline in *Ephelota* sp. numbers on *L. salmonis* during seawater temperature rises in July and August. Other stalked ciliates from free-living copepods have shown a seasonal decline in numbers (Turner *et al.*, 1979; Nagasawa, 1988), but this coincided with a change in host copepod population density, and was therefore not likely to be solely a seasonally driven event.

Udonella sp. showed very high rates of infestation at this site, independent of sea temperature, having a percentage prevalence of 100% in March 2001 when the sea temperature is at its lowest (6ºC), and maintaining a prevalence of 100% in July with the sea temperature at 14ºC, nearing its maximum. The dramatic fall in *Udonella* prevalence from 100% in July to 22% in August 2001 coincided with the administration of a sea lice control chemotherapeutant given to the fish at the end of July 2001. The chemotherapeutant used was the organophosphate azamethiphos. This organophosphate is reported to be efficacious in the removal of adult and pre-adult sea lice, but is not effective against chalimus stages (Roth & Richards, 1992). Its action is to inhibit the activity of the enzyme acetylcholinesterase in the nervous system causing the lice to become detached (Baillie, 1985).

This removal of sea lice from the salmon will also result in the removal of their udonellid epibionts that are attached to the affected lice. Any *Udonella* attached to unaffected adults and chalimus larval stages could also be removed as monogenea are also affected by organophosphates (Halton *et al.*, 1993). Organophosphates are also known to be toxic to ciliates (Saini & Saxena, 1986), which may account for the decrease in prevalence seen in *Ephelota* sp. in the months following chemical lice treatment. This decline in ciliate prevalence is possibly more likely to be a seasonal effect as the population did not recover until the following spring despite there being no further chemical treatment, whereas the *Udonella* population recovered by November.

Minchin (1993) reported that *Udonella* were absent from his samples during the summer months, which is the time most farm sites would be chemically treating for sea lice control, which might explain the high degree of variability in his percentage prevalence data.

Figure 3.5 Filamentous cyanobacteria growing in small clumps attached to the marginal membrane of the cephalothorax (x400), scale bar = 20µm.
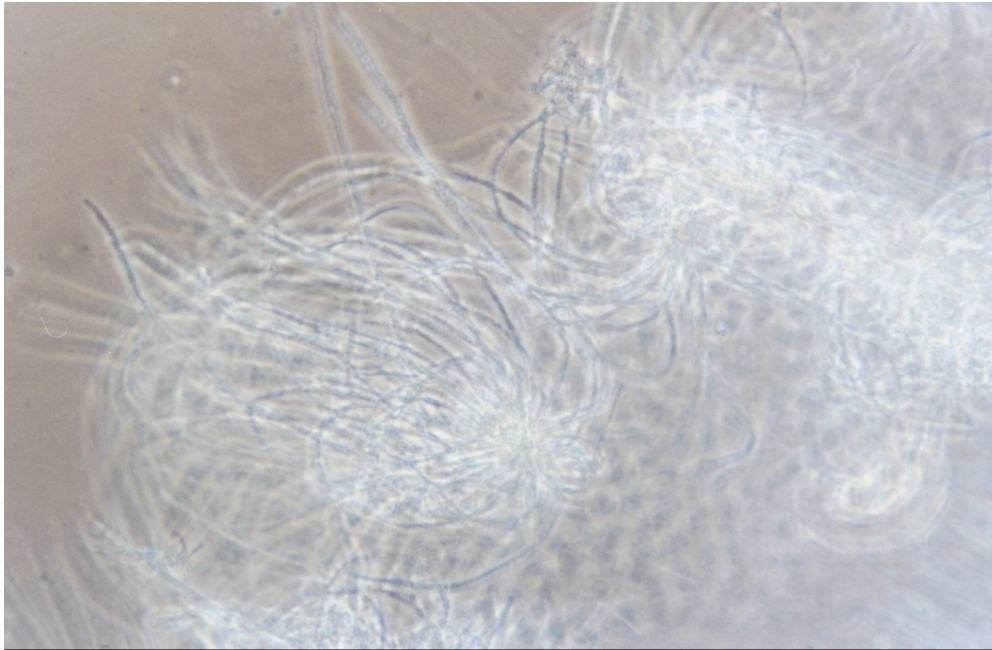
Figure 3.6 Adult female *L. salmonis* with large trailing filaments of brown and green algae attached to the dorsal surface of the cephalothorax, scale bar = 2mm.

Figure 3.7 Brown filamentous alga found attached to sea lice. Note the brown discoid chloroplasts (arrowheads) (x200), scale bar = 20µm.
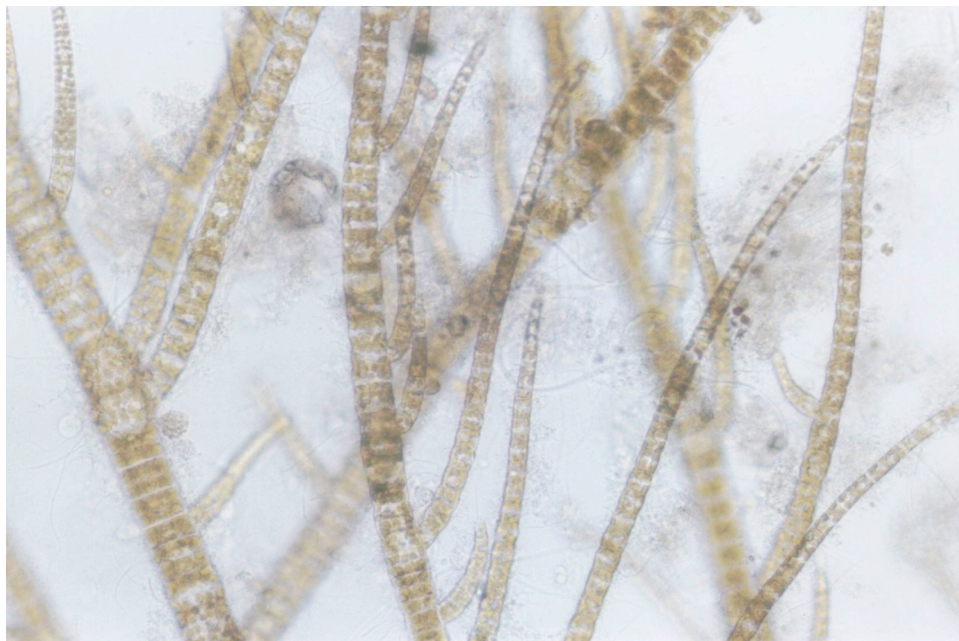
Figure 3.8 Red alga attached to the abdomen of an adult female *L. salmonis* (arrowhead), scale bar = 1mm.
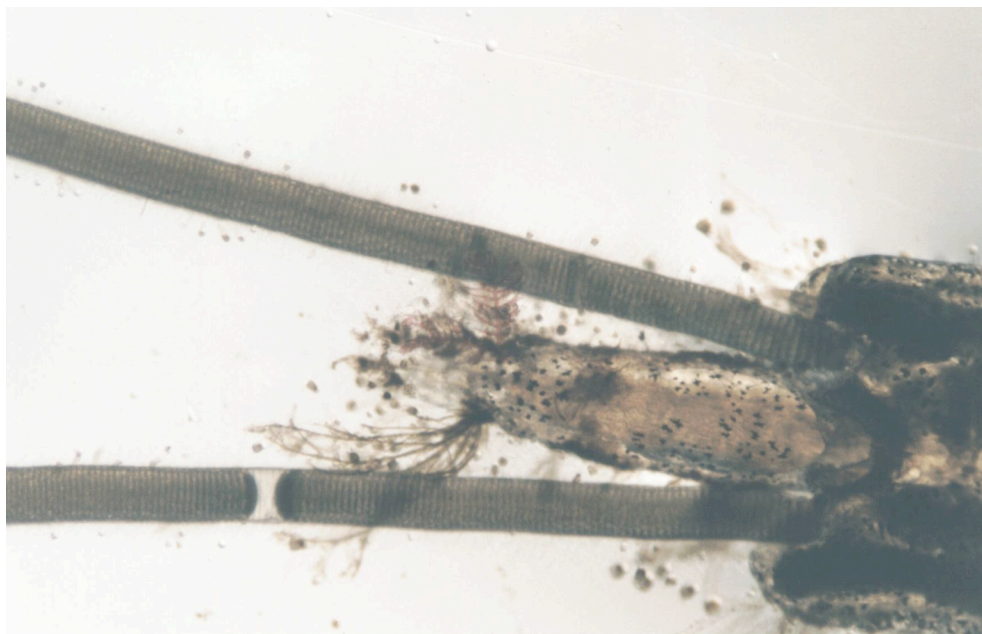
Figure 3.9 Stalked diatoms growing amongst filamentous cyanobacteria attached to the marginal membrane of the cephalothoracic shield (arrowhead) (x400), scale bar = 20µm.
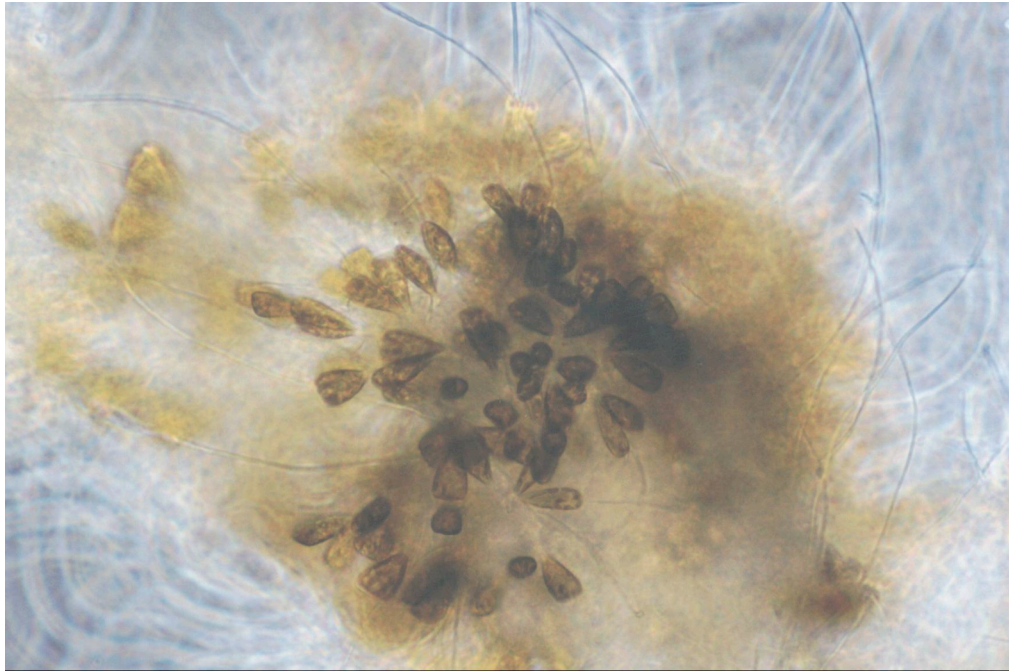
Figure 3.10 Adult female *L. salmonis* with the stalked suctorian ciliate *Ephelota sp*. attached to the genital segment and abdomen, scale bar = 2mm.

Figure 3.11 Adult male *L. salmonis* with the stalked suctorian ciliate *Ephelota sp*. attached to the genital segment and abdomen, scale bar = 1mm.
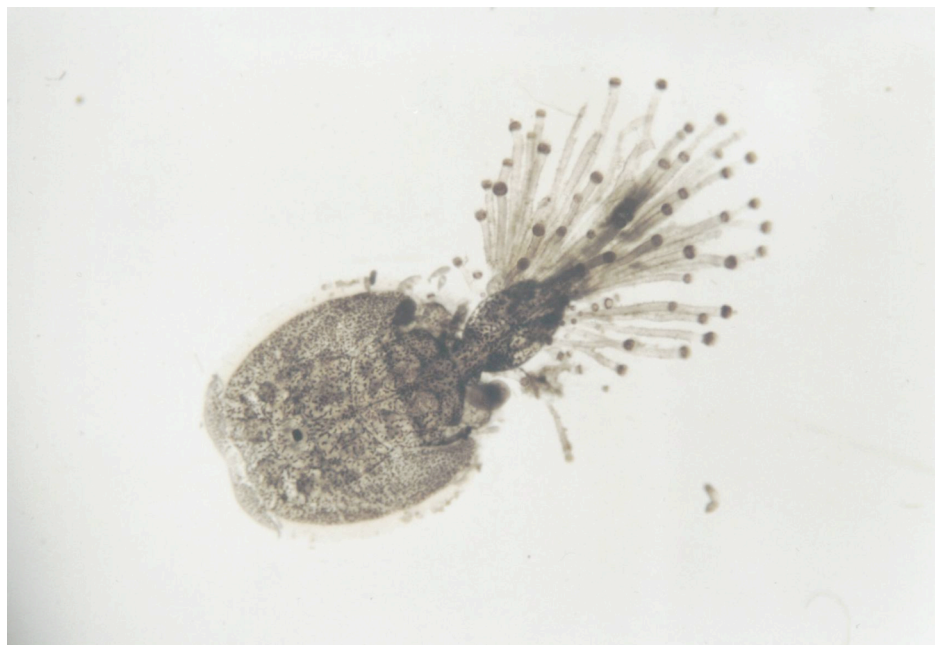
Figure 3.12 Very high numbers of *Ephelota sp*. infesting a mature egg string of *L. salmonis*, scale bar = 1mm.

Figure 3.13 Adult female *L. salmonis* with a successfully hatched pair of egg string despite the dense infestation of *Ephelota sp*. on the egg strings, scale bar = 2mm.
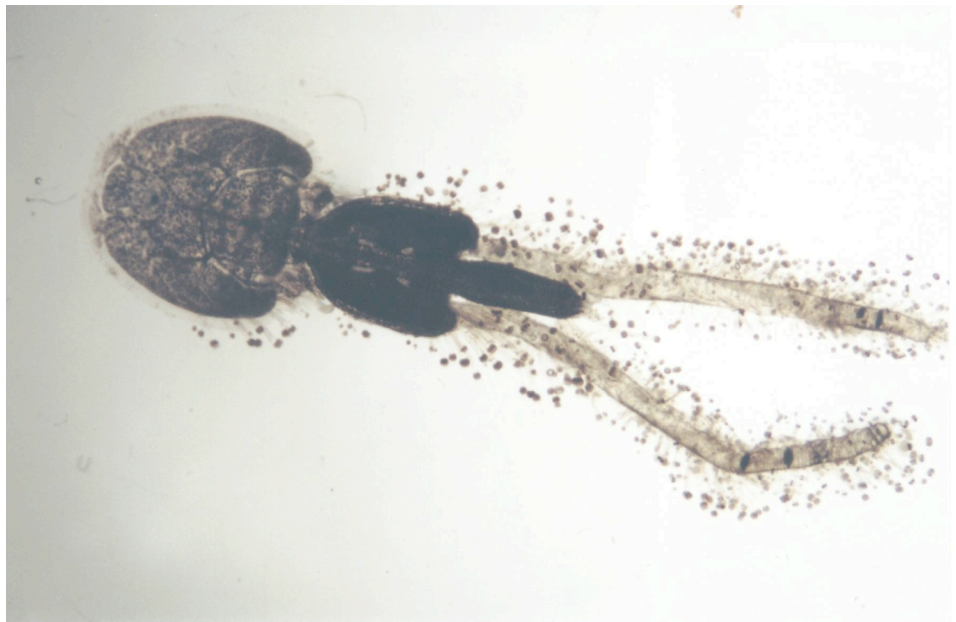
Figure 3.14 *Ephelota gemmipara* attached to *L. salmonis*. Note the characteristic suctorian tentacles (arrowheads) and striated stalk (x200), scale bar = 100 µm.
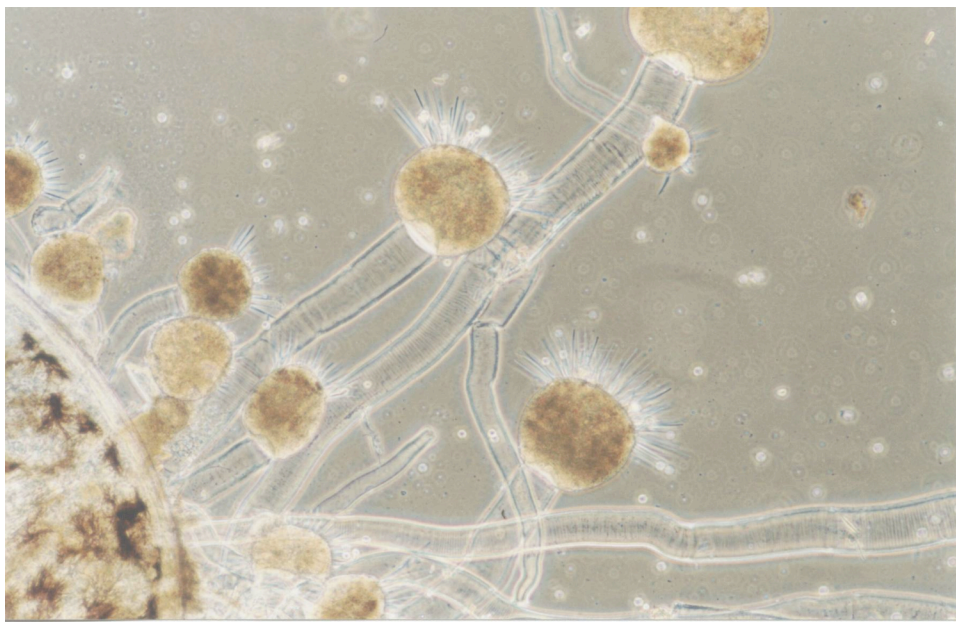
Figure 3.15 A cyrtophorine ciliate *Trochilioides* sp. attached to the marginal membrane of the cephalothorax and ventrally on the appendages (x400), scale bar = 10µm.
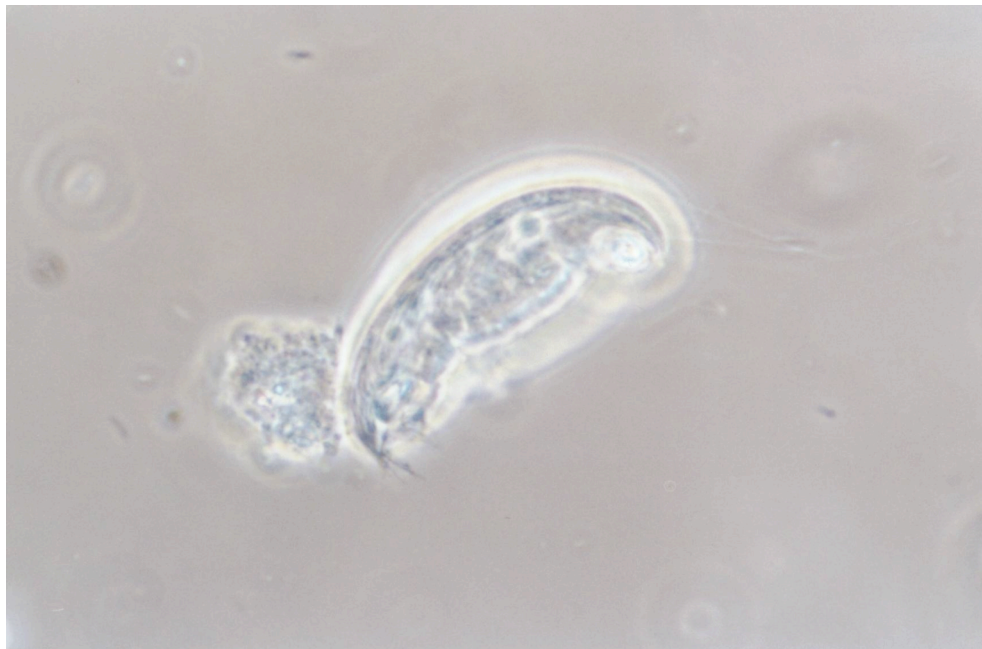
Figure 3.16 Adult Female *L. salmonis* infested with mature *Udonella* sp. typically attached around the margins of the cephalothorax, scale bar = 1mm.

Figure 3.17 Higher magnification of Fig.3.16 showing developing egg sting inside the genital segment. Juvenile *Udonella* sp. are also present (arrowhead), scale bar = 1mm.

Figure 3.18 Adult female *L. salmonis* with a large number of attached udonellid eggs; note the mature egg string (arrowhead), scale bar = 1mm.
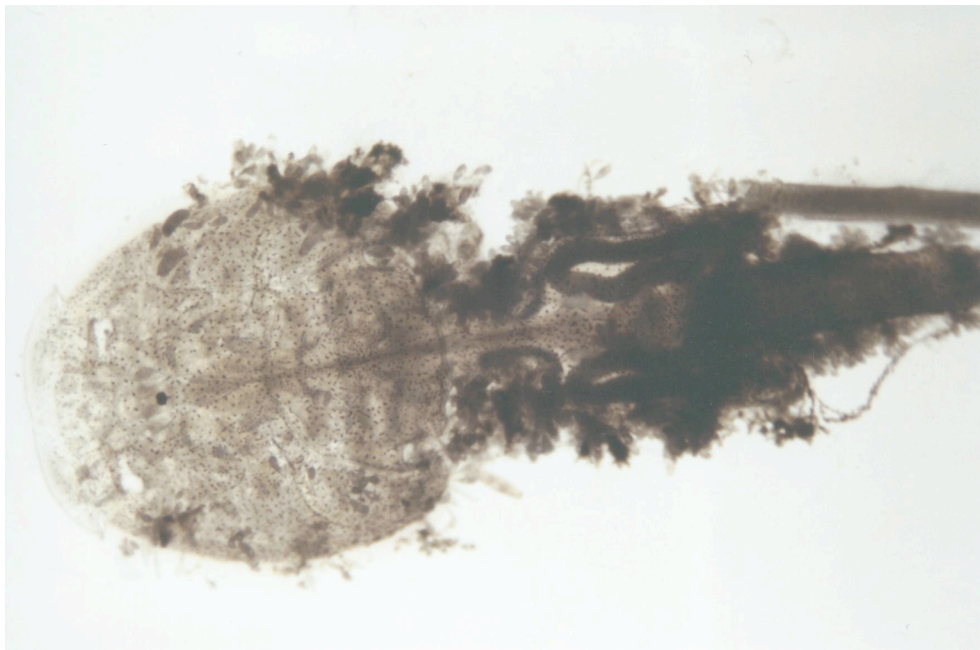
Figure 3.19 Higher magnification of figure 3.18 detailing the clusters of udonellid eggs (arrowheads) on the genital segment and abdomen; note the normal development of egg strings inside the genital segment (double arrowhead), scale bar = 1mm.
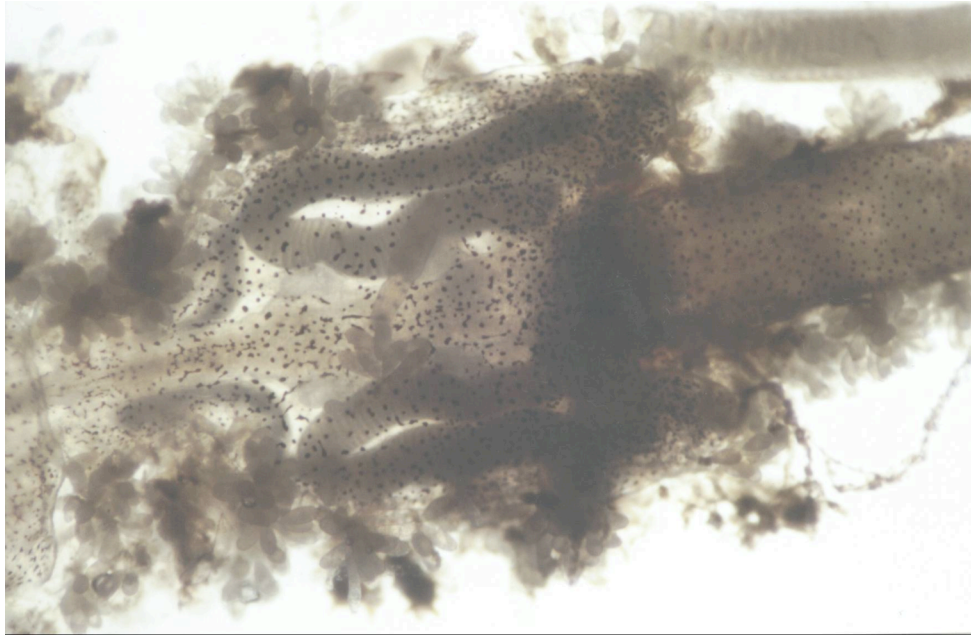
Figure 3.20 Adult male *L. salmonis* infested with adult (a) and juvenile (arrowheads) *Udonella* sp. around the margins of the cephalothoracic shield and on the abdomen, scale bar = 1mm.

Figure 3.21 Adult female *L. salmonis* infested with an extremely high number of udonellid egg clusters (greater than 500 eggs). Note the development of egg strings inside the genital segment (arrowhead), scale bar = 2mm.

Figure 3.22 Adult female *L. salmonis* infested with *Ephelota* sp. and *Udonella* sp. note the hatched egg strings, scale bar = 2mm.
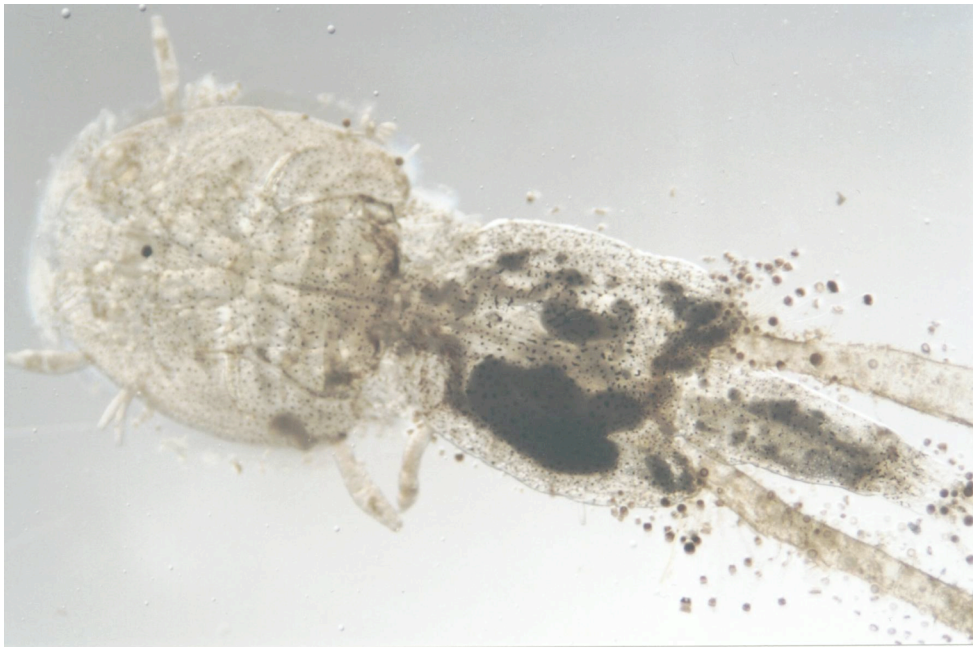
Figure 3.23 Adult female *L. salmonis* with a stoloniferous hydroid completely smothering the genital segment, note the hatched egg strings (arrowed), scale bar = 2mm.
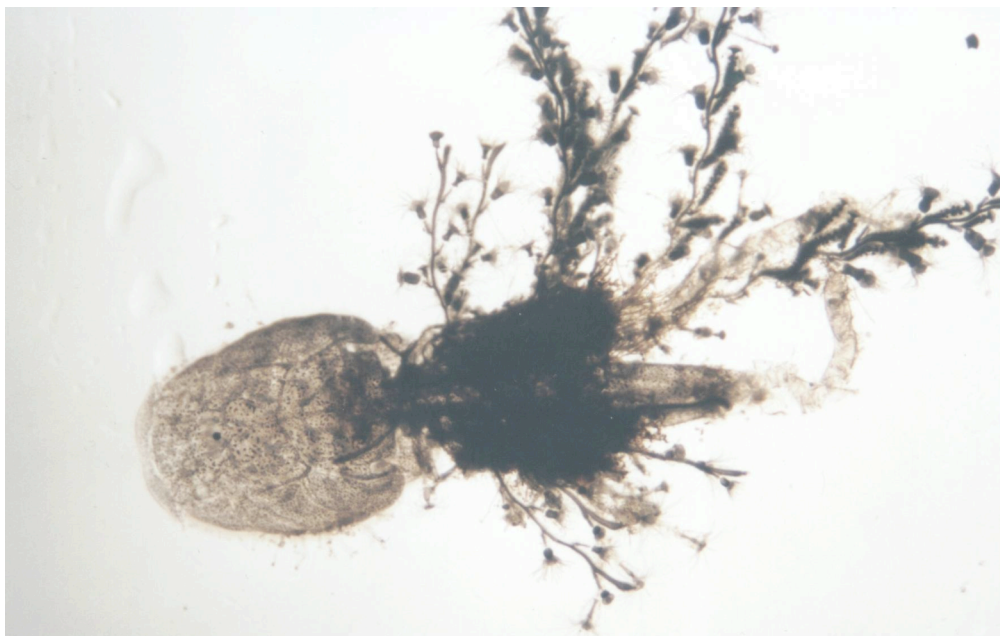
Figure 3.24 Higher magnification of figure 3.22, showing the reproductive head of the hydroid, scale bar = 1mm.

Figure 3.25 SEM of adult *Udonella* sp. attached to the cuticle of *L. salmonis* by its opisthaptor (h), with two eggs (e) attached to the cuticle via their filaments, scale bar = 100 µm.
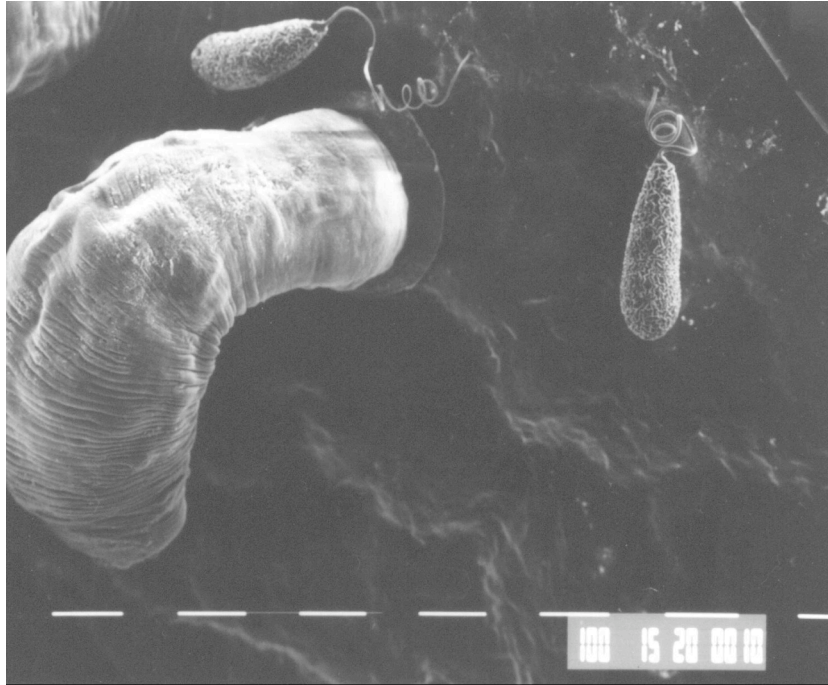
Figure 3.26 SEM of the opisthaptor of *Udonella* sp. attached to the cuticle of *L. salmonis*. Note the absence of damage to the louse's cuticle and the high concentration of bacteria over the udonellid's surface compared to that of the surrounding cuticular area, scale bar = 10 µm.
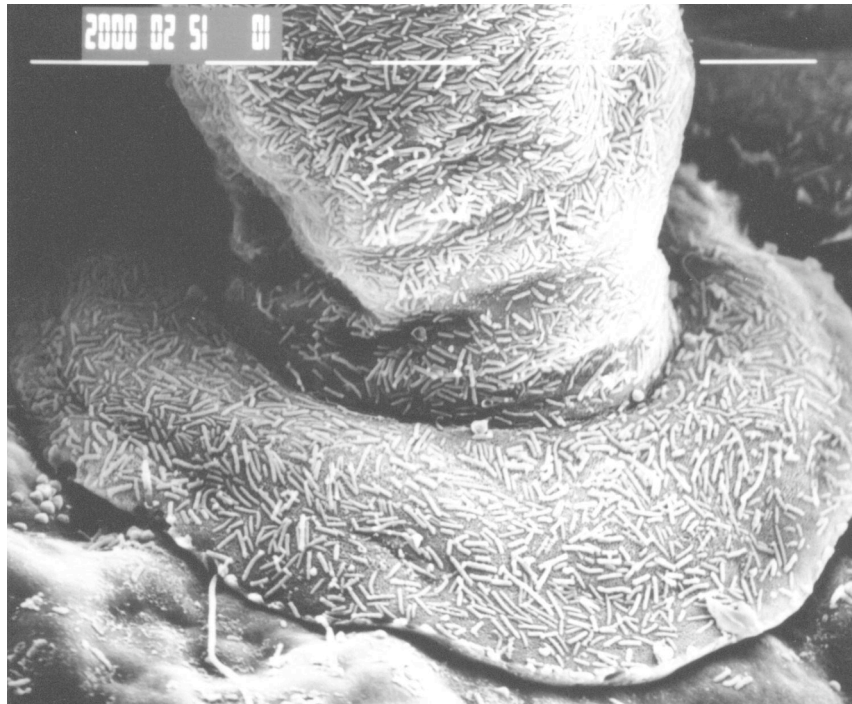
Figure 3.27 Attachment disc of *Udonella* egg filament. Note the depression made in the epicuticular layer of the sea louse, the multi-stranded arrangement of the egg filament (f), and the presence of a few bacteria (arrowed) on the attachment disc and surrounding areas, scale bar = 10 µm.
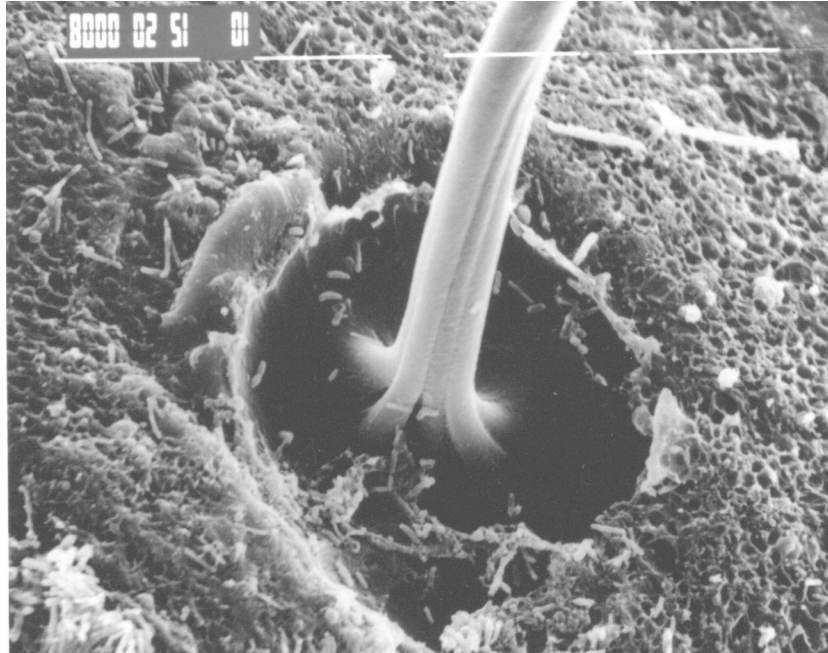
Figure 3.28 SEM of *Ephelota* sp. attached to the cuticle of *L. salmonis*. Note the appearance of bacterial / micro-organismal growth on the cuticle surrounding the colonising *Ephelota*, and the site of a previously attached *Ephelota* (arrowed) leaving no apparent cuticular damage, scale bar = 100μm.

Figure 3.29 SEM of *Ephelota* sp. attached to the cuticle of *L. salmonis*. Note the increase in bacterial numbers surrounding the site of attachment, and on the stalk (s) itself, scale bar = 10 μm.

Figure 3.30 SEM of an attachment site of *Ephelota* sp lifted away from the sea louse's cuticle, revealing that no cuticular damage or bacteria are present beneath the attachment site, scale bar = 10 µm.
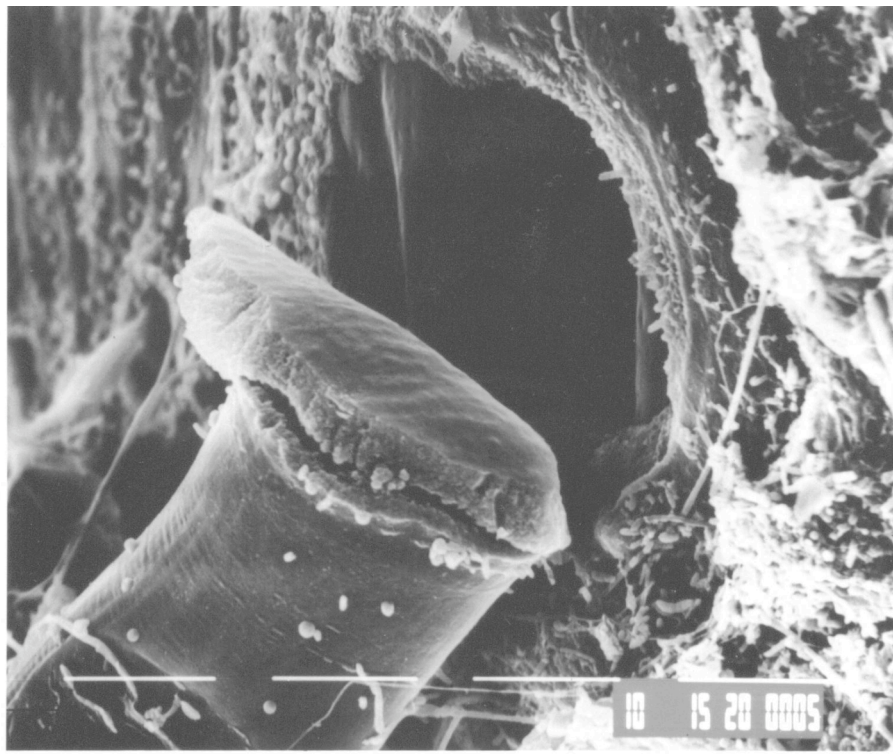
CHAPTER 4

ENDOBIONTS ASSOCIATED WITH SEA LICE

4.1 INTRODUCTION

There are numerous accounts in the scientific literature of aquatic crustacea being internally populated by symbionts and parasites. Apart from opportunistic pathogenic microorganisms, most of these symbionts and parasites appear to exhibit some degree of host specificity. In the current literature, there are no reports of internal symbionts or internal parasitic infections in the salmon louse *Lepeophtheirus salmonis*. Therefore, a literature review of known endobionts from other crustacean species was undertaken to provide information on the potential endobiont genera that might exist in the salmon louse.

4.1.1 Parasitic dinoflagellates, Paradinida and Ellobiopsidae

Of over 2,000 recognised species of dinoflagellates, approximately 140 are parasites of other organisms (Drebes, 1984). Marine crustaceans such as copepods, amphipods, mysids, euphausiids, and decapods are parasitised by two orders of dinoflagellates, the Blastodinida and the Syndinida. Two other orders that are similar taxonomically to the dinoflagellates; the Paradinida and the Ellobiopsidae, are also parasitic in crustacea. These parasites of marine crustacea inhabit the eggs, gut, soft tissues and the haemal sinuses of their hosts. An excellent review on these organisms and their related pathology to crustacea was compiled by Shields (1994).

4.1.1.1 Blastodinida

The order Blastodinidia consists of two families, the Blastodinidae and the Chytrodinidae. The Blastodinidae is represented by a single genus, the *Blastotodinium*, all members of which live as parasites in the stomachs of copepods. Although most of the species described have retained chloroplasts, they are estimated to obtain 50% of their energy budget from their hosts (Pasternak *et al.*, 1984). Infection leads to the lack of, or poor development of, the gonads and oviducts in their hosts (Chatton, 1920; Sewell, 1951; Jepps, 1937). This host castration is suggested to be caused by the physiological drain imposed on the host by the parasite and the mechanical pressures exerted on the internal organs of the copepod caused by the complete occupation of the alimentary canal by the parasites (Shields, 1994).

The Chytrodinidae consists of four genera whose taxonomic relationship within the family is confusing as they are only separated by small differences in their reproductive strategies. However, all genera within the family live as ectoparasites on the eggs of copepods, euphausids and penaeid shrimp (Dogiel, 1906). The generalised life cycle consists of a dinospore that attacks the host egg forming a stalk, which produces a peduncle-like organelle that penetrates and consumes the embryo. The parasite then undergoes sporulation and releases more dinospores. The epizootics of the Chytrodinidae are poorly understood but as a single chytriodinid can kill an embryo (Drebes, 1984), they are suggested to be capable of exerting considerable population regulation to their hosts, as with other egg-killing parasites (Wickham, 1986).

4.1.1.2 Syndinida

Members of the Syndinida are osmotrophic parasites living in the haemolymph, cytoplasm or body cavities of their hosts (Shields, 1994). The Syndinidia contains

four genera that parasitise crustacea: The *Actinodinium, Hematodinium, Syndinium* and *Trypanodinium*.

4.1.1.2.1 Actinodinium

No significant research of this genus has been undertaken, the only described species, *Actinodinium apsteini* (Chatton & Hovasse), develops in the gut wall of its copepod host *Acartia clausi*, where it projects numerous cytoplasmic rays into the haemal sinuses and lumen of the stomach (Chatton & Hovasse, 1938). The parasite is not reported to cause host castration, but its complete life cycle and pathogenicity to its host remain unknown.

4.1.1.2.2 Hematodinium

Members of this genus are primarily parasites of decapods, which are presumed to become infected by ingesting infectious spores or by cannibalism and subsequent ingestion of infectious trophonts (Shields, 1994). The parasite proliferates in the haemolymph of several decapod species, causing significant alterations in host physiology (Taylor *et al.*, 1996), biochemistry (Stentiford *et al.*, 1999; Stentiford *et al.*, 2000), locomotory performance (Stentiford *et al.*, 2000) and behaviour (Stentiford *et al.*, 2001a). Heavy infections are characterised by discoloured haemolymph that no longer clots, and can lead to high mortalities (Shields & Squyars, 2000; Stentiford *et al.*, 2001b; Field *et al.*, 1998). The infection of commercially important species such as, the Norwegian Lobster *Nephrops norvegicus* L., around the west coast of Scotland with *Hematodinium* sp. is also estimated to be causing high mortality rates in natural stocks (Stentiford *et al.*, 2001b).

4.1.1.2.3 Syndinium

Members of the *Syndinium* are primarily parasites of the radiolarians, however, some species parasitise crustacea where they inhabit the haemal sinuses and soft tissues of their hosts. The type species *Syndinium turbo* (Chatton) was described from the haemal sinuses of three copepod species (Chatton, 1910; Chatton, 1920). Another species was more recently described from the haemal sinuses of the amphipod *Gammarus locusta* L. (Manier *et al.*, 1971).

Copepods are assumed to acquire the infection by ingesting dinospores, which penetrate the gut wall and develop into plasmodia in the intestinal epithelial cells. A host capsule forms around the plasmodium from which the parasite eventually erupts, entering the body cavity, where it infects the nerve ganglion and invades and destroys the gonad (Chatton, 1920; Jepps, 1937). Host castration via the destruction of the gonads is noticeable in late stage copepodites and adult females; males are rarely infected (Chatton, 1920; Jepps, 1937), however, Manier *et al* (1971) did not report host castration in gammarids. *Syndinium* infections are considered to be more pathogenic to their copepod hosts than those of the *Blastodinium* spp.. *Syndinium* spp. have been reported in *Calanus finmarchicus* (Gunnerus) from the Firth of Clyde and from Loch Striven in Scotland, where prevalences of up to 30% have been reported (Jepps, 1937; Marshall *et al.*, 1934).

4.1.1.2.4 Trypanodinium

Trypanodinium ovicola (Chatton) is an endoparasite of the eggs of various copepod species. Host eggs are assumed to become infected when they come in to contact with swimming dinospores, which enter the egg and develop into plasmodia (Shields, 1994). Little is known about the potential effect these parasites have on their host population, but *Trypanodinium* sp. was reported as being responsible for up to 30%

mortalities in the eggs of its host shrimp *Pandalus borealis* (Krøyer), (Stickney, 1978).

4.1.1.3 Paradinida

Members of the order Paradinida are plasmodial parasites that infect the body cavities of copepods. These parasites, although sharing similarities with the aforementioned parasitic dinoflagellates, are separately classified on the basis of their nuclear development and flagellar organisation (Shields, 1994). Their life cycle has features distinct from that of the dinoflagellates. An amoebula develops from a bodonispore, which is presumed to be ingested by the copepod. It penetrates into the intestinal epithelium and develops in to an amoeboid plasmodium. The plasmodium has a bipolar spindle-like shape with fine filopodial extensions (Chatton, 1920). As these filopodia develop, they fuse and ramify throughout the body cavity, invading nerves and gonads, developing into huge plasmodia, which fragment into spores, which in turn migrate into the digestive tract and are expelled through the anus as multinucleate gonospheres (Chatton & Soyer, 1973). The gonospheres secrete a mucoid wall and attach to the caudal rami of the copepod. The resulting orange-coloured cyst can sometimes be longer than the host (Jepps, 1937). The gonosphere undergoes sporulation to form free-swimming bodonispores (Chatton & Soyer, 1973).

The *Paradinium* have a similar pathology to that of the *Syndinium* (Jepps, 1937). The early plasmodium is encapsulated by the host, but evades complete encapsulation to invade the nervous tissues, muscle and gonads. Host castration occurs as a result of the invasion of the gonad and feminisation of males has been observed, but is not a necessary outcome of infection. Some copepods can survive the infection, but it is assumed that they will significantly affect the host populations (Shields, 1994).

4.1.1.4 Ellobiopsidae

The Ellobiopsidae have previously been classified as members of the dinoflagellates (Chatton, 1920) and the fungi (Jepps, 1937). The zoospores of some members of this family are biflagellate, which is consistent with the dinoflagellates but other family members are uniflagellate (Galt & Whisler, 1970). It is the heterogeneity in this family that has led to confusion over the taxonomic placement of its diverse members. The Ellobiopsidae are primarily external parasites of pelagic crustaceans. There are four genera that parasitise crustaceans: *Ellobiocystis*, *Ellobiopsis*, *Parallobiopsis* and *Thalassomyces*.

4.1.1.4.1 Ellobiocystis

The genus *Ellobiocystis* consists of four described species that primarily infect shrimp and mysids (Chatton, 1920; Boschma, 1959). *Ellobiocystis* spp. spore stages are thought to settle on or adjacent to a seta of a buccal appendage of its host and develop into a trophomere (Fage, 1936). The trophomere is attached to the host via a non-invasive mucoid stalk and does not penetrate the body of its host. The locations of *Ellobiocystis* spp. around the mouthparts of its host, coupled with its non-invasive life style have suggested it to be a harmless commensal (Chatton, 1920).

4.1.1.4.2 Ellobiopsis

The genus Ellobiopsis consists of three species that primarily infect copepods (Boschma, 1959). In *Ellobiopsis* the trophomere possess a root-like organelle that penetrates the host cuticle and is suggested to be involved with absorption. Little is known about the pathology of *Ellobiopsis* spp., although, infection leads to a reduction in fecundity and can result in host castration depending on the numbers of parasites present per host (Jepps, 1937; Wickstead, 1963). *Ellobiopsis* sp. has been

observed as parasitic on the free-living copepod, *Calanus finmarchicus*, in the Firth of Clyde, Scotland (Jepps, 1937).

4.1.1.4.3 Parallobiopsis

The Parallobiopsis is represented by a single described species, *Parallobiopsis courtieri* (Collin), which attaches to the pleopods, gills and folds of the cephalothorax of its leptostracan host, *Nebalia bipes* (Fabricus), (Collin, 1913). *Parallobiopsis courtieri* has a trophomere with a sucker like holdfast equipped with an invasive peduncle, but little is known about its pathogenicity to its copepod host.

4.1.1.4.4 Thalassomyces

The genus *Thalassomyces* is the best-documented ellobiopsid as its members have large trophomeres that protrude extensively from the bodies of their hosts. The genus contains 12 species known to infect various crustacea, but not copepods. *Thalassomyces* have a specialised organ of fixation, equipped with protoplasmic rootlets that produce multiple trophomeres, each in turn producing multiple gonomeres. A detailed account of the genus *Thalassomyces* is reviewed by Shields, (1994). Pathogenicity varies as a result of the location of the organ of fixation, but regardless to its site of fixation the rootlets have an affinity for nervous tissues, but also penetrate the connective tissue, muscles and gonads (Wing, 1975). This invasion of the nervous tissues and gonad can lead to host castration, but is not an inevitable outcome of infection in all host species (Boschma, 1949; Kane, 1964).

4.1.2 *Rickettsia*, Rickettsia like organisms (RLO's) and *Wolbachia*

Rickettsia, rickettsia-like organisms and *Wolbachia* are obligate intracellular gram-negative bacteria that infect a wide range of hosts including arthropods, fish and

mammals (Alciati *et al.*, 2001; Fryer & Mauel, 1997; Wang & Gu, 2002; Werren, 1997).

4.1.2.1 *Rickettsia* and Rickettsia like organisms

Bacteria from the genus *Rickettsia* are pathogenic to mammals and humans, utilising ticks, mites and fleas as arthropod vectors, causing typhus and various fever related illnesses (Roux & Raoult, 1995; Alciati *et al.*, 2001). Whilst terrestrial arthropod vectors are seemingly unaffected by *Rickettsia* and RLO's, various crustacea have been observed to suffer from infections caused by RLO's in cultured and wild populations, in both the marine and freshwater environments (Romero *et al.*, 2000). Infection of the cultured freshwater Chinese mitten crab, *Eriocheir sinensis* (Milne-Edwards), with an RLO is reported to cause 'tremor disease' and mortalities of up to 90% (Wang & Gu, 2002). Stained prawn disease (SPD) of the marine shrimp *Pandalus platyceros* (Brandt), diagnosed by signs of black discolouration of the cuticle and of the hepatopancreas is caused by an RLO, and is causing mass mortalities in wild populations. Laboratory studies have shown that the pathogen is transmitted via contaminated water and by cannibalism, (Bower *et al.*, 1996). An RLO is reported as the most significant pathogen to cultured redclaw crayfish *Cherax quadricarinatus* (von Martens), in Ecuador, where growth rates are greatly reduced and mortality rates are as high as 80% (Jimenez & Romero, 1997; Romero *et al.*, 2000).

Infection of the fresh water amphipod *Crangonyx floridanus* (Bousfield), by a RLO is reported to produce a pale green iridescence in infected individuals, with up to 90 % mortality rates observed (Federici *et al.*, 1974).

4.1.2.2 Wolbachia

Wolbachia are maternally inherited, intracellular proteobacteria from the alpha sub-division that infect a wide range of arthropods. These bacteria are transmitted through the egg cytoplasm and alter reproduction in their arthropod hosts in various ways (Werren *et al.*, 1995). *Wolbachia* have been found to be associated with post-zygotic reproductive incompatibility (termed cytoplasmic incompatibility or CI) in a wide range of insects (O'Neill *et al.*, 1992; Breeuwer *et al.*, 1992), parthenogenesis in wasps (Stouthamer *et al.*, 1993) and feminisation of genetic males in isopods (Rousset *et al.*, 1992). These modifications of host reproduction impart a selective advantage for the bacteria (Turelli, 1994). *Wolbachia* occur in all major insect orders, and have been found in crustacea, mites and nematodes (Jeyaprakash & Hoy, 2000). This potential of *Wolbachia* to interfere with its host's natural reproductive cycle and sexual development could have implications for pest population management, if it were found in or could be introduced into sea lice.(Bouchon *et al.*, 1998; Breeuwer & Jacobs, 1996; Taylor & Hoerauf, 1999)

4.1.2.2.1 Cytoplasmic incompatibility (CI)

Wolbachia induced CI is a reproductive incompatibility between sperm and egg, which typically results in zygotic death in diploid species (Yen & Barr, 1971) or male production in haplodiploid species (Breeuwer & Werren, 1990). The bacteria are transmitted in eggs but are not (except in rare cases) transmitted through sperm (Hoffmann & Turelli, 1988; Bressac & Rousset, 1993). CI takes two forms, unidirectional and bidirectional. Unidirectional incompatibility typically occurs when the sperm from a *Wolbachia*-infected male fertilises an uninfected egg. The reciprocal cross (uninfected male and infected female) is compatible. Bidirectional incompatibility typically occurs when a male and female harbour different strains of

Wolbachia which are mutually incompatible (O'Neill & Karr, 1990; Mercot *et al.*, 1995). Although the exact mechanisms for CI are not fully understood, incompatibility is suggested to involve a two-component system, the bacterial modification of the sperm and the bacterial rescue of the egg. In this system the bacteria that modify the sperm must be then present in the egg to rescue this modification. If this rescue does not occur, then incompatibility between the egg and sperm results (Werren, 1997).

4.1.2.2.2 Parthenogenesis inducing (PI)

Wolbachia are also associated with parthenogenesis (reproduction from a female gamete without fertilisation by a male gamete). PI *Wolbachia* strains have been found in a wide range of parasitic wasps, including *Trichograma*, *Aphytis*, *Encarsia*, *Leptopilina* and *Muscidifurax* (Werren, 1997). Antibiotic treatment to these infected wasps reverts to production of male progeny; the causative bacteria have been identified as *Wolbachia* (Stouthamer *et al.*, 1990; Stouthamer & Luck, 1991). Whether or not *Wolbachia* induce parthenogenesis outside of the Hymenoptera is not yet known, as the cytogenic mechanism may bias their distribution to haplodiploid species. The mechanisms by which *Wolbachia* induce PI are well studied; meiosis is normal, in the first mitotic division but, the chromosomes condense properly in the prophase but fail to segregate in the metaphase, resulting in diploidisation of the nucleus, this is known as gamete duplication and results in homozygosity at all loci, subsequent mitotic divisions are normal (Stouthamer & Kazmer, 1994).

4.1.2.2.3 Feminising

In most diploid animals a balanced sex ratio is maintained by Mendelian inheritance, as one sex produces heterozygous haploid gametes, and the other homozygous. This is

almost always accompanied by anisogamy (unequal size of the gametes: males small with little cytoplasm, whilst females produce large gametes containing a large amount of cytoplasm) (Bull *et al.*, 1992). In some organisms sex determination is complicated by the influence of environmental factors, e.g. temperature (in some reptiles, fishes and crustacea), photoperiod (in some crustacea), and crowding (in nematodes) (Adams *et al.*, 1987; Ginsburger-Vogel & Magniettemergault, 1981), but can also be affected by inherited intracellular microorganisms such as *Wolbachia* (Bouchon *et al.*, 1998).

While the advantage for a microorganism to live in a host can be easily understood in terms of securing a favourable habitat, the advantage of inducing feminisation in the host, apart from that of anisogamy, is that inherited microorganisms are predominately transmitted vertically through the egg cytoplasm, and not the sperm. The male represents a dead-end host for such microorganisms, unless it is able to escape and be horizontally transmitted to another host (Rigaud *et al.*, 1997).

An example of such a distortion is *Wolbachia*-mediated feminisation of genetic males into functional females in some crustaceans. Twenty-two isopod species have been found to carry these bacteria. Although mainly terrestrial, one estuarine species and one shore species have also been found to carry *Wolbachia* (Bouchon *et al.*, 1998).

The phenomenon of thelygeny is also known in amphipods, which, as in isopods, results in vertically transmitted microorganisms inducing feminisation in juveniles. However, a number of differences between isopod and amphipod feminisation exist. Firstly, the causative agents of feminisation are different. While isopods are feminised solely by *Wolbachia* bacteria, feminisation in amphipods is associated with transovarially transmitted microsporidia (Smith & Dunn, 1991) and paramixydia (a protist closely related to microsporidia) (Ginsburger-Vogel & Desportes, 1979).

Secondly, *Wolbachia* and paramixydia are scattered throughout the host tissue, whereas transovarially transmitted microsporidia are only present in the female germline (Dunn *et al.*, 1995).

Other crustaceans have also been reported to have female-biased sex ratios. An unknown cytoplasmic substance or symbiont may control the duration of the egg incubation in the copepod *Tigriopus japonicus* (Mori), which in turn influences the sex of the larvae (Igarashi, 1964). Feminisation of males has also been reported in the decapod crab *Leptomithrax longipes* (De Hann), and is suggested to be caused by a bacterium present in the haemolymph (Roper, 1979), but the transmission strategies of these two microorganisms are currently unknown.

4.1.3 Microsporidia

Microsporidians are obligate intra-cellular, spore-forming, protist parasites that infect every major animal group, especially arthropods, fish and mammals (Sprague & Vavra, 1977). They are sufficiently unique to be classified in a separate phylum, the Microspora, Sprague 1977; and, although previously considered to represent primitive eukaryotes, are now recognised as highly evolved organisms related to fungi (Mathis, 2000; Van De Peer *et al.*, 2000).

Transmission of microsporidia occurs through a number of different routes, which can be categorised as horizontal transmission, vertical transmission or a combination of both. Horizontal transmission occurs between hosts of the same or different generation, usually through direct invasion of the host epithelium. Transmission can occur in this way between host species when an intermediate host is included in the parasites life cycle. Vertically transmitted parasites are passed from generation to generation and hence do not require an intermediate host. Vertical transmission can

take place via a number of routes in vertebrates, but the most significant vertical route amongst parasites of invertebrates is the transovarial route (Dunn *et al.*, 2001).

In microsporidia horizontal transmission is thought to be the most common method of transmission, where the infective spores are ingested by the new host, which then germinate in to the gut epithelium and spread to target organs throughout the body (Canning & Lom, 1986). However, microsporidia are also transovarially transmitted to new hosts. This transovarial route can be a minor route supplementing the main route of horizontal transmission, or can be the sole route of transmission and hence crucial for population maintenance (Terry *et al.*, 1998).

Records of microsporidan infections in crustacea include numerous genera that infect, crabs (Olson *et al.*, 1994; Zhu *et al.*, 1994), *Artemia* sp. (Martinez *et al.*, 1992), *Daphnia* spp. (Refardt *et al.*, 2002), ostracods (Diarra & Toguebaye, 1996), shrimps (Azevedo *et al.*, 2000; Azevedo, 2001; Bell *et al.*, 2001; Clotilde-Ba & Toguebaye, 2001), amphipods (Dunn *et al.*, 1995) and copepods (Voronin, 1996; Bronnvall & Larsson, 2001a).

Microsporidia are responsible for highly pathogenic diseases in several genera of shrimp known as 'cotton shrimp disease' (Ramasamy *et al.*, 2000), and are also responsible for poor survival and low egg production in *Artemia* culture (Martinez *et al.*, 1992). The transovarially transmitted microsporidian, *Nosema* spp., are reported to be responsible for feminisation in gammarid amphipods (Terry *et al.*, 1999; Terry *et al.*, 1998). Some species of microsporidia infecting terrestrial arthropods are also reported as being highly pathogenic; *Nosema bombycis* (Naegeli), causes severe gut pathology in commercial colonies of the silkworm *Bombyx mori* L. and *Nosema apis* (Zander) causes severe pathology in honeybees (Dunn *et al.*, 2001). High mortalities

of aquatic snails and the extinction of a land snail have also been attributed to infections with microsporidia (Cunningham & Daszak, 1998).

4.1.4 Fungi and yeasts

Aquatic invertebrates, including crustacea, have been previously reported as suffering from fungal epidemics which can seriously affect their population densities (Unestam, 1973). Most fungal parasites of crustacea belong to the water moulds (Phycomycetes), but members of the fungi imperfecti and the yeasts are also reported as parasitic in crustacea.

4.1.4.1 Water moulds (Phycomycetes)

An epidemic disease caused by the water mould *Hyphochytrium peniliae* (Karling) almost eliminated the planktonic cladoceran *Penilia avirostris* (Dana) during 1963 and 1964 in the Black Sea. The population density decreased by 98% in a few weeks and infected individuals were found to be filled with hyphae especially in the gut region (Artemchuk & Zelezinskaya, 1969).

Coelomomyces psorophorae is a water mould in the order Blastocladiales, which parasitise mosquito larvae. Infected mosquito larvae die and release hundreds of flagellate zoospores which must enter a copepod intermediate host before an infective zygote can be produced to infect further mosquito larvae (Travland, 1979). Preliminary field trials have suggested that *Coelomomyces psorophorae* may have potential as a biological control agent for mosquitoes (Whisler *et al.*, 1975). Typically, *Coelomomyces* spp. infect mosquito larvae where they develop in the haemocoel. Larvae are killed by the fourth instar, by which time hyphae have ramified throughout the lymphatic spaces causing substantial cell lysis and fat body depletion (Shoulkamy & Lucarotti, 1998).

Lagenidium callinectes (Oomycetes: Lagenidiales) infects the egg mass of the blue crab *Callinectes sapidus* (Rathbun), destroying up to 50% of the eggs in each mass; prevalence varies from year to year, but can be as high as 95%. Laboratory studies have also shown that the zoeal larval stages become infected and die (Rogers-Talbert, 1948). *Lagenidium callinectes* is also responsible for causing epizootics in cultured eggs and larvae of penaeid shrimp, *Cancer magister* (Dana) and *Homarus americanus* L., with up to 90% mortalities observed (Johnson, 1983). *Lagenidium* sp. has been noted as a virulent parasite of the freshwater calanoid copepod *Diaptomus novamexicanus* (Wilson), destroying the eggs and causing up to a 48% reduction in potential recruitment in the copepods (Redfield & Vincent, 1979). Whereas, *Lagenidium giganteum* has been evaluated as a biological control agent against mosquito larvae from the genera *Anopheles*, *Aedes*, *Culex*, *Culiseta* and *Psorophora*, with up to a 77% reduction in adult emergence being achieved in some experimental systems (Cuda *et al.*, 1997; Bisht *et al.*, 1996).

The related genera *Haliphthoros* and *Halocrusticida* (Oomycetes: Lagenidiales), have been isolated from moribund marine shrimp *Metapenaeus ensis* (De Haan), (Izumikawa *et al.*, 1999) and associated with mortalities in juvenile marine rock lobster *Jasus edwardsii* (Hutton), (Diggles, 2001).

Leptolegnia spp. (Oomycetes: Saprolegniales) have been suggested to cause mass mortalities in the marine copepod *Eurytemora hirundoides* (Nordqvist) and the freshwater cladoceran *Leptodora kindtii* (Focke), (Vallin, 1951; Petersen, 1910). *Leptolegnia caudata* caused 100% mortalities 7 days after inoculation with zoospores in laboratory experiments conducted with the mosquito larvae *Anopheles culicifacies* (Giles), (Bisht *et al.*, 1996).

Fungi from the genus *Aphanomyces* are largely parasites of freshwater fish and crustacea, although they have been associated with diseased marine fish in low saline and estuarine waters (Blazer *et al.*, 2002). *Aphanomyces ovidestruens* is parasitic on the freshwater copepods *Boeckella dilatata* and *Diaptomus gracilis* (Storch & Pfisterer) from New Zealand, attacking the egg sac through primary hyphal growth on the abdominal segment of female copepods (Burns, 1980). However, *Aphanomyces astaci* has been the focus of much attention, as it has been the cause of 'crayfish plague' and the near extinction of the European crayfish *Astacus astacus* L., since its recent introduction via the plague resistant American crayfish *Pacifastacus leniusculus* (Dana). Zoospores germinate when they come in to contact with the crayfish cuticle, penetrating the epicuticle by lipolytic action. Once inside the chitinolytic hyphae grow within the cuticle, and death results within a few days (Johnson, 1983).

4.1.4.2 Fungi imperfecti

Few Deuteromycetes (fungi imperfecti) are found parasitising crustacea, and are normally restricted to larger decapods such as lobsters and crabs in the marine environment, and crayfish in freshwater. Burn spot disease found on some crayfish and crab species, is caused by the fungus *Ramularia astaci*. After destroying the exoskeleton the fungus penetrates the gills and muscles, infecting populations with up to 85% prevalence causing significant mortalities (Mann & Pieplow, 1938; Järvekülg, 1958). The common soil saprophyte, *Fusarium* sp. has been responsible for mortalities in captive populations of penaeid shrimp and homarid lobsters, causing black gill disease, which is associated with high rates of mortality (Lightner, 1977).

4.1.4.3 Yeasts

Yeasts of the genus *Metschnikowia* were first isolated from diseased *Daphnia magna* (Straus) by Metschnikoff (1884). *Metschnikowia bicuspidata* was described as a parasite of *Daphnia magna*, its free needle-shaped spores were observed piercing the intestinal lining of its host (Metschnikoff, 1884). Another species, *Metschnikowia artemiae* was reported from the brine shrimp *Artemia salina* L., spores were reported as being liberated from the asci by naturally occurring dehiscence (Kamienski, 1899). Since then *Metschnikowia* spp. have been reported to parasitise the brine shrimp *Artemia salina* in commercial culture and in natural populations (Lachance *et al.*, 1976; Codreanu & Codreanu-Balcescu, 1981), marine copepods *Calanus plumchrus* (Marukawa) and *Eurytemora velox* (Lilljeborg) (Seki & Fulton, 1969; Fize *et al.*, 1970), the freshwater shrimp *Macrobrachium rosenbergii* (De Man) (Liu-Cheng, 1994) and *Daphnia* spp. (Codreanu & Codreanu-Balcescu, 1981; Ebert *et al.*, 2000). *Metschnikowia* spp. have been reported to be highly pathogenic in all the aforementioned cases, and are suggested to play a significant role in controlling copepod biomass in the sea (Seki & Fulton, 1969). *Metschnikowia bicuspidata* is indigenous to the UK and was first isolated from a Welsh salt marsh (Spencer & Spencer, 1979).

Other yeasts have been reported as pathogens of crustacea; *Candida sake* is reported to infect *Macrobrachium rosenbergii* in Taiwan causing cloudy white haemolymph and tissue opacity, resulting in death 4-5 days after clinical signs appear (Lu *et al.*, 1998). The marine yeast *Kluyveromyces penaeid* is found infecting the heart tissue in the shrimp *Penaeus chinensis* (Osbeck), but little is known about its pathogenicity (Tong & Miao, 1999). *Kluyveromyces penaeid* is reported not to grow in chemically defined media and is hence assumed to be parasitic, being similar in cell morphology

and ascospore number to *Kluyveromyces blattae* found parasitising the intestinal tract of the cockroach *Blatta orientalis* L. (Barnett *et al.*, 1990).

4.2 OBJECTIVES

The aim of the present study is to ascertain what endobionts are associated with the salmon louse *Lepeophtheirus salmonis* (and when available *Caligus elongatus*). The study is intended to develop and apply a protocol to screen sea lice, looking for endosymbiotic and endoparasitic organisms. If endobionts are present, their percentage prevalence will be estimated and their seasonality in occurrence studied. Sea lice will also be examined from wild caught salmon to maximise the potential for isolating pathogens from a wider range of environments.

4.3 METHODS

4.3.1 Louse collection and examination

Sea lice were collected from farm sites as detailed in chapter 2, paragraph 1.1.

Fifteen farms sites were visited over a period of three years (Figure 3.1, chapter 3), with a total of 46 farm visits in total. Wild salmon were caught using seine nets in Loch Duich and beach nets at the mouth of the North Esk, Montrose bay.

All sea lice collected per site visit (between 500 and 2000 depending on conditions and infestation rates on fish) were observed in aquaria for unusual characteristics, such as differences in colour, high opacity or the presence of inclusion bodies. This was facilitated by placing the aquaria on various backgrounds, such as white, grey and black, to accentuate any colour or opacity differences, and to assist in observing inclusions. One hundred randomly chosen adult female *L. salmonis* were also microscopically examined from each collection site using a dissecting microscope, to assist in the detection of any abnormalities. If abnormalities were present the percentage of lice infested with each suspected endobiont was calculated. When endobionts were found to be present on adult female sea lice, adult male sea lice were also examined to establish if any preference existed between female and male lice by the endobionts.

If endobionts were observed, infected tissues were dissected out, and fresh tissue squashes performed on microscope slides using physiological saline (0.85%) and cover slips. Fresh tissue squashes were analysed using a compound microscope as described in chapter 2, paragraph 2.1 to assist in endobiont identification. Zeiss KS 300 ver.3.0 (Imaging associates Ltd, Thame, UK.) image analysis software using a JVC KY-F30B 3CCD camera mounted on the Olympus BH-2 compound microscope,

with an interfacing 2.5x top lens and a 100x oil immersion lens (accuracy 0.01μ), were utilised to measure the dimensions of any endobionts observed.

Caligus elongatus, when present, was also screened for the presence of endobionts.

4.3.2 Isolation and sporulation of fungi

From the first 20 sea louse collections during this study (11 different farm sites), a sample of 20 adult female *L. salmonis* from each collection site was examined for fungal infection. Individual lice were washed five times to remove any external fungal spores in autoclaved seawater containing 0.5% tween 20. This was achieved by placing them in a 50 ml container with the autoclaved seawater and shaking moderately. The sea lice were finally washed in distilled water containing 50 units of penicillin and 100 units of streptomycin per ml to discourage bacterial growth. Whole lice were then placed on pre-prepared yeast-malt extract (YM) agar plates poured into 90 mm petri dishes (50 ml per plate) and incubated at 15 ºC for 10 days. Any subsequent fungal growth of interest was sub-cultured on to (YM) plates, marine agar (MA) plates and potato dextrose agar (PDA) plates, incubated at 15 ºC and periodically checked microscopically for the formation of fungal spores. A range of culture conditions was utilised in an attempt to induce sporulation, including: varying of media salinities, higher and lower temperatures for culture, UV light, and nutrient deficient media were all used to induce sporulation in the fungal isolates.

4.3.3 Screening for *Wolbachia* and RLO's

Rickettsia and rickettsia-like organisms were screened for using the light microscope as described in chapter 4, paragraph 3.1.

As *Wolbachia* are only present in reproductive tissues and not detectable using the light microscope, diagnostic PCRs were utilised to screen for the presence of these symbionts. One egg string from each female was taken and pooled with others, to

maximise the chances of finding infected individuals. Egg strings from approximately 1000 female *L. salmonis* from 15 farm sites were extracted in batches of 20 for total DNA as detailed in chapter 2, paragraph 3. PCR amplifications were carried out as detailed in chapter 2, paragraph 3.1, using the primer set (V1 and V6) specific for *Wolbachia*, described by O'Neill *et al* (1992) and a universal primer set (18 S-1 and 18S-2) specific for ribosomal DNA, described by Ellis *et al* (1986) used as control primers to check for DNA quality (Table 4.1).

Table 4.1 Primer details for PCR amplification of *Wolbachia* and ribosomal DNA

Primer	Seqnence
V1 (*E.coli* position 76-99) Fwd	(5′-TTGTAGCCTGCTATGGTATAACT-3′),
V6 (*E.coli* position 1012-994) Rev	(5′-GAATAGGTATGATTTTCATGT-3′).
18S-1 Fwd	(5'-GGGCAAGTCTGGTGCC-3')
18S-2 Rev	(5'-GGTCTGTGATGCCCTT-3')

A *Wolbachia* infected ladybird obtained from Dr. Majerus, Department of Genetics, University of Cambridge, was extracted for total DNA as described in chapter 2, paragraph 3 and used in all PCR amplifications as a positive control for *Wolbachia*. Other marine and terrestrial crustacea were also sampled. Free-living copepods were hand netted from farm sites; isopod species were taken from the intertidal zone and terrestrial isopods (woodlice) were also screened for the presence of *Wolbachia*.

After an initial denaturation at 95 ˚C for 5 min, samples were subjected to 30 cycles of amplification (Table 4.2) followed by a 5 min terminal extension at 72 ˚C.

Table 4.2 Temperature profiles for PCR amplifications were as follows:

V1 and V6 primer set	18 S-1 and 18S-2 primer set
5 min at 95 °C	5 min at 95 °C
1 min 95 °C	30 sec 95 °C
1 min 52 °C } x30	30 sec 55 °C } x30
1 min 72 °C	90 sec 72 °C
5 min 72 °C	5 min 72 °C

Amplified products were separated on a 1% agarose gel (0.5x TAE see appendix 1), visualised by staining with ethidium bromide (0.5μg/ml) and photographed on an ultraviolet transilluminator. Two DNA markers were run on the gel (100 ng/lane) in order to calculate the product size (Table 4.3).

Table 4.3 Table showing size range of DNA markers used to calculate PCR product sizes

DNA Marker	Marker size range (base pairs)
Lambda DNA-*Hind* III digest	23,130 to 2,027
ΦX174 DNA-*Hae* III digest	1,353 to 72

4.3.4 Screening for Transovarially-transmitted microsporidia

The DNA samples obtained from extracted sea lice egg strings for *Wolbachia* screening in section 4.3.3 were used as template DNAs for further PCR reactions with a microsporidian universal primer set (530f-580r), described by Vossbrink *et al* (1993) (Table 4.4) to screen for transovarially transmitted microsporidia.

Table 4.4 Primer details for PCR amplification of rDNA

Primer	Sequence
530f	(5´-GTGCCATCCAGCCGCGG-3´),
580r	(5´-GGTCCGTGTTTCAAGACGG-3´).

A microsporidian, *Glugea* sp., taken from an external cyst containing many hundreds of spores, from a stickleback, *Gasterosteus aculeatus aculeatus* L., was extracted for total DNA as described in chapter 2, paragraph 3 and used in all PCR amplifications as a positive control for microsporidia.

After an initial denaturation at 95 ˚C for 5 min, samples were subjected to 30 cycles of amplification (Table 4.5) followed by a 10 min terminal extension at 72 ˚C.

Table 4.5 Temperature profiles for PCR amplifications were as follows:

530f 580r primer set

5 min at 95 °C

30 sec 95 °C
30 sec at 43 °C } x30
1 min 72 °C

10 min 72 °C

Amplified products were separated on a 1% agarose gel (0.5x TAE see appendix 1), visualised by staining with ethidium bromide (0.5μg/ml) and photographed on an ultraviolet transilluminator. Two DNA markers were run on the gel (100 ng/lane) in order to calculate the product size (Table 4.3).

4.4 RESULTS

4.4.1 Isolation and sporulation of fungi

Fourteen fungal isolates of interest were cultured from sea lice, of which four had a similar appearance. Despite utilising a wide range of media and culture conditions only one isolate sporulated. This isolate was identified as *Trichoderma* sp. (N. Dix pers comm.) and was eliminated from further study due to its non-pathogenic nature.

Figure 4.1 Fungi growing on YM media from two adult female *L. salmonis*, two different isolates are shown, the isolate to the left is totally covering the sea louse.
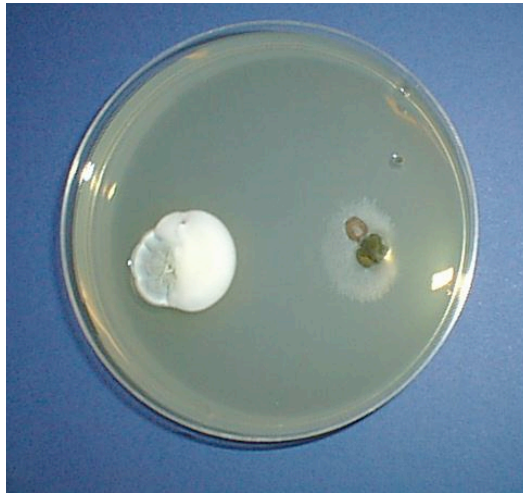
The isolates which appeared similar in culture and were most frequently observed, were deemed to be of most importance (N. Dix (senior mycologist University of Stirling) pers. comm.), and were sent for formal identification at the International Mycological Institute (IMI) London. The IMI were unable to induce sporulation under any conditions, and hence no formal identification of the isolates was deemed possible.

No invasive yeast species were observed or cultured from *L. salmonis*.

4.4.2 Wolbachia and RLO's

From 46 salmon louse collections from 15 different farm locations, no signs of rickettsia or RLO's were observed grossly or microscopically.

DNA samples extracted from adult female *L. salmonis* egg strings (1000 females, extracted in batches of 20 egg strings), from 15 different farm locations and the additional free-living marine and terrestrial crustacean species were screened for the presence of the maternally inherited rickettsia like organism, *Wolbachia*, using PCR reactions as detailed in section 4.3.3.

The PCR products were visualised on an agarose gel (as described in section 4.3.3) using the DNA marker ΦX174 DNA-*Hae* III digest (Table 4.3) as a size marker to locate the positive control of approximately 900 base pairs from the ladybird DNA.

No *Wolbachia* was detected in any of the DNA samples from *L. salmonis* (Figure 4.2), or from the free-living marine and terrestrial crustacean species

Figure 4.2 Agarose gel image of PCR targeting *Wolbachia* in DNA samples from extracted egg strings of *L. salmonis*.
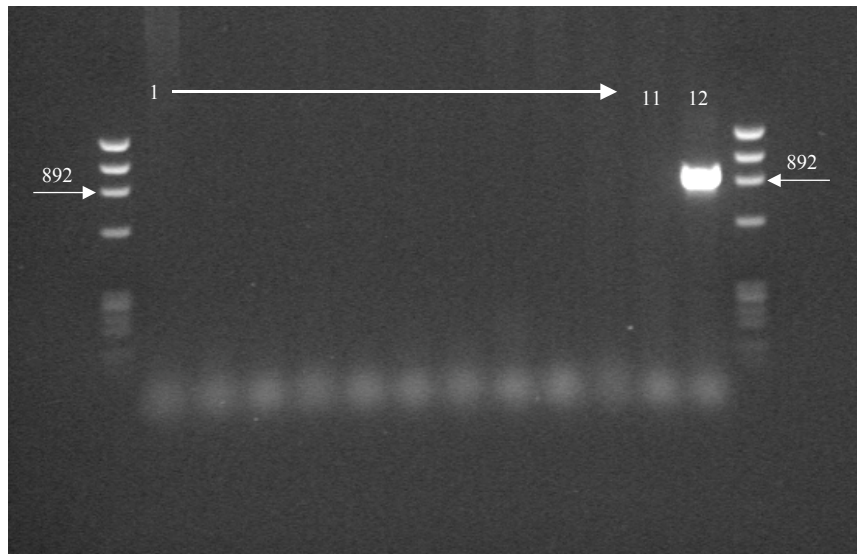
Lanes 1 to 11 each contain DNA extracted from 20 egg strings, each from a different individual female *L. salmonis*. Lane 12 contains the positive control which produced a

band at the expected size, approximately 900 base pairs. All DNA samples were successfully amplified using a universal primer set (as described in section 4.3.3), to check for DNA quality (See Figure 4.3).

Figure 4.3 Agarose gel image of universal primers to check for DNA quality
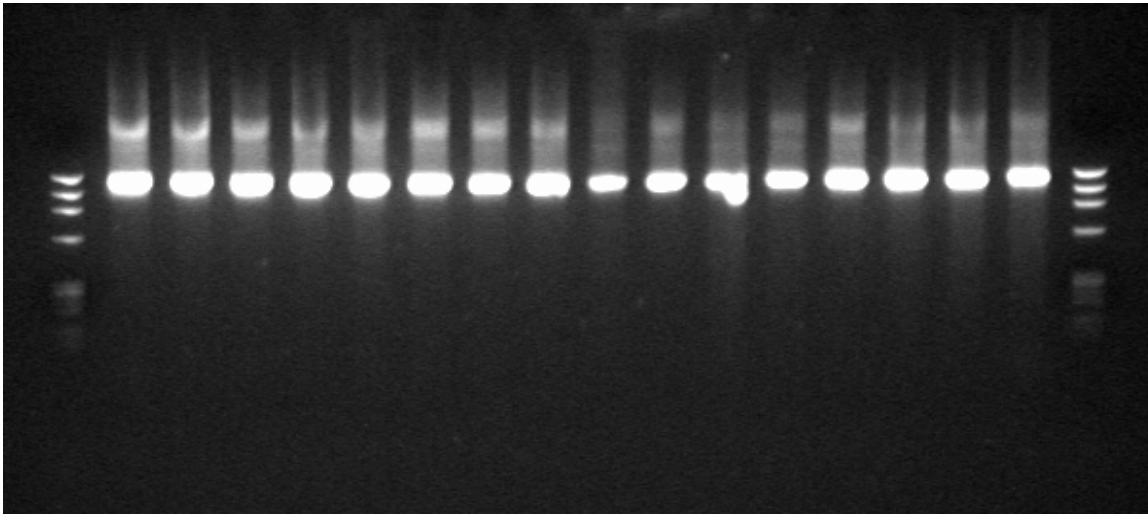
4.4.3 Parasitic dinoflagellates and related organisms

From 46 salmon louse collections from 15 different farm locations, no signs of parasitic dinoflagellates or related organisms were observed, either viewed grossly or microscopically

4.4.4 Transovarially-transmitted microsporidia

The same DNA samples used in the analyses for *Wolbachia*, from *L. salmonis* egg strings were also used in PCR reactions (as detailed in section 4.3.3) to detect transovarially-transmitted microsporidia that may be maternally transmitted in the eggs. From approximately 1000 female *L. salmonis* individuals tested, no transovarially-transmitted microsporidia were detected (Figure 4.4).

Figure 4.4 Agarose gel of PCR products from DNA samples of *L. salmonis* screened for transovarially-transmitted microsporidia.
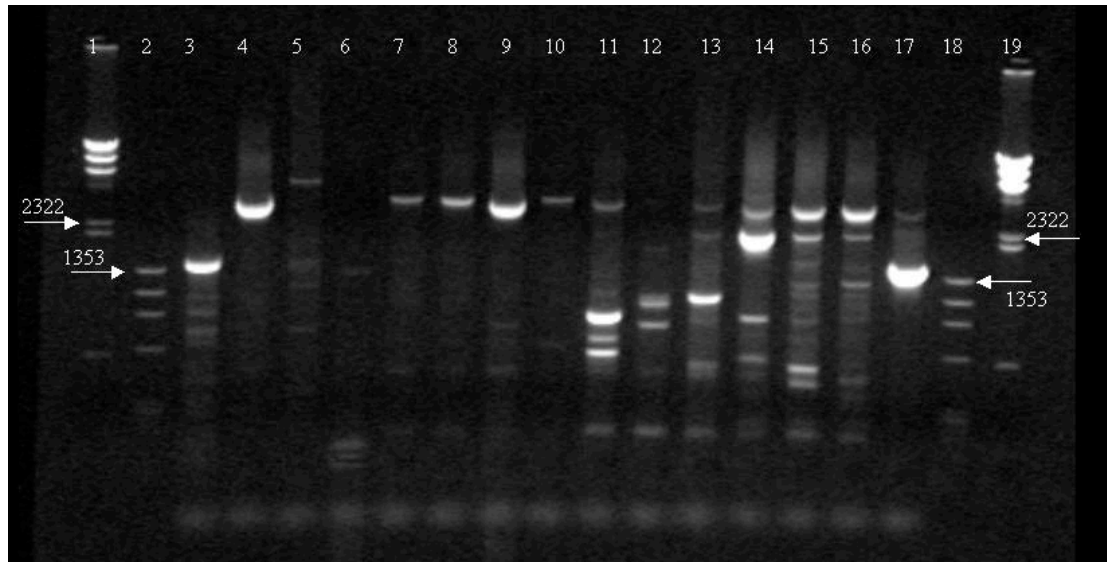
Table 4.6 Table describing lane positions for DNA samples for Figure 4.4

Lane number	Sample
1 and 19	DNA marker Lambda DNA-*Hind* III digest
2 and 18	DNA marker ΦX174 DNA-*Hae* III digest
3 and 17	*Glugea anomola* (Positive control)
4 to16	DNA from *L. salmonis* egg strings

The positive controls in lanes 3 and 17 produce a band at approximately 1400 base pairs, which is the expected size for microsporidia with the primer set used. No bands were observed at this size for the DNA samples from *L. salmonis* egg strings. The other bands present are to be expected, as this primer set also amplifies the gene region in other eukaryotes, but the band size is significantly larger at approximately 2500 base pairs.

4.4.5 Non transovarially-transmitted microsporidia

From three of the fifteen farm sites visited, salmon lice were observed with opaque internal inclusions, distributed throughout their bodies. Figure 4.6 shows an

uninfected louse and a heavily infected individual. Microscopic examination of freshly dissected tissue squashes revealed, uniform sub-spherical spore-like structures 2.34µm by 1.83 µm (±0.1µm) in dimension, consistent in appearance with that of microsporidia when viewed under a phase contrast light setting.

The number of sea lice infected with this type of infection per site varied between 1% and 10%, with an average of 5% of adult female sea lice having the microsporidian infection when present. The infection varied from a few dark internal inclusions to complete body opacity due to extensive infection as shown in Figures 4.7 and 4.8. Infection did not appear to be site specific, occurring in the cephalothorax, genital segment, abdomen and appendages, although inclusions were almost always present in the posterior lobes of the cephalothoracic shield in the early stages of infection as shown in Figure 4.7. Infection with the microsporidian was often associated with poorly developed or aborted egg strings as shown in Figures 4.7 and 4.8, but fresh squash preparations of these aborted egg strings did not indicated the presence of microsporidian spores. When the microsporidian was present in adult female sea lice, adult male sea lice from the same population were also infected with similar prevalence, no juvenile sea lice were observed with visible internal inclusions of microsporidia.

One farm site was sampled 17 times over a 22-month period, yielding seasonal data as detailed in Figure 4.5. The infection was first observed in December 2000, and persisted until March 2001, the infection was then absent until August 2001, when it returned and was present until January 2002. The infection was most prevalent (7%) from January to March 2001, when the sea temperatures was at its annual lowest (6-7 ºC), and again in October and November 2001 when the sea temperature was close to its annual high.

Figure 4.5 shows how the percentage of adult female salmon lice infested with a microsporidian infection varied on one farm site over a period of 22 months. The blue line indicates sea temperature. When the site was visited more than once in any given month the data was averaged.
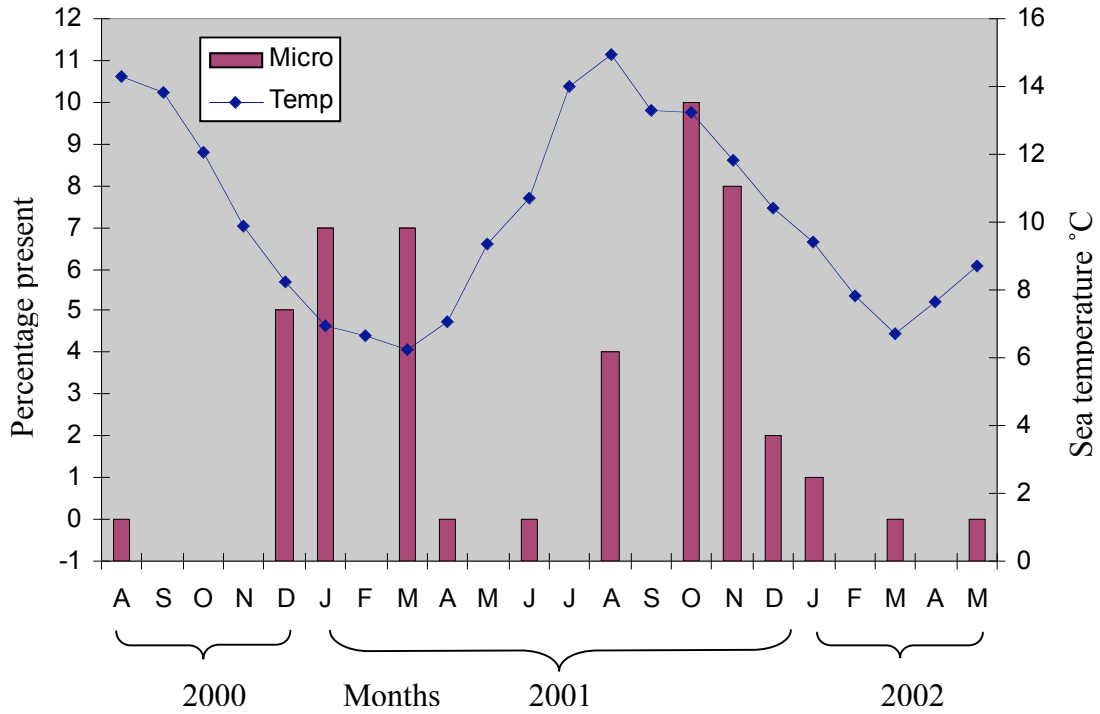
Figure 4.6 Two adult female *L. salmonis*; on the right hand side very heavily infected with microsporidia and nearly totally opaque, one on the left hand side uninfected, scale bar = 1 mm.
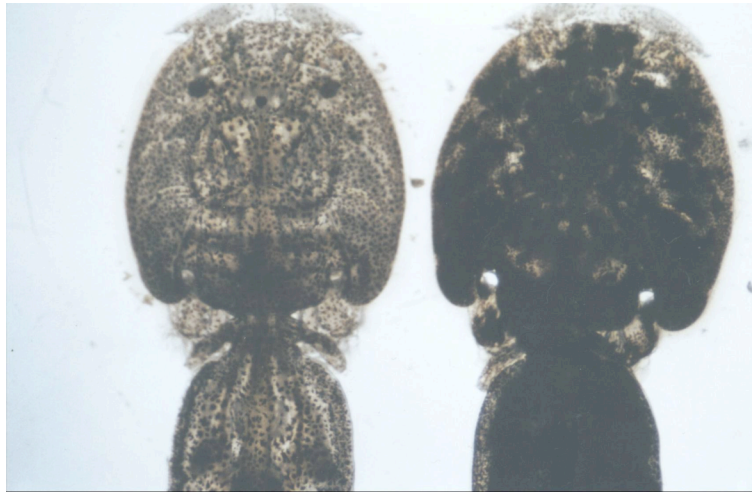
Figure 4.7 Adult female *L. salmonis* infected with microsporidia. Note the dark inclusions in the posterior lobes of the cephalothoracic shield (arrowheads), and the aborted egg string (large arrowhead), scale bar = 2mm.

Figure 4.8 Adult female *L. salmonis* very heavily infected with microsporidia. Note the dark inclusions throughout the entire body, and the aborted egg string (large arrowhead), scale bar = 2 mm.
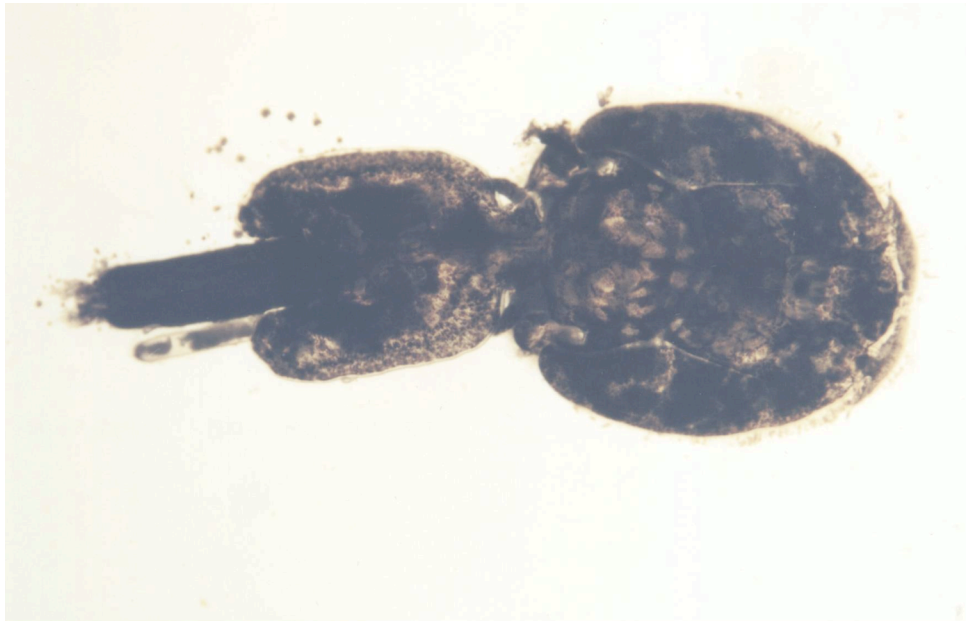
4.4.6 *Lepeophtheirus salmonis* from wild caught salmon and *Caligus elongatus*

Both *L. salmonis* and *C. elongatus* sampled from wild caught salmon, and *C. elongatus* sampled from farmed salmon were never observed with endobionts.

4.5 DISCUSSION

4.5.1 Parasitic dinoflagellates and related organisms

The absence of parasitic dinoflagellates and related organisms in the salmon louse is possibly due to the high degree of host specificity exhibited by these organisms. Not only are most parasitic dinoflagellates highly host specific, but the majority also rely on the ingestion of dinospores for transmission to a new host (Shields, 1994). This mode of transmission would possibly be far more effective in a filter-feeding copepod host than in a copepod parasitic on fish.

The parasitic dinoflagellates that attack host eggs are also well adapted to suit the eggs of their respective hosts. The Chytrodinidae only target copepods and euphausids

that have a very thin egg coat with little perivitelline spaces. The eggs of *L. salmonis* are surrounded by a double membrane layer, which in turn is protected from the external environment by a thick laminate layer (James Bron pers. com.), and may hence be impenetrable to the infective stages of these parasitic dinoflagellates.

4.5.2 Rickettsia like organisms and Wolbachia

No rickettsia or rickettsia like organisms have been previously reported infecting copepoda. In aquatic crustacea their observed host range is seemingly limited to larger decapods such as crabs and shrimp, with the notable exception of the freshwater crangonid amphipod from Florida (Federici *et al.*, 1974). Horizontal transmission between hosts is reported to occur primarily via the cannibalism of dead and moribund individuals (Bower *et al.*, 1996), and hence infection may be limited to crustacea that exhibit this type of feeding behaviour.

The only crustaceans reported in the scientific literature to harbour the maternally transmitted *Wolbachia* bacteria are from the Isopoda (Bouchon *et al.*, 1998), and more recently from the Amphipoda (Cordaux *et al.*, 2001). However, *Wolbachia* have been detected in fresh water copepods from paddy fields in Asia (Scott O'Neill pers. comm.). *Wolbachia* primarily infect terrestrial and freshwater arthropods, but have been detected in semiaquatic marine isopods and intertidal amphipods (Cordaux *et al.*, 2001). A number of free-living copepod species (approx 20) from the coastal waters around the UK have previously been examined for the presence of *Wolbachia* without any success (Henk Braig pers. comm.). Hence, the only known arthropods infected with *Wolbachia* in the marine environment are those that have an intimate association with the land / sea interface or are semiaquatic shore isopods. Thus it is widely accepted that *Wolbachia* is a bacterial symbiont of terrestrial origin that may horizontally transfer to arthropod species in the intertidal zone (Bouchon *et al.*, 1998).

Although *Wolbachia* rely on vertical transmission for propagation, infectious (horizontal) transmission is also known to occur between different host species (Rigaud *et al.*, 2001). Two distant strains of *Wolbachia* have been recently discovered in intertidal amphipods; the strains were closely related to different coastal isopod symbionts, which suggests that *Wolbachia* transmission may occur between distantly related crustacean hosts living under the same ecological conditions (Cordaux *et al.*, 2001). It may not be surprising therefore that *Wolbachia* has not yet managed to transfer horizontally naturally to *L. salmonis*, as prior to the onset of the salmon farming industry in the 1970's, *L. salmonis* parasitic on Atlantic salmon would have spent a limited time in this coastal zone, in which horizontal transmission to new hosts reputedly takes place.

Successful experimental transfers of *Wolbachia* by microinjection from one host to another has also been achieved in the laboratory within terrestrial isopods (Juchault *et al.*, 1994), and from the mosquito *Aedes albopictus* into *Drosophila simulans* (Braig *et al.*, 1994), suggesting horizontal transfer between host species is indeed possible, however, the natural mechanisms of these horizontal transfers remain unclear. It also remains unclear; as to how similar a *Wolbachia* infected crustacean (the donor) would have to be to the uninfected recipient crustacean for the microinjection transfer of *Wolbachia* to be successful. Cordaux (2001) suggests that natural horizontal transfer between isopods and amphipods may occur, however other researchers have met with varied success when trying to replicate this in the laboratory, using microinjection technology between two distant species. Therefore if an attempt to transfer *Wolbachia* to *L. salmonis* were to be made using microinjection technology, due to the lack of a suitable marine copepod donor, infected marine isopods and amphipods could

potentially lead to a successful transfer, or *Wolbachia* infected fresh water or terrestrial copepods.

Wolbachia have attracted recent interest for several reasons. Studies of their role in rapid speciation and population dynamics and their ability to alter early development and mitotic processes are being researched. But of more interest here is their potential role in biological control, as a microbial natural enemy, or as a vector for spreading desirable genetic modifications in arthropod populations.

Early studies considered the use of *Wolbachia* to introduce cytoplasmic incompatibility (CI) and to potentially eradicate host populations in a method analogous to sterile-male release (Laven, 1967). The strong evidence for horizontal transfer between species that are distantly related, based on non-congruence of host and bacterial phylogenies (O'Neill *et al.*, 1992; Werren *et al.*, 1995), is a good indication that a particular strain of *Wolbachia* is not restricted to one host, and that the mechanism of CI must affect a relatively general and conserved target. Implying that transfer of *Wolbachia* into previously uninfected host species should result in the expression of CI, as demonstrated by (Braig *et al.*, 1994).

The introduction of such a CI may result, as expected, in a reduction in population numbers (Werren *et al.*, 1995), but could also potentially act as a genetic marker assisting in epidemiological studies between discrete population of sea lice, for example the interaction of sea lice between wild and farmed salmon.

It is generally accepted that *Wolbachia* alone is unlikely to cause mass mortality in an infected population; as to do so would not be beneficial for self-survival. Of more interest is the CI between populations and the possible integration into future pest control strategies. However, one strain of *Wolbachia* detected in *Drosophila melanogaster* referred to as the 'popcorn' strain, is latent during the fly's development

but begins to massively proliferate in the adult causing widespread degeneration of tissues, including brain, retina and muscle, resulting in premature death. Tetracycline treatment of the hosts removes the *Wolbachia* and restores a normal life span (Min & Benzer, 1997).

A more ambitious use for *Wolbachia* in the future involves genetically engineered organisms, using plasmid DNA to spread desirable genetic modifications into arthropod populations, although this has greater potential application in the removal of pathogenicity in vector arthropods such as mosquitoes (Werren *et al.*, 1995).

4.5.3 Microsporidia

Although no microsporidian infections have been previously reported in the scientific literature from marine copepods, microsporidia are known to infect every major animal group, with numerous reports of infections in arthropods. With more than 10,000 species of copepod currently described from both fresh and marine aquatic habitats (Humes, 1994), it is not surprising therefore that a microsporidian parasite is found infecting a marine copepod.

The manner in which this microsporidian infection is distributed throughout the body of *L. salmonis*, suggests that the main method of host transfer is likely to be that of horizontal transmission. Infected lice frequently have poorly developed or aborted egg strings, however spores were not observed inside them. Histological studies will need to be undertaken to establish if this microsporidian invades a particular host cell type and whether vertical transmission is also utilised. Vertical transmission is unlikely since the screening programme utilised in this study examined over 1000 egg strings for the presence of microsporidia using PCR, and no parasites were detected in them. TEM studies are also necessary to describe the spore ultrastructure and different life stages present.

No clear seasonal trends have emerged for the presence of this microsporidian, however it was not sampled in the spring and early summer months. The maximum percentage prevalence of 10% may in fact, be significantly higher, as the figure of 10% represents animals that were showing visible signs of infection, and many more may have been recently or more mildly infected. Future studies should incorporate a PCR screening program for known infected sites, to determine the actual percentage of lice infected.

Microsporidia infecting freshwater copepods are sometimes involved in complicated life cycles involving more than one host species. A DNA phylogeny for this microsporidian parasite will assist in the identification of any potential intermediate host and identify which know microsporidia are similar with respect to their ribosomal DNA sequences. A molecular phylogeny will also assist in its ultimate identification and taxonomic placement.

4.5.4 Fungi and yeast

The fungal isolates obtained in this study would not sporulate in laboratory culture, and are assumed to be of terrestrial origin, but altered by their contact with the marine environment so sporulation could not be induced. No living sea lice were observed with visible hyphal growth, hence the isolates are assumed to be harmless and only present due to run off from near by land.

4.5.5 *Lepeophtheirus salmonis* from wild caught salmon and *Caligus elongatus*

L. salmonis from wild caught salmon and *C. elongatus* from farmed and wild caught salmon were never observed with the microsporidian infection. *C. elongatus* was often sampled along side *L. salmonis* infected with the microsporidian on farmed fish, but *C. elongatus* never appeared infected. Microsporidia are often host specific, which could explain why infected *C. elongatus* were not found. Very few sea lice were

examined from wild caught salmon and a larger sample size may reveal endobionts

not reported in this study.

CHAPTER 5

HISTOLOGY AND ULTRASTRUCTURE OF MICROSPORIDIAN HYPERPARASITE

5.1 INTRODUCTION

Currently there are more than one thousand species of microsporidia described from some 140 genera, infecting a wide range of hosts (Cali & Takvorian, 1999). All known members from the phylum Microspora form spores, the features of which are indispensable in microsporidian systematics (Sprague *et al.*, 1992). Figure 5.1 shows a representative spore, which contains the specialised apparatus in which infection is initiated in all microsporidia, a unique anteriorly attached polar filament. The polar filament is a hollow tube coiled inside the spore and, depending on the species, may have a few to more than 30 coils. Microsporidian spores are generally small, oval or pyriform structures that can vary in length from about 1 to 12 μm. With the exception of those undergoing transovarial transmission, microsporidian spores must spend a part of their life cycle in the environment outside the host (Kramer, 1976). This external environment may be harsh and spores may have to survive for long periods of time before encountering a suitable host. Some microsporidian spores can remain viable for up to 10 years out with their host, depending on environmental conditions such as temperature and desiccation (Kramer, 1970).

Under suitable environmental conditions, the polar filament of a mature spore is extruded. This extruded polar filament pierces the host cell plasma membrane and the

sporoplasm (contents of the mature spore) is deposited inside the host cell, initiating

the proliferative stage of development, as shown in figure 5.1.

Figure 5.1 Diagram of a microsporidian spore and representative life cycle (merogonic and sporogonic stages vary among different genera).
Taken from Franzen & Müller (1999) Clinical Microbiology Reviews vol 12 No 2 pp243-285
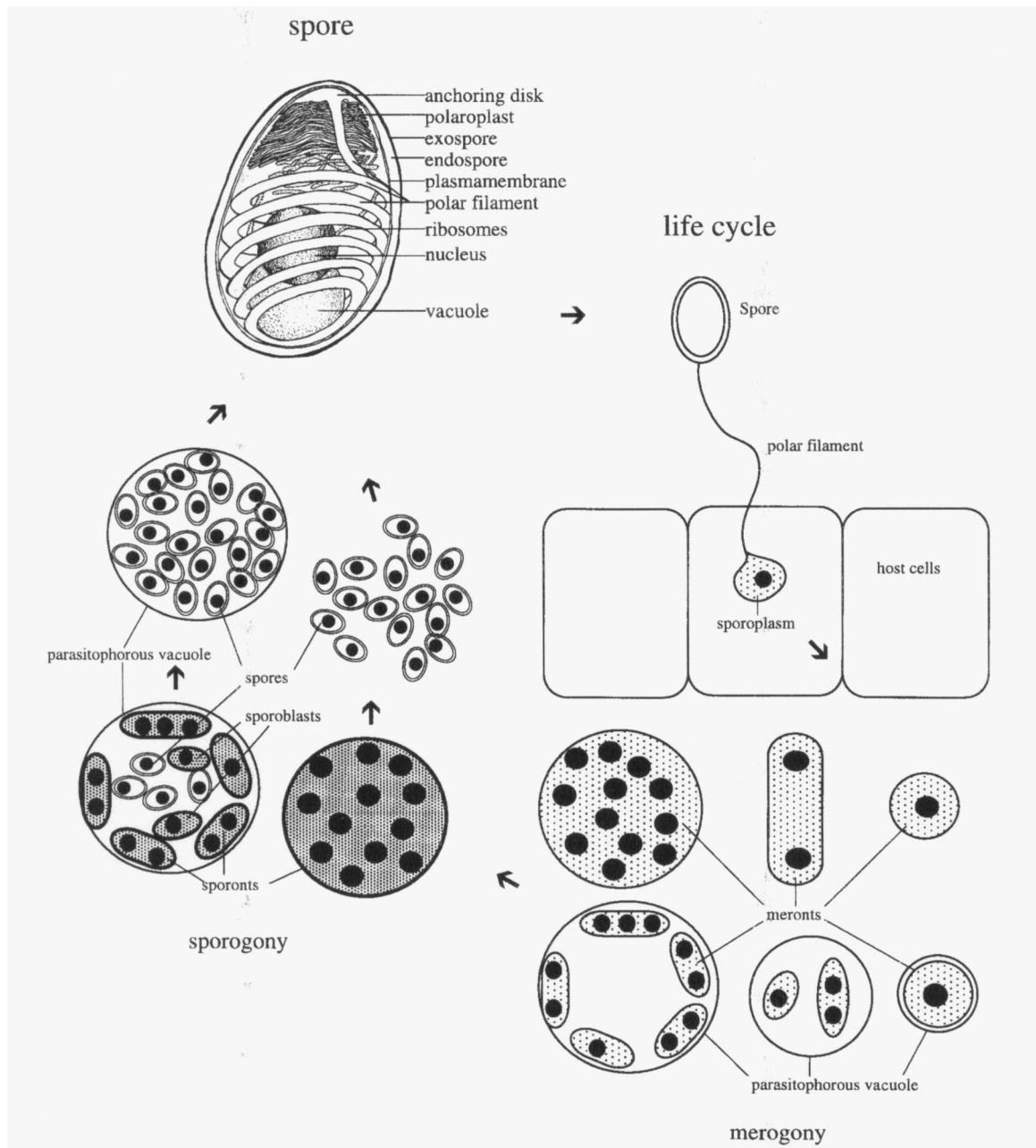
Proliferation includes all cell growth and division from the initiation of infection to

mature spore formation. This phase has previously been referred to as schizogonic or

merogonic by different authors, depending on differing types of nuclear activity.

However, schizogony and merogony have also been considered to be synonymous by other authors (Vavra, 1976). Such alternating use of taxonomic terminology has led to confusion when describing microsporidian developmental cycles and the terminology used in descriptions should be explained to avoid future misunderstanding. A generalised microsporidian life cycle includes a proliferative, vegetative phase, in which vegetative cells, meronts (schizonts) undergo multiplication by merogony (schizogony) and proliferate throughout the infected tissue or organism. After which, a stage of spore formation, sporulation, is reached, where sporoblasts undergo sporogenesis to form spores. During sporulation in some microsporidia, a proliferative process called sporogony occurs, in which sporonts or sporogonial plasmodia divide into numerous sporoblasts at the beginning of sporogenesis (Cali & Takvorian, 1999). In the present study the terms meront and merogony are preferred, as defined by Canning & Lom (1986). The study describes two proliferative processes, the first, merogony (the proliferation of meronts), the products of which enter the second cycle, sporogony, during which sporonts form and divide to produce sporoblasts, which subsequently mature to form spores.

There are many features that may occur during proliferation, which have taxonomic value. Thus it is necessary to describe the microsporidian developmental cycle in order to provide a formal identification. Many microsporidia are uninuculeate, but some are diplokaryotic at one or more stages of their life cycle. In some microsporidia, multinucleate plasmodia are formed when cytokinesis does not directly follow nuclear division. These plasmodia may be multinucleate ribbon like stages, which eventually divide by progressive fragmentation into smaller multinucleate stages, and finally by multiple fission into cells containing one nucleus or diplokaryon, or they may form large rounded multinucleate plasmodia, which divide

by plasmotomy. Some microsporidian species produce a typical number of spores during sporogony, which may be packaged in a sporophorous vesicle, whilst others may develop in direct contact with the host cell cytoplasm.

Entire developmental cycles are not known for many microsporidian species. Many microsporidia were described prior to the introduction of electron microscopy and the development of molecular techniques, descriptions being limited to 'oldfashioned' light microscope parameters, such as how cells divide and to spore morphology. Whereas, more recent descriptions detail molecular phylogeny and spore ultrastructure but many overlook these essential light microscope characteristics, making comparisons between old and new descriptions virtually impossible.

Other important information is also required when describing the systematics of a microsporidian parasite, such as the host organism and the site of infection. Some microsporidia are highly host specific whilst others may infect more than one host species. Microsporidia may be highly tissue specific, infecting only certain cell types, whilst others are less tissue specific, or may infect a tissue type that is less locally specialised and hence more widely distributed throughout the body. Therefore, histological sections of the host are required to locate the parasite in its chosen host cell and to evaluate potential pathogenicity caused by infection, such as the formation of tumour-like anomalies, referred to as xenomas (Larsson, 1999). A xenoma can be defined as a hypertrophid cell containing many intra-cellular parasites (Canning & Lom, 1986), but has more recently been applied to a parasitic complex that has a highly integrated nature and exhibits a conspicuous or spectacular feature (Sprague & Becnel, 1999).

5.2 OBJECTIVES

The aim of this study is to investigate the morphology of the microsporidian infection in the salmon louse *Lepeophtheirus salmonis*, reported in the previous chapter (chapter 4), using histological sectioning and transmission electron microscopy (TEM). The study aims to locate the site or sites of infection within the salmon louse and identify the cellular type or types infected. Enabling a preliminary evaluation as to the level of pathogenicity this parasite causes to its host. The study also aims to describe the ultrastructure of the mature spore, and attempts to locate pre-spore stages, in order to compile the developmental sequence of this microsporidian within the salmon louse. Finally, preliminary identification of the microsporidian was attempted, using the aforementioned morphological and ultrastructural data.

5.3 METHODS

5.3.1 Histology

Whole microsporidian-infected sea lice were prepared for wax histology, cut and mounted as detailed in chapter 2.2.1.1. Sections were dewaxed and stained with haematoxylin and eosin (H&E) or Geimsa or Gram stain as detailed in chapter 2.2.1, and examined under the light microscope for signs of microsporidian infection.

5.3.2 Transmission electron microscopy (TEM)

Infected salmon lice were dissected as required and prepared for TEM as described in chapter 2.2.2. Semi-thin sections were also cut and stained for examination using the light microscope according to the method in chapter 2.2.1.

5.4 RESULTS

5.4.1 Histology

L. salmonis adult female lice with gross signs of infection (described in chapter 4) were processed for histological cutting. H&E stained histological sections confirmed the presence of numerous discrete membrane bound cysts containing sub-spherical spore-like structures. These cysts were distributed somewhat randomly throughout the body cavity of infected salmon lice, occurring in the cephalothorax, genital segment, abdomen and appendages. Figures 5.2 and 5.3 show these structures staining positively with Giemsa and Gram stains. The cyst-like structures were compartmentalised within the haemocoel, each cyst-like compartment containing from a few to many thousand of spores-like structures. The cyst-like structures on closer examination conformed to the typical xenoma formation described by Canning and Lom (1986), during which host cells become massively hypertrophic, containing many thousands of spores. Xenomas were observed from a few microns in size up to 300 μm in section diameter; developing directly under the cuticle or along the haemocoelic separations (structures separating haemal sinuses / lacunae) of the haemocoel and appeared to be associated with the epidermal tissue layer. Figures 5.4, 5.5 and 5.6 are semi-thin sections, transversely cut through the abdomen of an infected adult female louse, xenomas can be seen completely filling the haemocoelic cavity between the centrally located gut and the cuticle. Figure 5.6 shows smaller xenomas intimately associated with the epidermal tissue layer of the haemocoelic separations. Infection was not only restricted to the central body compartments of the lice; figure 5.7 shows a xenomas forming from infected cells in the haemocoelic region of an appendage. Infection was not observed in the muscles or gonads, despite

large xenomas developing in close proximity (figures 5.2 and 5.8). The identity of the host cell parasitised by the microsporidian could not be identified at the light level. In heavily infected individuals, free spores were observed distributed throughout the haemocoel and, in some cases, in close association with the forming egg string within the genital segment (figure 5.9).

5.4.2 TEM

TEM confirmed that the infection arose in the epidermal cells lying beneath the cuticle and along the haemocoelic separations between the haemal sinuses. Infection, however, was not observed throughout the epidermal layer itself, and was mainly observed in the innermost cells (basal portion) of the epidermal layer as shown in figures 5.10 and 5.11. The infection was seemingly contained by the basal lamina. Host cell remnants could sometimes been seen in large xenomas. Figure 5.12 shows a single host cell nucleus, endoplasmic reticulum and mitochondria at the margin of the xenoma. However, smaller xenomas appeared to contain multiple nuclei, nuclear fragments or multi-lobed nuclei as shown in figure 5.13.

TEM showed conclusive evidence that the spore like structures were indeed microsporidian spores. Figure 5.14 shows a mature sub-spherical (round to ovoid) spore, with a single nucleus and a thickened electron lucent endospore wall measuring between 150-250 nm thick. The polar filament had between 5 and 8 turns, normally in a double coil, and was of the isofilar type, being of a similar diameter (65-85 nm) along its entire length. The spore has typical features of a microsporidian spore; the polar filament is attached at the anterior end of the spore via the anchoring disc, which is surrounded by the laminar structure of the polarplast (figure 5.15).

Figures 5.16 and 5.17 show the earliest developing merogonic stages (meronts) observed, which were diplokaryotic in nuclear arrangement, but showed little other

cellular organisation. The diplokaryotic nuclei appeared to divide as a pair producing two new pairs. Cytokinesis was linked to this nuclear division and the cells divided by binary fission, producing two new meronts each with a diplokaryon (figure 5.18). The diplokaryotic nuclear arrangement was not observed after this point in the developmental phase. Hence, nuclear dissociation is assumed to have occurred, this was followed by multiple nuclear divisions, which were not linked to cytokinesis, resulting in the formation of a paucinucleate rounded merogonial plasmodium, which ultimately divides by plasmotomy as seen in figure 5.19. The plasmodium contained granular cytoplasm, with nuclei showing electron-dense laminate bodies lying on the nuclear membrane, which may be indicative of recent nuclear activity, from either nuclear dissociation or division (figure 5.20). The resulting uninuculeate stages, sporonts, represent the beginning of the second cycle of proliferation, sporogony. Sporonts had a higher degree of cellular organisation than meronts. Early sporonts showed a precocious development of the polar filament and a modest thickening of the plasmalemma prior to cytokinesis by binary fission as detailed in figure 5.21. During early sporogony, tubules, described as microsporidian appendages by Cali (1999), appear in the hyaloplasm (host cell cytoplasm), which persisted throughout sporogony (figure 5.21). Other structures also became apparent in the hyaloplasm during sporogony, figures 5.22 and 5.23 show membranous secretions of about 30nm in width with a central core of electron dense particles that appeared to develop at the external surface of developing spores; these membranous secretions were occasionally seen as enclosed concentric membranous whorls (figure 5.24). During sporogony, the polar filament and its attachment apparatus (anchoring disc) continued to develop and are clearly well developed prior to the final binary division into sporoblasts (figures 5.25 to5.27). During this phase of proliferation from early sporont

to sporoblast, large membrane bound electron lucent inclusions (ELI's) were formed, which often appeared to have associated with large electron dense particles at the membrane (figures 5.26, 5.28 and 5.29). As sporoblasts developed, the plasmalemma significantly thickened and the electron lucent inclusions decreased in size (figure 5.29). Sporoblasts matured into spores without further cell division.

This microsporidian developed in direct contact with the host cell cytoplasm throughout its entire proliferative cycle and did not form a complete interfacial envelope of either parasite-derived or host-derived origin that contained a typical number of spores and therefore may be considered polysporous.

Host cell nuclei of microsporidian-infected cells were frequently observed with intranuclear inclusions, which were of a crystalline appearance with a regular pattern, as shown in figures 5.30 and 5.31.

Very occasionally spore dimorphism was seen to occur, much larger (2-3 x) spores were observed with up to 12 turns of the polar filament as seen in figure 5.32. These were sometimes found in association with the large disorganised spore-like syncytial cells shown in figure 5.33.

5.5 DISCUSSION

Although approximately 50 species of microsporidia have been described from copepods (Bronnvall & Larsson, 2001b), none have been described from a marine copepod. The majority of microsporidians described from copepods have complex life cycles utilising freshwater copepods as intermediate hosts and mosquito larvae as definitive hosts (Andreadis, 1994). In these species ovarian tissue is the main target organ for infection in the copepod (Becnel & Andreadis, 1999).

The present study found no microsporidian infection in mature eggs, developing eggs or in the ovaries, suggesting that the main route of transmission is horizontal, not vertical. The infection formed in the basal portion of the epidermal tissue throughout the body of the copepod. This type of infection has previously been reported in freshwater copepods from Sweden, where the epidermal and fat tissues become infected with a microsporidian from the genus *Agglomerata* (Bronnvall & Larsson, 2001b). Bronnvall & Larsson describe the infection of similar tissues and free spores in the haemocoel, as reported in this study, but the developmental sequence and ultrastructure of the spores are considerably different from the microsporidian found in *L. salmonis*. There are currently no described species of microsporidian infecting copepods that are similar to the microsporidian in the present study.

Extensive microsporidian infections developed from the basal portion of the epidermal layer in *L. salmonis*, but were not observed throughout the epidermal layer, suggesting that only the epidermal cells that adjoin the basal lamina and hence border the haemocoel become infected, indicating that infection is possibly initiated from within the haemocoel. The infectious spores or sporoplasms possibly being distributed from the initial portal of entry, to new or target sites for infection, in the haemolymph or contained within cells in the haemolymph. The epidermal layer in copepods is described by Bresciani (1986) as a flattened cellular layer containing a great number of membrane-bound vesicles and mitochondria, with a very complicated spatial arrangement. The basal portion of the cells rests on a glycocalyx similar in structure to the glycocalyx bordering muscle cells; hemidesmosomes are present, joining the epidermal cells to the glycocalyx. This glycocalyx is also referred to as the basal lamina and functions as the basement membrane separating the epithelial layer from the underlying tissues (Boxshall, 1992). The microsporidian in *L. salmonis* infects this

basal portion of the epidermal tissues bordering the basal lamina where it forms large xenomas. These xenomas may be neoplastic xenomas (Canning & Lom, 1986), formed when a single host cell becomes massively enlarged containing many thousands of spores, as in the *Glugea*-type cysts often reported in fish. Or they may be formed between the basal lamina and the epidermal layer, proliferation of the parasite forcing the basal lamina away from the epidermal layer in a condition more reminiscent of a syncytial xenoma (Weiser, 1976), where the fusion of host cells forms the xenoma.

Host cell remnants were observed in some xenomas, comprising a single nucleus, mostly normal in appearance, in close association with other host remnants of ER and mitochondria. However, occasionally small immature xenomas were observed with what appeared to be multiple host nuclei. As multiple host nuclei were never observed in mature xenomas these multiple nuclei were assumed to be all part of one grossly hypertrophic, branched host cell nucleus, caused by the huge cellular modifications occurring during the early development of the xenoma. Canning and Lom (1986) report this phenomenon of hypertrophic host cell nuclei in xenomas from microsporidia-infecting fish. The xenomas observed in *L. salmonis* are suggested to be neoplastic xenomas, formed from a single host cell, which is highly hypertrophic.

As microsporidia are obligate intracellular parasites, the host parasite interface is of taxonomic importance. A number of microsporidian genera have developmental cycles lacking any type of interfacial envelope, ie. developing entirely in contact with the host cell cytoplasm, *Nosema* in insects, *Unikaryon* a hyperparasite in trematodes, *Ameson* in crabs and *Enterocytozoon* and *Brachiola* in humans (Cali & Takvorian, 1999). In most of these genera there is a precocious thickening of the plasmalemma at the commencement of sporogony, which is suggested to assist in avoiding host

mechanism by which phagosomes are formed around them. Instead, these parasites are often found in close association with host cytoplasmic organelles, mitochondria and nuclei (Vavra & Larsson, 1999). However, in the microsporidian from *L. salmonis* there is only a modest thickening of the plasmalemma at the commencement of sporogony, the majority of which occurs at the late sporoblast stage, as is observed in the developmental cycle of *Enterocytozoon bieneusi* (Desportes), (Cali & Owen, 1990).

The microsporidian infection in *L. salmonis* does indeed complete its developmental cycle lacking any type of complete interfacial envelope. However, during late sporogony there were membranous secretions regularly observed near the outer surface of sporoblasts, occasionally in multiple layers. These are suggested to be parasite-derived secretions, as no host cell remnants were associated with them. These membranous secretions were never seen completely isolating the sporoblast from the hyaloplasm, and hence cannot be considered as an interfacial envelope. The structures were also observed as continuous concentric circular membranous whorls, whose function was not apparent. These membranous secretions are possibly partially formed parasite derived interfacial envelopes. Similar non-persistent parasite derived interfacial envelopes have been reported from *Vairimorpha necatrix* where envelopes are formed during sporogony, which only sometimes develop to fully enclose the sporoblasts (Mitchell & Cali, 1993).

Numerous tubules were also observed in the hyaloplasm during sporogony, Takvorian and Cali (1983) reviewed the form and function of microsporidian appendages, suggesting four different types. The appendages observed in this study most closely resemble the type I tubules Takvorian and Cali describe from *Glugea stephani*, but the present study never found convincing evidence that the tubules were continuous with

the parasite plasmalemma as demonstrated by Takvorian and Cali. The function of these appendages remains an enigma, but they are suggested to facilitate host-parasite interchange of materials (Takvorian & Cali, 1983). However, this study did not distinguish tubules attached to the parasite plasmalemma as would be expected in the exchange of materials, suggesting that other possible functions should be considered, such as a potential role in the production of the membranous secretions with which they co-exist.

The most significant features of the microsporidian developmental cycle in *L. salmonis* is the precocious development of the polar filament and the possession of electron lucent inclusions lined with electron dense particles. The first signs of the polar filament developing can be observed during early sporogony, with its development being almost complete upon final division to sporoblasts (figures 5.26 and 5.27). The ELI's are present throughout much of the developmental cycle and are at their largest during late sporont and early sporoblast formation, and reduced or absent in mature spores. Cali (1990) suggested that the ELI's observed in *E. bieneusi* are storage vacuoles and probably involved in the formation of the polar filament and other spore structures. Figure 5.29 shows ELI's from the microsporidian-infecting sea lice in close association with cytoplasmic cisternae and the developing polar filament, supporting Cali's theory. The ELI's may also be associated with the formation of the posterior vacuole, as they are positioned posteriorly in the developing spore, ultimately where the posterior vacuole develops.

These features of microsporidian development have only been reported from members of the family Enterocytozoonidae, which includes the genera *Enterocytozoon* infecting humans and *Nucleospora* infecting salmonid fish. The spores from these genera also share similar characteristics to the microsporidian spores in *L. salmonis*.

Enterocytozoon bieneusi has spherical to ovoid spores of a similar size (1.5 x 0.8 µm) with 4-7 coils of the polar filament and *Nucleospora salmonis* (Elston) has ovoid to pyriform spores (2 x 1µm) with 8-12 coils of the polar filament (Desportes-Livage *et al.*, 1996). This compares with the microsporidian in *L. salmonis* having round to ovoid (sub-spherical) spores (2.34 x 1.83 µm) with 5-8 coils of the polar filament; all three species are also polysporous and lack an interfacial envelop at all developmental stages.

Although there are clear similarities between the microsporidia in *L. salmonis* and both the genera *Enterocytozoon* and *Nucleospora*, there are also some major differences. Large differences exist in the choice of host species and the types of cells infected, *Enterocytozoon* infects human intestinal epithelium and *Nucleospora* infects the nucleus of haematopoietic cells in salmonid fish, although *Nucleospora salmonis* has been reported in pronephrotic cells (early kidneys cells) of epithelial origin in rainbow trout (Desportes-Livage *et al.*, 1996). Other morphological differences also exist, the diplokaryotic arrangement of nuclei is not seen in the Enterocytozoonidae, although it was reported in the initial description of *Enterocytozoon bieneusi* (Desportes *et al.*, 1985), but was later rejected by Cali (1990). Both *Enterocytozoon* and *Nucleospora* develop sporogonial plasmodia. However, the microsporidian-infecting sea lice develop merogonial plasmodia, and no sporogonial plasmodia have been observed. The formation of microsporidian tubules in the host cytoplasm is also not observed in the Enterocytozoonidae, and indeed is rare amongst microsporidia that develop in direct contact with the host cell cytoplasm, only being previously reported for *Brachiola vesicularum* (Cali *et al.*, 1998) and *Nosema algerae* (Avery & Anthony, 1983). However, the plasma membrane in early developing stages of *E. bieneusi* has been closely associated with laminar appendages that extend throughout

the host cell cytoplasm, suggested to correspond to cisternae of the host cell's endoplasmic reticulum (Desportes-Livage *et al.*, 1996).

The intranuclear inclusions observed in infected host cells had an appearance consistent with either crystalline inclusion or viral like particles (VLP's) (Ghadially, 1997). Microsporidian infections have previously been associated with simultaneous viral infections. Both genera from the family Enterocytozoonidae have been reported to be associated with retroviral infection of their hosts. *Nucleospora salmonis* has been found in fish infected with the retrovirus that is believed to be the cause of plasmacytoid leukaemia (Eaton & Kent, 1992; Kent & Dawe, 1993), and *E. bieneusi* is found predominately in HIV-infected patients, although this is largely accepted as opportunistic infections due to a lowering of the immune status caused be the viral infection (Kotler & Orenstein, 1999). The Gypsy moth *Lymantria dispar* L., has been found infected with the microsporidian *Nosema* sp. and nuclear polyhedrosis virus (Bauer *et al.*, 1998). Bauer experimentally infected moths, demonstrating significantly higher mortality rates in a population of moths infected with both pathogens than with just one. Intranuclear and intracytoplasmic crystalline inclusions are frequently associated with other cell abnormalities such as tumour cells and leukaemic conditions; intranuclear crystalline and paracrystalline inclusions have also been regularly associated with virally infected cells (Ghadially, 1997).

Spore dimorphism, although common amongst microsporidian species that have a two-host life cycle, is not well documented for species with only a single host life cycle. The occasional spore dimorphism seen in the microsporidian infecting *L. salmonis* has not been previously reported from the family Enterocytozoonidae. But some single host microsporidia have been reported to form two populations of spores, those that are autoinfective (infect adjacent cells in the same host) and those that are

environmental (spores which leave the host to find a new one), which can differ in morphology (Cali & Takvorian, 1999). A third type of spore, a macrospore, has been described from *Pleistophora* sp., so called as it is about two times the size of typical spores of that species (Canning & Hazard, 1982), and may explain the occasional macrospore (large spore) observed during this study.

Preliminary identification would place this microsporidian in the family Enterocytozoonidae, based on the similarities is spore morphology, the precocious development of the polar filament, the presence of electron lucent inclusions during sporogony, the lack of an interfacial envelope during the entire developmental cycle and its polysporous nature. However, many differences still exist between this microsporidian and other members of the family, and additional information will be required, such as ribosomal DNA sequences, before a genus or family can be formerly assigned to this parasite.

It is hard to imagine that an organism can be so heavily infected with parasitic spores and remain unaffected. Large xenomas were observed completely blocking the haemocoelic cavity in the abdomen, cephalothorax and appendages. Such obstructions must restrict the flow of haemolymph to such areas reducing the normal distribution of essential nutrients and compounds.

Infection has only been observed in the epidermal tissue beneath the cuticle, which are the cells responsible for the secretion of new cuticle during the molting process. As salmon lice do not molt again once they have reached the adult stage, a microsporidian infection in the molting juvenile stages may inhibit the secretion of new cuticle and interfere with the molting process, resulting in a higher degree of pathogenicity in juveniles than in adults. As the ovaries and developing egg strings were not found to be infected with the microsporidian, the aborted egg string reported

in chapter 4 could potentially be caused by a lack of nutrition and a reduced general fitness or may in fact simply represent a senescent louse. Pathogenicity trials will need to be conducted to fully evaluate any pathogenicity caused by this infection, concentrating primarily on the presence of infection in juvenile lice and their ability to successfully molt, the production of normally developed egg strings in young infected adult female lice and evaluating what role if any the viral like particles play in etiology.

Figure 5.2 Giemsa stained section, showing an area of the haemocoel filled with blue staining spores (s) adjacent to uninfected ovary tissue (o), scale bar = 20µm.
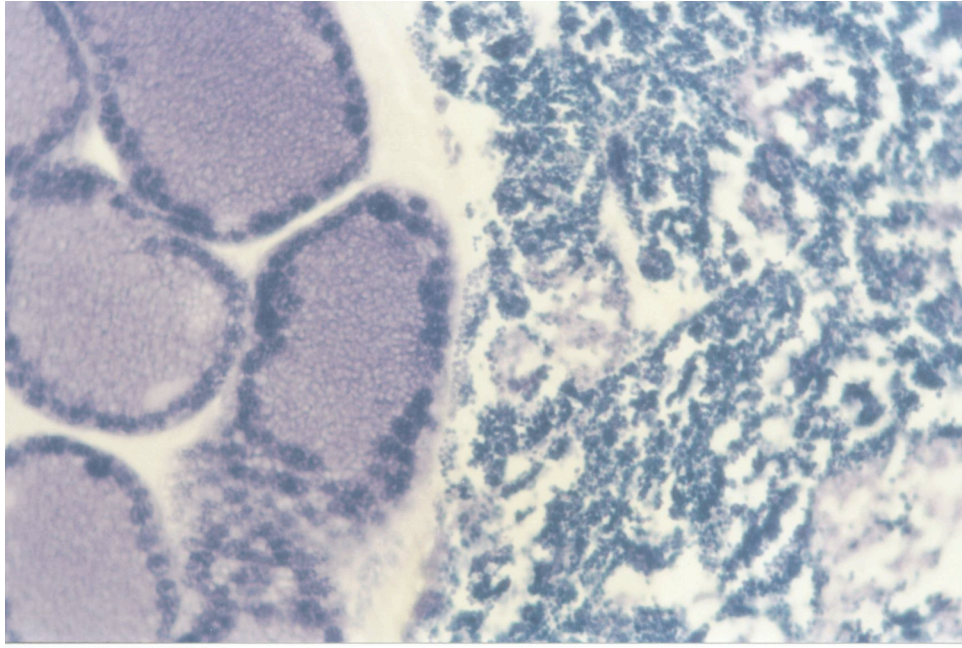
Figure 5.3 Gram stained section through the genital segment; large spore masses (s) stained Gram positively, which filled the segment, scale bar = 20µm.
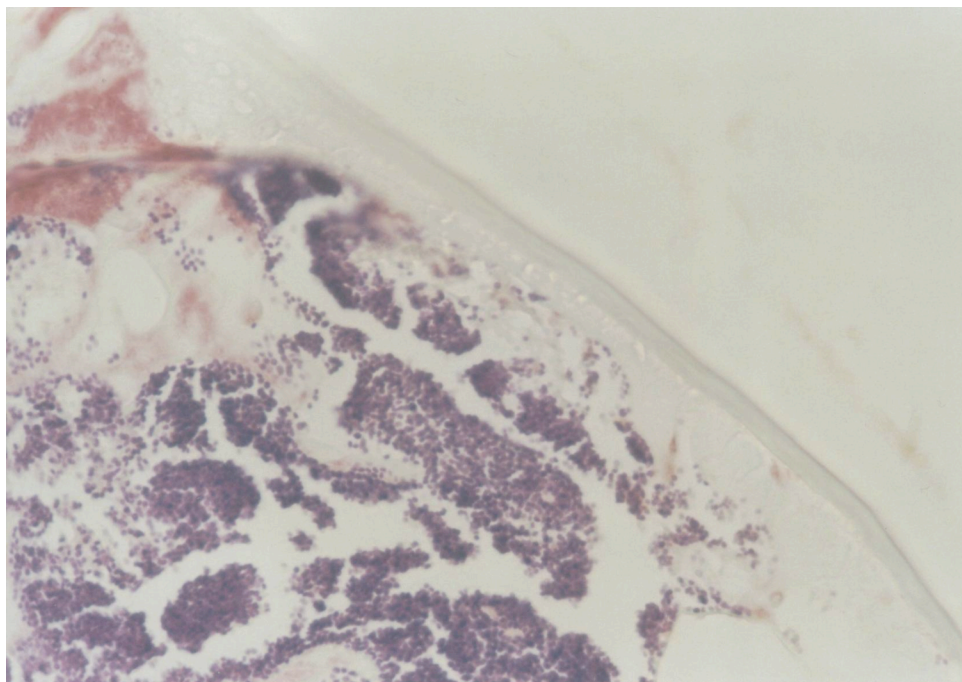
Figure 5.4 Transverse semi thin section (x 40) through the abdomen, showing xenomas of varying size (x) forming in the haemocoel (h) between the cuticle (c) and the gut (g), and along the haemocoelic separations in the haemocoel (hs), scale bar = 100µm.
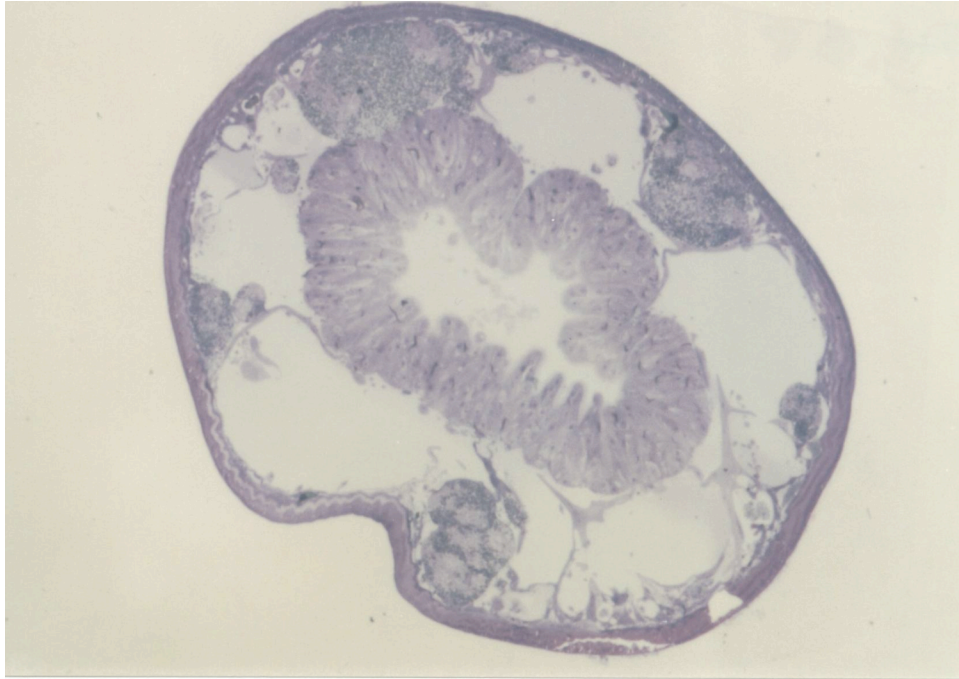
Figure 5.5 Higher power magnification (x 200) of figure 5.4, showing a xenoma (x) which completely occupies the haemocoelic space between the cuticle (c), the gut (g) and two haemocoelic separations (hs), scale bar = 20µm.
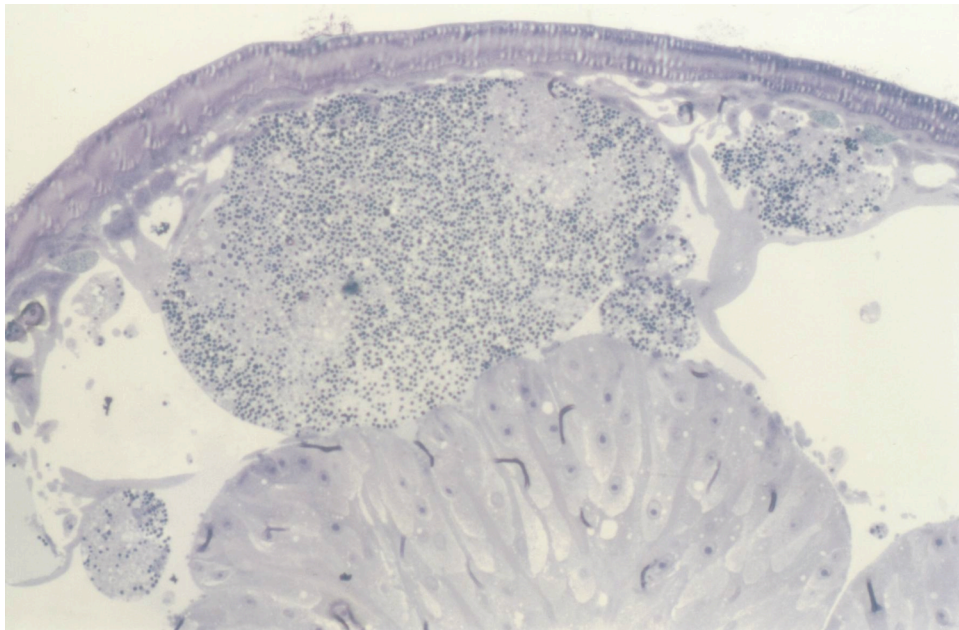
Figure 5.6 Higher power magnification (x400) of figure 5.4, showing the xenoma (x) developing directly under the cuticle (c) and the xenoma (xx) developing directly from the haemocoelic separation in the haemocoel (hs), scale bar = 20µm
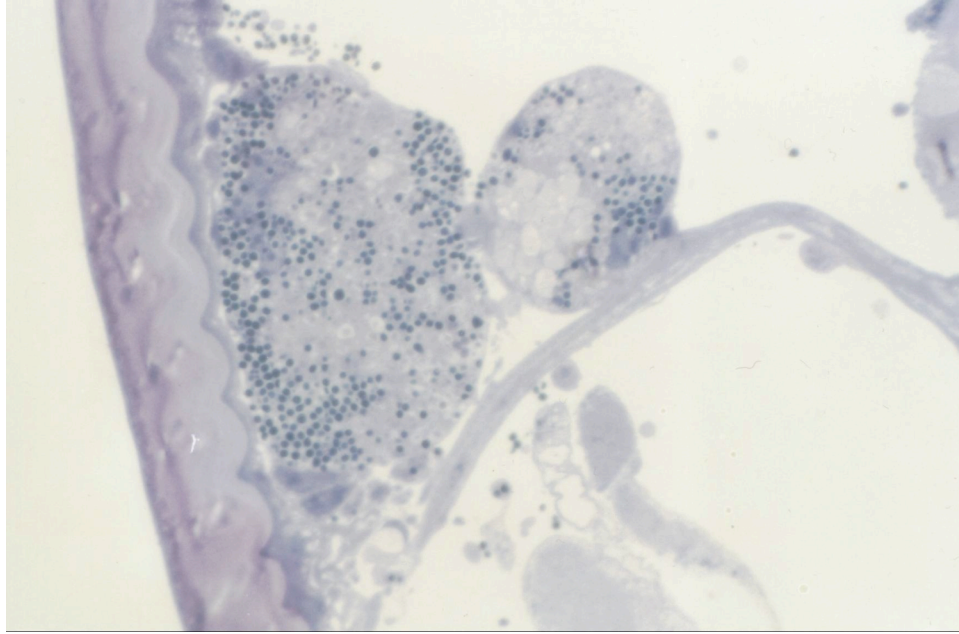
Figure 5.7 Semi thin section through a maxilliped, showing spore filled xenomas (s) under the cuticle, lying adjacent to uninfected muscle (m), scale bar = 50µm.
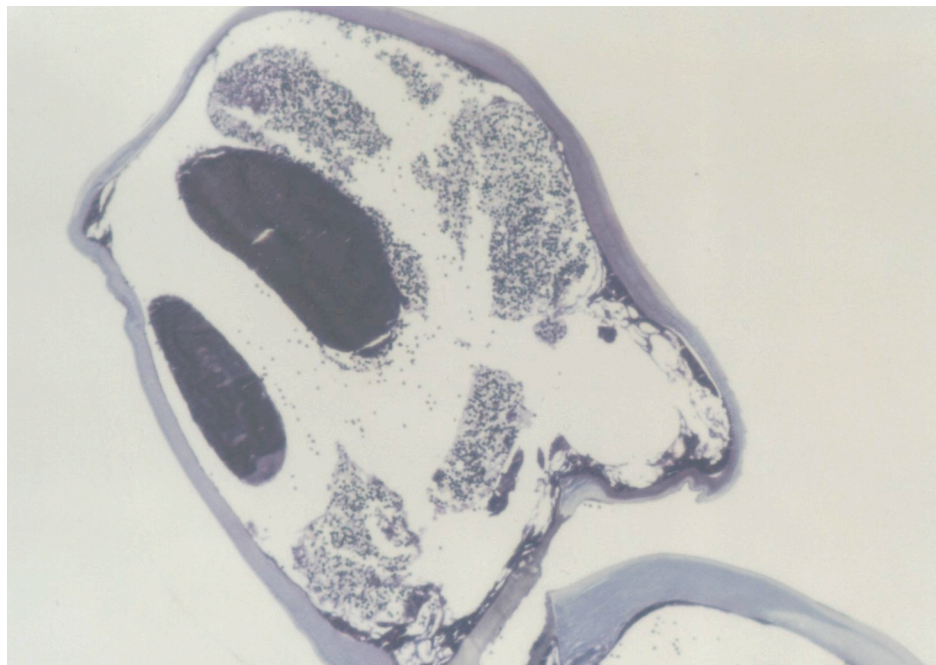
Figure 5.8 Giemsa stained section of a xenoma (x) forming between uninfected muscle (m) and a developing egg string (es) within the genital segment, scale bar = 20µm.
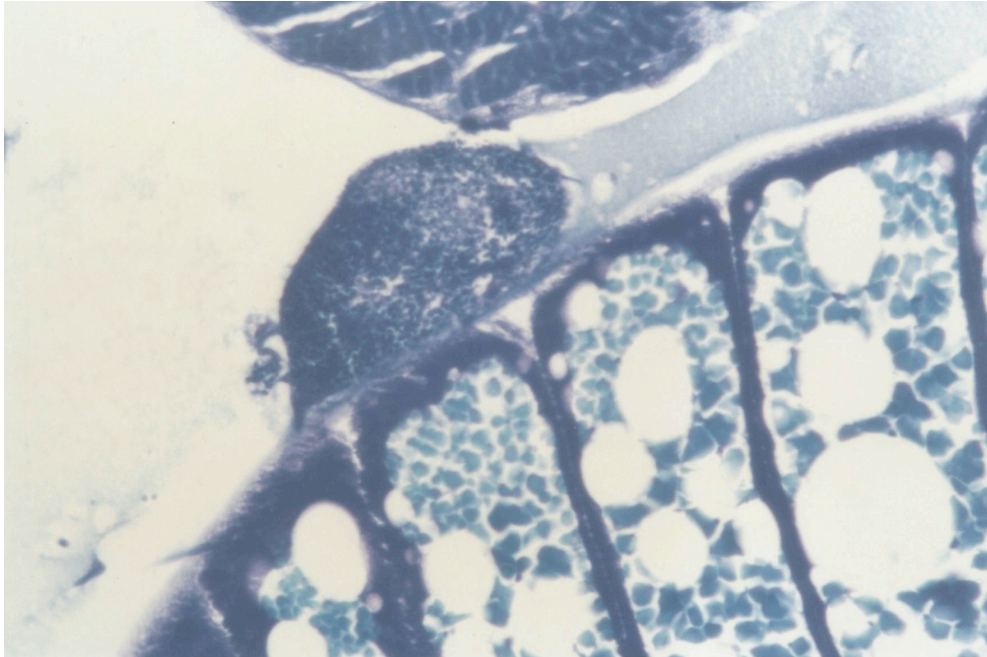
Figure 5.9 Giemsa stained section showing free spores (s) in the haemocoel, in close association with the developing egg string (es), in the genital segment, scale bar = 40µm.
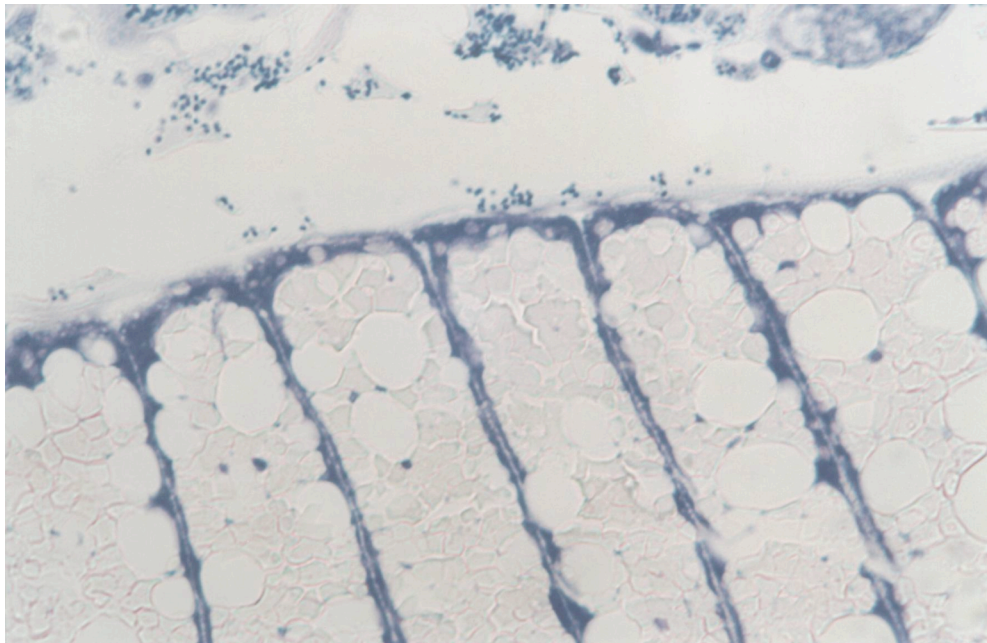
Figure 5.10 TEM showing the relationship between the procuticle (p), the epidermal layer (ep) and the site of microsporidian infection (m), scale bar = 5µm.
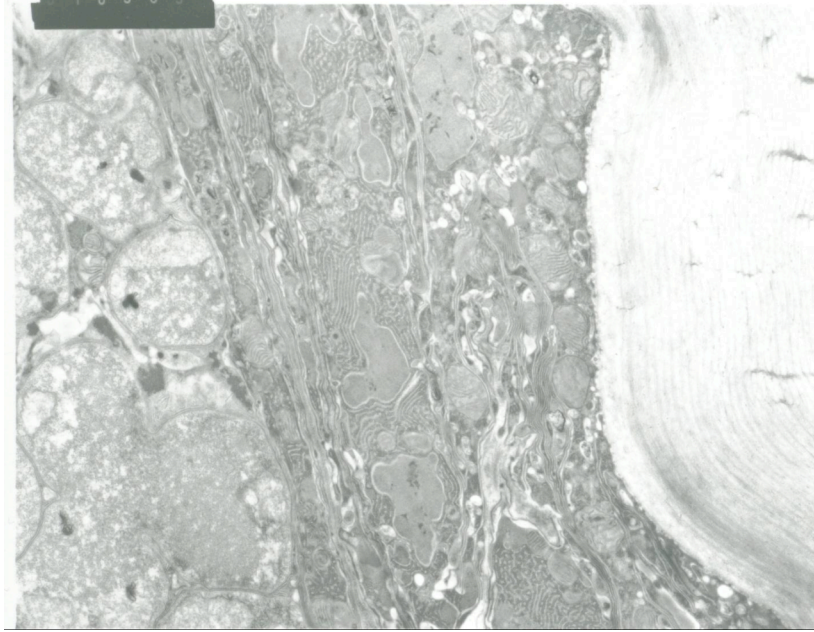
Figure 5.11 Higher magnification of figure 5.10; note the elongate cells of the epidermal layer containing abundant rough endoplasmic reticulum (rer), with an early proliferative stage of the microsporidian (m) within a host cell closely associated with the basal portion of the epidermal layer, note host cell mitochondria (arrow heads), scale bar = 1µm.
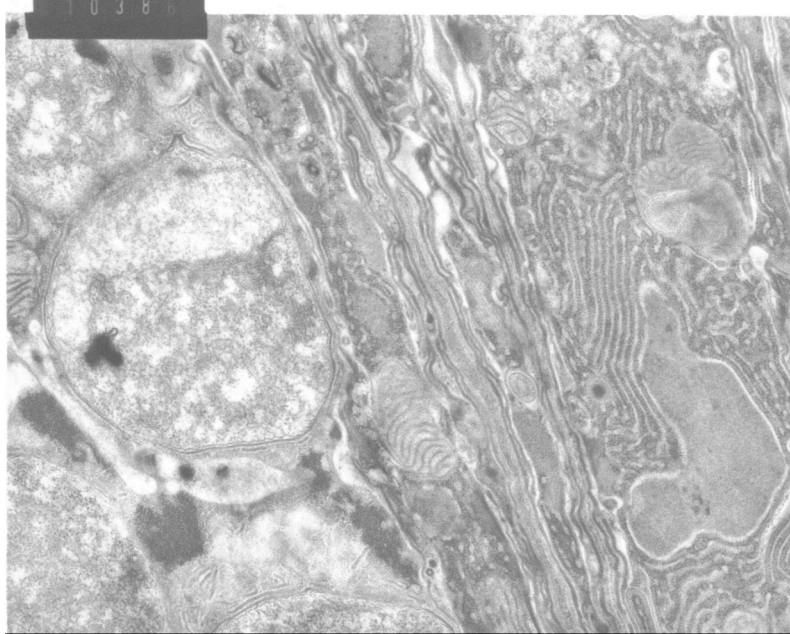
Figure 5.12 TEM of host cell remnants at the margin of a large xenoma contained by the host cell plasma membrane (pm), note the host cell nucleus (hn) the host endoplasmic reticulum (er) and host mitochondria (arrow heads) scale bar = 2µm.
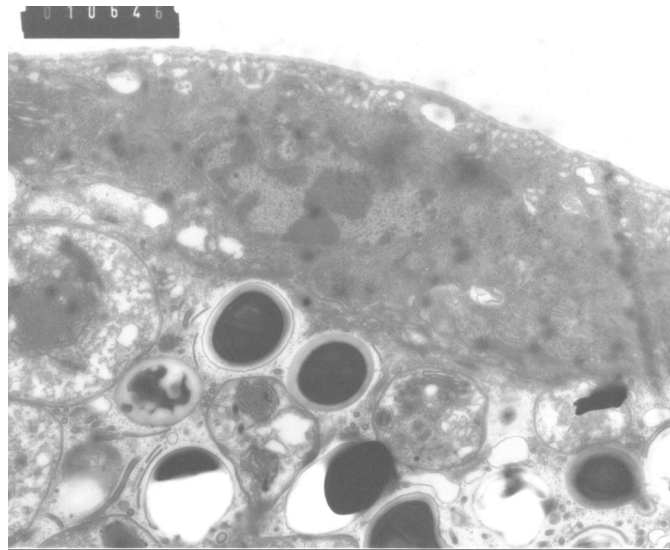
Figure 5.13 TEM of small xenoma, note the multiple host nuclei (HN) scale bar = 5µm.
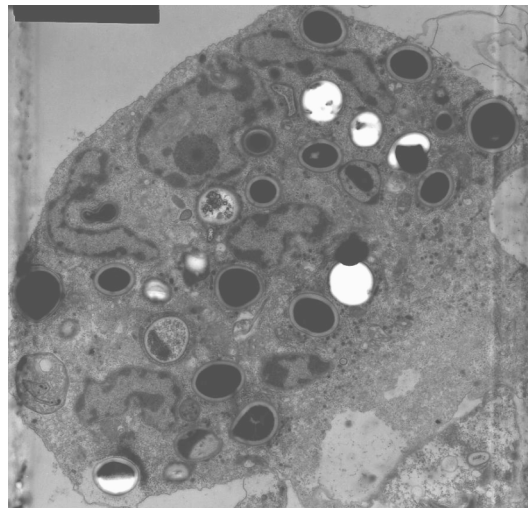
Figure 5.14 Transverse section TEM of a mature spore, showing thickened electron lucent endospore (en), 7-8 turns of the polar filament (pf), nucleus (n) and polarplast (pl), scale bar = 1µm.
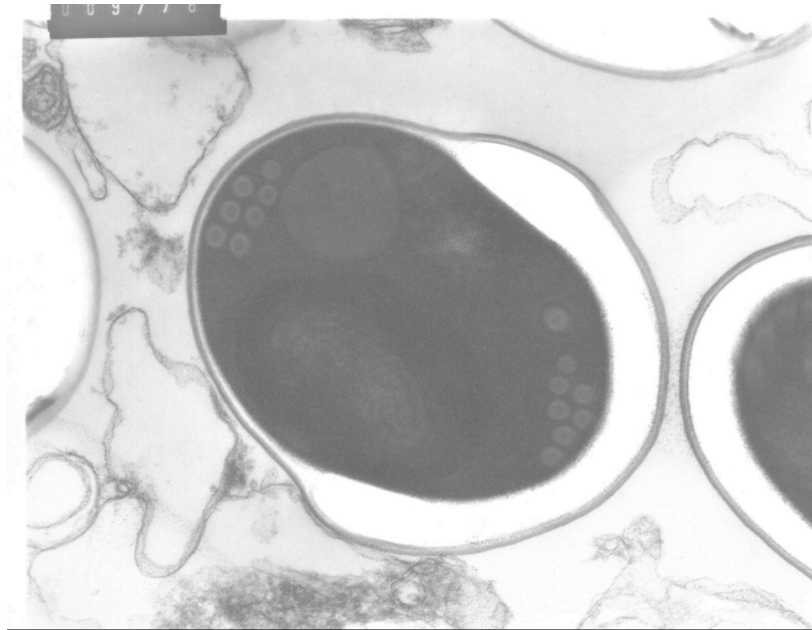
Figure 5.15 TEM of mature spore, showing the anchoring disc (ad) of the polar filament (pf) and the lamellar polarplast (pl), scale bar = 1µm.
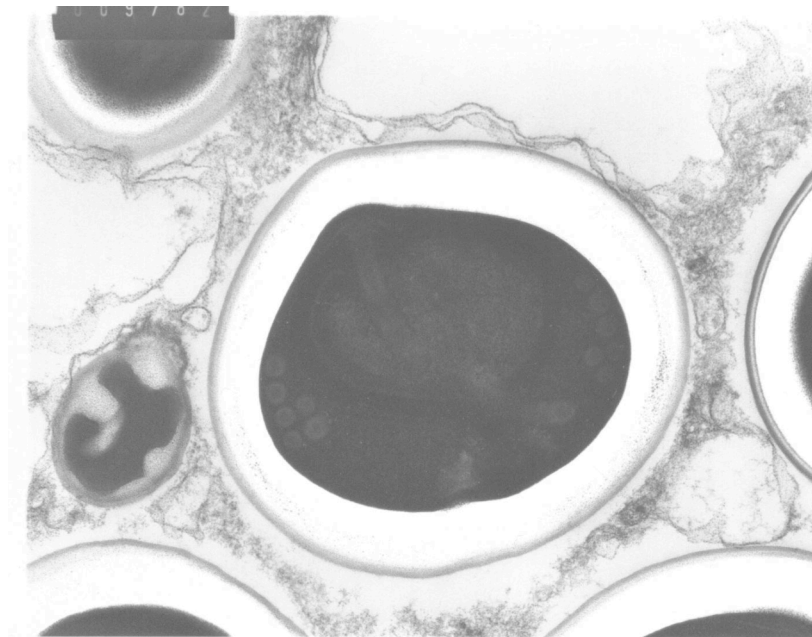
Figure 5.16 TEM of early proliferative stages, containing nuclei (n) in typical diplokaryotic arrangement, scale bar = 2µm.
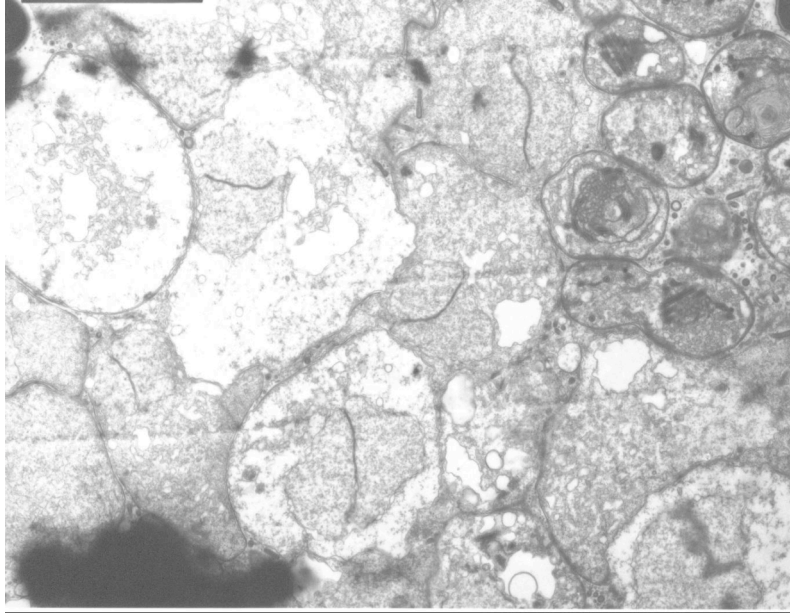
Figure 5.17 Higher power magnification of the diplokaryotic arrangement of the early proliferative stage, scale bar = 2µm.
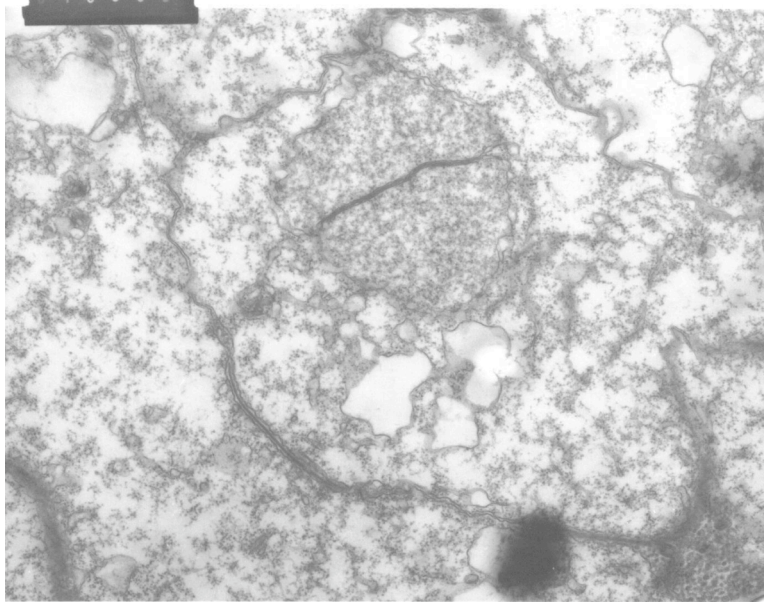
Figure 5.18 TEM of a cell undergoing binary fission (arrow heads), post replication of the diplokaryotic nuclei (n), scale bar = 2µm.
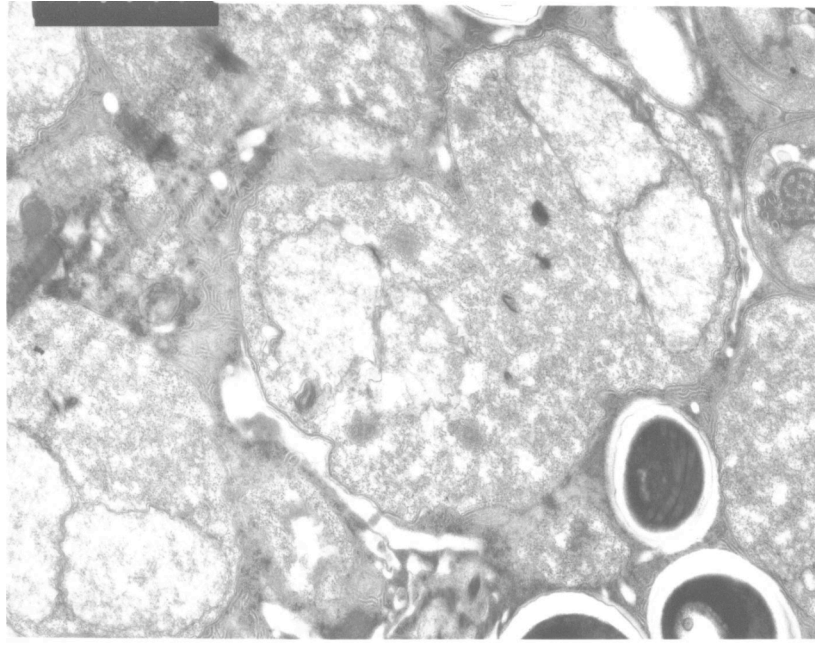
Figure 5.19 TEM of a multinucleate plasmodium, with five nuclei (n) clearly visible, dividing by plasmotomy, scale bar = 2µm.
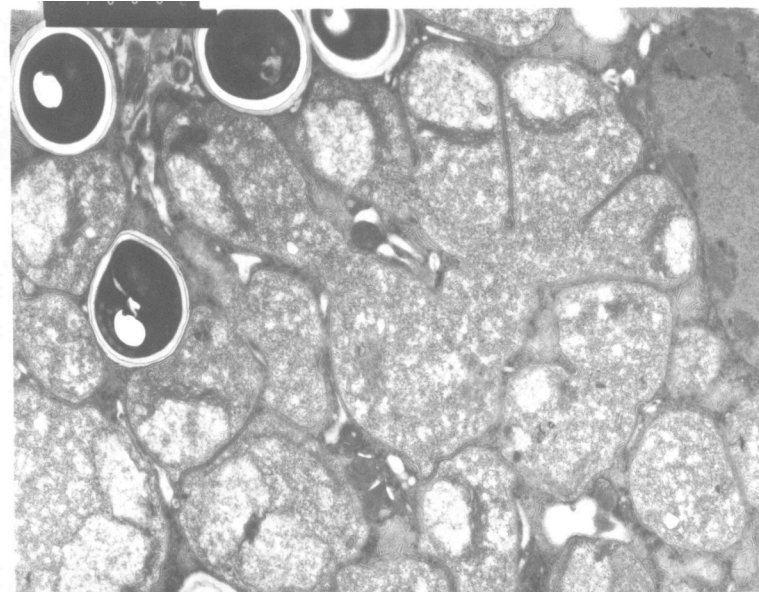
Figure 5.20 Higher power magnification of figure 5.18, showing plasmodium dividing via plasmotomy, with nuclei showing darkly staining laminate-bodies on the nuclear membrane (arrow heads), scale bar = 2µm.
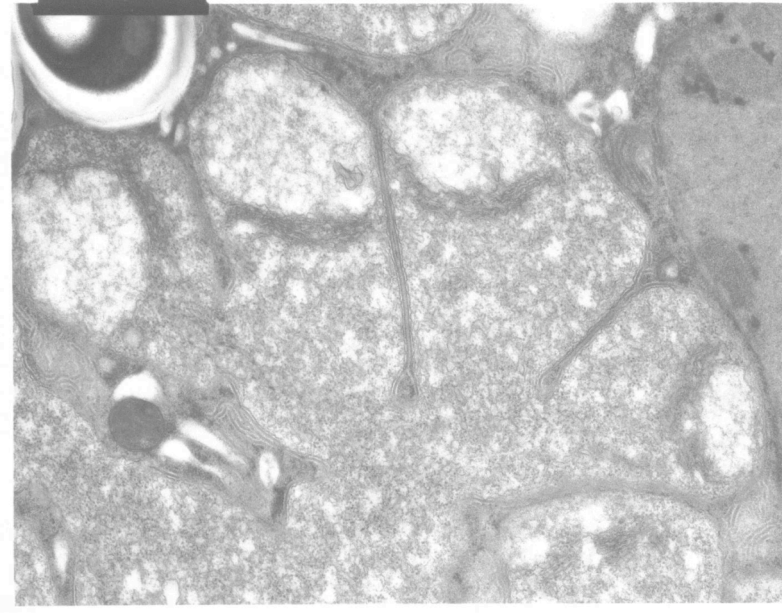
Figure 5.21 TEM of a sporont dividing by binary fission; note the tubules (t) in the hyaloplasm, and the precocious development of polar filament precursors (p) and modest thickening of the plasmalemma (arrow heads), scale bar = 1µm.
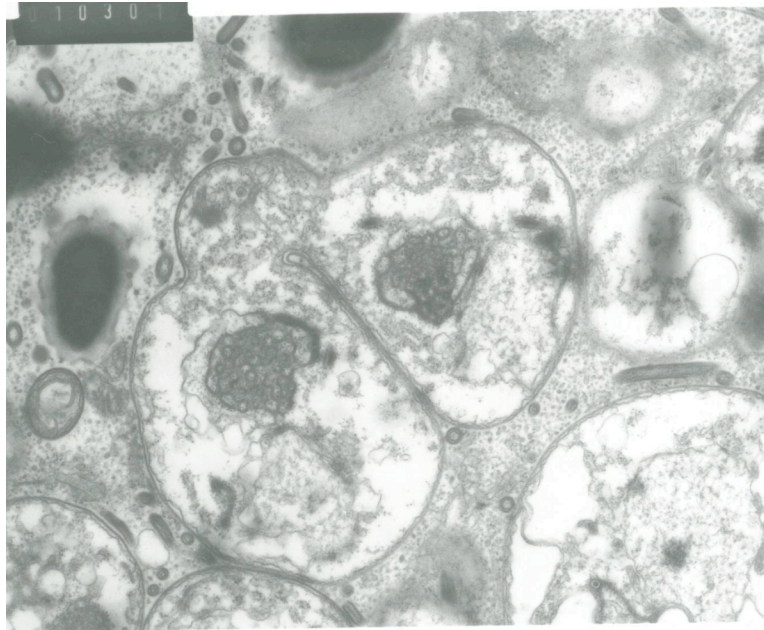
Figure 5.22 TEM of maturing sporoblast with layered membranous secretions (arrow heads) from the exospore in the hyaloplasm, scale bar = 1µm.
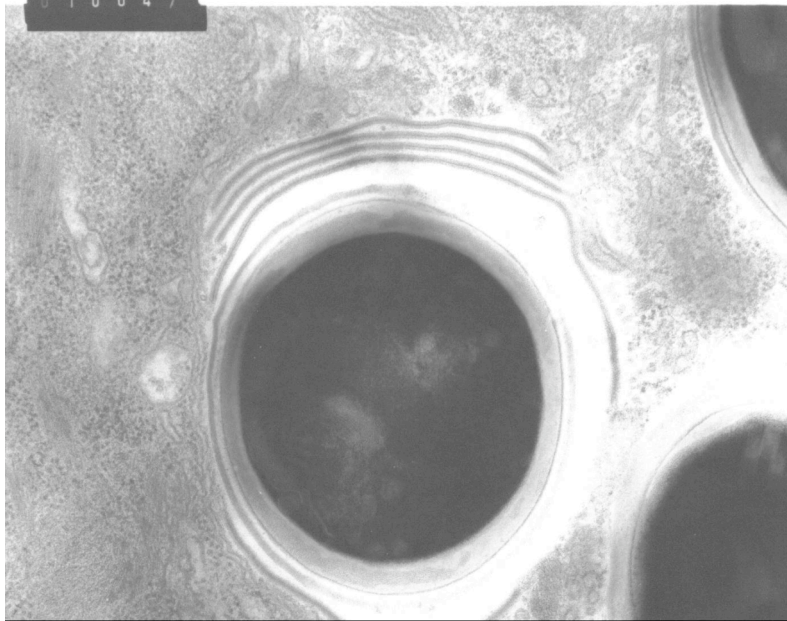
Figure 5.23 TEM of membranous secretion; note the central core of electron dense particles (arrow heads), scale bar = 0.5μm.
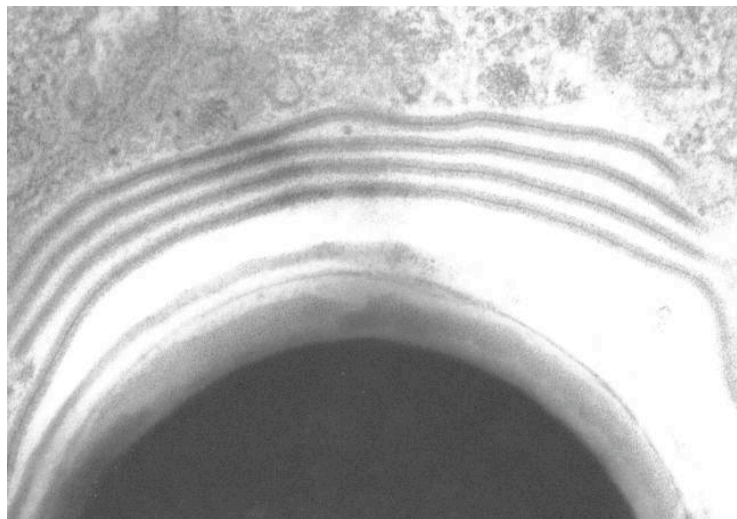
Figure 5.24 TEM of membranous secretions that have formed enclosed concentric whorls (w) in the hyaloplasm, note the membrane (arrow head) enclosing the xenoma, scale bar = 1μm.
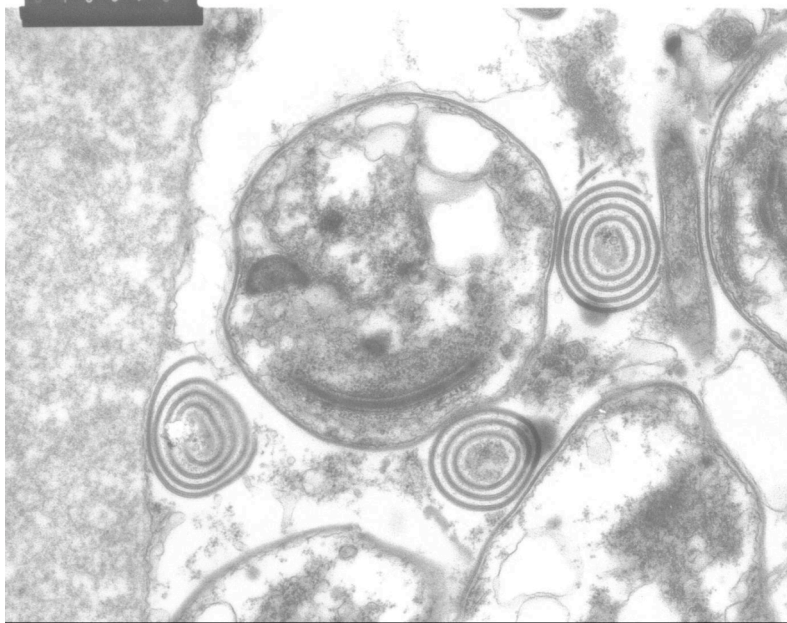
Figure 5.25 TEM of a sporont or sporoblast at the extremity of a xenoma, showing the formation of the anchoring disc (ad) and of the polar filament (pf); note the tubules (t) in the hyaloplasm, scale bar = 1µm.
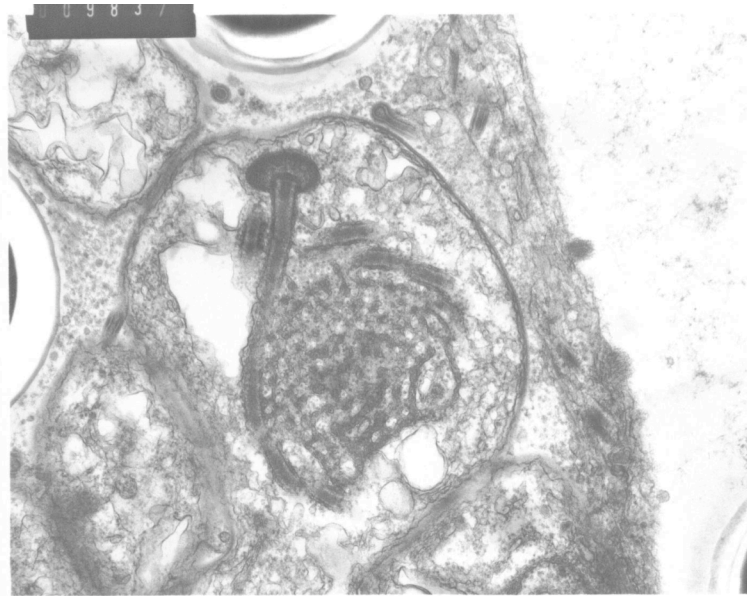
Figure 5.26 TEM of a late sporont prior to division to sporoblasts, with two sets of well-developed polar filament coils (pf); note the electron lucent inclusions (ELI's), scale bar = 1µm.
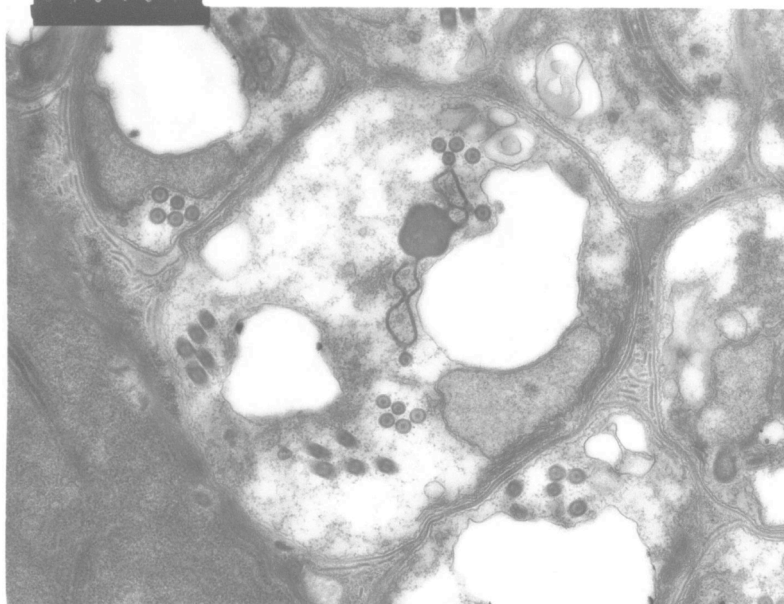
Figure 5.27 TEM of a late sporont dividing into two sporoblasts; note the advanced development of the polar filaments (pf), the much smaller ELI's (arrow heads) and a membrane whorl (w) in the hyaloplasm near the cytoplasmic isthmus of the dividing sporonts, scale bar = 1µm.
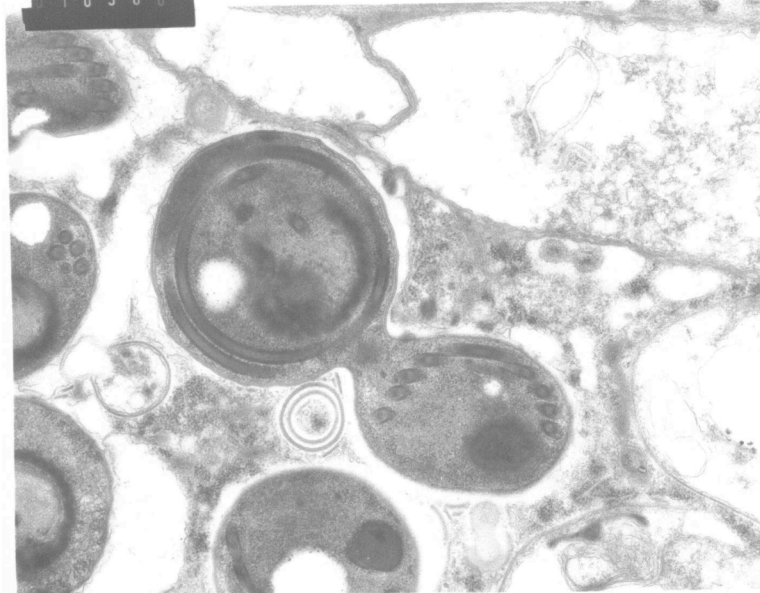
Figure 5.28 TEM of early sporoblasts, note the electron dense particles (arrow heads) associated with the electron lucent inclusions (ELI's), scale bar = 1µm.
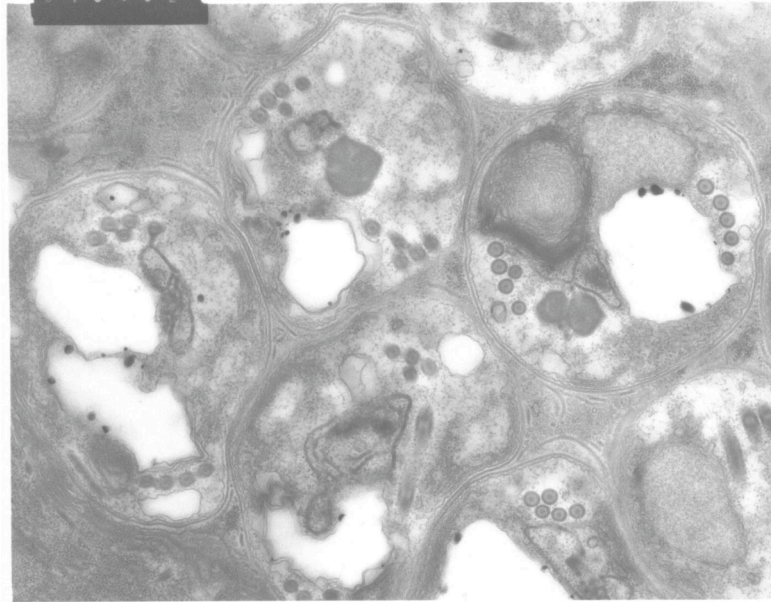
Figure 5.29 TEM of more mature sporoblast; note a reduction in the size of the ELI's, the cytoplasmic cisternae (arrow heads) associated with the developing polar filament (pf) and the thickening of the plasmalemma (double arrow heads), scale bar = 1µm.
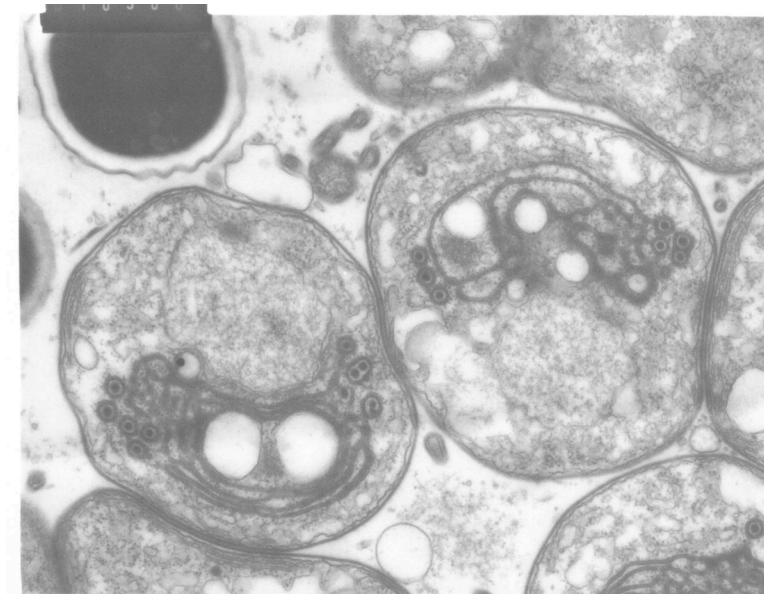
Figure 5.30 TEM of a host cell nucleus (hn) from a microsporidian-infected host cell; note the regular shaped rectangular intranuclear inclusion (arrow head), scale bar = 1µm.
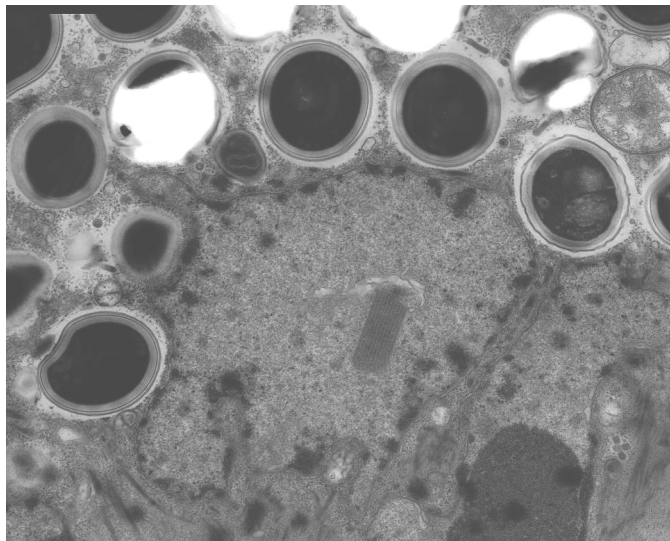
Figure 5.31 TEM of host cell nucleus with intranuclear inclusion of crystalline appearance; note the regular pattern, scale bar = 0.5µm.
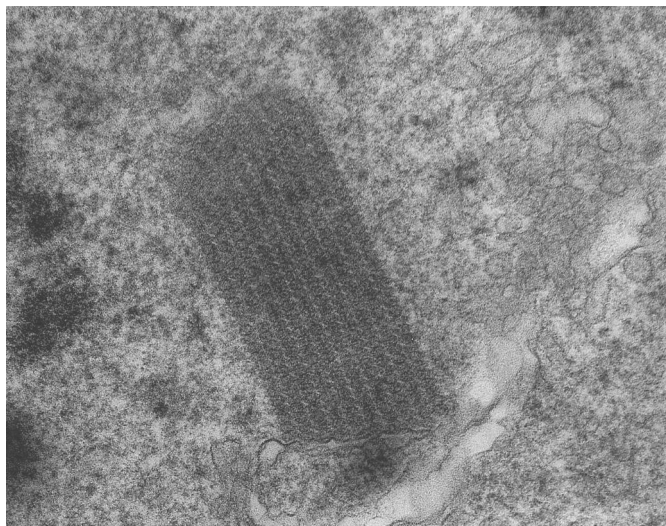
Figure 5.32 TEM of a mature spore, much larger in size and showing 12 turns of the polar filament, scale bar = 1µm.
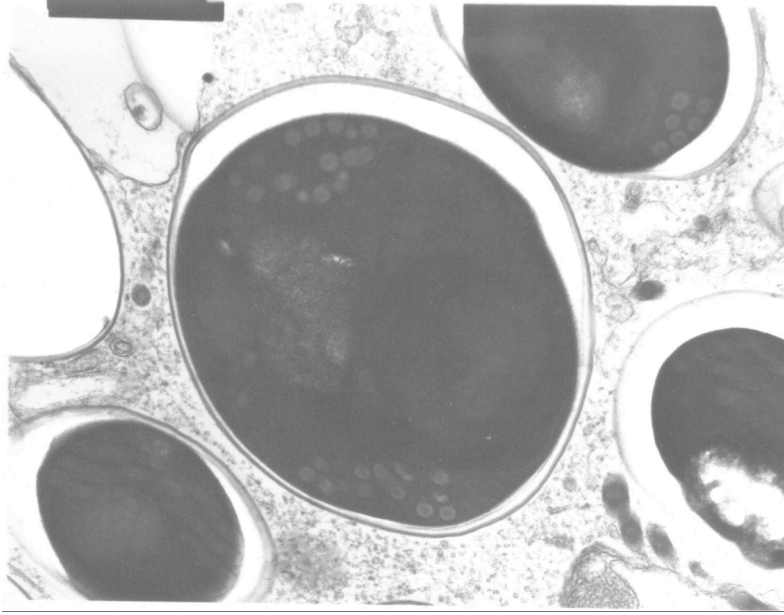
Figure 5.33 TEM of large electron dense 'spore-like' cell, showing highly disorganised cell contents, scale bar = 2µm.
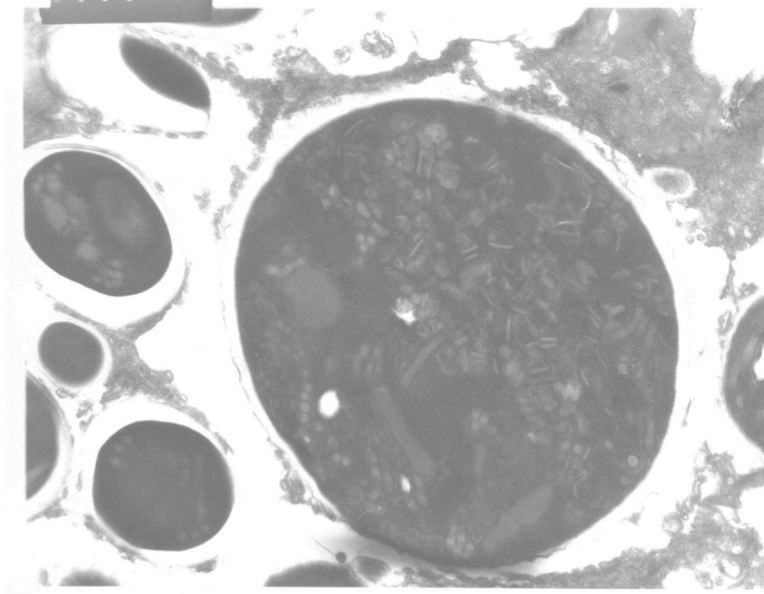
CHAPTER 6

MOLECULAR PHYLOGENY OF THE SALMON LOUSE-INFECTING MICROSPORIDIAN

6.1 INTRODUCTION

Having confirmed the presence of a microsporidian hyperparasite of *L. salmonis* using light and electron microscopy (chapter 5), further characterisation of the hyperparasite using molecular phylogenetic techniques is required in order to confirm the taxonomic position suggested by the histological and ultrastructural data and complete the full taxonomic description of this novel organism.

Contemporary studies have revealed large discrepancies between microsporidian systematics based on molecular and traditional characteristics. These differences have resulted in confusion as to the specific taxonomic value of certain developmental and morphological features (Baker *et al.*, 1994; Baker *et al.*, 1995; Weiss & Vossbrinck, 1998). Consequently, molecular based analyses may reveal relationships that were not previously apparent. Weiss *et al*. reviewed the usefulness of microsporidian rDNA in diagnosis and phylogeny and concluded that the 530f-580r region of the gene, shown in figure 6.1, extending from within the small subunit (SSU), through the internal transcribed spacer (ITS) region and into the large subunit (LSU), contained sufficient information to be useful for molecular phylogenetic studies of the Microspora (Weiss *et al.*, 1994). Initial rDNA-based analyses were typically restricted to a few genera (Baker *et al.*, 1994) or focused on those species isolated from humans (Vossbrinck *et al.*, 1993; Weiss *et al.*, 1994; Zhu *et al.*, 1994; Baker *et al.*, 1995). Recently, however,

the increased amount of microsporidian rDNA sequence data available has been utilised to re-examine the phylogenetic status of established species (Nilsen *et al.*, 1998; Nilsen, 2000; Muller *et al.*, 2000) and to investigate the systematic placement of members of the unclassified genus, *Microsporidium* spp., infecting the musculature of marine fish (Bell *et al.*, 2001; Yokoyama *et al.*, 2002). More recently, Terry *et al* (2002) used SSU rDNA to examine the origins of vertical transmission of microsporidia in marine and freshwater invertebrates.

6.2 OBJECTIVES

In the present study, rDNA (530f-580r) sequence data will be gathered for an unidentified microsporidian isolated from the salmon louse *L. salmonis,* and compared with equivalents available in the databases, in an attempt to identify the systematic position of this previously unrecorded microsporidian. A broader taxonomic perspective will be achieved by analysing partial SSU sequences of 38 species belonging to at least 22 different genera.

This data may reveal phylogenetic relationships otherwise unapparent, and assist in future life cycle and transmission studies. Furthermore, specific PCR primers will be designed and used in future diagnostic PCR assays, facilitating the identification of potential intermediate or alternative hosts that may be involved.

This data, combined with the histological and ultrastructural data from the previous chapter will enable this microsporidian to be placed in the correct systematic position within the Microspora.

6.3 METHODS

6.3.1 DNA extractions

Sea lice were screened microscopically for gross signs of the presence of the microsporidian infection, which appeared as opaque internal inclusions throughout the body, as described in chapter 4. Whole infected adult sea lice were washed in sterile seawater prior to extraction. DNA extractions were performed as previously described in chapter 2.3, except for the addition of 0.4g of 0.5mm glass beads (Sigma) to the homogenising buffer, and vortexed in a manner to maximise shearing directions for 1 min, facilitating the mechanical disruption of mature spore walls. Negative and positive control tissues were also extracted from uninfected sea lice and from the stickleback, *Gasterosteus aculeatus aculeatus* L., infected with the microsporidian *Glugea* sp..

6.3.2 DNA amplification

Targeted DNA (c. 1400 bp) was amplified using the universal PCR primers described by Vossbrinck *et al* (1993). Two further internal regions of approximately 925 bp and 580 bp in length, located within the 530f-580r rDNA target amplicon were also amplified from the original genomic DNA extractions using additional primers (Table 6.1 and Figure 6.1). These intermediate and internal primers were designed to be more specific to the sea louse-infecting microsporidian, and therefore could be used diagnostically. ClustalX, a multiple sequence alignment program (Thompson *et al.,* 1997), was used for comparing initial sequences determined with the original 530f-580r universal primer set, with comparisons made from blast data base searches. The intermediate and internal primers were designed from these initial sequence comparisons, targeting regions of maximum disparity.

Table 6.1 Primer details for PCR amplification of rDNA.

Primer	Seqnence
530f	(5′-GTGCCATCCAGCCGCGG-3′),
580r	(5′-GGTCCGTGTTTCAAGACGG-3′).
Intermediate fwd	(5'-GTCTGTGGATCAAGGACGAA-3')
Intermediate rev	(5'-ACTGATATGCTTAAGTTCAGG-3')
Internal fwd	(5'-CTGCTTAATTTGACTCAACAC-3')
Internal rev	(5'-CAGTCCTACTACTCATATACT-3')

After an initial denaturation at 95 ˚C for 5 min, samples were subjected to 30 cycles of amplification as detailed in table 6.2, followed by a 10 min terminal extension at 72 ˚C. Amplified products were separated on a 1% agarose gel (0.5x TAE see appendix 1), visualised by staining with ethidium bromide (0.5μg/ml) and photographed on an ultraviolet transilluminator.

Table 6.2 Temperature profiles for PCR amplifications were as follows:

530f 580r primer set	intermediate primer set	internal primer set
5 min at 95 °C	5 min at 95 °C	5 min at 95 °C
30 sec 95 °C	30 sec 95 °C	30 sec 95 °C
30 sec at 43 °C } x30	30 sec at 55 °C } x30	30 sec at 52 °C } x30
1 min 72 °C	1 min 72 °C	1 min 72 °C
10 min 72 °C	10 min 72 °C	10 min 72 °C

Two DNA markers (Biolabs) were run on each agarose gel (100 ng/lane) in order to calculate the product size (Table 6.3).

Table 6.3 Size range of DNA markers used to calculate PCR product sizes.

DNA Marker	Marker size range (base pairs)
Lambda DNA-*Hind* III digest	23,130 to 2,027
ΦX174 DNA-*Hae* III digest	1,353 to 72

6.3.3 DNA sequencing

PCR products of the predicted size were excised with a clean scalpel blade and purified using GFX spin columns (Amersham Pharmacia Biotech).

Sequences were determined directly from the PCR products. Cycle sequencing reactions using the BigDye Terminator^{TM} Sequencing Kit (Perkin-Elmer Corporation) and incorporating the same primers as those used in the initial PCR's were performed according to the manufacturer's instructions. Sequencing products were run on an ABI Prism 377 automated sequencer (Perkin-Elmer Corporation). Sense and anti-sense strands were sequenced for all PCR products (full 530f-580r region and both internal amplicons) and four replicates (PCR products amplified from separate DNA extractions) performed for each gene region.

6.3.4 consensus sequence compilation, sequence comparisons and generation of phylogenetic trees

The consensus sequence was generated using the alignment packages Clustal X (Thompson *et al.*, 1997) and BioEdit (Hall, 1999), allowing at least 100 bp overlap between amplicons on both sense and antisense strands. The consensus sequence was submitted to the standard nucleotide-nucleotide BLAST (blastn) NCBI database (Altschul *et al.*, 1997) to identify the closest known sequence matches. Clustal W (Thompson *et al.*, 1994) was used for initial sequence alignments with default settings for gap and weighting values. Regions of ambiguous alignment were identified visually and removed prior to analyses. Alignment files were converted into distances by the Kimura 2 parameter and trees constructed using the Neighbour-Joining (N-J) algorithm (Saitou & Nei, 1987) within the Phylogeny Inference Package (PHYLIP version 3.57; Felsenstein, J., 1993. PHYLIP: phylogeny inference package, version 3.5c. University of Washington, Seattle, WA.). In addition, cladograms were

produced from the alignment files using the Maximum-Likelihood (M-L) algorithm within PUZZLE (Olsen *et al.*, 1994; Strimmer & Vonhaeseler, 1996). Numerical values at the nodes indicate either 1000 (N-J) or 1000(M-L) bootstrap replicates that support the observed tree. *Amblyospora stimuli* (Andreadis) that occupies a basal phylogenetic position within the Microspora and *Tritrichomonas foetus* (Levine) a distantly related protozoan parasite of cattle were used as outgroups in the cladistic analyses.

For ease of interpretation, sequence comparisons were made by percentage identities rather than by distance matrices.
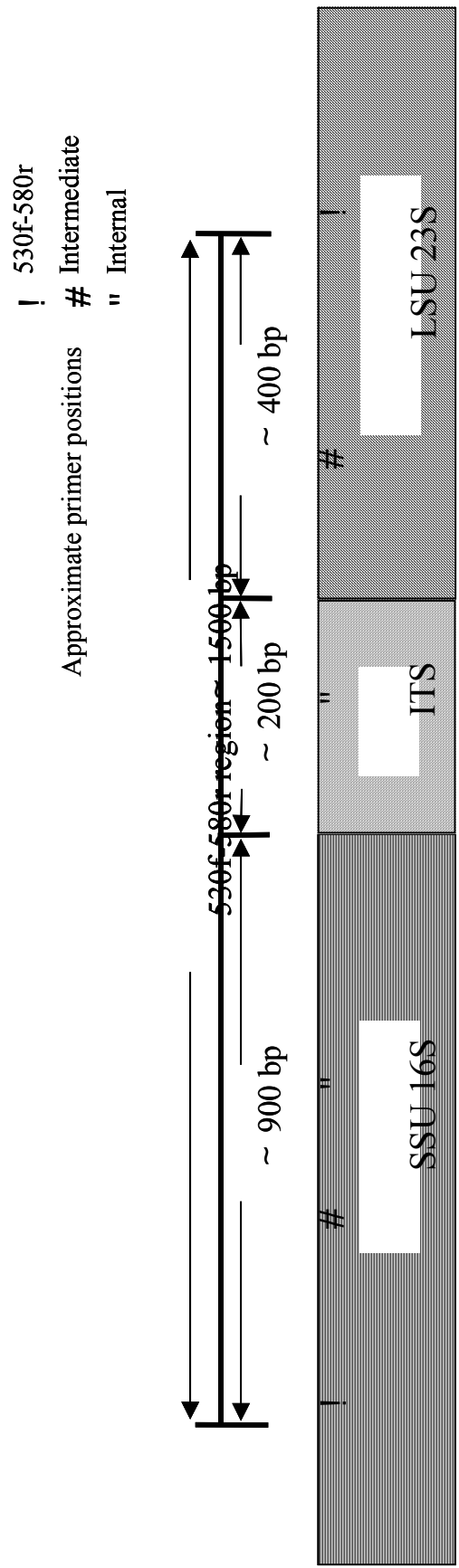
Figure 6.1 Schematic drawing of the rRNA gene from microsporidia. Note the single ITS (Internal Transcribed spacer) region and lack of 5.8s sequence. This gene will have multiple copies in the ribosomal DNA of all microsporidia. Marked are the approximate binding sites for the three primer sets used.

6.4 RESULTS

6.4.1 PCR amplification

DNA was extracted from four microsporidian-infected sea lice. PCR amplifications were successfully completed for each sample using the 530f-580r universal primers set. Figure 6.2 shows an image of the agarose gel from the PCR products, the positive control, *Glugea* sp. in lane 3 shows a band of approximately 1400 bp, the expected product size for amplified microsporidian DNA using this primer set. In lane 4, the negative control shows no amplification, and no band is present. The DNA obtained from the microsporidian-infected sea lice in lanes 5-8 all show a product band of similar size (c. 1400 bp). The uninfected sea lice in lanes 9 and 10 show no band of this size, but host (salmon louse) DNA yields a larger product of about 2500 bp.

The original genomic DNA extractions were successfully used in further PCRs to amplify specific / targeted internal regions of the gene. Figure 6.3 shows an agarose gel from the PCR products of the four microsporidian-infected sea lice producing a band at 925 base pairs with the intermediate primer set and a 580 base pair product with the internal primer set. The positive control, *Glugea* sp. from infected fish, used in the first PCR now fails to yield a product, due to the specificity of the new primer sets (lanes 2 and 8, figure 6.3).

Figure 6.2 Agarose gel of PCR products from microsporidian infected sea lice with negative and positive controls. Amplifications were achieved using the 530f 580r primer set.
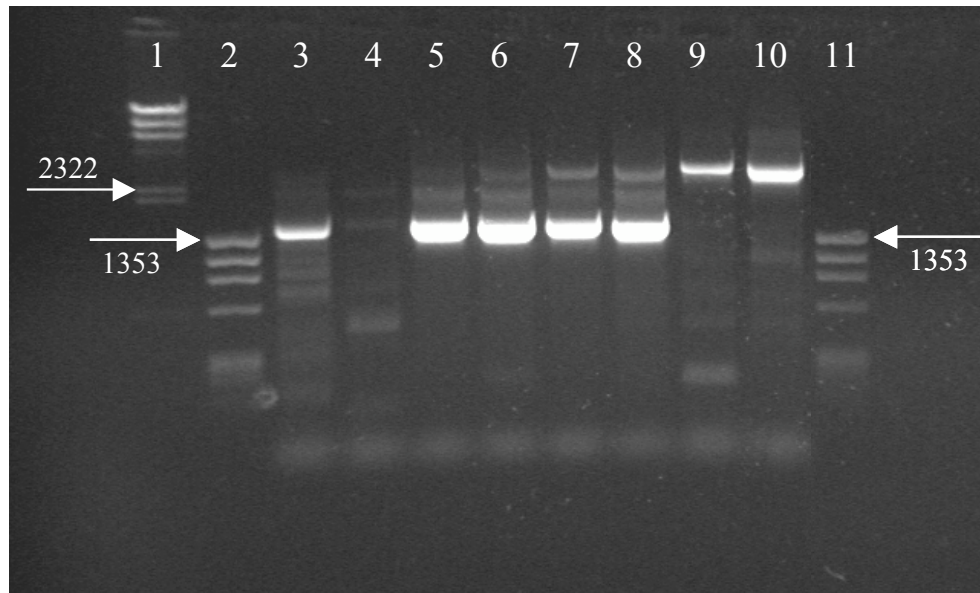
Table 6.4 Table describing lane positions for DNA samples for Figure 6.2.

Lane number	Sample
1	DNA marker Lambda DNA-*Hind* III digest
2	DNA marker ΦX174 DNA-*Hae* III digest
3	*Glugea* sp. infected fish tissue
4	-VE control (terrestrial insect)
5	Microsporidian infected sea louse ♀
6	Microsporidian infected sea louse ♀
7	Microsporidian infected sea louse ♀
8	Microsporidian infected sea louse ♂
9	Non-infected sea louse ♂
10	Non-infected sea louse ♀
11	DNA marker ΦX174 DNA-*Hae* III digest

Figure 6.3 Agarose gel of PCR products from microsporidian infected sea lice with negative controls, amplifications were achieved using both intermediate and internal primer sets.
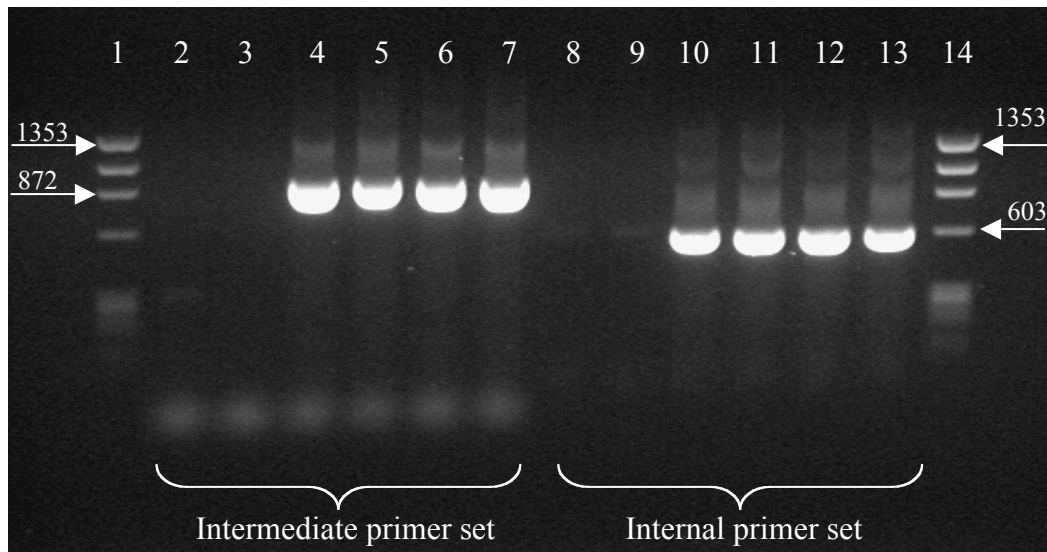
Table 6.5 Table describing lane positions for DNA samples for Figure 6.3.

Lane number	Sample		
1	DNA marker ΦX174 DNA-*Hae* III digest		
2	*Glugea* sp. infected fish tissue		
3	-VE control (terrestrial insect)		
4	Microsporidian infected sea louse ♀		
5	Microsporidian infected sea louse ♀	Intermediate Primer set	
6	Microsporidian infected sea louse ♀		
7	Microsporidian infected sea louse ♂		
8	*Glugea* sp. infected fish tissue		
9	-VE control (terrestrial insect)		
10	Microsporidian infected sea louse ♀		
11	Microsporidian infected sea louse ♀	Internal Primer set	
12	Microsporidian infected sea louse ♀		
13	Microsporidian infected sea louse ♂		
14	DNA marker ΦX174 DNA-*Hae* III digest		

6.4.2 530f-580r DNA sequence

The microsporidian rDNA from the four infected sea lice were successfully sequenced with 100% homology over a region of 1411 bases from within the 530f-580r region of the gene (appendix 3). Due to direct sequencing of the PCR products, the exact length of the entire 530f-580r region is unknown, but is estimated to be between 1480-1500 bp in length after comparisons were made with suitable available sequences in gene databases.

Table 6.6 shows the percentage identities and the gene region lengths of the 530f-580r gene from the *L. salmonis*-infecting microsporidian sequence with the equivalent 530f-580r gene region sequences of eight selected microsporidians from seven different genera. *Nucleospora* sp. infecting English sole (*Pleuronectes vetulus* Giraid) and *Nucleospora salmonis* infecting salmonids, demonstrate the highest percentage identities of 86.9% and 83.9% respectively, and the most similar gene length of 1487 and 1496 bases respectively. The human pathogen *Enterocytozoon bieneusi* (Desportes) demonstrates 76.6% sequence identity with the sea louse-infecting microsporidian, but has a much longer gene region length of 1631 bases due to an insertion in the ITS region. *Pleistophora typicalis* (Gurley), *Glugea anomala* (Moniez), *Loma salmonae* (Putz, Hoffman & Dunbar), *Nosema apis* (Zander) and the *Microsporidium* sp. (Bell) infecting fish and invertebrates all have shorter 530f-580r gene regions and lower percentage identities of 62.3%, 62.1%, 61.7%, 60.3% and 58.8% respectively.

The sea lice-infecting microsporidian shares the highest percentage identities over the 530f-580r region of the gene with the two *Nucleospora* spp. infecting fish and the human pathogen *Enterocytozoon bieneusi*. These three microsporidia all belong to the family Enterocytozoonidae. Table 6.7 shows the inter-relationship between these

three, most closely related species and the sea louse-infecting microsporidian. The most two closely related species are the *Nucleospora* sp. infecting sole and the microsporidian-infecting sea lice, with a percentage identity of 86.9% over the gene region. The most distantly related two species are *Nucleospora salmonis* and *Enterocytozoon bieneusi*, with a percentage identity of 75.7% over the gene region.

Table 6.6 Percentage identities of selected microsporidia with the microsporidian from sea lice over the 530f-580r region of the rRNA gene. Table also shows host type and length of gene region.

Microsporidian	Host	Estimated length of 530f-580r region	% identity across number of bases
Present study	Crustacea sea louse	1480-1500	100% 1411
Nucleospora sp.	Fish English sole	1487	86.9% 1411
Nucleospora salmonis	Fish chinook salmon	1496	83.9% 1406
Enterocytozoon bieneusi	Mammal rhesus macaque	1631	76.6% 1397
Pleistophora typicalis	Fish shorthorn sculpin	1437	62.3% 1318
Glugea anomala	Fish stickleback	1434	62.1% 1320
Loma salmonae	Fish chinook salmon	1396	61.7% 1314
Nosema apis	Insect honeybee	1392	60.3% 1263
Microsporidium sp.	Crustacea metapenaeid shrimp	1381	58.8% 1279

Table 6.7 Percentage identities between equivalent microsporidian rDNA sequences from the 530f-580r region of the rDNA gene. Below the diagonal is the number of bases compared and above the diagonal is the percentage identity.

	Sea Lice Microsporidian	*Nucleaspora salmonis*	*Nucleospora* sp. (sole)	*Enterocytozoon bieneusi*
Sea Lice Microsporidian		83.9	86.9	76.6
Nucleaspora salmonis	1406		84.2	75.7
Nucleospora sp. (sole)	1411	1462		76.8
Enterocytozoon bieneusi	1397	1419	1459	

6.4.3 Phylogenetic analyses

Twenty one 530f-580r microsporidian sequences from seven different genera and thirty eight SSU sequences from twenty two different genera, were each aligned and used in phylogenetic analyses using the Maximum-Likelihood and Neighbour-Joining methodologies, cladistic analyses of which are shown in figures 6.4 and 6.5. Analyses based on both the 530f-580r and SSU gene sequences placed the *L. salmonis*-infecting microsporidian with the clade containing members of the family Enterocytozoonidae. This grouping is independent of the tree building method used and is supported by high bootstrap scores in both cases and full congruence between cladistic analyses. Within the family Enterocytozoonidae, the sea louse-infecting microsporidian groups within the clade containing the genus *Nucleospora* and is most closely related to the *Nucleospora* sp. infecting the English sole, again supported by high bootstrap scores irrespective of method used. This clade forms a sister group to that containing the genus *Enterocytozoon*.

6.4.4 Accession numbers

The sequence determined in the current study was assigned the accession number: AJ431366. For GenBank accession numbers of additional sequences utilised in the analyses see appendix 3.

6.5 Discussion

The microsporidian from *L. salmonis* groups within the molecular phylogenetic clade containing members from the family Enterocytozoonidae, specifically the genera *Enterocytozoon* and *Nucleospora*. The grouping of this sea louse-infecting microsporidian within this clade is supported by 100% bootstrap values. The

Nucleospora spp. are the only known fish-infecting microsporidians that group away from the monophyletic clade containing all other fish-infecting microsporidian parasites (Nilsen, 2000; Bell *et al.*, 2001).

The family Enterocytozoonidae (Cali & Owen, 1990) was created after the description of developing stages of the previously reported pathogen infecting immunocompromised humans, *Entercytozoon bienuesi* (Desportes *et al.*, 1985). There are currently two genera within this family, *Nucleospora* and *Enterocytozoon*; however, the actual number of species assigned to each genus remains unclear. The intranuclear microsporidian, *Nucleospora salmonis*, was originally described from chinook salmon (*Oncorhynchus tshawytscha,* Walbaum), (Hedrick *et al.*, 1991), but was shortly thereafter renamed as *Enterocytozoon salmonis* (Chilmonczyk *et al.*, 1991). Subsequently, two other intranuclear microsporidian parasites of fish, *Enterocytozoon* sp. from Atlantic halibut (*Hippoglossus hippoglossus* L.), (Nilsen *et al.*, 1995) and *Enterocytozoon* sp. from lumpfish (*Cyclopterus lumpus* L.), (Mullins *et al.*, 1994) were also assigned to the genus *Enterocytozoon*. However, more recently, Docker *et al* suggested that, in the absence of significant reasons for the suppression of the generic name *Nucleospora*, the original name, *Nucleospora salmonis* is valid, as supported by the International Code of Zoological Nomenclature (Docker *et al.*, 1997). Furthermore, Docker *et al*. suggested that the intranuclear microsporidians Enterocytozoon sp. from Atlantic halibut and Enterocytozoon sp. from lumpfish also be reassigned to the genus Nucleospora, leaving only one non-intranuclear member in the genus Entercytozoon, the type species Enterocytozoon bieneusi. It is worth noting that fourteen genotypes of E. bieneusi have recently been reported, infecting other mammals in addition to humans (Dengjel et al., 2001).

Nucleospora salmonis has been detected in at least five species of salmonids from three different continents. SSU rDNA studies of isolates representing these distant geographical locations and different salmonid hosts suggests that they represent a single species (Gresoviac *et al.*, 2000). However, the SSU rDNA of the *Nucleospora salmonis*-like specimen from the non-salmonid host, English sole, was sufficiently different, with a variation of 12.72% when compared to that of the genotype from chinook salmon, that it was assumed by Gresoviac *et al* (2000) to be a separate species.

More recently, a second species of *Nucleospora, Nucleospora secunda,* has been described from the intestinal enterocytes of the fresh water aquarium fish *Nothobranchius rubripinnis* (Seegers, 1986) (Lom & Dykova 2002). Lom and Dykova (2002) describe the ultrastructure of this intranuclear microsporidian of the enterocytes but unfortunately were unable to sample DNA for phylogenetic studies.

One other intranuclear microsporidian species has been described from rodlet cells of salmonid fish, *Microsporidium rhabdophilia* (Modin, 1981). As *Nucleospora salmonis* has since been observed in rodlet cell nuclei (Chilmonczyk *et al.*, 1991), Lom and Dykova (1992) suggested that they could be the same species. Modin, however, performed thorough histological examinations and reported that the parasite showed a great degree of host cell specificity being present in the nuclei of rodlet cells only. No signs of disease were ever observed with *M. rhabdophilia* infections, unlike *N. salmonis* infections which are associated with acute anaemia (Elston *et al.*, 1987), lymphoblastosis (Morrison *et al.*, 1990) and leukaemia (Hedrick *et al.*, 1990). Nevertheless, due to the lack of DNA and ultrastructural studies on *M. rhabdophilia* further classification is not possible.

The Enterocytozoonidae represents an intriguing group of microsporidian parasites, consisting of only two described genera and five species. These parasites are known to infect humans, other mammals, fish and a crustacean, demonstrating the diverse host range and possible opportunistic nature of these organisms. To date *Nucleospora salmonis* and *Nucleospora secunda* remain the only true species attributed to the genus *Nucleospora*, as precise cellular location of the species infecting English sole has not yet been determined (Khattra *et al.*, 2000). There are, presently, limited rRNA gene data available for members of established genera that are closely related to *Nucleospora* within the Enterocytozoonidae. Consequently, it remains unclear as to the percentage variation that might be expected within a species or between species of this genus. Thus, it seems entirely possible that the 2.14% variation described by Gresoviac *et al* (2000) between putative *N. salmonis* isolates may actually represent a number of closely related *Nucleospora* species, especially given the environmental and geographical isolation of several of the fish hosts. These uncertainties emphasise the need, highlighted by Cheney *et al* 2001, to examine additional, more rapidly evolving, protein coding genes when investigating intra-generic and sibling species relationships within the Microspora (Cheney *et al.*, 2001), as well as the problems associated with assigning species status to parasites in general (Kunz, 2002).

The fact that, according to phylogenetic analyses, the most closely related species to the microsporidian-infecting sea lice are members from the genus *Nucleospora*, which exclusively infect fish, and predominately infect salmonids, is worthy of further investigation.

Nucleospora salmonis is found within the nuclei of lymphoblasts, predominately in the haemapoetic tissues but also in circulating blood (Hedrick *et al.*, 1990). As *L.*

salmonis take blood meals from their salmonid hosts (Brandal *et al.*, 1976b), there is a potential route for the microsporidian to be transmitted to the sea lice.

The natural host reservoirs and modes of transmission for *Enterocytozoon bieneusi*, the most frequent microsporidial species identified in humans, are still unknown. Several genotypes are known, and have been found in the faeces of animals, including pigs, cats and cattle (Dengjel *et al.*, 2001). In a recent study half of the water samples screened contained microsporidia pathogenic to humans, including *E. bieneusi*, suggesting that they may be waterborne pathogens (Dowd *et al.*, 1998). To date serious illness due to infection with *E. bieneusi* in humans has predominately been reported to occur in immunosuppressed individuals (Weber & Bryan, 1994). This susceptibility whilst immunosuppressed, might suggest that humans are not the primary hosts for *E. bieneusi*, but humans become infected by these opportunistic pathogens due to a lowering of their immune status.

Although invertebrates possess an immune system which is effective in combating some infections, it is often regarded as primitive when compared to the immune systems of vertebrates (Turner, 1994). They have limited humoral components to their immune system in comparison and lack the 'anticipatory' immune responses exhibited by vertebrates (Klein, 1989).

Although very little is known about microsporidia infecting marine copepods, these parasitic copepods may resemble an immunocompromised situation with respect to a fishes more advanced immune system, and transmission of the microsporidia to a new host could potentially occur.

Due to the molecular phylogenetic positioning of this microsporidian hyperparasite, combined with the histological and ultrastructural data, it should be placed in the family Enterocytozoonidae. However, at this time it is not possible to assign it to

either of the two existing genera within this family. It groups most closely with the intranuclear microsporidians (*Nucleospora* spp) infecting fish, and more distantly to the human parasite, *E. bieneusi*, isolated from the intestinal epithelium. However, histological studies in chapter 5 reveal that the microsporidian in sea lice is neither an intranuclear parasite nor a parasite of the intestinal enterocytes.

The intermediate and internal primer sets should now be utilised to investigate the prevalence of this parasite in the salmon louse. Previous studies (chapter 4) suggest that up to 10% of certain salmon louse populations may be visibly grossly infected. Sensitive PCR screening may reveal a much higher percentage prevalence for this disease, particularly in juvenile stages and young adults.

This parasite could potentially have either a single host life cycle, utilising only the salmon louse as a host, or, it may be utilising the copepod as an intermediate / alternate host in a two-host life cycle including the fish, as is frequently observed with freshwater copepod-infecting microsporidia, or, lastly may have been transmitted to the sea louse purely by opportunistic chance. The aforementioned PCR's should also be used to assist in the transmission studies of this parasite and to evaluate the role that Atlantic salmon play in this parasites' life cycle, if any.

CHAPTER 7

MICROSPORIDIAN TRANSMISSION AND POTENTIAL ALTERNATIVE HOST

7.1 INTRODUCTION

In order to determine the life cycle and to assess potential pathogenicity of the microsporidian found in the salmon louse, *L. salmonis*, experimental transmission of the environmental spores liberated from infected sea lice is necessary. This will ultimately demonstrate spore infectivity and viability, and eliminates the potential involvement of an obligate intermediate host. Experimental extrusion of the polar filament apparatus is frequently used to assess spore viability (Keohane & Weiss, 1999), but the most reliable way to demonstrate spore viability is a bioassay, reinfecting naïve individuals with the spores from previously infected members of the same species (Undeen, 1997; T. Andreadis, pers. comm.). The most common route of transmission in the microsporidia is the oral ingestion of spores. Infection is via the gut epithelium, but is not limited to this site and can spread to various target organs and tissues throughout the host's body (Cali & Takvorian, 1999). The mechanisms by which this spread of infection occurs are not fully understood, but it is generally accepted that the initial infection from the gut epithelium is spread throughout the body via migratory mesenchyme cells such as macrophages and histocytes (Weissenberg, 1968). However, recent studies have confirmed the occurrence of an early sporulation sequence in several species of microsporidia. These studies suggest that a 'primary spore' forms within a day or two of the initial infection in the gut

epithelium. This primary spore is proposed as a mechanism to spread the infection within the host organism to various target tissues, and is suggested as an explanation as to why infection is not always seen in the gut epithelium, even if the gut epithelium is known to be the initial point of entry into the host species (Solter & Maddox, 1998; Vossbrinck, 2002).

Experimental horizontal transmissions of many microsporidian species have been successfully achieved for both terrestrial and aquatic insects, fish and mammals. Various techniques such as co-habitation, oral ingestion of spores, and the direct injection of spores have been utilised (Didier & Bessinger, 1999; Becnel & Andreadis, 1999; Shaw & Kent, 1999).

The developmental and ultrastructural features combined with the gene sequence reported earlier in this study, suggest that the microsporidian from sea lice is more closely related to known species of microsporidians infecting fish, especially salmonids, than any other known microsporidians. These facts warrant a careful investigation to the fish's potential role as an intermediate / alternate host in the life cycle of this parasite.

7.2 OBJECTIVES

The aim of the current study is to attempt the experimental transmission of the microsporidian infection, from infected *L. salmonis* to uninfected *L. salmonis* using mature spores from infected individuals. The study will evaluate the percentage of lice successfully infected and will evaluate the number of mortalities caused by infection. The study will also analyse for the same microsporidian infection occurring in experimental and naturally infected farmed Atlantic salmon.

7.3 METHODS

7.3.1 Extraction of spores from infected salmon lice

Whole infected sea lice with advanced microsporidian infections were crushed manually in a ceramic pestle and mortar, using physiological saline (0.9%) at 10ºC as a diluent. The resulting homogenate was passed through a 5 ml syringe body, plugged at the outlet end with absorbent cotton wool to remove large particles and debris. The syringe body was rinsed through with 10 x volume of saline, using the plunger to increase the flow rate through the cotton wool plug when necessary. The resulting filtrate was centrifuged at 1000g for 5 min, the pellet resuspended and washed in saline a further three times, before the final undisturbed pellet was stored at 4ºC under 200µl of penicillin (250 units/ml) and 200µl of streptomycin (250µg/ml).

7.3.2 Counting microsporidian spores

Prior to infection, the pelleted spores previously stored at 4ºC were resuspended in saline and washed twice before final resuspension in 10ml of saline. The spore suspension was counted using a haemocytometer with a chamber depth of 0.1mm, and a large central square volume of 0.1µl, using a compound microscope set at phase contrast light setting. Only highly refractile spores were counted (developing spore stages and germinated or dead spores are not as refractile) to maximise the number of potentially infective spores. Counts were conducted in triplicate, and averaged for final spore numbers. Whenever possible fresh preparations of spores from infected lice were used in the infections, spores stored for more than one month were not used in experimental infections.

7.3.3 Experimental infection of Atlantic salmon and salmon lice with microsporidian spores

Thirty-four Atlantic salmon of approximately 300g in weight were previously infected with *L. salmonis* as described in chapter 2.4. The fish were maintained in 4 one meter diameter round tanks (0.8 m depth) with a constant flow through of ambient (7-10 $^{\circ}$C) temperature seawater (approx. 15 L min^{-1}.). Tanks 1.1 and 1.2 contained 9 and 8 fish respectively and were designated test tanks; tanks 1.3 and 1.4 contained 8 and 9 fish respectively and were designated control tanks. Each tank contained one tagged fish for repeat sea louse sampling, and all four tanks also contained two shore crabs, *Carcinus maenas* L., (approximately 50mm in carapace width).

7.3.3.1 Infection with microsporidian spores

Test tanks were infected with 1.25 x10^{9} microsporidian spores per tank (approximately 140 million spores per fish). During infection the water supplies were switched off for the first two hours to minimise the dilution effect. Additional aeration was supplied to the water during this time using an oxygen cylinder and diffuser stone, after two hours, the water supply was returned to the normal flow rate and the additional air supply removed. Water supplies were also switch off in the control tanks for the same period of time. Infections with microsporidian spores were carried out three times: firstly, on day 0 when the sea lice were at the chalimus II stage; secondly at day 15 when the sea lice had matured to chalimus III / IV and thirdly at day 36, when all sea lice were either late pre-adults or adult lice.

7.3.4 Sampling of experimental salmon and salmon lice

Three random fish from each tank were periodically anaesthetised (day 0, 15, 21, 36, 55, 84, 133) for total sea louse counts to be made. The tagged fish from each tank were also anaesthetised to permit the removal of sea lice for the later detection of the

microsporidian infection. On day 133 all lice were removed from all fish and three fish from each tank were sacrificed for the *post mortem* detection of the microsporidian infection. The remaining fish were returned to their relevant tanks without sea lice for long-term observation, awaiting final blood and tissue samples to be taken. The crabs were also sacrificed on day 133 for the detection of the microsporidia.

All fish were anaesthetised in seawater containing 0.01% MS222 (3-aminobenzoic acid ethyl ester) (Sigma A-5040).

7.3.4.1 Detection of microsporidia in salmon louse samples

During each sampling of the tagged fish, six sea lice were removed for analysis. All six lice were examined microscopically for gross signs of the microsporidian infection. Two lice were subsequently fixed in 10% formalin for wax histology, two lice were fixed in 2.5% gluteraldehyde for TEM and two lice were homogenised in high concentration urea buffer for DNA extraction and subsequent diagnostic PCR.

7.3.4.1.1 Wax histology

Sea lice samples were prepared for wax histology, cut and stained with haematoxylin and eosin, and Giemsa, and viewed under the light microscope for microsporidian infection as detailed in chapter 2.2.

7.3.4.1.2 TEM

Sea lice samples were dissected and prepared for TEM as described in chapter 2.2.2

7.3.4.1.3 PCR detection of the microsporidian infection in experimental sea lice

DNA extractions were carried out as detailed in chapter 2.3, diagnostic PCR amplifications were performed according to chapter 2.3.1, using the specific

intermediate and internal primer sets described in chapter 6.3.2. Nested PCRs were performed using 1µl of PCR product from the intermediate PCRs as the template DNA for the internal primer set PCRs, to increase detection sensitivity. Positive bands of the correct size were directly sequences as detailed in chapter 6.3.3.

7.3.4.2 Detection of microsporidia in experimental fish samples

On completion of the microsporidian infection experiment (day 133), the three sacrificed fish from each tank were dissected *post mortem*, and samples taken from the kidney, heart, liver, spleen, intestine, gill, muscle, eye and brain. Each fish tissue was treated as previously described for the sea lice samples (section 7.3.4.1), for subsequent wax histology, TEM and diagnostic PCR, to detect the microsporidian.

7.3.4.3 Detection of microsporidia in experimental shore crabs

On completion of the microsporidian infection experiment (day 133), the two shore crabs from each tank were removed and bled from the arthroidial membrane of an appendage joint, using a fine needle and syringe. The crabs were subsequently frozen, and later dissected to remove gill, muscle, cuticular epithelium and digestive gland. The haemolymph removed (50-200µl) and the tissue samples were used in a diagnostic PCR as detailed in section 7.3.4.1.3. to test for the presence of the microsporidia.

7.3.5 Sampling of farmed salmon for microsporidian infection

During routine salmon louse collections from salmon farm sites known to harbour the microsporidian infection in sea lice, harvest size fish (3-6 kg) were chosen to be sampled for the presence of the microsporidian infection. Fish were sampled that were observed as being parasitised by more than two adult female sea lice heavily infected with the microsporidian. Tissue samples were taken from the kidney, heart, liver and

gills. Each fish tissue was treated as previously described for the experimental fish samples (section 7.3.4.2), for subsequent wax histology, TEM and diagnostic PCR to detect the microsporidian. In addition 5ml of blood was taken in a heparinised syringe from the caudal artery shortly after death.

7.3.5.1 Blood sample preparation

The 5ml of heparinised blood was mixed with 15ml of saline, floated over 10ml of histopaque (Sigma 1077-1) and centrifuged at 400g for 30 minutes. The mononuclear cell layer was retrieved with a pipette and washed several times in sterile saline. The resulting pellet was divided for diagnostic PCR and TEM as detailed in section 7.3.4.1.

7.3.6 Direct injection of the midgut in experimental *L. salmonis* with microsporidian spores

Adult female sea lice collected from a farm site with no previous record of the microsporidian infection, were microscopically checked for gross signs of infection and damage caused by the mechanical removal process. Sea lice deemed healthy were starved for 48 hours and injected via the anus and hindgut into the midgut (anal gavage), with either a 10µl saline (0.9%) solution containing 1×10^5 microsporidian spores or a control injection of 10µl of saline solution. Injections were performed using a Narishige IM-6 microinjector, with a Narishige GD-1 (1mm bore) glass needles, previously stretched to a fine point ($< 50\mu m$) using a heated needle stretching apparatus (Research Instruments Ltd.). Lice were immobilised in a petri dish containing blind-ended plastic mesh channels secured with Blutak®. The petri dish was cooled from beneath with ice to reduce louse movement and prevent warming of the seawater during injection, which was facilitated with a dissecting microscope. Post-injection, lice were allowed to recover for 48 hrs; healthy-looking survivors were

returned to Atlantic salmon of about 200g in weight and maintained in 1m re-circulating seawater systems at 10 $^{\circ}$C, 34 ppt. Twenty lice from each group were used, 5 lice being placed of each experimental fish. Lice were examined weekly for up to 6 weeks for visible signs of microsporidian infection, and prepared for TEM examination as previously described in section 2.2.2.

7.4 RESULTS

7.4.1 Experimental transmission of the microsporidian to uninfected sea lice

Microsporidian spores were successfully extracted from infected sea lice taken from farmed Atlantic salmon, counted and used in experimental infections with Atlantic salmon that had been previously infected with naïve sea lice. Each experimental fish had an average of 35 settled copepodites per fish when counted two days post louse infection. Sea lice numbers had increased by day 15, as fist motile stages had moved from the gills and opercular cavity; lice numbers were reduced to 30 per fish on day 21 to avoid unnecessary stress to the fish. However, the fish began to show signs of early lice damage and numbers were further reduced to 22 lice per fish on day 36. Table 7.1 shows the number of lice present per fish over the 133-day experiment, the figures represents the average number of lice from three randomly chosen fish per tank at each sample day.

The number of sea lice per fish did not differ markedly between the test and control tanks throughout the experiment. Lice numbers decreased proportionately in test and control tanks until only a few male lice remained at day 133.

After initial observation and microscopic analysis of the salmon lice removed from the tagged fish throughout the 133 days of the experimental transmission trial, none showed gross visible signs of the microsporidian infection. Furthermore, stained wax

histological sections and TEM studies performed on these sea lice could not detect the

presence of the microsporidian, in either the test or control groups.

Table 7.1 Average numbers of lice per fish during the 133 days of the experimental transmission of the microsporidian-infecting the salmon louse. Lice numbers were reduced on day 21 and again on day 36. �֍ represents the challenge dates with microsporidian spores.

		Mean number of lice per fish. Test Tanks		Mean number of lice per fish. Control Tanks	
Day	Louse Stage	Tank 1.1	Tank 1.2	Tank 1.3	Tank 1.4
0 �֍	Chalimus 2	35	35	35	35
15 ✷	Chalimus 3/4 & Pre-adult 1	79	103	110	88
21	Chalimus 4 & Pre-adult 1	68 Reduced to 30	84 Reduced to 30	92 Reduced to 30	71 Reduced to 30
36 ✷	Pre-adult 2 & Adult	34 Reduced to 22	29 Reduced to 22	31 Reduced to 22	36 Reduced to 22
55	Adult ♀'s all gravid	16 ♀11 ♂5	22 ♀8 ♂14	10 ♀5 ♂5	13 ♀6 ♂7
84	Adult ♀'s all gravid	13 ♀7 ♂6	19 ♀8 ♂11	14 ♀7 ♂7	9 ♀5 ♂4
133	Adult No ♀'s present	3 ♀0 ♂3	3 ♀0 ♂3	3 ♀0 ♂3	2 ♀0 ♂2

Diagnostic nested PCRs using the specific primers designed in chapter 6 did detect the target microsporidian DNA, albeit, in nested samples only. Figure 7.1 shows these positive bands, however, the negative control in lane 18, also amplified a positive band in the nested PCR reaction.

Figure 7.1 Agarose gel of PCR products from diagnostic PCRs using the intermediate primer set shown in the upper 20 lanes and the internal primer set, in a nested PCR shown in the lower 20 lanes. Template DNA was from extracted sea lice from days 84 and 133 from the microsporidian transmission experiment. Table 7.2 shows which samples the lane numbers below represent.
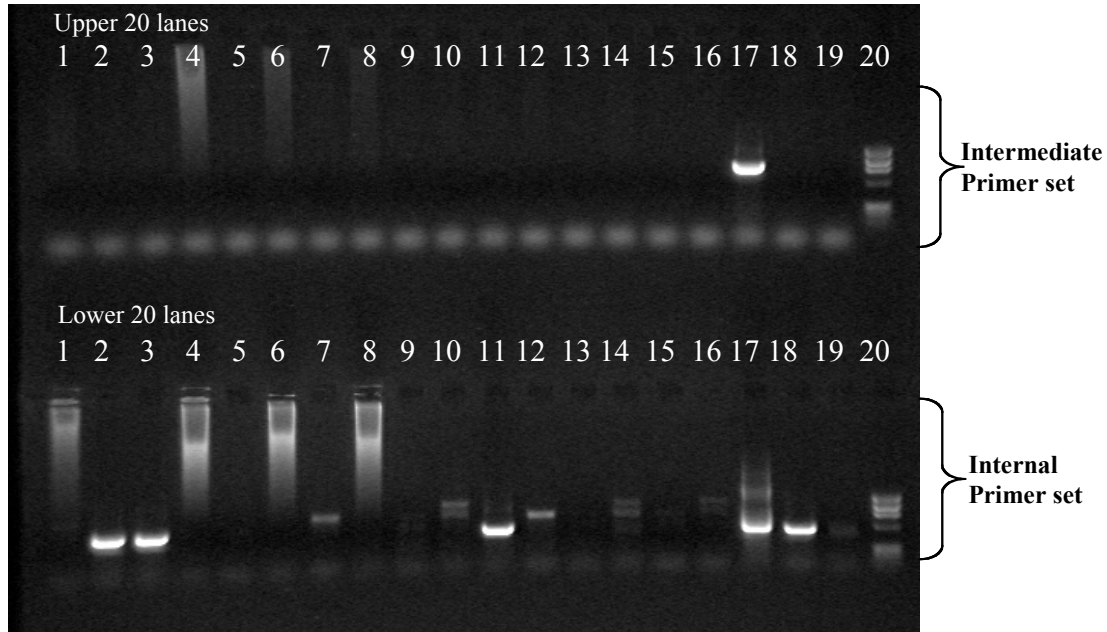
The PCR products obtained using the intermediate primer set in the upper 20 lanes show no positive bands except for the positive control in lane 17. In the nested PCR reaction, in the lower 20 lanes, lanes 2, 3 and 11 are all positive. Lanes 2 and 3 are both DNA samples from male sea lice taken form test tanks 1.1 and 1.2 respectively on day 84 of the trial and lane 11 is a DNA sample from a female louse sampled from test tank 1.1 on day 133 of the trial. These three samples produce a band of the correct size with the internal primer set, that corresponds with the positive control in lane 17, however the negative control in lane 18 also shows a band of the same size.

Direct sequencing of these positive PCR products from the nested reaction revealed an amplicon of approximately 400 bases in length, that has 100% homology to the corresponding part of the rRNA gene from the microsporidian gene sequence described in chapter 6 infecting the salmon louse.

Table 7.2 Table describing lane positions for DNA samples for Figure 7.1

Lane number	Sample
1	Day 84 Tank 1.1 (test) ♀ louse
2	Day 84 Tank 1.1 (test) ♂ louse
3	Day 84 Tank 1.2 (test) ♂ louse
4	Day 84 Tank 1.2 (test) ♀ louse
5	Day 84 Tank 1.3 (control) ♂ louse
6	Day 84 Tank 1.3 (control) ♀ louse
7	Day 84 Tank 1.4 (control) ♂ louse
8	Day 84 Tank 1.4 (control) ♀ louse
9	Day 133 Tank 1.1 (test) ♂ louse
10	Day 133 Tank 1.1 (test) ♀ louse
11	Day 133 Tank 1.2 (test) ♀ louse
12	Day 133 Tank 1.2 (test) ♂ louse
13	Day 133 Tank 1.3 (control) ♀ louse
14	Day 133 Tank 1.3 (control) ♂ louse
15	Day 133 Tank 1.4 (control) ♀ louse
16	Day 133 Tank 1.4 (control) ♂ louse
17	+VE control
18	-VE control
19	Water blank
20	DNA marker ΦX174 DNA-Hae III digest

7.4.2 Transmission of the microsporidian to experimental Atlantic salmon

Tissue samples taken from three fish from each experimental tank upon termination of the experiment, all tested negative for the presence of the microsporidian. PCRs were performed on all tissue DNA samples (figure 7.2, lanes 10-13) and the subsequent nested PCRs performed on the products of the primary PCR also remained negative. Due to the absence of the microsporidian DNA in these tissue samples the histological and TEM studies were not performed.

7.4.3 Transmission of the microsporidian to experimental shore crabs

DNA samples extracted from the experimental crab tissues and haemolymph all tested negative for the microsporidian infection in both standard and nested PCR's.

7.4.4 Long-term observation of the remaining experimental fish

The long-term observation of the remaining experimental fish continued for 9 months after the experiment had terminated. None of the fish showed any symptoms of disease. Final blood and tissue samples were extracted and screened using the diagnostic and nested PCRs, but tested negative for all fish.

7.4.5 Screening of farmed salmon for microsporidian infection

7.4.5.1 Diagnostic PCR of kidney and other organs

Initially one fish (designated fish 1) was investigated for the presence of the microsporidian found infecting the salmon louse. A maturing male Atlantic salmon (grilse) was observed during a routine fish harvest at a farm site being parasitised by four visibly microsporidian-infected adult female sea lice. A diagnostic PCR (intermediate primer set) of the internal fish organs' DNA yielded PCR products of the same size (approximately 900 bp's) as the microsporidian from sea lice, which was used as the positive control. Figure 7.2 is an agarose gel of the PCR products, showing a band of the same size as the positive control (lane 2) in lanes 5, 6, 8, and 9, which correspond to kidney, heart, gill and liver tissues from fish 1. The negative control in lane 3 shows no product band, and no amplification took place in the fish tissues in either lanes 4 or 7, which correspond to fish gonadal tissue and spleen tissue. The gel also shows experimental fish kidney (section 7.4.2) in lanes 10 to 13, which were all negative. Table 7.3 denotes the lane positions for the DNA samples used in the gel.

Figure 7.2 Agarose gel from a diagnostic PCR using the intermediate primer set of tissues from fish 1, arrow indicates the 872 base pair marker.
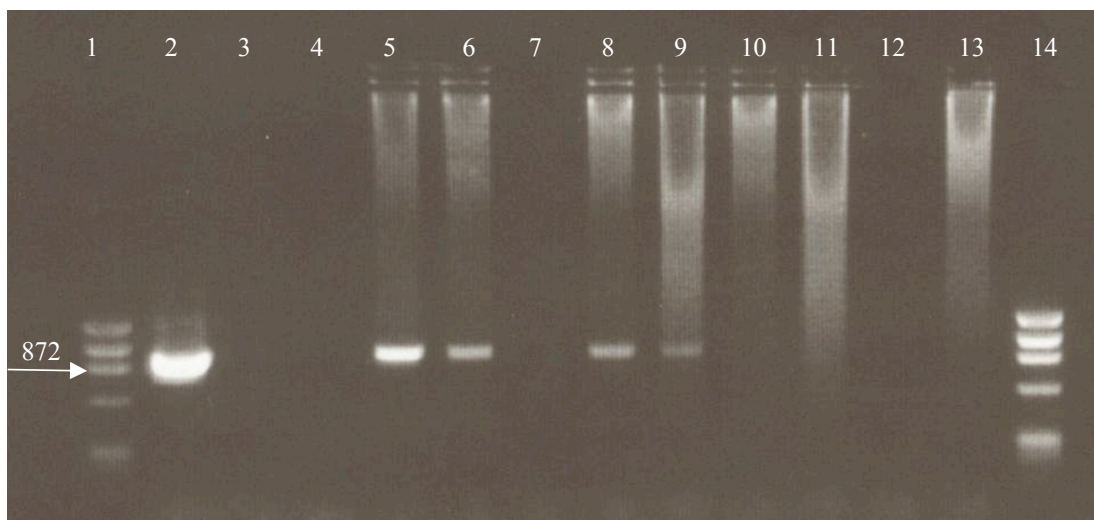
Table 7.3 Table describing the lane positions for DNA samples in Figure 7.2

Lane number	Sample
1	DNA marker ΦX174 DNA-*Hae* III digest
2	+VE control (microsporidian-infected louse)
3	-VE control (terrestrial insect)
4	Fish 1 gonadal tissue
5	Fish 1 kidney tissue
6	Fish 1 heart tissue
7	Fish 1 spleen tissue
8	Fish 1 gill tissue
9	Fish 1 liver tissue
10	Experimental fish, tank 1.1 (test) kidney tissue
11	Experimental fish, tank 1.2 (test) kidney tissue
12	Experimental fish, tank 1.3 (cont) kidney tissue
13	Experimental fish, tank 1.4 (cont) kidney tissue
14	DNA marker ΦX174 DNA-*Hae* III digest

On a subsequent salmon harvest, five further fish (designated fish 2 to fish 6) were sampled that were observed harbouring at least two microsporidian-infected sea lice. Fish 3 was a grilse, the other fish not yet showing secondary sexual characteristics. A

diagnostic PCR (intermediate primer set) using the DNA from the kidney tissues showed a strong positive band in fish 3 and a weakly positive band in fish 5 (figure 7.3). A subsequent nested PCR (internal primer set) showed strongly positive bands in fish 2, 3, 5 and 6 (figure 7.4). Figure 7.4 also shows the nested PCR products from kidney and heart tissues from fish 1. Tables 7.4 and 7.5 denote the lane positions for the DNA samples used in the gels.

Figure 7.3 Agarose gel of PCR products from kidney DNA from fish 2 to 6.
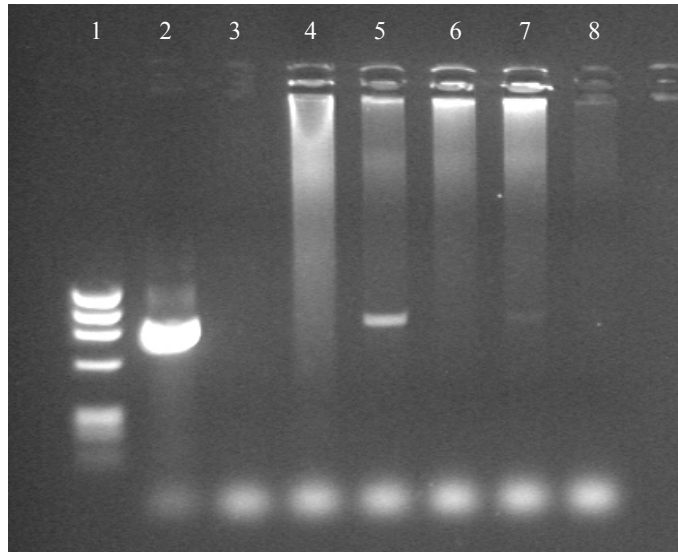
Table 7.4 Table describing the lane positions for DNA samples in Figures 7.3

Lane number	Sample
1	DNA marker ΦX174 DNA-*Hae* III digest
2	+VE control (microsporidian-infected louse)
3	-VE control (terrestrial insect)
4	Fish 2 kidney tissue
5	Fish 3 kidney tissue
6	Fish 4 kidney tissue
7	Fish 5 kidney tissue
8	Fish 6 kidney tissue

Figure 7.4 Agarose gels of nested PCR's. Lanes 4 and 5 tissues from fish 1, lanes 7 to 11 kidney tissues from fish 2 to 6.
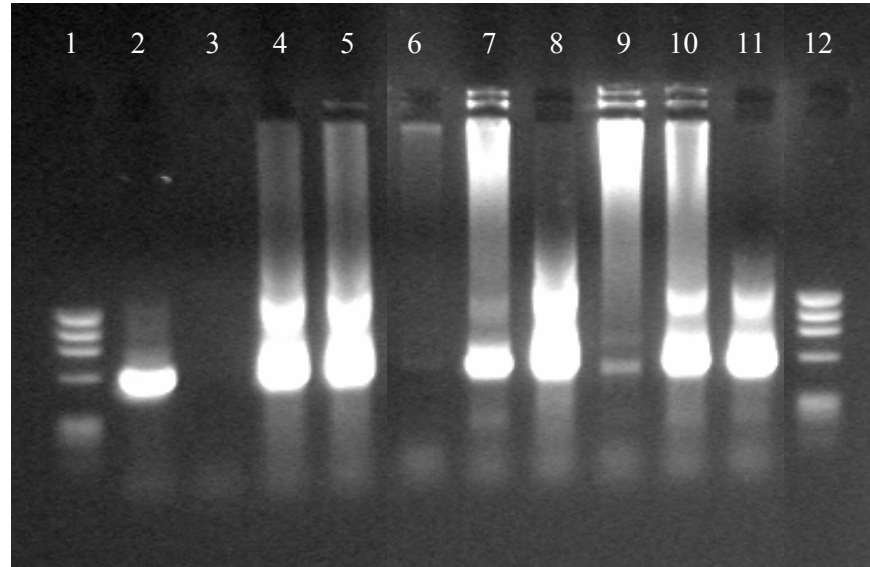
Table 7.5 Table describing the lane positions for DNA samples in Figures 7.4

Lane number	Sample
1	DNA marker ΦX174 DNA-*Hae* III digest
2	+VE control (microsporidian-infected louse)
3	-VE control (terrestrial insect)
4	Fish 1 kidney tissue
5	Fish 1 heart tissue
6	-VE control non-infected fish muscle
7	Fish 2 kidney tissue
8	Fish 3 kidney tissue
9	Fish 4 kidney tissue
10	Fish 5 kidney tissue
11	Fish 6 kidney tissue
12	DNA marker ΦX174 DNA-*Hae* III digest

Primary PCR products (non-nested samples only) from the kidney and heart tissues of fish 1 and kidney tissues from fish 3 and 5 were DNA sequenced directly. A consensus sequence of 860 base pairs was constructed for the four samples, commencing from base 260 and continuing to base 1120 on the sea louse-infecting

microsporidian sequence (chapter 6). This consensus sequence shared 99.4% similarity over the 860 bases with the consensus sequence described in chapter 6 from the salmon louse-infecting microsporidian, differing in only 5 bases. In the SSU there is an insertion of a thymine (T) base in the sequence isolated from fish after position 706 of the sea louse sequence; a substitution of an adenine (A) base in the lice sequence for a T base at position 738 in the fish isolate and in positions 850, 851 and 852 of the ITS region, the sea louse sequence of TAA is replaced with ATT in the fish isolate (table 7.6).

Table 7.6 Consensus sequences differences between the microsporidian isolates from the salmon louse and the Atlantic salmon. Base numbers represent the position on the sea louse-infecting microsporidian sequence.

Isolate	Bases 704-710 SSU	Bases 734-741 SSU	Bases 847-854 ITS
Sea louse	TAG*AACT	TGCG**A**CCC	ACG**TAA**GG
Atlantic salmon	TAG**T**AACT	TGCG**T**CCC	ACG**ATT**GG

7.4.5.2 Diagnostic PCR comparing kidney tissue and circulating white blood cells.

Diagnostic PCR's (intermediate primer set only) performed on DNA extracted from $1x\ 10^6$ circulating white blood cells and kidney tissue from a further ten farmed fish (designated fish I to fish X) are shown in figures 7.5 and 7.6. Table 7.7 denotes the lanes positions on the gels for each DNA sample.

Table 7.7 Table describing the lane positions for DNA samples in figure 7.5 and 7.6

Lane number	Sample
1	DNA marker ΦX174 DNA-*Hae* III digest
2	+VE control (microsporidian-infected louse)
3	-VE control (terrestrial insect)
4	Fish I
5	Fish II
6	Fish III
7	Fish IV
8	Fish V
9	Fish VI
10	Fish VII
11	Fish VIII
12	Fish IX Grilse
13	Fish X
14	DNA marker ΦX174 DNA-*Hae* III digest

Figure 7.5 Agarose gel of PCR products from kidney (upper 14 lanes) and white blood cells (lower 14 lanes). Arrow indicates the size of the positive control band of approximately 872 bases.
Figure 7.5 shows a strong positive band of the correct size from fish IX (grilse) in
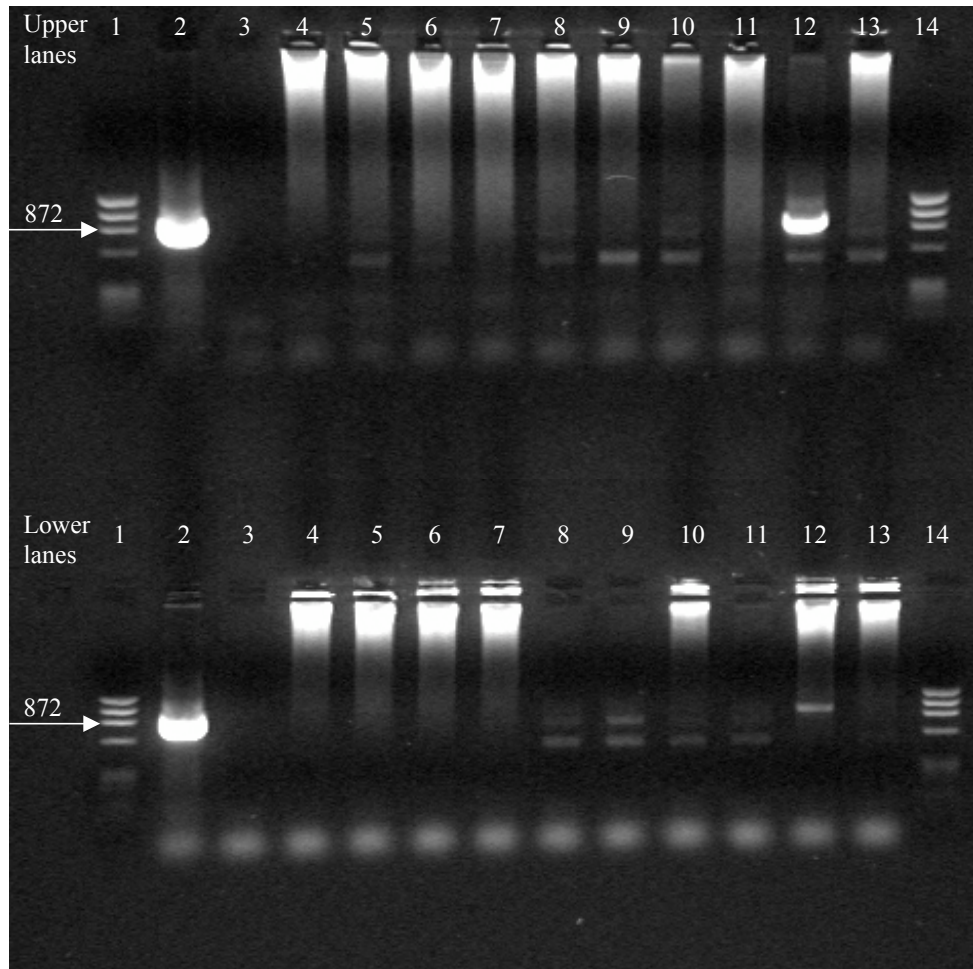
lane 12 (upper lanes) from kidney tissue, and a much weaker band of the correct size (lower lanes) also in lane 12 from circulating white blood cells. No other fish produced a positive band in either the kidney or circulating white blood cell DNA samples.

Figure 7.6 shows an agarose gel of the products from a nested PCR reaction using the products of the primary PCR (from figure 7.5) and the same intermediate primer set. Fish IX again shows a positive band of the correct size in both kidney and white blood cell DNA, but of much greater intensity; all other fish remain negative.

Figure 7.6 Agarose gel of PCR products from kidney (upper 14 lanes) and white blood cells (lower 14 lanes). Arrow indicates the size of the positive control band of approximately 872 bases.
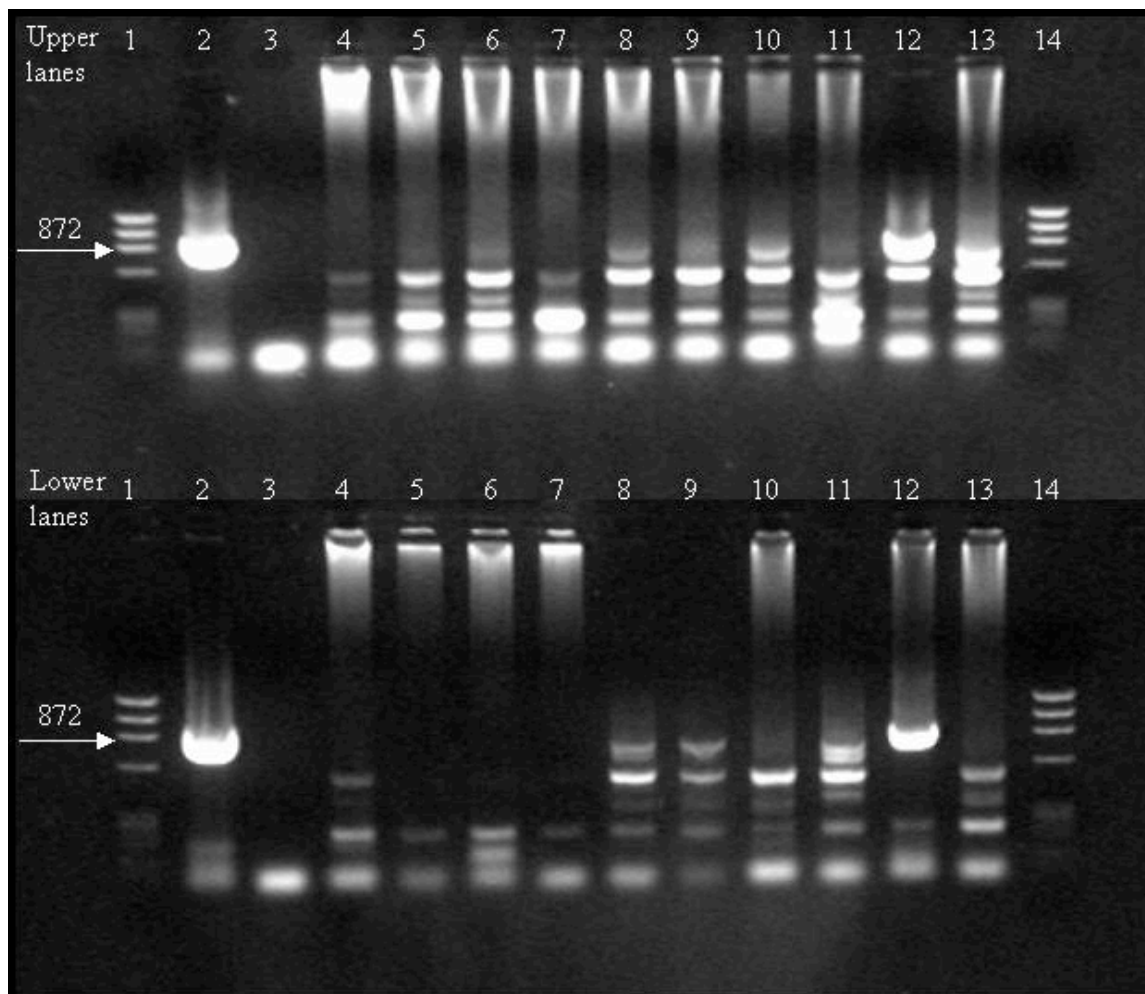
This experiment was repeated on a further eight fish, 5 grilse and 3 fish not displaying secondary sexual characteristics. From the eight fish one grilse produced a positive

band of the correct size in both kidney and circulating white blood cell DNA samples, the former of much greater intensity, as seen in the above example. A nested PCR yielded a marked increase in the intensity of the product band from circulating white blood cell DNA, and all other fish tested remained negative.

7.4.5.3 Tissue histology and TEM of farmed Atlantic salmon

Fish confirmed as positive for the presence of the microsporidian using the diagnostic PCR, appeared externally normal except for mild gill pallor. However, the kidneys of some fish (fish 1, 3, 6 and fish IX) were enlarged and discoloured to a greenish-grey containing numerous white flecks. H & E stained histological sections of the kidney tissue showed moderate hyperplasia in the renal haematopoietic tissue (interstitium), with numerous mitotically activity immature white blood cells. Heart tissue showed endocardial activation and hyperplasia with the occasional hypertrophy of myocardial nuclei. Other tissues appeared normal except for the occasional reticular cell proliferation of ellipsoids in the spleen (H. Ferguson (aquatic veterinary pathologist) pers. comm.).

Giemsa and Gram stains of the tissue sections failed to reveal the presence of any positively staining parasite bodies.

TEM examination of kidney, heart, liver and spleen tissues from fish 1, kidney tissues from fish 3 and 5 and lymphocyte pellets from fish IX, failed to observe microsporidia in any of the tissue examined.

7.4.6 Direct injection of the midgut in experimental *L. salmonis* with microsporidian spores

Injected lice had poor survival rates in both test and control groups. No lice remained in the control group after 4 weeks of the study and only 3 lice survived the full six weeks in the test group. Two randomly chosen lice were sampled every week for the

first 4 weeks for the control group and for the full six weeks for the test group. None of the sampled lice showed any gross signs of the microsporidian infection in any area of the body. Moribund and dead lice removed from the aquaria also showed no signs of the microsporidian infection, for either group. TEM and semi-thin sections of transverse abdominal sections showed no signs of microsporidian infection in either the intestinal enterocytes or the epidermal tissues beneath the cuticle, in either test or control groups for any of the time points sampled.

7.5 DISCUSSION

7.5.1 Transmission of the microsporidian to experimental sea lice, Atlantic salmon and shore crabs

The experimental transmission of the microsporidian parasite using spores taken from previously naturally infected sea lice to naïve sea lice was not achieved. Furthermore, neither the experimental fish nor the decapod crustacean became infected with the microsporidian. The positive results observed from the nested PCR products in figure 7.1 from sea lice sampled on days 84 and 133 from the experimental transmission test tanks (1.1 and 1.2) may therefore represent a falsely positive result or contamination. These positive results cannot be considered reliable as no other signs of infection were observed either in the histological sections or in the TEM from these samples. As nested PCRs are extremely sensitive, and the rRNA gene is a multiple copy gene, it is possible that spores used in the last experimental challenge, day 36, were still present on the external surfaces of the sea lice, causing a positive band in the nested PCR reaction; or that contamination / aerosols with 'perfect' template DNA has occurred from the positive control. The false positives obtained from these samples, including the negative control, highlight the potential dangers when using nested

PCR's as a diagnostic tool, when no corresponding weak band is present in the primary PCR and without supporting histological evidence.

Nevertheless the lack of successful transmission of the microsporidian parasite to uninfected sea lice is puzzling and could be due to a number of reasons. Firstly the microsporidian spores may not have been ingested by the sea lice, as sea lice feed on the surface of the fish and do not filter feed particulates from the water column as free-living copepods do. The oral ingestion of spores (*per os*) is considered to be the main route by which microsporidian infection is transmitted for horizontally transmitted microsporidia in fresh water copepods, mosquito larvae, *Daphnia* and marine decapod crustacea. This is achieved either through the filter feeding of particulates or by the cannibalism of infected / moribund individuals (Voronin, 1996; Andreadis, 1985; Sweeney *et al.*, 1985; Ebert, 1995; Micieli *et al.*, 2000; Ramasamy *et al.*, 2000). Secondly the spores used in the transmission experiment may not have been viable; which is difficult to determine without the aid of a functional bioassay. However, prior to experimental infection, the spores were checked using phase contrast microscopy and had a highly refractile appearance reminiscent of viable spores, according to (Undeen, 1997)Undeen (1997). Spores had also previously been observed with everted polar filaments under high salt conditions; this germination of spores is often regarded as an acceptable check for spore viability (Keohane & Weiss, 1999). Thirdly, it may be possible that the spores from sea lice are not able to directly infect naïve sea lice, but require an intermediate / alternative host to continue the next phase in their life cycle, as is widely reported with microsporidian infections in fresh water copepods (Andreadis, 1985; Sweeney *et al.*, 1985).

Experimental shore crabs and Atlantic salmon were not infected with the microsporidian during the transmission experiment. Microsporidia are generally

regarded to be host specific; hence it may be accepted that a microsporidian infecting a copepod would not also necessarily infect a decapod crustacean or a fish unless it was somehow involved in the parasite's life cycle as an alternate or intermediate host. Unless host fitness or host immunosuppression facilitated such an opportunistic invasion, as is frequently reported in immunocompromised mammals (Didier & Bessinger, 1999). However, this assumes that both crabs and fish were subjected to the correct exposure procedure for transmission to occur. It may be safe to assume that the crabs would have probably ingested some spores contained within settled detritus from the tank floor and hence did not become infected, despite *per os* ingestion of spores. Most fish infecting microsporidia are transmitted *per os*, either by the direct consumption of infected material (cannibalism), via a paratenic host such as a small crustacean, or via close co-habitation (Kent *et al.*, 1995; Baxa-Antonio *et al.*, 1992; Olson, 1976; Olson, 1981; Shaw *et al.*, 1998; Shaw & Kent, 1999). Hauck (1984) suggested that transmission of *Loma salmonae* to chinook salmon can occur via direct phagocytic uptake of spores by the gills, however, other researchers have not been able to reproduce this type of transmission experimentally (Shaw *et al.*, 1998). Experimental transmission of microsporidian infections in salmonids have been successfully performed using anal gavage, *per os*, intravascular, intramuscular and intraperitoneal injections of spores (Shaw & Kent, 1999). The experimental fish in this study may not have ingested spores *per os* and hence Atlantis salmon cannot be ruled out as potential alternate hosts for this microsporidian. Experimental injections of spores and the use of paratenic hosts or infected food pellets should be performed on experimental fish to clarify this uncertainty.

7.5.2 Anal gavage of experimental *L. salmonis* with microsporidian spores

The failure to transfer the microsporidian infection to naïve lice using the anal gavage of spores into the midgut suggests that the spores were either not viable, or that environmental spores from infected sea lice are not able to infect naïve sea lice directly but require an alternate or intermediate host to proceed with their life cycle, or that transmission into the sea lice is via an unknown route. Assuming that transmission is *per os* and that the spores are viable, this data combined with the data from section 7.5.1, suggests that sea lice may potentially obtain the microsporidian infection from another source other than previously infected sea lice.

7.5.3 Microsporidian infection in farmed Atlantic salmon

Five farmed Atlantic salmon, 4 grilse and one non-grilse, of harvest size have been diagnosed as positive for the microsporidian using the primary PCR. A further 2 fish were diagnosed as positive with the more sensitive nested PCR. DNA sequencing was only performed on non-nested PCR products from the kidney and heart tissues of fish 1 and the kidney tissues of fish 3 and 5. Sequencing of these products produced a consensus sequence with 99.4% similarity over 860 bases when compared to the sequence obtained from sea lice in chapter 6. The two sequence differences are represented by only 5 base changes. These differences could be potentially due to either sequence reading errors, or represent small base changes between the two-microsporidian isolates. There are presently uncertainties as to the percentage variation that might be expected within a species, however, work conducted on a microsporidian from the same family, Enterocytozoonidae, by Gresoviac (2000), suggested that a 2.14% variation over a similar region of the rRNA gene between five different isolates of *Nucleospora salmonis* could represent a single species. The 0.6% variation in the gene sequence seen in this study between the microsporidian DNA

isolated from sea lice and the microsporidian DNA isolated from fish suggests that the two microsporidian isolates are potentially the same species. This theory is supported by the intimate host / parasite relationship between the two organisms, which creates a possible route for microsporidian transmission to take place between the two species. However, a microsporidian species has never previously been reported that infects both invertebrate and vertebrate hosts (Larsson, 1999).

The fact that five fish tested positively with the primary PCR reaction, and some fish tested positively in more than one tissue, without inconsistent or falsely positive results, suggests that these positive PCR bands are not due to contamination. The absence of the microsporidian in the spleen and gonadal tissue of fish 1, were most probably caused by a poor DNA extraction from the spleen tissue (personal observation) and the fact that the microsporidian is possibly not present in reproductive tissues. The absence of the microsporidian in reproductive tissues suggests that infection is transmitted via a horizontal and not a vertical route.

So far the microsporidian has been detected in kidney, liver, heart, gill and circulating white blood cells from farmed Atlantic salmon. The salmonid microsporidian infection *Nucleospora salmonis* is found in similar tissues, being an intranuclear parasite of immature white blood cells, predominately found in the haematopoietic tissues of kidney and spleen and to a lesser extent in circulating white blood cells (Elston *et al.*, 1987; Hedrick *et al.*, 1990; Chilmonczyk *et al.*, 1991). From the above description of the microsporidian *Nucleospora salmonis*, it would appear to share similar characteristics to those of the microsporidian found in this study; the kidney tissues appear to be most seriously infected followed by other tissues and to a much lesser extent the circulating white blood cells. *N. salmonis* is known to cause serious mortalities in juvenile chinook salmon, however, apart from slight gill pallor and

modest renal hyperplasia, the infected Atlantic salmon in this study appeared normal, showing no signs of acute anaemia as reported from chinook salmon infected with *N. salmonis* (Elston *et al.*, 1987). It is also possible that the infection under study in Atlantic salmon is more severe at the hatchery level, as is the case for *N. salmonis* in chinook salmon, or that the infection is latent, not causing significant clinical signs in infected fish. Experimental studies performed on mammals have demonstrated that long-term almost asymptomatic or chronic microsporidian infections occur. These conditions have been described as balance host-parasite relationships because the host survives and the parasite persists. However, the same microsporidian infections in immunosuppressed experimental individuals leads to acute disease symptoms often resulting in host death (Didier & Bessinger, 1999). It is extremely interesting therefore, that four out of the five fish that have tested positive by diagnostic primary PCR are grilse. Fish that are considered to have immune deficiencies, such as the inability to produce certain antibodies that are readily produced in sexually immature fish and increased plasma hormone levels, such as cortisol and testosterone, which reduce antibody production and are known to reduce disease resistance in salmonid fish (Slater & Schreck, 1997; Kaattari & Tripp, 1987; Pickering & Pottinger, 1989). It may be possible that Atlantic salmon have this balanced host-parasite relationship with this species of microsporidia, showing no clear clinical signs. However, the disease in grilse becomes more apparent in the absence of a competent immune system and parasite numbers increase. This might reflect why only low levels of infection were found in positively testing fish by diagnostic PCR, and probably indicates the reasons why the histology and TEM has not so far been able to visualise this parasite in fish tissues.

Future work is needed to confirm the findings of this study. Firstly fish at a hatchery level need to be evaluated for acute signs of infection. The parasite will also need to be located in infected fish tissues, by either furthering histological studies or by using more sensitive techniques such as *in situ* hybridisation, using labelled specific PCR primers to detect the parasite. Transmission studies will need to be conducted, attempting transmission from known infected fish to naïve lice, which will enable the pathogenicity trials to be conducted on sea lice. It also remains to clarify the life cycle of this parasite. If the microsporidian spores from sea lice do not infect either lice or fish, there could potentially be a third host involved in the life cycle. However, the microsporidian infection seen in sea lice could simply represent the opportunistic invasion by a fish-infecting microsporidian, which has entered a new host through the blood feeding of the sea lice. If indeed a microsporidian species has managed to infect both a vertebrate and an invertebrate hosts, it may seem more plausible that a microsporidian originally from a vertebrate host might find the immunological defences of an invertebrate less challenging, than in the reverse situation.

The microsporidian infection may indeed prove to be pathogenic to *L. salmonis*, considering is gross pathology described in chapter 5; however, the sea lice could represent a dead-end host for the microsporidian.

CHAPTER 8

CONCLUSIONS AND FURTHER WORK

This study has yielded some useful and interesting information. Many new organisms have been observed in association with the salmon louse *L. salmonis*. A new microsporidian hyperparasite has been recorded for the first time that represents a new genus and species.

8.1 EPIBIONTS

The filamentous cyanobacteria and filamentous green and brown algae were ubiquitous in their distribution and are suggested to have a facultative epibiosis with sea lice. The stalked ciliates *Ephelota* spp. showed a seasonal pattern on sea lice in farmed fish, reaching maximum percentage prevalence in the spring months and decreasing over the summer and winter periods. *Ephelota* sp. was always absent from sea lice sampled from wild caught fish and is possibly not normally an epibiont of parasitic copepods on wild salmonids but flourishes on farmed salmon. It is confirmed to have a phoretic relationship with its sea louse host, being carried to areas of potentially greater nutrition. *Udonella* sp. was observed on both farmed and wild salmon. *Udonella* was shown to be unable to survive and reproduce on parasitic copepods that have been removed from their fish hosts. *Udonella* sp. does not show a seasonal pattern in its occurrence on farm sites and its population decline in the summer months coincides with the administering of sea lice control chemotherapeutants. *Udonella* spp. have an obligate relationship with parasitic

copepods and are confirmed to be ectocommensals feeding mainly from the fish mucus provided during copepod feeding activity.

Adult female *L. salmonis* were usually more densely populated with epibionts than adult males; this could be attributed to the greater life span of the female and its more sedentary behaviour after mating. Juvenile *L. salmonis* were sometimes populated with epibionts, but at much lower densities, which is possibly due to the shedding of existing epibionts during ecdysis. Sea lice do not moult further, once they become adults, therefore an increase in epibionts numbers would be expected.

Caligus elongatus was observed to have the same diversity of epibionts as *L. salmonis* when present in the same sample population, but as much lower densities.

Farm site specificity may exist in epibionts of sea lice, but could not be concluded from this study. The large differences observed in epibiont load between sea lice in different locations could be due to differing farm management strategies and sea lice control measures.

Surprisingly, egg string production and hatching success rate were not significantly affected by the presence of dense populations of epibiont species on either the genital segment or the egg strings.

An SEM study of the cuticle of *L. salmonis* infested with epibionts revealed that no damage was caused by the presence of epibionts, although bacterial numbers may increase at the areas of attachment in stalked ciliates and a depression in the cuticle appeared at the site of egg filament attachment in *Udonella* sp.

The epibionts observed in this study did not appear to have any detrimental effects on their sea lice hosts at any stage or season and hence could not be considered individually as potential biological control agents.

However, further research on the utility of *Udonella* sp. as a vector for spreading bacteria could be justified. The surface of udonellid worms was shown in this study to be capable of supporting high bacterial numbers, some of which inevitably colonise the sea lice cuticle. Marin *et al* (2002) and Minchin & Jackson (1993) have both reported that udonellid population size remains constant and is not related to the intensity of copepod infection on host fish. Aken'Ova *et al* (1996) observed *Udonella* spp. moving freely from copepod to copepod during copepod contact. The ability to persist in the environment irrespective of copepod host density and to move freely between copepods are both desirable features for a biological control agent. These features of udonellid worms could potentially assist in the dissemination of any newly developed bacterial biological control agent to parasitic copepods in the future.

8.2 ENDOBIONTS

The lack of endobionts observed in this study may be due to the moderate sample sizes studied, or due to the fact that the niche environment of a salmon farm is not suitable for such organisms to sustain their populations; assuming that an endobiont sea louse relationship may be due to evolutionary development, formed over long periods of time from coexistence with wild oceanic salmon. Or simply that many of the aforementioned endobionts are highly host specific, many relying on oral ingestion of infective stages to propagate and hence do not readily infect parasitic copepods.

However, this study concentrated on epibionts / hyperparasites and fungi, and did not screen for pathogenic bacteria or viruses. There are many crustacean diseases reported with viral and bacterial aetiologies, and this area of research may prove fruitful in the search for potential biological control agents for the salmon louse.

8.3 MICROSPORIDIAN

The microsporidian parasite reported in this study, is the first report of a microsporidian in sea lice, and indeed from marine copepods; it is also the fist report of a hyperparasitic microsporidian in crustacea.

No clear seasonal trends have emerged for the presence of this microsporidian and it has not been observed in *L. salmonis* sampled from wild caught salmon, or *C. elongatus* coexisting with infected *L. salmonis* on farmed fish.

Histological sections and TEM confirmed that microsporidian infection occurred throughout the copepod's body; large xenomas forming between the basal portion of the epidermal layer and the basal lamina. The developing eggs and egg strings were not observed to be infected with the microsporidian; horizontal transmission to a new host is expected to be the most likely route of transmission.

There are no similar microsporidian infections occurring within the copepoda, however, some of the developmentally distinct features observed in this species are also reported from other members from the family Enterocytozoonidae. These morphologically distinct features, the rRNA gene similarities to known existing *Nucleospora* spp. isolated from fish, combined with strong molecular evidence that some farmed Atlantic salmon harbour the same microsporidian found in salmon lice, suggests that this microsporidian infects both *L. salmonis* and Atlantic salmon.

Microsporidia are considered to be opportunistic but still basically host specific parasites, and there are certainly considered to be two distinct barriers that microsporidian genera cannot traverse. Firstly there are the borders between invertebrate and vertebrate hosts, and secondly the borders between poikilothermous and homoiothermous vertebrate hosts (Larsson, 1999). Research on microsporidia has increased significantly over the last fifteen years, largely due to the fact that they have

been recognised as opportunistic pathogens in AIDS patients. The sources of infection and mechanisms of transmission of these organisms in humans are mostly uncertain, but are thought to be zoonotic in nature (Dengjel *et al.*, 2001). The transmission of invertebrate microsporidia to mammals is normally considered to be impossible, as the temperature differences are too great to overcome. Therefore, the opportunistic infections seen in AIDS patients are assumed to originate from other homoiothermous mammalian hosts. Nevertheless, some experimental exceptions to this rule do exist. The microsporidian species *Nosema algerae* from culicine mosquitoes has been injected into the 'colder tissues' (feet pads and tails) of immunosuppressed mice and successfully caused infection (Trammer *et al.*, 1997). *Nosema algerae* has also recently been successfully cultured in human fibroblasts at temperatures of 31 ºC and 38 ºC (Trammer *et al.*, 1999). However, since no natural infections without experimental intervention or *in vitro* cell culture are documented, these examples should be considered the exception to the rule.

Temperature differences between hosts are still regarded as one of the main obstacles that microsporidia must overcome in order to infect new hosts. Immunosuppression in the novel host can facilitate this transition, however, species that maintain similar body temperatures are more likely to successfully share microsporidian parasites. Atlantic salmon are ectotherms that share a similar body temperature with their parasitic sea lice, which may improve the likelihood of this event occurring between these two species.

The results of this study strongly suggest that the microsporidian found in *L. salmonis* is also found in Atlantic salmon. However, no previously reported natural occurring microsporidian infection is known to exist between vertebrate and invertebrate hosts. The origin of the microsporidian infection under study is not known and could have

potentially originated in either the fish or the copepod host. There are currently no other microsporidian species described from marine copepods, therefore only comparisons to known fish-infecting microsporidian species were possible. There were similarities to the previously mentioned microsporidian parasite associated with salmonid fish, *Nucleospora salmonis*. Although *N. salmonis* is a recognised disease in salmonid fish from both marine and freshwater environments, successful experimental transmission between fish has only ever been achieved in freshwater. The lack of successful transmission of *N. salmonis* in the marine environment between fish of the same species, suggests that *Nucleospora* spp. may be predominately parasites of freshwater fish. Considering that, Atlantic salmon start their life cycle in freshwater and salmon lice are exclusively marine organisms, this supports the theory that the microsporidian under study originates in Atlantic salmon and not in the marine copepod. It may also seem more plausible that a microsporidian originally from a vertebrate host might find the immunological defences of an invertebrate less challenging, than in the reverse situation.

The discovery of this microsporidian parasite in the salmon louse, together with its suspected fish host origins, are both exciting and inspiring. This study has uncovered a previously unreported host parasite relationship, which requires further study, in which to begin to answer the many questions now raised. Firstly it remains to be shown without any doubt that this microsporidian infects Atlantic salmon. No significant unexplained mortalities with suspected microsporidian aetiologies have been reported in cultured Atlantic salmon from either freshwater hatcheries or marine net pens farms in Scotland. Furthermore, no clinical signs resembling those from *Nucleospora* infected salmonids have been reported from Atlantic salmon in Scotland. Assuming the indications of this study are correct, and the microsporidian under study

does indeed infect Atlantic salmon, then it remains to be ascertained whether the infection is initiated at a freshwater hatchery level or in the marine net pen environment. Allegedly infected harvest sized fish appeared ostensibly asymptomatic and the proportion of fish that carry this microsporidian will also need to be assessed.

If this microsporidian is acknowledged as a latent parasite of Atlantic salmon, transmission and pathogenicity trials to the salmon louse can be attempted using microsporidian infected host fish and naïve sea lice. This has the potential to be a unique delivery mechanism for a biological control agent. If transmission were only achieved from the blood feeding of sea lice on infected fish then a highly efficient delivery system, and one extremely host specific, would be accomplished. These are both highly desirable characteristics for a biological control agent to posses. However, many questions as yet remain unanswered and it remains to be seen, out with scientific experimentation, whether one potential parasitic disease could be knowingly given to farmed animals with the expectation of suppressing another parasite later in the production cycle. Furthermore, the increasing awareness of microsporidiosis in humans, would probably not advocate the use of such organism in the production food for human consumption. However, it is unlikely that a microsporidian infecting Atlantic salmon and sea lice at temperatures between 6 ℃ and 13 ℃ could ever infect homoiothermic mammals.

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APPENDIX 1

Crystal violet solution

Crystal violet	2 g
95% ethanol	20 ml
ammonium oxalate	0.88
distilled water	300 ml

Dissolve the dye in the ethanol, the ammonium oxalate in the water and mix the two solutions together. Filter before use. This stain is stable for 2-3 years.

Gram's iodine

Iodine crystals	1 g
Potassium iodide	2 g
Distilled water	300 ml

Dissolve the potassium iodide in 5 ml of water, and then dissolve the iodine crystals in this. Dilute to 300 ml for Gram's iodine.

1% aqueous neutral red

Neutral red	1 g
Distilled water	100 ml

Filter before use

Mayer's haematoxylin

Haematoxylin	2 g
Sodium iodate	0.4 g
Potassium alum	100 g
Citric acid	2 g
Chloral hydrate	100 g
Distilled water	2000 ml

Allow the haematoxylin, alum and sodium iodate to dissolve overnight in the distilled water. Add choral hydrate and citric acid, then heat until boiling, continue to boil for 5 minutes.

1% acid alcohol

To 1980 ml of methylated spirits add 20 ml concentrated hydrochloric acid (38%).

Scott's tap water substitute

Sodium bicarbonate	3.5 g
Magnesium sulphate	20 g
Tap water	1000 ml

Dissolve by heating if necessary. Add a few crystals of thymol, which will act as a preservative.

Eosin

Eosin 1%

Eosin yellowish	20 g
Distilled water	2000 ml

Putt's eosin

Eosin yellowish	4 g
Potassium dichromate	2 g
Saturated aqueous picric acid	40 ml
Absolute ethanol	40 ml
Distilled water	320 ml

Dissolve the eosin and dichromate in the ethanol. Add the distilled water, then the picric acid.

For the **final staining solution**, mix one part Putt's eosin with eight parts 1% eosin

Haematoxylin and eosin stain

Mayers haematoxylin (0.1%)	5 min
Wash in tap water	
Acid alcohol	3 quick dips
Wash in tap water	
Scott's tap water	1 min
Wash in tap water	
Eosin	5 min
Wash in tap water	
Methylated spirit	30 sec
Absolute alcohol II	2 min
Absolute alcohol I	1.5 min
Xylene (clearing)	5 min
Coverslip	

Giemsa stain

1 part Giemsa R66 to 9 parts distilled water (filtered)   20 min

Blot on filter paper

Methylated spirits   few seconds

Differentiate in colophonium alchol (colophonoim saturated methylated spirits)

Methylated spirits   30 sec

Dehydrate very rapidly in absolute alcohol

Xylene (clearing)

Coverslip

Gram stain

100% ethanol   5 min

Methylated spirit   1.5 min

Wash in running tap water

Crystal violet   2-3 min

Wash off crystal violet with gram's iodine   2-3 min

Wash running tap water

Differentiate in acetone

Wash immediately running tap water

Counterstain using 1% neutral red   1 min

Wash running tap water

Blot dry on filter paper

Rapidly dehydrate and differentiate in absolute alcohol

Xylene (clearing)

Coverslip

0.5 M EDTA

Dissolve 93.05 g of disodium salt dihydrate EDTA in 500 ml of distilled water, pH to 8.0

Tris-acetate buffer x 50 (TAE)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

Dissolve tris in 500 ml of distilled water, add the acetic acid and EDTA solution, and make up to 1 litre with distilled water, pH to 8.0

Dilute 1 part TAE x 50 in 100 parts distilled water for working solution.

YM media

Yeast malt extract (difco 0711-17-1)	11.5 g
Agar	5 g
Distilled water	500 ml

Autoclave media, then allow to cool to approximately 50 ^{0}C, add 10 ml of antibiotic solution (to give a final concentration in the media of: penicillin 100 iu / ml and streptomycin 100 µg / ml) then pour the media (approx. 50 ml) in each 90 mm petri dish.

Antibiotic solutions

Penicillin / streptomycin (Gibco 15070-022)

Penicillin	5000 iu / ml
Streptomycin	5000 µg / ml

APPENDIX 2