Larval Settlement and Epidemiology of *Lepeophtheirus salmonis* Krøyer, 1837(Copepoda; Caligidae)

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By

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DECLARATION

I hereby declare that this thesis has been composed by myself and is a result of my own investigations. It has neither been accepted, nor submitted for any other degrees. All sources of information have been duly acknowledged.

C.S. Tucker

Dedication

To my Family and Friends,
for their unbending love and support

" IN A SCIENTIFIC PURSUIT THERE IS CONTINUAL FOOD FOR DISCOVERY AND WONDER"

MARY SHELLEY (1818)

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CONTENTS

List of Tables

Chapter 6

List of Figures

Chapter 5

Chapter 6

List of Plates

ABSTRACT

This study has been carried out to investigate the biological and environmental parameters influencing the settlement and post-settlement survival of the infective stages of *Lepeophtheirus salmonis* Krøyer 1837.

The abiotic factors investigated were temperature and salinity. Temperature was found to have a significant effect on the settlement success of the copepodids with an inverse relationship between temperature and settlement. Survival of the louse at 10 days post infection showed a decrease at the reduced temperature. Temperature was also shown to have a direct relationship on lice development; higher seawater temperatures resulted in faster development. Regression analysis of temperature and settlement shows a significant correlation.

A constant reduced salinity, 24‰, resulted in a reduced ability of the copepodid to infect its host compared with 34‰. Post-settlement survival in 24‰, at approximately 13°C resulted in 5.8% survival of lice to day 10 post-infection compared to 79% in 34‰ salinity. When this experiment was repeated but with elevated seawater temperatures of up to 18°C, survival at the reduced salinity was found to be 75.3%, higher than the ambient control group. The developmental rate at day 10 post-infection of *L.salmonis* larva at 24‰ was shown to be slower than development at 34‰.

Distribution of the *L.salmonis* copepodid on its host showed the highest settlement on the gills and on the fins, particularly the pectoral and dorsal fins. Examination of *L.salmonis* survival at day 10 post-infection indicated the highest losses on the gills and the pelvic, caudal and dorsal fins. Settlement on the pectoral fins showed the highest settlement and the greatest survival.

The infective copepodid has a reduced ability to infect its host after 7 days following the moult from nauplius 2, compared to copepodids aged 1 and 3 days following the nauplius 2 moult. For copepodids of all ages, once settlement had been achieved, survival at 10 days post-infection was approximately 50% in all groups. Copepodids of all ages did not show any difference in the development rate at 10 days post infection. Highest settlement was found to be on the gills and pectoral and dorsal fins. The effects of varying dose rates of copepodids, has shown that a finite percentage of lice settle and survive the first five days post-infection. Settlement distribution was found to be highest on the body, gills and pectoral and dorsal fins. In serial infections of fish there was a reduced settlement count with second infections, possibly through intraspecific competition. Experiments using different host stocking densities showed that with an increased number of hosts the intensity of the infection of individual fish was reduced.

Smaller fish appear more susceptible to settlement of *L.salmonis* than larger fish, and this is associated with the relatively greater fin area of those fish compared to larger fish. *L.salmonis* exhibits a preference for the fins as an area of settlement in all sizes of fish. Comparison of copepodid settlement on salmon and sea trout showed that in single populations of fish salmon had the highest intensities of infection whilst in mixed populations of fish sea trout had a higher intensity. Settlement distribution of *L.salmonis* on salmon showed greatest settlement on the body, pectoral and dorsal fins, whilst on sea trout settlement was highest on the body, pectoral, pelvic, caudal and dorsal fins. The comparative development of *L.salmonis* between the two species of host fish showed an increased rate of development on salmon.

The calculated energy levels for *L.salmonis* larval stages show a decrease in available energy within each developmental stage. After 6 days from the nauplius 2 moult the

copepodid starts to show a sharp decline in energy levels which coincides with the reduced ability of the copepodid to infect the host. Post-settlement energy levels remain constant even though the copepodid is actively feeding, as seen by SEM examination at 2 days post-infection. The principal lipid class found within *L.salmonis* larval stages as energy reserve is triacylgylcerol (37.6% of the total lipid).

A preliminary epidemiological model for sea lice population dynamics is proposed. This is a differential equation compartmental model that has been designed to examine the flow of *L.salmonis* developmental stages on the host. The model was able to predict the timing of the maximum number of pre-adult 1 lice stages to within one day. The difference between the observed data and the model output is probably due to the considerable variability in the parameters used in the model construction.

CHAPTER 1. GENERAL INTRODUCTION.

1.1 GENERAL BACKGROUND

With the advent and success of the furunculosis (*Aeromonas salmonicida*) vaccine the most serious disease problem currently facing the Scottish salmon industry is the sea louse, *Lepeophtheirus salmonis* Krøyer, 1837. In recent years the controversy attached to "sea lice" and the Scottish salmon industry has been widely publicised by the media. The sea lice problem has become a very emotive public issue with the expansion of the salmon industry, through the perceived environmental threat caused by the use of new chemical treatments and the decline of wild sea trout populations.

The rapid commercial expansion of the salmon culture industry began in the 1960's in Norway, followed in the 1970's in Scotland and Ireland (Costello, 1993a). Epizootics of sea lice were reported for Norway in the 1960's (Brandal, Edigius & Romslo, 1976; Brandal & Egidius, 1977) and Scotland in the 1970's (Rae, 1979; Wootten, Smith & Needham, 1982). Cusack & Johnson (1990) reported serious outbreaks in Canada with high mortalities. The first major Scottish epizootic occurred at Lochailort in 1976 (G. Rae, pers. comm. 1995) although there was a presence of sea lice on farmed fish prior to this date (Wootten *et al.*, 1982). Salmon lice are now considered to be the major problem in the culture of marine salmonids and untreated epizootics can lead to considerable economic loss.

Through sport fishing for ascending migratory adult salmon *L.salmonis* has gained widespread recognition and notoriety. "There can be few other parasites that invoke such mixed emotions as that of *Lepeophtheirus salmonis*. On the one hand causing euphoria, to game fishermen lice show the recent transitional return to freshwater of the migrating

salmon, but to salmon farmers the heartache of the destruction of their salmon stock by this parasite" (Pike, 1989). The importance and significance of this ectoparasite grows substantially with the increases in salmon culture production.

1.2 BIOLOGY AND LIFE CYCLE.

Lepeophtheirus Nordmann, 1832 is the second largest genus within the copepod family Caligidae with the name being derived from the Greek word meaning "scale" and "louse" (Bron, 1993). Within this thesis the common descriptive terminology used for *Lepeophtheirus salmonis* (Krøyer, 1837) will be "sea or salmon louse", although a second species of caligid copepod is found to infect Atlantic salmon, *Caligus elongatus* Nordmann, 1832, and is often included under this collective name. This second species, although smaller than *L. Salmonis*, does inflict damage to the host, requiring chemotherapeutic treatment but in comparison to *L. salmonis* this is minor, although it should not be ignored (Treasurer & Grant, 1994).

Lepeophtheirus salmonis has a circumpolar distribution in the northern hemisphere and occurs on most species of salmonids of the genera *Salmo, Salvelinus* and *Oncorhynchus* in their marine phase. It is common on wild salmonids (White, 1940a; Tully, 1989; Berland, 1993) There are records of non-salmonid hosts (Bruno & Stone, 1990) although these must be considered unusual and possibly does not offer the opportunity for full development and survival of this species of caligid (Kabata, 1979).

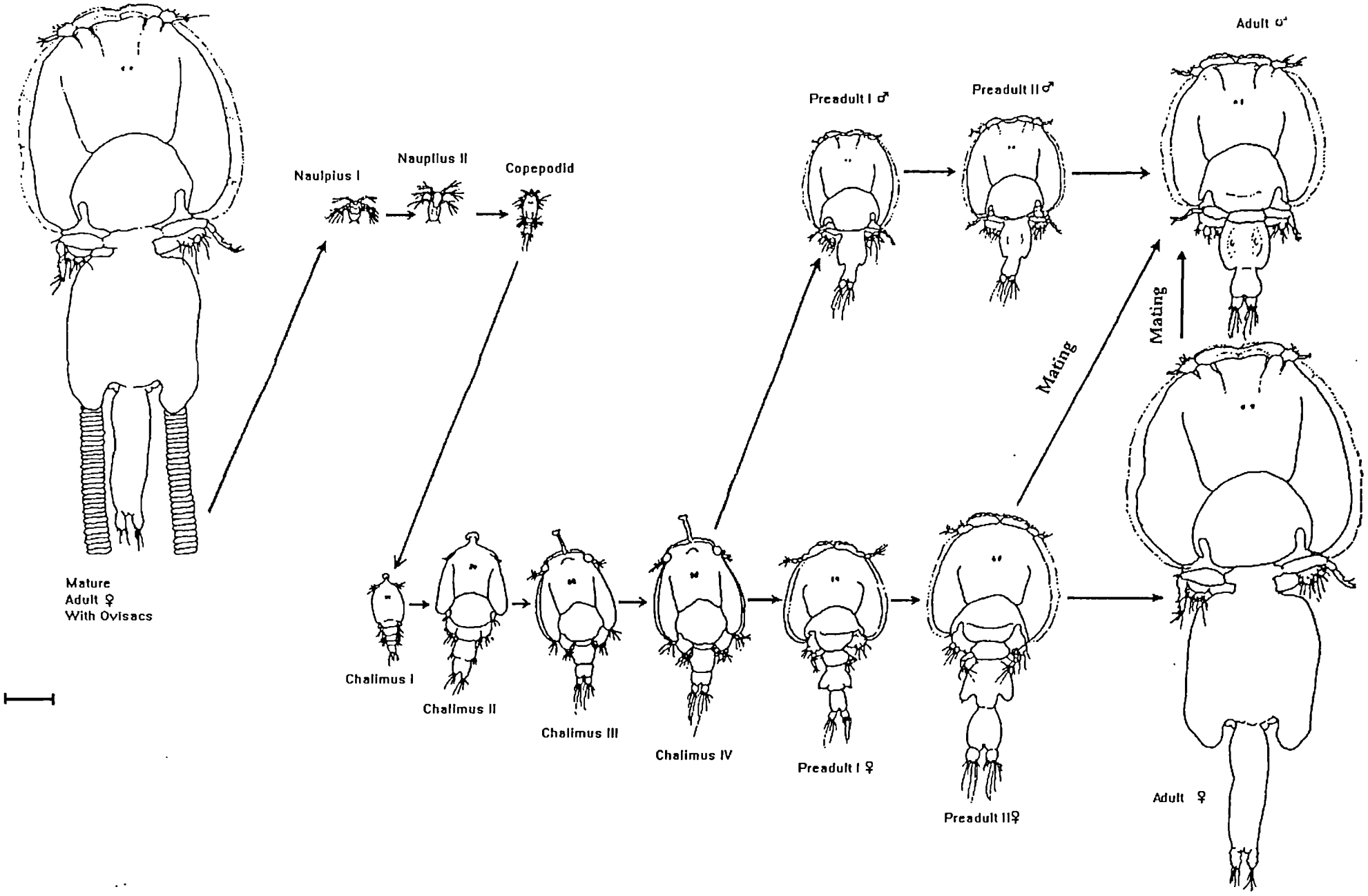
L.salmonis is a large dark brown ectoparasite, in which the adult female reaches lengths of 18 mm (excluding egg strings) and adult males up to 7 mm in length (Pike, 1989). The life cycle of *L.salmonis* comprises 10 individual life stages each separated by a moult, in 5

phases, nauplii, copepodid, chalimus, preadult and adult. (Kabata, 1972; Schram, 1993) See Figure 1.1. These ten stages consist of two free-living, planktonic, lecithotrophic nauplii stages and one lecithotrophic semi-planktonic copepodid stage, the latter being infective to the fish. Then follows four attached chalimus stages that utilise a frontal filament for attachment to the host, two motile preadult stages and one motile adult stage (Kabata, 1972). At 10°C the generation time is six weeks for *L.salmonis* and therefore it is estimated that it is possible to complete four generations in Scottish waters between May and October when sea temperatures are between 9-12°C (Wootten *et al.*, 1982).

Bron, Sommerville, Jones & Rae (1991) and Bron (1993) describe the settlement pattern of the infective copepodid stage as being similar to that of barnacles in their close searching phase, as described by Crisp (1976) and Walker, Yule & Nott (1987). There is a searching phase after the copepodid has made initial contact with the host, moving over a small area, gripping with its maxillipeds but probing with its first and second antennae. At this stage, the settlement is still reversible and the copepodid can re-enter the water column. Once a suitable site has been located the primary attachment phase begins. In this phase the parasite remains gripped with the maxillipeds whilst the second antennae are driven into the host epidermis, with a repeated stabbing action. This is followed by the secondary filament attachment phase in which the frontal filament is extruded and attaches the copepodid to the host, this is rapidly accompanied by the moult to chalimus 1 (Bron *et al.*, 1991). Attached chalimi adhere to the basement membrane of the host via a glue-like secretion. This secretion is injected under the epithelium where it spreads along the line of weakness between the basement membrane and the epidermal cells overlying it to form a basal plate (Bron *et al.*, 1991).

All members of the Caligidae are ectoparasitic and are similar in shape. All chalimi are

Figure 1.1 Life cycle of *Lepeophtheirus salmonis* (Krøyer, 1837).
Scale bar = 1mm.



permanently attached to the host by a frontal filament (Kabata, 1981). Preadult and adult sea lice attach to host surfaces by the cephalothoracic sucker (Kabata, 1981). This mode of attachment allows free movement over the host and depends mainly on suction. The concavity of the cephalothorax, sealed around most of its margin by a membrane applied to the host surface, is partially evacuated of water. A fall in pressure within the enclosed space creates suction, which presses the copepod firmly against the surface of the host. The hold is enhanced by the modified swimming legs sealing off the rear of the concavity and the assistance of the second antennae (Kabata, 1981). Certain stages, preadult and adults are capable of free movement over the host surface (Kabata, 1979). When attached to the fish host caligid copepods are always orientated in the direction in which the fish is moving, i.e. they are directed with the anterior end against the flow of water (Kabata & Hewitt, 1971).

1.3 FEEDING ON THE HOST

There are no published reports of direct ingestion by *L.salmonis* until the chalimus 1 stage, although the copepodid stage may become a trophic phase once attached to the fish (Bron, 1993). The earlier true lecithotrophic naupliar stages have a finite energy reserve to provide sustenance whilst planktonic. The contents of the nauplius "yolk cells" are currently under investigation but are believed to be lipid (Gravil, 1996). Feeding during the chalimus phase is considered to be mainly on host mucus around the point of attachment (Brandal & Egidius, 1977). Jones, Sommerville & Bron, (1990) considered that the specific damage to the epidermis beneath the chalimus oral cone and the presence of food material or sloughed cells seen in the alimentary canal suggest the gut to be active.

The mode of feeding of *Lepeophtheirus salmonis* adults has been extensively described by Kabata (1974) and Andrade-Salas (1997). The tissues of the fish are gouged out by the movements of the strigil and the debris is then cut and picked up by the mandibles, which initiate its passage into the buccal cavity. The strigil consists of a divided dentiferous bar at the tip of the labrum. Transport of food into the buccal cavity is assisted by contractions of the labral musculature, capable of creating a propelling, peristaltic wave (Kabata, 1974). The strigil is reported to be missing in copepodids (Johnson & Albright, 1991a), although Bron (1993) from a single specimen, suggests that a strigil is present in the oral cone of copepodids.

The alimentary canal of adult *L.salmonis* consists of a short, tubular foregut, a large midgut running from the anterior part of the cephalothorax and into the abdomen, and a short tubular hindgut (Nylund, Økland & Bjørknes, 1992; Andrade-Salas, 1997). Bron, Sommerville & Rae (1993a) describe a similar structure for the larval stages of *L.salmonis.* Brandal *et al.* (1976) demonstrated spectrophotometrically that host blood constitutes an important component of the diet of *Lepeophtheirus salmonis*, especially for adult females, whose larger size may allow them to penetrate deeper into the dermal tissues (Wootten *et al.*, 1982). White (1940a) suggests that *L.salmonis* feeds upon skin and subcutaneous tissues, including mucus and blood.

1.4 PATHOLOGY.

The pathogenesis of sea lice is possibly entirely due to the feeding activity of all host associated phases, adults causing most injury and damage (Pike, 1989). The damage caused by *L.salmonis* can be severe. Chalimus stages cause only local ulceration around

the point of attachment, 0.5 cm in diameter (Jones *et al.*,1990), the extent of the damage being governed by the length of the frontal filament. This area will increase as the chalimus develops and may be as much as 3 mm by the chalimus 4 stage (Pike, 1989).

Jones *et al.* (1990) report four main areas of interaction associated with pathological changes between host and the chalimus stages of *L.salmonis*. These are second antennal attachment, filament attachment, maxilliped activity and feeding activity. The skin changes involved were initially mechanical disruption followed by epidermal hyperplasia associated with the mouth tube. Little reaction to the chalimus filament was seen. Erosion of epidermal layers by feeding activity forms a ring (although not 360°) of erosion and later these become melanized leaving a tell-tale sign of an old chalimus site (Jones *et al.*, 1990).

Rae (1979) considered the most damaging and serious phase of sea lice to be that of the motile preadults and adults. These stages are larger, therefore capable of greater damage and over a larger area of the host. The adult female is considered to cause the most serious damage since it tends to be relatively immobile, remaining in one place for some time whilst on the host (Bron, 1993). The adult and preadult phases are found distributed widely over the dorsal and ventral surfaces of the host. Distinctive grey patches can be seen on the cranial and dorsal surfaces where copepods have been actively feeding. However reports of the most serious damage centre around the dorsal cranial region (White, 1940a; Håstein & Bergsjö, 1976; Wootten *et al.*, 1982). Håstein & Bergsjö (1976) suggest that this is due to the relative thinness of the dermal tissue over the cranium, which is devoid of scales. Kabata (1970) reports cases of erosion and penetration to the cranial tissues are a common finding in severely affected fish in salmon cages.

In adult *L.salmonis* Jónsdóttir, Bron, Wootten, and Turnbull (1992) found an oval imprint

corresponding to the attachment of the margin of the parasite cephalothorax on the host skin and also found lice wedged under the scales. General pathological changes include oedema, hyperplasia, sloughing of cells and cellular inflammation at the point of feeding and attachment. These authors also report that, in gross lesions, serious damage occurs to the host epidermis with scale loss and haemorrhage. Wootten *et al.* (1982) also report subepidermal haemorrhages occurring in the perianal region. These authors document evidence of head abrasions so severe that the cranium is exposed. Subdermal tissues around feeding sites become grossly haemorrhaged and the epidermis sloughs away (Wootten, Needham and Smith, 1977). Fish probably, ultimately die from osmoregulatory failure.

The number of lice required to cause serious and significant pathology is variable and depends on the size of the fish and the species of lice (Wootten *et al.*, 1982). These authors also state that as few as five adult *L.salmonis* lice are capable of causing serious pathology on newly introduced salmon smolts. Over 2000 *L.salmonis* have been recorded on a single specimen of farmed salmon in Norway (Brandal & Egidius, 1977), but this must be considered exceptional; more commonly lice numbers in the order of 10-100 may be found on a caged salmon.

It has been suggested that the presence of sea lice is associated with outbreaks of bacterial disease, especially *Vibrio* infections (Wootten *et al.*, 1982). It is not clear whether lice cause the secondary infection by providing a route of entry for *Vibrio* invasion or whether the lice are carriers (Wootten *et al.*, 1982). These same authors report the belief of some salmon farmers that the bacterial invasion is incidental as lice infested fish damage themselves on the cage and thus allow entry for secondary pathogens. Sea lice do stress fish and the resultant elevated cortisol levels may lead to some immunosuppression

(MacKinnon, 1993).

Nylund *et al.* (1992) considered *L.salmonis* a vector for bacterial pathogens. *Aeromonas salmonicida* has been isolated in high numbers from *L.salmonis*, possibly within the gastrointestinal tract (Nese & Enger, 1993). The potential for transmission (although not proven in farming conditions) of infectious salmon anaemia (ISA agent) and *A. salmonicida* has been demonstrated in the laboratory and that *L.salmonis* could serve as a possible vector and reservoir for such opportunistic invaders (Nylund *et al.*, 1992).

1.5 THE SCOTTISH SALMON INDUSTRY AND THE HOST

Atlantic salmon is now farmed in 17 countries, north and south of the equator (FAO, 1992). Scottish salmon production has risen from 31,500 tonnes in 1990 (FAO, 1992) to 83,121 tonnes in 1996, an increase of 51,261 tonnes (over 62%) over seven years (SOAFD, 1993 and SOAEFD, 1996). Scottish salmon farming continues to grow with a predicted increase of 17% in production for 1997 (SOAEFD, 1996). The production figures from the last decade show an increase of 87.3% of salmon harvested over this period (SOAEFD, 1996). Scottish aquaculture production of Atlantic salmon is the third highest in the world, behind that of Norway and Chile (FAO, 1992; Anon, 1995a).

The fish species of primary concern to the British aquaculture industry is the Atlantic salmon, *Salmo salar* (L.). Salmon ongrowing takes place primarily in cages with volumes commonly ranging from 1000 m^3 to 3000m^3 (Costello, 1993a) but which can be as much as 12,500m^3 (Sedgewick, 1988). The average stocking density safely held in floating cages is 10 kg m^{-3} according to Sedgewick (1988) although some salmon companies increase this stocking density to 20 kg m^{-3} (G. Dear, pers. comm. 1997). Atlantic salmon appear (from

scale readings) to feed continuously throughout their marine life but more intensively in the spring and summer. Temperature and salinity have a marked effect on growth. Salmon prefer full oceanic (33-34‰) salinity with an average current flow rate 100-500 mm s^{-1} (Sedgewick, 1988). Beveridge (1987) gives figures of 100-600 mm s^{-1}, higher than Sedgewick's maximum value.

In terms of financial outlay the costs of the treatment chemicals and the costs in man hours of such labour intensive treatment methods are substantial (G. Rae, pers. comm. 1996). 1996 estimates of the costs incurred due to *L.salmonis* in the Scottish salmon industry are in the range £12-15 million have been suggested (Anon 1998a). The annual expenditure of lice control measures within Ireland's salmon farming industry is £1.5 million, 23% of the industries total health care budget (Anon. 1998a). One of the larger salmon companies in Scotland put the cost of *L.salmonis* at 7.2p kg^{-1} salmon (G. Dear, pers. comm. 1997). Costs incurred include, medicines, fish mortalities and loss of growth. Due to stress and irritation caused by the ectoparasites, the fish perform less well in their food conversion and therefore slower growth results (Kabata, 1970; Roberts & Shepherd, 1986). Further economic loss is due to the depreciated market value of damaged fish with gross lesions, such red lesions will detract aesthetically and therefore the fish will be down-graded (Kabata, 1970; Boxshall, 1977; Roberts & Shepherd, 1986).

1.6 CONTROL METHODS AND MANAGEMENT.

While the original infestations of sea lice in farmed salmon were presumably introduced from wild populations the main source for transmission after establishment is from within farms or between adjacent farms (Bron, Sommerville, Wootten & Rae, 1993b). Theoretically, cultured systems may provide a reservoir of sea lice for infections to other

farm systems and back to wild salmonid populations (Costello, 1993a). Farm systems are dominated by lice transmission via self-infection and cross-infection (Costelloe, Costelloe & Roche, 1996). Once established in a farm the parasite numbers slowly increase until epizootic proportions are reached and hence serious pathogenesis (Bron *et al.*,1993b). Jaworski & Holm (1992) suggest that intensity of infection would increase with size of host and reflect the duration of exposure and duration of infection. However they found that the intensity per unit area of farmed salmon did not change with size of host. These authors suggested that intraspecific competition for preferred sites on the host may limit the lice densities.

Lice infestations until recently have been controlled by bath treatments of the organophosphate, Dichlorvos being for many years the only licensed treatment in the U.K.. Hydrogen peroxide is now widely used, being granted an Animal Exemption Certificate in 1993, with success against the preadult and adult phases. Other control strategies used in the control of sea lice in caged salmon culture are the use of wrasse, fallowing and stocking of single year classes (for a comprehensive review see Costello, 1993a). Wrasse, cleaner fish of the genus *Centrilabrus* have been shown to be successful for the continuous *in situ* control of sea lice (Bjordal, 1988, 1990) if stocked at densities 1:50-150 (Costello, 1993b). Wrasse can show cleaning rates of removing 78 lice per wrasse per day (Costello, 1991), cleaning of heavy infestations can be achieved in days with goldsinny wrasse (Rae, 1991). Fallowing of salmon farms permits the recovery of the seabed and prevents the carry over of infectious agents to the next production cycle (Grant & Treasurer, 1993). Fallowing of a site will break the cycle of caligid infestation and lead to low numbers of sea lice on newly introduced fish for several months after stocking (Bron, Sommerville, Wootten & Rae, 1993c). The recommended period of fallowing for a site by Bron *et al.* (1993c) must exceed the maximum survival time of the female adult (34 days) and the

development of the larval stage to the infective copepodid. This will be temperature dependent. The longer the period of fallow for a site the better the prospects of lice control (Bron *et al.*, 1993c). Fallowing of an entire loch system will require management agreements between companies located at adjacent sites (Grant & Treasurer, 1993). Maintenance of a single year class stocking of salmon will prevent infestation of smolts from infected previous year class salmon by cross contamination, lice are however rapidly acquired, in large numbers on newly introduced fish (Bron *et al.*, 1993b).

1.7 EPIDEMIOLOGY.

The study of sea lice population dynamics and epidemiology in farms is confused by the need for farm sites to treat fish against epizootics and thus the normal lice population dynamics will be disrupted. Considerable variability in lice population dynamics has been shown in adjacent farm sites and even between adjacent cages on the same site. (Bron *et al.*,1993b).

Lepeophtheirus salmonis can be found on Scottish salmon farms throughout the year. Observations of continuous recruitment have been reported by Wootten *et al.* (1982) and Ritchie, Mordue (Luntz), Pike & Rae (1993). Wootten *et al.* (1982) reported highest lice numbers are found in late summer and early autumn where a single generation time can be six weeks at temperatures of 9-12°C. This is due to louse numbers building up through the warm summer months with an increased rate of reproduction (Wootten *et al.*, 1977; Bron *et al.*,1993b).

It is possible to find all host associated phases of the life cycle on the host at all times of the year (Wootten *et al.*, 1982; Wootten, 1985). Lice numbers fall with the onset of winter.

Low winter temperatures will retard development times but with cultured salmon this kind of remission may occur less with obvious consequences (Pike, 1989). Rae (1979) suggests that a spring peak in lice numbers can be observed. when examining the population dynamics of sea lice.

Paperna (1980) observed that the maximum prevalence of *Caligus minimus* Otto, 1821 on sea bass was found in early spring, but throughout the year distinct cohorts of infections could be seen. Boxshall (1974c) noted a similar trend when certain stages of *Lepeophtheirus pectoralis* Müller, 1776 were grouped together, suggesting simultaneous infection by infective larvae. Bron *et al.* (1993b) and Bron (1993) in a 20 month study of *Lepeophtheirus salmonis*, although often disrupted by organophosphate treatments, showed a difference in population dynamics between adjacent cages. There was however, considerable concordance in the number and timings of the peaks between sampled cages on a single site. Peaks were seen in March, May, July, November, in that particular study site.

Rae (1979) suggested that there was a spring peak of lice numbers on farmed fish and this spring peak is now the target of an industry-wide sea lice initiative (Anon. 1998a). Previously, in loch systems where adjacent salmon farms were located, based on agreement between farms, a strategy of simultaneous lice treatments and fallowing existed, but was not compulsory. The aim of this new initiative is to conduct and co-ordinate lice treatments at the optimal timing, i.e. late winter/early spring (preferably March) within a given area. All salmon cages within a designated area will be treated at the same time. This new strategy aims to disrupt the reproductive capacity of the female lice and the subsequent generations. When such a targeted sea louse strategy was applied in a limited area, fish mortalities were reduced by 85% and fish downgrades at harvest were cut by

80% (Anon. 1998a). Co-ordination of sea lice treatments shows the requirement of predictability of the timing of susceptible lice stages on the host, presently this information is not available. Our understanding of *Lepeophtheirus salmonis* population dynamics is still incomplete.

This timing of treatments with this initiative will coincide with the "spring run" of wild salmon and sea trout, preventing cross-infection, if any. In recent years wild salmon and sea trout populations in the west coast of Scotland and Ireland have declined. Returning fish have been found to be heavily infected with sea lice (Birkeland, 1996) leading to accusations that parasites originating from farm fish are responsible. McGeorge & Sommerville (1996) in their survey of parasitic fauna on wild fish, in the vicinity of four Scottish salmon farms found little evidence of interaction between wild fish and farmed fish. Their conclusion was that the transfer from farmed fish to wild fish was not significant and unlikely to contribute greatly to the parasitic fauna on wild fish (McGeorge & Sommerville, 1996). *L.salmonis* has been shown to be very species specific to salmonids. Nagasawa (1985) in his study of wild caught salmonids in the Pacific Ocean found some species more susceptible than others, chinook salmon showing the highest parasite abundance and prevalence.

A comparison of infestations on wild and farmed salmonids usually shows a higher prevalence and abundance on the latter, indicating enhanced transmission of the sea lice in the farm environment (Costello, 1993a). Epizootic events in wild populations of salmon are rare (Tully, Poole, Whelan, & Merigoux, 1993). One of the few reports of such an epizootic is by White (1940a) in wild Atlantic salmon off the coast of Nova Scotia, Canada. This was believed to have resulted through elevated water temperature. A similar Canadian epizootic occurred to migrating coho salmon that had accumulated in a river

system prior to migration up river. Here a large natural reservoir of potential hosts had been established and mass mortalities through sea lice infestation occurred (Johnson, Blaylock, Elphick, & Hyatt, 1996). A recent review of the issues involved in the interactions of sea lice and salmonids can be found in ICES CM1997/M:4, Ref:F (Anon, 1997).

1.8 STUDY OBJECTIVES.

Although sea lice have been extensively studied over the last twenty years, serious gaps in our knowledge of the infection process still exist. A better knowledge of the infection process, and through an understanding of its influences and consequences, will lead to a better understanding of sea lice population dynamics and therefore better pest management for the industry and the environment.

This study has been conducted to increase our knowledge of sea lice infection processes and to contribute towards the development of improved pest management strategies.

The study aims are:

1. To examine the abiotic environmental variables of temperature and salinity that influence the settlement success and survival of *L.salmonis* on its salmonid host.

2. To examine the biotic variables that influence the settlement and survival of *L.salmonis*. The biotic variables considered are

a) Age of copepodid on infection

b) Dose rates of copepodid on infection

c) Effects of the stocking density of the salmonid host on infection

d) Effects of secondary infection of the salmonid host on the settlement success

e) Effects of host surface area presented for infection

f) Influence of the host species on settlement.

3. To examine the energy requirements for infection and the energy reserves available to the free-swimming stages of *L.salmonis*.

4. The development of a mathematical model for the predictability of sea lice population dynamics.

CHAPTER 2. GENERAL MATERIALS AND METHODS.

2.1 INTRODUCTION.

This chapter details the materials and methods that were commonly utilised throughout this work. Methodologies particular to specific areas of study are detailed in their relevant chapters.

2.2 MATERIALS AND METHODS.

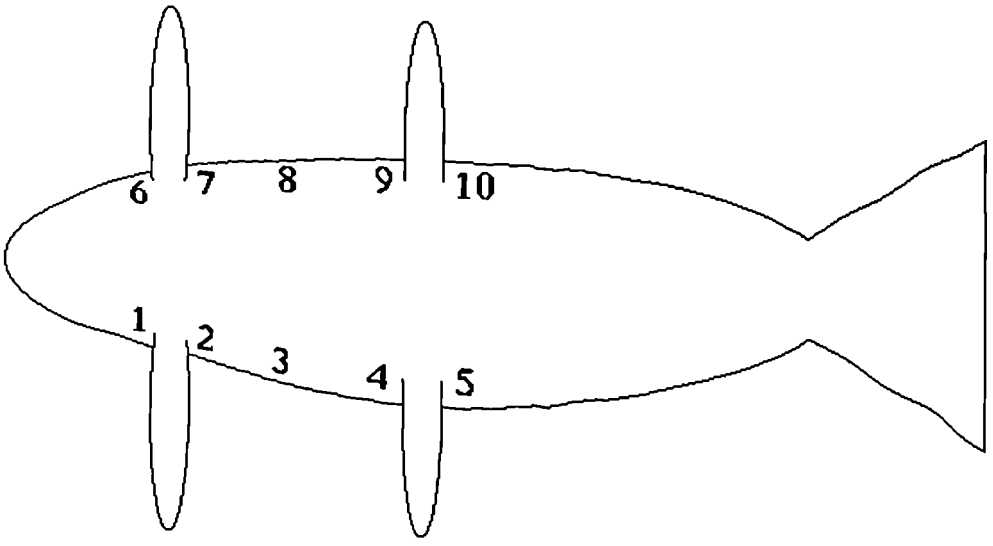
2.2.1 FISH HUSBANDRY.

Sea lice experimental infection experiments were conducted at the Marine Environmental Research Laboratory, Machrihanish, Argyll. Experiments took place in a variety of appropriate sized fibreglass tanks; 70 litre circular tanks, 300 litre square tanks and 736 litre circular tanks (see chapters 3 and 4 for specific details). Experimental systems had flow through natural sea water (except chapter 3, salinity experiment). All experimental tanks had opaque lids to prevent undue disturbance and stress to the fish. Daily sea water temperature, salinity and flow rates were recorded in each experiment. Experimental tanks received a constant air supply. Temperature, salinity and oxygen levels were recorded by a Dryden Aquaculture OxyGuard Handy Mk.2 or hand-held salinometer and thermometer. Flow rates were measured by timing a known volume of sea water on inflow. Flow rates within individual experiments were kept constant at 2 l min^{-1} [and adjusted where

necessary]. Machrihanish Marine Environmental Research Laboratory has a sub-surface pump ashore sea water supply to a main collection header tank, where prior to storage sea water passes through a 65μm filter. This sea water supplies all experimental tanks. The Marine Laboratory is situated on the west coast of the Kintyre peninsula specifically to prevent transmission of disease from neighbouring fish farms, the closest fish farm is a pump ashore site, c.20 miles away by sea.

All fish for the experimental purposes were supplied from the Institute of Aquaculture Howietoun hatchery, Stirling, as smolts before transfer to Machrihanish Marine Laboratory for rearing. Fish had not previously been exposed to sea lice infection and thus were naive. Two weeks prior to the start of an experiment, all fish were randomly removed from stock, weighed to the nearest gram, measured (body length) to the nearest mm and then panjet marked, injected under pressure with Alcian blue dye (1% w/v). In most cases a single dot marking scheme, on the ventral surface was used, as shown in Figure 2.1. When necessary, for numbers greater than 10, a combination multiple dot marking scheme was utilised.

Figure 2.1. Schematic diagram of fish numbering system. Numbers indicate the corresponding position of the panjet mark on the ventral surface.



After the fish had been introduced into their respective tanks statistical analysis of the body size and weight was carried out to ensure there was no significant statistical difference between experimental groups.

Fish were fed throughout at a daily regime of 1% wet body weight with a commercial dry pellet diet, BOCM Pauls Fulmar Hyper 2.5 and 3.5mm.

2.2.2 LICE SAMPLE COLLECTION.

Gravid female *L.salmonis* were collected at harvest from several commercial Atlantic salmon farms on the west coast of Scotland. Fish were killed, prior to handling by a blow to the head. Once the host had been culled, all lice were gently removed by lifting them off with the use of curved forceps. Lice that had been picked off the fish were placed in clear plastic bags (63 x 48 cm) containing on-site sea water, at approximately 200 lice per bag. Before departure from the site, the sea water was completely changed to ensure that lice were transported in uncontaminated sea water. Any ovisacs detached from the lice after removal from the host and in the plastic bag were caught by use of a mesh sieve at the time of water change. Once the water had been changed the bag was double bagged and both were sealed and placed in insulated cool boxes on crushed ice for transport. Journey time from harvest site to either Machrihanish or Stirling was no longer than three hours.

2.2.3 OVISAC INCUBATION AND HATCHING.

Incubation of the ovisacs was undertaken either in a constant temperature room (10°C) at the Institute of Aquaculture, Stirling University or at ambient sea water temperatures at the

Machrihanish Marine laboratory. On arrival at either location, gravid female lice and detached ovisacs were transferred and held just beneath the surface of their incubation vessels in mesh sieves positioned over containers of static sea water. Incubation vessels were supplied with fresh (60µm filtered) sea water and provided with a constant trickle air supply. Incubation vessels utilised for all the settlement experiments were plastic buckets (9 litre volume) or, for smaller volume incubations, plastic aquaria (2.5 litre volume). All incubation vessels were held in water baths to maintain the correct sea water temperatures.

i) For incubation at the Institute of Aquaculture, Stirling University. Salinity was maintained at full strength sea water (34/35‰) and measured by use of an ATAGO hand held salinometer/refractometer. If the salinity of the sea water from the Institute supply had to be reduced, fresh tap water was used for the dilution but only after the water had air bubbled through it to remove chlorine. Whenever possible, sea water from the farm site from which the lice were obtained was used for the daily water changes. Incubation at the laboratory was under a 12 hr light : 12 hr dark timed lighting regime and under constant temperature conditions at 10°C.

ii) For incubation at Marine Environmental Research Laboratory, Machrihanish. Ambient sea water conditions, which were monitored daily, were used for incubation unless specific temperature and salinity experiments were being carried out. In these cases the incubation regime mirrored the experimental regime (see Chapter 3). Where age-determined copepodids were required for experimental infections, each day's hatch was separated to allow careful observation and assessment of the larval development (see Chapter 4). Ambient light was used although the incubations took place within tanks with opaque fibre glass lids. Where a controlled temperature environment was not required for the incubations of the ovisacs and larvae, the incubation vessels were kept bathed in a constant

flow of ambient temperature sea water within larger tanks.

Lice stages were checked daily for development by observation under a dissecting microscope. If large numbers of larvae hatched in a 24 hour period, the density was reduced by separating the copepodids by volume and diluted to facilitate counting. The use of plastic sieves during incubation facilitated the removal of dead or moribund lice (noted by the pink coloration) and the spent egg strings and allowed any newly hatched larvae to fall through into the water column of the incubation vessels. The sieves also allowed the quick and easy transfer of the live ovigerous female lice to a new incubation vessel to separate each day's hatch, when necessary. The daily removal of dead female lice and spent egg strings was necessary to maintain the optimum quality of the water during incubation. To further maintain the water quality, a daily 75% water change with filtered sea water (60μm mesh filter) was carried out.

2.2.4 EXPERIMENTAL INFECTION PROCESS.

On daily examination of the incubation vessels, a number of 10ml sub-samples of larvae were taken to determine the life stages present and their numbers (see below). Once it had been determined that sufficient copepodids were present (>60% of the total larval population), a more accurate count of the incubation vessel population was carried out. Copepodid counts of 7 x 10 ml aliquots were taken to determine the numbers present. A 10ml Bogorov tray, under a dissecting microscope, was used during examination to correctly identify the larval stages and the number of each stage present. Only active larval stages were recorded. Once counts of the stages present were complete, an estimate of the total numbers of copepodids present in the incubation vessel could be calculated. The

highest and lowest values (outlier values) from the sub-sample were discarded to allow the calculation of an average within the incubation vessel.

Eq 2.1 Calculation of sea lice life stages from aliquots taken from incubation vessels

$$\frac{\text{No. of Lice stage present in sub-sample}}{\text{Vol. of sub-sample}} \times \frac{\text{Water Vol. in incubation container}}{1}$$

Once sufficient numbers of copepodids (or of the correct age, see Chapter 4) were found to be present in the incubation vessels, they were then utilised for experimental infections of fish. An approximate infection dose rate of 200 copepodids per fish was used with copepodids no more than two days old after the nauplius 2 moult. If the incubation vessel was found to contain too many copepodids, copepodids were removed (by volume) to give the required number for infection and the water level returned to the standard 8 litres volume. All copepodids for the experimental infection of fish were introduced on the same day and at approximately the same time.

Before any infection, the inflow to the tank was shut off, and the water level was lowered to reduce the volume of tank water to accommodate the introduced volume of water with the copepodids. This also prevented any possible loss of copepodids to the outflow and allowed maximum contact between the copepodids and the fish. The required number of copepodids was introduced, by gentle pouring, into the tank of fish. At the introduction of the copepodids into the experimental tank, initial fish behaviour, was observed for signs of stress such as jumping and rapid swimming.

The experimental infection process was carried out over a period of 8 hours, during which the water supply remained off. The water was kept aerated throughout the infection period

to ensure sufficient oxygenation for the survival of the fish and to increase the homogeneous mixing of the copepodids throughout the experimental tanks. The infection process was monitored periodically to ensure no water loss occurred from the tank stand pipes and that the fish were not unduly stressed, as judged by their behaviour. During the summer months, at higher sea water temperatures, a greater number of observations were undertaken. If water levels during infection were found to be low the tanks were replenished to the correct tank volume, although not to over flowing. Feed for the fish was withheld on the day of infection and on days of sampling.

At the end of the infection period the water supply to all tanks was turned back on and the tanks flushed to remove any remaining unattached copepodids. After the tanks had been flushed, the flow rates were measured and returned to 2 l min^{-1}. Fish behaviour was observed again to determine any signs of undue stress of the fish.

2.2.5 FISH ANAESTHESIA.

All examinations of fish, either at initial handling for introduction into an experiment or sampling for sea lice, was conducted under light anaesthesia, i.e. the operculum showing slight movement. Two anaesthetics were used in the experiments, initially Benzocaine and then later MS222. Benzocaine (Sigma, $C_9H_{11}NO_2$, Ethyl p-aminobenzoate) was found to have a pernicious effect on lice survival (Unpublished data, Institute of Aquaculture) and its use was discontinued. A stock solution of Benzocaine (100g Benzocaine in 1l of absolute alcohol) was prepared and stored in a dark glass bottle. For anaesthesia, 10ml of Benzocaine in absolute alcohol (10% solution w/v) was added to 10 litres of filtered fresh sea water. Benzocaine acts as a surface anaesthetic of the ester type (Stoskopf, 1993).

MS222, Tricaine Methane Sulfonate (Sigma, $C_9H_{11}NO_2 \cdot CH_4SO_3$, 3-Aminobenzoic Acid Ethyl Ester) is a fine, white crystalline powder which is highly water soluble and used at a dose of 100 ppm. MS222 acts on the muscular activity of the organism (Stoskopf, 1993).

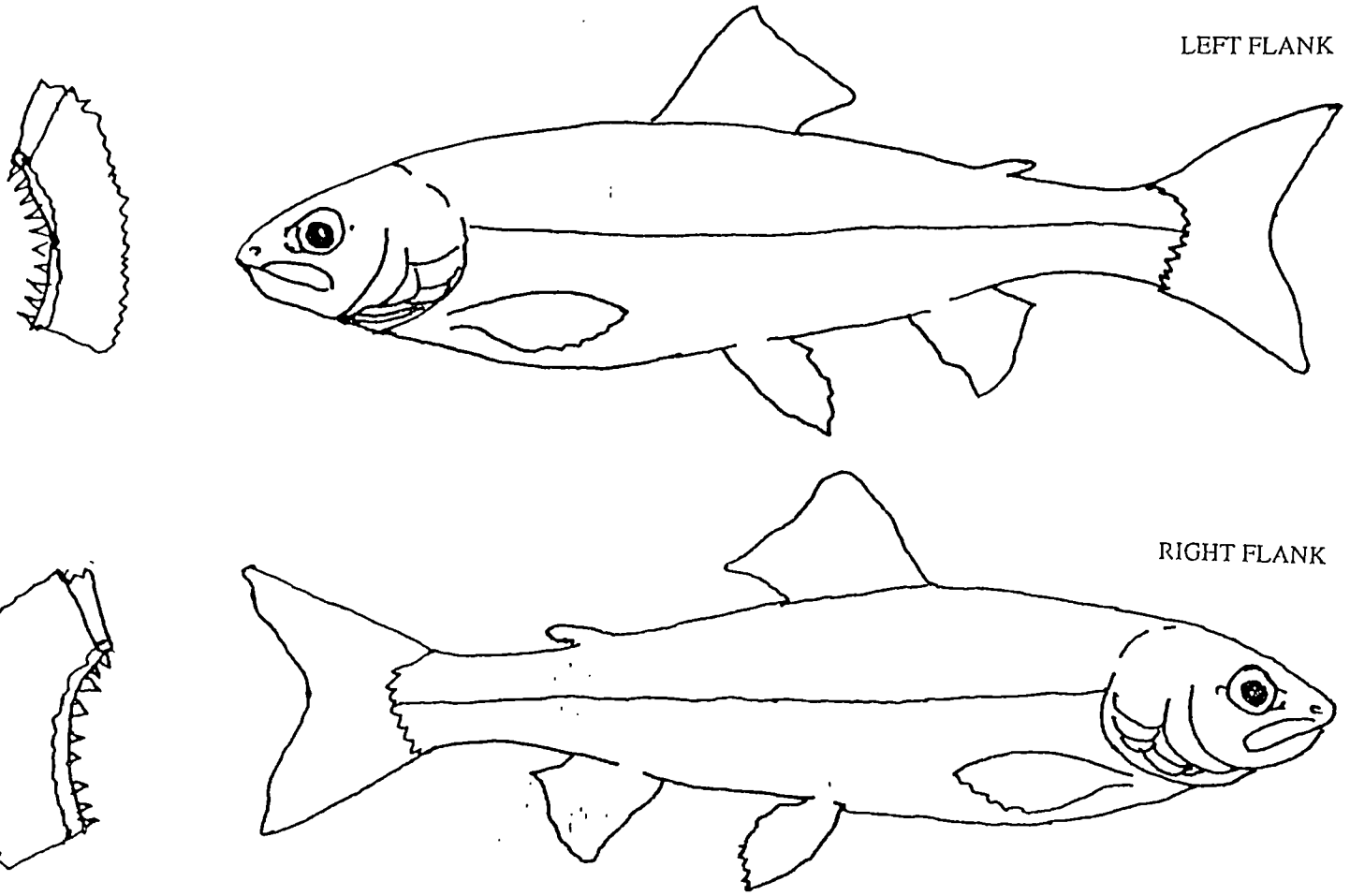
All fish anaesthesia and euthanasia was conducted in buckets containing 10 litres of anaesthetic at the appropriate concentration. At higher sea water temperatures, the anaesthetic was periodically aerated to prevent fish mortalities. Within an individual experiment, either Benzocaine or MS222 was used and thus any effects due to the change in the anaesthetic used during this study on sea lice would be uniform within that experiment.

Once fully examined, the fish were placed in a recovery tank with fresh aerated and flowing sea water.

2.2.6 FISH SAMPLING PROCESS.

All fish in the settlement experiments were examined at post-infection day 5 (D.P.I. 5) and post-infection day 10 (D.P.I. 10). Using a dissecting microscope for examination (see section 2.2.7) the number, developmental stage and position of each *L.salmonis* on the host was recorded on a fish map (see Figure 2.2). At D.P.I. 5 fish were examined under anaesthesia, whilst at D.P.I. 10 all fish were individually killed with an overdose of anaesthetic and then examined. Fish under examination (D.P.I. 5 and 10) were maintained in their respective experimental environmental conditions, correct experimental temperature and salinity regimes were maintained during anaesthesia and euthanasia. If preadult or adult life stages were found to be present on a fish, then the anaesthetic container was filtered, after each fish had been sampled, to collect any lice that may have

Figure 2.2 Schematic diagram of a fish map utilised for sea lice examination counts during abiotic and biotic settlement experiments.



KEY:		
C – copepodite	7 = Pre adult 2 male	G = Gravid female
1-4 = Chalimus 1-4	8 = Pre adult 2 female	
5 = Pre adult 1 male	9 = Adult male	
6 = Pre adult 1 female	10 = Adult female	

been detached during anaesthesia. The detached lice were counted but their former position of attachment on the body could not be ascertained.

2.2.7 MICROSCOPY.

2.2.7.1 LIGHT MICROSCOPY.

All fish examined in the settlement experiments and post settlement energy level experiments (Chapters 3, 4 and 5) were inspected, under anaesthetic, by use of an Olympus SZ30 binocular dissecting microscope, at x10 magnification with incidental light, Olympus TL2 or an Olympus highlight 300 fibre optic incidental light. Compound microscopes used were an Olympus CH-2 and BH-2 with planar lenses and standard interface facilities for histological examination and photography. An Olympus SC35 camera with Fujichrome T-64 slide film was used for all photomicroscopy.

2.2.7.2 SCANNING ELECTRON MICROSCOPY (SEM).

Specimens for SEM were initially fixed in 1% glutaraldehyde in 0.1M sodium cacodylate at 4°C for 1 hour and then transferred to 3% glutaraldehyde in 0.1M sodium cacodylate at 4°C for further fixation and storage prior to processing. After fixation, samples were washed in sodium cacodylate buffer for one hour before post-fixing with 1% osmium tetroxide in buffer for two hours. If fish mucus was present on specimens an additional step of washing with 16% glycerol in a rotator for 8 hours and then a further wash with 20% ethanol, (three changes of two hours each), to remove the glycerol was included.

The specimens were dehydrated through a graded ethanol (30%-100%) series before

transferring directly to HMDS (Nation, 1983) for drying. Dried specimens were mounted on aluminium stubs with double-sided stickers before they were gold sputter-coated with an Edwards S150B sputter coater, specimen distance 3cm, a current of 40mA and voltage of 0.8Kv. SEM examination was performed with a Philips 500 SEM running at 12Kv or ISI60A at 15Kv. All SEM photographs were taken with Ilford FP4 film.

2.2.8 STATISTICAL ANALYSIS.

As many parasitic communities have a negative binomial distribution (Anderson & May, 1995), statistical analysis was carried out with this in mind. Before the application of a statistical test, the variance ratio of the data was determined to test for data distribution. The Kruskal-Wallis test, Mann-Whitney U-test and Dunn's test were used for non-parametric data. If data were found to be normally distributed then ANOVA and T-tests were employed.

2.2.8.1 ESTIMATION OF THE MEAN.

The sample or arithmetic mean ($\bar{x}$) gives the best estimate of the population mean μ. This is however only true of normally distributed data. Use of the mean value and standard deviation, although possibly incorrect for those data sets that are not normal, allows the summary comparison of the data with each other. The arithmetic mean and standard deviation have been used to summarise (rather than analyse) the data presented, regardless of its distribution, allowing an overview of the data and comparison with the literature.

2.2.8.2 HOMOGENEITY OF VARIANCES.

F-test

The F-Test establishes the departure of the variance ratio of two samples from unity. Where the homogeneity of a number of samples was required, only the largest and smallest variances were tested since, if these samples are not significantly different from each other, then the others cannot be. The sample calculated value of F_s was then compared with the tabled value of the distribution of F at P, probability = 0.05 (5%). If the calculated value of F_s was less than the tabled value of F then the sample variances were considered to be homogeneous and therefore the data was not suitable for non-parametric analysis. Should the variances be equal then the data is considered to be normally distributed.

2.2.8.3 TWO SAMPLE COMPARISON.

T-test (small samples) or Z-test (large samples).

After testing for the variance ratio, a T-test (<30 observations) or Z-test (≥30 observations) was applied if the data were found to be normally distributed. If the calculated value for the T-test or Z-test, with the appropriate degrees of freedom for the test, exceeded the corresponding tabulated value at P = 0.05 (5%) the difference between means was considered to be statistically significant.

Mann-Whitney U-test.

This test is a non-parametric technique for comparing the difference in medians of two unmatched samples, an analogue of the T-test. It may be used for as few as four observations in each sample. The Mann-Whitney U-test was employed on ranked data

from independent samples. Although this test is non-parametric it assumes that the distributions of the two data sets are similar.

2.2.8.4 MULTIPLE SAMPLE COMPARISON.

ANOVA

Where sample variances were found to be homogeneous and the data therefore normally distributed, one way analysis of variance ANOVA was employed. This test allows a comparison between any number of sample means in a single test.

Kruskal-Wallis test

This test is a non-parametric technique which allows the comparison of medians of several samples, although the sample sizes do not have to be equal. If there are only three samples then there must be at least five observations in each sample.

Dunn's test

The limitation of the Kruskal-Wallis test is that it does not indicate where differences occur between the samples, merely that there is a statistically significant difference in treatments. To pinpoint where these differences occur, a pairwise Mann-Whitney U-test could be used but this would increase the probability of a Type 1 error, i.e. raise the probability of rejecting the true null hypothesis. Dunn's procedure provides a multiple comparison procedure for comparing treatment medians and indicates where the differences lie between the samples (Zar, 1984).

2.2.8.5 CONFIDENCE LIMITS OF A PERCENTAGE OR PROPORTION.

Where multiple comparisons of calculated percentages between experimental groups was required, 95% confidence limits were utilised according to Fowler & Cohen (1992). The calculation of confidence limits is executed by multiplying the standard error of the sample proportion by 1.96 (for 95% C.I.). The estimate of the standard error from a sample proportion is derived from the formulae:

$$\text{S.E.} = \sqrt{\frac{p(1-p)}{(n-1)}} \quad \text{or} \quad \text{S.E.} = \frac{S.D.}{\sqrt{n}}$$

Where p = sample proportion

n = number of units in the sample

S.D. = standard deviation

95% confidence intervals are obtained by multiplying the standard error by the appropriate *z-score* as follows:

95% confidence intervals $\bar{x} \pm 1.96$ S.E.

Calculation of the 95% confidence limits allows graphic comparison of the percentage losses of parasites from specific host body regions and statistical analysis of proportionally determined data.

2.2.8.6 COMPUTER PROGRAMMES USED AND REFERENCES FOR STATISTICAL TESTS.

The major sources of information for the statistical tests utilised for this study were Sokal & Rohlf (1981), Fowler & Cohen (1992), McKenzie, Schaefer & Faber (1995) and

Gardiner (1997). Most statistical tests were conducted using the student edition of Minitab® for Windows statistical software, but for the Dunn's non-parametric test, a computer program stprog3.exe, provided by Dr J. Bron, Institute of Aquaculture, was used.

CHAPTER 3. ABIOTIC SETTLEMENT FACTORS.

3.1 INTRODUCTION.

The environment within which any organism lives must be close to its optimum to ensure survival. As *Lepeophtheirus salmonis* is a poikilothermic and stenohaline organism all metabolic functions will be governed by its external environment and therefore sea water temperature and salinity will have distinct influences. Sub-optimal environmental conditions will retard growth and development or be lethal. Any organism in its natural environment is subject to a variety of changes and will respond to the total resulting stimuli or stresses rather than single environmental fluctuations. The response of the organism to the complex interactive environment is multidimensional (Kinne, 1970a) and is therefore difficult to study and interpret. However, in the laboratory the importance of individual environmental factors can be determined experimentally in controlled environments both separately and in combination.

3.1.1 TEMPERATURE.

All biological processes require heat and are restricted by ranges of temperature tolerance. With regard to life on Earth, temperature is -next to light- the most potent environmental component (Kinne, 1970a). Marine poikilothermic organisms are decisively affected by the temperature of the surrounding body of water. Temperature is the environmental parameter that fluctuates considerably over the salmon production cycle and thus affects *Lepeophtheirus salmonis* and its development. Seasonal variability of sea water

temperatures on the west coast of Scotland can range from 4-5°C in winter to 14°C+ in the summer and extremes up to approximately 19°C.

Much information is available on the effects of temperature on developmental rate of *L.salmonis*, both pre and post hatching. Wootten *et al.* (1982) suggested that at temperatures between 9-14°C, summer/autumn sea water temperatures, the life cycle of a single generation takes approximately six weeks. Other authors extended this time to 7.5-8 weeks at a constant 10°C (Johnson & Albright, 1991b). Temperature effects on individual life stages have been examined by a number of authors, Wootten *et al.* (1977), Tully (1989), Johnson & Albright (1991a, b) and Johnson (1993), all agree that there is an inverse relationship between the development time of *L. salmonis* larval stages and temperature. Johnson & Albright (1991a) state that at 10°C development from egg to adult female takes 52 days. Johannessen (1978) observed the robust nature of copepodids when in a single day temperature fluctuated from 19°C down to 10°C rising again to 21°C, with no visible adverse effects. Temperature is the environmental variable that is most likely to fluctuate over the generation time of *L. salmonis* and thus effect the infective and parasitic stages of the parasite and the response of lice to such changes will be an important consideration in the planning of fish farm management strategies.

3.1.2 SALINITY.

Kinne (1970b) states that invertebrates considered euryhaline tolerate salinity ranges of 10‰ to 30‰ where prerequisites of high cellular tolerance and high capacities for osmoregulation are required. Salmon have an optimum salinity for growth of 33-35‰ (Sedgewick, 1988). Any change in the ambient salinity will not only have an effect on the

salmon host but also on the parasite. There is anecdotal evidence that salmon farm sites situated with a large fresh water run-off suffer less from epizootics of sea lice, *i.e.* Loch Leven (Marine Harvest McConnell Site) and Loch Fyne (Lighthouse).

It has long been considered that sea lice seen on salmon running up river is indicative of a fresh run fish and is therefore viewed with favour by sports fishermen. Such euphoria may be generated by the misguided belief that salmon lice quickly die once the salmon host has entered fresh water. The louse life stages that would be directly visible to the sports fisherman would be the preadult and adult stages and recent studies have been conducted on their survivability. McLean, Smith & Wilson (1990) conducted two studies with a total of 10 estuary caught salmon to examine the duration of survival of lice on the host entering fresh water. When fish were quickly transferred to fresh water (1.5‰ and 0.5‰) at summer water temperatures (12.8-16°C) less than 20% of lice remained after 48 hours, the remainder lasted 6 days. The maximum number of lice, at the start of the experiment, on a single fish was 43 but the senility of the lice was unknown, as were the pre-capture movements of the host. Hutton (1923) suggested that females could remain attached for 7.5 days at 7.5°C. Ashby (1951) suggested a survival time of 25 days at a low temperature of 8°C; however these lice were reported to be under the mucous layer of the host. Finstad, Bjørn & Nilsen (1995) used captured charr (*Salvelinus alpinus* L.) to assess the survival of *L.salmonis* motile stages in fresh water. In this study, Finstad *et al.* found that 21% of *L.salmonis* survived for two weeks in fresh water, although two charr had lice remaining at three weeks. Survival over the two week experimental period of the control lice in sea water (34‰) was 100%. Kabata (1981) highlights misgivings over the study by Berger (1970) who found 100% mortality of adult lice held for four hours in water with a salinity of 8‰, which was in contradiction with the finding of lice on *Oncorhynchus nerka*, 60 miles upstream in full fresh water, an impossible distance to cover in four hours. Wootten

et al. (1982) agreed with Kabata (1981) concluding that *L.salmonis* can survive for long periods in fresh water. There are therefore many contrasting reports on the survival of *L.salmonis* in fresh water, but all these studies have been conducted on salmonid hosts that have been caught and not on hosts that have been artificially infected with a lice population of a known history.

Kinne (1970b) states that early ontogenic stages of many invertebrates exhibit lesser tolerances to salinity than their respective later and adult stages. The sensitivity of *L.salmonis* free-swimming stages has been investigated by several authors. Wootten *et al.* (1977) found that, at 12°C, *L.salmonis* eggs hatched at all salinities above 10‰ but that development to copepodids only occurred above 24‰. Johannessen (1978) demonstrated that between 5-12°C, all *L.salmonis* eggs were aborted at 11.5‰. Johnson & Albright (1991a) tested the development, growth and survival of *L.salmonis* at a number of temperatures (5-15°C) and salinities (10-30‰). These authors found that copepodids only survived one day at 10‰ whilst all survived at 30‰. *L.salmonis* eggs developed but failed to produce nauplii at 15‰, however when transferred to higher salinities survival to the copepodid was increased (Johnson & Albright, 1991a). Berger (1970) suggests that the free-swimming lecithotrophic planktonic stages are less sensitive to salinity changes above 8‰ than the adult stages, an opposite situation to that which is true of other parasitic copepods (Kabata, 1981). Heuch, Parsons & Boxaspen (1995) suggests that copepodids are very sensitive to salinity changes and utilised water stratification, accumulating at the halocline interface. Johnson & Albright (1991a) concluded that in low salinities (<15‰) *L.salmonis* may be excluded due to reduced hatching success and larval survival.

In these experiments the ovisacs, nauplii and copepodids are being exposed to a continuous salinity of 24‰ and 34‰ prior to infection, to assess their ability to infect the host.

3.2 STUDY AIMS.

The work in the present chapter sought to investigate the effects of temperature and salinity on settlement and survival of *L.salmonis*. Previous works have described the influence of temperature and salinity on hatching and the post-hatching phase prior to infection and settlement on the host. No work has been done on the effect on settlement and survival. The understanding of these two important environmental variables will give a better comprehension of the transition from the free-swimming phase to the parasitic phase and the subsequent effects on the population dynamics.

3.3 MATERIALS AND METHODS.

3.3.1 FISH HUSBANDRY FOR TEMPERATURE AND SALINITY EXPERIMENTS.

Fish were maintained at their respective experimental regimes for three weeks prior to infection to allow acclimation to the experimental conditions. Any alteration from ambient was achieved slowly, by a reduction of 2°C per day in the case of temperature or 2‰ per day in the case of salinity, to prevent distress to the fish. S½ salmon smolts were utilised for all temperature and salinity experiments, 30 per experimental group and 10 fish per replicate. Fish, each approximately 100g were prepared for inclusion in an experiment as described in chapter 2.2.1.

For the temperature experimental conditions ambient winter sea water was used for the cold water supply whilst a heating system (Imperuio 3KW Polaris submersible heating

element) was installed in a flow through experimental header tank to provide sea water at the elevated temperature. For the purpose of this study the cold ambient supply of sea water will be referred to as cooled water and the raised heated supply of sea water will be referred to as heated sea water. The heated water temperature mirrored that of the ambient system in that any rise in the ambient temperature would result in a comparable rise in the heated system. Daily recordings of sea water temperature were taken at mid morning and mid afternoon to give a daily mean value. With thermostatic control of the raised temperature an approximate difference of 5°C was maintained between the two temperature regimes. Fish for the temperature experiment were held in flow through systems with a flow rate of 2 l min^{-1}.

The salinity experiment was conducted at two constant salinities, 25‰ and ambient 34‰. A re-circulation system was used to achieve a constant lowered salinity of 25‰ whilst the control group received flow through sea water of ambient salinity. To reduce the salinity sea water was diluted with fresh water that had been stored for 36 hours and that had air bubbled through it to remove any chlorine agents. Salinity and temperature was recorded in both experimental and control groups by use of a hand-held salinometer and thermometer. The experimental environmental conditions for sea water temperatures were recorded twice per day to obtain a daily mean value whilst salinity was recorded at the time of water exchange.

Daily sea water changes (>75%) were made in the re-circulation system to provide fresh sea water, thus preventing the accumulation of waste metabolites. Nitrite (NO_2^-) and ammonia (NH_4) were monitored daily by use of Tetra Werke water test kits (TetraTest Nitrit NO_2^- and TetraTest Ammonia NH_3/NH_4^+). Due to high ambient air temperature in the summer a chilling unit (Grant Instruments C2G Series No.4) was installed, in the

experimental header tank of the re-circulation system to depress sea water temperatures to that of ambient sea water.

In the 1997 experiment with the reduced salinity regime the chiller unit failed, due to mechanical breakdown, which resulted in an increase in water temperature in the 24‰ salinity regime. A continuous flow rate of 2 $lmin^{-1}$ was maintained for both experimental groups in each experiment.

For each experiment, temperature effects on settlement and salinity effects on settlement were repeated in different years to confirm results. The temperature experiments were carried out in March 1996 and March/April 1997, the salinity experiments were conducted in July 1996 and July 1997

3.3.2 SAMPLE COLLECTION OF LICE.

Sea lice used to provide copepodids for each experiment were collected from a single naturally infected salmon farm site as described in chapter 2.2.2. Incubation of lice for the individual experiments was conducted at the specific experimental environmental conditions.

3.3.3 INCUBATION OF OVISACS FOR TEMPERATURE EXPERIMENT.

Incubation of the ovisacs was carried out at the same sea water temperature as those for the experimental regime and the acclimatisation of the experimental fish. The static incubation vessels were insulated with polystyrene and positioned, to be bathed in the outflow water from the experimental tanks to maintain the correct sea water temperature. Any change in

the ambient temperature required for incubation and experimental purposes was achieved slowly, at a reduction of 2°C per day, to allow full acclimation of the lice. Daily sea water changes were carried out in the static incubation system to maintain optimum environmental conditions for incubation of the ovisacs. Ambient salinity was utilised throughout for the incubation of eggs in the temperature experiments.

3.3.4 INCUBATION OF OVISACS FOR SALINITY EXPERIMENT.

Environmental conditions for the incubation of ovisacs was maintained to coincide with the correct experimental salinity regime. The static incubation vessels were insulated and bathed in the outflow water from the experimental tanks to maintain the correct sea water temperature. Incubation was carried out in sea water which was transferred from the experimental tanks to maintain the correct constant experimental salinity conditions. Required reduction in salinity was carried out at a rate of 2‰ per day during the incubation.

3.3.5 INFECTION AND EXAMINATION PROCEDURE FOR THE TEMPERATURE AND SALINITY EXPERIMENTS.

Once sufficient copepodids were found to be present in the incubation vessels (as calculated in chapter 2.2.4) the standard infection procedure and examination were carried out as described in chapter 2.2.4. to 2.2.6. For each experiment six 70 litre tanks were used, 30 fish per experimental group which were held in three tanks, giving three replicates per group. Fish were maintained in their respective experimental environmental conditions during anaesthesia for examination D.P.I. 5 and 10. Settlement was assessed from the numbers of lice present at D.P.I. 5 and survival, those that remained at D.P.I. 10. At D.P.I.

10 fish were killed by an overdose of anaesthetic and lice counted. If preadult or adult stages were found to be present, then the anaesthetic was filtered after each fish to collect any lice that may have been removed during anaesthesia. For all experiments the anaesthetic used was MS222.

3.3.6 STATISTICAL ANALYSIS.

All statistics have been calculated on the numerical values with their respective (95%) confidence limits. General methods utilised for statistical analysis have been described in chapter 2.2.9. All replicates within experimental groups were tested for statistical analysis to ensure there were no statistically significant differences within the data before being pooled.

3.3.7 HISTOLOGY OF HOST.

Sections of host epithelium, taken from the mid body over the lateral line and fins were removed at D.P.I. 10 for examination of the presence and number of mucous cells. All specimens were fixed in 10% neutral buffered formalin prior to embedding for histological staining and examination. Specimens were dehydrated through a series of methanol, ethanol and chloroform in a Reichart-Jung Histokinette 2000 automatic processor. Sections were wax embedded, cut and stained, after drying overnight at 40°C, with PAS (Periodic Acid-Schiffs reagent, Drury & Wallington, 1980) for mucopolysaccharides and mounted on slides with Pertex mounting medium. Examination of the prepared slides was undertaken with a compound light microscope.

3.4 RESULTS.

3.4.1 SALMONID HOST.

3.4.1.1 HISTOLOGY OF THE HOST EPITHELIUM.

To exclude the possibility that host epithelial structure varied between experimental groups histological samples were taken for direct comparison. Host epithelium was examined to determine any differences in the number of mucous cells present between the host experimental groups. No examined differences in the number of mucous cells present were found (plates 3.1-3.4) between the experimental groups. Dr. H. Rodgers (Disease diagnostics, Institute of Aquaculture), confirmed these findings.

3.4.1.2 SALMON MEASUREMENTS.

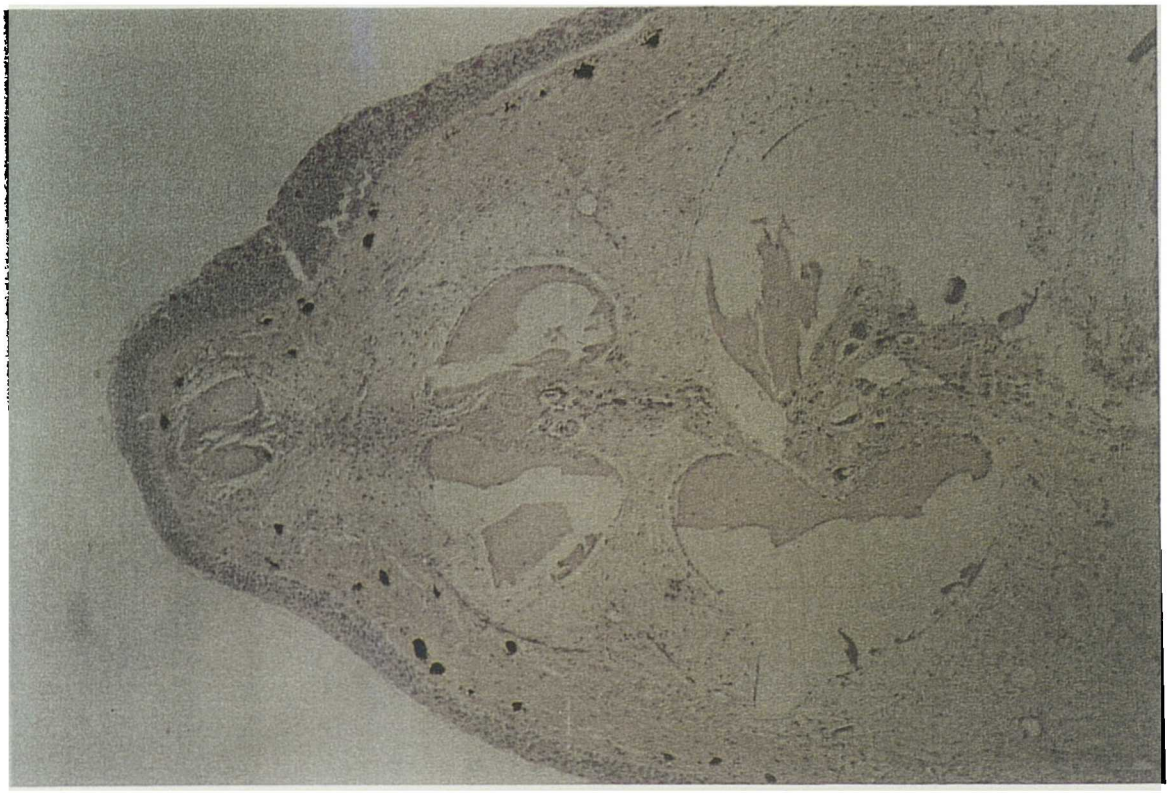
Statistical analysis of the mean fish weight showed no statistically significant difference ($p<0.001$) between the experimental groups in all experiments.

Plate 3.1 Histological section of the epidermis of salmon held in cooled water (≈7°C) for 5 weeks stained with PAS to show mucous cells.

Mag = x 40

Plate 3.2 Histological section of the epidermis of salmon held in heated water (≈12°C) for 5 weeks stained with PAS to show mucous cells.

Mag = x 40



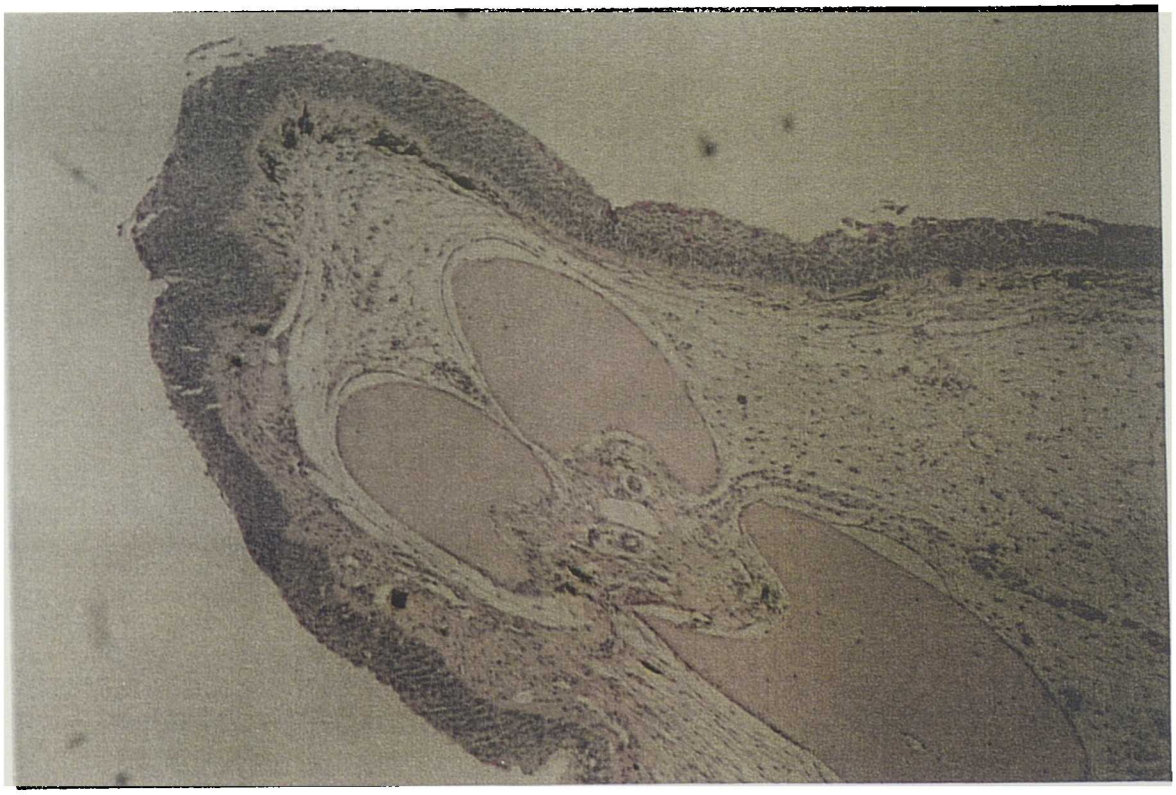
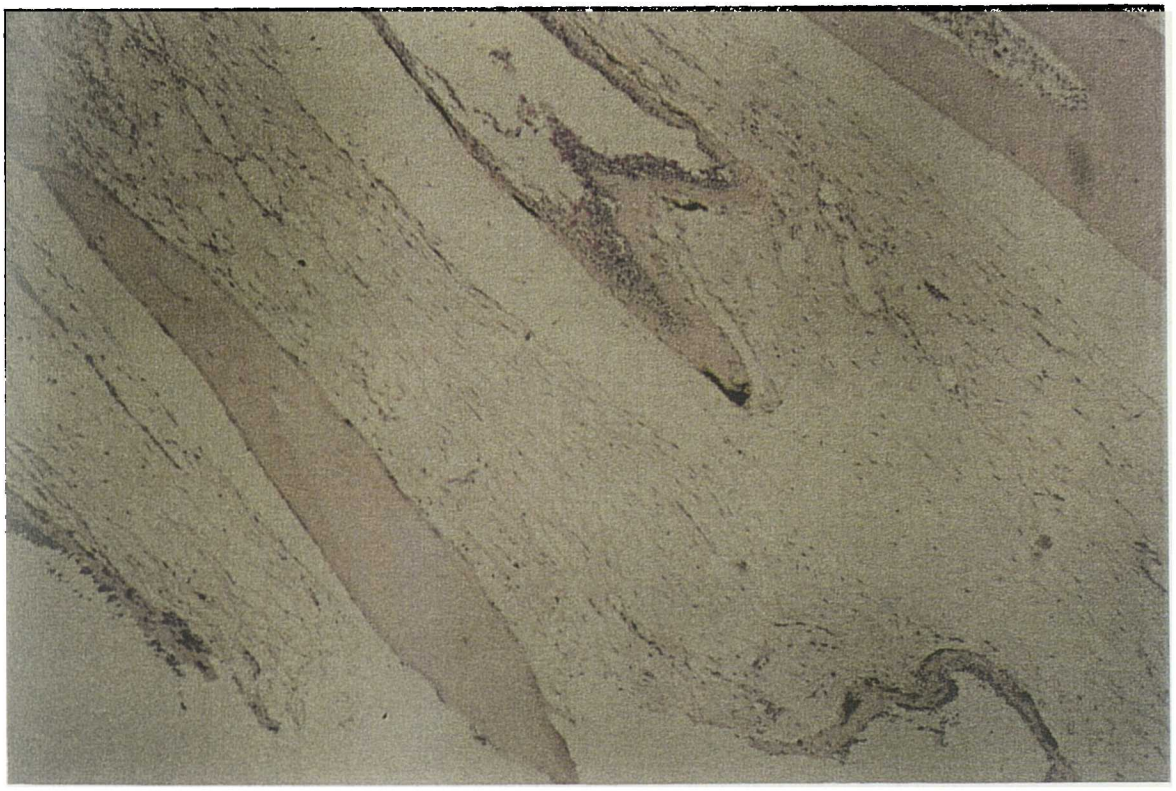


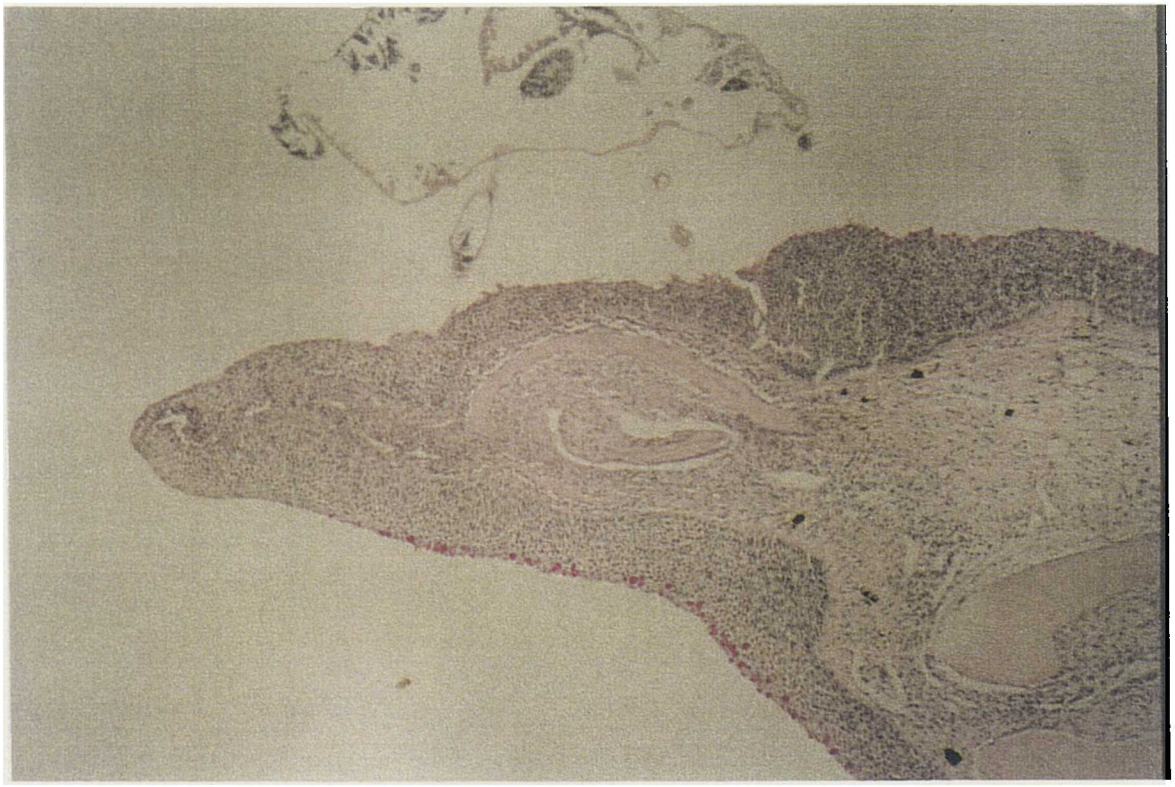
Plate 3.3 Histological section of the epidermis of salmon held in 24‰ salinity for 5 weeks stained with PAS to show mucous cells.

Mag = x 40

Plate 3.4 Histological section of the epidermis of salmon held in 34‰ salinity for 5 weeks stained with PAS to show mucous cells.

Mag = x 40





3.4.2 EFFECTS OF TEMPERATURE.

3.4.2.1 TEMPERATURE, ENVIRONMENTAL VARIABLES.

Sea water temperatures for experiments 1 and 2 are given in Table 3.1. Ambient salinity was used throughout both experiments.

Table 3.1. Environmental conditions during temperature experiments 1 and 2.

	Cooled Temp (°C)	Heated Temp (°C)	Salinity (‰)
Exp. 1 (1996)	7.1 ± 0.5	11.7 ± 0.6	34
Exp. 2 (1997)	6.9 ± 1.3	12.8 ± 1.0	34

3.4.2.2. TEMPERATURE EFFECTS ON SETTLEMENT AND SURVIVAL.

In both experiments when initial settlement and survival of the *L.salmonis* larvae were examined, a clear difference in the initial settlement success (D.P.I. 5) and subsequent survival (D.P.I. 10) between the two experimental groups can be seen (Table 3.2 and Figure 3.1 and Figure 3.1a).

Table 3.2. Percentage settlement and survival for both years of the temperature experiment.

	Cooled Water Settlement (%)	Heated Water Settlement (%)	Cooled Water Survival (%)	Heated Water Survival (%)
Expt1 (1996)	3.20	44.44	33.39	79.16
Expt2 (1997)	8.95	15.84	49.72	55.56

Figures 3.1 and 3.1a clearly illustrate the differences for the settlement and survival in both

Figure 3.1. Effects of temperature on the settlement and survival of *L.salmonis* on Atlantic salmon (1996/1)

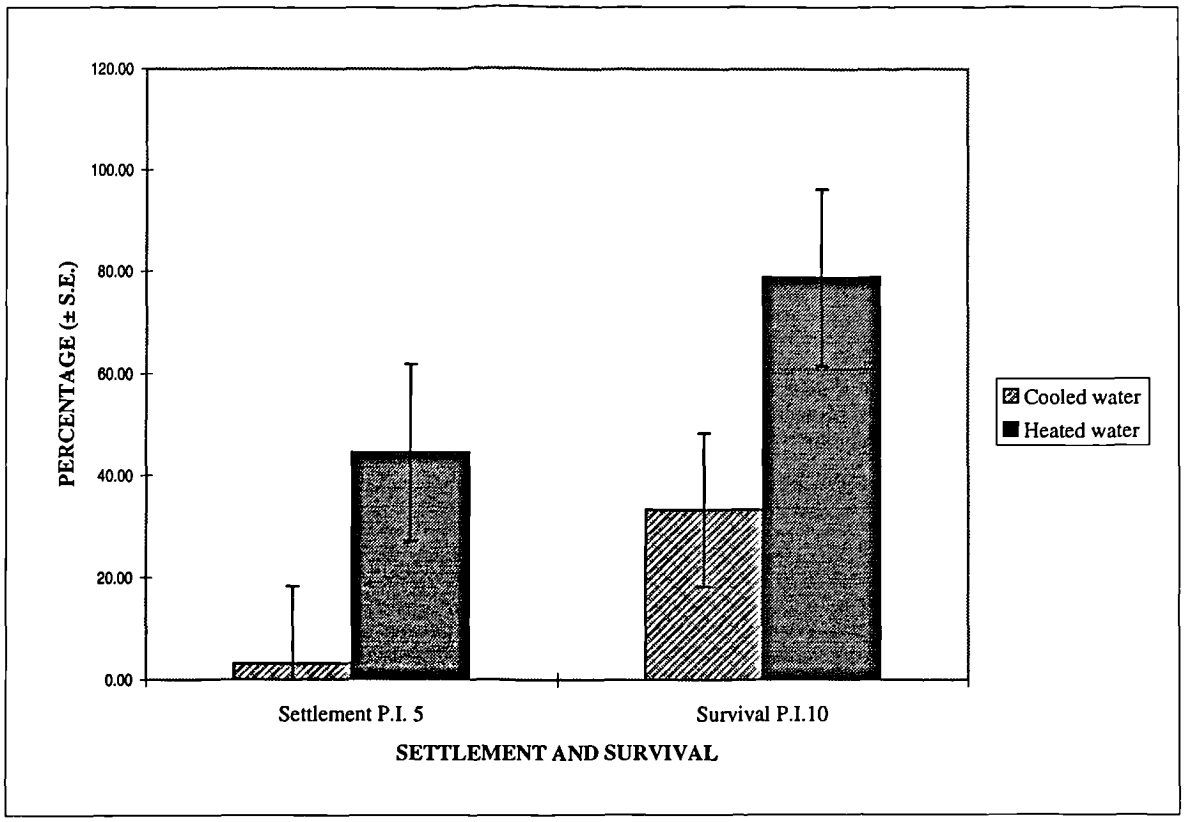
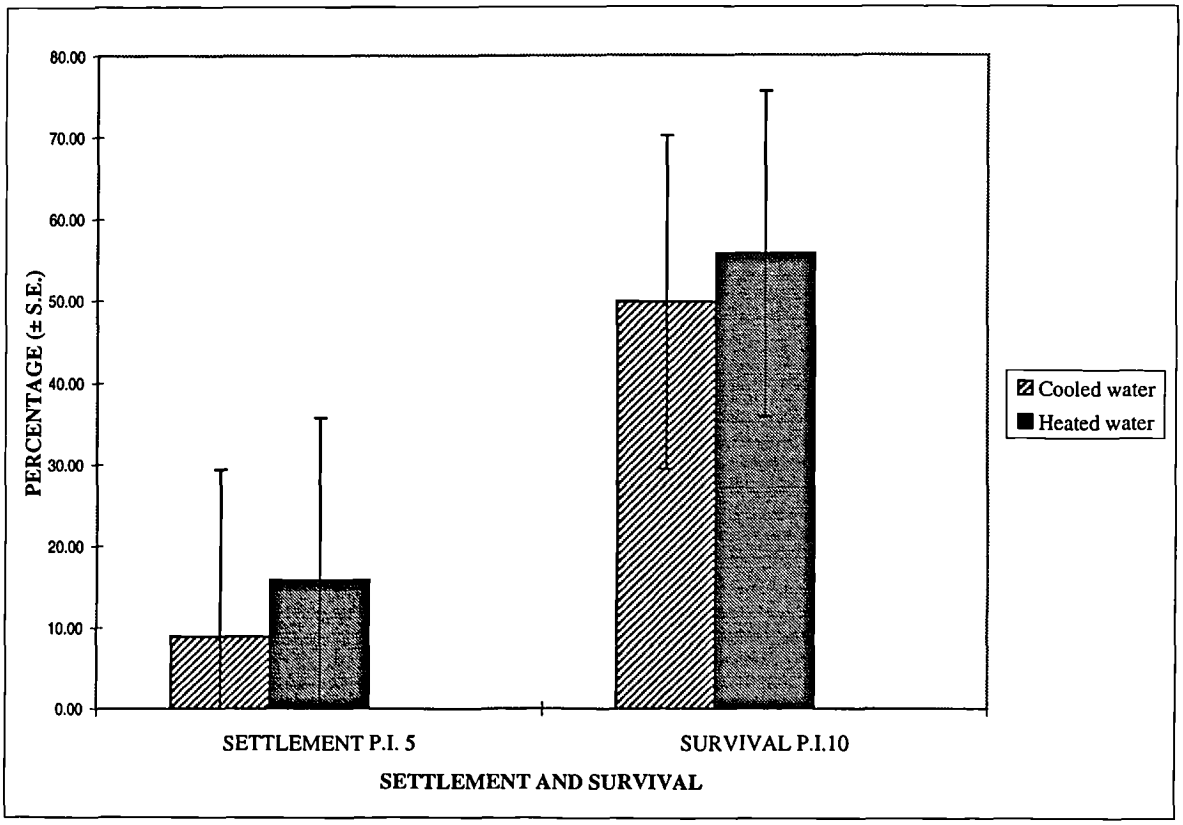


Figure 3.1a. Effects of temperature on the settlement and survival of *L.salmonis* on Atlantic salmon (1997/2)



years the experiments were conducted. In the 1996 experiment (Figure 3.1) a statistically significant difference ($p<0.001$) was found between the settlement and survival in the two different temperatures, the percentage number of lice settling on the host being 3.2% and 33.39% for the cooled and heated sea water temperatures respectively, a difference of 30.19%. A statistically significant difference ($p<0.001$) was also found in survival of larvae at day 10 D.P.I. where there was a difference of 45.77% between the two experimental groups.

In the 1997 experiment (Figure 3.1a), although the values were much closer a statistically significant difference of 6.5% ($p<0.001$) in settlement between the two experimental groups occurred. A statistically significant difference ($p<0.001$) was also found in the survival in the 1997 experiment with a difference of 5.94% between the two groups.

3.4.2.3. TEMPERATURE EFFECTS ON THE DISTRIBUTION OF THE PARASITE ON THE HOST BODY SURFACE.

Examination of host body regions for settled copepodids showed that they had preferred sites for settlement (Figures 3.2.and 3.2a.), particularly the gills and pectoral and dorsal fins. In the 1996 experiment (Figure 3.2), due to the low numbers of lice recovered in the cooled water regime there was a statistically significant difference ($p <0.05$) observed between the two temperature regimes for all the host body regions. The cooled water system has the highest settlement on the gills (>45%), pectoral and dorsal fins in both experimental years. However, the low numbers of copepodids settling in the cooled water system may introduce bias and therefore the results of the heated water system, where larger numbers have settled may be more representative of the natural settlement pattern.

Figure 3.2. Percentage settlement of *L.salmonis* by host body region at D.P.I. 5 (1996/1)

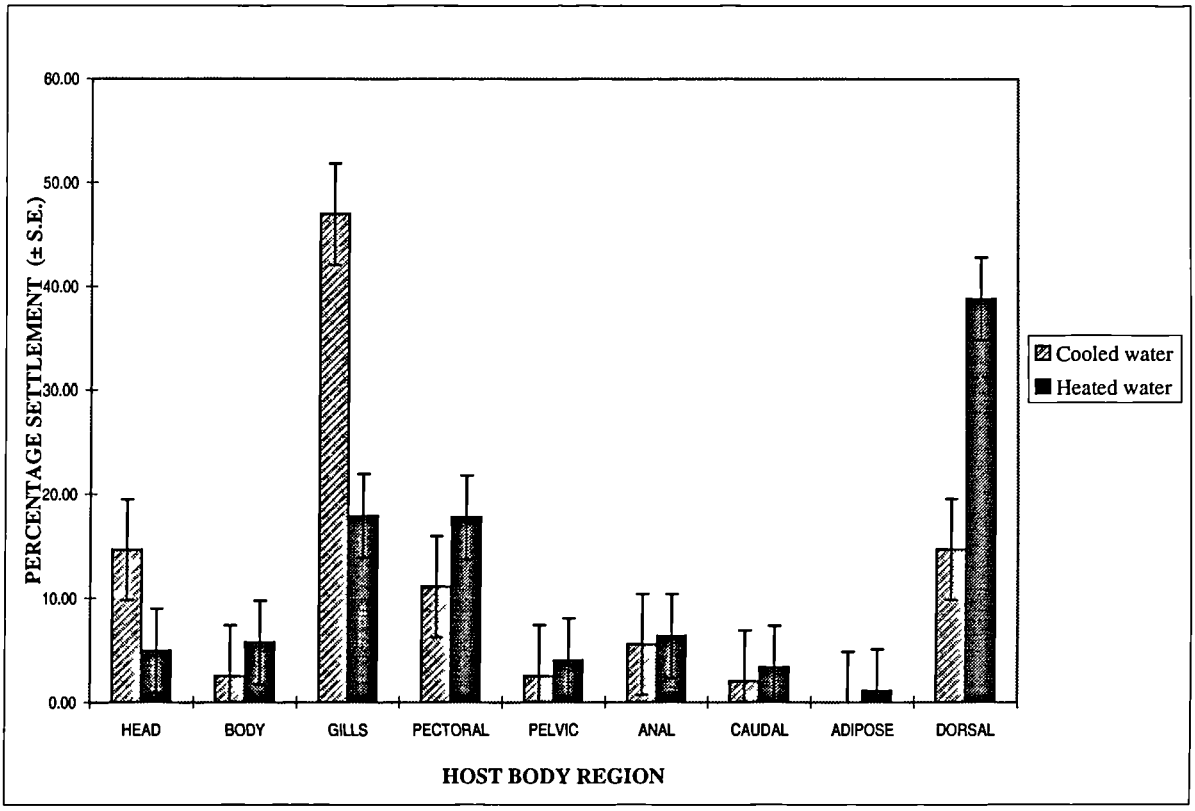
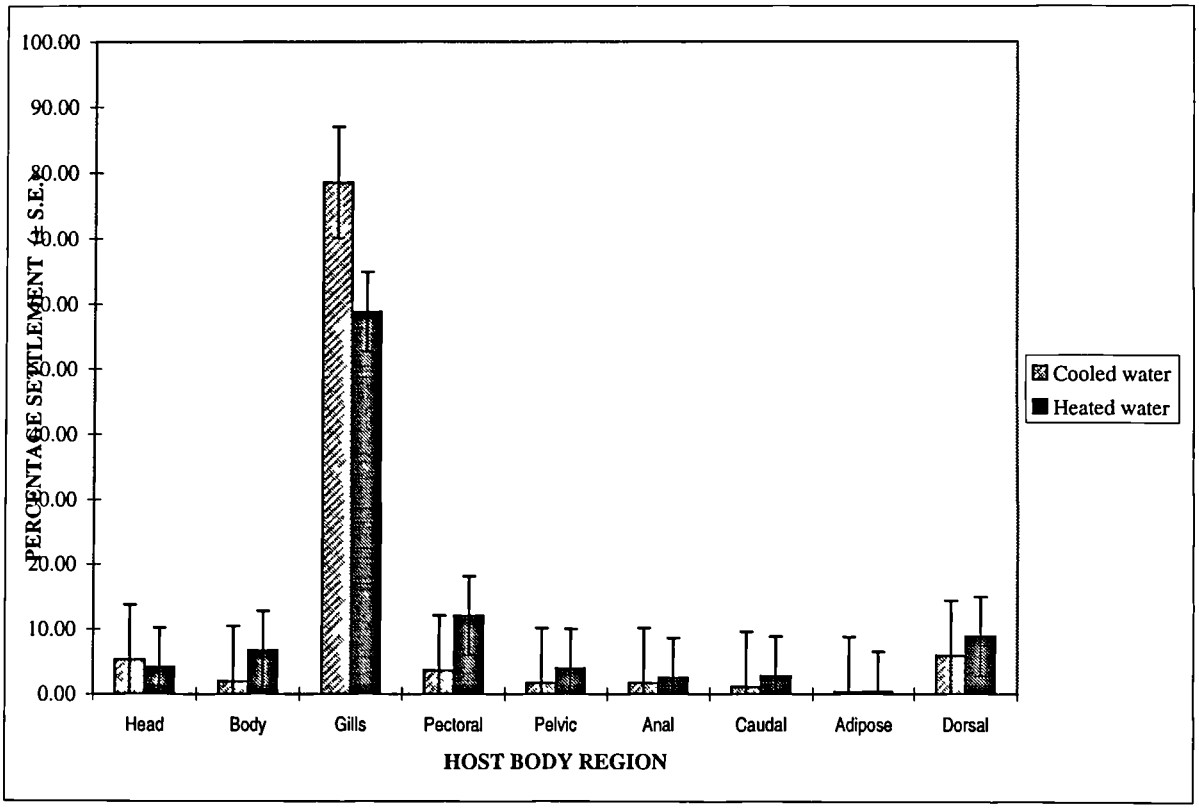


Figure 3.2a. Percentage settlement of *L.salmonis* by host body region at D.P.I. 5 (1997/2)



In the heated water system (1996) the favoured sites of settlement were the gills (17.91%), the pectoral fins (17.78%) and the dorsal fin (38.79%). Examination of the external settlement sites, after removal of the gill settlement data, shows that fin site selection accounts for 71.36% of settlement. Statistical analysis (Dunn's test) of settlement between the host body regions shows statistically significant differences ($p<0.05$) within each temperature regime. In the cooled sea water regime settlement in the gill region was statistically significantly different from all the other body regions, whilst the head and dorsal fin region showed a significant difference from other regions but not from each other. The pectoral fins showed a significant difference ($p<0.05$) from the gills, head and dorsal fin regions but not from the other body regions. For the heated regime a significant difference ($p<0.05$) was found between the dorsal fin and all other host body sites. A further significant difference was found between the gill and pectoral fin groups and all other host body regions. No statistically significant difference was seen between the remainder of the host body groups.

For the 1997 experiment (Figure 3.2a), due to the very low numbers of lice settling on the fish host within the cooled experimental regime a settlement pattern, although present, is difficult to distinguish. The 1997 experiment had a lower overall (<16%) settlement for both experimental regimes, however there was a statistically significant difference ($p <0.05$) between all body regions at the two temperatures. Examination of the heated experimental regime showed a more distinct settlement pattern, with particular affinity for the fins which have 31% of the total settlement.

In the 1997 experiment the preferred sites of settlement in the heated regime are the gills (58.76%), pectoral fins (12.04%) and the dorsal fin (8.82%). Removal of the gill data gives an overall settlement on the fins of 73.70%. The heated sea water regime again shows a

statistically significant difference ($p<0.05$) between the gills and all other body regions. The body and dorsal fin regions show a significant difference ($p<0.05$) from all other regions except the pectoral fin region, which is itself significantly different to all remaining body regions. With the cooled sea water regime there is a statistically significant difference between the gill region and all other host body regions, although the remainder show no significant difference ($p>0.05$) between them.

3.4.2.4.TEMPERATURE EFFECTS ON SURVIVAL BY HOST BODY REGION.

The percentage loss of parasites from specific host body regions is shown in Figures 3.3 and 3.3a.

Due to the low numbers of copepodids present in both the temperature cooled sea water experiments it is difficult to draw valid conclusions from the values obtained for the percentage losses. Any loss from a body region with a low settlement count will result in a large and potentially biased percentage being derived i.e. pelvic fins in the cooled sea water regime of the 1996 experiment (Figure 3.3) showed 100% loss with the removal of 18 parasites. However, examination of the gill regions for both experimental groups (1996) shows a large percentage loss. The losses are 52.7% and 47.4% for the cooled and heated regimes respectively. The 1997 experiment (Figure 3.3a) shows a similar pattern with losses of 49.1% and 59.0% for the cooled and heated regimes respectively. In the 1996 and 1997 experiments there was a large initial settlement on the gills and a large subsequent loss.

Due to the possible bias caused by low settlement counts producing large calculated percentage losses the pattern of survival of *L.salmonis* has been examined only in the

Figure 3.3. Effects of temperature on the percentage loss of *L.salmonis* by host body region at D.P.I. 10 (1996/1)

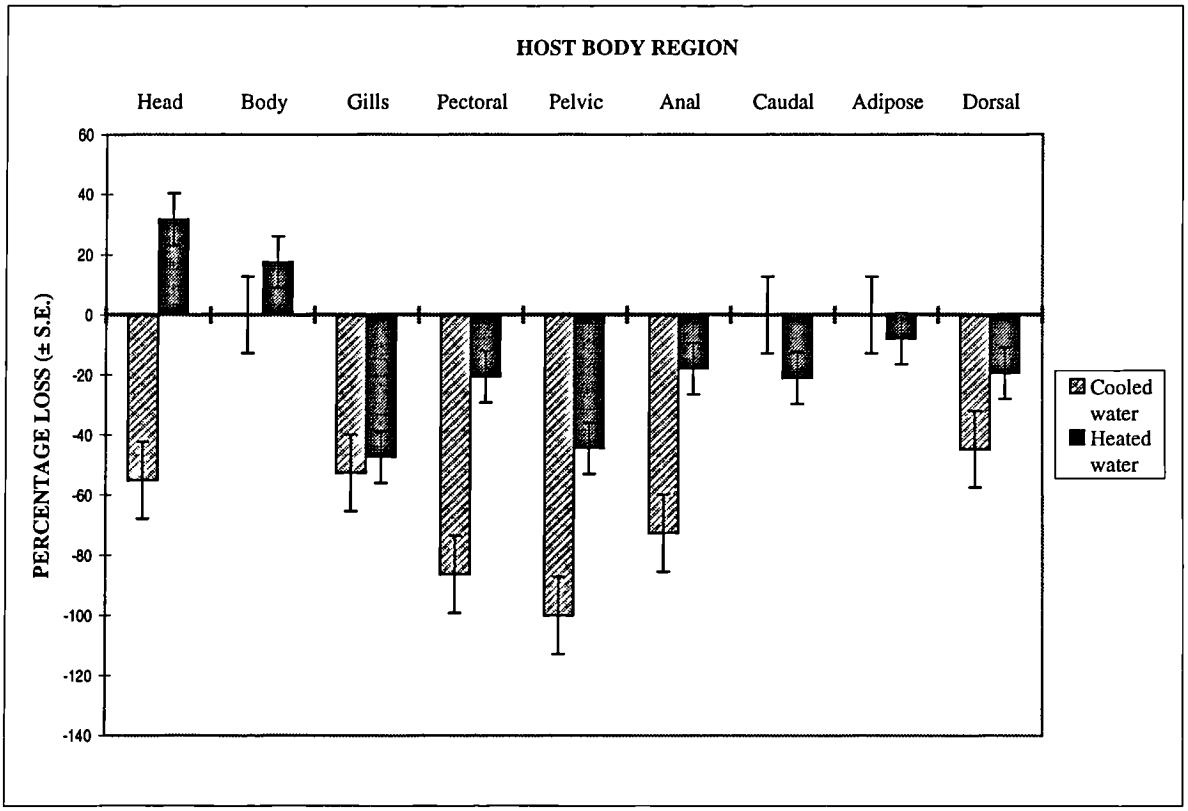
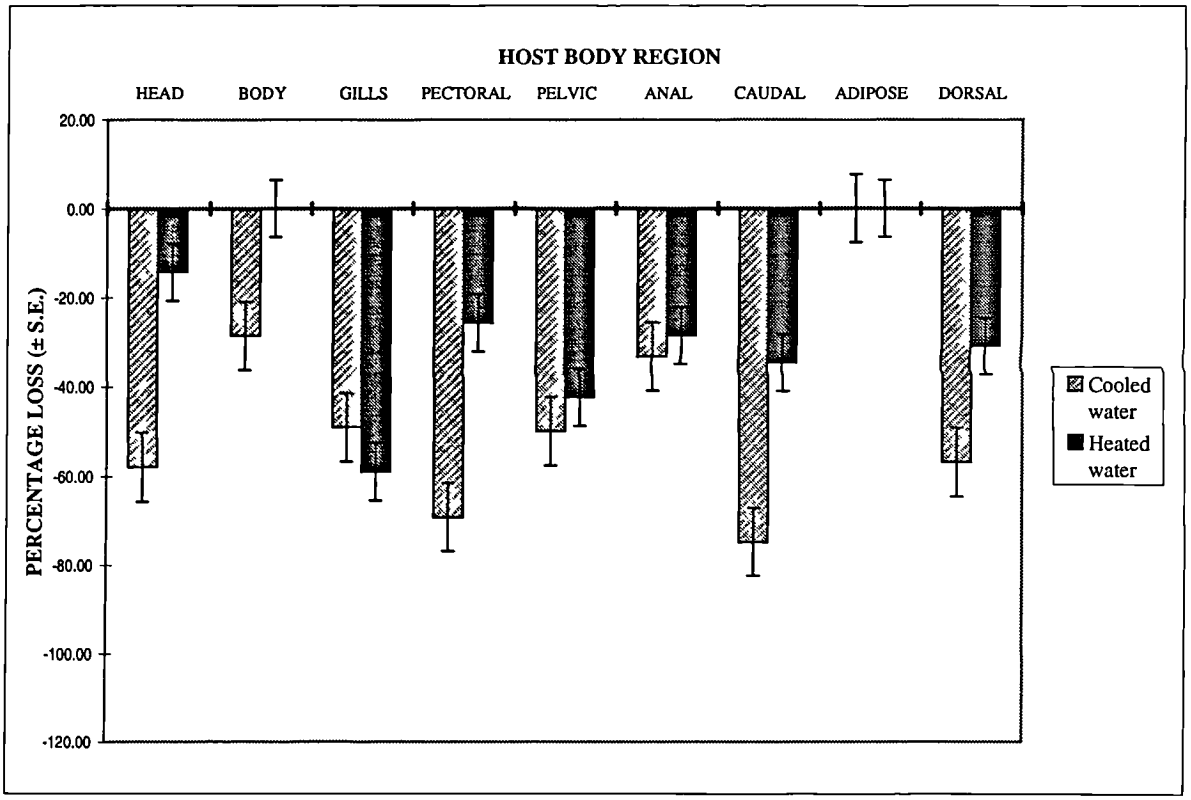


Figure 3.3a. Effects of temperature on the percentage loss of *L.salmonis* by host body region at D.P.I. 10 (1997/2)



heated regime (Figures 3.4 and 3.4a). In the 1996 heated regime experiment (Figure 3.4) there was a statistically significant difference (95% C.I.) in the loss of copepodids from the head and body regions and between these and all other body groups; numbers on the head and body had increased. An increase in *L.salmonis* numbers, 36 parasites on the head and 23 on the body, was found on examination at post-infection day 10. The adipose fin shows a statistically lower loss than all fin and gill regions and statistically higher loss than the head and body. The gill and pelvic fin regions also show a statistically significant difference (95% C.I.) in higher losses than all remaining body groups, the gills have a loss of 47.43%. The pectoral and dorsal fin have losses of only 20.69% and 19.41% respectively.

In the 1997 heated regime experiment (Figure 3.4a) the gill and pelvic fin regions again showed a statistically significant higher (95% C.I.) loss between each other and all other body groups, the gills and pelvic fins having a loss of 59.03% and 42.42% respectively, while the pectoral and dorsal fin have losses of only 25.74% and 31.08%. The body and adipose fin show no losses but have low settlement counts and also some immigration of parasites, giving the false impression of greater louse survivability in these regions.

3.4.2.5. TEMPERATURE EFFECTS ON DEVELOPMENTAL RATE.

Examination of the effects of temperature shows a clear difference in developmental rate of *L.salmonis* after 10 days in both experimental years. In the 1996 experiment (Figure 3.5), at 10 days D.P.I. the majority of lice (74.2%) in the heated system had developed to the chalimus 2 and 3 stage, 43.3% and 30.9% respectively. A small number of chalimus 4 stages were present (0.15%). In the cooled system, no parasites had developed past the

Figure 3.4. Effects of temperature on the percentage loss of *L.salmonis* by host body region at D.P.I. 10 (1996/1)
HEATED SYSTEM ONLY

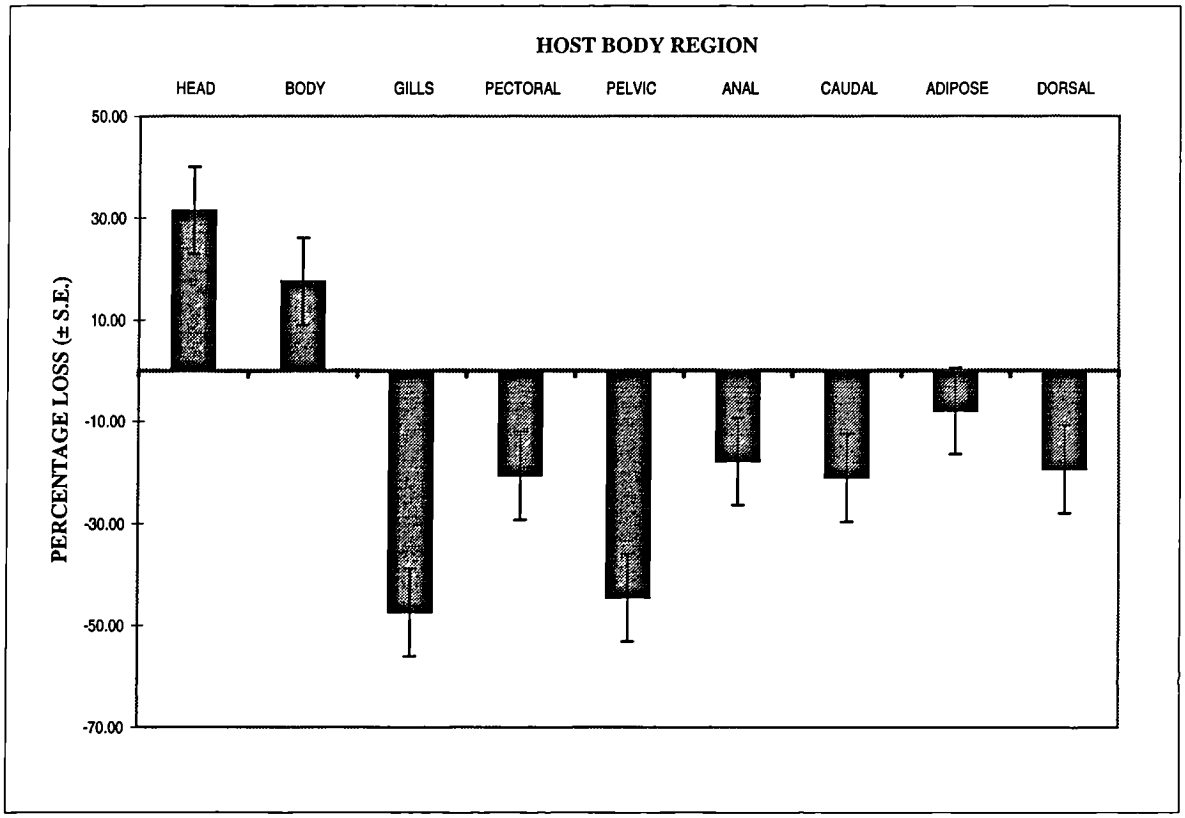
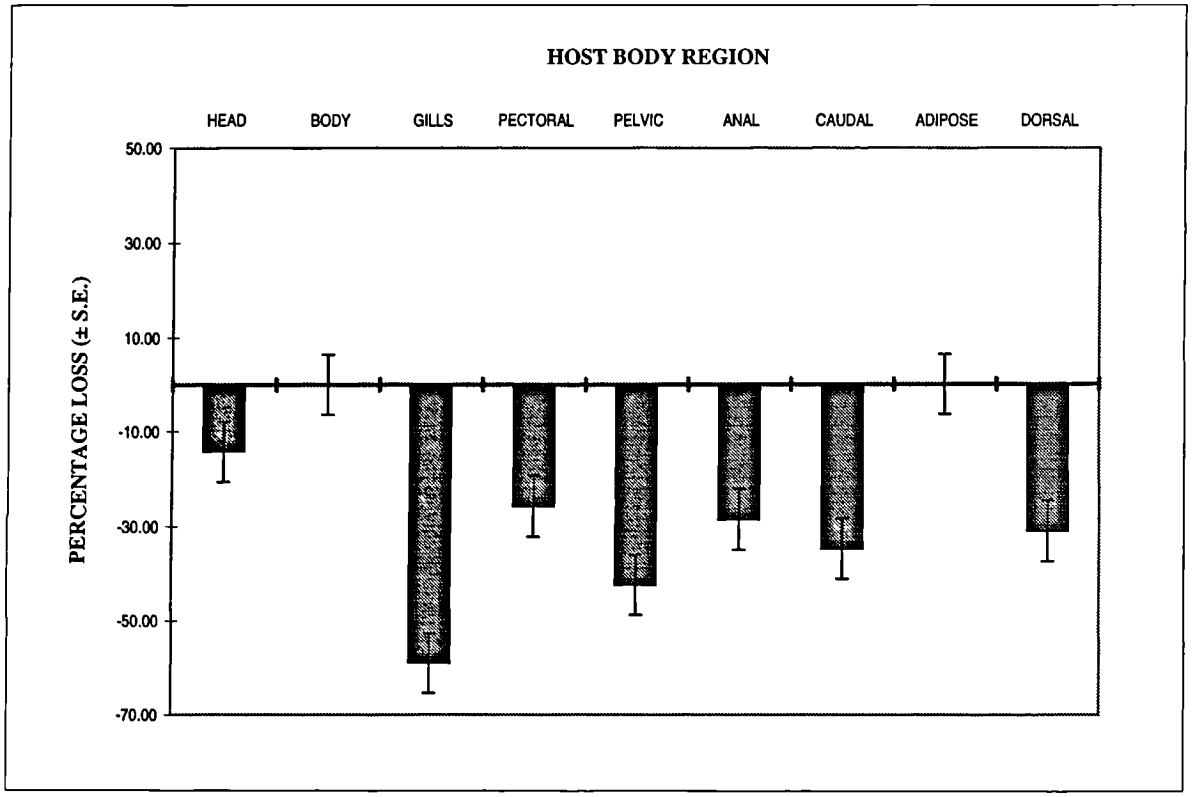


Figure 3.4a. Effects of temperature on the percentage loss of *L.salmonis* by host body region at D.P.I. 10 (1997/2)
HEATED SYSTEM ONLY



infective stage and thus remained as copepodids.

In the 1997 experiment (Figure 3.5a), at 10 days D.P.I., the majority (69.0%) of the *L.salmonis* stages in the heated system had developed to the chalimus 2 and 3 stage, 33.2% and 35.8% respectively. As in 1996, a small number of chalimus 4 stages were present (1.6%). In the cooled system 3.4% of the copepodids had developed to the chalimus 1 stage, whilst the remainder had still not developed past the copepodid stage (96.6%).

3.4.2.6. THE EFFECT OF A SEASONAL RANGE OF TEMPERATURES ON SETTLEMENT.

The experiments described in this chapter have been part of a series examining the abiotic and biotic (chapter 4) effects on the settlement of *L.salmonis* infective stages on its salmonid host. The control data, for these experiments with ambient temperature and salinity, were collated and the mean values calculated for specific temperatures. An apparent pattern of increasing settlement with increasing temperature is evident (Figure 3.6). The variability in the settlement data is demonstrated by the regression analysis of the temperature/settlement data (Figure 3.6a). The scatter of the data points and the low R^2 value (0.3) reflect the variable settlement pattern with temperature. However there is a statistically significant relationship ($p<0.001$) between temperature and settlement.

Figure 3.5. Effects of temperature on the development of *L.salmonis* at D.P.I. 10 (1996/1)

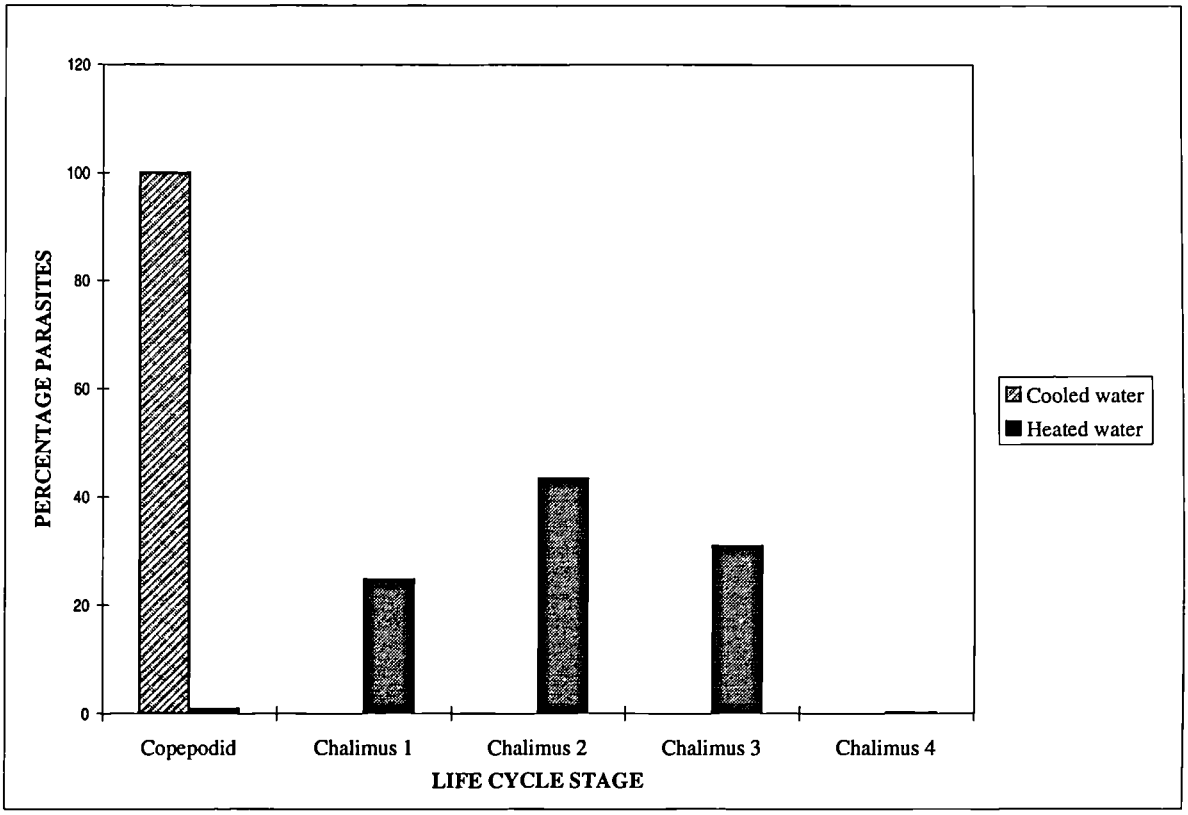


Figure 3.5a. Effects of temperature on the development of *L.salmonis* at D.P.I. 10 (1997/2)

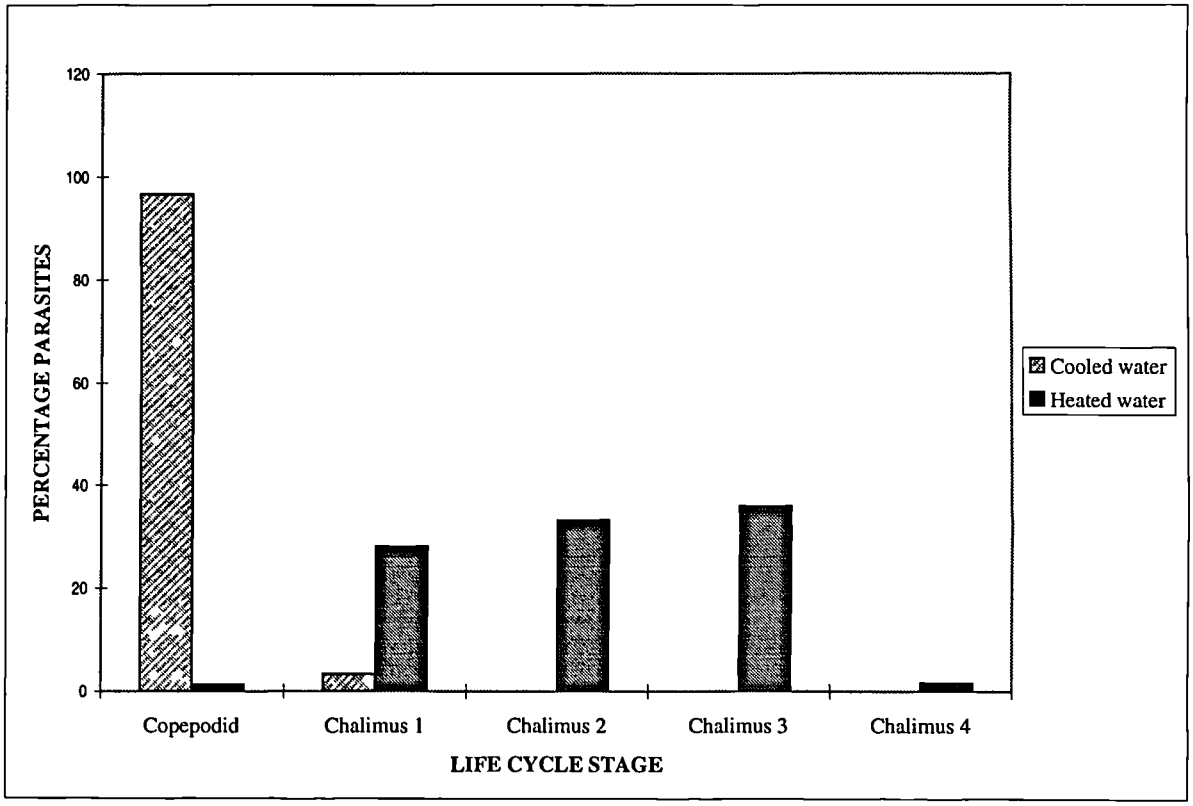


Figure 3.6. Mean percentage settlement of *L.salmonis* on Atlantic salmon over a range of temperatures

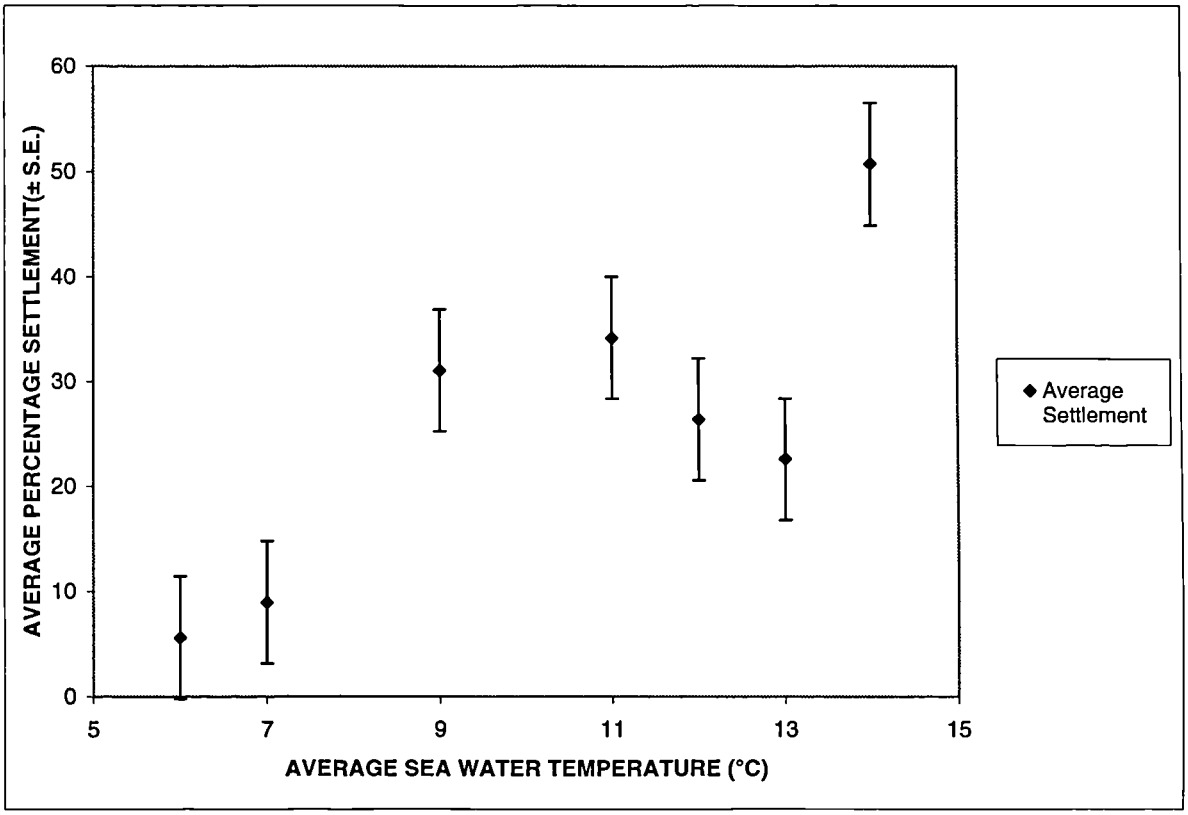
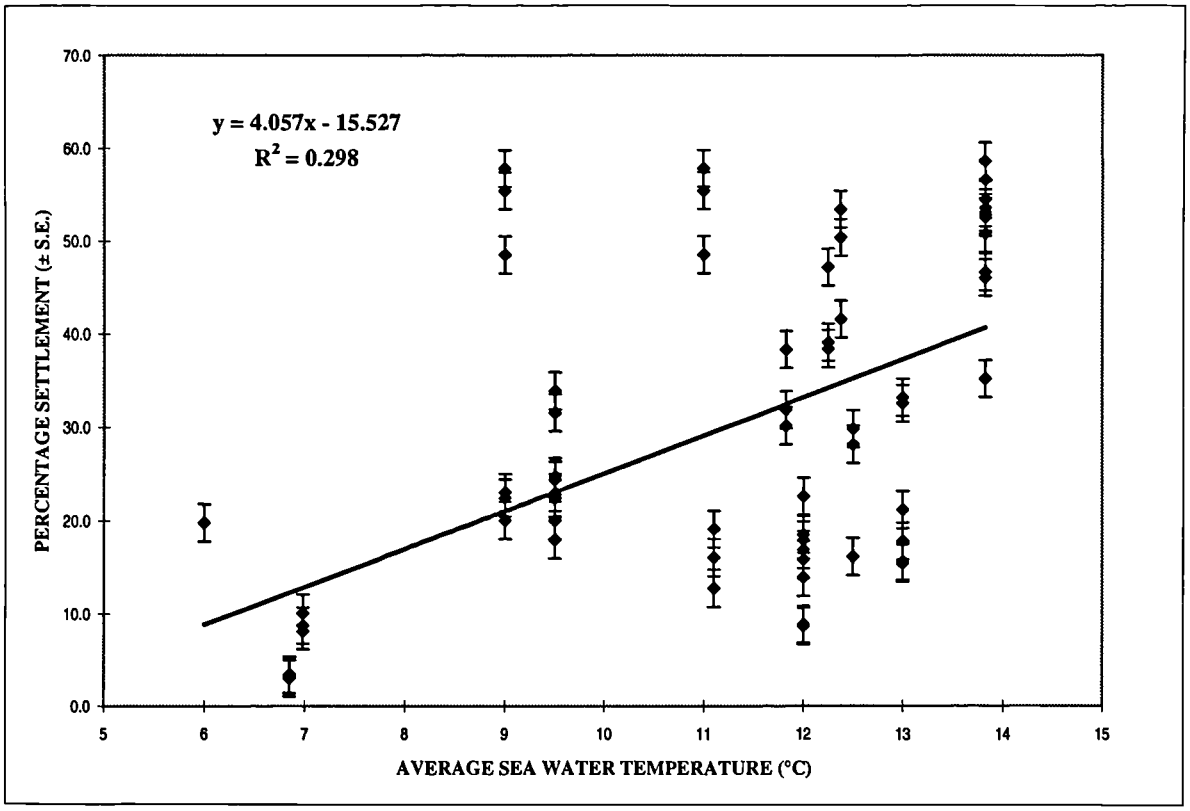


Figure 3.6a. Regression analysis of the percentage settlement of *L.salmonis* on Atlantic salmon over a range of temperatures



3.4.3 EFFECTS OF SALINITY.

3.4.3.1 SALINITY, ENVIRONMENTAL VARIABLES.

Salinity was maintained at a constant level throughout the experimental period. Ambient salinity was 34‰ during the experimental period whilst the re-circulation system, for the reduced salinity regime was maintained at a constant 25‰. The environmental parameters for the salinity experiments are given in Table 3.3.

Although the temperatures within the 24‰ salinity re-circulation system were higher than the 34‰ system there was no statistically significant difference ($p < 0.05$) between the two temperatures within each experiment. The higher temperature in the 24‰ salinity group (1997) was as a result of a mechanical breakdown in the chiller and did reach a maximum of 18°C on three days.

Table 3.3. Environmental conditions during salinity experiments 1 and 2.

	34‰ Salinity Temperature (°C)	24‰ Salinity Temperature (°C)	Salinity (‰)
Exp. 1 (1996)	12.6 ± 0.9	13.2 ± 0.9	34/25
Exp. 2 (1997)	14.7 ± 0.9	15.3 ± 1.4[1]	34/25

Nitrite (NO_2) and ammonia (NH_3) were monitored daily and did not exceed the recommended safety levels for salmonids. Nitrite levels did not rise above 0.33 mg $NO_2\ l^{-1}$ whilst Ammonia (NH_3) did not rise above 0. Harmful concentrations for nitrite toxicity are

[1] Due to a mechanical breakdown of the chiller unit day time temperatures within the 24‰ salinity re-circulation on three consecutive days did reach a high of 18°C. See conclusions.

considered to be 1.0 mg NO_2 -N l^{-1} (Wickens, 1980). The harmful levels of ammonia to salmonids are 0.02 mg l^{-1} NH_3 (Roberts & Shepherd, 1986). No difference in nitrite and ammonia levels was found between the experimental groups, all groups had levels below harmful concentrations.

3.4.3.2 EFFECTS OF SALINITY ON SETTLEMENT AND SURVIVAL.

The effects of salinity on settlement of *L.salmonis* can be clearly seen in Figures 3.7 and 3.7a and Table 3.4.

In both the experiments there is a statistically significant ($p<0.001$) greater settlement of the *L.salmonis* on the host at the higher salinity. In the 1996 experiment this represents a difference of 30.38% and in the 1997 experiment a difference of 52.59%.

Table 3.4. Percentage settlement and survival for both salinity experimental years.

	24‰ Settlement (%)	34‰ Settlement (%)	24‰ Survival (%)	34‰ Survival (%)
Exp. 1 (1996)	18.04	48.42	5.80	78.54
Exp. 2 (1997)	24.63	77.22	75.30	73.25

In the 1996 experiment (Figure 3.7) the significant difference ($p<0.001$) between the experimental groups remained when survival of the parasites was examined at 10 D.P.I.. However, in the 1997 experiment, there was no significant difference between the experimental groups at 10 D.P.I. In the 1996 experiment 1040 lice (94.20%) were lost in the 24‰ salinity regime resulting in an overall survival of this group of only 5.8%. In the ambient regime (34‰) there was a loss of 21.46% only. The 1997 experiment (Figure 3.7a) shows a different result. In this experiment survival in the reduced (24‰) salinity regime was marginally higher than the ambient salinity, and the percentage losses were

Figure 3.7. Effects of salinity on the settlement and survival of *L.salmonis* on Atlantic salmon (1996/1)

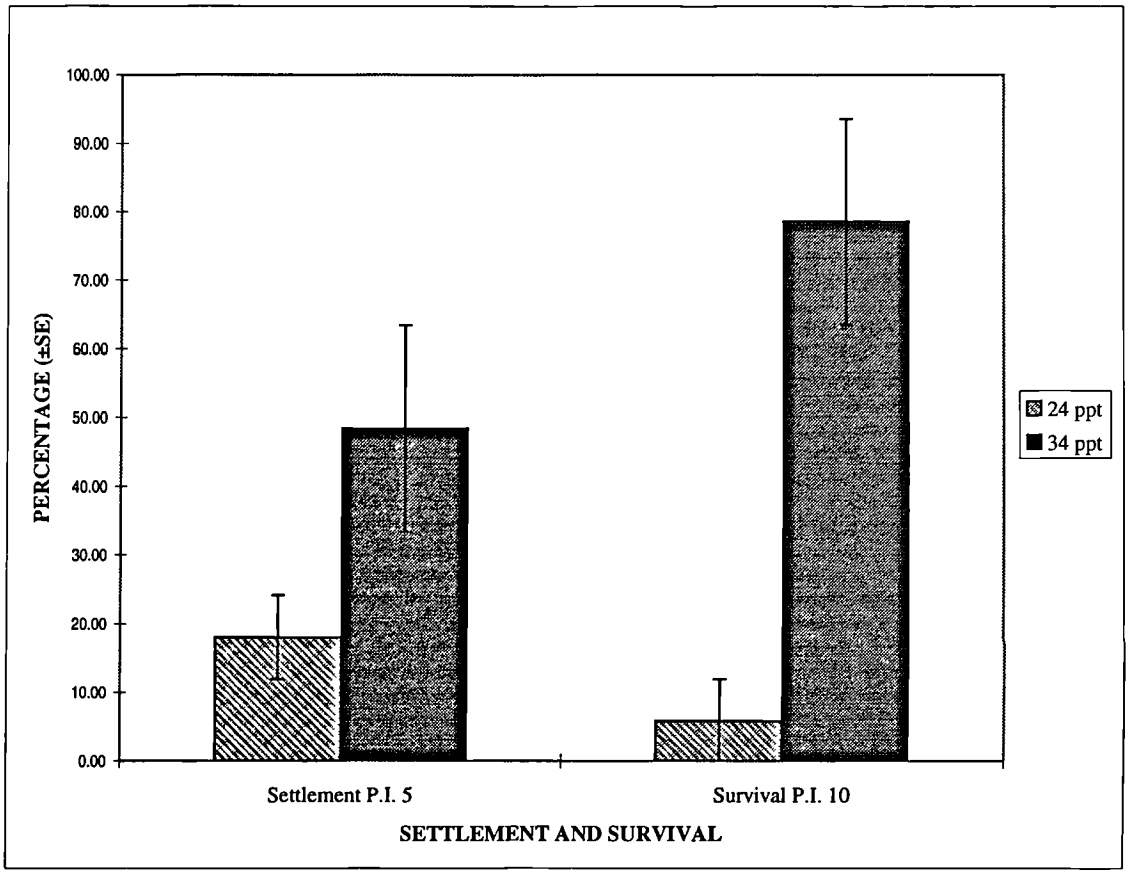
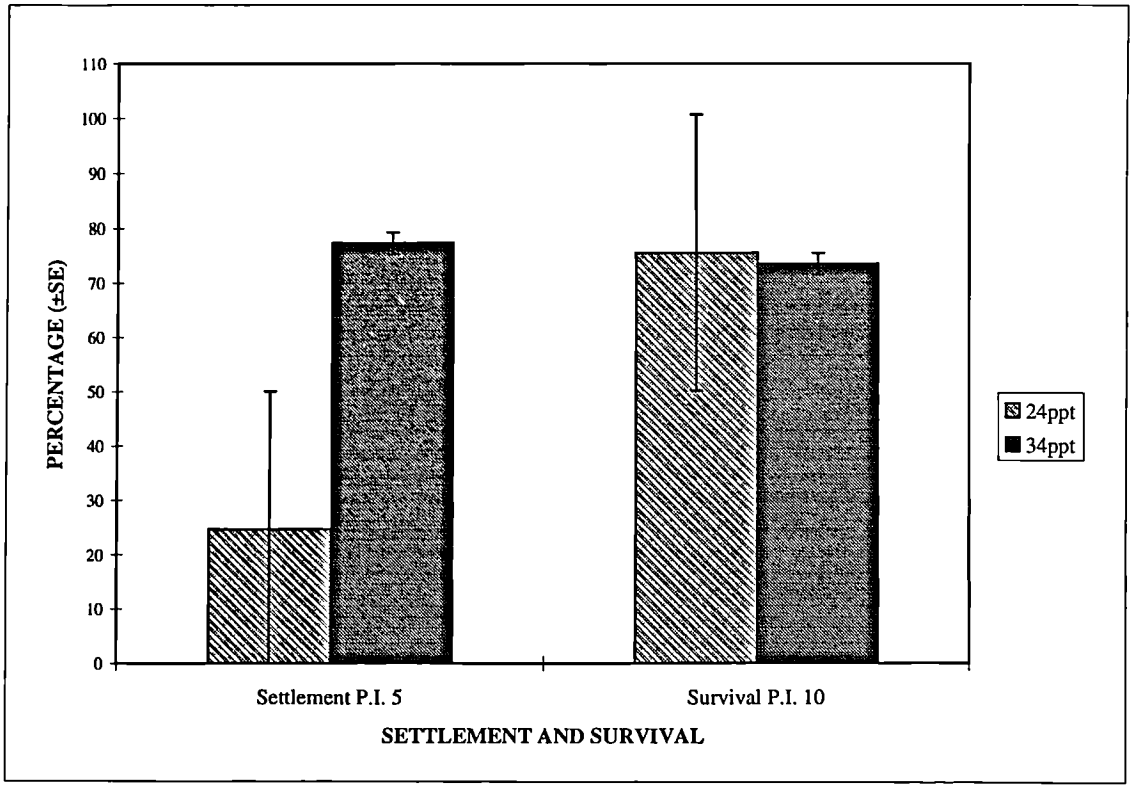


Figure 3.7a. Effects of salinity on the settlement and survival of *L.salmonis* on Atlantic salmon (1997/2)



similar being 24.70% and 26.75% for the reduced and ambient salinity regimes respectively. Although the number of lice settling on the fish host in the reduced salinity regime was initially low, 75.30% survived to 10 D.P.I..

3.4.3.3. SALINITY EFFECTS ON DISTRIBUTION ON THE HOST BODY SURFACE.

In both experiments there was a statistically significant difference (95% C.I.) in the settlement distribution of lice between host body regions in each salinity regime (Figures 3.8 and 3.8a).

In the 1996 experiment shown in Figure 3.8, the highest settlement in the reduced salinity regime (24‰) was found on the gills (6.24%), pectoral fins (3.38%) and dorsal fins (4.75%). The percentage of overall settlement for all the fins combined was 52.08%, with 45.11% of the total settlement on the pectoral and dorsal fins. If the gill data are removed then fin settlement accounts for more than 68% of the total number of lice settled in both salinity regimes.

In the 24‰ salinity regime a statistically significant difference (95%C.I.) was found between the gills, pectoral and the dorsal fins and all other body regions. In the 34‰ salinity regime settlement was highest on the body (9.10%), pectoral fins (8.10%) and dorsal fin (12.21%). The percentage of overall settlement for the fins was 61.69% with 42.15% on the pectoral and dorsal fins. In the ambient salinity regime a statistically significant difference (95%C.I.) was found between the settlement on the dorsal fin and all other host body regions. The pectoral fins and body showed a significant difference (95%C.I.) in settled parasites from all host body regions, except the gill and head regions.

Figure 3.8. Percentage settlement of *L.salmonis* by host body region at D.P.I. 5 (1996/1)

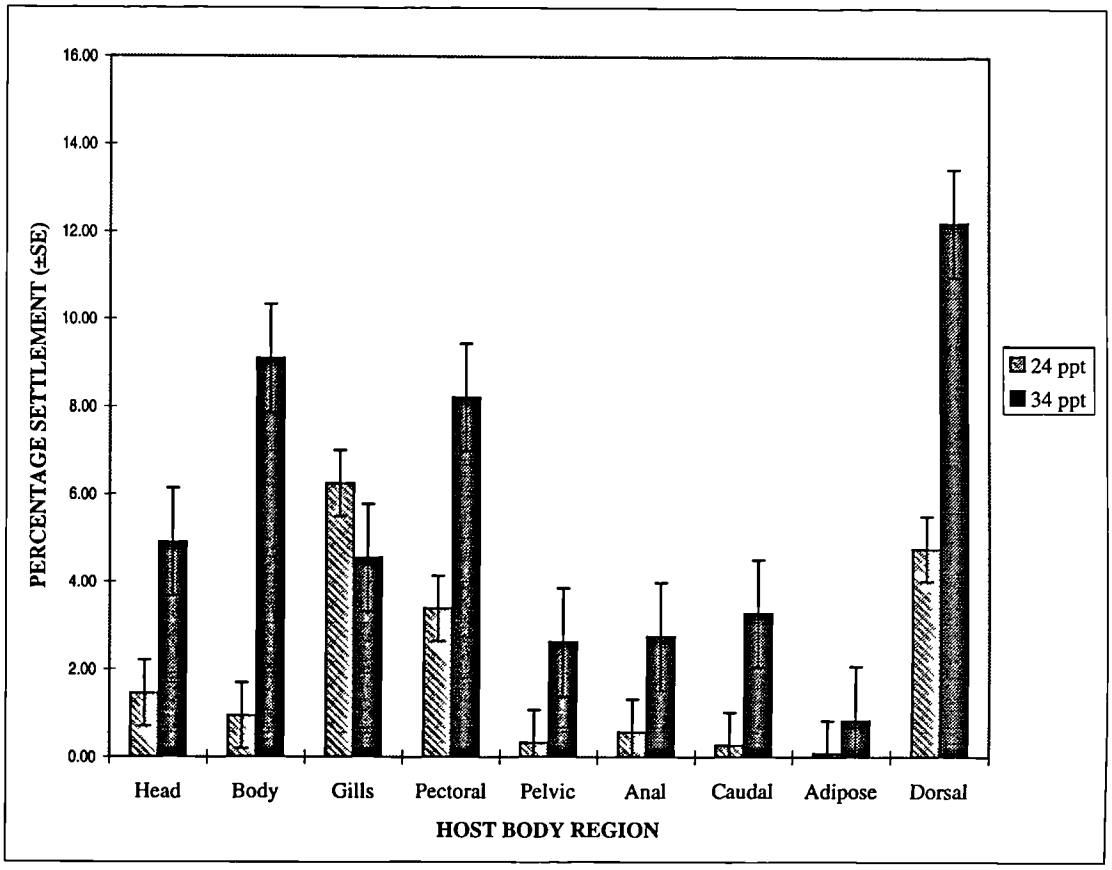
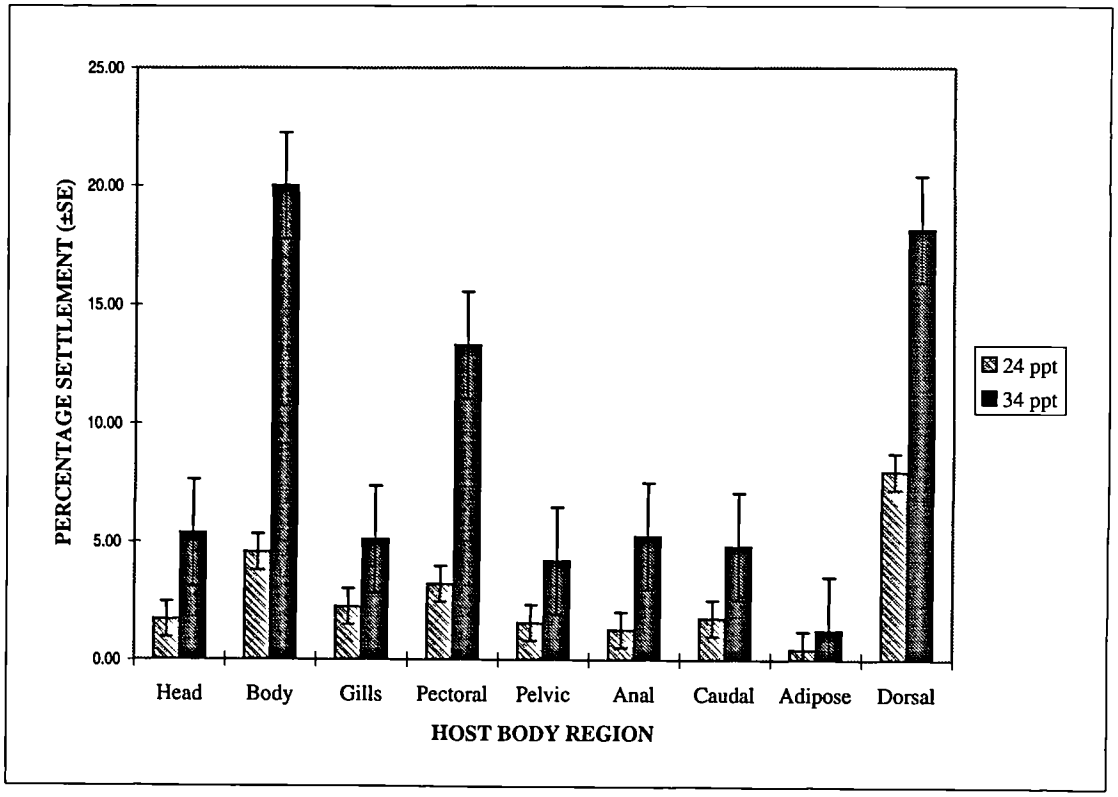


Figure 3.8a. Percentage settlement of *L.salmonis* by host body region at D.P.I. 5 (1997/2)



In the 1997 experiment (Figure 3.8a) a similar pattern is seen; the highest settlement in the 24‰ salinity regime was found on the body (4.55%), pectoral fins (3.20%) and dorsal fins (7.91%). Overall settlement on the fins in the reduced salinity regime was 65.41% with 45.11% of the total settlement on the pectoral and dorsal fins. Again, if the gill data is removed then fin settlement is greater than 64% in both salinity regimes. In the reduced salinity regime a statistically significant difference at the 95% C.I. is found between the higher settlement on the body and dorsal fin and all other host body regions.

In the 34‰ salinity regime settlement was also found to be highest on the body (20.0%), the pectoral fins (13.27%) and the dorsal fin (18.22%). Percentage of overall settlement in the ambient salinity regime for the fins was 60.57% with 40.65% of the total settlement on the pectoral and dorsal fins. In the ambient salinity regime a statistically significant difference (95%C.I.) was found between the body, pectoral fins and the dorsal fin and all other host body regions.

3.4.3.4. SALINITY EFFECTS ON SURVIVAL BY HOST BODY REGION.

Figures 3.9 and 3.9a show the percentage losses from individual host body regions in both experimental years. As previously noted (section 3.3.3.1) survival in the 1996 (Figures 3.9) experiment was low in the reduced salinity regime, with an overall loss of 94.20%, whereas in the 1997 experiment overall survival was high, at over 73% in both salinity regimes. Statistical analysis of the 1996 experiment of the proportional 95% confidence limits for losses within salinity regimes showed that in the reduced salinity regime there was no statistically significant difference between the pelvic, anal, caudal and adipose fins. However there was a significantly higher difference between these areas and all other body

Figure 3.9. Effects of salinity on the percentage loss at D.P.I. 10 of *L.salmonis* by host body regions (1996/1)

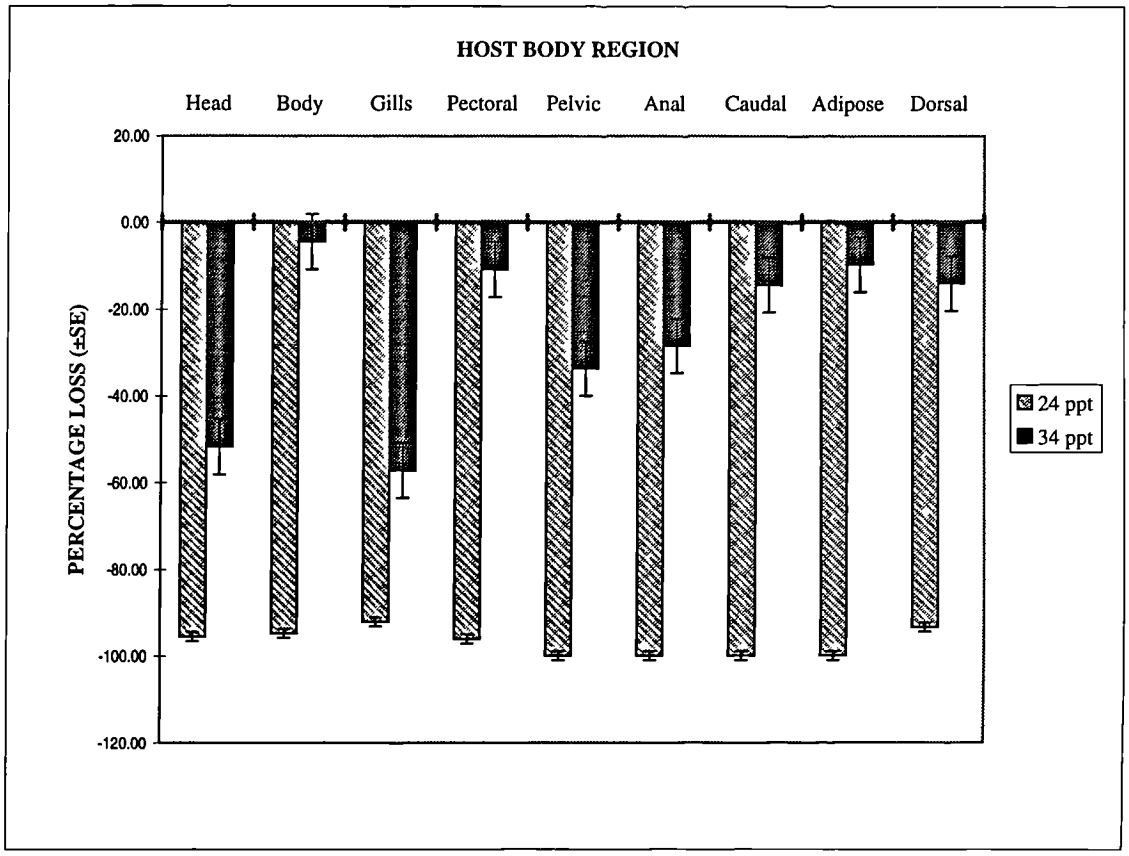
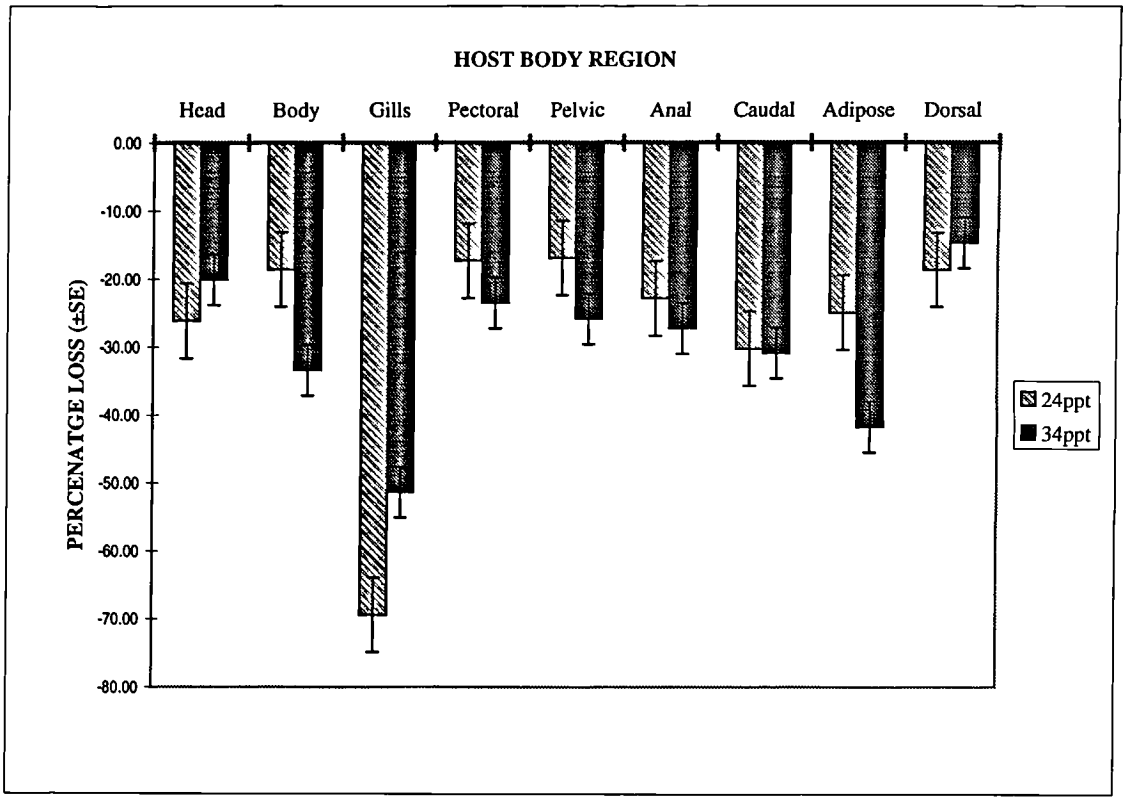


Figure 3.9a. Effects of salinity on the percentage loss at D.P.I. 10 of *L.salmonis* by host body regions (1997/2)



regions. There was no statistically significant differences among numbers in the other body regions. In the 1996 experiment in the ambient salinity regime there was a statistically significant difference between survival on the head, body, gills, pelvic and anal fins and between these and all other body regions. The head and gills showed the highest losses at 51.67% and 57.19% respectively. Survival was highest on the pectoral and dorsal fins with less than 15% losses.

In the 1997 reduced salinity experiment (Figure 3.9a) the gills, with a loss of 69.41%, were significantly different (95% C.I.) from all other body regions. A statistically significant difference (95% C.I.) was found between the head and caudal fin and all other body regions. No statistically significant difference was found between the remaining body regions. In the 1997 ambient salinity regime (Figure 3.9a) a statistically significant difference (95% C.I.) was found between losses from the gill and adipose fin (51.40% and 41.86% respectively), and between these regions and all other groups. However losses from the adipose fin represent only 18 parasites. A statistically significant difference (95% C.I.) was also found between the body, caudal and dorsal fins and all other body regimes, the dorsal fins having the smallest losses (14.73%).

3.4.3.5. SALINITY EFFECTS ON DEVELOPMENTAL RATE.

Reduced salinity was seen to have a marked effect on developmental rate at 10 days post infection as shown in Figures 3.10 and 3.10a. In the 1996 experiment (Figure 3.10), the majority (54.84%) of the developmental stages in the reduced salinity regime remained as copepodids. The other 45.16% had developed to the chalimus 1 (19.45%), chalimus 2 (9.68%) and chalimus 3 stages (16.13%). In contrast, in the ambient salinity regime the

Figure 3.10. Effects of salinity on the development of *L.salmonis* D.P.I. 10 (1996/1)

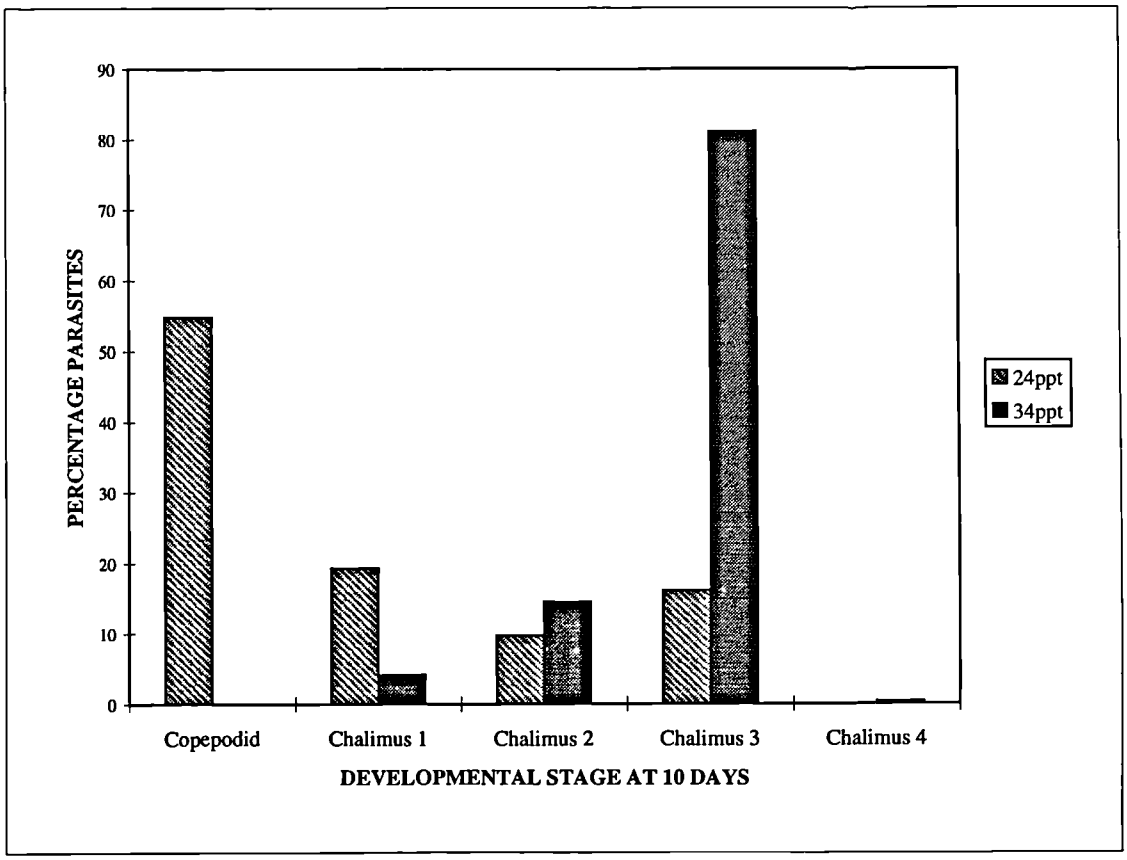
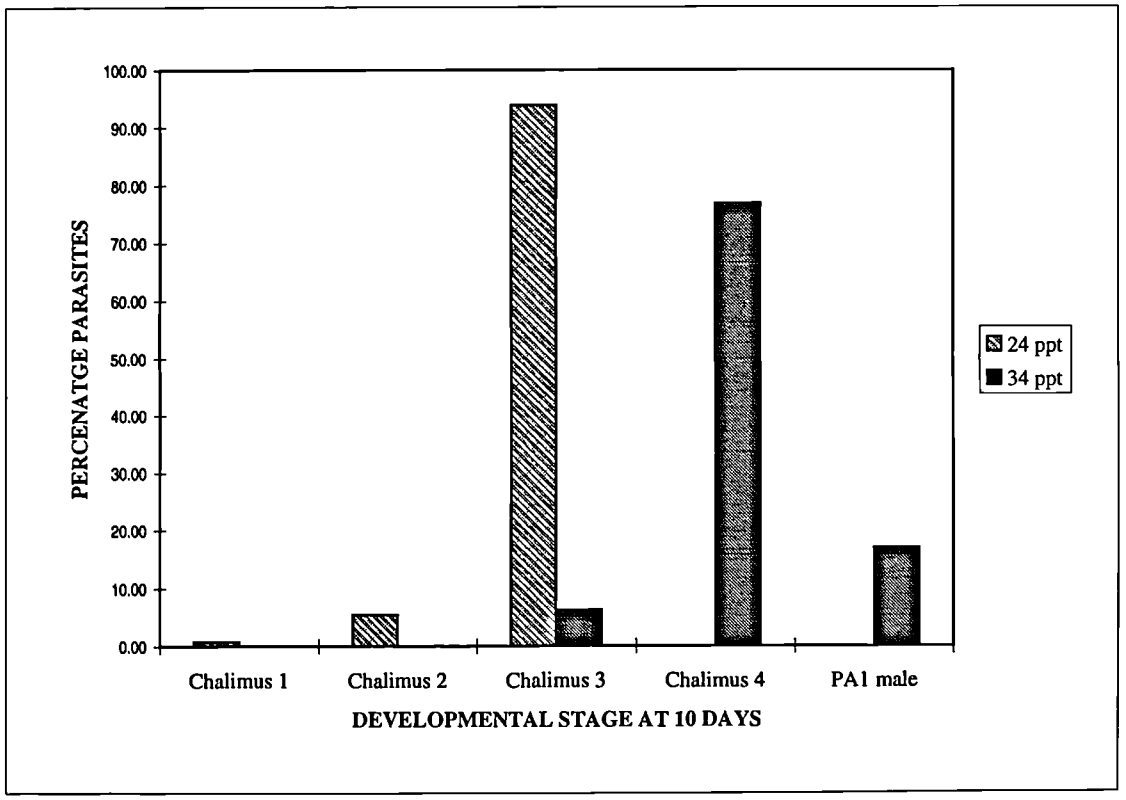


Figure 3.10a. Effects of salinity on the development of *L.salmonis* at D.P.I. 10 (1997/2)



majority of lice had progressed to the chalimus 3 stage (81.09%) with the appearance of some chalimus 4 stages (0.30%).

In the 1997 experiment (Figure 3.10a) most lice in the reduced salinity regime had reached the chalimus 3 stages (93.87%) with the remainder (<7%) present as chalimus 1 and 2 stages. In the ambient system the majority of the stages were chalimus 4 (76.75%) with the presence of preadult 1 males (16.98%), the other stage was chalimus 3. Although development had progressed further in the 24‰ salinity regime in 1997 than the same salinity regime in 1996 it was still a slower rate of development than the ambient (34‰) salinity regime.

3.5 DISCUSSION.

It can be clearly seen from the settlement experiments described in this chapter that both temperature and salinity have marked effects on the ability of *L.salmonis* to infect and survive on its host. The influence of ambient temperature on biological processes is well documented and it is accepted that all organisms function within an optimum temperature regime. Parasitic copepods are no exception (Kabata, 1981). Any environmental temperature change will directly affect poikilothermic invertebrates, decreasing or increasing all metabolic activities. Previous literature (Johannessen, 1978; Wootten *et al.*, 1982; Tully, 1989; Johnson & Albright, 1991a, b; Johnson, 1993) reports temperature effects on the free-swimming stages of *L.salmonis* yet this study is the first to consider the effects on settlement and early post settlement survival.

Conley & Curtis (1993) state that hatch duration and success in the parasitic copepod *Salmincola edwardsii* are unaffected by temperature. On the other hand, Gravil (1996)

found that temperature does affect the hatching success of *L.salmonis* but with an increase in temperature there is a decrease in the hatching period. Johannessen (1978) found that temperature prior to and during embryogenesis influences the egg bearing period of *L.salmonis* and also found that copepodids were not obtained in the laboratory below 8°C, although they were sampled in the sea at 5-6°C, in low numbers. Gravil (1996) agrees with Johannessen (1978) that nauplius II stages fail to moult to the copepodid stage at 5°C. In the present study of the effects of temperature, sufficient copepodids were obtained for the experimental infection protocol after incubation at 7°C. Gravil (1996) found high numbers of copepodid mortalities at 7.5°C. In this study settlement was achieved at 7°C although it was low (<10% settlement), reflecting the extreme robustness of the copepodid and nauplii stages. Although a winter population of lice was used, settlement and survival of the copepodids was enhanced at the higher temperature i.e. 12°C indicating that it is capable of coping with substantial environmental fluctuations. Scottish winter sea water temperatures are often below 7°C and settlement is known to occur on salmon farms in these circumstances.

Conley & Curtis (1993) further observed that an increase in temperature had a significant effect on the duration of copepodid swimming activity and survival, inversely related to water temperature, in *Salmincola edwardsii.* Gravil (1996) states that spontaneous swimming activity in *L.salmonis* larval stages is greatly increased at higher temperatures. Similarly, in the present study, copepodid activity of *L.salmonis* increases and duration of activity decreases with increasing temperature. Copepodids reared under the higher temperature regime showed greater swimming activity when sampled. In the colder sea water temperatures in both experimental years copepodids were observed, pre-settlement activity to be very inert which may have lead to the observed reduced settlement.

The data presented indicates that temperature affects the ability of the infective stage to settle and develop on its host. With increased water temperature prior to initial settlement there was an increased capability to infect the fish host, although there was considerable variation observed. There was a statistically significant difference between the two temperature regimes for the numbers of lice settling on the salmonid host in both experiments, repeated in consecutive years. Lice utilised in the temperature experiments were winter generation lice and therefore any thermal shock would have been received by the lice introduced into the higher sea water temperatures. No such effect was seen; the winter population lice exposed to higher sea water temperatures had settlement counts above those of the lice exposed to ambient sea water temperature. Once attached to the fish host there was approximately 55% or more survival up to D.P.I. 10 (Figure 3.1 and Figure 3.1a). There was considerable variation in settlement between the two experimental years and although the first 6000 copepodids, those with highest energy reserves, were utilised the reason for this variation is unknown. Collection of the salmon lice from a farmed fish does mean that the senility of the lice and the egg batch number of the ovisacs is unknown and may therefore contribute to the success of settlement.

Combining the results for mean values of several experimental infections shows that the settlement of *L.salmonis* increases with increasing in sea water temperature (Figure 3.6). However when the regression analysis of the settlement data is examined the variability in settlement is readily apparent (Figure 3.6a). The general trend is clearly observed but until we have a better understanding of the cause of the variability in the settlement process, it is the effects of temperature on the generation times that are most significant. There will be reduced generation times with increased temperatures. This data is from tank trials examining the abiotic and biotic effects on settlement but as the history of the salmon lice utilised is unknown this may be a contributing factor to the variability. Epidemiological

data (Institute of Aquaculture, unpublished data) of adjacent sea cage sites show considerable variability in the lice settlement counts between salmon ongrowing cages and the conclusive reason for which is unknown.

Undoubtedly, temperature is the single most important factor affecting growth of copepods (Klein Breteler & Schogt, 1994). According to Kinne (1970a), in several species of crustacea, shedding of the cuticle is blocked by low temperature, although life processes continue. Survival was reduced in the colder sea water temperatures in both experimental years and development was slowed, possibly through the prevention of moulting. Only copepodids were present at day 10 D.P.I. at the colder temperature and these are probably more easily detached as no frontal filament had been produced. The losses within the heated sea water temperatures were considerably lower after 10 days as the lice had moulted through to chalimus 2 and 3 stages and were permanently attached by their frontal filaments. Johnson & Albright (1991a) state that 50% development from copepodid to chalimus 1 takes 7 days at 10°C. In the present study, at average sea water temperature of above 11°C the majority of the life stages were beyond chalimus 1stage at day 10. In both experiments more than 70% had reached chalimus 2 or beyond at this time. Again the use of winter generation lice did not seem to inhibit the effect of increased temperature on developmental rate of the sea lice.

Johannessen (1978) and Kabata (1981) also observed an inverse relationship between development time and temperature for *L.salmonis* with higher water temperatures resulting in faster development (Kabata, 1981). Shields & Tidd (1968) reported that the rate of development of larval *Lernaea cyprinacea* L. also shows a decrease in development with increase in temperature.

Generation times greatly decrease with the increase in sea water temperatures. Wootten *et*

al., (1982) report that at 9-12°C a complete generation takes six weeks, whilst Johnson & Albright, (1991a) report generation times of 7.5-8 weeks at 10°C. Development from chalimus 1 through to gravid female takes 3-4 weeks at 12°C but 4-5 weeks at 9.5°C (Wootten *et al.*, 1977). Pike, Mordue (Luntz) & Ritchie (1993) reported that the duration of each stage of *Caligus elongatus*, a related parasitic copepod, also shows an inverse relationship with temperature. Experimental infections of salmon with *L. salmonis* (Institute of Aquaculture, unpublished data) have shown a two-fold increase in the generation time from infection to gravid adult female between summer and winter. In winter with an average water temperature of 6.3°C, the generation time was found to be approximately 71 days whereas the generation time in summer was approximately 31 days with an average water temperature of 13.8°C.

Salinity effects on settlement and survival of *L.salmonis* are also marked. There was a statistically significant difference in the settlement of *L.salmonis* at 24‰ and 34‰. In both experiments the reduced salinity regime resulted in lower settlement rates of less than 25% (Figure 3.7 and 3.7a). However, the survival of *L.salmonis* differed between the two experimental years. In the 1996 experiment, survival fell dramatically to only 5.8% of those originally settling on the host by day 10. Interestingly, in the reduced salinity regime in the 1997 experiment survival was 75.3%, slightly higher than among the ambient salinity group despite the replication of the experimental design. The main difference between the experimental years was the sea water temperature. In the second year the average temperature was higher and this was compounded by the chilling unit, servicing the re-circulation system which failed through mechanical breakdown on three consecutive days. The temperature in the re-circulation system reached a daily average of 18°C over this period. Survival and the developmental rate in 1997 experiment was higher and had progressed further than the 1996 experiment, apparently as a result of the increased

temperature. Interactions of salinity and temperature have been analysed by Lance (1963) who found that *Acartia tonsa* tolerates low salinities better at high temperatures. Miliou (1993) found that development of another copepod, *Tisbe holothuriae* was primarily effected by temperature and secondarily by salinity variations. Sokal & Rohlf (1969) stated "that the effects of two factors are not simply additive but that any given combination of levels of factors...contributes a positive or negative increment to the level of expression of the variable" i.e. there is an interrelationship of such influences. In the 1997 experiment the survival of the louse has been enhanced by the increase in temperature possibly through an increase in metabolism.

Hahenkamp & Fyhn (1985) demonstrated that the osmotic response of *L.salmonis* to reduced salinity is not dependent on the osmoregulatory ability of its host. Attached lice can osmoregulate, replacing salts by actively feeding on the mucus and body fluids/tissues of the host. They found that free swimming lice or unattached lice quickly dehydrated and died. The isosmotic condition between the haemolymph of the attached parasite and the blood plasma of the host in fresh water further suggests a close dependence of the parasite on the salmon for the maintenance of water and ionic balance under these conditions (Hahenkamp & Fyhn, 1985). It appears, therefore that active feeding is important under sub-optimal conditions. Unattached and free-swimming stages, i.e. those not actively feeding die after only 8 hours in fresh water (Hahenkamp & Fyhn, 1985) providing an explanation why survival in the 1996 experiment in the reduced salinity regime was so low (where only 45% of the lice had developed to the chalimus stages). In the 1997 experiment the chalimus present predominately belonged to chalimus 3 stage in the reduced salinity regime compared with chalimus 4 stages in the higher salinity regime. Thus the effects of the reduced salinity can also be seen in the development of the louse which was retarded compared to the ambient salinity regimes in both experimental years.

In all experiments the favoured sites of settlement were the fins particularly the pectoral and dorsal fins. This agrees with the work of Wootten *et al.*, (1982) and Johnson & Albright (1991a). Wootten *et al.*, (1982) reported favoured sites being the dorsal and the pelvic fins, although the gills were not examined, whilst Johnson & Albright, (1991a) state the preferred sites to be the gills (53% settlement) and the fins (33% settlement). The favoured fin regions by the latter authors are pelvic fins (14%), pectoral (9%) and the anal fin (7%). Bron *et al.* (1991) report the preferred site of settlement to be the fins. In the present study the preferred site of settlement was the fin regions. In the 1996 temperature experiment fin settlement represents 35.86% and 71.36% of the total settlement in the cooled and heated regime respectively whilst in the 1997 experiment the fins represent 14.25% and 30.39% of the total settlement for the cooled and heated regimes. The latter experiment had a much lower overall settlement count with percentage settlement on the gills of more than 58%. In the salinity experiments the fin settlement was greater than 52% in all salinity regimes. Within the fin region the highest settlement, in each experiment was the pectoral and dorsal fins. The fins represent approximately 20% of the total body surface area (Chapter 4) but, with the removal of the gill data retain greater than 64% of the settlement in all experiments. Histological examination of the host epithelium showed no significant difference between the experimental groups in the number of mucous cells present. Further Turnbull (1992) found that normal and naïve Atlantic salmon show no histological difference in the epithelial structure between the fins and the host body surface. Therefore all host surfaces, irrespective of experimental treatment will be the same. However Pickering & Richards (1980) suggest that the salmonid epidermis is dynamic as it is mediated by the endocrine system and therefore can respond rapidly to environmental changes. No such changes were observed in these experiments.

There must be specific advantages to the dorsal and pectoral fins for settlement, either as a

site of passive preferential settlement or a site of increased survival, but as yet these are undetermined for *L.salmonis*. No histological differences in the epithelial structure were found between the fins and body surface of Atlantic salmon by Turnbull (1992) and therefore it would suggest that hydrodynamics of the fin surface play an important role in settlement and survival. Anstensrud & Schram (1988) suggest that larval *Lernaeenicus sprattae* (Sowerby, 1805), although initially randomly distributed over the host will actively migrate to the host fins within two hours of infection. Anstensrud & Schram (1988) further suggest that the guidance mechanism by which *L.sprattae* "homes in" on the fins is by means of water currents. Kabata & Cousens (1977) explained the disproportionate settlement distribution of *Salmincola salmoneus* Dana, 1852 as a result of preferential attraction to respiratory and fin currents. Bron *et al.* (1991) suggest that *L.salmonis* settlement distribution may result from advantageous local current speeds and increased ability of the copepodid to secure itself to fin rays. Settlement sites may therefore be viewed as sites that offer better protection/shelter than sites of preferential settlement; site selection through survival rather than active site selection. Pickering (1974) found that the mucous cell distribution of *Salmo trutta* (L.) and *Salvelinus alpinus* (L.), although highly variable did show a significant difference in the numbers of cells per mm^2 in specific body regions, cell counts highest in the anterior regions and significantly fewer cells in the fins. Harris & Hunt (1975b) found that higher mucous cell counts in the non-scaled areas than scaled areas of *Salmon salar*. In adult Atlantic salmon the epidermis of the head is thicker, 10-15 layers of cells compared to scaled areas 6-12 layers of cells (Harris & Hunt, 1975a). Van Oosten (1957), Pickering & Richards (1980) and Hawkes (1983) all state that the epidermis and its mucous secretions form an immediate interface against a potentially hostile environment and infection. The copepodid attaches to the host by use of its second antennae, penetrating the host epidermis and therefore mucous

secretions will have little part to play in the survival of *L.salmonis*.

Examination of the parasite losses after ten days in the heated regime of the temperature experiment and the ambient salinity regime, where development had advanced past the copepodid stage, shows a particular pattern of loss. In the heated water system the distribution of losses shows a similar pattern between the two experiments with losses being greatest from the gills, pelvic and caudal fins, in declining order of importance. The reduction in parasite numbers from the fin sites after 10 days may be due to intraspecific competition for space, through parasite size and competition for a food source. The gills show a large percentage settlement at D.P.I. 5 in all groups, which agrees with Johnson & Albright, (1991a) but this body region also shows the largest reductions at D.P.I. 10, approximately 50% in both experiments. Post-settlement development on the gills is possible (Johnson, 1993; A. Shinn, pers. comm. 1995, pers. obs.) through the various life stages to the motile preadults where they emerge onto the host body surface (Shinn, pers. comm. 1995). Every available surface of the salmonid host is utilised by *L.salmonis* for settlement, even the vent (pers obs.) with successful development. Bron *et al.* (1991) report the presence of *L.salmonis* on the gills and buccal cavity, although these authors found only small numbers of parasites on each fish. Bron *et al.* (1991) further suggest that although *L.salmonis* were found on the gills this may be an experimental artefact of tank trials, resulting from slow current speeds. Settlement on the gills is presumably the result of larvae being inhaled with the respiratory current, and to that extent is an opportunistic and incidental settlement site. Whether apparently preferred settlement sites are actively sought at the time of infection or result from increased survival of larvae in these sites is unclear and still to be debated.

Temperature is of primary importance to the settlement and survival of *L.salmonis*, whilst

salinity is secondary, particularly if reduced salinity is compensated by higher sea water temperatures. For *L. salmonis*, in its natural environment both environmental variables will play a significant and interacting role. Kabata (1981) suggests that in general parasitology the parasite should have a broader tolerance to environmental conditions than its host to be successful, the sensitivity of the free-swimming and infective stages being the limiting factor. This study has suggested that the preferred salinity for settlement is above 25‰ although epizootic outbreaks in Scotland and Norway have occurred in salinities of 17‰ (Stuart, 1990).

The settlement counts were conducted in *in vivo* environments and therefore possibly may not necessarily be representative of a host situation in its natural environment. However, sea cages act as a reservoir of potential hosts and such high settlement rates may be possible at warmer sea water temperatures, particularly with multiple infections of the host.

Presently, as our understanding of the dynamics of the infection process are limited, it is the effect of temperature on the generation time of the parasite that is of most significance. Due to the large variability, at a given temperature in *L.salmonis* settlement it is the generation time of the parasite, once affected by any increases in temperature, that will have the most significant impact on the subsequent generations and the impact on salmon farming. It is the presence of motile stages that cause significant damage to the salmonid host and therefore reduced generation times will have profound effects on the actions of the salmon farmer. Temperature effects on development are shown to be consistent and therefore predictable, not highly variable like the settlement counts observed. Although copepodids show a loss of infectiousness with temperature, epizootic outbreaks of winter population lice are possible. Metabolic pathways will be affected by fluctuations in ambient sea water temperatures and therefore diligence is required in the control of sea lice

with the increase in sea water temperatures.

CHAPTER 4. BIOTIC SETTLEMENT FACTORS.

4.1 INTRODUCTION.

There are many biological variables that affect the interactions of sea lice and its salmonid host, many of which interact forming a complex, combined effect. Biological variables influence the host and parasite individually or as a combined effect. Understanding the biological interactions will increase our knowledge of host parasite interactions.

Factors to be examined include:

1. The effect of the age of copepodid on infection.
2. The effect of dose rates of copepodid on infection.
3. The effect of stocking density of the host at the time of infection.
4. The effect of multiple wave infections on the host.
5. The effect of presented surface area for infection.
6. The effect of alternative hosts on infection.

4.1.1 AGE OF COPEPODID ON INFECTION.

A study of the energetics of the barnacle cyprid has been conducted by Lucas *et al.* (1979) who showed that the older the larval stage prior to settlement the less likely it is to survive and successfully metamorphose. The energy budget of the lecithotrophic cyprid is linked to its internal energy reserves accumulated by the earlier naupliar stages (Waldock & Holland, 1978). The biochemical composition of energy reserves within barnacle cyprids comprise lipids, with 9.2% (of total dry weight) neutral lipid (Holland & Walker, 1975).

Triacylglycerols (TAG), account for 63% of the barnacle cyprid neutral lipid fraction (Waldock & Holland, 1978). If free-swimming cyprids are prevented from settling then survival can be up to 8 weeks (at 8°C) before the exhaustion (90% loss) of the lipid reserve (Holland & Walker, 1975). However there is rapid lipid depletion after 4 weeks, with 60% of neutral lipid reserve lost (Holland & Walker, 1975). A study of barnacle cyprid energetics has shown that for successful (>90%) settlement and metamorphosis to the juvenile barnacles (Lucas *et al.*, 1979) settlement must take place before the end of week 4 at 10°C. Between week four and five settlement and successful metamorphosis decrease from approximately >90% in week four to less than 50% in week five. After week four the levels of energy reserves needed for successful metamorphosis have been depleted in many individuals, although the energy requirement for settlement in sheltered microhabitats is lower than exposed sites (Lucas *et al.*, 1979).

L.salmonis copepodids are believed to be lecithotrophic (Bron, 1993) and therefore there is finite energy available to find and settle on the host. Johannessen (1978) suggested that copepodids may live up to one month, if attached to a substratum, however Wootten *et al.* (1982) suggest that such a duration is unlikely given its presumed dependence on energy reserves. Temperature will have a profound effect on longevity and survival. Gravil (1996) found a maximum longevity of *L.salmonis* copepodids to be 17 days at 5°C and 9 days at 15°C. Johnson & Albright (1991a) suggest copepodid survival to be 4 days at 5°C and 6 days at 15°C, whilst Wootten *et al.* (1982) report copepodids remain active for 4 days at 12°C. 50% mortality of copepodids occurred within 4.8 days at 5°C and 3.4 days at 15°C according to Gravil (1996), longevity and metabolism of the copepodid being directly linked to temperature. Infection experiments with aged copepodids were conducted by Gravil (1996), who found that seven day old copepodids had a reduced infection success

compared to one day old copepodids, the difference in percentage settlement being 8%.

4.2.2 DOSE RATE OF COPEPODID.

No literature appears to be available on the effects of varying dose rates of infective stages of parasitic crustaceans on their settlement on the fish host. Cage aquaculture provides a large potential reservoir of hosts for infection. The infection dynamics of *L.salmonis* would appear to be governed by self-infection and therefore fish will be exposed to ever increasing numbers of lice. During the salmon production cycle sea lice population numbers will be increased, capable of reaching epizootic proportions, which will result in increased numbers of potentially infective larvae. During the production cycle fish will be exposed to varying numbers of copepodids. The effect of increased numbers of copepodids on the host requires investigation.

4.1.3 STOCKING DENSITY OF THE HOST.

Stocking densities of Atlantic salmon have fallen to one-fifth of their 1970's stocking densities (Beveridge, 1996), now being about 10 kg m^{-3} (Sedgewick, 1988; Laird & Needham, 1988) although densities of 35 kg m^{-3} can occur at harvest (Pillay, 1993). Although the stocking density has reduced the cage sizes have increased from 100-150 m^3 in the 1960's and 1970's to fifty times that size now (Beveridge, 1996).

Parasites cause stress in their hosts; aquaculture also causes stress in most species being cultured. Stress is accumulative and the stress of moderate parasitic infection levels plus crowding can be lethal (Burt & Mackinnon, 1997). High stocking densities cause

environmental stress to fish species and therefore increase the susceptibility of the fish to disease outbreak (Snieszko, 1974). Mawdesley-Thomas (1972) suggests that "disease, *per se*, is not an entity or an end in itself. Disease is the end result of an interaction between a noxious stimulus and a biological system and to understand disease is to understand all aspects of the biology of the species". It is important to understand and consider the relationship between the "noxious stimulus" and the outbreak of disease. Fish rearing operations are frequently subject to parasitic diseases as a result of a decrease in the resistance of the fish caused by stress. In addition high stocking densities offer the opportunity for the rapid transmission of pathogens by contact (Davydov & Isayeva, 1990). Stocking density effects the degree of infestation of carp as reported by Davydov & Isayeva (1990), the stocking density produces a direct (due to the transmission dynamics and the proximity of healthy fish to diseased fish) and indirect effect (via its influence on the morphophysiological and biochemical status of the fish). At high stocking densities the abundance of parasites with a direct development cycle increases (Davydov & Isayeva, 1990). Excessive crowding with high stocking densities of fish will therefore increase the potential for epizootic outbreaks.

4.1.4 SECONDARY INFECTION OF COPEPODID.

Cage aquaculture will result in a continuous exposure of fish to pathogens originating within and outside the cage system. All other experiments within this study, have been conducted to examine the influence of various factors on settlement and survival of the copepodid larvae of *L.salmonis,* using only single discrete experimental infections, whereas in a cage culture system fish would be constantly exposed to the infective stage of parasites. Lester & Adams (1974) demonstrated that sticklebacks exposed to multiple

infections of *Gyrodactylus alexandri* showed a reduced parasite burden after a period of recovery (1 week) by the host.

4.1.5 SURFACE AREA OF THE HOST.

Margolis, Esch, Holmes, Kuris & Schad (1982) provided the parasitological definition, amongst others, for the term density as "number of individuals of a particular parasite species per unit area, volume or weight of infected host tissue or organ". The surface area of a potential host will have a marked effect on the numbers of ectoparasites and the resulting pathology. Large fish are generally better able to withstand an infection of a given size than a small fish infected with the same number of parasites. It is generally the case of parasitic infections, that there is an increase in intensity of infection with increase in host size; due to an increase in the size of surface available for attachment (Dogiel, Petrushevski & Yu, 1958). Hanek & Fernando (1978) clearly indicate that there is an increase in intensity of infection with Monogenea and Copepoda with the age and size of *Lepomis gibbosus.*

Jaworski & Holm (1992) in their paper on the distribution of post-chalimus sea lice on the fish examine the effects of host surface area on parasite intensity. These authors devised a method of expressing parasite intensity on fish of different sizes, with a surface area model which calculates either percentage parasite coverage of the host or parasite density in 8 specific regions. Their model calculations, however, do not take into consideration the preferential settlement of *L.salmonis* on the fins. Jaworski & Holm (1992) report that larger fish have a higher parasite intensity due to the increased surface area available and that surface area is a more reliable means of reporting lice intensity than merely percentage

parasites when fish of varying sizes are considered. Jaworski & Holm (1992) suggest two methods of describing the parasite distribution on the host: 1. the number of lice in each body region is expressed as a proportion (or percent) of the total number of lice on the fish; 2. the infestation in each body region is expressed as a ratio of the local parasite intensity to the average for the whole fish body. Both methods express in parasites in terms of numbers per unit area (cm^2). The interpretation of host surface area and therefore parasite density is an important consideration when comparing fish of differing sizes.

4.1.6 ALTERNATIVE HOST.

The decline of the sea trout populations in the West of Ireland and West of Scotland is a very controversial issue. The establishment of salmon farms is said to have led to a proliferation of sea lice numbers some of which have infected migrating sea trout and caused their death or premature return to freshwater. The collapse of the sea trout population has not just been associated with the culture of salmon in Ireland and Scotland. The problems first became apparent on the west coast of Ireland in the late 1980's (Whelan, 1993) and continue. In May sea trout post-smolts have been recovered with heavy infections of sea lice and adult fish, during the angling season appeared thin and displayed poor gonadal development (Whelan, 1993). Rod and line catches of sea trout in north-western Scottish rivers have been on the decline since the 1950's, with unprecedented low catches from 1989 to 1992 (Walker, 1993). In Norway ascending sea trout post-smolts and adults have had heavy infestations of sea lice and were in poor physical condition (Birkeland, 1996). In Scotland a sharp drop in sea trout catches began in 1989 with *L.salmonis* suggested as the major cause by Northcott & Walker (1996) although this study concentrated its analysis on the west and north-west coastal regions

with only three sites sampled outside salmon farming regions.

From preliminary analysis Walker (1993) suggests that the primary cause of sea trout decline in west highland rivers is due to increased marine mortality. A decline in sea trout catches was also evident in England and Wales during 1989 (Anon, 1994b) in regions where salmon cage culture is not present. The collapse of the sea trout population may be a natural phenomenon. Determinant population dynamics from short-term studies can introduce a presumptuous conclusion.

Other possible causes for the collapse of the sea trout population are:

1. poor smolt adaptation to salt water due to physiological stress,
2. the effects of increased acidity on the physiology of migrating parr or smolts,
3. the presence of disease or the effects of increased internal or external parasite loading development (Whelan, 1993).

Dawson, Pike, Houlihan & McVicar (1997) demonstrated a greater susceptibility of sea trout when infected with *L.salmonis* copepodids compared to Atlantic salmon. In their study of single and mixed populations of fish, infected with a low dose rate of copepodids the sea trout populations showed the highest infection intensity.

4.2 STUDY AIMS.

The work in the present chapter sought to evaluate the effect of specific biotic effects on the settlement and survival of *L.salmonis*. Those biotic factors considered include:

1. The age of copepodid on infection

2. Varying dose rate of copepodid on infection

3. The effects of stocking density of the host on infection

4. Settlement and survival of repeated infections

5. Effects of surface area of the host on infection

6. Differential settlement and survival on salmon and sea trout.

Very little literature is available on such parameters and therefore an understanding of these important biological variables will give a better understanding of the transmission and population dynamics.

4.3 MATERIALS AND METHODS.

4.3.1 FISH HUSBANDRY FOR ALL BIOTIC FACTOR EXPERIMENTS.

All fish were maintained at ambient sea water temperatures and salinity as described in chapter 2.2.1. Experimental tanks used for this series of experiments were:

For surface area experiments: 1 x 800 litre circular tanks

For alternative hosts (mixed populations): 2 x 300 litre square tanks

For stocking density: 3 x 300 litre square tanks

All other experiments were conducted in 3 x 70 litre circular tanks, 30 fish per experimental group, 10 fish per replicate.

S½ salmon smolts of approximately 100g weight were used for all experiments, except the

surface area experiment where fish of differing sizes were used. The anaesthetic used in all experiments was MS222 except for the 1995 experiments, age of copepodid (age exp. 1) and dose rates of copepodid (dose rate exp. 1) where Benzocaine was used.

4.3.2 SAMPLE COLLECTION OF LICE.

Sea lice were collected from a single naturally infected salmon farm site as described in chapter 2.2.2.

4.3.3 INCUBATION OF OVISACS FOR ALL BIOTIC FACTOR EXPERIMENTS.

All incubations of ovisacs were at ambient sea water temperature and salinity for all experiments described in this chapter. Incubation and hatching procedures were as described in chapter 2.2.3.

4.3.3.1 AGE OF COPEPODID.

To obtain copepodids of a known age each day's hatch of a batch of eggs was separated to give a 24-hour cohort of larval stages. Daily examination of the progress of the incubation of the batch of eggs was conducted to determine the hatching and development of the larvae. When an incubation vessel contained >60% copepodids this was registered as day 1. Once sufficient copepodids of age 1 day, age 3 days and age 7 days had been accumulated and numbers calculated as described in chapter 2.2.4, the experimental infections were carried out.

These experiments were conducted in May 1995, experiment 1; August 1997, experiment 2 and March 1996, experiment 3. Experiments 1 and 2 were carried out in 70 litre flow-through tanks, three tanks per experimental group, 10 fish per tank. Experiment 3 was carried out in three 300 litre flow-through tanks, again 30 fish per experimental group. The anaesthetic used in experiment 1 (1995) was Benzocaine whilst in experiments 2 (1997) and 3 (1996) MS222 was used. Fish in all experiments were approximately 100g.

4.3.3.2 DOSE RATE OF COPEPODID.

Available copepodid numbers varied greatly depending on the success of the hatch and incubation. The aim of this experiment was to use a two-fold increase in parasite numbers between the experimental groups (Table 4.1).

Table 4.1 Dose rates (number of copepodids) per fish administered to salmon for dose rate experiment.

	Low Dose per Fish	Medium Dose per Fish	High Dose Per Fish	Very High Dose per Fish
Exp. 1 (1995)	100	250	400	N/A
Exp. 2 (1996)	50	150	300	600
Exp. 3 (1996)	120	240	396	900

N = 30 for each experimental group except the excessive dose rate where only 10 fish were used.

All experiments were carried out in 70 litre flow-through tanks, three tanks per experimental group, 10 fish per tank. These experiments were conducted in July 1995, experiment 1; August 1996 for experiments 2 and 3. The anaesthetic used in experiment 1

(1995) was Benzocaine whilst in experiments 2 (1996) and 3 (1996) MS222 was used. Fish in experiment 1 and 2 were approximately 45g whilst fish in experiment 3 were 120g.

4.3.3.3 STOCKING DENSITY OF THE HOST.

Copepodids were introduced into three 300 litre tanks containing 5, 15 or 25 fish, respectively. Using fish of the same size (95.8g ± 9.95) resulted in stocking densities of 1.64 kg m^{-3} (SD5), 4.48 kg m^{-3} (SD15) and 7.42 kg m^{-3} (SD25) in experiment 1 and 1.79 kg m^{-3}, 4.54 kg m^{-3} and 7.61 kg m^{-3} in experiment 2.

Experiments were conducted in June/July 1996 and the anaesthetic used was MS222 for both experiments. An approximate copepodid dose rate of 5000 was introduced into each tank.

4.3.3.4 SECONDARY INFECTION OF COPEPODID

Multiple infections were administered to experimental fish. The infection protocol required a small initial infection then the main infection which was administered at the same time to both experimental groups. Dose rates used were:

Single inf. = Single infection of approximately 8000 copepodids

Double inf. = Double infection of copepodids, initial dose of approximately 2500 followed by approximately 5500 copepodids.

The initial dose of approximately 2500 copepodids was administered to one fish group, then five days later a second application of 5500 was introduced to the experimental group.

Three 70 litre flow-through tanks were used experimental group with 10 fish per tank. These experiments were conducted in July 1996 and June 1997 with all fish of approximately 80g. The anaesthetic used in both experiments was MS222.

4.3.3.5 SURFACE AREA OF THE HOST.

A surface area experiment had been conducted to quantify the surface area of salmon, using image analysis to examine and calculate surface area of fish of different sizes. The areas of the fish examined included the individual fins and the total body surface area. Fish were dissected and photographed to allow the tracing of the surface area by image analysis. It was therefore possible to estimate total area of a specific experimental group of fish in tanks from weight before infection.

Three experimental groups were utilised, 5 large fish (mean wt. 730g), 10 medium size fish (mean wt. 179g), and 30 small fish (mean wt. 38g). Surface areas of the fish body and fins were determined by Image Analysis. Prior to infection the approximate surface area of 5 large fish (ave. 644.4g ± 61.6), 10 medium sized fish (ave. 172.9g ± 31.8), and 30 small fish (ave. 42.8g ± 4.1), were calculated and found to be ≈ 2800 cm^2 for large fish, ≈ 2600 cm^2 for medium fish and ≈ 2800 cm^2 for small fish.

The simultaneous infection of all 45 fish took place in a single tank, 736 litres therefore giving an approximate copepodid concentration of:

Expt. 1 (ISO 5) = 10.32 copepodids per litre. Expt. 2 (ISO 6) = 10 copepodids per litre

For examination of the fish the anaesthetic MS222 was used. These experiments were conducted in May 1996.

4.3.3.6 ALTERNATIVE HOST.

The alternate host utilised for this experiment was the sea trout, *Salmo trutta.* 30 sea trout were used for each experiment. In the first experiment 30 salmon and 30 sea trout were kept in separate tank populations whilst in the second experiment 30 salmon and 30 sea trout were kept in mixed populations. The was no significant difference in fish lengths between the two species ($p>0.05$). The mean length of sea trout in experiment 1 was 181.6 mm ± 1.51 whilst that of salmon was 170.4 mm ± 0.85. In experiment 2, the mean length of sea trout was 181.3 mm ± 12.63 whilst that of salmon was 179.6 mm ± 10.59.

Two populations of sea trout (*Salmo trutta*) and salmon (*Salmo salar*) were utilised for the infection experiments, one a separate population and the second a mixed population, in both 30 fish (≈ 180g) of each species were used. In the separate population experiment fish were held in 3 x 70 litre tanks, 10 fish per experimental group and experimentally infected with 6000 copepodids per group. In the mixed populations, fish were held in 2 x 300 litre tanks, 15 fish per species per tank. A dose rate of 6000 copepodids was administered to each tank.

Both experiments were conducted in July 1997 and the anaesthetic used was MS222.

4.3.4 INFECTION AND EXAMINATION PROCEDURE FOR THE BIOTIC FACTOR SETTLEMENT EXPERIMENTS.

Once sufficient copepodids were found to be present in the incubation vessels (as calculated in chapter 2.2.4) the standard infection procedure and examination was carried out as described in chapters 2.2.4. to 2.2.6. Fish held under anaesthetic for examination

(post-infection day 5 and 10) were maintained in ambient conditions. At post-infection day 10 (D.P.I. 10) fish were killed by an overdose of anaesthetic and lice counted. If preadult or adult life stages were found to be present, then the anaesthetic container was filtered after each fish to collect any lice that may have been removed during anaesthesia.

Data from age of copepodid (experiment 2) was also analysed for the effects of aged copepodid on development, settlement adjacent to the fins and for any settlement differences in the dorsal and ventral surface of paired fins. The comparative rate of *L.salmonis* development was analysed for the sea trout and salmon populations.

The percentage losses by host body region for these experiments have not been calculated as they were largely carried out at higher sea water temperatures which resulted in faster development and motile stages were always present at D.P.I. 10. As the motiles can freely move about the host body surface and between fish calculating percentage losses from specific host body regions was not possible.

4.3.5 STATISTICAL ANALYSIS.

All statistics have been calculated on the numerical values with their respective (95%) confidence limits. General methods utilised for statistical analysis are described in chapter 2.2.9. Graphical representation of the data has been presented as percentages, unless stated, whilst all statistical analysis has been conducted on the numerical values except where the 95% confidence intervals have been calculated from the standard error (see chapter 2.2.9).

Statistical analysis was conducted on the settlement and survival data of each experiment. The settlement distribution data was analysed for statistical differences between the experimental groups and each host body region, further analysis was conducted between

the host body regions within each experimental group.

Experimental data from replicates was tested for statistical analysis before the data could be pooled. Only data that was not statistically difference, within the replicates could be pooled.

4.4 RESULTS.

4.4.1 AGE OF COPEPODID.

4.4.1.1 SETTLEMENT AND SURVIVAL OF COPEPODIDS OF DIFFERENT AGES.

The effect of the age of copepodid on infection was examined on three occasions, twice at summer sea water temperatures (mean 10°C ± 0.7 and 15°C ± 0.5 for exp. 1 and 2) and once at winter sea water temperature (6.5°C ± 0.6). In each experiment the effects of *L.salmonis* copepodid age on settlement can be clearly seen, Table 4.2 and Figures 4.1a-c.

Table 4.2 Percentage settlement and survival of aged copepodids of *L.salmonis*

	Cop 1 day Settlement	Cop 3 day Settlement	Cop 7 day Settlement	Cop 1 day Survival	Cop 3 day Survival	Cop 7 day Survival
Exp. 1 (1995)	75.42	74.70	53.90	75.65	83.94	82.34
Exp. 2 (1997)	80.74	81.94	44.56	90.34	72.01	58.81
Exp. 3 (1996)[1]	19.76	19.11	8.87	46.39	51.23	47.29

Key: Cop 1 day = copepodid aged 1 day from NII moult,
Cop 3 day = copepodid aged 3 day from NII moult,
Cop 7 day = copepodid aged 7 day from NII moult.

[1] Exp. 3 (1996) Age of copepodid on infection experiment conducted at winter sea water temperatures.

Figure 4.1a Effects of age of *L.salmonis* copepodid on settlement and survival (1995/1)

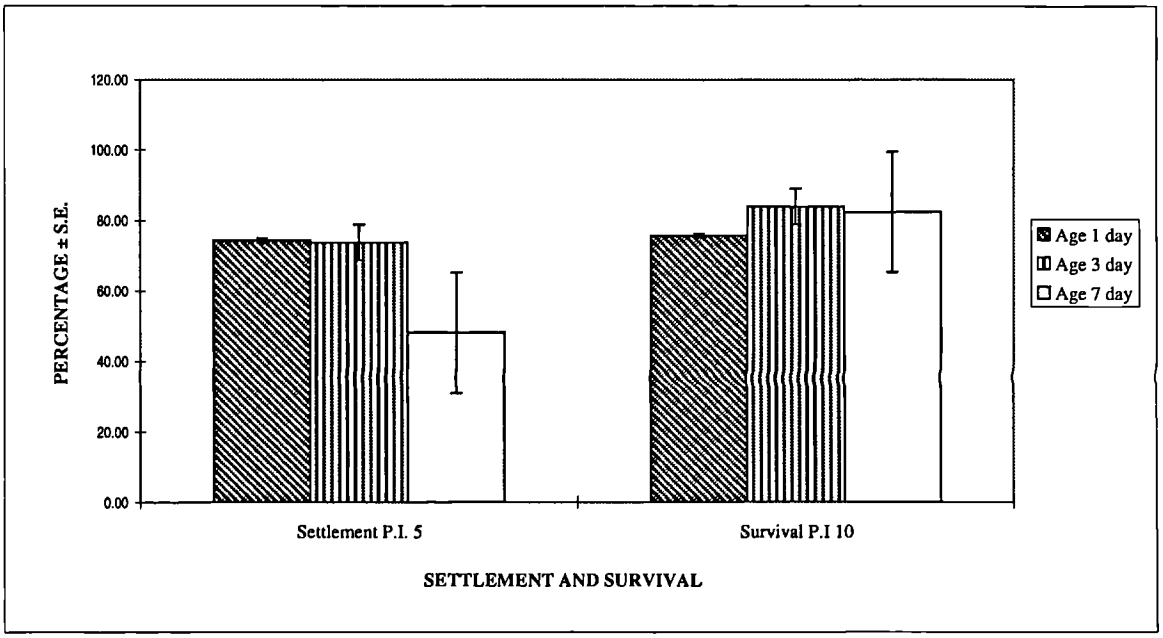


Figure 4.1b Effects of age of *L.salmonis* copepodid on settlement and survival (1997/2)

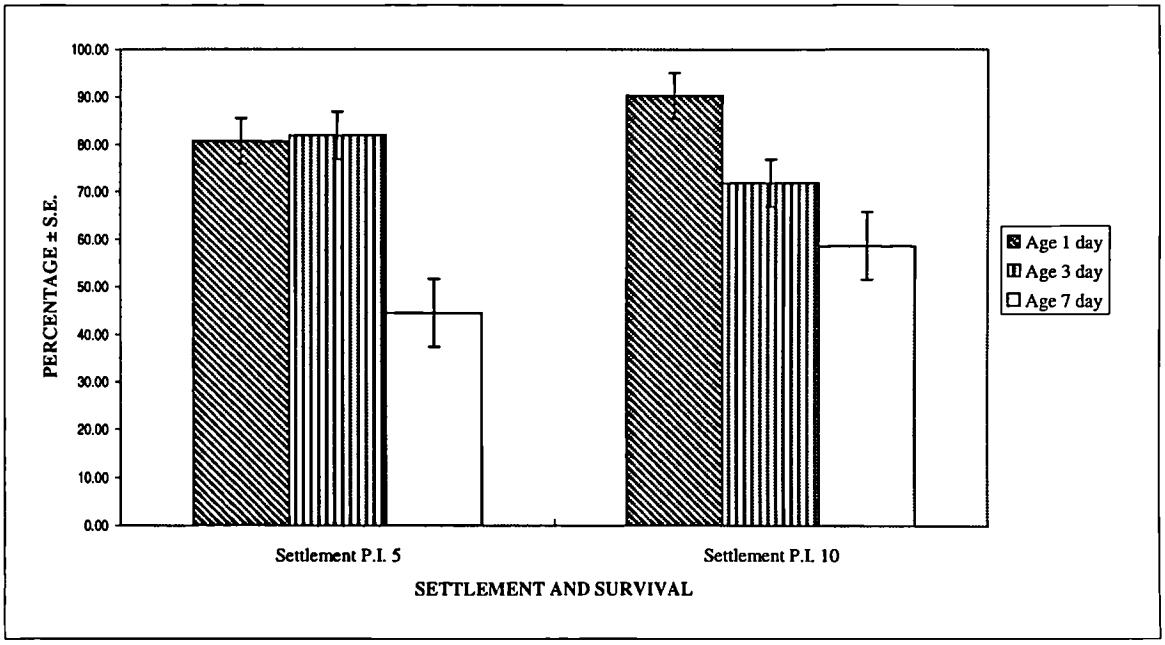
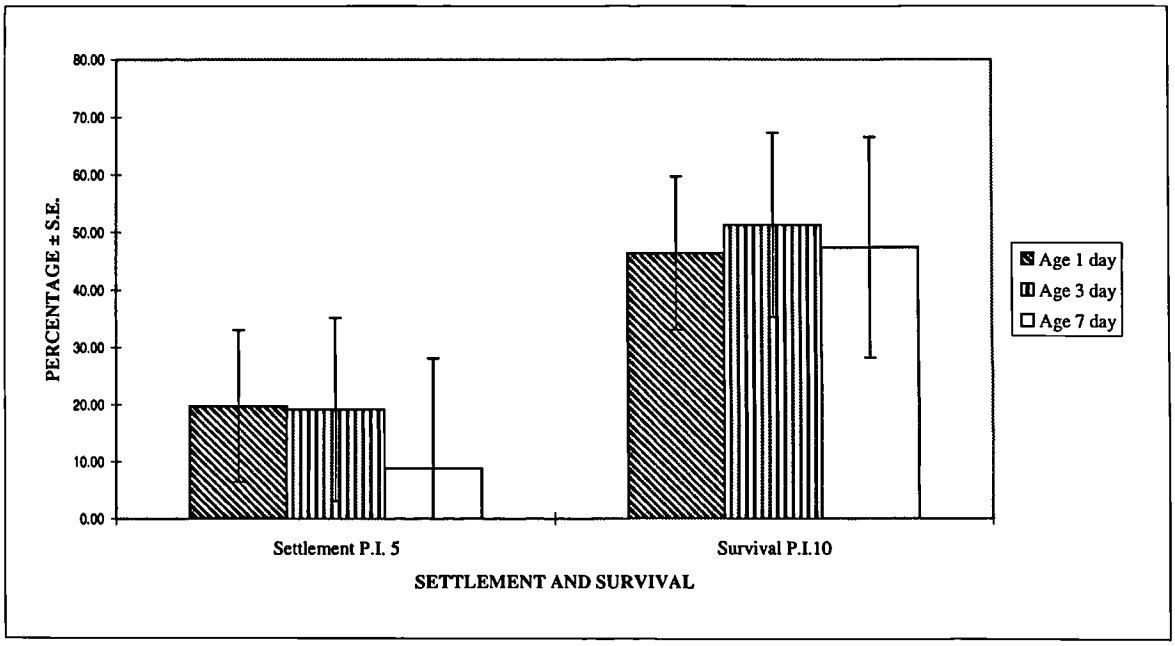


Figure 4.1c Effects of age of *L.salmonis* copepodid on settlement and survival (1996/3)



Key: Age 1 day = Copepodid aged 1 day, Age 3 day = Copepodid aged 3 day, Age 7 day = Copepodid aged 7 day.

There is a statistically significant difference in settlement success ($p<0.05$) between the 1 day and 3 day old copepodids and the 7 day old copepodids. The older copepodids showed a reduced ability to settle on the host. No statistically significant difference in settlement success was found between 1 and 3 day old copepodids.

Examination of the survival data for these experiments also showed a trend towards reduced survival post settlement with increasing age of copepodid. In experiment 1 a statistically significant difference was only found between 3 and 7 day old copepodids, in experiment 2 a statistically significant difference was found copepodids aged 1 day and 7 day and also between copepodids aged 3 day and 7days. In experiment 3, conducted at winter temperatures a statistically significant difference was found copepodids aged 1 day and 7 days and also between copepodids aged 3 days and 7 days. The percentage settlement of the youngest copepodid age groups in experiment 1 and 2 is high compared to experiment 3, reflecting differences in temperatures at which these experiments were conducted.

4.4.1.2 EFFECTS OF AGE OF COPEPODIDS ON SETTLEMENT DISTRIBUTION ON THE HOST.

Examination of the copepodid settlement distribution for each experiment shows (Figure 4.2a-c) that the body, gills, pectoral fins and the dorsal fin are preferred by all copepodid age groups, although there are differences between experiments.

In experiment 1, comparing the settlement between experimental groups (Figure 4.1a), significantly more parasites were found between the head and body region between copepodids aged 1 days and 3 days compared with the copepodids age 7 days ($p<0.05$).

Figure 4.2a Percentage settlement of *L.salmonis* aged copepodid by host body region at D.P.I. 5 (1995/1)

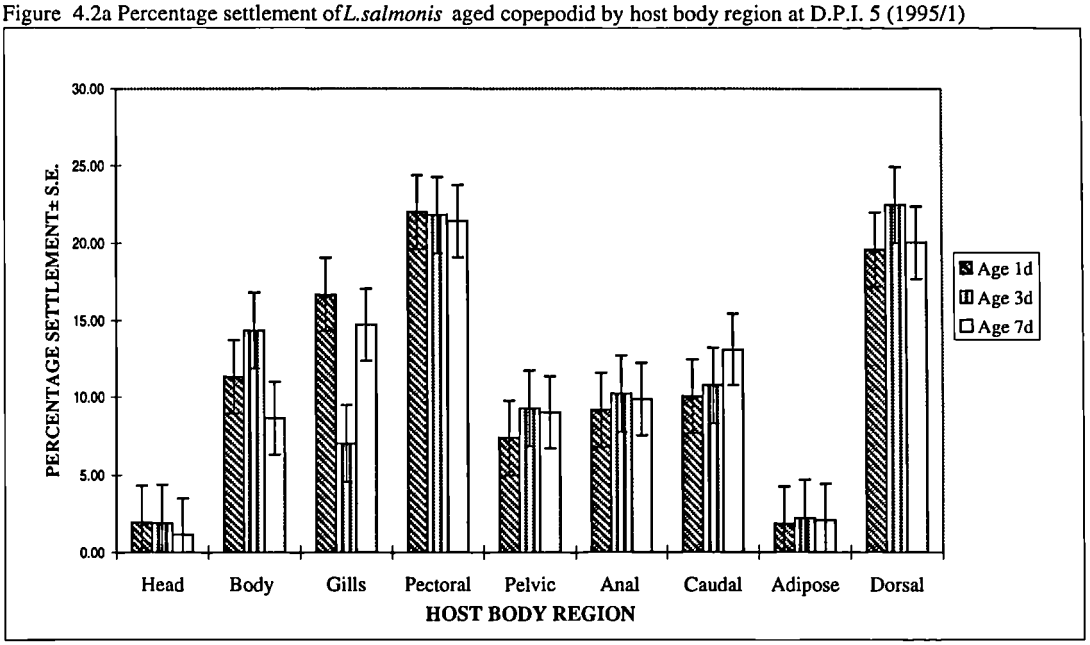


Figure 4.2b Percentage settlement of *L.salmonis* aged copepodid by host body region at D.P.I. 5 (1997/2)

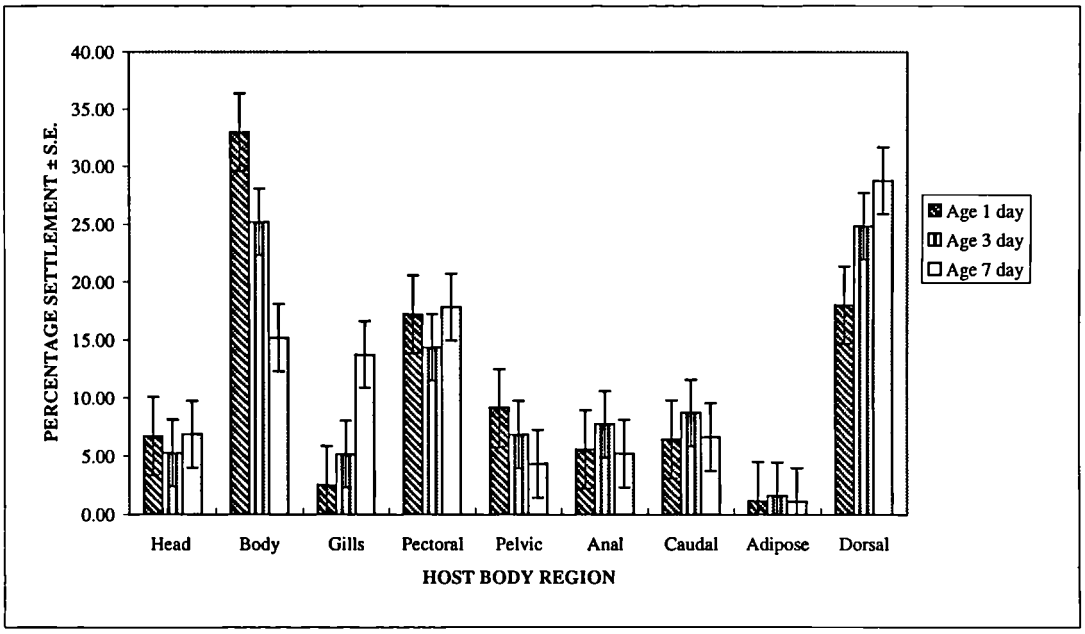
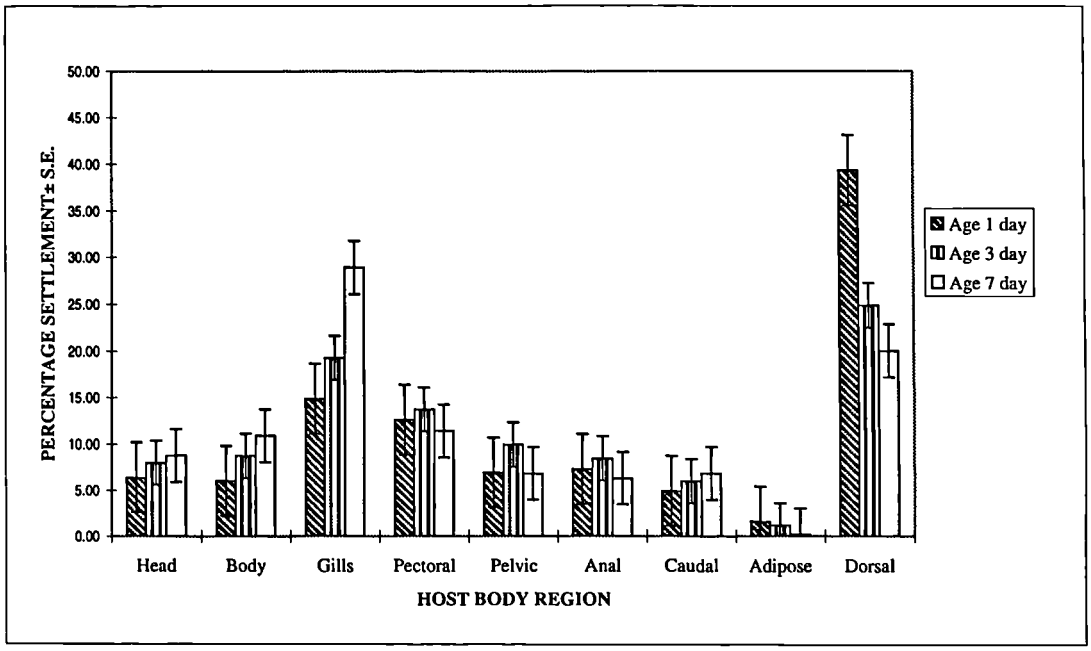


Figure 4.2c Percentage settlement of *L.salmonis* aged copepodid by host body region at D.P.I. 5 (1996/3)



Key: Age 1 day = Copepodid aged 1 day, Age 3 day = Copepodid aged 3 day, Age 7 day = Copepodid aged 7 day.

A statistically significant difference ($p<0.05$) in settlement on the gills was found between all copepodid age groups. No statistical difference ($p>0.05$) was found between the pectoral, pelvic, anal, caudal and adipose fins. Significantly more 1 day and 3 day old copepodids settled on the dorsal fin than copepodids age 7 days ($p<0.05$).

Examination of the statistical analysis of the settlement distribution in experiment 2 (Figure 4.2b) shows the statistically significant difference in the head region between the copepodids age 1 day and copepodid age 7 day only. The body region shows a statistically significant difference with higher settlement in copepodids age 1 day and 3 days than copepodids age 7 days. The gills show statistically higher settlement of the 7 day old copepodids than the other two groups. The pectoral and pelvic fins have a significantly higher settlement between the copepodids age 1 and 3 days than copepodids age 7 days. The anal fin shows a statistically higher settlement between the copepodids age 3 days and the other two experimental age groups. The caudal and dorsal fins show a significant statistical difference between all age groups. The adipose fin only shows a significant difference between the higher settlement of copepodids age 3 days with the copepodids age 7 days. While the dorsal fin region shows only a significant difference between the copepodids age 1 days and copepodids age 3 days.

In experiment 3 (Figure 4.2c), using winter population lice the settlement of aged copepodids on the pectoral, pelvic and adipose fins is statistically lower between the copepodids age 7 days and the other two experimental age groups. The head region has statistically more settlement in the copepodid age 7 days than the other two groups. The body and gills and anal and caudal fins show no statistical difference ($p>0.05$) in settlement between any of the copepodid age groups. The dorsal fin region shows statistically significant differences ($p<0.05$) in settlement between all copepodid age

groups.

Examination of settlement within specific age groups of copepodids for statistical differences shows a distinct pattern. In experiment 1 (Figure 4.2a) statistical analysis of copepodids age 1 day and copepodids age 3 days both show there is a significantly lower settlement ($p<0.001$) on the head and adipose fin than on the other body regions. Further there is a statistically higher settlement between the pectoral and dorsal fin, with a third group encompassing the remaining body regions, body, pelvic, anal, caudal fins where there is no statistically significant difference. Settlement on the gills is not statistically significantly different than that on caudal fin, although it is by comparison with the other body regions in this group. The copepodids age 7 day there is the same settlement pattern but the gills show a statistically significant difference ($p<0.05$) with all other body regions.

In experiment 2 (Figure 4.2b) within age group statistical analysis a different settlement pattern can be seen. There is a significant difference ($p<0.05$) between the high settlement of the 1 day old copepodids on the gills and all other body regions. There is also a statistically significant difference between pectoral and dorsal fins show a significant difference with all other body regions. The remaining host body regions show no statistically significant difference ($p>0.05$) among each other. Three day old copepodids show significantly higher ($p<0.05$) settlement on the body and dorsal fin than on all other body regions. The pectoral fins also show a significant difference with all other body regions. The remaining host body regions, except for the adipose fin show no statistically significant differences ($p>0.05$) among each other which is statistically significantly different ($p<0.05$) from all other body regions except the head and pectoral fin. Examination of copepodids age 7 day shows a significant difference ($p<0.05$) between the pectoral and dorsal fin region with high settlement and all other host body regions. The

body, gills and pectoral fin show a statistically significant difference ($p<0.05$) with all other body regions. The remaining host body regions show no significant difference to each other ($p>0.05$).

In experiment 3 (Figure 4.2c), settlement of 1 day old copepodids (winter population lice) is statistically higher ($p>0.05$) on the dorsal fin region than on the other host body regions. The gills and pectoral fins also show a statistically significant difference ($p>0.05$) between these regions and all other host body regions. The remaining body regions show no significant difference from each other ($p>0.05$). Three day old copepodids show a significantly higher settlement ($p>0.05$) between the dorsal fin and all other host body region except the gills. The gills show a statistically significant difference ($p>0.05$) with all other host body regions except the pectoral and dorsal fin. Of the remaining host body regions there is no significant difference to each other ($p<0.05$) except the adipose fin is significantly different to the pectoral and pelvic fins ($p>0.05$). Settlement of 7 day old copepodids was statistically higher ($p>0.05$) on the gills and dorsal fin than on all other host body regions. The remaining body regions show there is no significant difference to each other ($p<0.05$), except for the adipose fin which shows a significantly lower settlement than on the body and pectoral fins ($p>0.05$).

As seen in chapter 3, the fins account for the greatest proportion of copepodid settlement; >70% in experiment 1, >51% in experiment 2 and >57% in experiment 3.

4.4.1.3 ON HOST DEVELOPMENT OF DIFFERENT AGES OF COPEPODIDS.

In experiment 2 (Figure 4.3) the developmental rate, at day 10 post-infection of *L.salmonis* copepodids of different ages was analysed. In all age groups, the majority of *L.salmonis*

Figure 4.3 Effect of aged copepodid on the development of *L.salmonis* at D.P.I. 10 (1997/2)

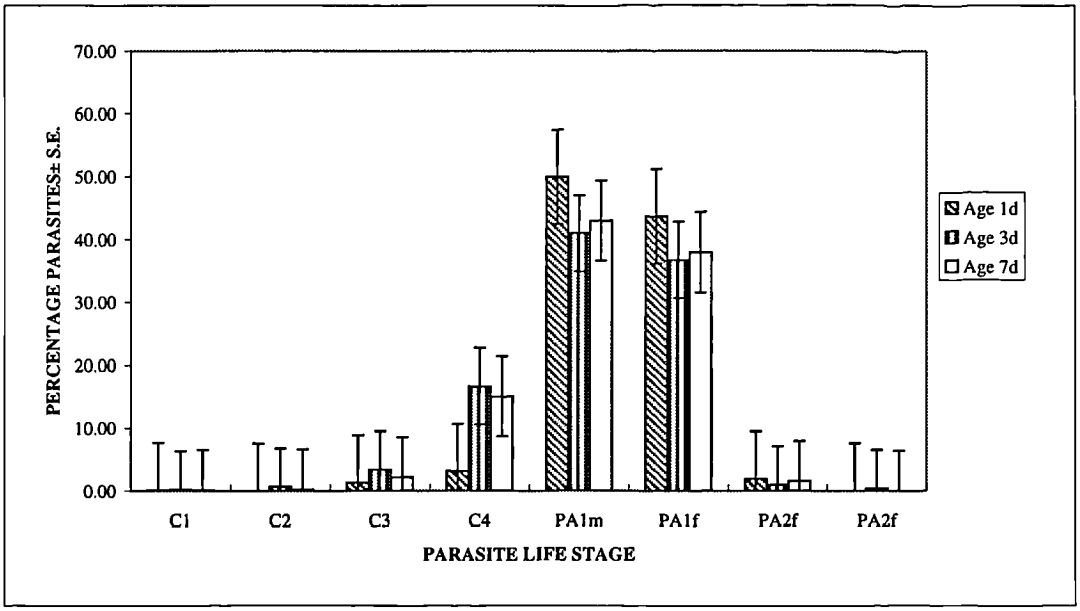


Figure 4.4 Effect of aged copepodid on the body settlement distribution of *L.salmonis* at D.P.I. 5 (1997/2)

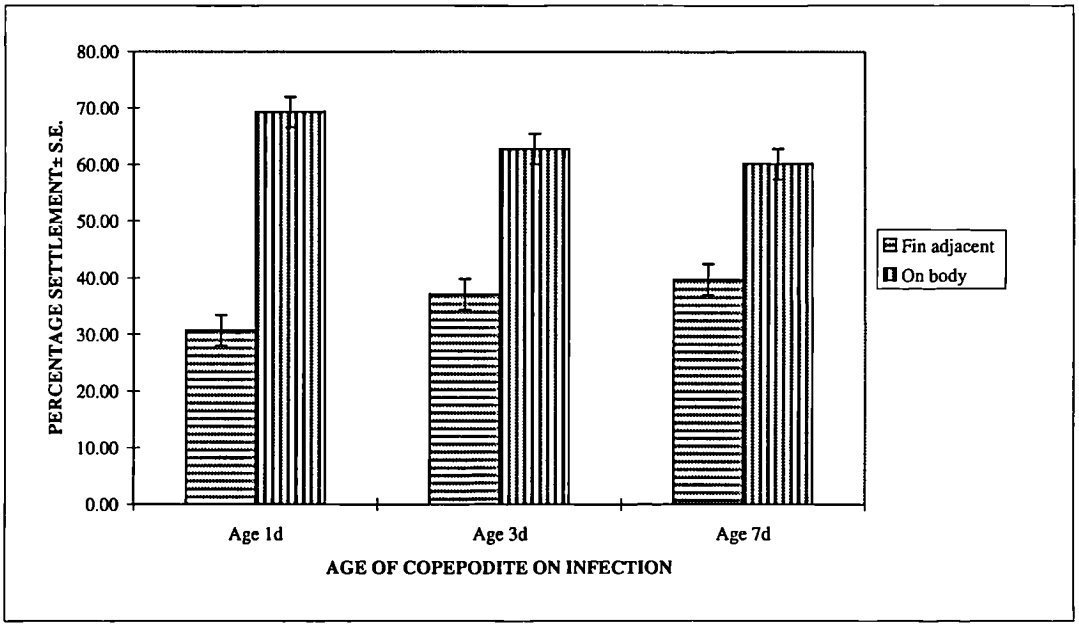
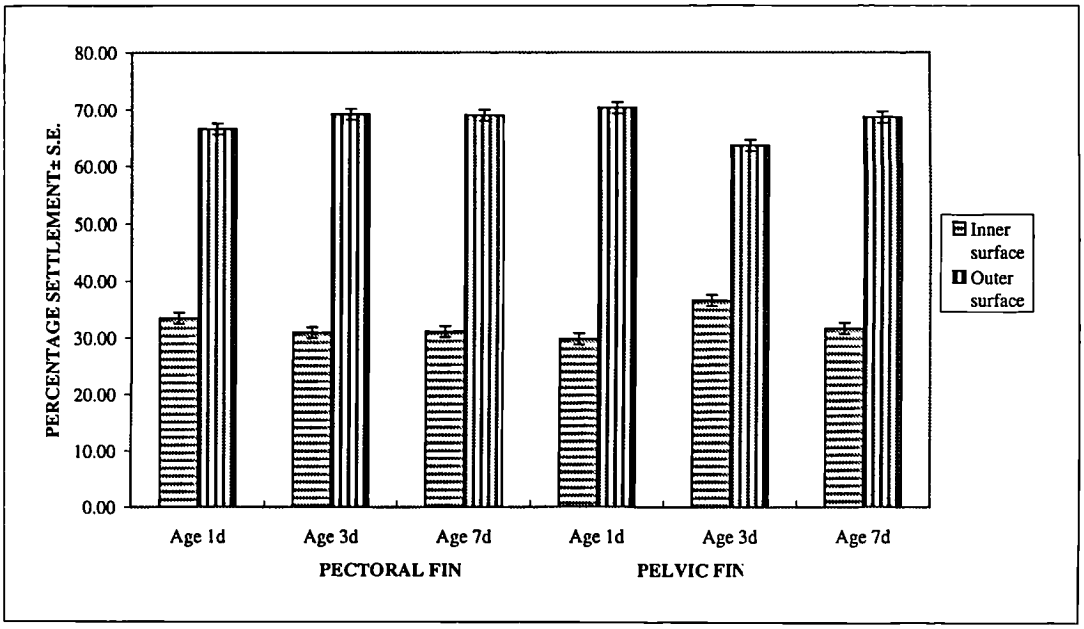


Figure 4.5 Effect of aged copepodid on the paired fin settlement distribution of *L.salmonis* at D.P.I. 5 (1997/2)



Key: Inner surface = fin inner dorsal surface; Outer surface = fin outer ventral surface

had progressed as far as preadult 1 males and females at D.P.I. 10, greater than 40% males in all groups and 36% females. No statistically significant difference was found between the proportion of parasites at different developmental stages and the age of copepodids at infection except for significantly lower numbers of chalimus 4 stage ($p<0.05$) on fish infected with 1 day old copepodids.

4.4.1.4 THE SETTLEMENT OF COPEPODIDS OF DIFFERENT AGES ON DIFFERENT PARTS OF THE HOST BODY SURFACE.

Analysis of settlement data from experiment 2 (Figure 4.4) suggests that copepodids on the body do not seek out sheltered areas adjacent to the fins. There is a clear statistically significant difference ($p<0.05$) between the settlement at the fin bases (>20%) and the remainder of the body of salmon by copepodids of all ages.

4.4.1.5 THE EFFECTS OF AGED COPEPODIDS ON FIN SETTLEMENT DISTRIBUTION.

The position of copepodids on the paired fins of Atlantic salmon was examined in experiment 2 to see if the inner surface, adjacent to the body or the outside surface had preferential settlement (Figure 4.5). With all copepodid age groups there is a statistically significantly greater number of lice ($p<0.05$) on the outer ventral surface of both pectoral and pelvic fins.

4.4.2 DOSE RATE OF COPEPODID.

4.4.2.1 EFFECTS OF DOSE RATE OF COPEPODIDS ON SETTLEMENT AND SURVIVAL.

The effect of varying copepodid dose rates on settlement and survival is shown in Figure 4.6 a-c and Table 4.3.

Table 4.3. Percentage settlement and survival for varying parasite dose rates

	Low Dose Settlement	Medium Dose Settlement	High Dose Settlement	Excessive Dose Settlement	Low Dose Survival	Medium Dose Survival	High Dose Survival	Excessive Dose Survival
Expt. 1 (1995)	38.67	37.60	56.61	N/A	92.58	90.58	90.37	N/A
Expt. 2 (1996)	22.33	21.80	29.26	22.35	96.06	89.19	86.40	84.32
Expt. 3 (1996)	55.50	48.51	52.89	35.07	81.98	82.83	83.62	80.12

Key: Low Dose = ≈ 100 copepodids per fish
Medium Dose = ≈ 250 copepodids per fish
High Dose = ≈ 400 copepodids per fish
Excessive Dose = ≈ 900 copepodid per fish

In experiment 1 (Figure 4.6a) there was a statistically significant difference ($p<0.05$) between settlement success at the low and medium dose rates and the high dose rate. Settlement at the low and medium dose rate was 38.67% and 37.60%, respectively whilst the high dose rate had a percentage settlement of 56.61%. In experiment 2 (Figure 4.6b) there was a significantly greater statistical difference ($p<0.05$) in settlement between the high does rate and all dose rates of parasites. In experiment 3 (Figure 4.6c) there was a statistically significantly higher settlement with ($p<0.05$) the low dose rate compared with the high and excessive dose rates. There was also a significantly higher settlement with the medium dose rate compared with the excessive dose rate.

Figure 4.6a Effects of dose rates of *L.salmonis* copepodite on settlement and survival (1995/1)

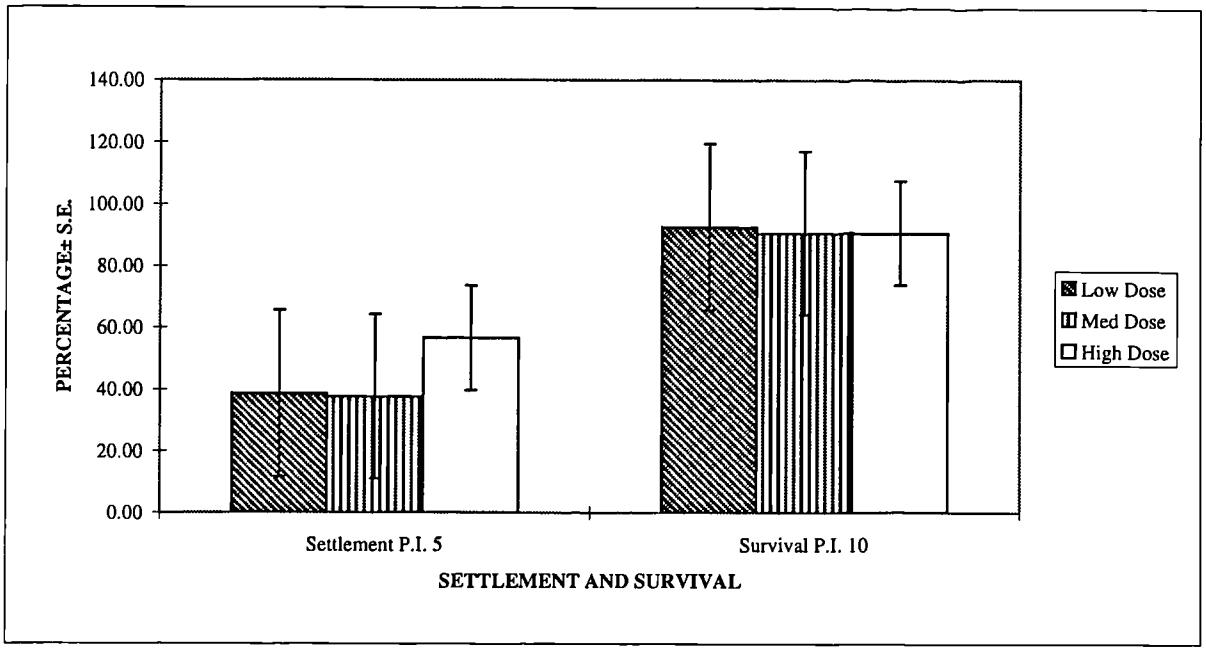


Figure 4.6b Effects of dose rates of *L.salmonis* copepodite on settlement and survival (1996/2)

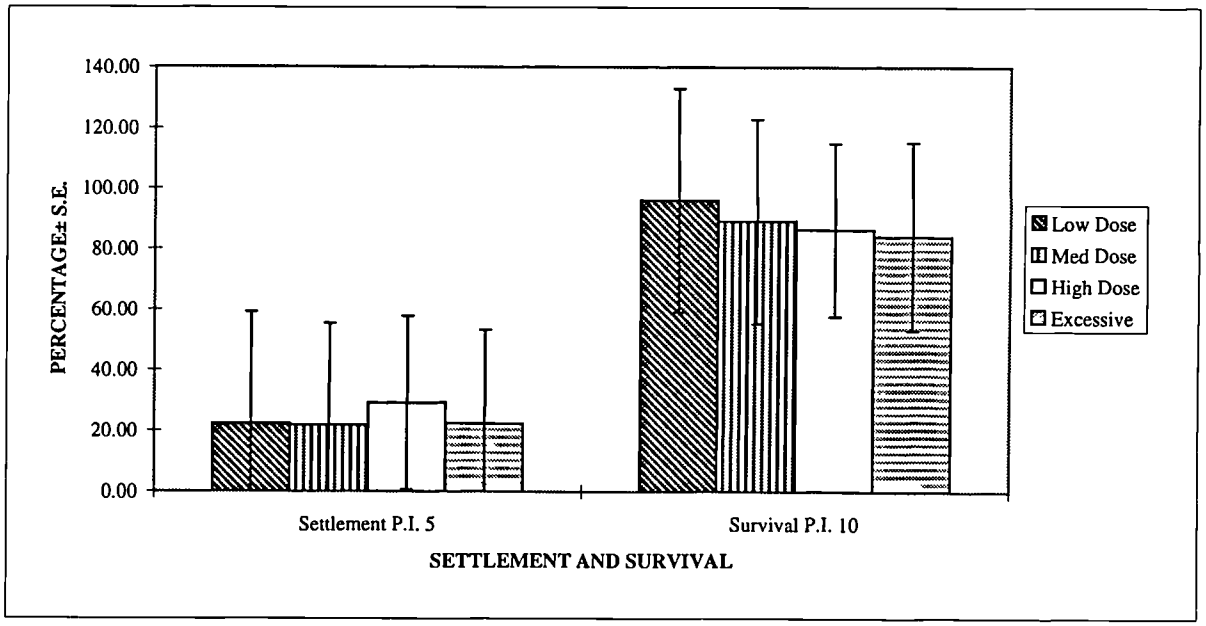
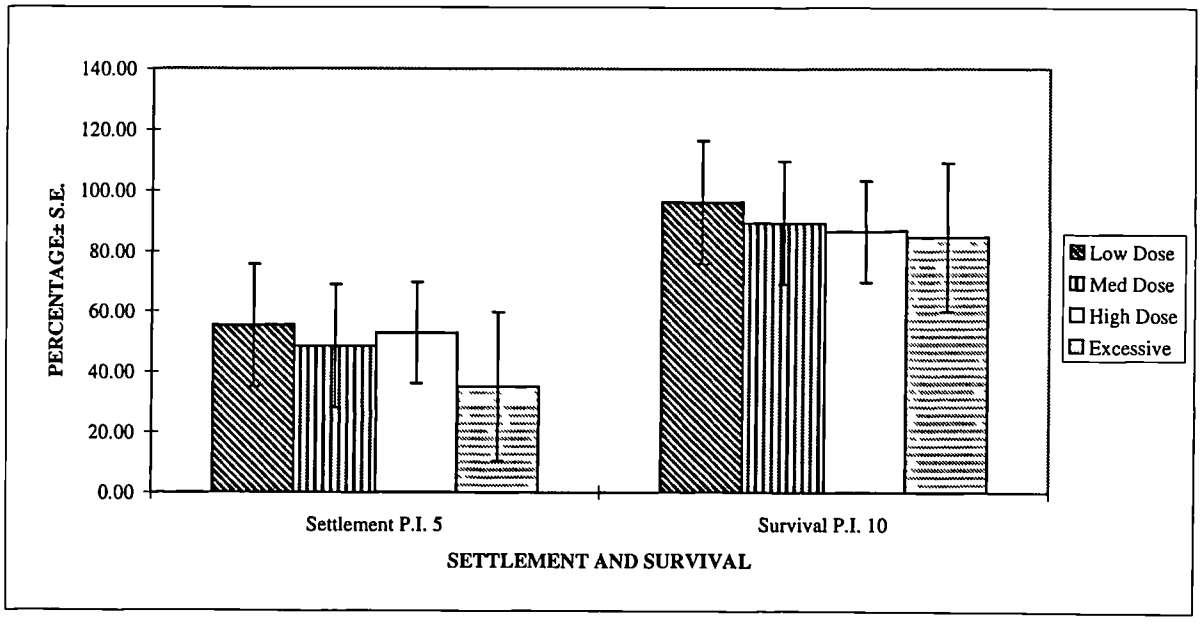


Figure 4.6c Effects of dose rates of *L.salmonis* copepodite on settlement and survival (1996/3)



There was no significant difference ($p>0.05$) in survival rate of *L.salmonis* at any dose rate in any of the experiments (Figure 4.6 a-c).

4.4.2.2 EFFECTS OF DOSE RATES OF COPEPODIDS ON SETTLEMENT DISTRIBUTION ON THE HOST.

Examination of the copepodid settlement distribution from varying dose rates shows preferred sites for settlement, (Figure 4.7a-c) in all experiments, particularly for the body, gills, pectoral fins and the dorsal fin, irrespective of copepodid dose rates.

In experiment 1 (Figure 4.7a) significant statistical differences were found between the head, body, gills, pectoral, pelvic, anal, caudal and dorsal fin regions between low and medium dose rates and the high dose rate group ($p<0.05$). On the adipose fin a significant difference was only found between the low and high dose rate groups ($p<0.05$).

Examination of the statistical analysis of the settlement distribution in experiment 2 (Figure 4.7b) shows the statistically significant difference ($p<0.05$) in the head, body, gills, pectoral, pelvic, anal, caudal and dorsal fin regions region between all dose rate groups except between the high and excessive dose rates. The pelvic fin regions show a statistically significant difference ($p<0.05$) between the low and medium and the high and excessive dose rate groups. The adipose fin shows significant statistical difference ($p<0.05$) between the high dose rate and the low and medium dose rates.

In experiment 3 (Figure 4.7c) the settlement of copepodids of varying dose rates on the head region shows a statistically lower settlement ($p<0.05$) between the low dose rate and the other three experimental dose rate groups. The body, pectoral, pelvic and caudal fins show a statistical difference ($p<0.05$) between the high and excessive dose rate sand the

Figure 4.7a Effects of dose rates on *L.salmonis* copepodite distribution at D.P.I. 5 (1995/1)

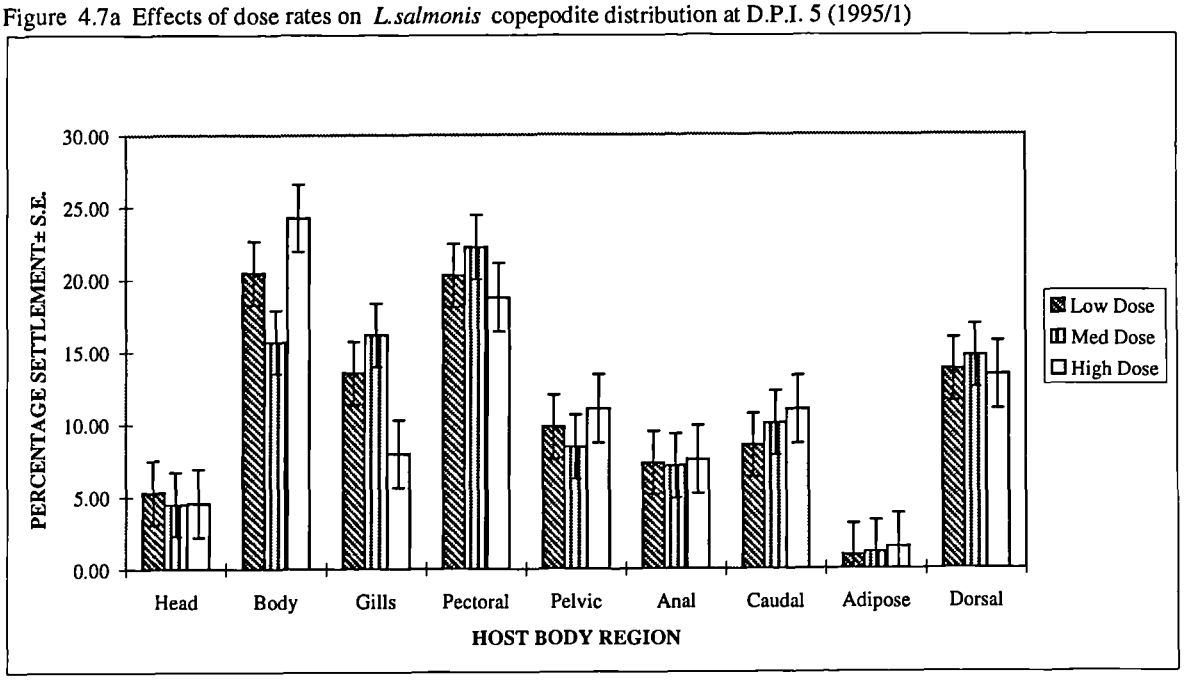


Figure 4.7b Effects of dose rates on *L.salmonis* copepodite distribution at D.P.I. 5 (1996/2)

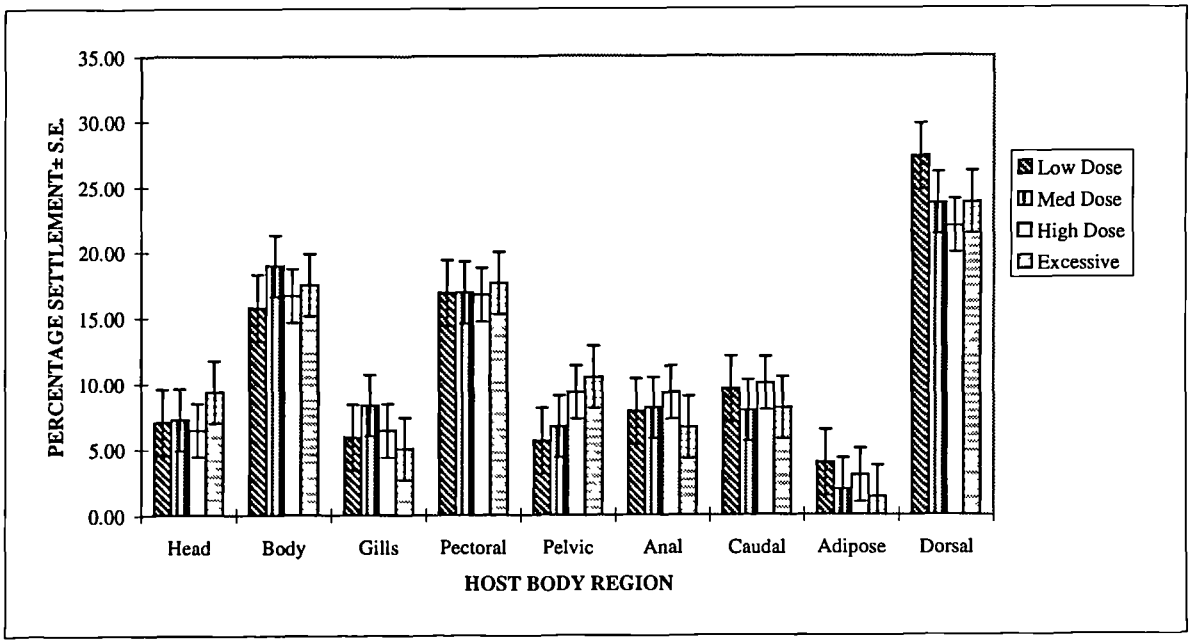
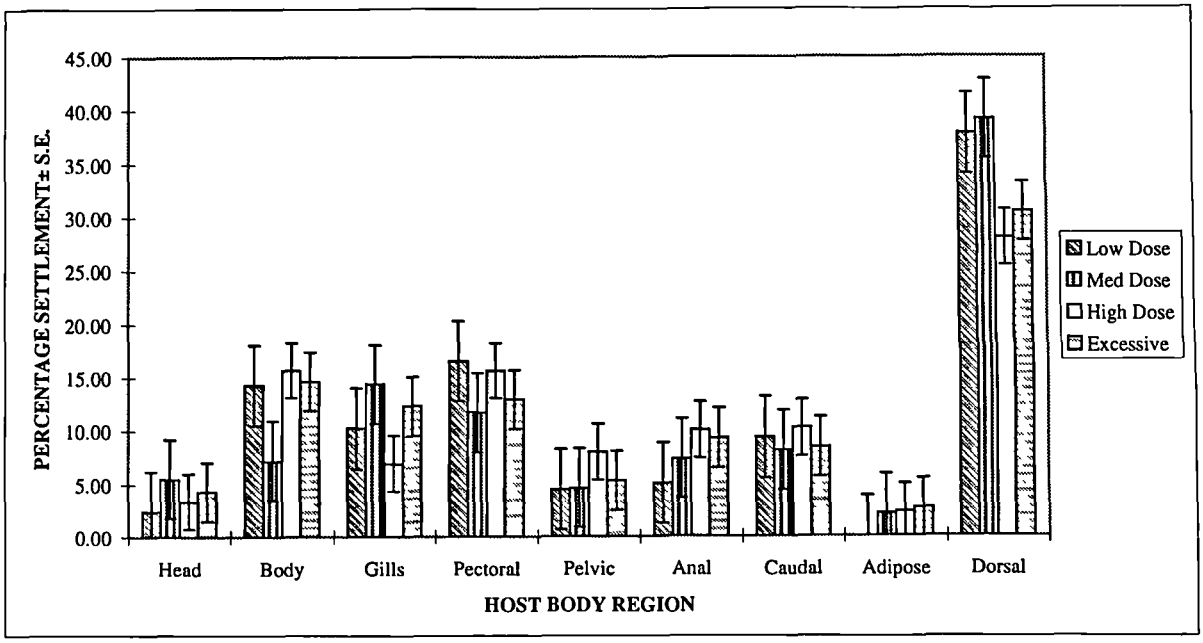


Figure 4.7c Effects of dose rates on *L.salmonis* copepodite distribution at D.P.I. 5 (1996/3)



low and medium dose rate groups.

There is a statistically significant difference ($p<0.05$) in settlement rates between the gills and adipose fin region for all copepodid dose rate groups. A statistically significant difference ($p<0.05$) was found between the low dose rate and all other dose rates and between the medium and excessive dose rate in the dorsal fin region.

In all experiments investigating the effects of varying copepodid dose rates on settlement, the favoured sites of settlement are the body, gills, pectoral and dorsal fins.

4.4.3 STOCKING DENSITY OF THE HOST.

4.4.3.1 EFFECTS OF HOST STOCKING DENSITY ON COPEPODID SETTLEMENT AND SURVIVAL.

The effects of various host stocking densities on the settlement and survival of *L.salmonis* copepodids is shown in Figure 4.8 (a-b) and Table 4.4. Three stocking densities, of fish of the same size (95.8g ± 9.95) were utilised for these experiments, to give stocking densities of 1.64 kgm^{-3} (SD5), 4.48 kgm^{-3} (SD15) and 7.42 kgm^{-3} (SD25), in experiment 1 and 1.79 kgm^{-3}, 4.54 kgm^{-3} and 7.61 kgm^{-3}, in experiment 2.

In both experiments a statistically significant lower settlement ($p<0.05$) was found with the highest stocking density (SD25) compared to the other two stocking densities (SD5 and SD15). Settlement in the lower stocking densities was found to be greater than 21% whilst percentage settlement in the highest stocking density was above 18% in both experiments. *L.salmonis* survival in experiment 1 and experiment 2 showed no statistically significant difference ($p>0.05$) between stocking densities. In experiment 1 survival was greater than

Figure 4.8a Effects of host stocking density on *L.salmonis* copepodid settlement and survival (1996/1)

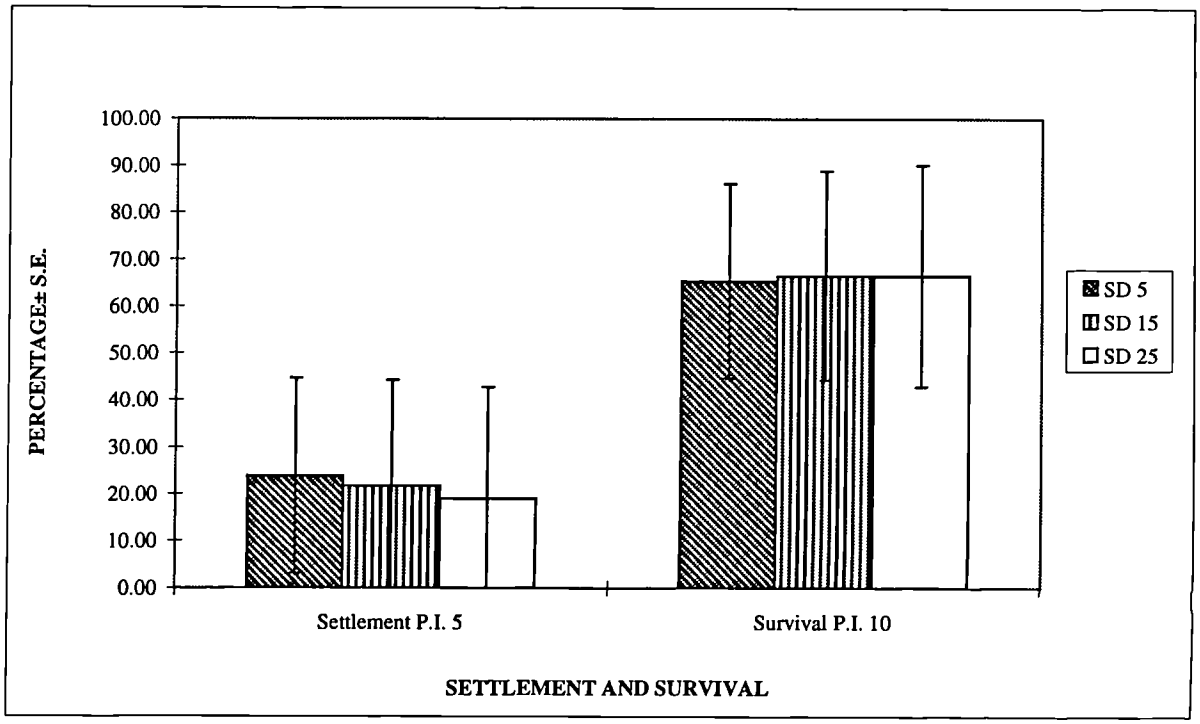
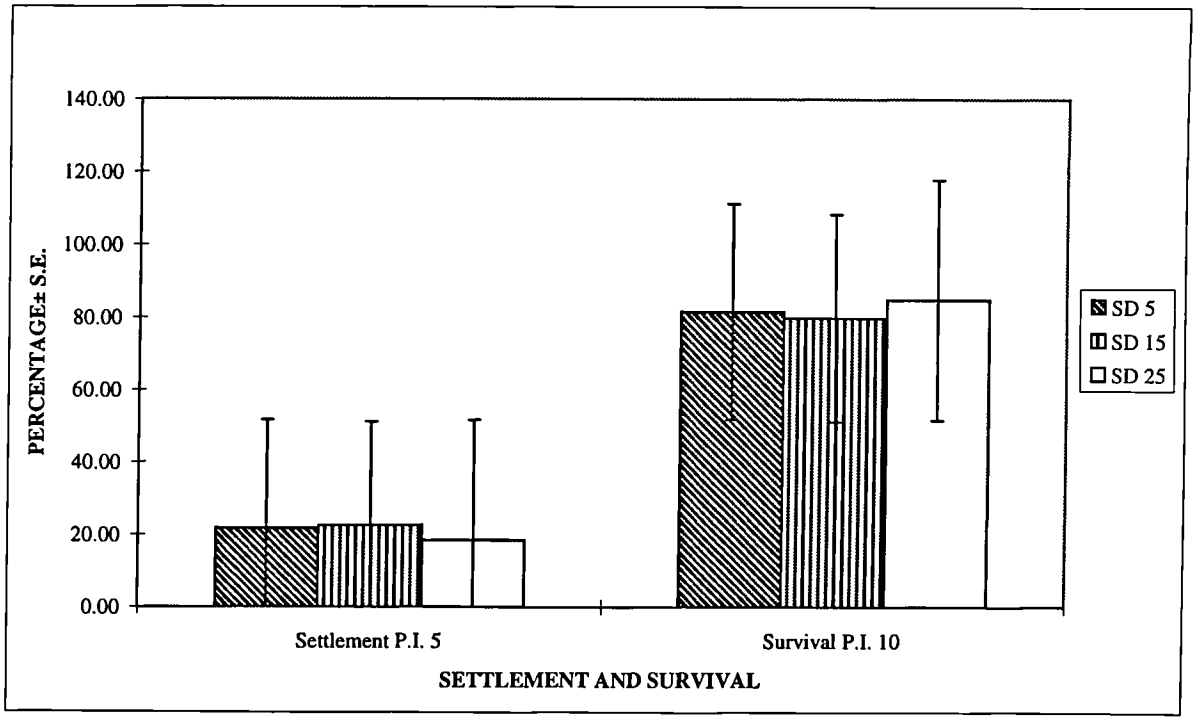


Figure 4.8b Effects of host stocking density on *L.salmonis* copepodid settlement and survival (1996/2)



Key: SD5 = stocking density 5 fish
SD15 = stocking density 15 fish
SD25 = stocking density 25 fish

65% in all groups whilst in experiment 2 survival was above 79%.

Table 4.4. Percentage settlement and survival of *L.salmonis* copepodids exposed to varying host stocking densities.

	SD5 % Settlement	SD15 % Settlement	SD25 % Settlement	SD5 % Survival	SD15 % Survival	SD15 % Survival
Expt. 1 (1996)	23.91	21.76	19.04	65.43	66.51	66.41
Expt. 2 (1996)	21.86	22.52	18.40	81.41	79.65	84.61

Key: SD5 = Stocking density of 5 fish
SD15= Stocking density of 15 fish
SD25= Stocking density of 25 fish

4.4.3.2 EFFECTS OF HOST STOCKING DENSITY ON COPEPODID SETTLEMENT DISTRIBUTION ON THE HOST.

In experiment 1 and experiment 2 (Figure 4.9 a-b) statistical analysis of the copepodid settlement distribution on hosts at different stocking densities shows a significant difference ($p<0.05$) between the highest stocking density (SD25) and the lowest stocking density (SD5) in each host body region. Whilst a significant difference ($p<0.05$) between the SD25 and SD15 was found in the body, gills pectoral, pelvic adipose and dorsal host body regions. A further statistically significant difference was found between the head regions at SD5 and SD15 fish. In experiment 2 a significant difference in copepodid settlement was also found in the anal fin region between SD15 and SD25.

Parasite distribution in experiment 1 (Figure 4.9a), within SD5 shows a statistically significantly higher settlement on the dorsal fin and all other host body regions. Of the remaining body regions there is no significant difference between each other, except for the adipose fin where settlement is significantly lower ($p<0.05$) than on the body and pectoral fins. In SD15 group settlement on the dorsal fin is also statistically higher

Figure 4.9a Effects of host stocking density on *L.salmonis* copepodid settlement distribution (1996/1)

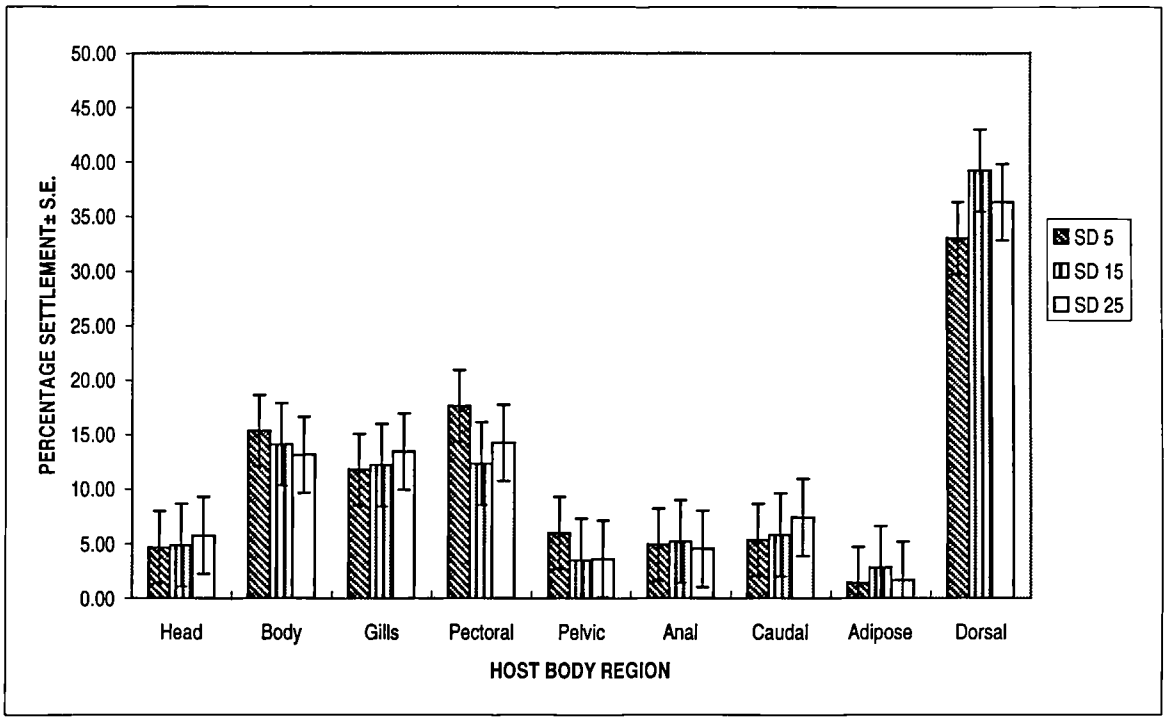
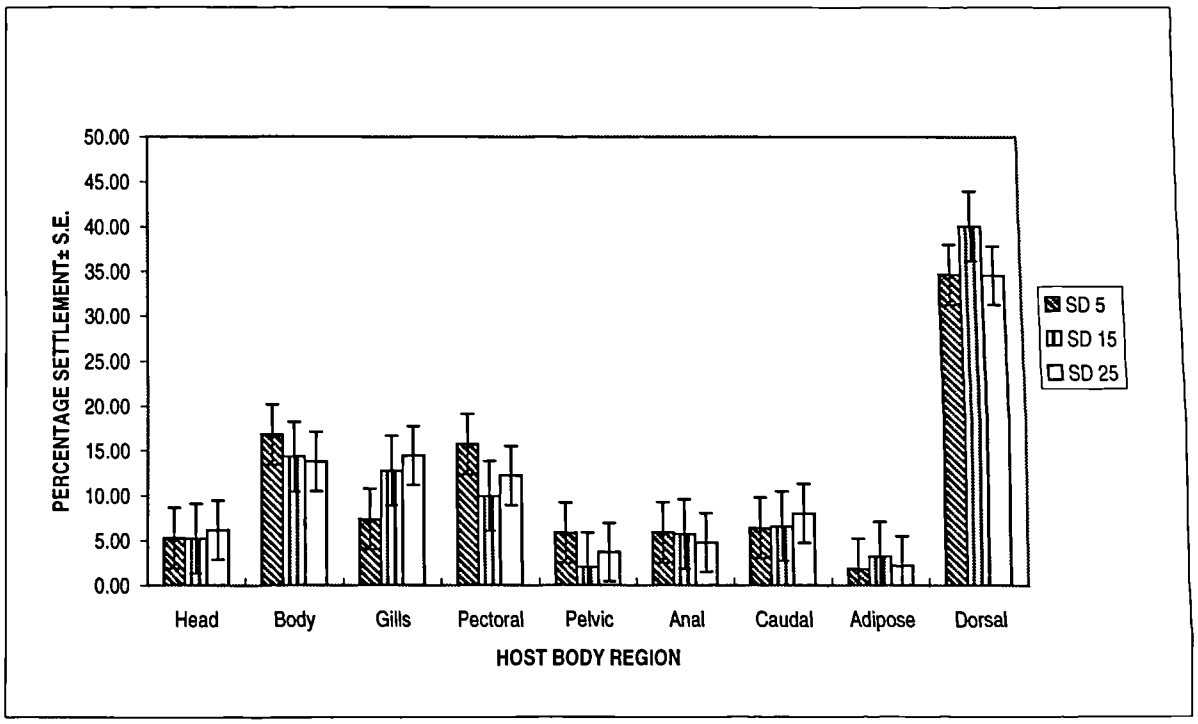


Figure 4.9b Effects of host stocking density on *L.salmonis* copepodid settlement distribution (1996/2)



Key: SD5 = stocking density 5 fish
SD15 = stocking density 15 fish
SD25 = stocking density 25 fish

($p<0.05$) than on other host body regions, whilst all other host body regions show no statistically significant difference from each other. The SD25 treatment again shows a significantly higher settlement on the dorsal fin and those on all other host body regions. The body, gills and pectoral fin show a significant difference with all other host body regions except the caudal fin. The caudal fin and the remaining body regions show no statistical difference from each other.

Experiment 2, within group analysis (Figure 4.9b) shows the dorsal fin, at all host stocking densities has significantly higher ($p<0.05$) settlement than on all other host body regions. Of the remaining body regions there is no statistically significant difference between the host regions at stocking densities SD15 and SD25, except for the statistically lower settlement found on the pelvic and adipose fins and the body and pectoral fin regions. In SD5 there is no significant difference between remaining the host regions except between adipose fins and the body and pectoral fin regions.

4.4.4 SECONDARY INFECTION OF COPEPODID.

4.4.4.1 EFFECTS OF INFECTION REGIMES ON COPEPODID SETTLEMENT AND SURVIVAL.

In this experiment two groups of fish were exposed to an approximate total of 8000 copepodids, either as a single infection or as a double infection involving an initial infection of 2500 copepodids followed by a second infection of 5500 copepodids. The percentage settlement and survival data are presented in Figures 4.10 a-b and Table 4.5. In experiment 1 (Figure 4.10a) there was no significant difference ($p<0.05$) between the two infection regimes, with an overall percentage settlement values of 41.52% and 33.79% for

Figure 4.10a Settlement and survival of *L.salmonis* copepodids in single and multiple infections (1996/1)

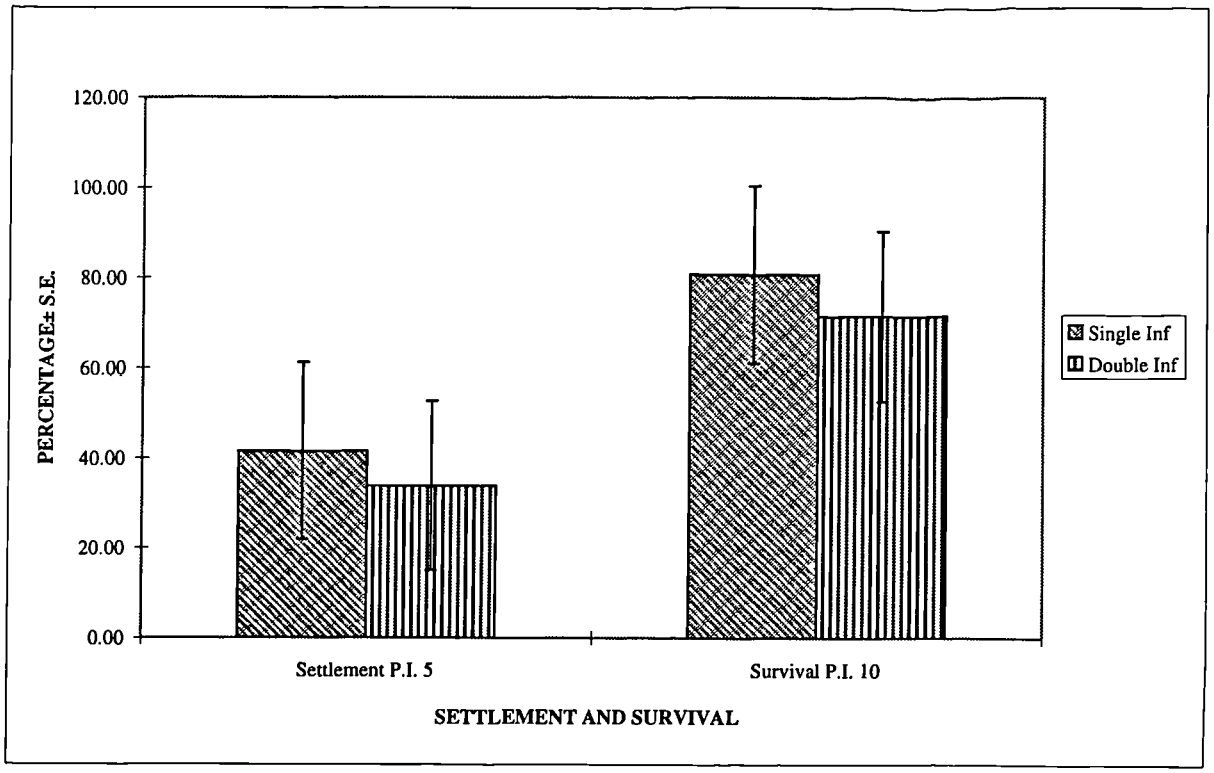
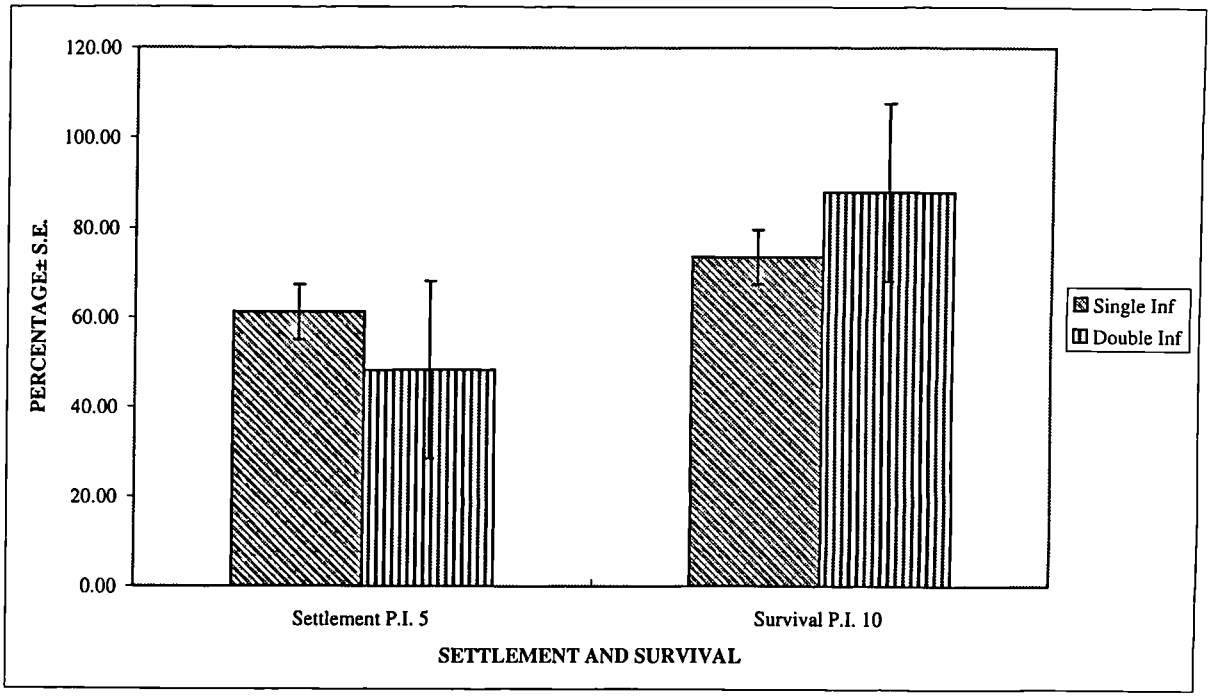


Figure 4.10b Settlement and survival of L.salmonis copepodids in single and multiple infections (1997/2)



Key: Single Inf. = single wave infection
Double Inf = multiple wave infection.

the single and double infection regimes respectively. In experiment 2 (Figure 4.10b) there was a statistically significant ($p<0.05$) higher (12.96%) settlement in the single infection regime. Analysis of the survival data of the two infection regimes shows no statistical difference in experiment 1, although survival was higher in then single infection regime (Figure 4.10a), but a statistically significant difference between the infection regimes in experiment 2 (Figure 4.10b), where there was greater (14.44%) survival associated with the double infection regime.

Table 4.5 Percentage settlement and survival of copepodids administered at single and double infections

	Single Inf. Settlement	Double Inf. Settlement	Single Inf. Survival	Double Inf. Survival
Expt. 1 (1996)	41.52	33.79	80.78	71.44
Expt. 2 (1997)	61.11	48.16	73.32	87.76

Key: Single inf. = Single infection of ≈8000 copepodids
Double inf. = Double infection of copepodids, initial dose of ≈2500

4.4.4.2 EFFECTS OF INFECTION REGIMES ON COPEPODID SETTLEMENT DISTRIBUTION ON THE HOST.

The favoured sites of settlement in both experiments and both infection regimes are the body, gills, pectoral and dorsal fin more than 12.4%, 9.8%, 8.5% and 27.1% for these regions respectively. In both experiment 1 (Figure 4.11a) and experiment 2 (Figure 4.11b) there is a statistically significant difference ($p<0.05$) between all host body regions and the two infection regimes, other than in experiment 1 where there is no statistical difference in the head and dorsal region, and with respect to the head region in experiment 2.

Figure 4.11a Effects of multiple infections on *L.salmonis* copepodite settlement distribution (1996/1)

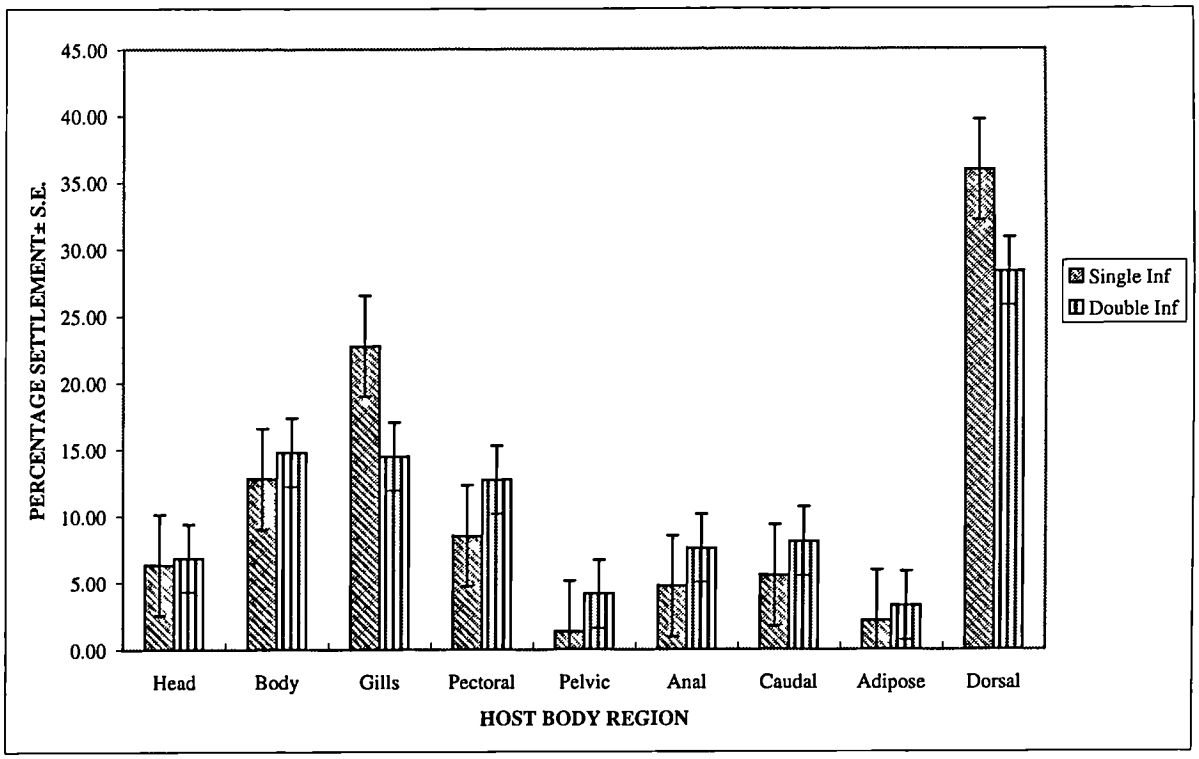
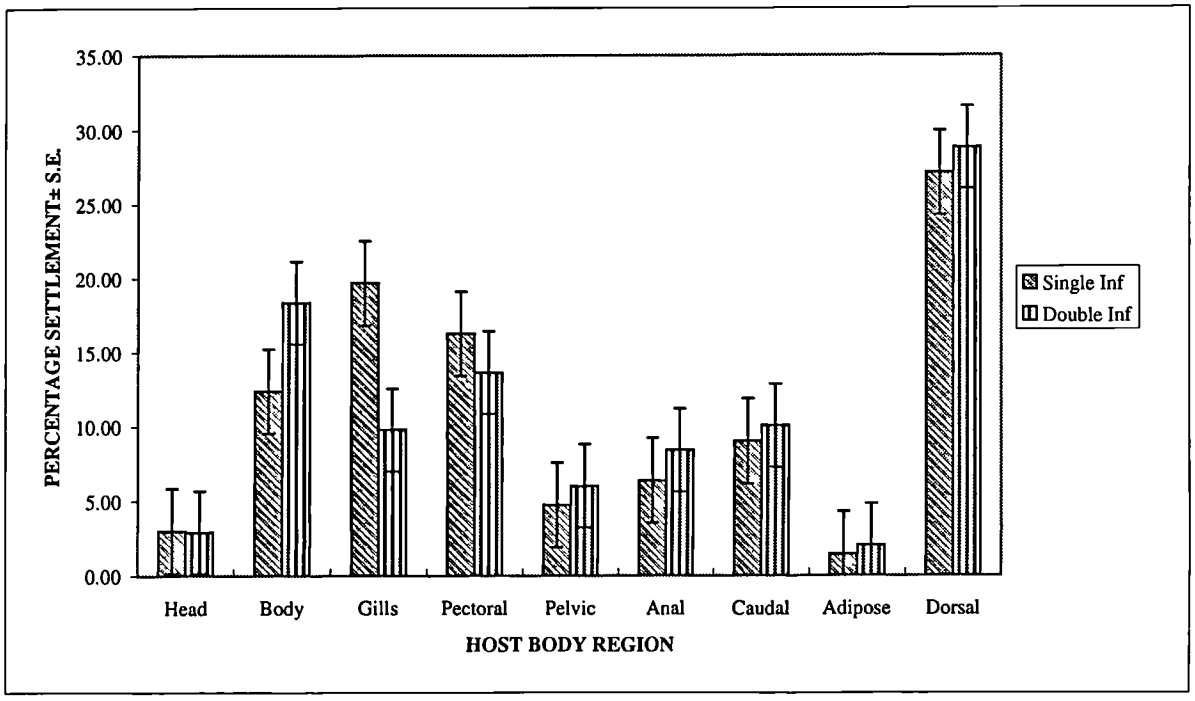


Figure 4.11b Effects of multiple infections on *L.salmonis* copepodite settlement distribution (1997/2)



Key: Single Inf. = single wave infection
Double Inf = multiple wave infection.

Within the single experimental infection regime of experiment 1 (Figure 4.11a) the dorsal fin and gills both show significantly higher settlement than all other host body regions. All other host regions show no statistically significant difference, except for the pelvic and adipose fins where there are significantly lower settlement rates than on the body and pectoral region. Within the double infection regime of experiment 1 the dorsal fin region has a statistically higher settlement than all other host body regions. The body, gills and pectoral fins show a significantly higher settlement than the remaining host body regions.

In experiment 2 (Figure 4.11b), within the single infection regime the dorsal fin shows a significantly greater settlement than all other host body regions. The body, gills and pectoral fins show a statistically higher settlement than the other host body regions except for the pectoral fin and the body and caudal fin. The double infection regime shows a significantly higher settlement between all host body regions and the dorsal fin. The body shows a statistically greater settlement between each other host regions, except for the pectoral fin. The pectoral fin has no statistical difference with the gills and caudal fins but is statistically higher than all other host regions. The head, pelvic and adipose fins show no statistical difference with each other but is significantly lower than all other body regions.

4.4.5 SURFACE AREA OF THE HOST

4.4.5.1 FISH SURFACE AREA.

The average surface area of different body regions (cm^2), for each size group of fish was calculated from image analysis are given in Table 4.6.

Table 4.6 Average fish surface area (cm^2) of Atlantic salmon of varying sizes.

Fish Size	Body	Pectoral Fin	Pelvic Fin	Anal Fin	Caudal Fin	Adipose Fin	Dorsal Fin
Small	63.49	4.62	3.02	2.63	11.74	0.45	1.92
Medium	193.05	8.23	6.47	5.96	25.67	0.92	7.57
Large	433.68	16.46	12.54	12.95	43.45	2.29	13.05

Percentage body region surface area for each group size of fish is given in Table 4.7

Table 4.7 Average percentage fish surface area of Atlantic salmon of varying sizes.

Fish Size	Body	Pectoral Fin	Pelvic Fin	Anal Fin	Caudal Fin	Adipose Fin	Dorsal Fin
Small	72.24 %	5.25 %	3.44 %	3.00 %	13.37 %	0.51 %	2.19 %
Medium	77.89 %	3.32 %	2.61 %	2.40 %	10.36 %	0.37 %	3.05 %
Large	81.15 %	3.08 %	2.35 %	2.42 %	8.13 %	0.43 %	2.44 %

It should be noted that small fish generally have a larger percentage fin surface area. Body surface area values will be underestimated as the image analysis process can only measure the surface area of a 2D and not a 3D image.

4.4.5.2 EFFECTS OF PRESENTED HOST SURFACE AREA ON COPEPODID SETTLEMENT AND SURVIVAL.

The percentage settlement and survival of copepodids on hosts of varying sizes can be seen in Figure 4.12 (a-b) and Table 4.8. In experiment 1 (Figure 4.12a) there was a statistically greater settlement on the small fish group compared to the larger fish. In experiment 2 (Figure 4.12b) there is a significantly greater settlement on the small fish group than on the other two fish groups. In the small fish groups the settlement was 0.25 and 0.45 parasites

Figure 4.12a Effects of host surface area on *L.salmonis* copepodid settlement (D.P.I. 5) and survival (D.P.I. 10) (1996/1) By host surface area of fish of varying size

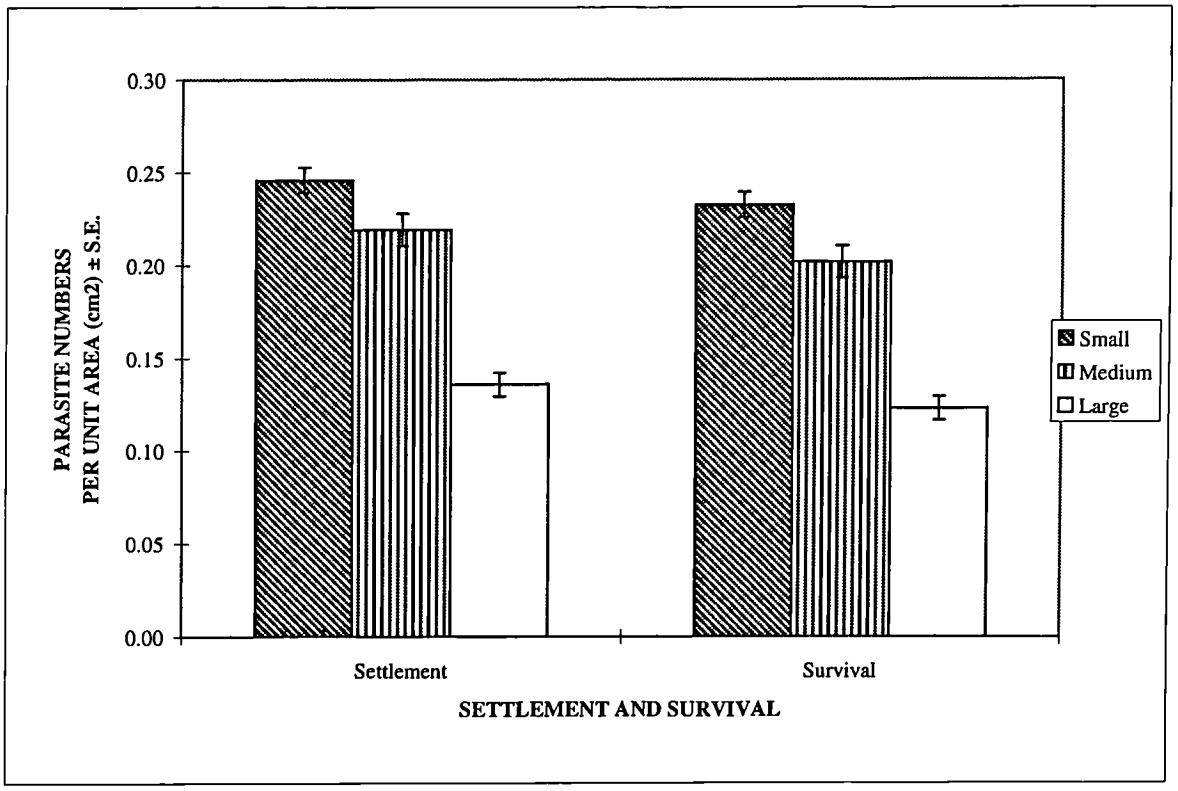
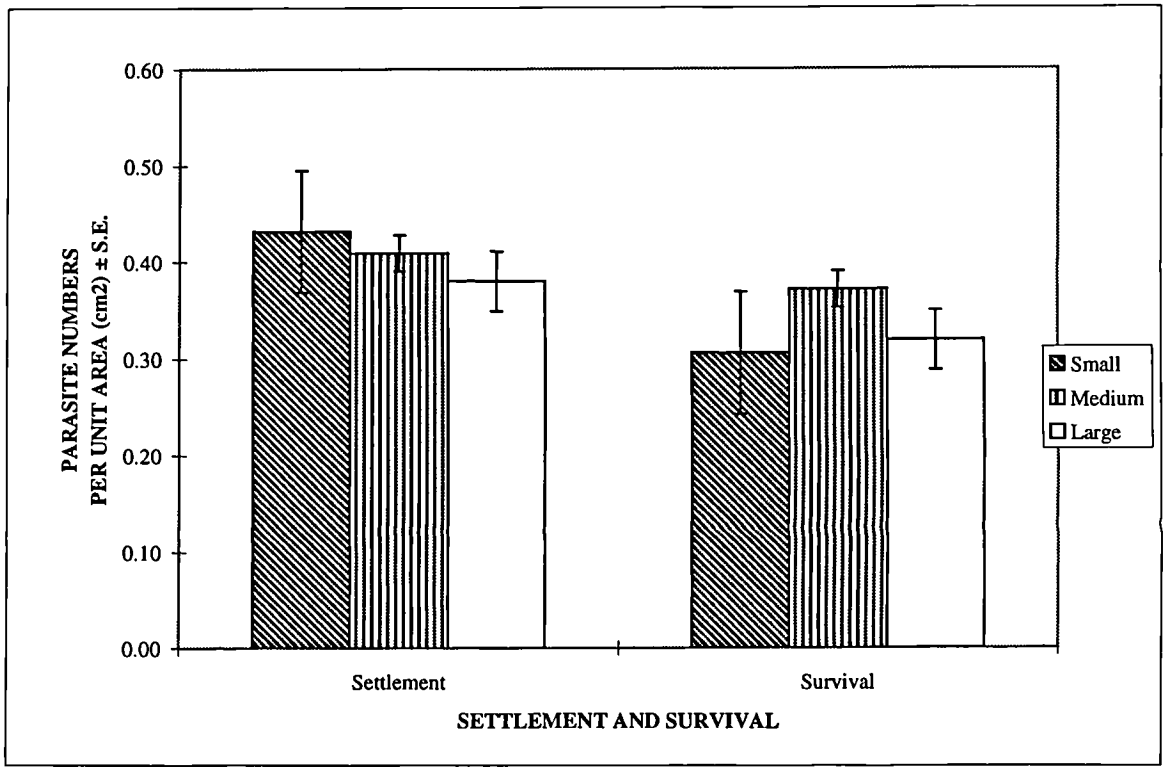


Figure 4.12b Effects of host surface area on *L.salmonis* copepodid settlement (D.P.I. 5) and survival (D.P.I. 10) (1996/2 By host surface area of fish of varying size



cm^{-2} for experiment 1 and 2 respectively. Although this group contains the largest number of fish it does not have the largest total available surface area.

Examination of the survival data shows the same pattern between fish groups. In experiment 1 there was a significantly greater survival at D.P.I. 10 on the small than on the large fish groups, whilst in experiment 2 there was a significantly greater survival on the small fish group than on the medium and large fish groups.

Table 4.8 Percentage settlement and survival of copepodids infecting fish of differing surface area.

	Small Fish Settlement	Medium Fish Settlement	Large Fish Settlement	Small fish Survival	Medium Fish Survival	Large Fish Survival
Expt. 1 (1996)	9.30	6.88	4.97	94.54	92.05	90.58
Expt. 2 1996)	17.04	13.94	14.55	70.81	90.74	83.75

Key: Small fish = 30 small fish ≈ ave. wt. 43g
Medium fish = 10 medium fish ≈ ave. wt. 148g
Large fish = 5 large fish ≈ ave. wt. 749g

Figure 4.13 (a-b) shows the percentage settlement per unit area and the overall percentage survival for fish of varying sizes from both experiments. The percentage survival was greater than 80% in all groups, except in experiment 2 where survival in the small fish groups was 70%. There is a significantly greater survival (95% C.I.) on the small fish group than on the larger fish groups in experiment 1, whereas in experiment 2 there is a statistically significant difference (95% C.I.) between survival in all fish groups.

Figure 4.13a Effects of host surface area on *L.salmonis* copepodid settlement (D.P.I. 5) and survival (D.P.I. 10) (1996/1) Settlement per unit area and percentage survival

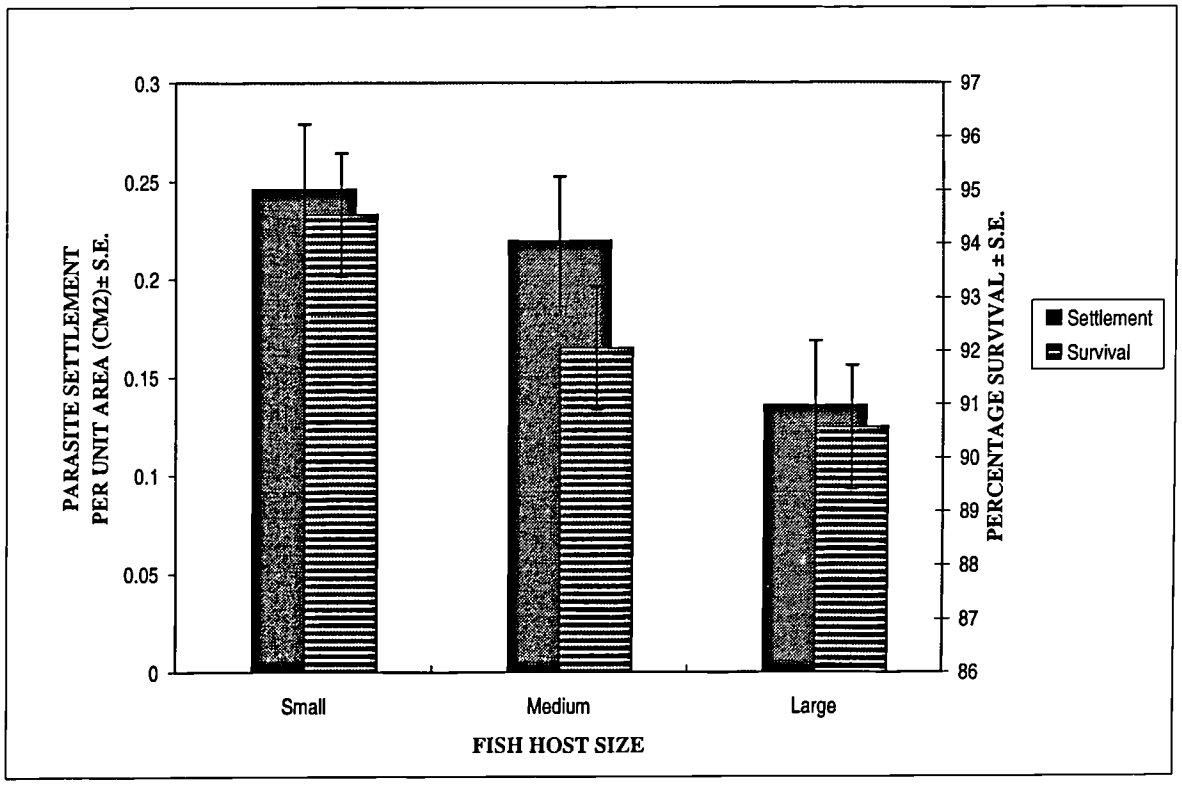
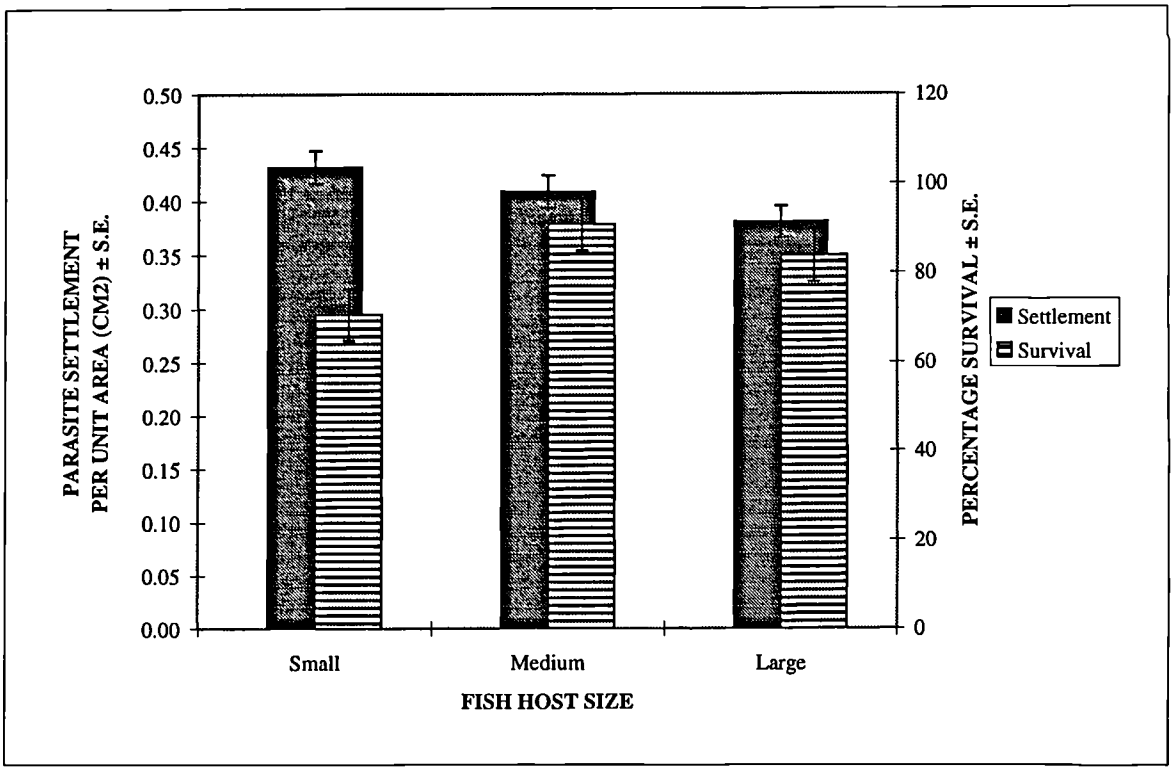


Figure 4.13b Effects of host surface area on *L.salmonis* copepodite settlement (D.P.I. 5) and survival (D.P.I. 10) (1996/2) Settlement per unit area and percentage survival



4.4.4.2 EFFECTS OF PRESENTED HOST SURFACE AREA ON COPEPODID SETTLEMENT DISTRIBUTION ON THE HOST.

The percentage settlement on the three main host body regions is shown. Although the values are similar, statistical differences are found in Figures 4.14 a-b. Figure 4.14 highlights the significance of *L.salmonis* parasite settlement on the fins as a primary site of settlement, greater than 60% in both experiments. In experiment 1 there is a significantly lower settlement (95%C.I.) between the medium size fish group and the other fish groups in the body region. In the fin region there is a statistically higher settlement (95%C.I.) between the medium size fish and the other two fish groups whilst in the gill region no statistically significant difference was found between the fish groups.

In experiments 2 there was a statistically lower settlement (95%C.I.) in the body region of the medium size fish and the other two fish groups. Again, there is no statistical difference in the gill region whilst in the fin region there is a significantly higher settlement (95%C.I.) between the small and medium size fish group.

Examination of the percentage settlement by host body regions shows a distinct pattern, (Figures 4.15 a-b). Percentage settlement is expressed in terms of the total settlement and the primary sites of settlement are the body, gills, pectoral and dorsal fin. Statistical comparison of the fish host sizes and the parasite distribution in experiment 1 (Figure 4.15a) shows no significant difference (95% C.I.) in settlement in the head, gills, anal and adipose fin regions. In the body, pectoral and caudal fin region there is a significantly higher settlement (95% C.I.) in the small fish group than in the other two fish groups whilst in the pelvic region there are significantly more parasites found in the small fish group than in the large fish group. With respect to the remaining area, the dorsal fin has a significantly higher settlement on the medium size fish than on the remaining two fish groups.

Figure 4.14a Effects of host surface area on *L.salmonis* copepodid settlement distribution (1996/1)

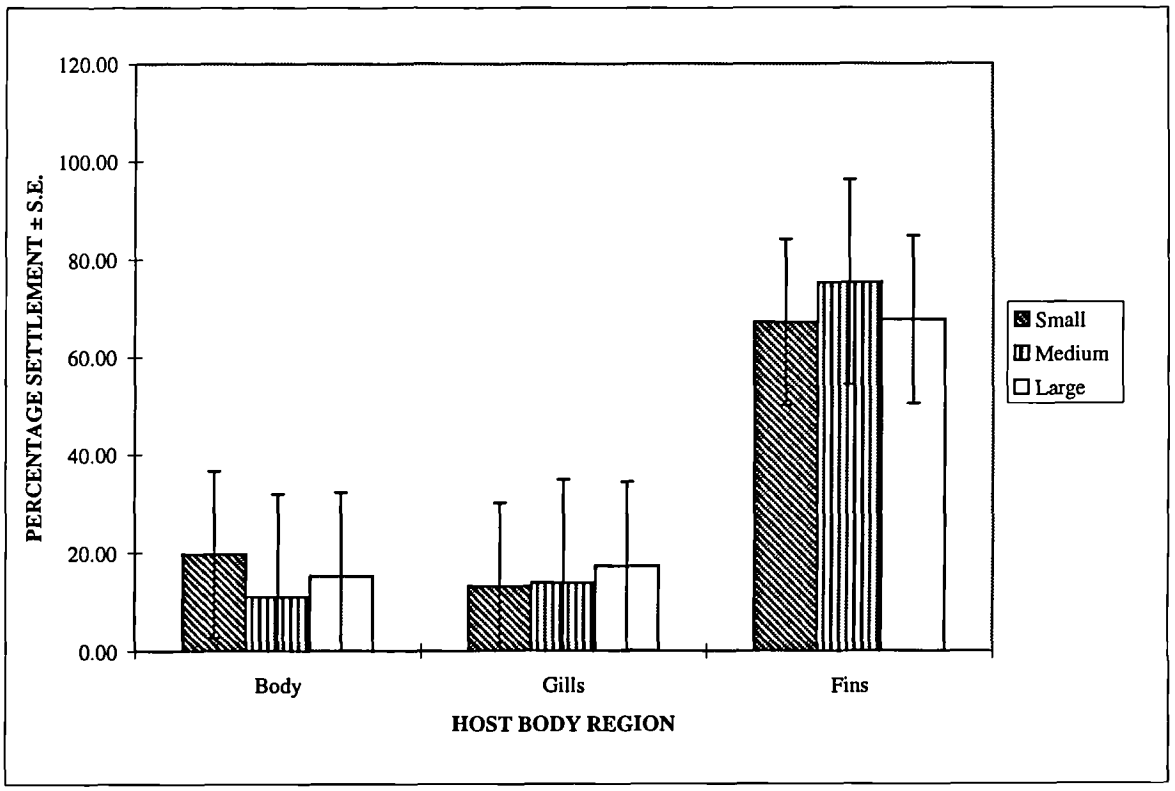
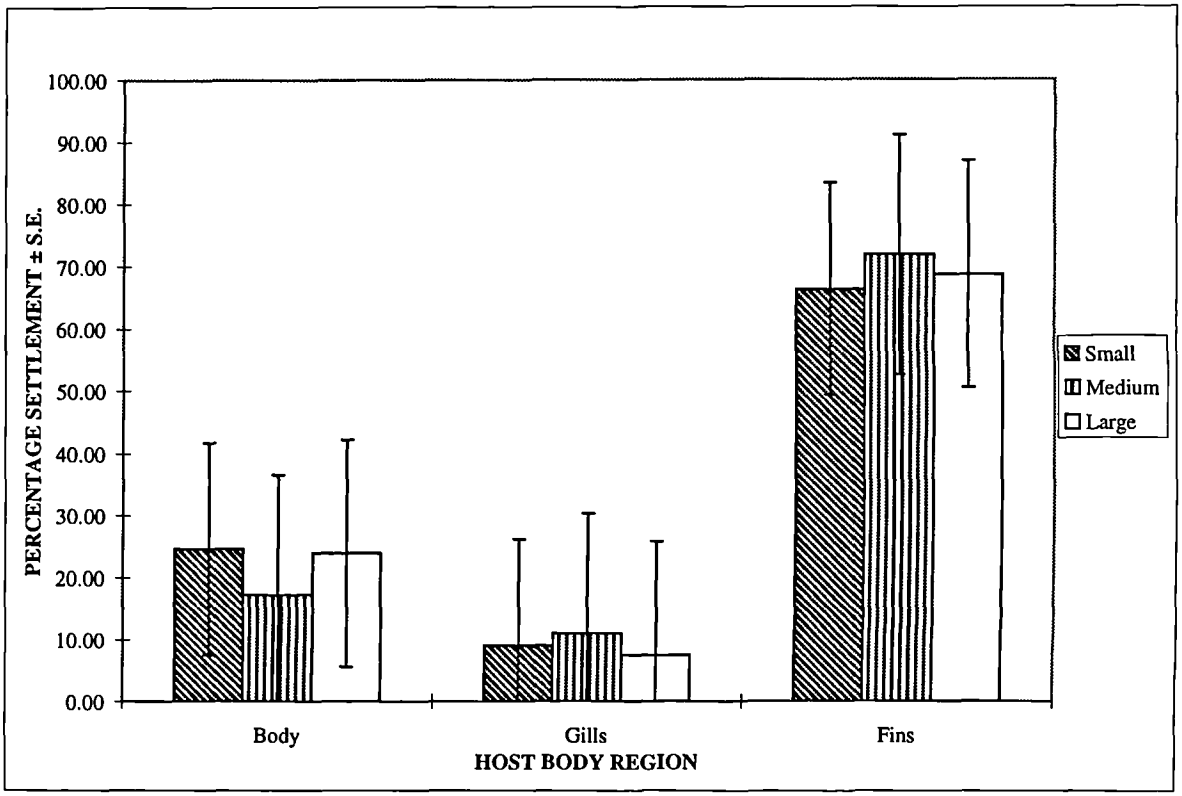


Figure 4.14b Effects of host surface area on *L.salmonis* copepodid settlement distribution (1996/2)



In experiment 2 (Figure 4.15b) no statistical difference was found in the head, gill, pelvic, and adipose fin region between fish size groups. In the body region a statistically lower settlement (95% C.I.) was found on the medium size fish and the other fish groups whilst in the caudal fin region a significantly lower settlement (95% C.I.) was found between the large fish group and the other remaining groups. In the pectoral and dorsal fins a statistically significant difference (95% C.I.) was found between the all fish groups.

When percentage settlement is calculated as parasite density (parasites per unit area) the settlement distribution has a different pattern (Figures 4.16a-b). Now the most significant settlement is on the dorsal and adipose fins. The head settlement counts have been included in the body counts to calculate the parasite density of the whole body surface. Previously, in direct settlement counts, the dorsal fin was an important site of larval settlement; now when parasite density is considered the dorsal fin remains the most important site of settlement with over 1.65 parasites cm^{-2} in experiment 1 and over 3.46 parasites cm^{-2} in experiment 2. The adipose fin shows a high parasite density, even with a low settlement count because of its small size.

In experiment 1 (Figure 4.16a) there is no statistically significant difference (95% C.I.) between the fish groups in the pelvic, anal and adipose fin region. In the body, pectoral and caudal fin region there is a statistically greater settlement (95% C.I.) in the small fish group than the other two groups. There is a significantly higher settlement (95% C.I.) in the dorsal fin region of medium and large size fish groups than the small fish. Experiment 2 (Figure 4.16b) shows there is no statistically significant difference (95% C.I.) between any of the fish size groups in any of the host body regions. All host body regions show similar parasite settlement densities.

Figure 4.15a Effects of host surface area on *L.salmonis* copepodid settlement distribution (1996/1)
All host body regions

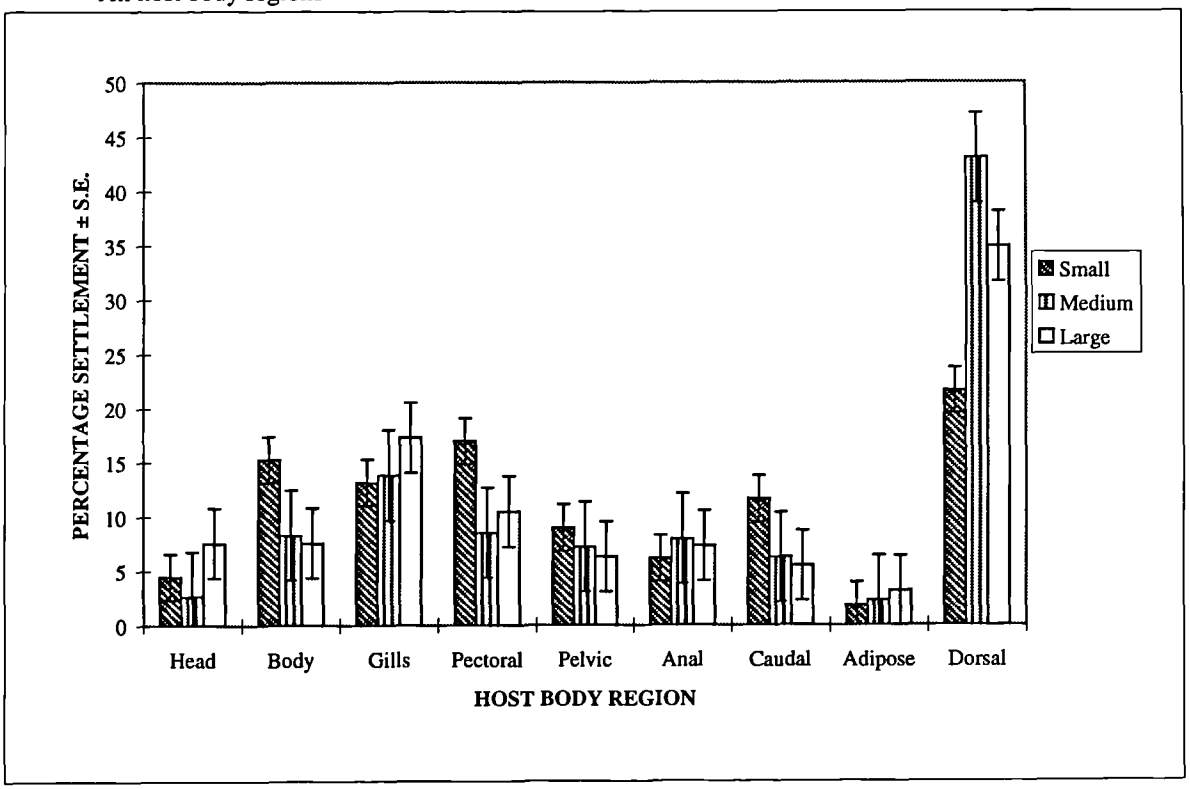


Figure 4.15b Effects of host surface area on *L.salmonis* copepodid settlement distribution (1996/2)
All host body regions

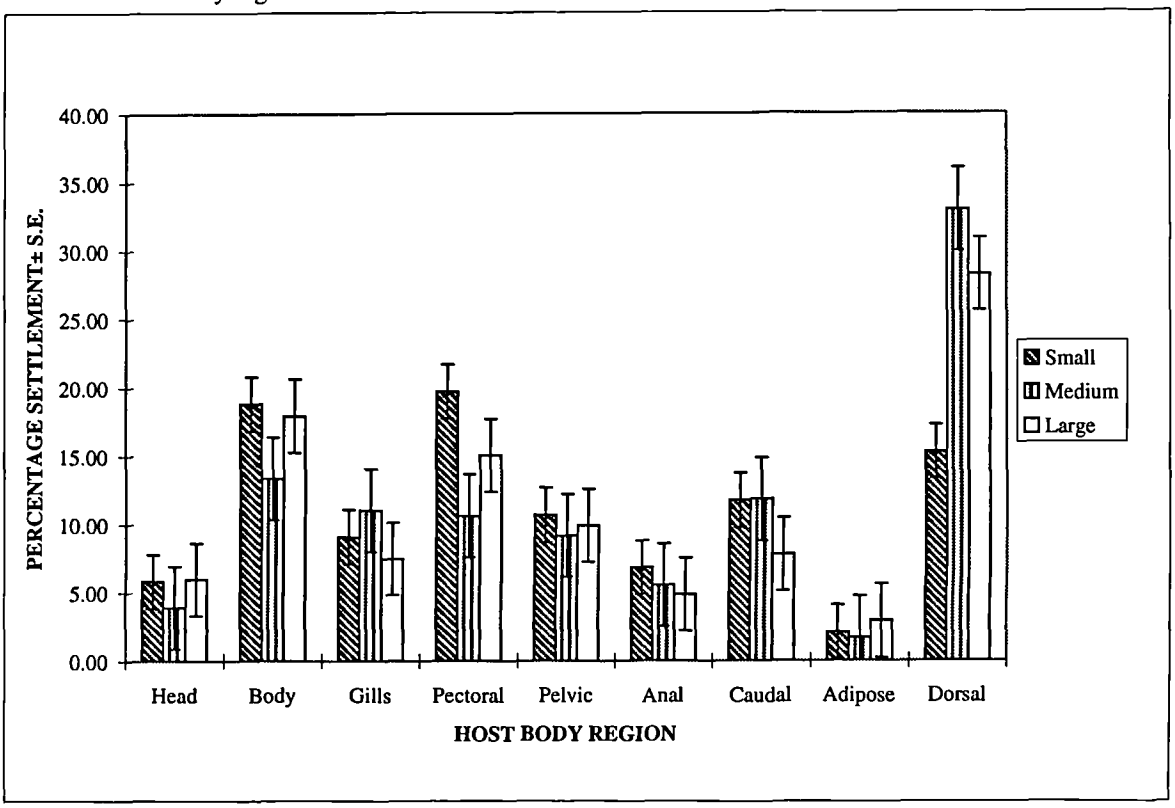


Figure 4.16a Effects of host surface area on *L.salmonis* copepodid settlement distribution (1996/1)
By area

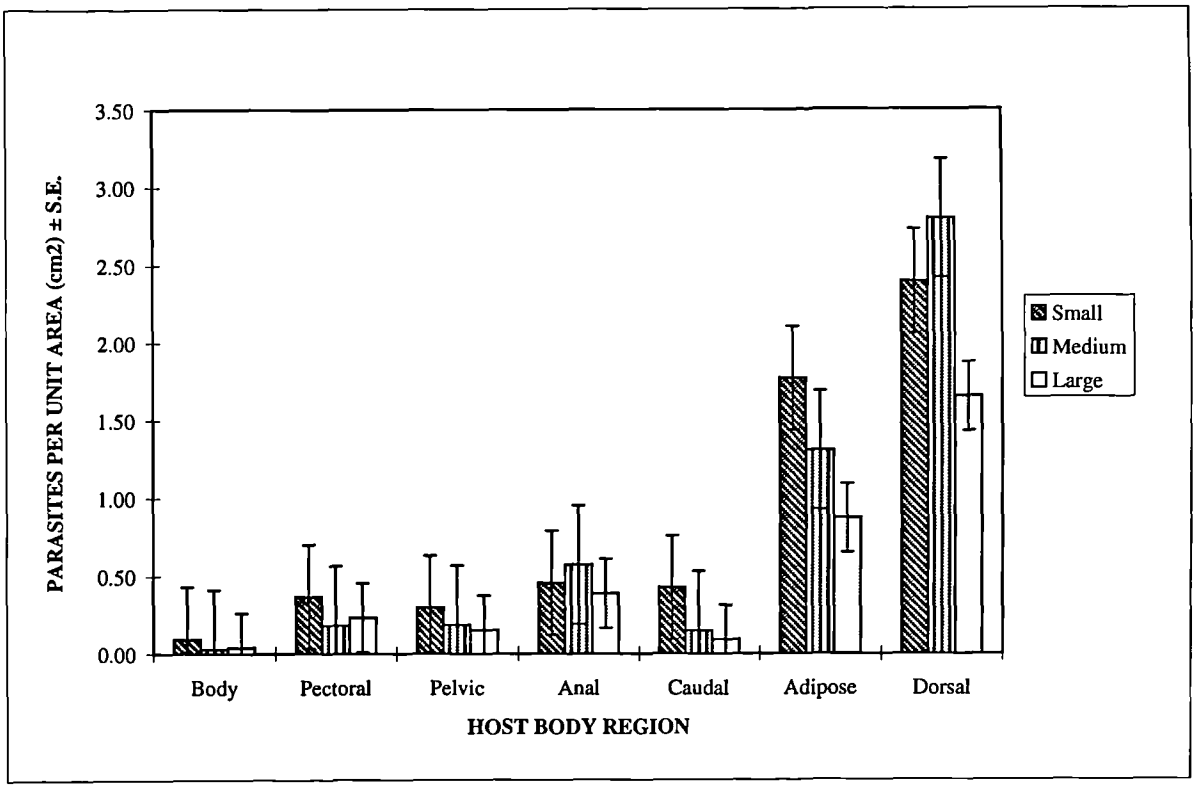
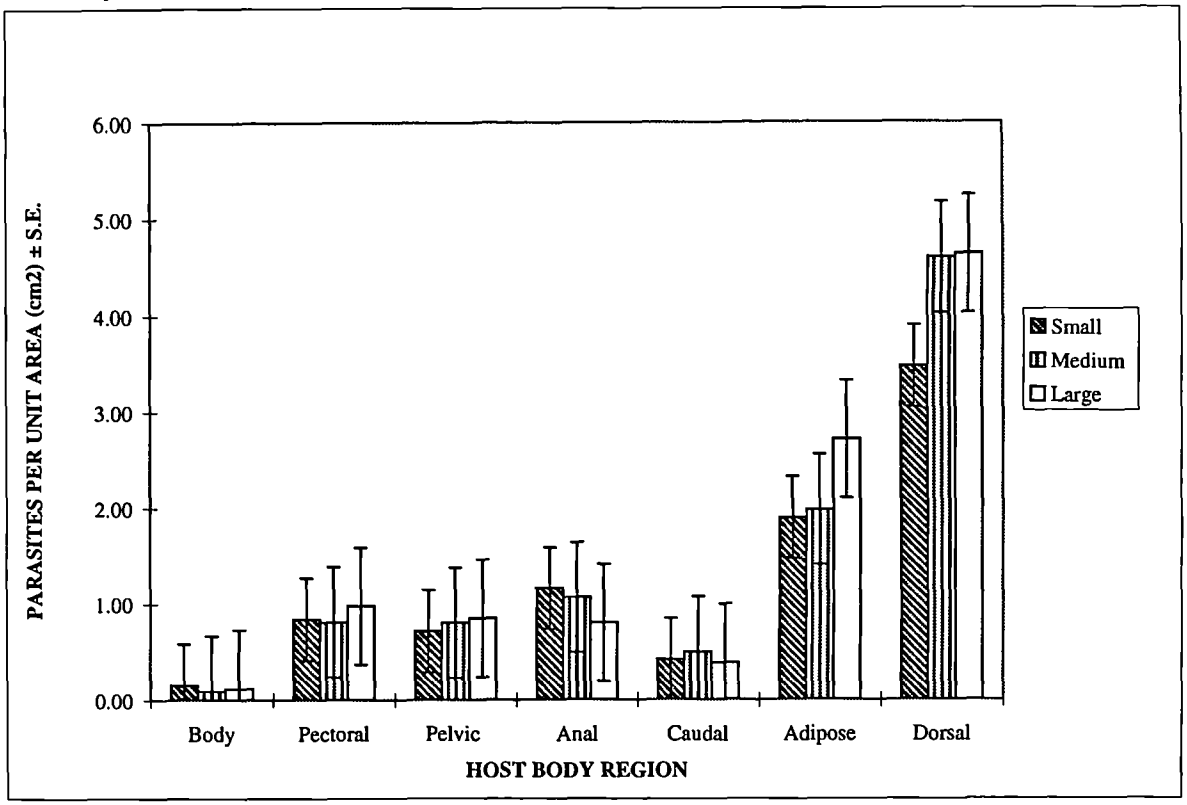


Figure 4.16b Effects of host surface area on *L.salmonis* copepodid settlement distribution (1996/2)
By area



4.4.4.3 REGRESSION ANALYSIS OF HOST BODY FACTORS AND HOST SURFACE AREA.

From the surface area analysis of the host a regression analysis against weight and standard length was conducted. Regression analysis of Atlantic salmon total body surface area against weight and standard length is given in Figures 4.17 and 4.18.

Regression analysis of fish weight and total surface area (including fins) gives the regression equation:

$y = 0.6131 x + 86.144$, with an R^2 value of 0.9871.

Analysis of fish standard length with total surface area gives the regression equation:

$y = 1.3311 x - 74.035$, with an R^2 value of 0.9267.

4.4.6 ALTERNATIVE HOST.

4.4.6.1. COMPARATIVE SETTLEMENT OF *L.SALMONIS* COPEPODIDS ON SALMON AND SEA TROUT.

In experiment 1 separate populations of fish were infected with copepodids and the settlement and survival data are shown in Figure 4.19a and Table 4.9. Settlement was highest in the salmon population with 73.7% settlement compared to 67.1% in the sea trout population. This is a significant difference ($p<0.05$) between the two fish populations. There is also a significant difference in survival between the salmon and sea trout in experiment 1 where 88.2% of the copepodids on the sea trout survived and 81.2% survived on the salmon at D.P.I. 10.

Figure 4.17 Regression analysis of fish weight and total surface area

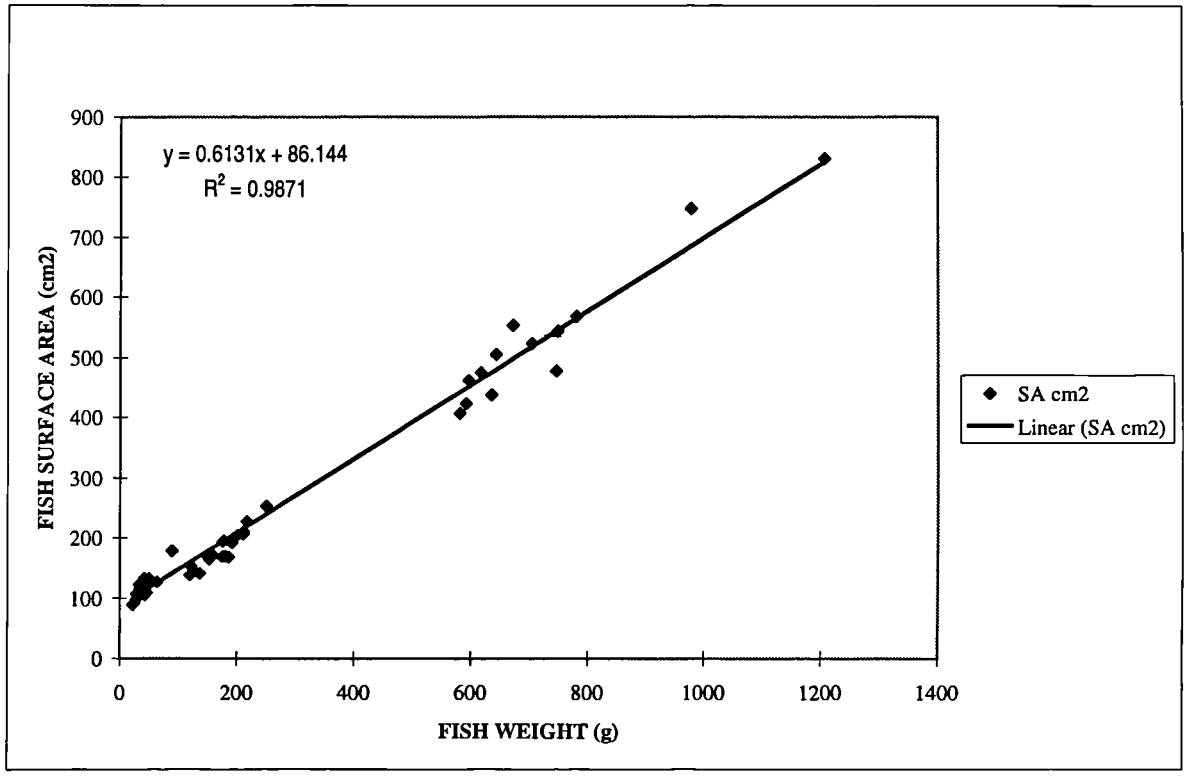


Figure 4.18 Regression analysis of fish length and total surface area

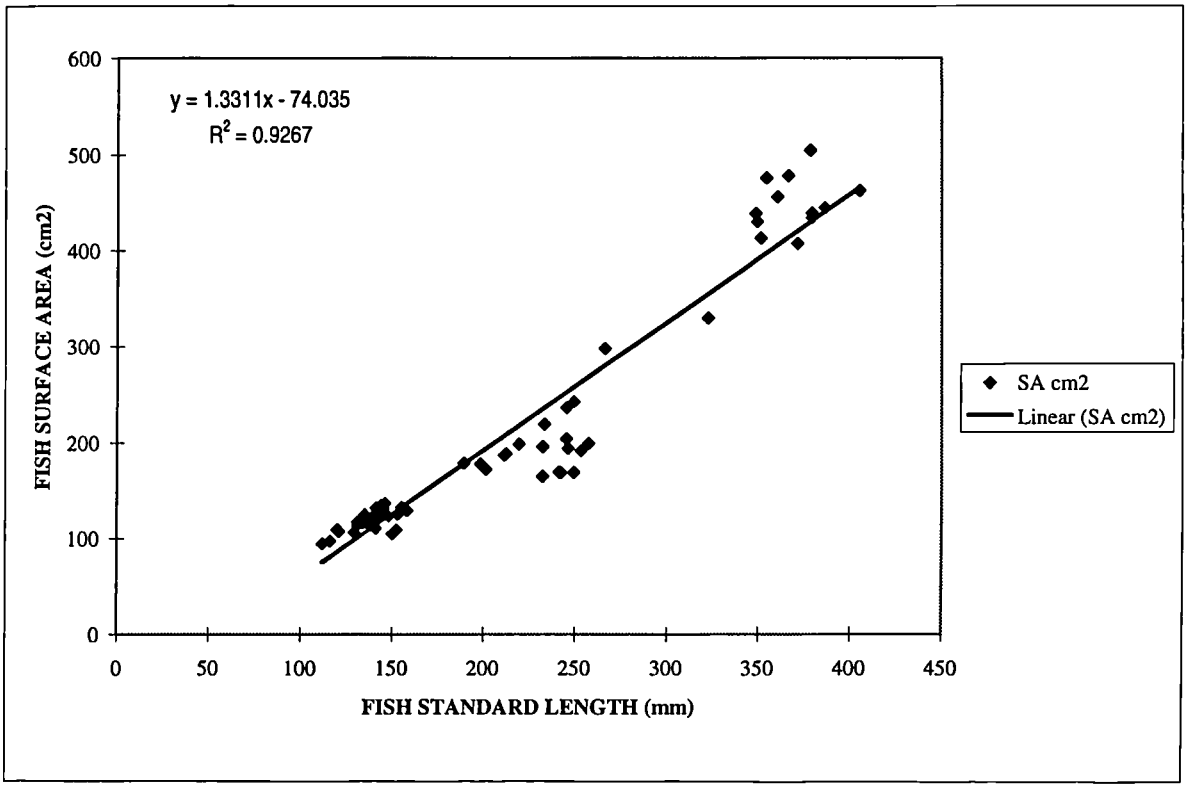


Table 4.9 Percentage settlement and survival of *L.salmonis* copepodids on alternate hosts.

	Sea Trout Settlement	Salmon Settlement	Sea Trout Survival	Salmon Survival
Expt. 1 (1997)[2]	63.14	73.69	88.24	81.19
Expt. 2 (1997)[3]	44.90	26.94	83.94	88.59

Key: Sea Trout n = 30
Salmon n = 30

Experiment 2 (Figure 4.19b) used mixed populations of sea trout and salmon and in this case the sea trout had the higher percentage settlement, 44.9%, compared with that on salmon, 26.9%. This is a statistically significant difference ($p<0.05$). The survival data for the mixed populations also shows a statistically significant difference ($p<0.05$), survival was highest (88.6%) in the salmon population whilst in the sea trout population survival was 83.9%.

4.4.6.2. EFFECTS OF ALTERNATE HOSTS ON *L.salmonis* COPEPODID SETTLEMENT DISTRIBUTION ON THE HOST.

The settlement distribution of experiment 1 (Figure 4.20a-b), over the host body surface shows a statistically significant difference ($p<0.05$) between both fish populations and with each host region. There was significantly higher settlement in the salmon group in the head, pectoral, gills and body whilst a higher settlement is found in pelvic, anal, caudal adipose and dorsal. A significant difference ($p<0.05$) was also found in all body regions (Figure 4.20b) between the mixed populations of fish except in the head and body region

[2] Sea Trout /Salmon experiment: separate fish populations

[3] Sea Trout /Salmon experiment: mixed fish populations

Figure 4.19a Effects of alternate hosts on *L.salmonis* copepodid settlement and survival (1996/1)
Seperate populations

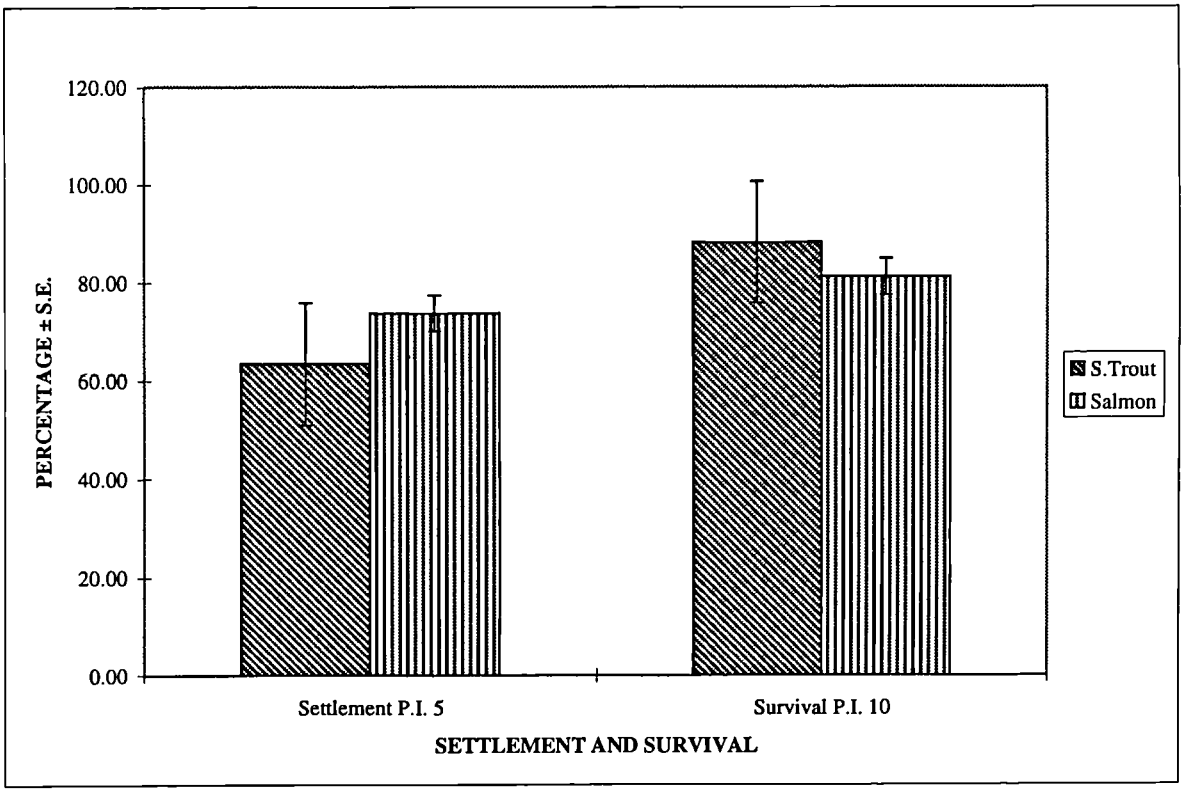


Figure 4.19b Effects of alternate hosts on *L.salmonis* copepodid settlement and survival (1996/2)
Mixed populations

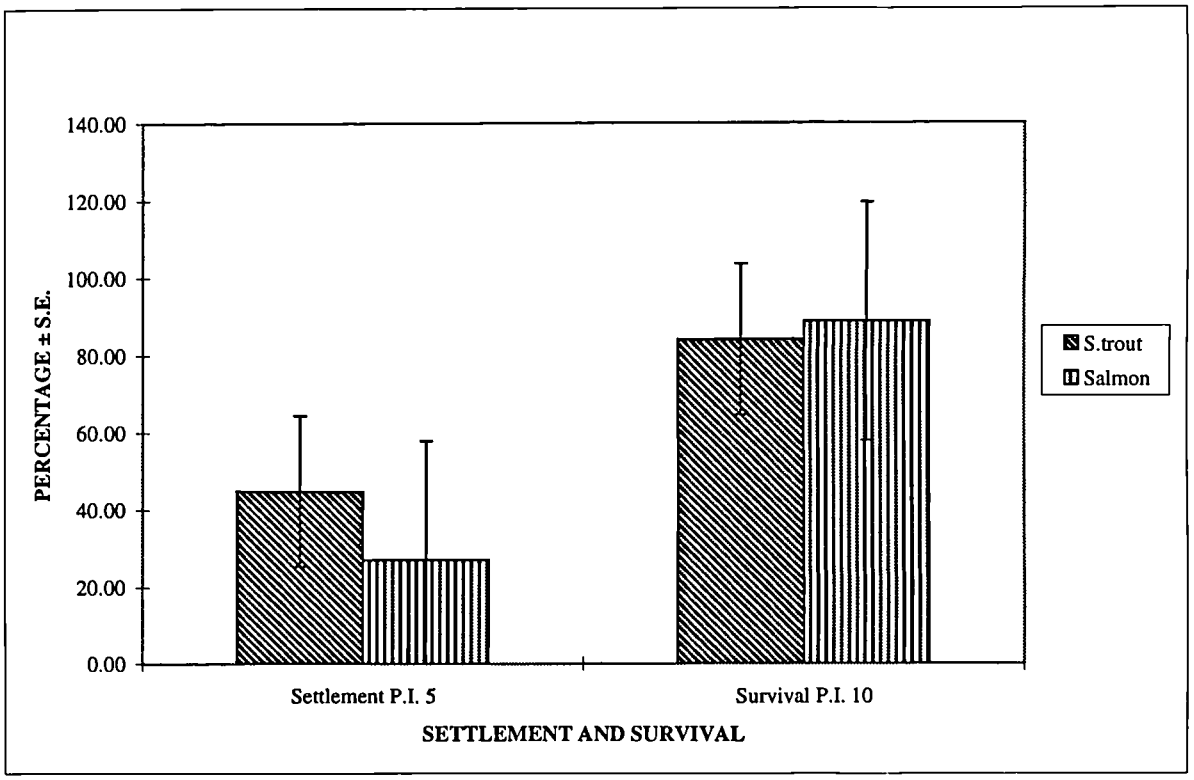


Figure 4.20a Effects of alternate hosts on *L.salmonis* copepodid settlement distribution (1996/1)
Seperate populations

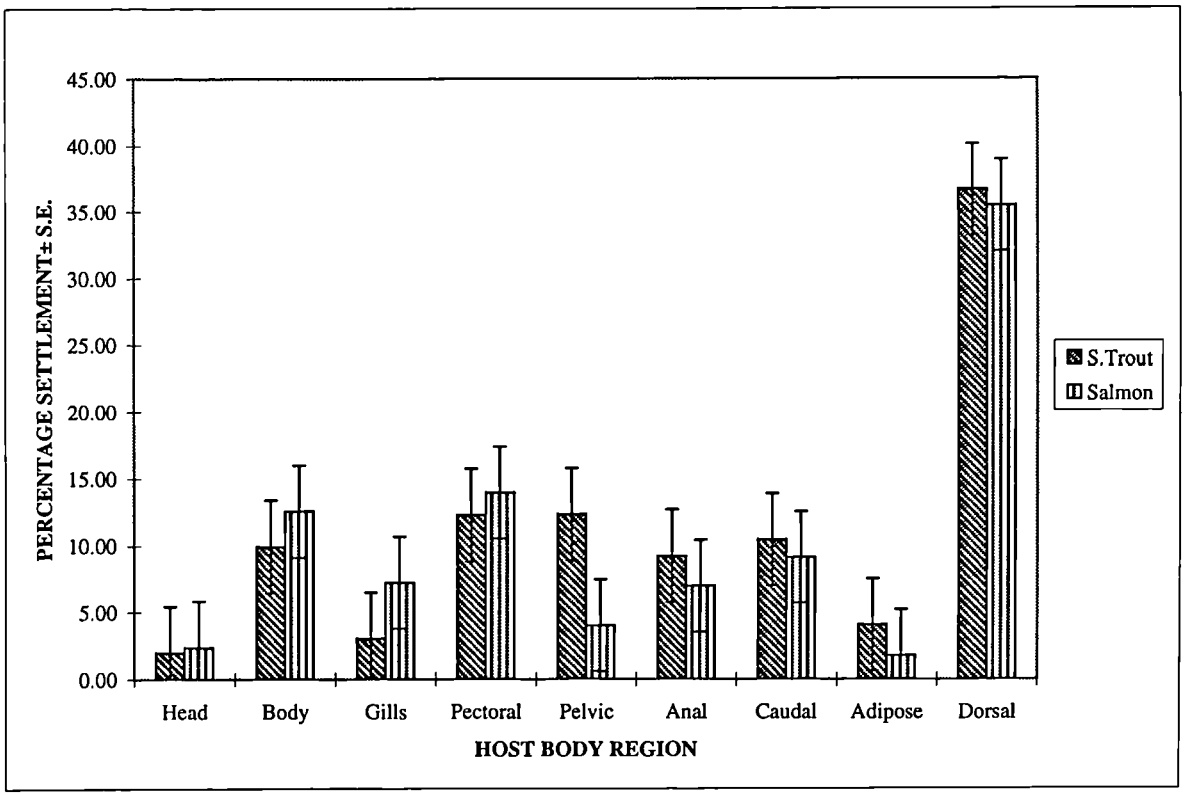
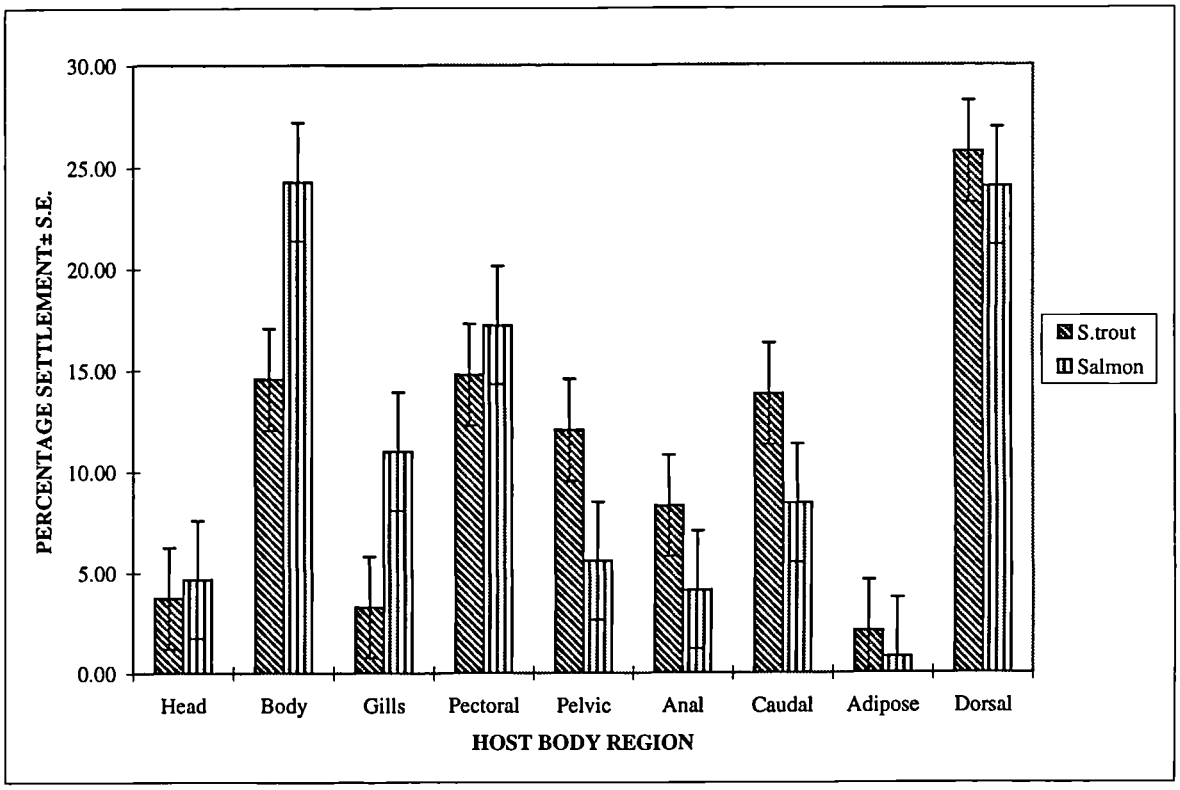


Figure 4.20b Effects of alternate hosts on *L.salmonis* copepodid settlement distribution (1996/2)
Mixed populations



where no statistical difference ($p>0.05$) was found.

Within fish species settlement distribution shows a distinct pattern in experiment 1 but a varied pattern in experiment 2. The sea trout population in experiment 1 shows a statistically higher settlement ($p<0.05$) on the dorsal fin than on all other host body regions. However there is no significant difference among ($p>0.05$) the remaining body regions There is also a significantly greater settlement ($p<0.05$) on the dorsal fin than on all other body regions in the salmon population of experiment 1. The pectoral fin region does not show significant difference with the body and caudal fin region but does show a significantly higher settlement ($p<0.05$) compared to all other host regions. The body did not show significant difference ($p>0.05$) among the gills, anal and caudal fin region but does show a significantly greater ($p<0.05$) difference than on all other host regions. Of the remaining host body regions no statistically significant difference ($p>0.05$) in the settlement distribution within a fish population was found.

In experiment 2 (Figure 4.20b), settlement distribution within the sea trout population shows a statistically significant difference, with higher settlement ($p<0.05$) between the dorsal fin and all other body regions. The body, pectoral, pelvic, anal, and caudal fins show a significantly higher settlement ($p<0.05$) than the remaining host body regions, except for the anal fin where there is no statistical difference ($p>0.05$). The salmon population settlement distribution shows a significant higher settlement ($p<0.05$) on the body and dorsal fin than on the remaining host body regions. The pectoral fin is statistically higher ($p<0.05$) than the remaining host body regions, except for the gills where settlement is not statistical different ($p>0.05$). The remaining host body regions do not show a statistical difference ($p>0.05$) to each other, except for the adipose fin where settlement is significantly lower ($p<0.05$) than on the gills and caudal fin regions.

4.4.6.3. EFFECTS OF ALTERNATE HOSTS ON *L.salmonis* COPEPODID RATES OF DEVELOPMENT.

Figures 4.21 (a-b) show the development rate of *L.salmonis* on salmon and sea trout in mixed and separate populations.

In experiment 1 (Figure 4.21a) there is no statistically significant difference (95% C.I.) in the proportion of chalimus 1 stage present between fish species although there is a statistically significant difference (95% C.I.) in each of the remaining developmental stages between fish hosts. In experiment 1 the majority of the developmental stages present on the sea trout at D.P.I. 10 were chalimus 3 (44.4%) with 24.4% at chalimus 4 and 21.6% at preadult 1 male. On salmon there were 57.2% at the chalimus 4 stage with 26.2% at preadult 1 male and 14.7% at chalimus 3. In experiment 2 (Figure 4.21b) no significant difference was found between the proportion of the chalimus 1 stage on each fish species but there was a statistically significant difference (95%C.I.) between the remaining stages at D.P.I. 10. On sea trout the majority of stages had reached the chalimus 4 stage (78.8%) with 3.2% preadult 1 male and 15.3% chalimus 3. On salmon 58.5% lice were at the chalimus 4 stage with 8.8% at preadult 1 male and 21.5% at chalimus 3. Development of *L.salmonis* on salmon was thus faster than on the sea trout at the temperature used in this study.

4.5 DISCUSSION

This study has explored the effects of some biotic factors on the settlement and survival of *L.salmonis*. The first factor to be examined was the age of copepodid on infection. In both winter and summer populations of lice 7 day old copepodids showed a loss of over 40%

Figure 4.21a Effects of alternate hosts on *L.salmonis* copepodite development D.P.I. 10 (1996/1)
Separate populations

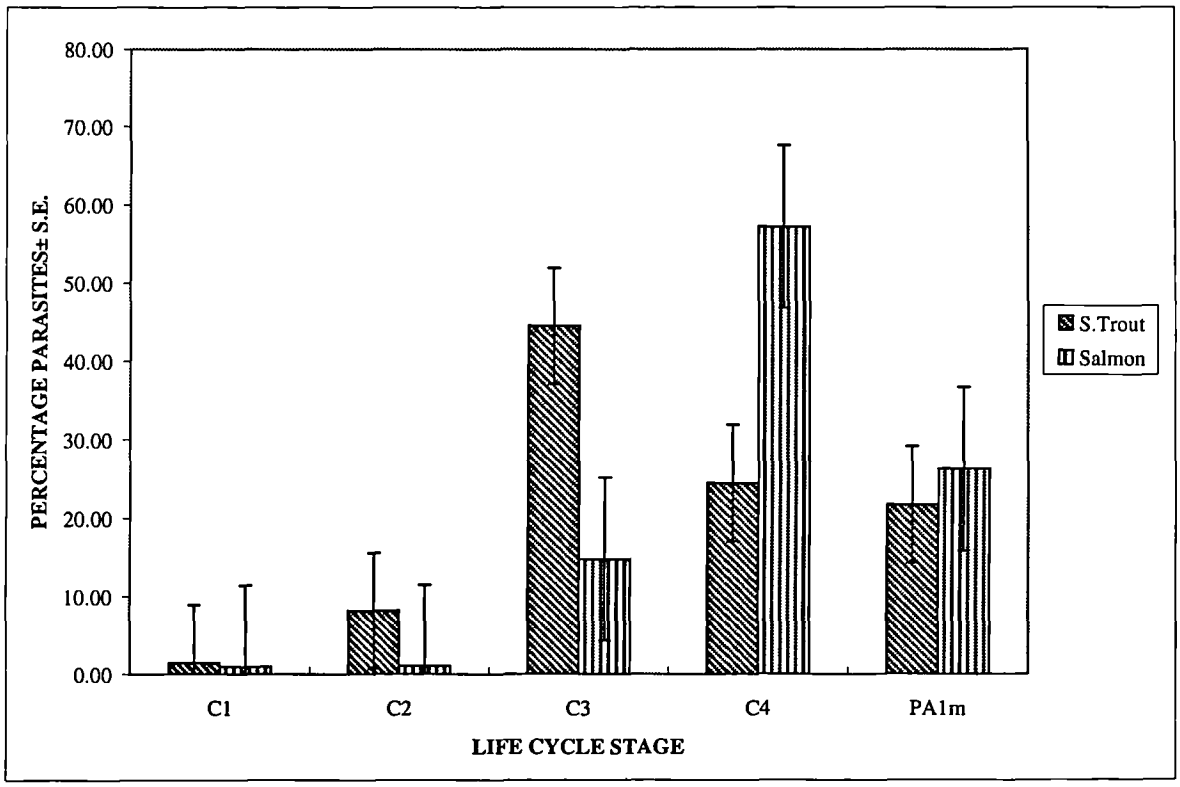
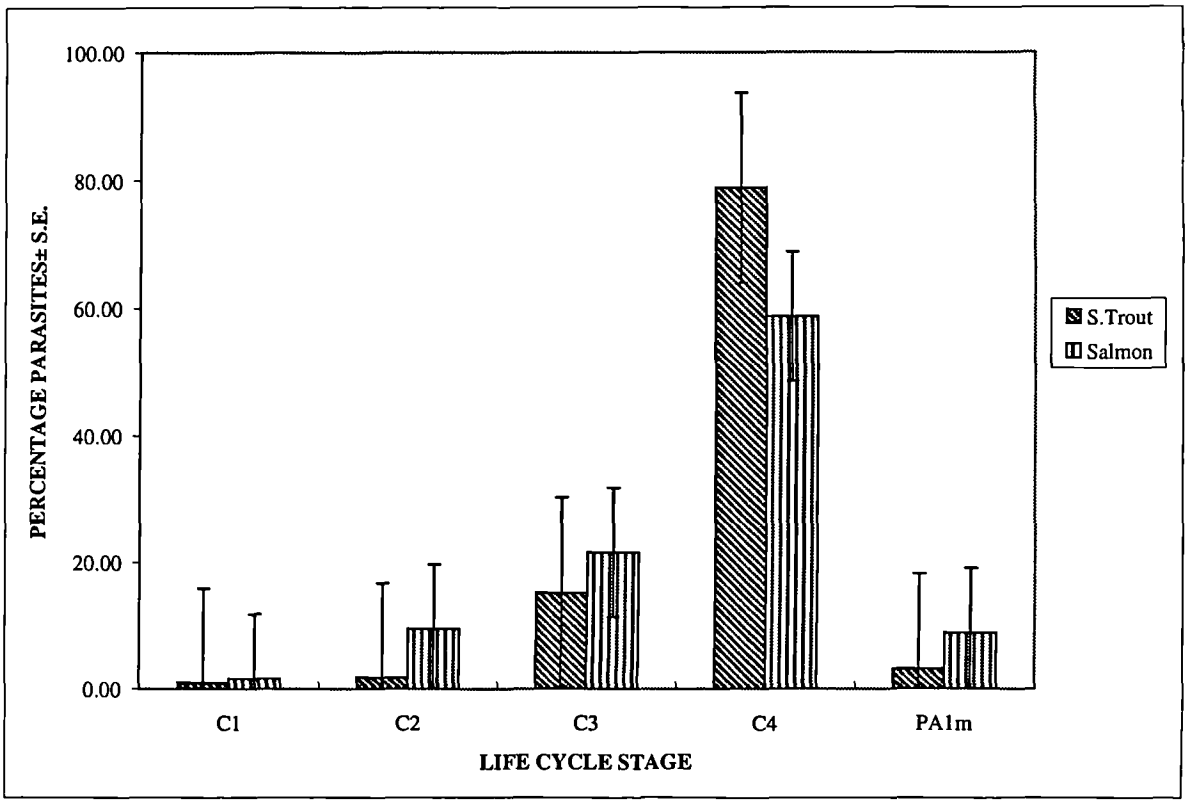


Figure 4.21b Effects of alternate hosts on *L.salmonis* copepodite development D.P.I. 10 (1996/2)
Mixed populations



infectivity compared with 1 and 3 day old copepodids. Gravil (1996) also demonstrated that 7 day old copepodids are less infective than 1 day old copepodids at D.P.I. 5 with an 8% difference in settlement between the two ages of copepodids.

Gravil (1996) demonstrated that the free-swimming copepodids could survive for 9-17 days whilst Johnson & Albright (1991a) gave survival figures of 2-8 days (dependent on temperature). However, the infectivity of a parasite infective may not be necessarily linked to its longevity (Anderson & Whitfield, 1975) and it is not possible to determine the ability of the parasite to infect its host from its longevity. It has been demonstrated for the barnacle cyprid that its longevity is almost twice the period of viable infectivity.

Holland & Walker (1975) report that the free-swimming cyprid can survive for 8 weeks before the complete utilisation of its lipid reserves. Lucas *et al.* (1979) in their study of the energy budget of free-swimming and metamorphosing cyprids report that settlement success and development to the juvenile declined sharply after week 4. After 4 weeks approximately 60% of the neutral lipid reserve is depleted therefore the energy required for metamorphosis must come from the remaining approximate 40% of energy available (Holland & Walker, 1975). The larva must retain sufficient energy reserves to allow metamorphosis to occur after settlement. There is no preferential fatty acid catabolism by barnacle cyprids during settlement and metamorphosis (Waldock & Holland,1978), although in the nematode *Strongylus vulgaris*, preferential fatty acid catabolism is observed suggesting specific energy reserve strategies related to transmission (Medica & Sukhdeo, 1997). Such knowledge of lipid utilisation is not available for sea lice but the depletion of the nauplii (Gravil, 1996; this study, chapter 5) and copepodid energy reserves (this study, chapter 5) have been investigated.

Holland & Walker (1975) suggest that the depletion and utilisation of energy reserves may

have some influence on the drive to settle in cyprids. This could be summarised as an increased drive to settle in unsuitable locations due to the lack of energy available to explore their environment fully. Crisp & Meadows (1963) found that the rate of settlement in cyprids increased with age, this is not observed in sea lice.

The same cannot be said to be true for sea lice. With the older aged copepodids there is a decrease in settlement ability although once attached to the host the copepodid can actively feed and replenish its energy reserves (this study, chapter 5). In this study once the older copepodids had attached to the host the survival was high. In experiment 1 and 2 survival was greater 58% in all age groups whilst in experiment 3, at winter sea water temperatures survival was approximately 50% in all age groups.

Copepodids of *L.salmonis* showed preference for the body, gills, pectoral and dorsal fins as sites of settlement. When very high settlement counts are observed the parasite numbers on the body increase significantly suggesting that the fins, although preferential settlement sites, may have a finite carrying capacity. The effect of crowding in ectoparasites of fish was observed by Jensen & Johnson (1991) in the monogenean *Gyrodactylus salaris* where distribution changed with increasing intensity of infection. With numbers of ≈100 *G.salaris* settlement was primarily on the dorsal fin but when numbers increased to 1000 the body surface was infected. Distribution of *L.salmonis* on the body was random and the proximity of the fins did not influence settlement adjacent to the fins. The gills are believed to be regions of incidental infection, through the inhalant current, as losses are also greatest in this region. Gill settlement was discussed in the previous chapter and may not play a significant role in copepodid survival. Of lice settled on the fins greatest survival was on the pectoral fins and the outer ventral surface shows preferential settlement, with over 60% in all age groups of copepodids.

In the previous chapter the preferential settlement on the fins was suggested to be due to attraction to fin currents (Kabata & Cousens, 1977). Fish hydrodynamics change from laminar flow in the boundary layer to turbulent flow with increase in fish swimming speed (Bond, 1996). The pectoral fins are utilised as gliding planes to produce *lift and glide down* (Bond, 1996) and as brakes, whilst the pelvic fins act to counteract braking by the pectoral fin (Jobling, 1995). To produce lift there must be fluid displacement to give disproportionate velocities of flow over the two surfaces; the velocity of flow must be higher above a lifting fin than below (Bone, Marshall & Blaxter, 1996). Pressure differences on the inner fin surface will be negative. Drag over the fin is also disproportionate, trailing edge flows separate over the surface (the Kutta condition) (Daniels, Jordon & Grunbaum, 1992) flowing over the fin towards the tip to create tip vortices (Bone, Marshall & Blaxter, 1996). The fins of a fish are dynamic zones of flow, pressure distribution and thrust. Boxshall (1976) suggests that preferential fin settlement by *Lepeophtheirus pectoralis* is due to these sites being associated with water currents and that copepodids move towards water currents. Settlement distribution reflects the attraction of water currents produced by the host. Fins, with their fin rays will also provide increased microhabitats for protection in settlement just as Crisp (1961) suggested for cyprids that actively seek presented hemispherical pits.

Considerable variability in settlement was found for all dose rates of copepodids used and the transmission dynamics of *L.salmonis* is obviously an area of further research. These experiments have been conducted at summer sea water temperatures and show the potential parasite loading on juvenile salmonids. In experiment 3 (Figure 4.6c) the fish in the high dose rate group had an average of 209 parasite per fish, whilst the excessive dose rate group had an average of 316 parasites per fish at D.P.I. 5. High dose rates lead to high settlement counts and as no serum response is found in naturally infected salmon (Grayson,

Jenkins, Wrathmell & Harris, 1991) a potentially fatal situation arises. Survival of lice in all experimental dose rates was exceptionally high with greater than 80% survival in all groups. Survival of *L.salmonis* through all the chalimus stages is approximately 23% (Shinn, 1994 unpublished data, Institute of Aquaculture) and therefore juvenile salmon receiving the high and excessive dose rates of lice recorded here could be exposed to a potentially high and fatal number of motile preadults.

With the environmental stresses associated with summer conditions, fish would soon die if such high parasite intensities were maintained. Severe infections on sockeye salmon resulted in high mortalities from high dose rates when the transmission dynamics in a crowded holding inlet allowed high *L.salmonis* intensities (Johnson *et al.*, 1996).

The effect of dose rates is the inverse principle of host stocking densities. In these experiments settlement was approximately 20% in all stocking density groups. When the parasite load is distributed between 25 fish the average intensity was approximately 38 parasites per fish whilst in the lowest stocking density the average intensity was approximately 224 parasites per fish. At the highest stocking density used in these experiments, settlement was 19 and 18.4% compared with over 21% in the other stocking density groups. When survival of lice was examined at D.P.I. 10 the highest survival was found in the highest stocking density compared to the other stocking densities used in both experiments, suggesting increased survival through reduced competition between the parasites. However, survival in the SD5 and SD15 groups was over 65% in experiment 1 and over 79% in experiment 2 showing high intensities of parasites in the presence of reduced numbers of fish.

The stocking density strategy employed by salmon farmers is to stock with high numbers of fish and grade the fish as necessary. This allows the separation of year classes and helps

disease control. The results of these experiments show that the stocking of sea cages with high numbers of juvenile salmon will distribute any parasitic infection, reducing the parasite mean intensity. Stocking densities (for land based rearing systems) suggest that a stocking density of above 125 kg m^{-3} could be maintained (Kjartansson, Fivestad, Thomassen & Smith, 1988). *L.salmonis* has a negative binomial distribution in a population of salmon and in a low stocking density whilst it will be overdispersed, a high stocking density will result in greater spread of the parasite population through the host population so there will be a greater chance of salmon survival. However, in a natural situation a low stocking density would lead to a reduced transmission rate and thus also reduced parasite numbers. Albert & Curtis (1988) demonstrated that a decrease in stocking density of Brook trout (*Salvelinus fontinalis*) would improve growth and reduce parasites although the reduction of parasites is due to the prevention of ingested parasites due to reduced competition for food.

There is no literature available on the effects of multiple infections of ectoparasites. All experiments to date have involved single wave experimental infections whereas in a cage culture environment fish would be constantly exposed to infective stages. The present experiments have demonstrated that multiple infections have lower settlement intensity than single infections. In experiment 1 there was a difference of 7.7% whilst in experiment 2 there was a difference of 13.0%. The subsequent infections must be in direct competition for space as the initial low infection had developed to the chalimus 2 and 3 stages at D.P.I. 5, the settlement sites were occupied by larger developmental stages. The survival of *L.salmonis* chalimus stages in these experiments was over 71% in all infection groups. In experiment 1 the survival was highest in the single infection regime whilst in the second experiment survival was highest in the multiple infection group.

The proposed model for sea lice infection densities suggested by Jaworski & Holm (1992), whilst adequate for the motile stages that migrate over the body surface, is inadequate for copepodid and chalimus stages. Their proposed model does not allow the differentiation of the fin surfaces from that of the body surfaces, which have been incorporated. Their model reports lice intensity as a percentage of the host surface area occupied by the louse. In their study larger fish would have higher intensities as they can accommodate a greater number of preadult and adult parasites, a small fish would have difficulty having a high intensity of preadult lice. Dogiel *et al.* (1958) agree with the principle that host size and parasite attachment are linked. A larger fish has lived longer and therefore it can accumulate more parasites, their size provides more opportunity for infection. Anstenrud & Schram (1988) found no size preference of host by the parasitic copepod *Lernaeenicus sprattae.*

Jaworski & Holm (1992) divided the host body area into eight regions that were orientated around the body. In the present study the fish was divided into the whole body surface and each separate fin group. The salmonid body:fin ratio changes with age, in juvenile fish there is a higher percentage fin area than in older fish. As settlement of *L.salmonis* copepodids is predominately on the fins this will have a marked effect. Fin settlement in all groups examined in this study was greater than 60%. In this study the small fin have the largest fin surface area, approximately 33% compared to 26% and 23% for the medium and large sized fish respectively. Settlement was highest, in the small fish group in both experiments, 9.3% in experiment 1 and 17.0% in experiment 2. Survival was high in all groups, in experiment 1 survival was greater than 90% whilst in experiment 2 survival was greater than 70%.

A second contributing factor to settlement on fish of varying sizes is fish swimming speed. All fish were infected in a single 800 litre tank. Jobling (1995) suggests fish optimum

swimming speeds of 1-3 body lengths s^{-1} for larger fish, moving faster will therefore be more difficult to infect by copepodids. Fish utilised for these experimental infections had a size range of 139-379 cm (standard length). Copepodid settlement is stimulated by rheotaxis and therefore the copepodid has to react to fish movement. Copepodid stimulated swimming speed is 6.84 cms^{-1} (Gravil, 1996) and thus the smaller fish are more likely to be infected, as they move slower in comparison to the larger fish. Host swimming speed will have a marked effect on copepodid ability to infect and settle on the host.

In this study the two experiments using sea trout and salmon have given apparently contradictory results when fish were infected with approximately the same number of copepodids. In experiment 1, using separate populations of fish a significantly higher settlement occurred on salmon (73.69%) as compared to sea trout (67.14%). In the second experiment, in using mixed populations of fish the sea trout had a higher intensity of infection (44.90%) than the salmon (26.94%). The values for lice survival at D.P.I. 10 are greater than 80% for both sea trout and salmon. Dawson *et al.* (1997) found higher intensities of *L.salmonis* on sea trout when infected as separate and mixed populations. These authors used a low infection dose rate, 1.3 copepodids per litre and a low stocking density, however sea trout retain a higher mean abundance than salmon.

Birkeland (1996) caught ascending and descending migratory sea trout and found 96% prevalence in post-smolt sea trout (94 fish) and 87% prevalence in older migrants (74 fish) with sea lice infestations. These fish are reported to have returned prematurely to their native river system and due to the sea lice infestation had a reduced growth rate and mass, thereby reducing fecundity and reproductive success (Birkeland, 1996). The post-smolt fish reported by (Birkeland, 1996) had approximately 88% copepodids and chalimus stages present whilst the older migrants had approximately 69%. Grinmes & Jakobsen (1996)

found that at high parasitic intensity post-smolt Atlantic salmon showed no severe physiological effects from attached chalimus stages but with the development of motile stages there was a sudden increase in fish mortalities, intensities of >30 motile lice per fish. Motile lice graze the epidermis and numbers of <30 lice on such a small surface area can cause severe disruption of the epidermis (personal observation).

In the first experiment there was a higher settlement on the salmon than sea trout, a difference of over 10%. In the second experiment the lice have shown a preference for sea trout, approximately 18% difference, as they have been given a direct choice. However, is this indicative of the situation in the wild and adjacent to salmon sea cages? The suggestion that salmon farm cages are the cause of the collapse of the sea trout populations relies on the fact that a free-swimming sea trout must come into contact with a cage source sea louse. The age of copepodid experiments have shown that sea lice copepodids lose their infective ability, through lack of energy reserves after 7 days. The local hydrodynamics of a cage culture site must be such that they allow contact and infection of the sea trout within this 7 day window, the probability of coincidence. Costelloe, Costelloe & Roche (1996) found that plankton dispersion of sea lice larvae associated with cultured salmon was less than 10% of the larval density inside the salmon cage, indicating high retention of larvae. Of those found outside the salmon cage the highest densities were found 10m from the salmon cage, 4.3 larvae m^{-3} whilst in the cage densities of 59.6 larvae m^{-3} were found. At a distance of 1 km from the salmon cage less than 1 larvae m^{-3} was found. Gravil (1996) in pump sampling of waters adjacent (2m away) to salmon cages, at various depths found a larval (only naupliar stages) concentration of 11.6 larvae m^{-3} in surface waters. No lice specimens were found at a distance of 2m from the salmon cages in pump sampling (Gravil, 1996). With such evidence the question must arise as to the source of sea lice as reported in such concentrations by Birkeland (1996). Natural aggregations of

fish can cause epizootics through cross infection. Johnson *et al.* (1996) report high mortalities of migrating salmon, assembled in an inlet had high intensities of *L.salmonis* which, through cross-infection, resulted in high mortalities.

Alternate hosts, like sea trout show variable intensities of sea lice. Of six Pacific salmon species Pink salmon was found to have the highest prevalence, mean intensity and abundance (Nagasawa, Ishida, Ogura, Tadokoro & Hiramatsu, 1993). The comparative susceptibility of Atlantic salmon compared with Pacific salmon shows that Atlantic salmon are more susceptible to infection (Johnson & Albright, 1992b). In comparing the developmental rate of sea lice growth on Atlantic and chinook salmon Johnson (1993) found that development was faster on Atlantic salmon and suggested that this was due to non-specific host responses. In this study it was found that the slower development of sea lice on sea trout was statistically different from Atlantic salmon, again possibly due to non-specific host responses, although this was not investigated. Survival over the 10 day period, where late chalimus stages were present, for the sea trout/salmon experiment was high in all tanks, greater than 80%. Dawson *et al.* (1997) suggest no difference in the developmental rate of lice on salmon and sea trout although their samples were conducted at weekly intervals and there was no differentiation of the chalimus stages.

The copepodid has a finite time within which to locate and infect any potential host, there is a rapid decline in its infective ability after seven days. Cage culture infections of *L.salmonis* are seen as self-infecting (Bron, Sommerville, Wootten & Rae, 1993) with few larval stages found beyond the salmon cage, a reduction of 97.7% of cage numbers being observed at 200 m (Anon, 1995b). Local hydrodynamics will govern the extent of the range of lice escapees from the salmon cages. As salmon cages are self-infecting the stocking density of the host will have marked effects on the mean intensity of any

infection. Stocking fish at the highest density, without undue stress will disperse an infection. Such knowledge of copepodid behaviour can help in the establishment of more effective management strategies.

CHAPTER 5. ENERGY LEVELS OF *L.salmonis* LARVAE.

5.1 INTRODUCTION.

Many scientific investigations have been undertaken to examine the calorific content of marine organisms. Such information is intrinsic to the understanding of population and community energetics, including the construction and interaction of food chains. A study of the energy flux is an important step in understanding the dynamics of any ecosystem. Every organism, irrespective of its chemical composition and size, corresponds to a given amount of chemically bound energy; this can be utilised internally by the organism itself or by other organisms using it as a food source (Båmstedt, 1986). Calculations of the energy available within an organism have been mostly derived from bomb or microbomb calorimeters, where the calorific value is detected directly from the combustion of samples.

Energy levels have often been investigated as part of seasonal studies of phytoplankton and organisms in higher trophic levels. As primary producers, phytoplankton are the starting point of any aquatic food chain. Platt & Irwin (1973) in their study of phytoplankton during an April spring bloom found energy values ranging from 2.062 - 3.746 cal mg^{-1} dry wt and state that a good conversion factor for phytoplankton is 1 mg = 11.40 calories. Marine microcrustacean energy levels were investigated by Comita & Schindler (1963) and they reported values for a number of microcrustacea species ranging from 4478 - 5672 cal g^{-1} dry wt. Comita, Marshall & Orr (1966) determined seasonal fluctuations in the calorific value of eggs, copepodids and male and female adult *Calanus finmarchicus*. Values for the eggs and copepodid were 4750 and 5478 cal g^{-1} dry wt, respectively. Schindler, Clark & Gray (1971) showed seasonal variation in energy levels of various freshwater zooplankton, particularly larval *Cyclops bicuspidatus thomasi* with values in the

range of 6830 - 7115 cal g^{-1} dry wt from an oligotrophic lake. Intraseasonal changes in calorific content for a number of freshwater invertebrates ranging from 3682 - 6687 cal g^{-1} dry wt have been given by Wissing & Hasler (1971). Similarly, Snow (1972) found that the calorific content of *Daphnia publicaria* ranged from 4900 - 9000 cal g^{-1} dry wt for summer and winter animals respectively. Seasonality will have effects through food availability, food type and body size on the energy availability.

The energy content per unit weight of an organism is determined by the proportion and the composition of the organic matter present (Båmstedt, 1986). Animal carbohydrates have an average energy content of 17.16, proteins 23.63 and lipids 39.35 Jmg^{-1} (Winberg, 1971 in Båmstedt, 1986). Mathews & Van Holde (1990) give values of energy content, from complete metabolic oxidation for carbohydrates and proteins of 17kJ g^{-1} whilst triacylglycerols yield 37 kJg^{-1}. Lipids therefore have the highest energy content available to an organism. Within lipid classes triacylglycerol (TAG) will yield 1.5 times as much energy per molecule as wax esters, TAG provides 3 acyl chains for subsequent oxidation whereas wax ester provides one acyl chain and one fatty alcohol, which is subsequently metabolised to a fatty acid before being utilised for catabolism. However, in terms of energy per gram, energy output during catabolism of both lipid classes can be regarded as similar (Bell, 1998 pers. comm.).

Benson, Lee & Nevenzel (1972) suggest that lipid metabolism in marine copepods is a most elegant example of environmental adaptation. Their world-wide function in grazing the photosynthetic algae of the sea and inland waters means that at least half of the earth's photosynthetic production is converted, for a time, to wax. The first isolation of wax ester from the marine copepod, *Gaussia princeps*, led to the recognition of wax ester metabolism as a major metabolic activity in nature (Lee, Nevenzel & Paffenhöffer, 1970a;

Benson *et al.*, 1972). Examination of the biochemical composition of free-swimming marine copepods at varying sea depths shows that lipid wax esters are synthesised faster than triglycerides and that their formulation may represent a biochemical mechanism for increasing the rate of lipid deposition from an excess of dietary constituents (Morris & Sargent, 1973). Morris & Sargent (1973) found that the rate of synthesis of wax esters appears to be governed by the rate of conversion of fatty acid to fatty alcohol. Radioactive labelling has shown that wax esters may be relatively stable compared to triglycerides, the latter being turned over at a faster rate than wax ester. Crustaceans are able to synthesise *de novo*, from simple dietary precursors, elaborate wax esters and other lipid classes. Consequently wax esters or free fatty alcohols are not obligatory dietary constituents for crustaceans.

Lee, Nevenzel & Paffenhöffer (1972a) and Ackman, Linke & Hingley (1974) also found that the dominant class of lipids in marine planktonic crustaceans is wax ester and deposition of this energy store takes place when food is freely available. Lipid deposition and storage in the copepod *Calanus hyberoreas* from the Arctic Ocean correlates well with phytoplankton blooms. Wax ester deposition, with summer phytoplankton-like fatty acid composition, follows active feeding by *Calanus hyberoreas* (Lee, 1974). Claus, Benijts & Vandeputte (1979) have shown that fed *Artemia* larvae are no exception to this rule and assimilate lipids from their diet.

Natural waxes are formed from the esterification of fatty acids and long-chain alcohols. The head group is weakly hydrophilic and attached to two hydrocarbon chains, these are completely water insoluble and hydrophobic (Mathews & Van Holde, 1990). Triacyglycerols (TAG) are three long hydrocarbon chains of fatty acid containing carbon in a fully reduced form, making them highly efficient for energy storage (Mathews & Van

Holde, 1990). These long saturated chains are often packed closely together.

Energy metabolism in marine organisms is generally focused on production and utilisation of lipids and these organisms resort to lipid metabolism for production of water (Benson *et al.*, 1972; Alberts, Bray, Lewis, Raff, Roberts & Watson. 1989). Jefferies (1970) found that the nutritional value of planktonic organisms is influenced by their physiological age and environment, and by the type of foods available. The lipid class distribution available to marine free-swimming copepods is very dependent on their vertical distribution in the marine environment (Lee, Hirota & Barnett, 1971a; Benson *et al.*, 1972). Not only does the depth at which the invertebrates are caught have profound effects on the rate of lipid deposition but so does the time of day and the season (Lee *et al.*, 1971a; Gatten & Sargent, 1973).

Copepod genera found in deep waters or cold surface waters have a higher proportion (>20%) of wax esters than tropical or temperate genera, which contain <10% (Benson *et al.*, 1972). A further example of this is given by Sargent & Henderson (1989). Gatten & Sargent (1973) examined depth aspects of lipid, wax ester content, and wax ester biosynthesis in *Calanus finmarchicus* and found less lipid and more wax ester biosynthesis at the ocean surface, which supports a *buoyancy/food deposition hypothesis i.e. wax esters* give better buoyancy. Ackman *et al.* (1974) also support this hypothesis as they suggest that the formation of wax esters is an economical method of buoyancy and means of accumulating long term energy reserves. Nevenzel (1970) suggests three possible functions for wax esters in marine organisms: 1. buoyancy 2. thermal insulation and 3. reserve energy storage. Lee, Nevenzel, Paffenhöffer and Benson (1970b) propose that phospholipids and cholesterols have a structural function whilst trigylcerides and wax ester serve as energy reserves.

Total neutral lipids (wax esters and triglycerides) in the four copepod species studied by Lee *et al.* (1971a) accounted for approximately 70% of the total lipid present. The pattern of active assimilation of wax esters during feeding in copepods shows a consistent general trend however, during starvation, a different lipid class is utilised. During starvation the copepod draws on two sources of energy, triglycerides and wax esters, but at controllable and differing rates (Benson *et al.*, 1972). Lee (1974), Lee *et al.* (1970a) and Lee, Nevenzel & Paffenhoffer (1971b) found that most copepod species readily utilised the triglyceride component of their stored lipid and metabolised their wax esters more slowly. Benson *et al.* (1972) concluded that the control of this process is governed by the relative activity of two enzymes, triglyceride lipase and wax ester lipase. No overlap of substrate specificity seems to occur between these enzymes. Activation of wax lipase by the stress of starvation controls the depletion of the organism's lipid reserves. Therefore, there is preferential catabolism of triglycerides over wax esters, which are consumed slowly after the triglyceride supply is exhausted. These results suggest the importance of enzymatic control to allow preferential utilisation of different substrates (Lee *et al.*, 1971a). Lipid analysis of *Gaussia princeps* during eight days of starvation showed that this copepod utilises triglycerides whilst wax esters remained relatively unchanged. During starvation the copepodid V of *Gaussia princeps* lost lipid at half the rate of the adult, only triglycerides being used during 120 hours of starvation (Lee *et al.*, 1971a). However, in long term starvation experiments (5 weeks) there was complete utilisation of triglycerides before wax esters, which depreciate from 67% to 25% wax ester of the total lipid (Lee *et al.*, 1971a). Wax ester therefore serves as a long-term energy store (Ackman *et al.*, 1974). Triglyceride is the reserve lipid in most invertebrates although in some species of copepod it comprises less than 20% of the percentage dry wt. (Lee *et al.*, 1971a).

The variation in amounts of wax ester and triglyceride within a copepod species can also

alter with the developmental stage. The early developmental stages have a greater proportion of triglyceride, but there is more wax ester in later developmental stages. The eggs of *Calanus helgolandicus*, a near surface copepod, contain triglyceride as 60% of total lipid present whilst the adults contain only 12%, but have wax ester as 41% of total lipid (Benson *et al.*, 1972). Benson *et al.* (1972) cite Lee, Hirota, Nevenzel, Sauerheber, Lewis, & Benson (1972b) as stating that the developmental stages which must require resilience in the face of environmental adversity contain the largest amount of wax ester. The lipid density is also an important factor in determining the type of lipid stored (Benson *et al.*, 1972). The density of wax ester is 0.86 whereas triglycerides have a density of 0.92 (Ackman *et al.* 1974). Eggs of *Calanus helgolandicus* have a high proportion of triglyceride and therefore float to the surface, where early nauplii stages can graze on microalgae. Lee *et al* (1972a) state that late developmental stages of some marine copepods after active feeding switch from triglycerides to wax ester as stored lipid.

Parasitic copepods have a different lipid class composition from free-living forms. Lee (1975) found that *Lepeophtheirus salmonis*, presumably adult lice (due to their low number of samples and the weight) on the skin of coho and pink salmon, had only trace amounts (<0.5%) of wax ester but the triglyceride composition was greater than 47% of total lipid. The lipid composition of the host skin also had triglyceride as the principal neutral lipid (Lee, 1975). The hydrocarbon fraction of the parasitic copepods studied by Lee was found to give the same gas-chromatograph pattern as that of the host skin hydrocarbons, suggesting parasite lipids were derived directly from the host skin. The lipid available from the host will therefore have impacts on the energy available to the organism and that which is passed on to the egg for oogenesis.

The free-swimming stages of *L.salmonis* are lecithotrophic, as suggested by the presence

of internal yolk reserves (Kunz, 1985) and lipophilic staining characteristics (Gravil, 1996). Further, TEM examination of the *L.salmonis* nauplii stages revealed the absence of a fully developed gut (Bron, 1997. pers. com.). Therefore, as the first three life stages of *L.salmonis* are lecithotrophic sufficient energy reserves must be laid down in the egg to allow development to the infective stage. These pre-settlement larval stages appear therefore to be reliant on this innate energy resource to develop to the infective copepodid and then locate a host to begin the parasitic phase of the life cycle. Excessive depletion of this resource, prior to the infection of the host, may result in a loss of viability and infectivity of the copepodid.

The study of free-living crustaceans may provide a valuable insight into the energetics and behaviour of larval *L.salmonis*. Similarities in settlement behaviour have been drawn between barnacle cypris larvae and the copepodid of *L.salmonis* (Bron, 1993). Bron (1993) and Bron *et al.* (1991) suggested the method of settlement followed by *L.salmonis* showed the same pattern as that of the barnacle cyprid on the substratum, i.e. the sequence of "attachment, exploration and fixation" as described by Crisp (1976), and modified and adapted by Bron *et al.* (1991). They suggest that the "close searching" phase of the cyprid behaviour is similar to that of *L.salmonis* copepodids on the host. The close searching phase can be broken down into three sequential events during settlement and attachment, as adapted by Bron *et al.* (1991).

1. Searching phase.

After initial contact with the host a period of close searching is undertaken. The maxillipeds grip the fish whilst the copepodid moves over a small area of the host surface, probing the surface with the anterior end of the cephalothorax and bringing the 1st and 2nd antennae into close contact with the host surface. At this stage settlement is still reversible

if the site is unfavourable.

2. Primary attachment phase.

Copepodids commencing attachment retain their grasp of the host with the maxillipeds whilst the 2nd antennae are driven into the host epidermis with a repeated stabbing action. Penetration of the 2nd antennae is as far as, and sometimes through, the basal membrane. The anterior edge of the dorsal cephalic shield, including the rostrum, is drawn down and forward causing lifting and aggregation of the host epithelium.

3. Secondary "filament" attachment phase.

The final phase of attachment is through production of a frontal filament to anchor the larva to the host. The filament is attached beneath the host epithelium, along the basal membrane and attachment was accomplished by the copepodid stage. Filament production must be rapidly followed by the moult to the chalimus 1 stage (Bron *et al.*, 1991).

Barnacle cypris larvae are also lecithotrophic (Rainbow & Walker, 1977) although the various preceding nauplius stages are active feeders. As with *L.salmonis* copepodids, they must find a suitable settlement site and survive to metamorphose, before their internal energy reserves become depleted.

Barnes (1965) found that in the early stages of barnacle egg development there was greater protein and carbohydrate loss but in the later stages of egg development lipid reserves were used. Holland & Walker (1975) demonstrated that the lipid energy reserves of *Balanus balanoides* cyprids accounted for 14% of the total dry weight, with neutral lipids accounting for 9.2%, i.e. 66% by weight of the lipid fraction. Of the neutral lipids in *Balanus balanoides* cyprids, no wax ester was detected in analysis but triacylglycerols were found to be the main energy reserve, accounting for 7.74% of ash free dry weight and

63% of the neutral lipid fraction (Waldock & Holland, 1978). These lipid reserves of triacylglycerol were utilised after eight weeks at 8°C, although this is an abnormally long time for the cyprid to remain in the plankton (Holland & Walker, 1975). Survival of the individual to adult may therefore be a shorter period to allow for the unknown energy cost of metamorphosis (Holland & Walker, 1975). These authors suggest that depletion of lipid reserves may influence barnacle cyprid settlement. Waldock & Holland (1978) demonstrated that triacylglycerol levels rapidly decreased from approximately 8% to 2% of ash free dry weight as the non-feeding larva developed into the young barnacle.

Using a lipophilic stain Gravil (1996) has shown that lipid is the main energy reserve in *L.salmonis* nauplius and copepodid stages. In the nauplius stages, where the lipid reserve is clearly visible, the overall size of the lipid reserve depreciates with time, as directly measured by Gravil (1996). The lipid reserves form distinct and discrete vesicles in the nauplius stage but in the copepodid these are distributed in the epithelial cells of the newly developed midgut lumen (Bron, 1993; Gravil, 1996). Bron *et al.* (1993c) have shown that the alimentary canal is clearly visible and complete in the copepodid and chalimus stage and that peristaltic movements of the mid and hind gut of these stages has been observed (Bron, 1993). Once settlement and attachment has been achieved, active feeding by the copepodid can apparently take place, although it has not been determined whether this actually occurs.

5.1.1 C:H:N ANALYSIS.

In the late 1960's and early 1970's energy availability was expressed as calories g dry wt^{-1} in energy budget studies, using SI units this is now given as kJ g^{-1} organic carbon.

Platt & Irwin (1973) found that the calorific value of an organism may be predicted with high accuracy from its carbon content as a percentage of its dry weight. The carbon content of marine copepods from various areas of the world oceans range from 28% to 63% dry wt dependent on latitude (Båmstedt, 1986). Carbon content is closely correlated with the energy content of aquatic organisms (Salonen, Sarvala, Hakala & Viljanen, 1976). New scientific methods have made it possible to determine the organic carbon content accurately for submicrogram samples, even those unsuitable for microbomb calorimetry (Salonen *et al.* 1976). Carbon:Hydrogen:Nitrogen analysis is one such method. Carbon analysis provides a method for energy estimation that requires less application but retains the same precision as direct calorimetry (Salonen, 1976).

5.1.2 HPTLC LIPID ANALYSIS.

Gravil (1996) stained the free-swimming larval stages of *L.salmonis* and concluded that the vesicles observed in these stages was lipid and she determined energy depreciation by direct measurement of these stained vesicles.

Gravil (1996) also calculated the percentage lipid present in the ovisacs of the *L.salmonis* as 29.69% but did not discriminate the extent of development of the ovisacs. The lipid classes within the larval stages of *L.salmonis* have not been determined. The extraction of lipid from biological specimens can be complex but the Folch, Lees & Stanley (1957) procedure is accepted as satisfactory (Geise, 1967; Wharton & McCarty, 1972) and used extensively in the analysis of brood stock egg quality (Bruce, Shields, Bell & Bromage, 1993) and fish flesh (Nickell & Bromage, 1998). The sample analysis of the lipid present is determined by HPTLC (High Performance Thin Layer Chromatography) and scanning

densitometry (Olsen & Henderson, 1989).

5.1.3 FEEDING APPARATUS OF THE COPEPODID OF *L.salmonis.*

The general anatomy of the oral cone of *Lepeophtheirus* sp. and other caligids has been well documented by Kabata (1974) and Boxshall (1985, 1990). Adult *L.salmonis* feed on mucus and epithelium and/or blood (White, 1940; Håstein & Bergsjö, 1976; Brandal *et al.*, 1976), whilst chalimus stages are believed to feed on mucus and epithelium (Jones *et al.*, 1990). It has not been determined whether the copepodid stage, once it has located and settled on a host, immediately feeds. The barnacle cyprid, once settled on the substratum, does not start to actively feed for at least two days after metamorphosis, and only after this period does it start to replenish lost energy reserves (Rainbow & Walker, 1977). Previous studies on *L.salmonis* copepodids have shown that the feeding apparatus is incomplete, with the strigil missing (Johnson & Albright, 1991b), although Bron (1993) suggests that it is present. Bron (1993) does agree that the strigil cannot be observed under SEM; however his illustration taken from a single specimen cut at 2.5µm could show the presence of a strigil. Neither authors specify the age of the copepodids used, although Bron (1993) suggests that his single specimen may be one that is ready to moult to the chalimus 1 stage. Copepodid moult to chalimus has never been observed off the host. In the chalimus stages the strigil is present and the only difference from the adult stages is the degree to which the labium and labrum unite (Jones *et al.*, 1990; Bron, 1993). Jones *et al.* (1990) did not rule out the possibility of external digestion of the host epithelium by the chalimus stages although the host damage observed is apparently of a mechanical nature. Bron (1993) states that feeding derived damage is seen beneath the oral cone of the copepodid and that host remains were found within the buccal cavity, although not in the midgut. Kabata

(1974) originally described the mode of feeding in adult *L.salmonis*, which has been revised and refined by Andrade-Salas (1997). The action of the insertion of the strigil into the host epithelium is intrinsic to the "scooping movement" for ingestion. Once the epidermal strip, excised by the strigil, has been lifted into the oral cavity it is then cut by the action of the mandibles (Andrade-Salas, 1997). Whether the disunited labium and labrum and missing strigil will have serious consequences for feeding by the copepodid has yet to be determined.

5.2 STUDY AIMS.

The work presented in this chapter sought to determine the energy requirements of *L.salmonis* copepodid larvae during the pre-settlement and settlement phases on the salmonid host. A knowledge of the energy requirements of the copepodid prior to settlement will give a better understanding of the infection process and the transition from the free-swimming phase to the parasitic phase. The energy available to the lecithotrophic larval stages will be determined by their chemical composition and therefore analysis of the lipid vesicles will give an indication of total energy availability and viability. Once settlement has been accomplished the copepodid must have sufficient energy to moult to the first chalimus stage so the timing of first feeding is important.

C:H:N analysis indicates percentage organic carbon present and allows the calculation of the energy available to the free-swimming stages of *L.salmonis*. Once the lipid classes have been separated by HPTLC, the use of scanning densitometry gives a quantitation of the lipid classes present in the nauplius 2 stage. A SEM study of aged copepodids will reveal the possible emergence of the strigil, if present. Examination of attached

copepodids, examined daily after infection will show the timing of first feeding on the host and the extent of the damage caused.

5.3 MATERIALS AND METHODS.

5.3.1 ENERGY AVAILABILITY.

Two methods for assessing the energy levels of larval *L.salmonis* were attempted, bomb calorimetry and the C:H:N analysis. The former is a direct method and the latter indirect. Unfortunately the bomb calorimeter available could not analyse the very small sample sizes required and therefore C:H:N analysis had to be used to infer energy levels. For the C:H:N analysis, groups of 150 nauplius and 100 copepodids were used, each group weighing approximately 0.4 and 0.3mg, respectively. The miniature bomb calorimeter apparatus described by Phillipson (1964) can combust samples of 5-100mg and was used by Salonen *et al.* (1976) in their study of aquatic invertebrates. It is from this paper that the derived regression equation for energy content as kJ g^{-1} organic carbon has been utilised. A second equation derived by Platt *et al.* (1969), also using a Phillipson miniature bomb calorimeter, gives energy content as calories g^{-1} dry weight. Platt *et al.* (1969) utilised 50g wet weight trawled zooplankton samples to derive their regression equation whilst Salonen *et al.* (1976) used 19 monospecific aquatic invertebrate samples.

Many earlier papers give the calorific content as calories g^{-1} dry weight and therefore both regression equations have been used within this study to calculate energy availability and to allow comparison with the literature.

5.3.1.1 C:H:N ANALYSIS.

5.3.1.2 COLLECTION OF MATERIAL AND INCUBATION.

Sea lice ovisacs were collected as described in Chapter 2. Incubation (see Chapter 2.2.2) was conducted at the Institute of Aquaculture in a constant temperature facility held at 10°C, also described in Chapter 2.

Pre-settlement energetics (experiment 1)

Specimens of a known age used for C:H:N analysis were harvested as follows at:

1. Nauplius 1 stage, 12 hours post hatch.
2. Nauplius 2 stage, 12 hours post moult, aged 1 day.
3. Nauplius 2 stage, aged 2 day.
4. Nauplius 2 stage, aged 3 day.
5. Copepodid, 12 hours post moult, aged 1 day
6. Copepodid, aged 2 day.
7. Copepodid, aged 2 day
8. Copepodid, aged 3 day.
9. Copepodid, aged 4 day.
10. Copepodid, aged 5 day.
11. Copepodid, aged 7 day.

These specimens were cultured and monitored daily for development until the required time of harvest and analysis.

Post-settlement energetics (experiment 2).

The experiment for post-settlement energy analysis used 10 salmon smolts that had been infected (as described in Chapter 2) with copepodids less than 2 days old. After infection the fish were left undisturbed for 24 hours before sampling to give copepodids at 1 day post settlement. The post-settlement *L.salmonis* copepodids, to be used to calculate the energy levels were sampled from the host 1-5 days post-settlement. All fish were examined under anaesthetic and parasites removed as described in Chapter 2.

5.3.1.3 PREPARATION OF SAMPLES FOR C:H:N ANALYSIS.

Pre-settlement larval *L.salmonis* were individually counted and removed from a Bogorov tray to a beaker containing 60 μm filtered fresh sea water. For nauplii 1 and 2 stages 150 individuals were used for each sample, for copepodids 100 individuals were used. Samples were taken in triplicate.

For post-settlement copepodids, 100 individuals were individually removed from an anaesthetised fish with fine forceps and placed in a beaker with 60 μm filtered fresh sea water. As part of the post-settlement analysis 100 pre-settlement copepodids, from the hatch used for infection were sampled before infection for analysis of energy levels. Sample sizes, of post settlement copepodids were restricted by the success of the infection and therefore samples were taken in duplicate.

Once the specimens had been collected they were prepared for C:H:N analysis. To prepare the specimens for drying and weighing each sample was filtered through 60μm mesh, which was then washed twice with distilled water to remove any contaminants, mainly salt. Inspection of the filtered mesh under a dissecting microscope allowed the removal of any

larger contaminants and a second count of the parasites present. Samples were then placed in a foil cap (0.07ml) that had been previously weighed using a Mettler MT5 microbalance.

Samples were then oven dried at 110°C overnight and re-weighed. Once the dry weight had been recorded, the samples were placed in a Perkin Elmer PE2400 series II CHNS/O elemental analyzer. CHNS/O analyzers can determine, by combustion, the carbon, hydrogen, nitrogen, sulphur and oxygen content of organic material in a sample. The C:H:N analyzer in operating mode uses a combustion method to convert the sample elements to simple gases, these gases are homogenised and allowed to de-pressurise through a column where they are separated in a stepwise steady state manner. Detection is as a function of the thermal conductivity (Perkin Elmer PE2400 series II CHNS/O Analyzer Users Manual, 1991).

A computer print out of the percentage organic carbon, hydrogen and nitrogen present in each sample was obtained.

5.3.1.4 CALCULATION OF ENERGY LEVELS FROM C:H:N ANALYSIS.

As energy has to be inferred and calculated from percentage organic carbon present two equations have been utilised, one in Joules and the other in calories.

The energy levels were derived from the following regression equations:

Eq 1: $Y = 30.67 + 0.29\ X$ kJ g^{-1} org.C. Salonen *et al.* (1976)

Eq 2: $Y = -227 + 152\ X$ cal g^{-1} dry wt Platt *et al.* (1969).

One international table calorie is equal to 4.1868 joules (Uvarov & Isaacs, 1993).

Salonen *et al.* (1976) give two regression equations for the calculation of energy levels. The second has been corrected for nitrogen which gives a higher r^2 value, but it has not been used here as such a correction is only applicable to energy levels derived from oxygen bomb calorimetry. Bomb calorimetry reaction products do not take into consideration that nitrogen compounds in the bomb are different from those of biological reaction products and therefore a correction is necessary (Kersting, 1972).

5.3.2 HPTLC ANALYSIS.

5.3.2.1 LIPID EXTRACTION.

The procedure of Folch *et al.* (1957) was used for the extraction of lipids. This procedure is biphasic with extraction using lipid solvents and then a washing with salt solution to give a lower phase of total pure lipid. The washing procedure removes all the non-lipid contaminants from the extract with only a small loss of lipid.

Nauplius II stages were used to determine the lipid classes found within the vesicles which stained with Sudan Black B. Nauplius II stages could be reared in enough numbers to give a sufficiently large, homogeneous sample for analysis. For lipid determination approximately 20,444 nauplius 2 larvae were used in each replicated sample, calculated as described in Chapter 2. Each sample of nauplius 2 larvae was homogenised mechanically (Ultra Turrax, $60s^{-1}$), in a glass teflon homogeniser tube in 2:1 chloroform-methanol (v/v) mixture with butylated hydroxytoluene (BHT 0.01%) on ice to a ratio of 1µg to 20µg. The homogenate was filtered through a prewashed (in 2:1 chloroform-methanol) Whatman No1

filter paper and washed again with 2:1 chloroform-methanol. Potassium chloride (KCl) salt solution (0.88%) was added at a ratio of 4:1 to the filtrate for biphasic separation. This solution was capped to allow it to be shaken, then the cap removed to allow centrifugation (taken up to 3000 rpm and immediately back down) to facilitate complete separation. The upper aqueous phase was then aspirated off. A clean glass tube, previously weighed, received the transferred bottom lipid layer which was then dried down with oxygen-free nitrogen (OFN_2) and held in a vacuum desiccator for 1 hour. On re-weighing the glass tube the total lipid weight present could be calculated.

For storage, if necessary, the lipid was resuspended in solvent plus BHT (0.01%), gassed with argon to seal, capped and stored at -70°C.

5.3.2.1 HPTLC LIPID CLASS ANALYSIS.

This analysis followed the procedure of Olsen & Henderson (1989) for the separation of marine lipids by high performance thin layer chromatography (HPTLC). In this study only the neutral lipids (the storage lipids) were separated and quantified although the procedure allows for separation and quantification of both polar and neutral lipids.

Lipid classes were analysed using HPTLC 10x10cm silica gel plates (E.Merck, Dramstadt, Germany). Plates were marked 1cm from the bottom with the spot origins, 3mm long at intervals of 12mm. This resulted in seven lanes for the development of the lipid classes, with the centre lane for a standard control. Plates were predeveloped, run to full length in hexane:diethyl ether (1:1 v/v) in a small chromatography tank with lid, to remove impurities. The plate was then activated by oven drying for 1 hour at 110°C. 1µl spot samples of lipid were found to give the best separation. Samples of lipid (1µl) were spotted

onto the marked origin of the silica gel plate lanes with a glass micro syringe. A standard of fish brain (1mg μl^{-1}) (provided by Dr G. Bell, NERC Unit of Biochemistry, University of Stirling) was spotted on the centre lane. The neutral lipid classes were developed in a solvent system of hexane:diethyl ether:acetic acid (90:10:1) to 0.5cm from the top of the plate. Separated lipid classes were detected after air drying and spraying the plate to saturation with 3% cupric acetate in 8% phosphoric acid (Fewster, Burns & Mead, 1969), followed by charring at 160°C for 20 minutes. Lipid class analysis was performed by quantitative scanning densitometry utilising a Shimadzu CS9000 densitometer.

All developments were performed at room temperature in standard saturate chambers for 10x10cm HPTLC plates loaded with HPLC standards and samples.

5.3.3 ELECTRON MICROSCOPY OF FEEDING APPARATUS (MOUTHPARTS) OF COPEPODIDS OF *L.salmonis* .

Copepodids of a known age (up to 7 days) after moult from the nauplius 2 stage and settled copepodids up to 5 days post-infection with areas of host epithelium were prepared for SEM as described in Chapter 2.

5.4 RESULTS.

5.4.1 C:H:N ANALYSIS OF ENERGY LEVELS OF *L.salmonis* (PRE-SETTLEMENT).

The mean energy levels for all pre-settlement larval stages of *L.salmonis* can be seen in Figure 5.1. and Table 5.1. Figures 5.2 and 5.3 show the mean energy levels for the nauplii and copepodid stages only of *L.salmonis* (pre-settlement), respectively. Each Y axis corresponds to the calculated energy levels ("Y1" from Salonen *et al.*, 1976 and "Y2" from Platt *et al.*, 1969) with the percentage carbon values given beside the plotted point. The pattern of the calculated energy levels between the two equations shows the same shape, with theoretical increase in percentage carbon after each moult. Examination of the energy levels, within a developmental stage show that the rate of loss of energy between nauplius 2 stages aged 1 and 2 days and between copepodids aged 5 and 7 days, exhibiting a sharp decline.

Table 5.1: Mean energy levels calculated as kJ g^{-1} org. C (from Salonen *et al.*, 1976) and as cal g^{-1} dry wt (from Platt *et al.*, 1969) for pre-settlement larval *L.salmonis* stages.

	N1 12hrs	N2 1d	N2 2d	N2 3d	Cop 1d	Cop 2d	Cop 3d	Cop 4d	Cop 5d	Cop 7d
Percent C	50.05	54.59	49.44	49.15	57.58	54.45	53.99	53.28	51.32	47.28
kJ g^{-1} org. C	45.18	46.50	45.01	44.92	47.37	46.46	46.33	46.12	45.55	44.38
S.D.	0.21	0.29	0.27	0.49	0.11	0.50	0.92	0.67	0.59	0.51
cal g^{-1} dry wt	7380	8070	7288	7243	8525	8048	7979	7871	7573	6959
S.D.	108.3	153.6	143.5	258.5	55.47	267.9	408.1	364.7	291.3	268.7
n =	7	3	6	5	3	10	10	11	9	2

Key: N1=nauplius 1; N2 1d=nauplius 2 days old; Cop 1d=copepodid 1 day-old.

n = number of samples.

Figure 5.1 Energy Availability for *L.salmonis* Larval Development (Pre Settlement)

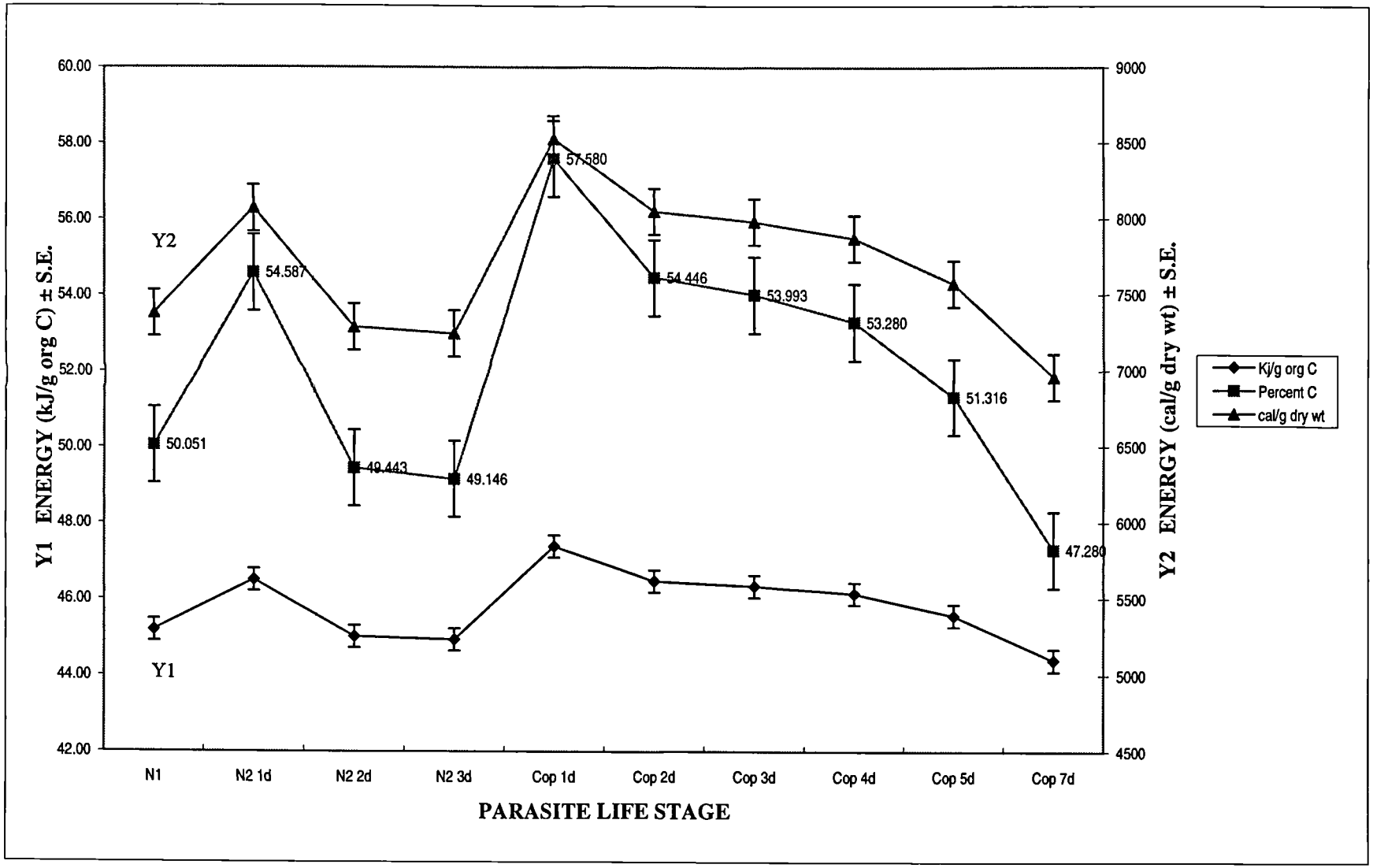


Figure 5.2 *L.salmonis* Nauplii Mean Energy and Organic Carbon levels (Pre settlement)

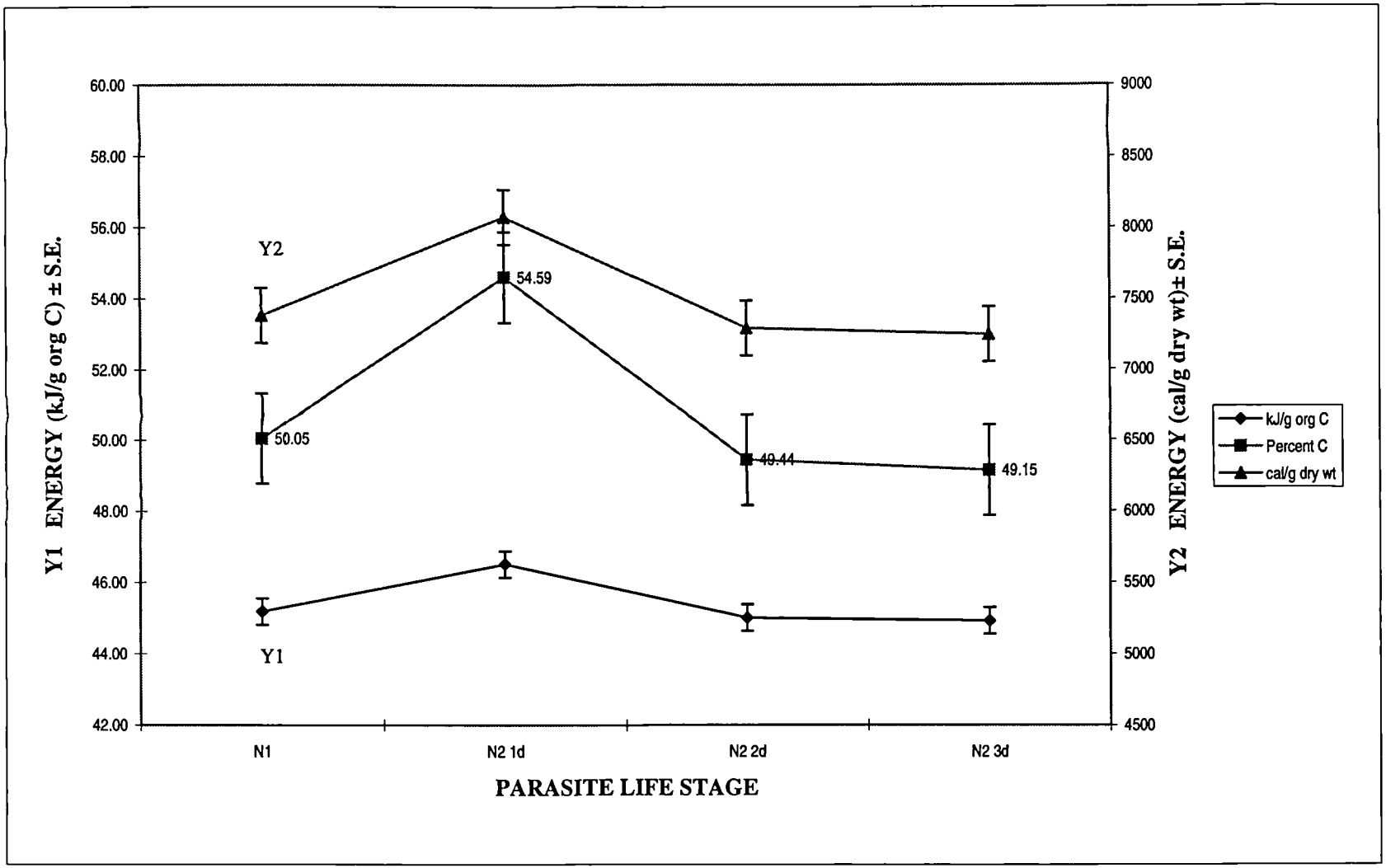
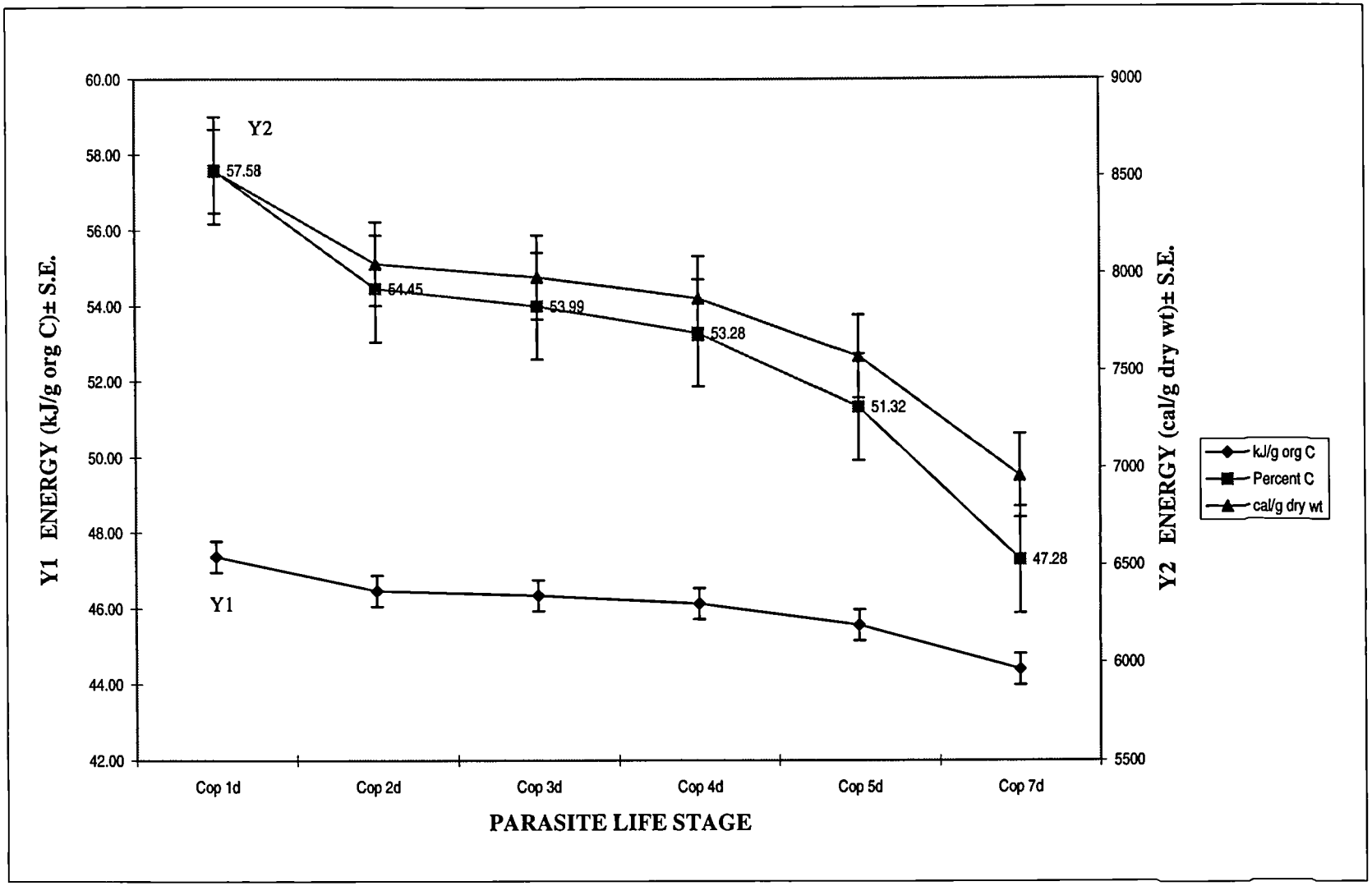


Figure 5.3 *L. Salmonis* Copepodid Mean Energy and Organic Carbon levels (Pre settlement)



As there are morphometric changes between the moulted stages of *L.salmonis*, only the same stages of different ages were compared statistically using the Mann-Whitney non-parametric test to determine any significant differences between the energy levels and the age of a particular stage. A statistically significant difference (p=0.028) was found between nauplius 2 (1 day-old) and nauplius 2 (2 days old). No significant difference was found between nauplius 2 (2 days old) and nauplius 2 (3 days old). Analysis of the copepodid stages shows a statistically significant difference between 1 day-old and 2 day-old copepodids (p=0.014) and also between 5 day-old and 7 day-old copepodids (p=0.045).

5.4.2 C:H:N ANALYSIS OF ENERGY LEVELS (POST-SETTLEMENT).

The mean energy levels for pre-settlement and post-settlement copepodids of *L.salmonis* can be seen in Figure 5.4. and Table 5.2. Samples of copepodids from the same ovisac batch were analysed for pre-settlement and post-settlement energy levels.

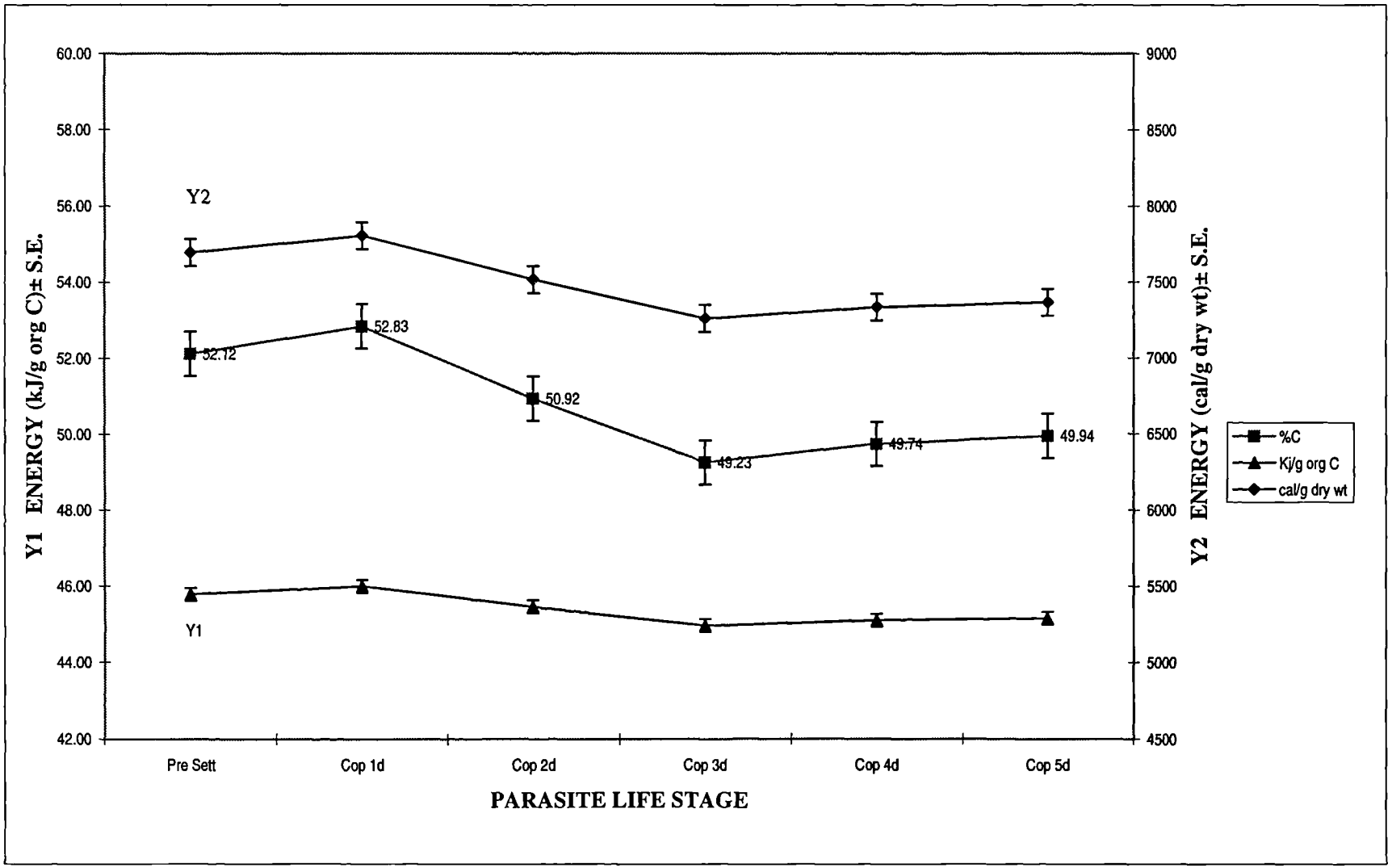
Table 5.2: Mean energy levels calculated as kJ g^{-1} org. C (from Salonen *et al.*, 1976) and as cal g^{-1} dry wt. (from Platt *et al.*, 1969) for pre- and post-settlement copepodid stages.

	Pre Sett 2d	Cop 1d	Cop 2d	Cop 3d	Cop 4d	Cop 5d
Percent C	52.12	52.83	50.92	49.23	49.74	49.94
kJ g^{-1} org. C	45.78	45.99	45.44	44.95	45.09	45.15
S.D.	0.363	0.007	0.757	0.495	0.071	0.276
Cal g^{-1} dry wt	7694.7	7803.1	7512.1	7256.0	7332.7	7363.9
S.D.	191.3	5.4	397.7	259.0	38.7	144.0
n =	3	2	2	2	2	2

Key: Pre Sett.2d = pre-settlement copepodids 2 days old, Cop 1d = post-settlement copepodid 1 day attached. n = number of samples.

Due to the very small sample sizes of post-settlement lice obtained, statistical analysis

Figure 5.4 Energy Availability for *L.salmonis* Copepodid Development (Post Settlement)



must be treated with caution. Mann-Whitney and Dunn's non-parametric tests on percentage organic carbon values, kJ g^{-1} org. C values and cal g^{-1} dry wt values show no statistically significant differences ($p < 0.05$) between the pre- and post-settlement lice. It should be noted that there is a difference in the percentage organic carbon present in the 2 day-old copepodid utilised in the pre-settlement (Table 5.1) and post-settlement (Table 5.2) calculations. In the pre-settlement group, experiment 1 the average percentage carbon is 54.45 % whilst in the pre-settlement group, experiment 2 the average percentage carbon is 52.12 %. Although these lice came from the same farmed site they were harvested at different times of the year and were therefore not compared. Examination of the numerical values for post-settlement shows, although not statistically significant, an initial rise, although not significant, followed by a decrease (547 cal g^{-1} dry wt) in energy availability to day 3, followed by constant energy levels.

5.4.1.3 C:H:N ANALYSIS - Energy Values (converted to compare results).

The conversion of the derived energy values for pre and post settlement stages of *L.salmonis* between kJ and calories is seen in Table 5.3 and 5.4. One international table calorie is equal to 4.1868 joules (Uvarov & Isaacs, 1993). This conversion allows comparison of results using the regression equations of Salonen *et al.* (1976) and Platt *et al.* (1969).

It should be noted that when the derived values for kJ g^{-1} dry wt. (from Salonen *et al.*, 1976) are converted from SI units to calories the values obtained are considerably smaller than those derived from Platt *et al.* (1969). The values obtained within this study from the use of the regression equation of Platt *et al.* (1969) are similar to those provided by the

literature in respect of free-living copepod larvae.

Table 5.3: Calculated energy levels converted between kJ and calories for pre-settlement *L.salmonis*.

Stage	Percentage org. C	kJ g^{-1} org C	Converted to calories	cal g^{-1} dry wt	Converted to joules
N1	50.05	45.19	189.18	7380.8	1762.9
N2 1d	54.59	46.50	194.69	8070.2	1927.5
N2 2d	49.44	45.01	188.44	7288.4	1740.8
N2 3d	49.15	44.92	188.08	7243.2	1730.0
Cop 1d	57.58	47.37	198.32	8525.2	2036.2
Cop 2d	54.45	46.46	194.52	8048.8	1922.4
Cop 3d	53.99	46.33	193.97	7979.9	1906.0
Cop 4d	53.28	46.12	193.10	7871.6	1880.1
Cop 5d	51.32	45.55	190.72	7573.0	1808.8
Cop 7d	47.28	44.38	185.82	6959.6	1662.3

Key: N1=nauplius 1; N2 1d=nauplius 2 days old; Cop 1d=copepodid 1 day-old.

Table 5.4: Calculated energy levels converted between kJ and calories for post-settlement *L.salmonis*.

Stage	Percentage org. C	kJ g^{-1} org C	Converted to calories	cal g^{-1} dry wt	Converted To joules
Pre Sett	52.12	45.78	191.69	7694.7	1837.9
Cop 1d	52.83	45.99	192.55	7803.1	1863.8
Cop 2d	50.92	45.44	190.23	7512.1	1794.2
Cop 3d	49.23	44.95	188.18	7256.0	1733.1
Cop 4d	49.74	45.09	188.80	7332.7	1751.4
Cop 5d	49.94	45.15	189.04	7363.9	1758.8

Key: Pre Sett. = pre-settlement copepodid plus 2 days old, Cop 1d = post-settlement copepodid plus 1 day attached.

5.4.2 HPTLC.

The amount of lipid extracted from an estimated 20,444 nauplius II was 6.6 mg dry weight, which gives lipid as 11% of total dry weight. Lipid per individual was calculated as 0.32μg.

HPTLC separation of the neutral lipid classes present in the nauplius II stage of *L.salmonis* as analysed by the Shimadzu CS 9000 densitometer is shown in Figure 5.5 and Table 5.5. Lipid class present in the fish brain standard are shown in figure 5.6. Figure 5.7 shows the scanned HPTLC plate with separation of the neutral lipid classes. It should be noted that no wax esters were detected.

Table 5.5: Percentage lipid classes present in analysis of *L.salmonis* nauplius II stage and fish brain standard.

	L. salmonis nauplius stage	Fish brain standard control
Polar Lipid	30.42 %	40.46 %
Monoacylglycerol	3.85 %	2.74 %
Cholesterol	5.65 %	28.94 %
Free Fatty Acids	22.53 %	4.97 %
Triacyglycerols	37.55 %	17.04 %
Sterol Esters	Nil	4.67 %

5.4.3 FEEDING APPARATUS OF COPEPODIDS OF *L.salmonis* .

SEM photographs of the copepodid, pre- and post-settlement have provided evidence of the dimensions of the attachment, feeding site and absence of specific feeding apparatus. Pre-settlement copepodids, aged 1-7 day-old provided the dimensions of the distance from

Figure 5.5 Neutral lipid scanning densitometry print out from a Shimadzu CS9000 densitometer for *L. salmonis* nauplius 2 stage.

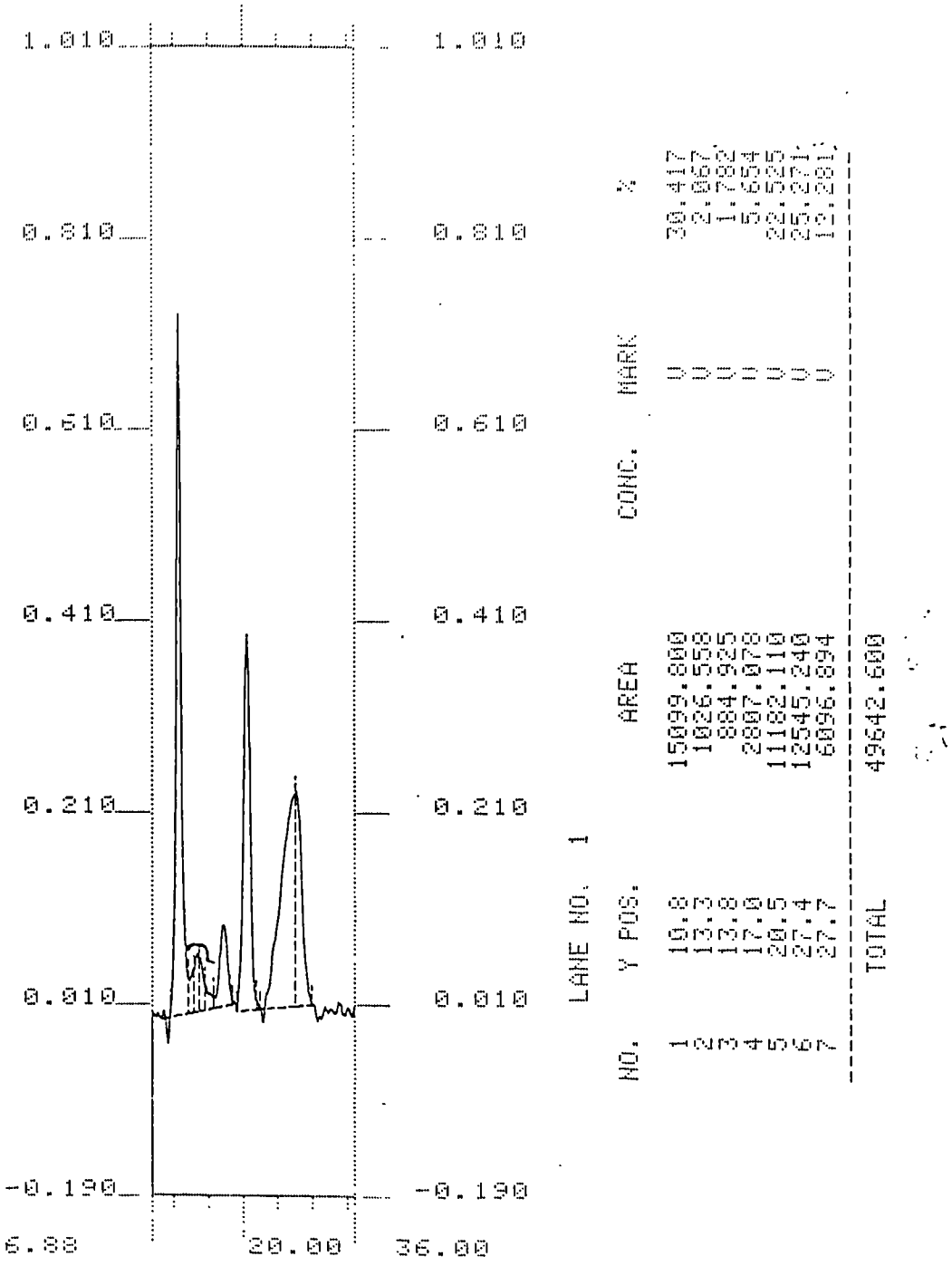


Figure 5.6 Neutral lipid scanning densitometry print out from a Shimadzu CS9000 densitometer for fish brain standard.

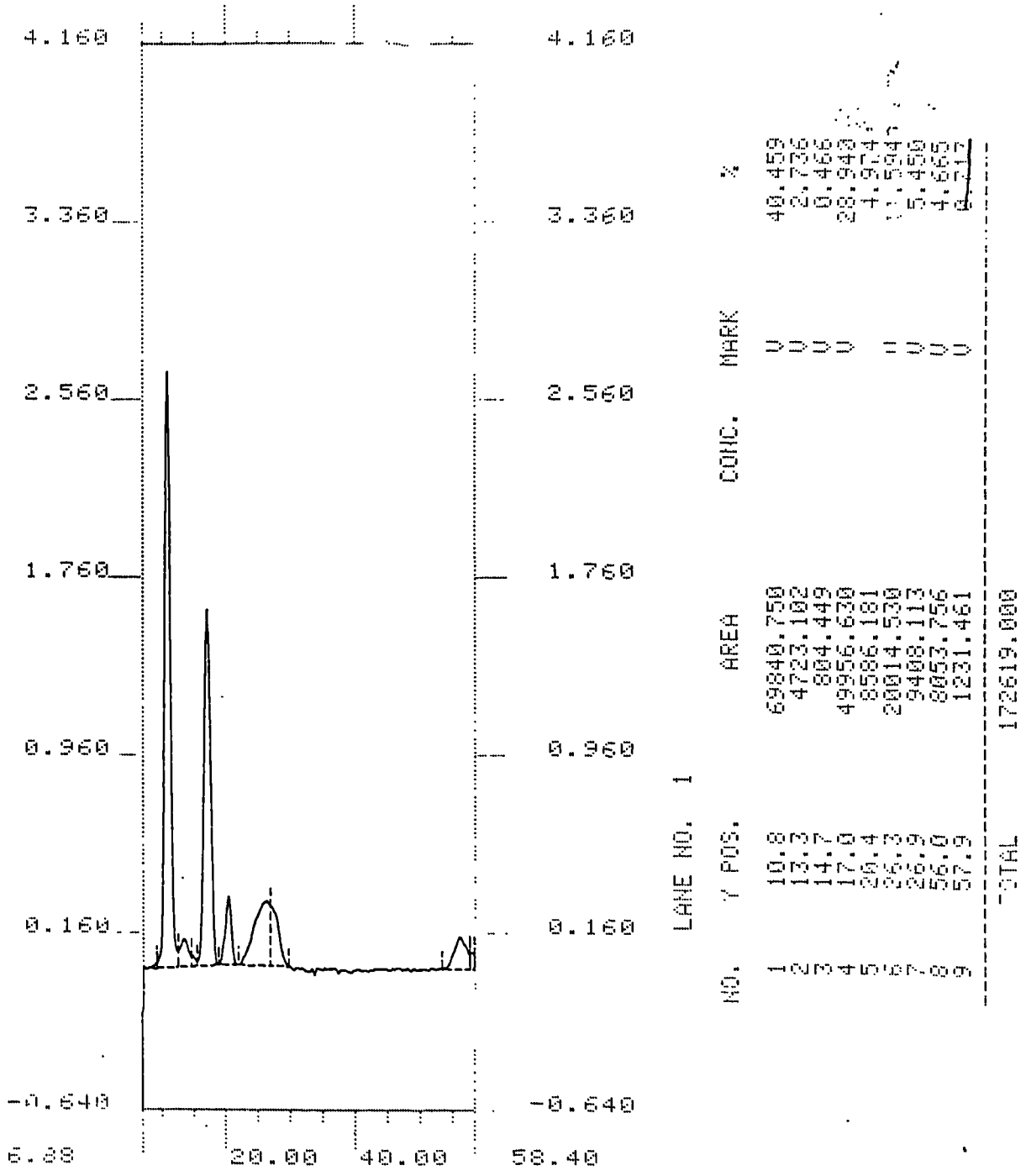
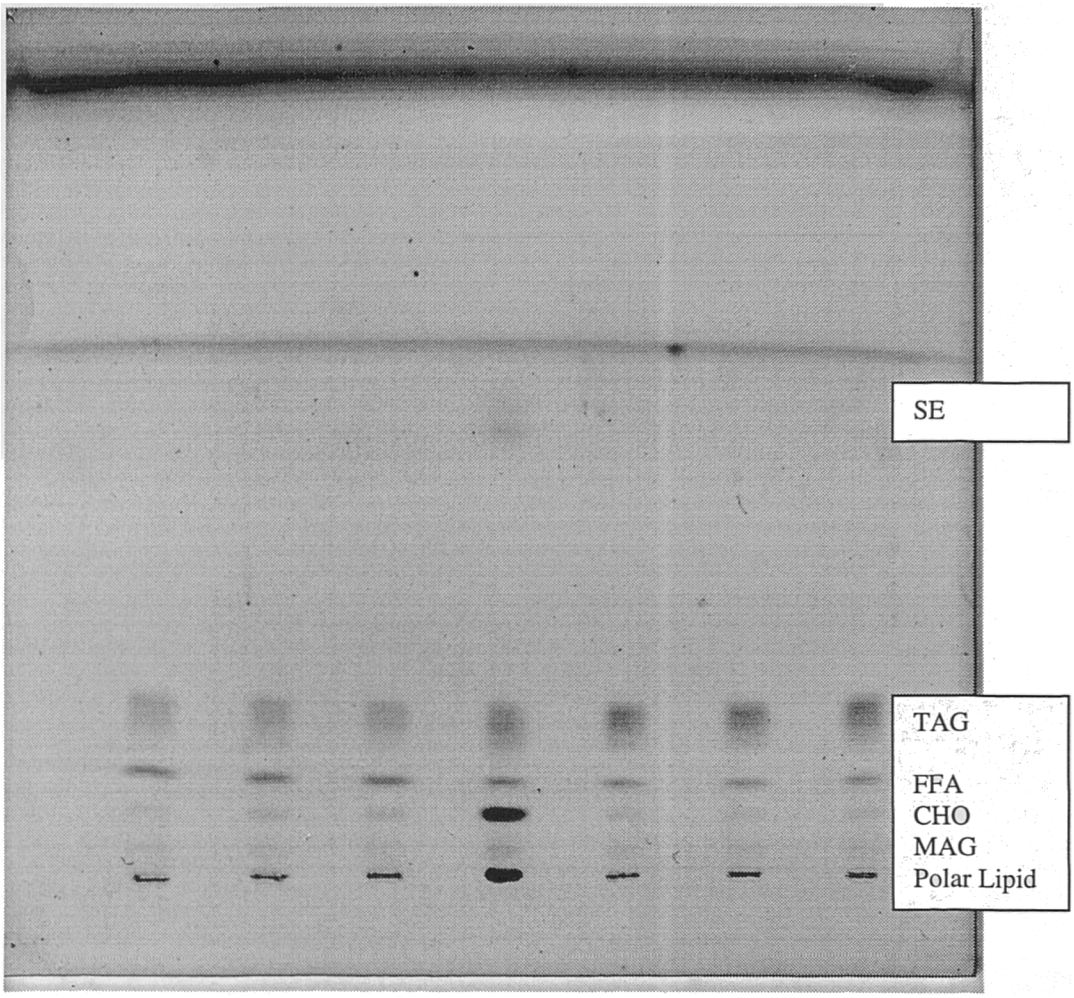


Figure 5.7. HPTLC plate for *L.salmonis* naulius II larvae, lane 1-3 and 5-7. Fish brain standard ran on centre lane.



Key:
SE = sterol ester; MAG = monoacylgylcerol; CHO = cholesterol; FFA = Free Fatty Acid;
TAG = triacylgylcerol;

the 2nd antenna to the oral cone, (Plate 5.1-5.2), a distance of approximately 102μm. An attached copepodid (Plate 5.3), with the 2nd antenna penetrated into the host shows the same measured distance as 129μm. Closer examination of the oral cone (Plate 5.4) shows that the mandibles have 10 denticles.

Evidence of feeding in an aged copepodid (2 days post-infection) on the dorsal fin is seen in Plates 5.5-5.6. The overall dimensions of the grazed area (Plate 5.5) are 167 x 88μm, with the distance from the puncture wound to the anterior and posterior edge of the feeding site being 92 and 254μm respectively. Plate 5.6 shows in greater detail the puncture wound site dimensions from the site of the 2nd antenna puncture wounds to the edge of the site of feeding, 150μm. The measured distance, 2nd antenna to the oral cone in the detached copepodid was found to be 140μm.

The feeding apparatus in the adult female clearly displays all mouth parts, the mandible and the strigil can be seen (Plate 5.7). However pre-settlement copepodids (Plates 5.9 and 5.10) of varying ages, up to 7 days did not reveal the presence of the strigil. Further, examination of detached copepodids (Plate 5.8) also did not reveal the presence of a strigil even though feeding sites had been observed.

Pre-settlement copepodids, when fixed display their oral cones in a vertical position. Measuring the dimensions from the centre of the 2^{nd} antennae to the centre of the oral cone gives a number of distances, the longest of which was 140μm, Plate 5.6.

Plate 5.1. 1 day-old *L.salmonis* copepodid showing 2nd antennae and oral cone. The distance from the centre of the 2nd antennae to centre of the oral cone is 102.5µm. The aperture of the oral cone 87.5 x 45.8µm.

SEM mag. 320x, scale bar = 10µm

Key: A1= first antenna, A2= second antenna, FD= filament duct, MX= second maxilla, MP= maxilliped, OC= oral cone, R= rostrum.

Plate 5.2. *L.salmonis* copepodid attached to the pectoral fin with the oral cone extended. The distance from the point of penetration of the 2nd antennae to the centre of the oral cone is 129.2µm.

SEM mag. 160x, scale bar = 100µm

Key: A2= second antenna, OC= oral cone.

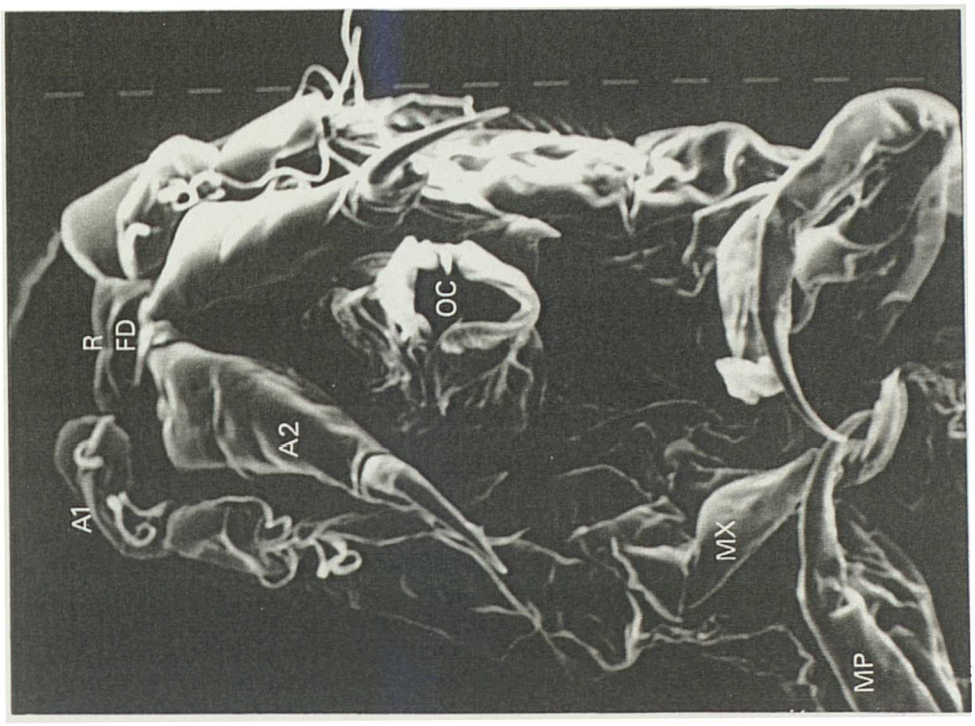


Plate 5.1. 1 day old *L.salmonis* copepodid showing 2nd antenna and oral cone.

Plate 5.2. *L.salmonis* copepodid attached to the pectoral fin with the oral cone extended.

Plate 5.3. *L.salmonis* 1 day-old copepodid oral cone. The 10 denticles on the mandible can clearly be seen.

SEM mag. 1250x, scale bar = 10μm

Key: LB= labium, LR= labrum, M= mandible.

Plate 5.4. 2 day-old *L.salmonis* copepodid feeding site on host dorsal fin showing puncture wounds from 2nd antennae. The disruption to the host epidermis can be seen and the basal membrane over which the epidermis has been grazed. The distance from the puncture wounds to the centre of the feeding site is 166.7μm. The dimensions of the feeding site are 166.7 x 87.5μm.

SEM mag. 160x, scale bar = 100μm

Key: FS= feeding site, PW= puncture wounds.

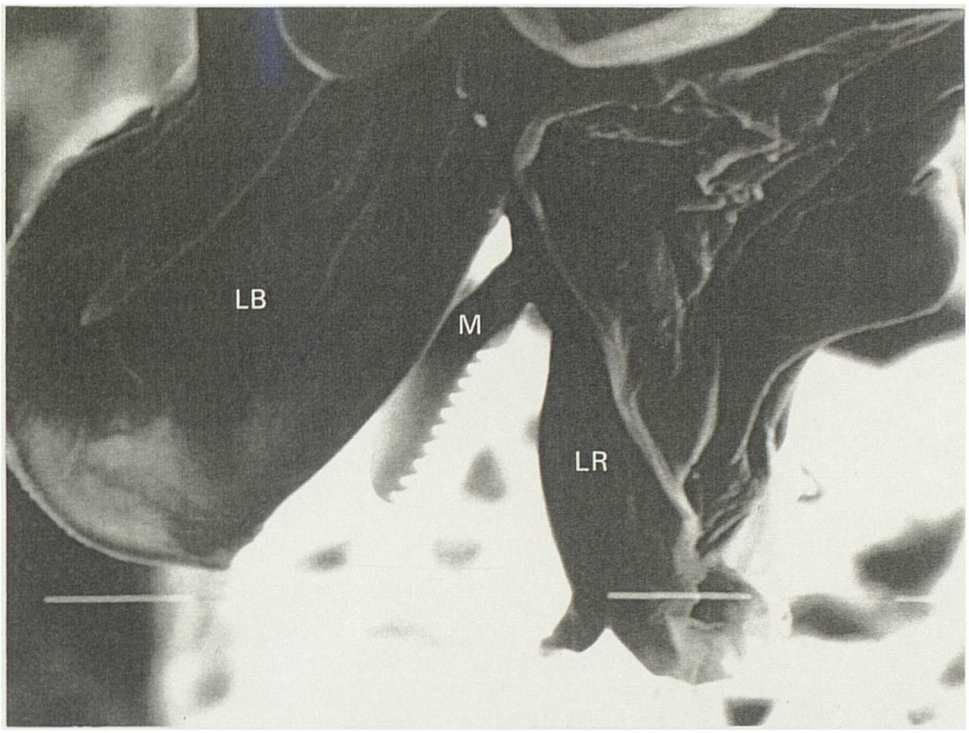


Plate 5.3 *L.salmonis* 1 day old copepodid oral cone.

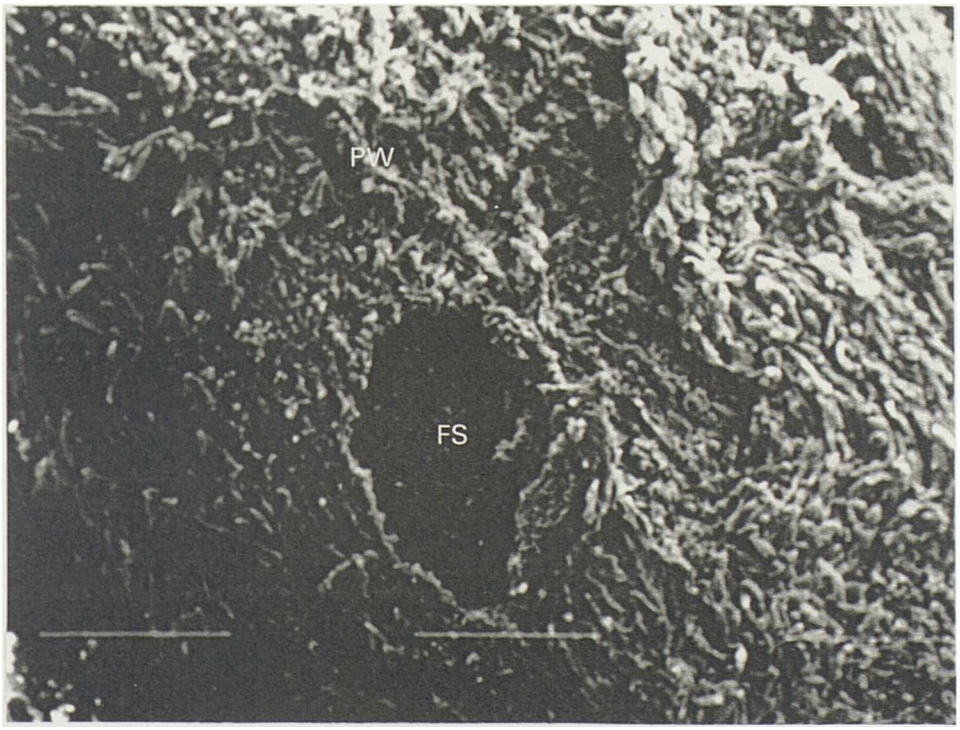


Plate 5.4. 2 day old *L.salmonis* copepodid feeding site on host dorsal fin

Plate 5.5. 2 day-old *L.salmonis* copepodid feeding site on host dorsal fin showing puncture wounds from 2nd antennae. The distance from the puncture wounds to the edge of the feeding site is 150μm

SEM mag. 320x, scale bar = 10μm

Key: FS= feeding site, PW= puncture wounds.

Plate 5.6. *L.salmonis* 2 day-old copepodid from feed site. The 2nd antennae snapped in removal. The distance from the centre of the 2nd antennae to the centre of the oral cone is 140μm.

SEM mag. 320x, scale bar = 10μm

Key: A1= first antenna, A2= second antenna, OC= oral cone.

Plate 5.5. 2 day old *L.salmonis* copepodid feeding site on host dorsal fin showing puncture wounds from 2nd antennae

Plate 5.6. *L.salmonis* 2 day old copepodid from feeding site.

Plate 5.7. Adult female *L.salmonis* oral cone showing mandibles and strigil.

SEM mag. 320x, scale bar = 10μm

Key: LB= labium, LR= labrum, M= mandible, S= strigil.

Plate 5.8. Oral cone of a detached *L.salmonis* copepodid post settlement. This copepodid had been actively feeding on the host but note the absence of the strigil.

SEM mag. 5000x, scale bar = 1μm

Key: LB= labium, M= mandibles.

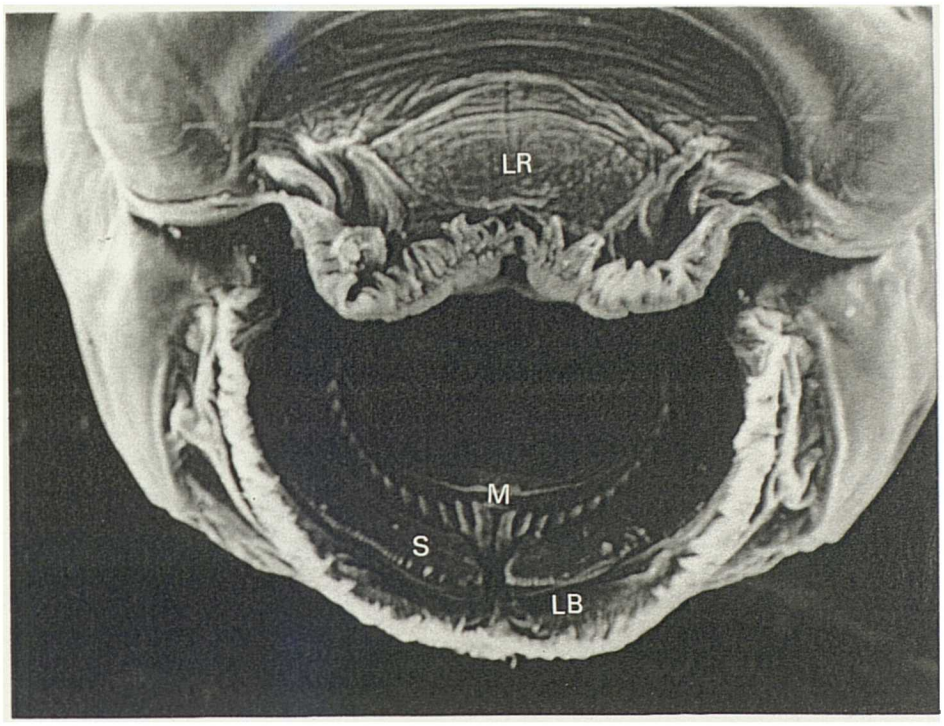


Plate 5.7. Adult female *L.salmonis* oral cone showing mandibles and strigil.

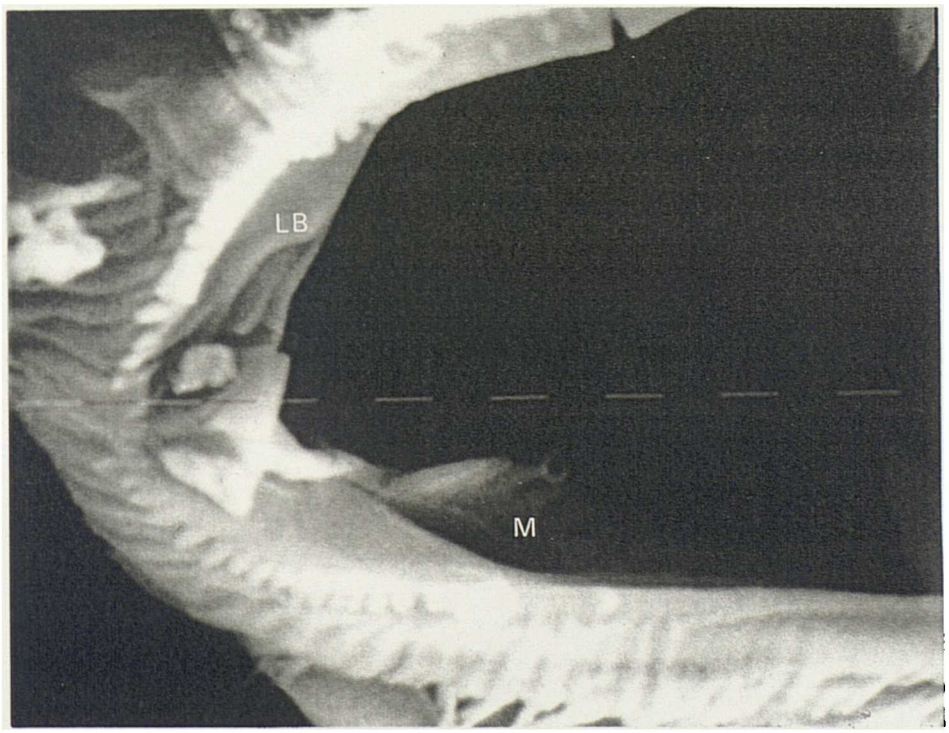


Plate 5.8. Oral cone of a detached *L.salmonis* copepodid post settlement.

Plate 5.9 Oral cone of pre settlement *L.salmonis* copepodid, aged 1 day. No observable strigil is seen.

SEM mag. 5000x, scale bar = 1μm

Key: LB= labium, M= mandibles.

Plate 5.10. Oral cone of pre settlement *L.salmonis* copepodid, aged 7 day. No observable strigil is seen.

SEM mag. 5000x, scale bar = 1μm

Key: LB= labium, M= mandibles.

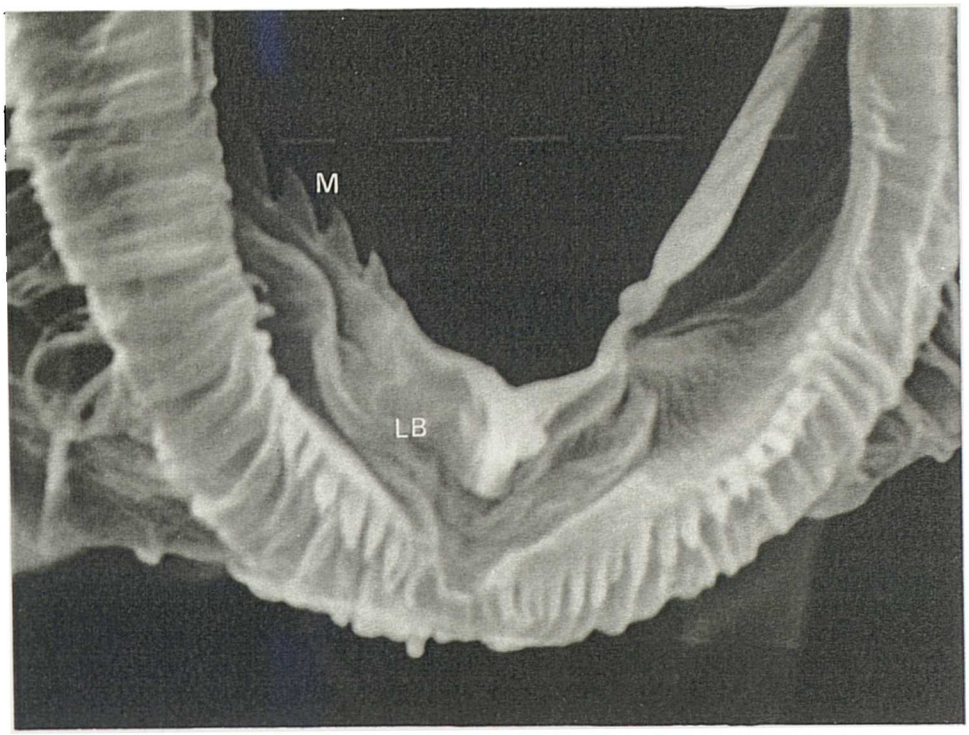


Plate 5.9. Oral cone of pre settlement *L.salmonis* copepodid, aged 1 day.

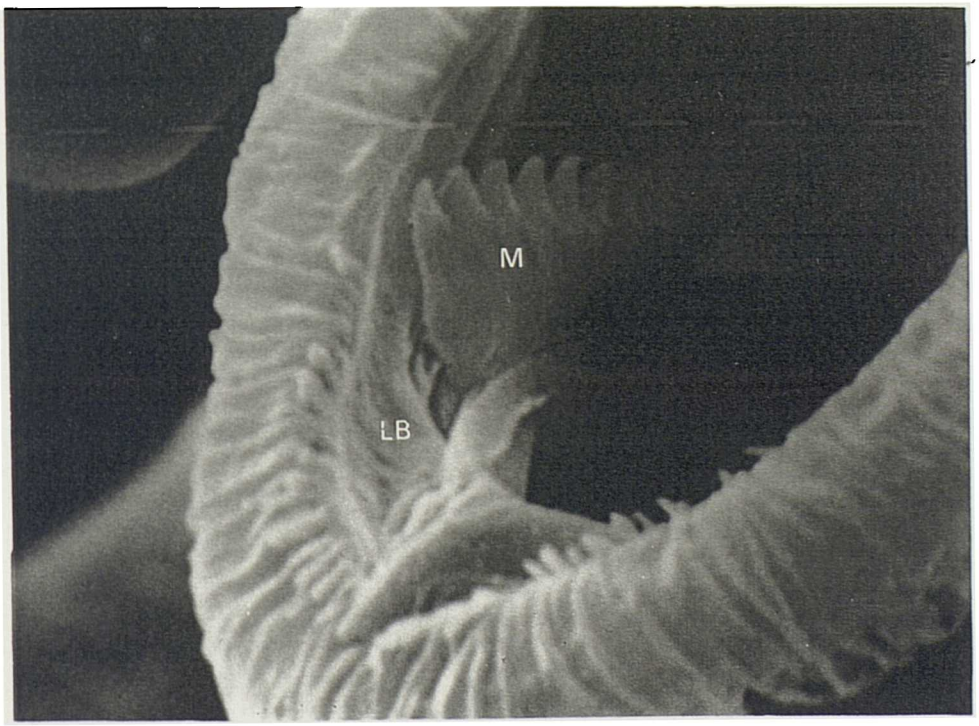
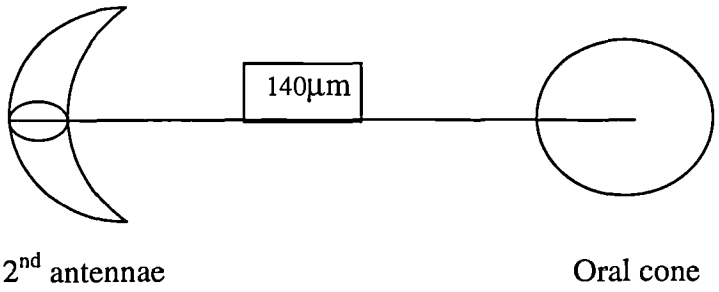


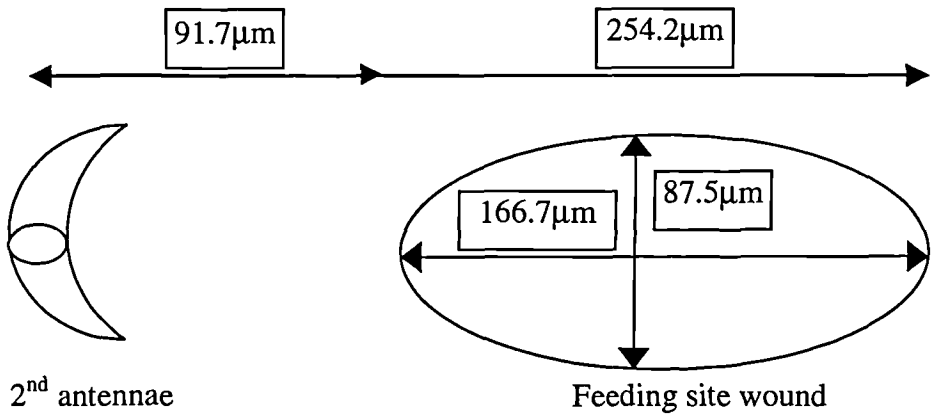
Plate 5.10. Oral cone of pre settlement *L.salmonis* copepodid, aged 7 day.

Figure 5.8 Schematic representation of the 2nd antennae and oral cone dimensions of a *L.salmonis* copepodid. From plate 5.1.



The dimensions of the puncture wounds caused by the 2nd antennae and the feeding site (Plate 5.4) were measured from the centre of the puncture wounds to the front edge of the feeding site and to the posterior edge, distances of 91.7µm and 254.2µm respectively. The dimensions of the feed site were taken at its widest margins being 166.7µm and 87.5µm respectively.

Figure 5.9 Schematic representation of the feeding site dimensions of an attached *L.salmonis* copepodid. From plates 5.8-5.10.



5.5 DISCUSSION.

All *L.salmonis* pre-settlement larvae are lecithotrophic and therefore reliant on internal energy reserves laid down during oogenesis. The energy levels of the pre-settlement *L.salmonis* larval stages show a gradual decrease with the age of the larval stage. The calculated energy levels for *L.salmonis* larvae are derived from the percentage organic carbon present. As the free-swimming stages of *L.salmonis* are lecithotrophic there should be a continuous decrease in energy levels over time. In this study, after each moult an apparent increase in percentage carbon is found and therefore there has been an increase in calculated energy levels. If this is a closed system there cannot actually be an increase in energy levels. It is probable that post-moult organic carbon has increased in proportion to the non-carbon products. The proportion of carbon left after a moult is higher due to a change in the ratio of carbon and non-carbon products within the larva. The amount of carbon per unit dry mass may have changed due to the losses of non-carbon products i.e. with the exuvium. Because of the changes in the percentage carbon present between different larval stages no comparison of energy levels should be made between different larval stages. Energy levels should be compared within larval stages e.g. between varying ages.

Comparing the energy levels within the same developmental stage shows a decrease with time. The pre-settlement energy levels of the aged nauplii 2 stage and the aged copepodids both show a decrease with time. The nauplius 2 stage shows a sharp decline 24 hours after the moult from nauplius 1 after which the energy decline slows between the 2 and 3 day nauplius 2 stages.

Examination of the pre-settlement copepodid stage indicates a rapid decline in energy availability over time, from 8525 cal g^{-1} dry wt to 6959 cal g^{-1} dry wt up to day 7. There is

a statistical difference in the energy levels between 1 day and 2 day-old copepodids after which there is a gradual decline. There is a further statistically significant decline in energy levels between 5 day and 7 day copepodids. In the infection experiments which used copepodids of different ages (see Chapter 4) there were a statistically significantly fewer 7 day-old copepodids than with 1 day and 3 day-old copepodids settling on the host. The maximum survival of *Lepeophtheirus pectoralis* copepodids is given by Boxshall (1976) as 6 days, although they can survive for only three days if free-swimming. By contrast, the longevity of *L.salmonis* copepodid is reported by Gravil (1996) as 13 days at 10°C.

Lucas *et al.* (1979) have demonstrated that *Balanus balanoides* cypris larvae can alter their oxygen consumption rate, reducing it from 1.17 ml O_2 h^{-1} g^{-1} in the swimming phase to 0.66 ml O_2 h^{-1} g^{-1} during the exploring phase. This unusually low metabolic rate would allow extensive exploration of a suitable substratum for settlement. Once settlement has taken place the oxygen consumption rate increases during the period of metamorphosis.

Lucas *et al.* (1979) in their study showed that cyprid age (time in water column) has an effect on metamorphosis success. Cyprids show 100% successful metamorphosis if the maximum time in the plankton is between 2.5 and 4 weeks, success falling sharply after this period. This coincides with the depletion of lipid energy reserves. It has not been possible to determine the depletion of lipid energy reserves for *L.salmonis* copepodids as culturing sufficient numbers of aged copepodids was not achievable. Gravil (1996) has shown the longevity of free-swimming copepodids to be 13 days at 10°C although this study has show a reduced ability to infect its host after 7 days.

Energy storage strategies may be intimately related to transmission strategies of some nematode parasites (Medica & Sukhudo, 1997). The infective L3 larvae of trichostrongyle and strongyle nematodes are non-feeding (Rogers & Sommerville, 1963) and therefore

these energy reserves must be used for host location and infection (Medica & Sukhudo, 1997). Depleted energy reserves lead to a loss of infectivity. Exhausted *Strongylus vulgaris* larvae had utilised 50% of their lipid reserves suggesting that the primary energy source is lipid (Medica & Sukhudo, 1997).

According to Kabata (1981) the most important aspect of locomotory activities of parasitic copepods is swimming during the dispersal phase, since this is directly responsible for the location of the host and survival of the parasite. The copepodids in this study were maintained in small aquaria with a constant trickle air supply, requiring them to constantly maintain their position in the water column with constant expenditure of energy. Larval stages of *L.salmonis*, in the laboratory, follow a pattern of "sink and swim" in the water column (Wootten *et al.*, 1982; Bron *et al.*, 1993a; Gravil, 1996). Such behaviour, according to Wootten *et al.* (1982), might have value in locating a swimming host that is likely to be in the upper layers of the water column. This behaviour would maintain all *L.salmonis* larval stages in the upper layers of the water column and conserve energy by covering a larger area whilst maximising energy control. Temperature will also have a marked effect on the development and metabolism of the infective copepodid (see chapter 3) and should not be ignored.

Copepodids are positively phototactic (Bron *et al.*, 1993), bringing the copepodid into the photic surface waters. Heuch *et al.* (1995) suggest a distinct diel vertical pattern in their behaviour based on field observations. Copepodids gathered near the surface waters during the day before dispersing to deeper waters at night. This migration pattern would allow increased parasite-host encounters as salmonids swim through populations of sinking (night time) and rising (dawn) copepodids every 24 hours according to Heuch *et al.* (1995), who considered that copepodid distribution is controlled by light intensity and not by either

salinity or temperature.

Copepodids are also rheotactic, stimulated by mechanical vibration and therefore respond to potential host movement by a combination of burst swimming and "looping" behaviour (Bron *et al.*, 1993) which helps to bring it into contact with a potential host. Measured swimming speeds of copepodids by Gravil (1996) measured an average swimming speed of 2.14 cm s^{-1} for copepodids, although they were capable of stimulated speeds of up to 6.84 cm s^{-1}. On successful grappling of the putative host the identification of the host is probably achieved by high threshold chemoreceptors on the antennules (Bron *et al.*, 1993).

The calculated energy levels of the pre-settlement stages of *L.salmonis* are comparable with those of larval stages of free-swimming copepods. However, due to the difficulty of obtaining monospecific samples of sufficient size few examples are available in the literature (Schindler *et al.*, 1971). Most energy values obtained from the literature are determined as cal g^{-1} dry wt. The energy values for *L.salmonis* copepodids are in the same range as the copepodid of *Calanus* sp. Energy values obtained from the literature and compared with *L.salmonis* energy values are given in Table 5.7.

Table 5.6. Comparative energy availability to copepod species (as cal g^{-1} dry wt). Average values used.

Species	NI's	NII's	Copepodid	Reference
L.salmonis	7381	7534	7826	This study
D.minutus	5487		5536	A
C.bicuspidatus	6832		7158	B
C.bicuspidatus			6549	B
Calanus spp.			7416	C
C.finmarchicus.			7749	D

Refs: A= Schindler *et al.* (1971), B= Schindler *et al.* (1971), C= Comita and Schindler (1963), D= Comita *et al.* (1966),

It would be interesting to determine for certain if *L.salmonis* larval stages have the potential to "feed" prior to settlement, to supplement their yolk sac energy reserves. Tester & Turner (1991) demonstrated that nauplius 1 stages of the copepod *Acartia tonsa* (Dana) have a functional mouth and gut and can drink and thus can osmoregulate. These *A.tonsa* N1 stages were exposed to flourescein isothiocynate dextran and this was found to be contained in the gut. A similar study for *L.salmonis* larval stages to determine whether there is any external energy input would indicate whether these stages are purely lecithotrophic.

The energy levels of *L.salmonis* larvae in this study show a progressive decrease with time within developmental stages. Seasonality will have a marked effect on derived energy values for *L.salmonis* as seasonal morphometric differences have been established for larvae (Gravil, 1996) which in turn will have marked effects on calculated energy values. The ovisacs and the developed larval stages for these experiments were obtained in winter when a larger size of *L.salmonis* larva is found (Gravil, 1996).

The study of post settlement energy levels of *L.salmonis* showed a small drop in energy availability after day one post-infection, after which energy levels stabilise. Statistically there were no significant differences between post settlement energy values. However, the small sample sizes mean that these findings should be treated with caution. In this study there was no indication of additional energy being stored. No large increase in energy values of post-settlement copepodids was observed, energy levels of settled copepodids appearing to be maintained at constant levels. Johnson & Albright (1991a) suggest that the long duration of the copepodid on the host may be necessary to allow the recovery of lost energy prior to the moult to the chalimus 1 stage. In this study no increase in energy availability was observed. The pre-moult energetics to the chalimus stage could not be

calculated as no infected fish were available. Johnson & Albright (1991a) suggest an alternative to the energy replenishment hypothesis in that additional time on the host by the copepodid may be required for the preparation and completion of the development of the frontal filament prior to moult. This study has shown that the attached copepodid is actively feeding on the host and therefore this second hypothesis may explain the post-settlement copepodid energetics. Energy obtained by feeding is used in the development of the filament and therefore no energy storage occurs, at least within the time frame of this study. A more extensive study of post-settlement energetics would clarify this point.

The dominant class of lipids found in marine planktonic crustaceans is wax ester and deposition of this energy store takes place when food is freely available (Lee *et al.* 1972a; Ackman *et al.*, 1974). Lipid deposition and storage in the copepod *Calanus hyberoreas* from the Arctic Ocean correlates well with phytoplankton blooms so that wax ester deposition, with summer phytoplankton like fatty acid composition, follows active feeding (Lee, 1974).

Lee (1975) states that the major lipid class found in *L.salmonis* on coho and pink salmon is triacylglycerol and suggested that this is host derived. In this study of *L.salmonis* on Atlantic salmon TAG was also found to be the major lipid class (37.55%) present. G. Bell (pers. comm. 1998) states that the principal lipid class of rainbow trout is TAG, which makes up 30-50% of the total skin lipid by weight and it seems reasonable to suggest that Atlantic salmon would have similar quantities of TAG in its skin. TAG comprises greater than 73% of all Atlantic salmon muscle groups, dark and white being associated with the myosepta (Zhou, Ackman & Morrison, 1995 and 1996). Therefore, it is very likely that lipid classes in *L.salmonis* on Atlantic salmon are host derived. The energy availability per molecule is greater with TAG and although host derived it will have important

implications for oogenesis and energy availability to the early developmental stages.

In this study whole animals were used for the extraction of lipids and the determination of lipid classes present in *L.salmonis*. Benson *et al.* (1972) withdrew oil directly by microsyringe from the lipid deposit of *Calanus helgolandicus* and found it to consist solely of wax ester. Triglycerides must be stored and located in separate sites of the copepod *C.helgolandicus*. No wax ester was found in *L.salmonis* nauplii. However if a similar microsyringe procedure was carried out on the discrete lipid vesicles observed then the lipid classes of *L.salmonis* could be determined. Determination of the lipid classes present in adult females and ovisacs would establish the source of lipid available prior to hatching. Clarke (1980) calculated that 60% of lipid in the triacylglycerol rich *Euphausia superba* accumulated in spring and summer is ultimately transferred to the eggs. TAG high energy density lipid class makes it a more important from of lipid class for energy storage and availability for developing embryos after fertilisation. It would therefore be interesting to examine the lipid classes present in adult *L.salmonis* ovigerous females with and without ovisacs.

Kabata (1974), Boxshall (1974a, 1974b) and Andrade-Salas (1997) have given descriptions of the caligid mouth parts and structures. In this study feeding in the post-settlement copepodid has been studied to ascertain the timing of energy input into larval energetics.

Both Kabata (1974) and Andrade-Salas (1997) described the action of the feeding of adult *L.salmonis*, and the particular function of the strigil. Kabata (1974) hypothesised that the strigil is revealed and brought into contact with the host skin surface by the compression of the oral cone on the skin, which would push away the labial fold. In the description by Andrade-Salas' (1997) it is the strigil that cuts a swathe of epithelium from the host which is then guillotined for removal by the mandibles and passed into the oral cone. It should be

noted that salmon skin starts to repair, with the migration of epithelial cells to cover any wound, within 1 hour of injury (Bullock & Roberts, 1992). Fresh wounds on the host caused by sea lice copepodids are difficult to observe.

The findings of this study agree with Bron (1993) and Johnson & Albright (1991b) in that the strigil cannot be observed in the oral cone of copepodids of *L.salmonis* under SEM. The strigil could not be observed in any of the samples of 1-7 day-old pre-infection copepodids. Copepodids that had been attached to a host but removed were also sampled and although these parasites were associated with feeding sites the strigil could not be observed (Plates 5.7 and 5.11). Examination of the *L.salmonis* copepodid mandibles reveals 10 denticles present, in agreement with Boxshall (1974b).

In fixed adults, Plate 5.1 the strigil can be clearly seen but whether it remains hidden in the copepodid or exists as some form of protostrigil is still to be established. It would seem unlikely that the strigil develops during the moult from copepodid to chalimus, but it can be found after the moult. Bron (1993) suggests that the strigil found in the single specimen that he observed may either have been due to the fact that the copepodid was close to moulting to the chalimus 1 stage or that it was a pre-formed strigil that is non functional in the copepodid. Kabata (1974) also states that the mouth tube (oral cone) must be perpendicular to the body when feeding to bring it into contact with the host. In Plate 5.7 the attached copepodid has the oral cone extended but whether this is the extended position for feeding at fixing or an artefact of preparation for SEM is unclear.

If attached copepodids removed sites of feeding grazed areas could be seen (Plate 5.8). Evidence of feeding by copepodids, two days post infection is seen in plates 5.8-5.10. This SEM showed that copepodids actively feed but the size of the skin area available to the oral cone is restricted by the position of the 2nd antennae. The oral cone can only excavate

in an anterio-posterial direction with little lateral movement due to the fixed position of the 2nd antennae. The 2nd antennae do not necessarily fully penetrate the skin (plate 5.5) and further reach of the oral cone may be obtained by pivoting through the base of the 2nd antennae. The epidermis is apparently grazed by mechanical damage down to the basal membrane, this is in agreement with Jones *et al.* (1990). The mechanism of feeding must be similar to that of the adult with probably some modification to allow for the absence of the strigil. It must also be mechanical as the size of the oral cone and the type and construction of mouthparts would not accommodate any other feeding mechanism. Blood feeders, i.e. praniza larva of the isopod *Gnathia maxillaris* have small, complex and highly modified (Davis, 1981) mouthparts that are strong serrate or pointed styles for anchorage and piercing host flesh. Such modifications are not seen in the copepodid of *L.salmonis* and the damage to the host is consistent with that described for later life-stages.

Feeding of both the copepodid and chalimus 1 stages is restricted to a limited area around the oral cone since both are firmly attached to the host and do not move (Jones, 1989, Bron, 1993). Once the copepodid has moulted into the chalimus stage where attachment is by the single frontal filament more movement is possible, although not through 360° (Jones *et al.*, 1990). Damage to the host epidermis, by the feeding copepodid will be restricted due to the small size of the oral cone as well as the restricted movement of the parasite.

Bron *et al.* (1993c) found the alimentary canal of copepodids to be fully contiguous in both free-swimming and settled stages. Once settled the copepodid can actively feed and no longer be reliant on its stored lipids. Bron (1993) found intact host epithelial cells, mucous cells and blood corpuscles in the oral cavity and more rarely in the mid-gut. The copepodid, once settled is thus not solely dependent on its on lipid reserves and it is no longer lecithotrophic.

Boxshall (1976) suggests that the time interval between *L.pectoralis* copepodid settlement and moult to the chalimus 1 stage is 2-3 days, although no temperature is specified. The duration of the *L.salmonis* copepodid stage is reported to be long, 6.7 days (at 10°C) for 50% of copepodids to develop into the chalimus 1 stage (Johnson & Albright (1991a) and is very temperature dependent. The time for development agrees well with this study (Chapter 6) where 50% development time to the chalimus 1 stage was 6 days (at 10°C). In this study (Chapter 5), at 10°C only copepodids were found to be present after 5 days. This relatively long phase is an opportunity for the copepodid to recover energy lost during the nauplii stages or the requirement of additional time or energy for completion of development prior to filament production and moulting to the chalimus 1 stage (Johnson & Albright (1991a). The trend of a short period for lecithotrophic pre-feeding naupliar stages (possibly due to the finite supply of energy reserves) followed by a relatively long period for the first feeding stage is seen in free-living copepods (Landry, 1983).

Prior to settlement the free-swimming copepodid has sufficient energy reserves for 7 days, after which its ability to infect its host is greatly reduced. C:H:N analysis and calculated energy levels indicate that the energy availability to pre-settlement free-swimming stages depreciates with age and this results in a loss of infectiousness at day 7. The principal lipid class present as the energy reserve for free-swimming stages of *L.salmonis* is triacylglycerol. TAG deposition from adult females feeding on salmonids, host derived lipids, are passed on during oogenesis.

Once attached to the host the copepodid can actively feed although some modification to the feeding mechanism may be necessary as the strigil is not observed in the copepodid, pre- and post-settlement. Feed site dimensions match those of the dimensions of the 2nd antennae and oral cone. Post-settlement analysis of calculated energy levels shows little

change over the period of the attached copepodid and therefore the additional time (approximately 6 days at 10°C) is required for completion of development prior to moult to the chalimus 1 stage.

CHAPTER 6. EPIDEMIOLOGY AND MATHEMATICAL MODELLING.

6.1 INTRODUCTION.

Epidemiology may be defined as the study of the patterns of disease that exist under field conditions. More specifically, epidemiology is the study of the frequency, distribution, and determinants of health and disease in populations (Martin, Meek & Willeberg, 1987). Focusing on the population biology allows a holistic approach and understanding of a disease problem through "population dynamics", be they host population dynamics, parasite population dynamics or host-parasite population dynamics (Scott & Smith, 1994). Infectious disease in population biology can be divided into two broad categories, those caused by microparasites and macroparasites (Anderson & May, 1995). This study concerns only macroparasites which, suggest Anderson & May (1995), are typically of a persistent nature, with hosts being continually infected.

Simple mathematical models of the transmission of infectious agents within biological communities can help to interpret observed epidemiological trends, to guide the collection of data and help design programmes for the control of infection and disease (Anderson & May, 1995). Sensibly used mathematical models are no more and no less than tools for understanding epidemiological processes, for thinking about things in a precise way (Anderson & May, 1995). The traditional approach to the emergence of a new disease entity is through seeking interventions which will prevent or cure disease at the individual animal level. This traditional paradigm requires the development of the understanding of the disease processes at the individual animal, organ, cellular and genetic level. Such a

reductionistic approach largely ignores the complex interactions that occur between individuals when aggregated in populations that must exist in an environment not always suitable for health and optimal production in intensive culturing of animals (Thrushfield, 1995). Primary health care, by its nature deals with population aspects of transmission and persistence of infections (Anderson & May, 1995).

Epidemiological studies have been successfully used in both human and veterinary medicine. A recent high profile example of the use of epidemiological modelling was the analysis of bovine spongiform encephalopathy (BSE) in British cattle (Anderson, Donnelly, Ferguson, Woolhouse, Watt, Udy, MaWinney, Dunstan, Southwood, Wilesmith, Ryan, Hoinvilles, Hillerton, Austen & Wells, 1996). These authors assessed the past and future trends of the epidemic and suggested an effective culling policy to the British government. Anderson *et al.* (1996) predicted the natural elimination of the disease by the year 2001 and also suggested, from model predictions, the best culling policy to remove infected animals from the food chain.

Mathematical modelling is susceptible to pitfalls in the predictions that the model forecasts. Models work on certain assumptions and therefore are only as good as the information that is available to build them. Epidemiological models try to infer predictions of complex biological systems and are therefore susceptible to inconsistencies. A second example of the effectiveness of veterinary epidemiological modelling is the study of French & Morgan (1996) on ovine cutaneous myosis (blowfly strike) using predicted abundance of *Lucilia sericata* and a pattern of sheep susceptibility. French & Morgan (1996) have shown in the application of their model that early season treatment, with a single application of insecticide against blowfly strike will be effective at controlling ovine cutaneous myosis. By comparison with field data the model predictions, similar in pattern,

show some discrepancies. These discrepancies could be related to the assumptions made within the model (French & Morgan, 1996). The process of model building and the need to make assumptions highlights important gaps in our knowledge and the need for areas of further specific investigation. Performance of the model will be improved by the incorporation of more precise parameters into the model.

Salmon culture in Scotland is restricted in the chemotherapeutants that are licensed for use against sea lice. Presently only two chemical treatments, the organophosphate Dichlorvos and hydrogen peroxide, are available to treat epizootic outbreaks of lice, both of which are only effective against the motile stages of the lice population. With sea lice costing the salmon industry an approximate £12-15 million each year, not only is there a need for an increased range of chemotherapeutants but an increased knowledge of the lice population biology to allow better predictability of the timing of the maximum number of treatment susceptible lice stages present on the host. Bron *et al.* (1993a; 1993b) and Bron (1993) have demonstrated the variability found in the numbers of sea lice present on salmon hosts, not only in differences between adjacent salmon farm sites, but even in adjacent salmon cages. Marine Harvest McConnell, the largest salmon producer in Scotland will introduce a new sea lice control strategy in 1998 with the early treatment of salmon in early spring, preferably March (Anon., 1998a). There is still much uncertainty as to when to treat fish and the future effects on the sea lice population of this proposed treatment regime. With the limited number of treatment regimes presently available and the costs involved there is an obvious need to plan and forecast the timing of any treatment required as accurately as possible.

A mathematical model would serve this purpose, counting the initial stages of lice present on the host and forecasting the time of the maximum number of treatment susceptible lice.

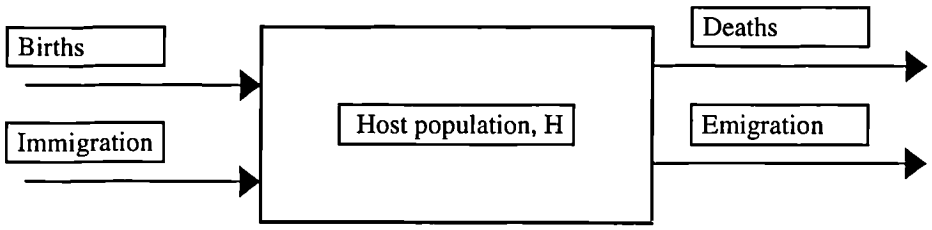
A mathematical model of the sea lice population dynamics would give predictability of lice numbers and could account through the knowledge of the efficacy of administered treatments future lice population dynamics.

There are a number of epidemiological modelling formats that can be adopted for models but for this study differential equation modelling has been used. This model format allows the translation of simple flow charts into corresponding models (Scott & Smith, 1994). The purpose of modelling is to understand how the system works and the interaction between a number of different processes; the method of modelling enumerates these interactions and represents them as a mathematical model. Differential equations provide a method of defining and combining these processes; the biological processes can be defined as rates and expressed simply within this model format.

6.1.1 DIFFERENTIAL EQUATION MODELLING APPROACH.

Differential equation approach for sea lice population dynamics allows the use of simple compartmental modelling. Each compartment, within a population will be influenced by four basic biological processes, determining how individuals in the population change with time: birth, death, immigration and emigration, and these can be represented diagrammatically by use of a flow chart (Scott & Smith, 1994).

Figure 6.1 Flow chart representing population dynamics of an animal population. H, number of animals in the population. From Scott & Smith (1994).



Each biological process has an influence on the parasite numbers found in each compartment on the host and each of these compartments will be influenced by time, its rate of change. Anderson & May (1995) suggest that there is a natural division in the epidemiological modelling of micro and macroparasites. A microparasite may be thought of as one that has direct reproduction - usually at very high rates - within a host (Anderson and May, 1979). They tend to be characterised by small size and a short generation time. Hosts that recover from infection usually acquire immunity against reinfection for some time, and often for life. The duration of infection is typically short compared to the expected life span of the host. For such infective agents, such as bacteria, viruses or fungi, the host population can be divided into relatively few classes, susceptible, infected and recovered/immune, the S.I.R. model. Microparasite infections are described by compartmental prevalence models, models which indicate the numbers or proportion of individuals in a set defined infection category (Anderson & May, 1995). The unit of study in this case is the host as it is difficult to measure burden of infection due to the direct mode of transmission.

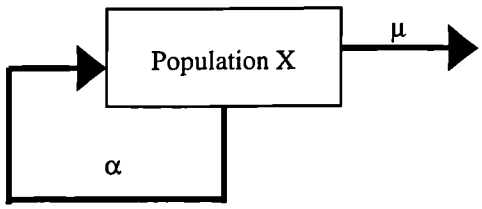
A macroparasite may be thought of as having no direct reproduction within the definitive host (Anderson & May, 1979). This category encompasses most parasitic helminths and arthropods. Macroparasites are typically large and have much longer generation times than microparasites and are of a persistent nature with the host being continually reinfected. The various factors characterising the interaction with the host can all depend on the number of parasites in a given host. The host population will have varying infection levels and therefore describing the host as infected is insufficient. The simple compartmentalised model of a microparasite is mathematically insufficient for a macroparasite model and therefore this model type must be replaced by a more complicated system that accounts for the distribution of the parasites among the hosts, a distributional model. The unit of study

is the parasite, not the host, and the distribution of the parasite numbers in the host population is closely linked to the effects of density dependence factors (Anderson & May, 1995).

Although sea lice behave like a macroparasite the model approach to be taken will be the microparasite model. A microparasite compartmental model would follow the number of lice stages through the developmental life cycle. A macroparasite model would be unsuitable for the establishment of a sea lice model as the parasite distributional burden on the host is immaterial to this study. In cage and tank culture systems there is 100% prevalence and as the timing of the appearance of the maximum number of treatment susceptible lice is wanted a model of the parasite and not the host is required. The fish host is presently immaterial, parasite burden is not required and therefore the model represents the population dynamics of the parasite. For this study a model based on the compartmental microparasite model, with each compartment representing one parasite developmental stage will be applied.

Once a model flow diagram has been established (see figure below) the development of the mathematical equations to translate the diagram can begin. Each compartment will be described by one equation with its inputs (births and immigration) and its outputs (death and emigration). The compartmentalised life cycle can be separated into discrete time divisions with all life processes and development being a function of time or rate of change. The compartment populations can be graphically represented as an increasing or decreasing number and are changing continuously as time varies, either by a positive or negative slope of the graph i.e. death rate over time. This plotted slope represents the overall rate of change over time, the derivative of which is: $\text{slope} = \frac{dX}{dt}$

A simple flow diagram for microparasite transmission processes would be:

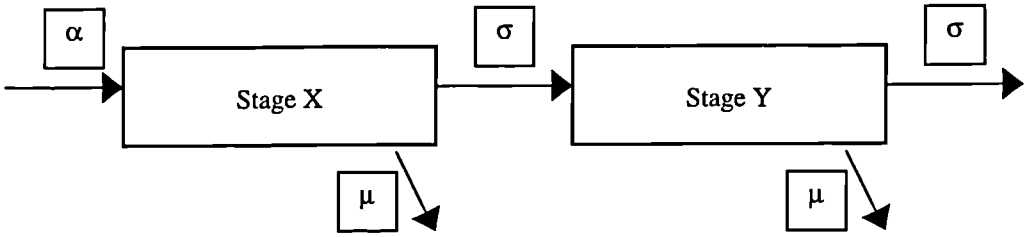


The rate of change in population density X would be represented by the differential equation of :

$\frac{dX}{dt} = \alpha X - \mu X$ Where α is the per capita birth rate and μ is the per capita death rate.

Population X is therefore increasing over time by the birth rate, αX, but decreasing by the death rate over time, μX.

Parasite numbers "flow" from compartment to compartment, influenced by the biological factors of birth, immigration, death and emigration.



Where α is the per capita birth rate, μ is the per capita death rate and σ is the recruitment rate into the next developmental stage.

Differential equations for each stage would be:

$\frac{dX}{dt} = \alpha X - \mu X - \sigma X$

$$\frac{dY}{dt} = \sigma X - \mu Y - \sigma Y$$

6.2.1 SEA LICE MODEL DEVELOPMENT.

For the sea lice population dynamics model the life stages have been compartmentalised to allow the determination of the parasite densities at each stage of the louse development cycle (Figure 6.2). Free-swimming stages, for the purpose of this model are unimportant.

Each life stage (compartment) is described by a differential equation of the same format as equation 6.1.

$$\frac{dL_i}{dt} = \sigma_{i-1}.L_{i-1} - (\mu_i + \sigma_i).L_i \qquad (6.1)$$

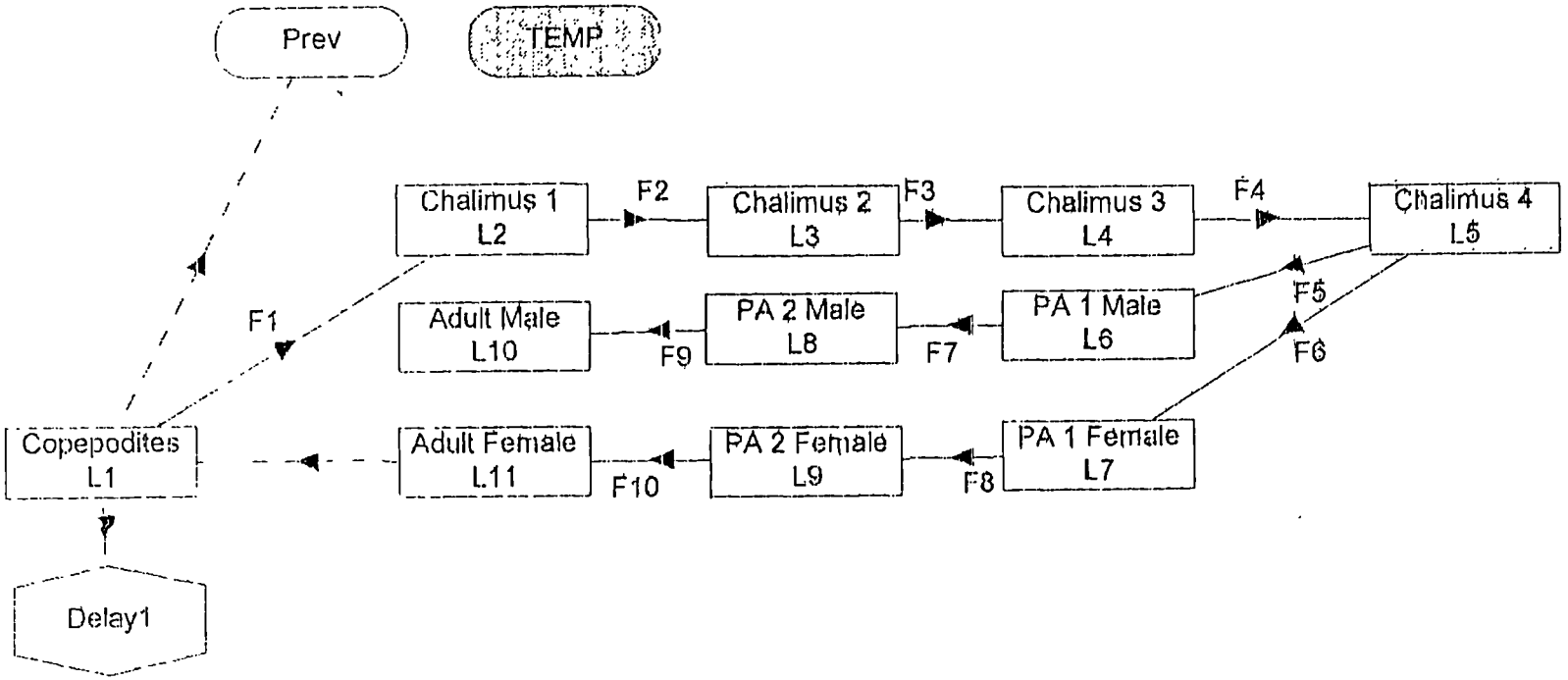
Where σ_i is the developmental rate from stage L_i to L_{i-1} and μ_i is the mortality rate of the stage L_i.

The parameters are calculated from the data as follows:

σ_i is calculated as $1/t$ and μ_i is calculated as $-ln\frac{(X_i/X_{i0})}{t_i}$ where X_i is the density of stage$_i$ at time t_i and X_{i0} is the density of stage$_i$ at time t = 0. X_{i0} was taken to be the density of parasites coming from the previous stage (i-1), X_i was taken to be the density leaving stage$_i$ and t_i was the time an average individual spends in stage$_i$.

The value of t used, for both σ_i and μ_i is assumed to be equal to the development of 50% of the lice stage.

Figure 6.2 Flow diagram for a tank culture sea lice model.



At the chalimus 4 stage there is sexual dimorphism and the assumed proportion for each sex is 0.5.

The full model is given in Appendix 1.

6.2.2 SEA LICE MODEL APPLICATION.

Once the flow chart has been translated into a system of mathematical equations the investigation of the model behaviour can be undertaken. It is assumed that the behaviour of the model represents the behaviour of the system to be studied. For this study four complete data sets were provided where three data sets will be used to estimate the model parameters and the final data set will test the model. The data set to be used as the test data was from tank 1:14 whilst the three remaining data sets were used for the calculation of the model parameters.

The data sets were provided by Anon (1994c, Unpublished data, Institute of Aquaculture internal report) where four tanks of ten fish had been experimentally infected with approximately 2000 or 5000 sea lice. Fish were infected and examined as described in the procedure in chapter 2 (Anon, 1994c, Unpublished data, Institute of Aquaculture internal report). Fish were anaesthetised for examination with Benzocaine every two days until all lice had died and the fish were purged of lice.

Table 6.1. Sea lice development and survival data. From Anon (1994c, Unpublished data, Institute of Aquaculture internal report) at 10°C. All timings are given in days. Mean values based on average of three data sets and 30 fish.

L.salmonis life stage	C.D.T. (days)	Development Time (days)	Duration (days)	Range (days)	Ave. Proportion	Ave. % Mort.
Copepodid			10[1]	0 - 10.7	0.069	88.7
Chalimus 1	8	7.8	7.3	5.6 - 14	0.446	55.4
Chalimus 2	14	5.4	12	9.3 - 24.7	0.691	68.8
Chalimus 3	22	9.1	10	16.3 - 26.7	0.796	74.6
Chalimus 4[2]	25.5	7.4	8	22.3 - 34.3	0.753	48.9
Pre adult 1 male	29.5	2.4	10	24.7 - 39.3	0.447	55.3
Pre adult 1 female	32.5	5	13.3	27 - 41.3	0.403	59.6
Pre adult 2 male	35	5.7	10	30.3 - 42.7	0.665	33.5
Pre adult 2 female	42	9.6	13.3	38 - 52	0.818	18.2
Adult male	38.3	12	27	41.5 - 87	0.832	16.8
Adult female	64	14.9	36	48.5 - 82	0.755	24.5

Key: C.D.T. = cumulative development time (time of peak number development of the population), Mort. Rate = mortality rate. Development time = lice stage 50% development. Ave. Proportion = the average proportion of lice in a stage as calculated by X_i/X_{i0}

Once the values for σ_i and μ_i had been calculated the differential equations and the parameter values were fed into the computer software programme ModelMaker© (version 2.) and the results analysed.

6.3 RESULTS.

The working hypothesis of this experiment was that the timing of the first treatment

[1] Table 6.1 Average settlement in three tanks of fish was 11.3 %.
[2] Table 6.1 Sexual dimorphism results in 14.9 % mortality in both pre adult 1classes.

susceptible lice stages, the preadult 1 stage, could be predicted from early chalimus counts from an examination of the fish.

Figure 6.3 shows the overall population dynamics of the test sea lice data. This is the original data and shows that the maximum number of preadult 1 males is found on day 25 post-infection whereas the maximum number of preadult 1 females is found on day 27. This is the target time to be predicted by the epidemiological model for validation. Figure 6.4 shows the original data areas of specific interest, *L.salmonis* pre adult stages, the time to maximum development and the numbers of lice present. Figure 6.5 is the predicted sea lice population dynamics from the model with Figure 6.6, the model predictions for the peaks in preadult stages, target stages. The specific days for predicted treatment are given in Table 6.2.

Table 6.2. Predicted days of required treatment against susceptible sea lice preadults stages.

Lepeophtheirus salmonis Life Stage	Data analysis (day No.)	Model prediction (day No.)	Difference in model data and original data
Pre adult 1 male	25	24	-1
Pre adult 1 female	27	26	-1

This model predicted days of treatment lower than the original test data. The predicted treatment day, by the model has forecast for the appearance of the maximum numbers of lice of the pre adult stage 1 day earlier than their appearance in the test data. It should be noted that presently the predicted number of sea lice is lower than the original test data. Examining the model output of peaks of maximum numbers present, all are lower, indicating the model mortality rate presently is wrong.

Figure 6.3 Population dynamics of *L.salmonis* showing the development profile, over time to be used to test and validate the model

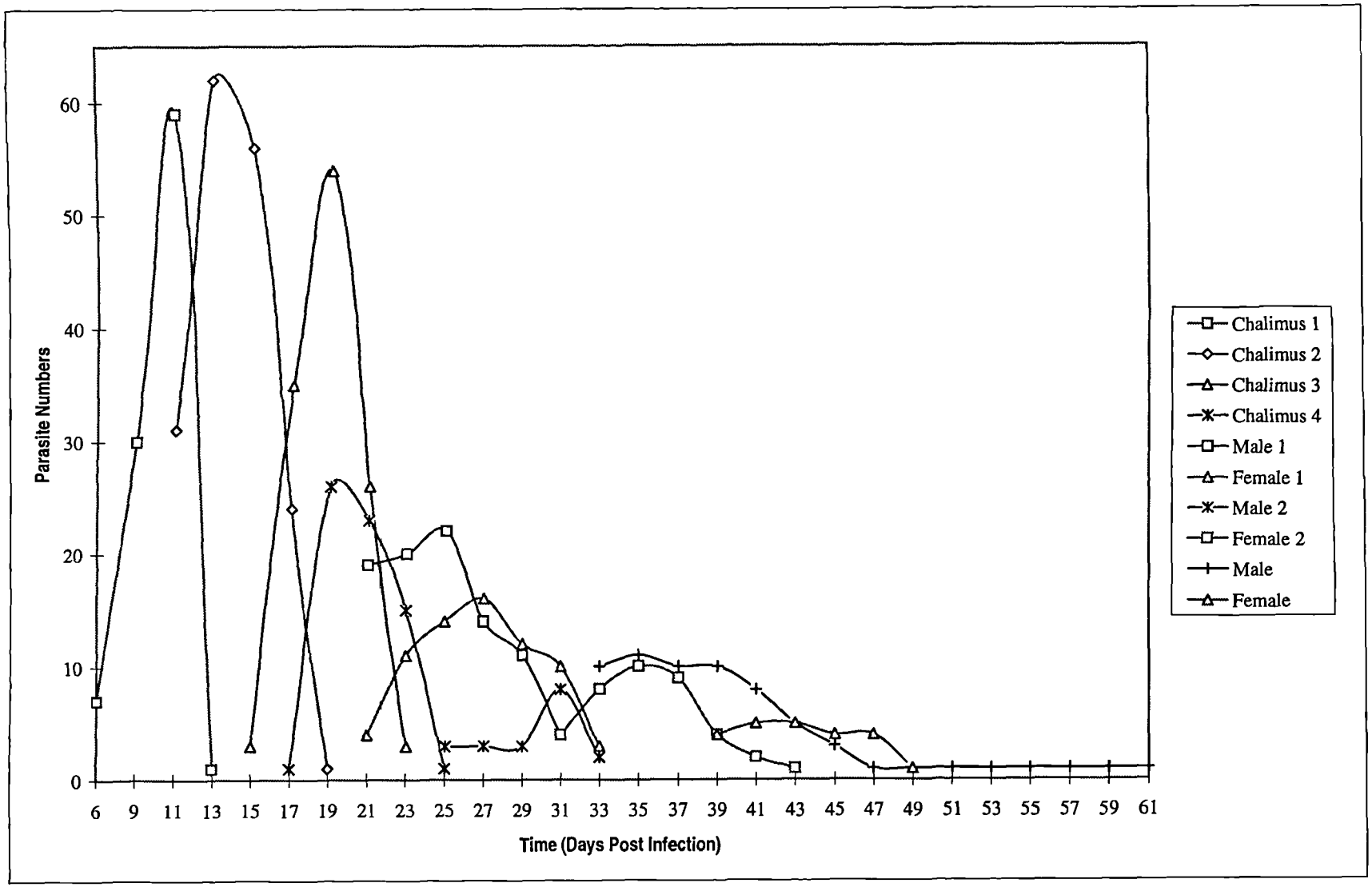


Figure 6.4 Target test data extracted from Figure 6.3. *L.salmonis* population dynamics with examination of targeted parasite life stage

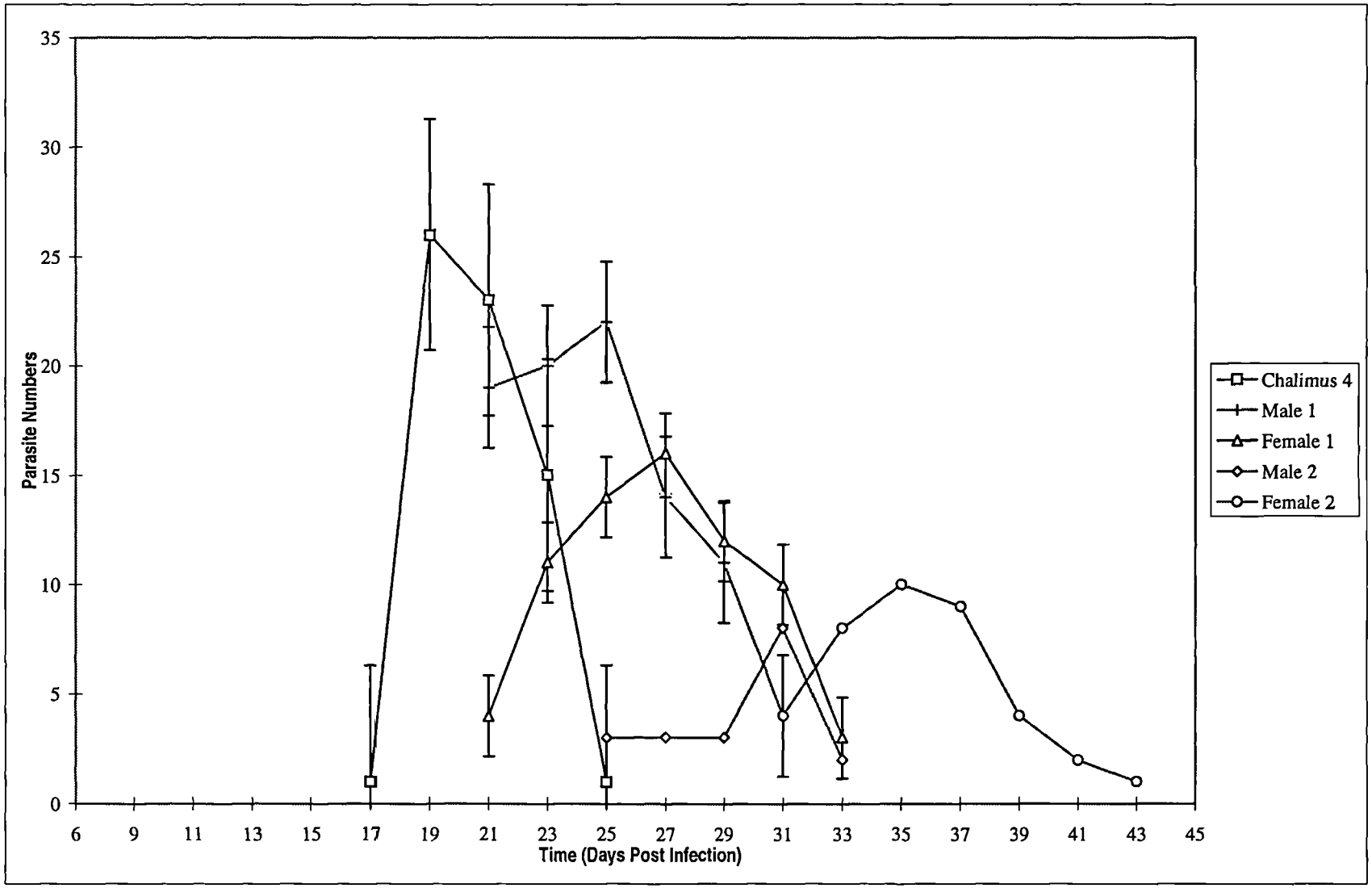


Figure 6.5 Epidemiological model of the generated population dynamics of *L.salmonis.*

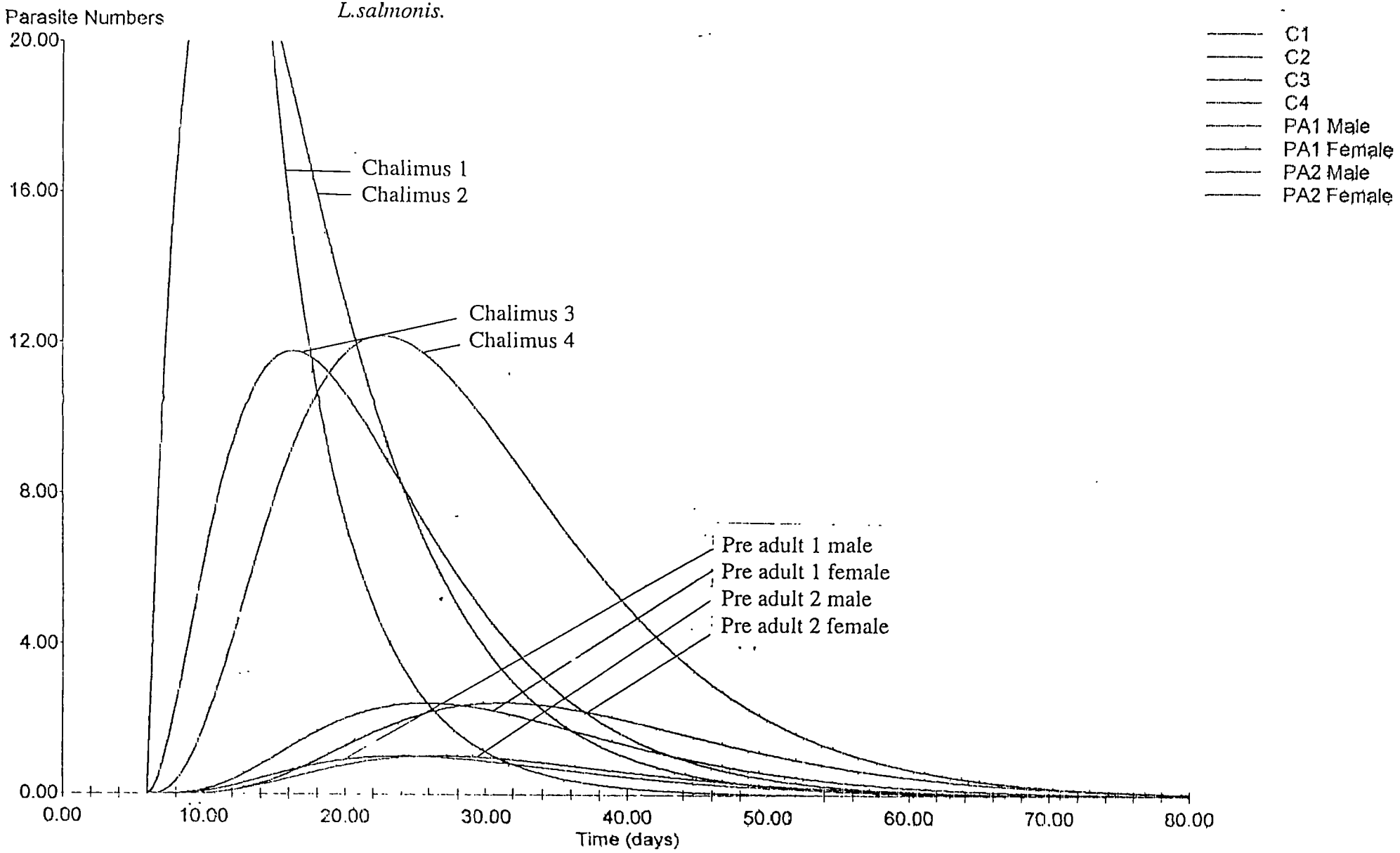
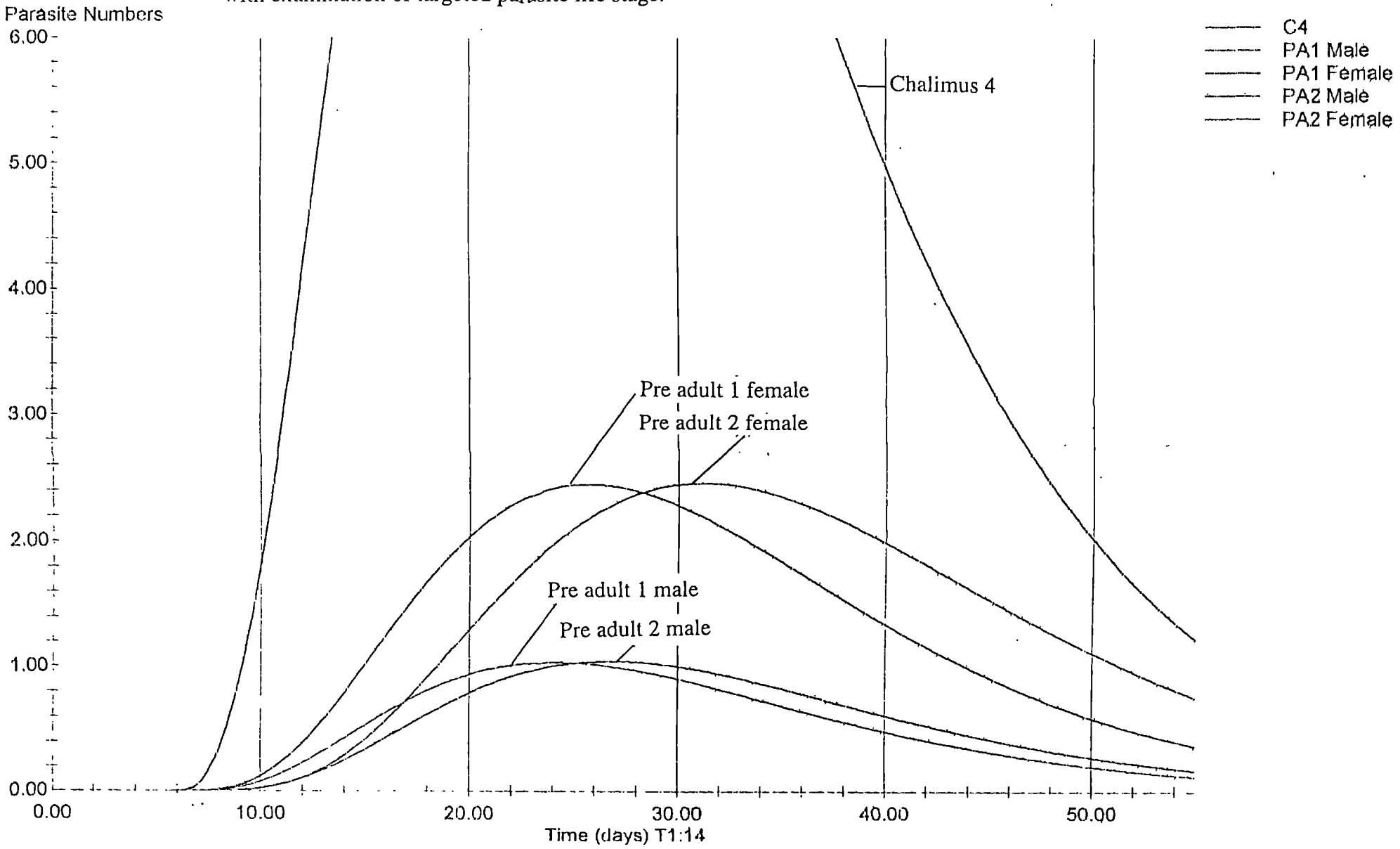


Figure 6.6 Epidemiolgical model of the generated population dynamics of *L.salmonis*, with examination of targeted parasite life stage.



6.4 DISCUSSION.

This preliminary model has predicted, to within one day, the time of the maximum target stages of *L.salmonis* compared to the test data. The prediction of the lice numbers in each stage by the model is considerably lower than the test data. The production of this model has emphasised the problems with this model, the variability of the data used to construct the model. Any mathematical model is only as good as the data on which it is built. The original trial data was conducted to provide a standard protocol for a sea lice challenge procedure. This data, although accurately assembled does demonstrate significant variability between tanks. Accurate information on the developmental times of the lice to a specific stage would enhance this model, presently these have been calculated from the time when 50% of a stage have developed. Within the original trial data there was considerable biological variation between the calculated developmental timings of the stages, the timed duration in a particular stage and the mortality rates for individual tanks of fish. Until this biological variation is understood the parameters of any mathematical model will be difficult to determine accurately.

Not only is there considerable variation in the natural biological processes, mortality rate and developmental rate but also the effects of the anaesthetic and fish handling on the host and lice population are not accurately known. Preliminary studies at the Institute of Aquaculture have shown that the type of anaesthetic used can have an effect on the lice population, especially Benzocaine that has a detrimental effect. A destructive sampling technique would eliminate the use of anaesthetic and constant handling of the fish.

Johnson and Albright (1991a) report the cumulative 50% developmental time (C.D.T.) and duration of various lice stages. A comparison of their values with those calculated for this model data is given in Table 6.3. Both experiments (Johnson and Albright, 1991a and

Anon, 1994c) have been conducted at 10°C and yet there is considerable variation in both the duration in a stage times and the C.D.T.

Table 6.3 Comparative values for 50% cumulative development time and duration in stage for *L.salmonis* at 10°C.

L.salmonis Life stage	C.D.T Ref A.	Duration Ref A.	C.D.T Ref B.	Duration Ref B.
Copepodid		10		10.3
Chalimus 1 (C1)	6.7	5	7.8	7.5
Chalimus 2 (C2)	10.3	5	13.2	11.5
Chalimus 3 (C3)	17.5	9	22.3	9.5
Chalimus 4 (C4)	19.7	6	29.7	8.0
Pre adult 1 male	26.7[3]	8	30.3	10
Pre adult 1 female		10	32.7	13
Pre adult 2 male	35.3[4]	9	36.0	9
Pre adult 2 female		12	42.3	13
Adult male			48.0	23
Adult female			57.2	30

Key: Ref A. Johnson and Albright (1991a), Ref B. Anon (1994c, Unpublished data, Institute of Aquaculture internal report)

Johnson and Albright (1991a) have conducted the experiment using a destructive sampling technique, thus eliminating the effects of anaesthetic although the total number of fish in each sample is not specified. In their study the chalimus 1 stages 50% of numbers developing in that stage appeared by day 6.7 post-infection and in the Anon (1994c) study chalimus 1 stages was on day 6 post-infection. Initially there is good agreement between the two studies; however in the Johnson and Albright (1991a) study there is a gradual acceleration in development resulting in a 10 day difference by the chalimus 4 stage. The reported duration and developmental times of each stage by Johnson and Albright (1991a) were lower than the present study and this may be a consequence of the adverse effects of

[3] This CDT value is only given as preadult 1 with no identification of its sex.
[4] This CDT value is only given as preadult 2 with no identification of its sex.

anaesthetic used by Anon (1994c) or other unknown biological factors.

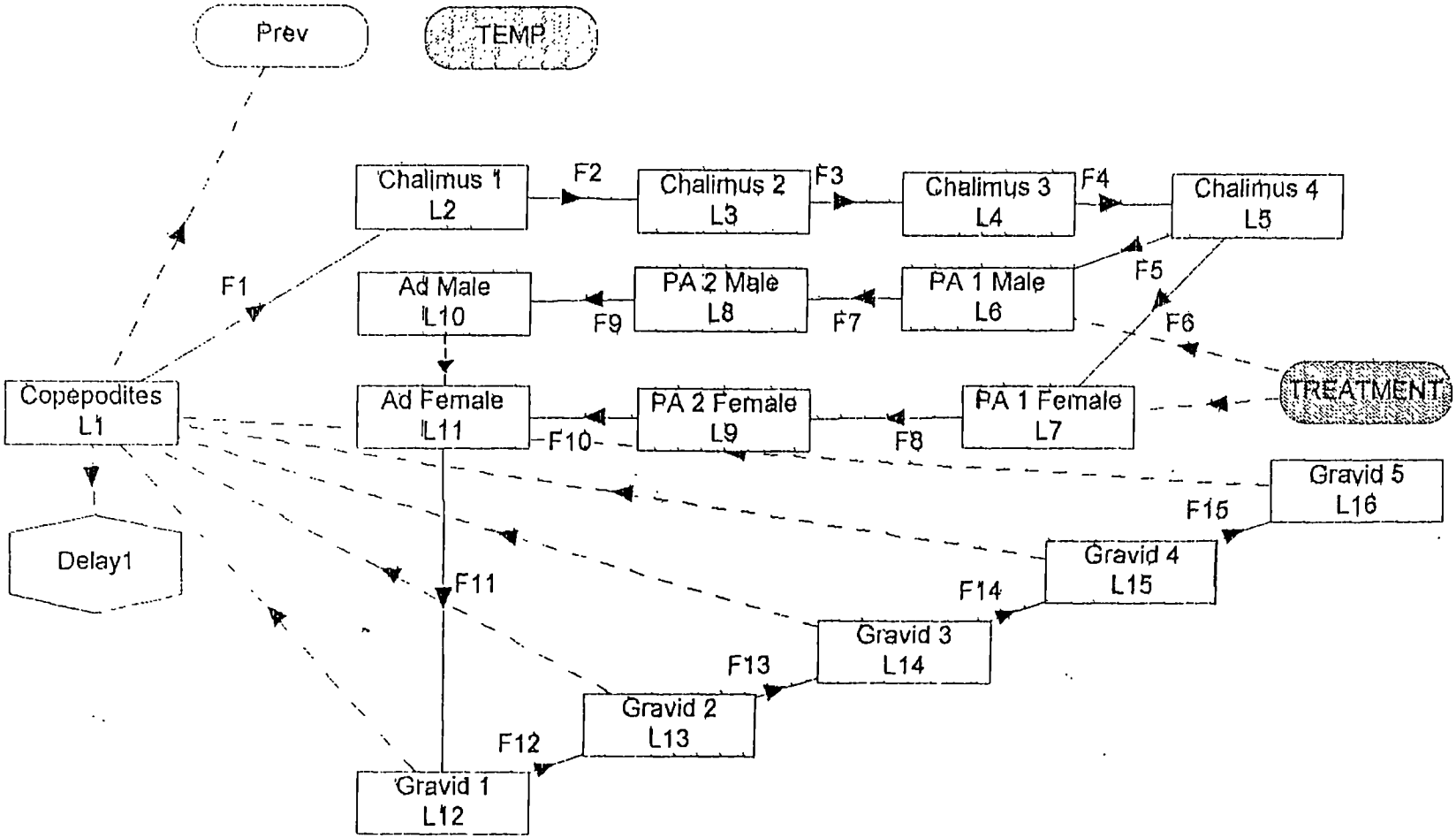
Further investigation of the model parameters with larger sample sizes would eliminate large errors and increase our understanding of the biological processes involved, allowing refinement of the model. French & Morgan (1996) suggest that, although there is a need to make assumptions within a modelling system, this does highlight the weaknesses of the model and the potential areas required for further investigation. When more precise estimates of model parameters are made these can then be incorporated into the model and therefore improve the performance of the model.

This preliminary model has been based on a tank culture system, within a controlled environment. Any future model with wider application to the salmon culture industry would have to be based on cage systems and include parameters such as flow rates of water, reinfections from ovigerous females and effects of treatments administered. Such a model flow diagram is seen in Figure 6.7.

This is the first attempt to model the sea lice problem in salmon culture and although presently flawed the model does demonstrate the validity of the initial investigation of the application of a mathematical model for the control of sea lice. This model is based on the ecology of the parasite and, as such, will lead to a better understanding of the parasite system and lead to better pest management control strategies.

The high costs of sea lice treatments to the salmon industry means that better control strategies are required. Accurate predictability of the timing of the susceptible lice stages would allow the treatments to be targeted with greater effect. The subsequent effects of the treatment on the lice population could also be monitored and the next treatment, for maximum efficacy could be predetermined. Mathematical modelling undoubtedly has its place within pest management strategies.

Figure 6.7 Flow diagram for a cage culture sea lice model.



CHAPTER 7. SUMMARY AND CONCLUSIONS.

With the present restriction of the number of chemotherapeutic treatments available there is a greater necessity to understand the biological and ecological requirements of this parasite and such knowledge could be useful in the development of control strategies.

The work presented in this thesis has sought to investigate the abiotic and biotic factors that affect settlement of the copepodid of *L.salmonis* on its fish host and its survival and development during the early post-settlement phase.

The first part of this study examined the effects of abiotic factors on copepodid settlement. The two factors studied were temperature and salinity. It was considered that flow rates, without the correct experimental equipment would be too difficult to validate. However this would be an important area of further research. Understanding the effect of flow rates in cage aquaculture and its influence on disease transmission would assist in the development of control strategies.

Although previous studies have examined the influence of temperature and salinity on the development and survival of pre-settlement *L.salmonis* larvae, this is the first study in which the influence of temperature and salinity on settlement has been examined. The effects of temperature were clearly seen; with an increase in temperature there is an increase in settlement. This is however highly variable, regression analysis of settlement over a seasonal range of temperatures shows that statistically temperature and settlement are significantly related, although the r^2 value obtained in the analysis was very low. The reason for the highly variable settlement pattern is not understood, but indicates the possible interactions of a number of variables. These results show the likelihood of

increased lice numbers on cage culture salmon in summer months. Within the temperature experiments survival of the louse was approximately 50% or greater in all groups and once settled the louse has a good survival rate at 10 days post-infection. The development of the *L.salmonis*, within the two temperatures examined showed a faster developmental rate at the higher temperature. The transmission dynamics of this ectoparasite is an area of potential future research, to determine the interaction of energetics, metabolism of the louse, the susceptibility of the host and environmental parameters.

Salinity also had a marked effect on the settlement and development of *L.salmonis* larvae. At a reduced constant salinity of 24‰ settlement counts were significantly lower, less than 25% of the values obtained in a constant 34‰. The survival of these lice at 10 days post-infection was approximately 6% and 78% at 24‰ and 34‰ respectively; the lice population in the reduced salinity had collapsed. In the second year of this experiment a different result was obtained, settlement at 24‰ was 24% and 77% at 34‰ with survival above 73% in both groups at 10 days post-infection.

These salinity experiments were repeated in different years. The first was conducted at approximately 13°C and the second was conducted at approximately 15°C with an increase of up to 18°C due to the mechanical failure of the chiller unit used. Where previously the louse population had collapsed now there was increased survival, higher than the ambient salinity regime. The reason for the increased survival could be due to the increased temperature encountered at 24‰ salinity through the breakdown of the chiller, the raised temperature resulting in increased metabolism and development. The reduced salinity had affected the lice population as development had been retarded in comparison to the ambient salinity regime. Hahenkamp & Fyhn (1985) suggest that lice can tolerate adverse conditions if attached to the host and feeding where they will osmoregulate by replacing

salts from a host source. The maintenance of osmotic balance under sub-optimal conditions is through active feeding. Sea lice are therefore primarily affected by temperature and secondarily by salinity.

These findings raise interesting questions on the sites selected for salmon culture. There is anecdotal evidence that shows that salmon cage sites with large fresh water run-off suffer less with lice infestation and yet maintain good salmon growth. Salmon cage sites in such areas would provide a natural constraint on sea lice populations.

In this study high stocking densities have been shown to dilute the effects of sea lice infestation. Similar numbers of lice were seen to infect the whole population of experimental fish but at high host densities the parasites were distributed among 25 fish compared with 5 at low host densities. The current practise of salmon farmers is to maintain high stocking densities of fish when smolts are introduced. The results of this study suggest that this will also be coincidentally advantageous for disease management as it will disperse the lice population. The investigation into the effect of multiple waves of infection reflects the natural situation of cage culture and the constant exposure of fish to this ectoparasite. This study has shown that subsequent infections of *L.salmonis* show a reduced settlement count on the host. This will affect the louse population dynamics and although single wave experiments show the effects of specific parameters, these will interact in cage culture. Understanding the interactions of multiple infections and their effects on lice numbers will be important to understanding future lice population dynamics. Intraspecific competition and interactions between lice on the host are presently not understood and are potentially an area for further research.

Host size is an important consideration from a disease perspective, larger fish having a

greater carrying capacity for lice. Small fish, in this study were found to have the heaviest burdens of lice per unit area. *L.salmonis* has an affinity for the fins of its salmonid host and small fish have been shown to have a greater fin surface area relative to fish of a larger size. For small fish the fin surface area is 33.5% of the total body surface area whilst the medium and large size tested had 26.5% and 23.0% respectively. Small fish also have a slower swimming speed and consequently run an increased risk of lice infection.

Salmon farming has recently been held responsible, in some quarters for the controversial decline of sea trout populations in eastern Atlantic waters. Comparative infections of sea trout and salmon has shown partly contradictory results. Infections of single populations of sea trout and salmon result in the highest intensities of lice settled on the salmon, whilst in mixed populations of fish sea trout have the highest intensities. The behaviour of the fish at the time of infection was not observed and this may have a significant bearing on the result i.e. swimming activity. Fish behaviour at the time of infection may have a marked effect of the intensity of infection. Examination of the development of *L.salmonis* on the two salmonid species has shown a slower development on sea trout. This is in contradiction to the results of Dawson *et al.* (1997) although they do not differentiate the chalimus stages. Knowledge of the physiological differences and the susceptibility to infection between these two host species would contribute to the understanding of the host parasite interactions. Studies of the genetic characteristics of the lice populations on wild salmonids would identify the source of the sea lice.

Many fundamental criteria must be met for any interaction of parasitic cross-infection to take place between wild populations of fish and cage cultured fish. Any wild population will have a low population density and be unrestricted by cages. Johnson *et al.* (1996) have reported the effects of natural reservoirs of hosts in migrating salmon resulting in epizootic

outbreaks and high mortalities. Natural reservoirs of lice from wild salmonids do exist. For cross infection the wild fish population must come into contact with the infective copepodid. Costelloe *et al.* (1996) found that 90% of sea lice larvae are retained within the salmon cages whilst, at a distance of 1 km *L.salmonis* parasite densities of 0.4 copepodid m^{-3} were found. Those larval stages that do escape only have a measurable time to develop and find a new host. This study, through experimental infections and examination of the energy available, has shown that once the copepodid stage has developed then it only has a finite time in which to find and infect its host. After 7 days the ability to infect its host declines rapidly. For cross infection of wild fish the probability of both the appropriate local hydrography and the proximity of a new host occurring must be low.

Investigations of the energy levels of *L.salmonis* larvae have shown a progressive decline in energy within all individual developmental stages. Energy levels have been inferred from the calculation of energy from percentage carbon present in the organism and although the calorific values are similar to those reported for free-swimming copepods, it would be more advantageous if these could be determined directly. Obtaining mono-specific samples of such small organisms in sufficient numbers was difficult, although large numbers of nauplius 1 and 2 stages could be obtained. If sufficient samples of between 5-100 mg dry weight could be collected direct analysis of the energy levels could be determined with the use of a microbomb calorimeter. These could be compared with those inferred in this study.

This study has shown that copepodids, after 5 days show a rapid decline in energy levels and this coincides with a reduced their ability to infect their hosts. Analysis of the lipid reserves of free-swimming *L.salmonis* larvae have shown that the principal lipid class present is triacylglycerol (37.6%). However it has not been determined whether this is

stored directly in the observed lipid vesicles. Benson *et al.* (1972) withdrew oil directly from the lipid deposit of free-swimming copepods by microsyringe. The lipid deposit was shown to consist solely of wax ester, other lipids were stored elsewhere in the copepod. The exact composition of *L.salmonis* lipid vesicles, if sufficient, could be collected and determined in the same way. This study is the first to examine the nature of the lipid reserves of the larval stage and this could be extended to include the lipid deposition of the attached chalimus and preadult stages. Waldock & Holland (1978) examined the fatty acid composition of TAG in barnacle cyprids for evidence of selective utilisation. Similarly Medica & Sukhdeo (1997) looked at the fatty acid composition in infective stages of strongylid nematodes. The former study found no evidence of selective fatty acid utilisation whilst the latter did. Examination of the lipid reserves and the fatty acid profile of the infective stages of *L.salmonis* would establish the TAG composition and its utilisation.

The SEM study of the attached copepodid has shown evidence of active feeding two days after infecting the host. This study, like those of Johnson & Albright (1991a) and Bron *et al.*(1993) did not show the presence of the strigil, although in this investigation aged copepodids pre-settlement and post-settlement were used. Copepodids that had been removed from feeding sites also did not show a strigil. The strigil is intrinsic to feeding in the latter stages of the *L.salmonis* (Andrade-Salas, 1997). Therefore some modification to the feeding behaviour of the copepodid must take place to allow it to feed effectively. The strigil may be recessed and hidden, as a protostrigil, and only utilised at the chalimus 1 stage. It would seem very unlikely that this piece of apparatus must develop on moulting to the chalimus stage.

This study has been the first to attempt an epidemiological model of sea lice populations.

The development of the mathematical model for the predictability of lice population dynamics was partially successful and was beneficial in that it highlighted areas of future research. This model has predicted the timing of the maximum number of treatment susceptible stages, to within one day although the actual numbers of predicted lice is much smaller than those in the test data. The variability in the data used to develop the model parameters probably accounts for the failure of the model to predict the timing of the maximum number of target lice stages. The current model requires an improved understanding of the variability in the data, to allow the calculation of precise parameters. The developmental rate in this model was assumed to be equal to the appearance of the maximum number of lice of a particular stage and this needs to be more accurately quantified. The mortality rate and the duration in stage are highly variable in the data used for the model. These parameters, properly defined would increase the efficiency of the model.

The preliminary model has been based on three data sets for a tank culture system with small fish sample sizes. A greater number of data sets with increased sample size would allow the calculations of the basic epidemiological data on which to determine the parameters of the model. Differential equation modelling is not the only type of mathematical model available, for further development of other model systems could be investigated, i.e. network modelling, stochastic modelling or distributional models.

An epidemiological model would not only allow the prediction of the optimum time for treatment against sea lice but would also allow the analysis of the effects of treatments on the parasite population and thus be important in the development of an effective management strategy against sea lice.

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Appendix 1.

Copepodite (L_1) $\quad \frac{dL_1}{dt} = b.L_{11} - (\mu_1 + \sigma_{10}).L_1$

Chalimus 1 (L_2) $\quad \frac{dL_2}{dt} = \sigma_{10}.L_1 - (\mu_2 + \sigma_2).L_2$

Chalimus 2 (L_3) $\quad \frac{dL_3}{dt} = \sigma_2.L_2 - (\mu_3 + \sigma_3).L_3$

Chalimus 3 (L_4) $\quad \frac{dL_4}{dt} = \sigma_3.L_3 - (\mu_4 + \sigma_4).L_4$

Chalimus 4 (L_5) $\quad \frac{dL_5}{dt} = \sigma_4.L_4 - (\mu_5 + \sigma_5 + \sigma_6).L_5$

Pre adult 1 male (L_6) $\quad \frac{dL_6}{dt} = \rho.\sigma_5.L_5 - (\mu_6 + \sigma_7).L_6$

Pre adult 1 female (L_7) $\quad \frac{dL_7}{dt} = (1-\rho).\sigma_6.L_5 - (\mu_7 + \sigma_8).L_7$

Pre adult 2 male (L_8) $\quad \frac{dL_8}{dt} = \sigma_7.L_6 - (\mu_8 + \sigma_9).L_8$

Pre adult 2 female (L_9) $\quad \frac{dL_9}{dt} = \sigma_8.L_7 - (\mu_9 + \sigma_{10}).L_9$

Adult male (L_{10}) $\quad \frac{dL_{10}}{dt} = \sigma_9.L_8 - (\mu_{10}).L_{10}$

Adult female (L_{11}) $\quad \frac{dL_{11}}{dt} = \sigma_{10}.L_9 - (\mu_{11}).L_{11}$