Studies on *Entobdella hippoglossi* (Müller, 1776) (Monogenea) and *Lepeophtheirus hippoglossi* (Krøyer, 1837) (Copepoda); Ectoparasites of Atlantic halibut (*Hippoglossus hippoglossus* L., 1758)

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DECLARATION

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

Signed _____ Date 20/12/01.

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Abstract

This study has elucidated aspects of the biology of two ectoparasites of Atlantic halibut (*Hippoglossus hippoglossus*).

The monogenean *Entobdella hippoglossi* can live for up to 146 days as an adult with a complete life span of up to 250 days. It becomes mature and commences egg production at 9mm in length but can reach 21mm. Egg production increases with size. A mean of 186 eggs can be laid *in vitro* and 122 *in vivo* over 24hours. Eggs take 27-54 days to hatch at 12°C and 5°C, respectively. Juvenile *Entobdella* were observed on halibut 85-93 days (8-11°C) after infection with adult parasites. Juvenile (<10mm) *Entobdella* were found on the tail and dorsal surfaces of the halibut whilst adults (≥10mm) were usually found on the anterior ventral portion of the body and head. They caused significant haemorrhagic lesions, particularly when congregated. A timing regime for formalin bath treatments was devised.

The life-cycle of the caligid copepod *Lepeophtheirus hippoglossi* consists of 10 stages and is completed from attachment to egg production in 29 days at 13°C and 62 days at 7.5°C. The timing for each of the life stages was ascertained at 13.1, 9.7 and 7.5°C. The morphology of each of the 10 stages is described. Settlement of the copepodid stage occurred mainly on the fins and body of the halibut and the motile stages were found on the dorsal anterior portion of the fish, particularly around the abdominal cavity. The relationship between *E. hippoglossi* and *L. hippoglossi* and the structure of the skin they parasitise is discussed. Severe haemorrhagic lesions occurred with as few

as 25 adult female *L. hippoglossi* on halibut of more than one kilogram. An in-feed treatment emamectin benzoate (SLICE) was trialed as a control method for *L. hippoglossi* and up to a 96.5% reduction in lice numbers was obtained.

FIGURES

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Chapter 1

General Introduction

1.1. Background

Recent years have seen an expansion in European aquaculture, with an increasing interest in the introduction of marine species new to aquaculture, including the turbot (*Scophthalmus maximus* L.), the Atlantic halibut (*Hippoglossus hippoglossus* L.) and the cod (*Gadus morhua* L.). The halibut and the cod have supported important capture fisheries in the North Atlantic and are considered to be potentially significant species for aquaculture (Bromage & Roberts, 1995).

The halibut has considerable potential for aquaculture as wild stocks have been overexploited (Haug, 1990) and its market price is very high, US$12 per individual fry (Olsen *et al.*, 1999). They are fast growing in cold waters (Haug, 1990; Haug and Tjemsland, 1986; Jakupsstovu and Haug, 1988) and can reach a mean weight of 3kg after three and a half years in captivity (Adoff *et al.*, 1993). Experiments on halibut hatching and larval culture commenced in 1974 (Solemdal *et al.*, 1974) and since 1988 these studies have led to the development of commercial sized hatcheries producing halibut juveniles in large numbers, thus providing the basis for an ongrowing industry (Olsen *et al.*, 1999). During this study the production output in Scotland has increased from 30,000 fry in 1997 to 170,000 fry in 1999 (R. Shields *pers comm.*).

1.2. The Halibut

The Atlantic halibut, *Hippoglossus hippoglossus* L. 1758 is a pleuronectiform flatfish. It has recently been classified by Ahlstrøm *et al.*, (1984) and Hensley & Ahlstrøm (1984) as part of the sub-family Pleuronectinae within the family Pleuronectidae. In the past the halibut has been known by other names, such as *Pleuronectes hippoglossus* L., 1758, *Hippoglossus vulgaris* Flemming, 1828, *Hippoglossus hippoglossus* Jordan & Everman, 1898 and *Hippoglossus americanus* Gill, 1864 (Andriyashev, 1954). The Pacific halibut (*Hippoglossus stenolepis*) and the Atlantic halibut have been considered to be the same species but recent studies using electrophoretically detectable protein variants (Grant *et al.*, 1984) proved that these are two distinct species, as suggested by Schmidt (1930).

Halibut are found in areas of the Arctic Ocean and in the northern parts of the Atlantic Ocean, sometimes as far south east as the Bay of Biscay and southwest as far as New York. They can also be found in the south western parts of the Barents Sea, as far north as Bear Island, and occasionally on the west coast of Spitzbergen. They are particularly common along the coasts of Norway, the Faeroe Islands and Iceland and they may also be found off southern Greenland, in the North Sea and the western Baltic Sea (Andriyashev, 1954). In North America, the halibut is found from Pierre Bank and Grand Bank in the south as far north as the Hudson Straits. They are mostly found on the continental shelf and on fishing banks (Bowering, 1986). Within these areas fish of different ages occupy different habitats. Young halibut (1-6 years) remain resident on nursery grounds at about 20-60 m (Vedel-Tåning, 1938; 1947; Sigurdsson

&Fridriksson, 1952; Sigurdsson, 1956; Haug & Sundby, 1987; Godo & Haug, 1988; Stobo *et al.*, 1988). After leaving the nursery grounds the fish migrate, often over very large areas. A great deal of mixing of the stock appears to occur during this period (Bowering, 1986; Godø & Haug, 1988; Stobo *et al.*, 1988). In the winter the sexually mature halibut gather together for spawning. They use clearly defined spawning areas which are not frequented outwith the spawning season. Halibut have been found in deep and shallow water, inshore and offshore (Devold, 1938; Mathieson & Olsen, 1968; Olsen, 1969; Haug & Tjemsland, 1986; Jakupsstovu & Haug, 1988).

1.3. Halibut Lifecycle

Sexually mature halibut have enormous reproductive potential, large females producing several million eggs (Fulton, 1891; Haug & Gulliksen, 1988). The halibut egg is large compared to other planktonic fish eggs (Russell, 1976), ranging from 3.06 to 3.49mm (Haug *et al.*, 1984; Vedel-Tåning, 1936; Jakupsstovu & Haug, 1988). Rollefsen (1934) found that eggs took 16 days at 6°C to hatch, while Lønning *et al.* (1982) found that hatching occurred after 18 days at 5°C and Blaxter *et al.* (1983) reported incubation times to 50% hatching of 20, 18 and 13 days at 4.7, 5 and 7°C, respectively. Little is known of the pelagic larval stage in the wild, presumably because the larvae disperse at low density over a wide area and are difficult to sample. Many observations on hatching, development, behaviour, nutritional and environmental requirements have been made during experiments on artificially fertilised eggs and larvae in culture tanks (Haug, 1990).

On hatching the larva is 6-7 mm long, has no mouth, pigmentation or functional eye and the yolk sac is still large (Rollefsen, 1934; Blaxter *et al.*, 1983; Haug, 1990). It takes approximately 50 days for the yolk sac to be fully absorbed although it is thought that food may need to be consumed before the yolk is completely absorbed (Haug, 1990). Benthic settling occurs when the larvae reach between 34 mm (the largest pelagic larva found) and 47 mm (the smallest benthic stage found) in length (Vedel-Tåning, 1936). Settling occurs before metamorphosis is complete. Berg & Øiestad (1986) observed that the larvae could settle on the bottom at day 95 post hatching. The final transformation into typical "flatfish" occurs on the seabed when they are between 34 and 47 mm (Vedel-Tåning, 1936).

Young halibut can survive in water below freezing although their feeding stops at around 2°C (Goff & Lall, 1989). The optimum feeding and growth rate seems to be within the range of 6-12°C, the larger fish having a lower optimum than smaller ones (Smith & Dye, 1989; Adoff *et al.*, 1993). Halibut are active predators and will feed upon most fish species, particularly gadoids and herring. They will also feed on crabs, octopus and bivalve molluscs. In nature juvenile halibut settle in nursery areas along the coast at 20-60m and remain there for up to 6 years (Vedel-Tåning, 1938; 1947; Sigurdsson & Fridriksson, 1952; Sigurdsson, 1956; Haug & Sundby, 1987; Godø & Haug, 1988; Stobo *et al.*, 1988). A length of 44-70mm is reached 5-7 months after hatching and after 2 years fish can reach approximately 25cm in length (Haug, 1990). Male and female halibut reach sexual maturity at different rates. The mean age, length and total weight at which the separate sexes reached sexual maturity in Faeroese waters

were 4.5 years, 55 cm and 1.7 kg for males and 7 years, 110-115 cm and 18 kg for females (Jakupsstovu & Haug, 1988). It is thought that sexual maturity in the halibut is a function of growth rate and size rather than of age alone (Roff, 1982). Bjornsson (1995) showed that males became sexually mature at a mean weight of 3.2 kg and the females at 12.7 kg when reared at 7°C. Halibut can reach up to 250kg in weight (Martinez Cordero *et al.*, 1994) and are known to be long lived.

1.4. Halibut Culture

Rollefsen (1934) carried out the first known halibut egg artificial insemination experiments in Norway. These eggs were taken from halibut in an aquarium and were incubated but the larvae only survived for 10 days. In Norway more extensive experimentation began in 1974 (Solemdal *et al.*, 1974). Eggs were gathered from females caught in gill nets on the spawning grounds, they were immediately fertilised and then brought back to the laboratory for incubation. In 1983 a brood stock was established (Haug, 1990) from adult fish caught using long lines outside the spawning season. The fish were kept in large, covered tanks with a continual flow of water and maintained at temperatures (5-6°C) and salinities (35‰) similar to those recorded at the spawning grounds (Rabben *et al.*, 1986). Rabben *et al.* (1986) observed that adult halibut can be kept alive, fed and stripped through several seasons in captivity. The first generation of mature fish bred in captivity have now begun to produce eggs both in Norway (Bromage & Roberts, 1995) and Scotland (P. Smith, *pers. comm*).

The brood stock halibut are normally fed to satiation two or three times a week (Haug *et al.*, 1989) on a moist pelleted diet or herring, mackerel, squid and trash white fish together with a vitamin mix. Halibut recruited to the brood stock which have been reared in captivity may also be fed on a dry pelleted food (Berge and Stockenbakken, 1991; Adoff *et al.*, 1993). The fish are not fed while they are spawning (Bromage & Roberts, 1995). Broodstock are maintained in circular tanks ranging from 3.5-10m diameter at stocking densities of up to 11kg m^{-3} and with 1-2 males per female (Shields *et al.*, 1999)

Female halibut are batch spawners and lay eggs at more or less regular intervals during the spawning season. Fish of 20-60 kg have been reported to release 6-16 egg batches per season (Holmefjord, 1991; Norberg *et al.*, 1991; Norberg & Kjesbu 1991). Each batch will have approximately 10-200 thousand eggs and batch size may be variable throughout the spawning season (Haug, 1990).

In captivity, stripped eggs are kept dry until they are fertilised, then eggs, milt and water are mixed at a ratio of 100:1:100 (Rabben *et al.*, 1986). Fertilisation rates of 90% and above are usually obtained (Mangor-Jensen & Jelmert, 1986; Rabben *et al.*, 1986; Jelmert & Rabben, 1987). In the UK it was found that the temperature was relevant to spawning performance, those fish maintained at 6°C before and during the spawning season produced more eggs with greater fertilisation and hatching rates compared to those held at ambient temperature (Brown *et al.*, 1995).

In Scotland eggs are maintained in cylindro-conical tanks supplied with a slow inflow of raised salinity water (35‰) at densities of 125 eggs per litre (Bromage & Roberts, 1995). These tanks are maintained at 5°C and hatching occurs within 19 days.

Once hatched the yolksac larvae are very delicate and susceptible to external factors such as temperature, salinity, light and water movement (Opstad & Raae, 1986), and this stage has been a bottleneck in the development of halibut farming. In the UK the larvae are moved to static water tanks at around 150 degree days to reduce mortalities during the later stages of development (Shields *et al.*, 1999). Haug (1990) stressed that larval density should be as low as possible, temperature should be low and disturbance kept to a minimum during the final stages of yolk sac absorption to ensure large and healthy larvae.

First feeding begins around 210-250 degree days but the yolk sac is not totally absorbed until approximately 330 degree days (Bromage & Roberts, 1995). In the UK first feeding is carried out in indoor tanks which are artificially lit. The larvae are put into "green water", i.e. seawater including a culture of micro-algae (Shields *et al.*, 1999). Initial experiments into intensive, hatchery based feeding, focused on the use of both rotifers and *Artemia*. However, it was found that rotifers were not advantageous to first feeding and so *Artemia*, including enriched metanauplii, are the main food given to larvae from day one (Shields *et al.*, 1999).

The main problems at first feeding are a high mortality rate and problems with pigmentation, metamorphosis and eye migration (Shields *et al.*, 1999). Calanoid copepods (*Eurytemora velox*) have been found to be nutritionally better than the branchiopod *Artemia*, even after enrichment, and are known to have a high concentration of free amino acids (Fyhn, 1989) and also highly unsaturated fatty acids (HUFA's) (Gara *et al.*, 1998). They are, however, more difficult to culture on a large scale (Shields *et al.*, 1999). *Artemia* are commercially available and can be reared in intensive, year round production (Shields *et al.*, 1999) but they have a different chemical composition from copepods. In experiments, fish reared on *Artemia* were found to have low growth rates, malpigmentation (Næss *et al.*, 1995) and mass mortality before and during metamorphosis (Holmefjord *et al.*, 1989). The enrichment of *Artemia* has focused on increasing the lipid composition of the prey and on optimising the presentation of the diets. Substances such as tuna orbital oil emulsion and spray-dried preparations of the marine thraustochytrid fungus *Schiztochytrium* have been tested (Gara *et al.*, 1998).

After metamorphosis the larvae are weaned on to a commercially available dry food at a wet weight of around 250mg (Opstad, 1995). Weaning is normally carried out around 800-1000degree days post-hatching using a crumbled formulated feed (Shields *et al.*, 1999). Recent improvements in hatchery survival rates, combined with greater egg availability, have enabled the annual UK production to rise to hundreds of thousands of juveniles (Shields *et al.*, 1999).

Once the halibut are weaned they are maintained in tanks with a constant supply of clean seawater and are fed continually with commercially available crumb feed. When they reach approximately 200-300g in weight they are transferred to cages. In Scotland the most common cage design is converted salmon cages (with a tarpaulin secured to the base of the cage), although other cage designs include those with multiple shelves to increase the carrying capacity (BHA workshop, 1997) have been used. The halibut are usually found on the bottom of the cages and only swim to the surface to feed or if the bottom of the cage is disturbed due to bad weather (Martinez Cordero *et al.*, 1994). When first put into cages the stocking density should be around 30kg m^{-2} increasing to 50kg m^{-2} for halibut over 1kg in weight. Halibut will take 39-46 months to reach a marketable size of 2-5kg (BHA workshop, 1997).

1.5. Parasites of Wild Halibut

There have been a number of studies into the parasite fauna of wild halibut (Ronald, 1960; 1963; Bray, 1979; Zubchenko, 1980; Schram & Haug, 1988; Scott & Bray, 1989; Lile *et al.*, 1994; Appleby & Mo, 1997). Wild halibut are naturally infected with a range of intestinal helminth parasites which apparently cause no harm to the adult fish, although the digeneans *Derogenes varicus* (Müller, 1784) and *Hemiurus luehei* (Ohdner, 1905) have been reported to cause mortalities in larvae (Appleby, unpublished data). Scott & Bray (1989) studied intestinal helminths of 272 halibut from the Scotian shelf. They found a total of 23 species of parasites, sixteen digeneans, 3 cestodes, 2 acanthocephalan and 2 nematode. The most common parasites were the digeneans *Derogenes varicus* and *Steganoderma formosum* (Stafford, 1904), but overall the

prevalence and intensity of parasites was low (<50% and <10 per fish respectively). This study suggested that the length of the fish affects the parasite fauna. In the largest length group there was a higher prevalence and intensity of the acanthocephalans *Corynosoma* sp. and *Echinorhynchus gadi* (Müller), an anisakid nematode and the digenean *Otodistomum veliporum* (Dollfus, 1937) than in the smaller length groups. In a study of the complete parasite fauna carried out by Zubchenko (1980) in the north west Atlantic, only 10 fish were examined but 23 species of parasites were found. There were 6 parasites with a direct development cycle, including the ectoparasitic copepods *Hatschekia hippoglossi* (Guerin-Meneville, 1837) and *Lepeophtheirus hippoglossi* (Krøyer, 1837) and 4 species of myxosporidians, the most common being *Ceratomyxa drepanopsettae* (Averintesev, 1907) (60% prevalence). The remaining species of parasites had indirect life cycles and the most common were *Derogenes varicus* (90% prevalence), *Scolex pleuronectis* (Müller, 1788) (70% prevalence) and *E. gadi* (60% prevalence). There were 11 species of digenean trematodes found in the fish and this rich fauna was thought to be connected to benthic organisms such as amphipods, molluscs and decapods upon which the halibut feed.

The caligid copepod, *Lepeophtheirus hippoglossi* was found on 66-78% of sexually mature wild halibut in north Norway (Schram & Haug, 1988). Those fish not infected tended to be male and immature. Most of the fish examined by Schram and Haug (1988) had only one or two copepods, the largest number on one fish was 38. Female copepods were much more common, comprising up to 81% of the population.

Entobdella hippoglossi is a capsalid monogenean which is common on wild halibut. Schram and Haug (1988) found that 50-60% of fish from north Norway were infected. This parasite can grow up to 20mm in length and is usually found on the ventral surface of the fish, where it can occur at densities as high as 500 per 'large' fish (Schram & Haug, 1988), although the mean number per fish was seven. Large fish (151-200cm in length) have more parasites and Schram and Haug (1988) suggest that smaller fish (<51cm) are perhaps not infected until they congregate for mating. Schram & Haug (1988) also observed that female halibut tended to have more parasites and suggested that this was due to the more active mating behaviour of the males which made it more difficult for the oncomiracidia to settle on them. They also observed no immature halibut that were infected.

The only parasite species of any known economic importance in wild halibut is the encysted plerocercoid larva of the tapeworm *Grillotia erinaceus* (van Beneden, 1858). The full life-cycle of the cestode is unclear but the final hosts for the sexually mature worms are skates and rays (Haug, 1990). The encysted plerocercoid larvae are known to infect common food fishes (Johnstone, 1912) and the earlier stages are found in Crustacea such as copepods (Ruskowski, 1934). The larvae of this parasite can occur anywhere in the host but normally form cysts on the gut, in the abdominal cavity or the liver (Appleby, unpublished data; Haug, 1990). It can occur in such high numbers that the presence of the cysts in the muscle can affect the saleability of the fish (Rae, 1958).

1.6. Parasites of Cultured Halibut

Occurrences of parasite-related fish diseases have increased since the advent of large scale aquaculture. This is probably due to high host density which favours transmission of parasites (Roberts, 1989) and increased stress of host populations which reduces the immune capacity of fish and makes them more susceptible to infection. Stress may result from one or more factors including: poor sanitation, poor nutrition, poor water quality, overcrowding or improperly designed cages or tanks (Roberts, 1989). It is uncertain which parasites will prove of greatest significance in halibut farming but those with a direct life cycle will be the most likely to cause problems because of their ability to establish self-sustaining populations in aquaculture systems (Appleby, unpublished data), as has occurred in other forms of mariculture. Those parasites which have more than one host in their life cycle are expected to cause fewer problems since they will be less readily established in farming systems (Appleby, unpublished data).

A number of potentially significant protozoan infections have been detected, especially in the larval and fry production stages of halibut. A new species of ciliate *Trichodina hippoglossi* was recorded in 1-3g larvae in a hatchery near Bergen, Norway. These fish became heavily infected when the temperature of their tank water was raised to 18°C, with up to 1000 *Trichodina* per fish. The trichodinids were found only on skin and fins, where they caused skin haemorrhages and the fish were greyish from excess mucus production (Nilsen, 1995). Trichodinid infections have been reported from a number of other species of cultured marine fish, including turbot, *Scophthalmus*

maximus L. (see Sanmartin Duran *et al.*, 1991), cod, *Gadus morhau* (L.) (see Jensen, 1986) and wolf fish, *Anarhichas lupus* (L.) (see Moksness, 1990).

A microsporidian of the genus *Pleistophora* caused muscle degeneration and macroscopic cysts in the abdominal cavity and the skeletal muscles in farmed halibut fry aged 2-4 months. Spores were also found in internal organs such as the pancreas, liver and kidneys. Ten percent of the fry were parasitised and the infection was assumed to be fatal, either by directly killing the larva or by impairing its swimming ability and thereby leading to starvation (Nilsen & Bristow, 1994). A second, unidentified microsporidian species, similar to the genus *Enterocytozoon*, was isolated from lymphoblasts from the kidney of farmed halibut larvae in Norway by Nilsen (1995). Infection led to the destruction of kidney tubules and haematopoietic tissue which caused swollen kidneys and spleen in 13% of the fish studied. The infection was frequently found in dead or moribund larvae.

Helminths of marine fish often have crustacean intermediate hosts and halibut larvae fed on wild plankton may be at risk of infestation (Appleby, unpublished data). Bristow (1990) (from Appleby, unpublished data) found 5 parasite species in halibut fry fed with wild plankton, although none were apparently fatal to the fish. However, (Appleby, unpublished data) suggested that the digeneans *Derogenes varicus* (Müller, 1784) and *Hemiurus luehei* (Odhner, 1905) and the nematode *Hysterothylacium aduncum* Rudolphi, 1802 cause mortalities in halibut larvae. The larvae of *Grillotia erinaceus* (Beneden, 1858) may also be able to infect farmed fish through larval feed

stuffs. Nilsen and Bristow (1994) reported that 10% of halibut larvae from a commercial hatchery were infected with the microsporidian *Glugea stephani* (Olsen, 1981). Heavy infections led to severe degeneration of skeletal muscle fibre. The use of cultured feeds, such as *Artemia* and copepods, instead of wild plankton would prevent such infections (Appleby, unpublished data).

1.7. Survey of Parasites in UK Halibut Production Facilities

In order to have a better understanding of the parasitic problems occurring in the UK halibut industry a questionnaire was sent out to each of 8 sites at the beginning of this study. The questionnaire was designed to ascertain which parasites had been found in UK production facilities, how often outbreaks occurred, treatments and basic details of the farm and surrounding areas. The most common parasite found was *Entobdella hippoglossi* (Müller, 1776), present on four out of eight sites. The ectoparasitic flagellate *Ichthyobodo* and the ciliate *Trichodina* were found infecting the skin and gills of halibut aged from 6 months to broodstock on 3 farms. These infections were treated with a formalin bath (1:2000) for 20 minutes. The parasites had presumably been transmitted from other fish species on the farms (e.g. turbot) or had originated from the wild, probably through the water inflow. *Ichthyobodo* has been reported from a number of marine species including plaice (*Pleuronectes platessa* L.), winter flounder (*Pseudopleuronectes americanus* Walbaum), haddock (*Melanogrammus aeglefinus* (L.)), dab (*Limanda limanda* (L.)), halibut and post-smolt salmon (Bruno, 1992). It can cause moderate to severe gill pathology, including lamellar fusion and hyperplasia of gill filaments in farmed salmon (Bruno, 1992). Two on-growing cage sites reported

the copepod *Caligus elongates* (von Nordmann, 1832), this is a non-host specific parasite and probably originated from wild fish found around the cages. This copepod has caused skin erosion, even at the chalimus stage, in farmed salmon when found in high numbers (Pike *et al.*, 1993). One farm reported that microsporidian cysts had been found in the muscle of a broodstock halibut that had died. This was a wild caught halibut and had presumably become infected before capture.

Entobdella hippoglossi was the only parasite reported to cause any damage to farmed halibut (skin erosion, haemorrhagic lesions on the ventral surface leading to the fish going off their food). On one farm very high intensities (150+ per fish) of the parasite were reported. It was only reported from broodstock fish, all of which had been wild caught. It is assumed that the infection was brought into the farms with these fish and had originally been missed due to the parasites mainly inhabiting the ventral surface of the fish. Once the eggs become established in the tanks it has proved very difficult to eradicate the infection. When the fish are treated the water level was dropped to a third of the normal volume and formalin was introduced at a concentration of 1:2000. The fish were left in the formalin for up to one hour. The formalin will not affect any eggs entangled on the walls of the tank, above the water level, and those within the water are well protected by their heavily tanned shells. Infections have re-occurred after repeated formalin baths and it is assumed that the eggs are not killed by this treatment.

Thus a number of parasitic species have been recorded at different stages of the halibut culture cycle, none have caused serious health problems and most are species with

direct life-cycles as expected. This number of species is likely to grow as the industry develops, particularly into ongrowing.

This study has been undertaken as part of the UK Government Technology Foresight Challenge, Halibut Farming Development project. The overall purpose of the project was to overcome the most significant problems facing halibut farmers in the UK and thus to encourage development of commercial scale production. This study concentrated on the monogenean *Entobdella hippoglossi* and the copepod *Lepeophtheirus hippoglossi* in relation to their potential role as pathogens in halibut culture. *Entobdella* is already established within some farms and is known to cause damage to valuable broodstock fish. *Lepeophtheirus hippoglossi* is a common parasite of wild halibut and it is quite likely that it may become established in farm stocks either from wild caught broodstock, or by infection of cage sites from local wild halibut stocks. *L. hippoglossi* is closely related to the salmon louse, *L. salmonis*, and may be expected to cause similar severe pathology to the host fish.

Chapter 2

Aspects of the biology of *Entobdella hippoglossi* (Capsalidae, Monogenea)

2.1. Introduction

Monogeneans frequently cause severe epizootics in captive and wild fish. Their direct life cycle enables them to reach epizootic levels very quickly at high host densities (Thoney & Hargis, 1991).

The best known example of a pathogenic monogenean skin parasite is *Gyrodactylus salaris* which has caused severe mortalities in natural populations of Atlantic salmon parr in Norway (Sterud *et al.*, 1998). Mortality is caused by excessively high *G. salaris* populations (sometimes in excess of 10 000 flukes per fish), associated with opportunistic secondary pathogens such as the fungus *Saprolegnia* sp. (Johnsen, 1978; Mo, 1994). In the New York Aquarium, the lemon shark (*Negaprion breviristris* Poey, 1868) is commonly infected with the capsalid *Dermophthirius nigrellii*, which causes skin lesions (Cheung *et al.*, 1982). *Benedenia seriolae* caused mass mortalities of yellow tail (*Seriola quinqueradiata* Temminck & Schlegel, 1845) (Hoshina, 1968; Egusa, 1983) and *Benedenia* spp. caused mortalities in Florida pompano (*Trachinotus carolinus* Linnaeus, 1766) (Lawler, 1977). In Australasia and Asia epizootics of species of *Anoplodiscus* have occurred on cultured and captive wild-caught sparid fish e.g. *A. cirrusspiralis* on *Pagrus auratus* (Bloch & Schneider), *A. tai* on *P. major*

(Temminck & Schlogel, 1844), *A. spari* on *P. auratus* and *A. australis* on *Acanthopagrus australis* Gunther (see West & Roubal, 1998a). These parasites have caused damage to the hosts but no mortalities have been reported. The gill parasite *Heterobothrium okamotoi* has been responsible for anaemia and large scale mortalities in tiger puffer (*Takifugu rubripes* Temminck & Schlegel, 1850) cultured in Japan (Ogawa & Inouye, 1997).

Entobdella hippoglossi (Müller, 1776) is a monogenean ectoparasite (Monopisthocotylea; Capsalidae) which is common on wild halibut. Schram & Haug (1988) found that 50-60% of fish examined from north Norway were infected. *E. hippoglossi* can reach at least 20mm in length and is usually found on the ventral surface of the fish, with as many as 97 parasites per large (200+cm) fish although the mean number per fish was nine in the wild (Schram & Haug, 1988). In cultured conditions infection intensities of 641±233.3 per fish (5-20kg broodstock halibut) have been observed (Gil-Ha, 1998). *E. hippoglossi* has a single-host life cycle. Eggs hatch at night and the ciliated oncomiracidia larvae remain infective for up to three days post-hatch at 4°C (Kearn, 1974a). Schram & Haug (1988) observed that female halibut tended to have more parasites and suggested that this was due to the more active mating behaviour of the males which made it more difficult for the oncomiracidia to settle on them. They also observed that immature halibut were uninfected, suggesting that young halibut are not infected until they congregate for mating.

Entobdella hippoglossi have caused lesions on brood stock fish but are relatively easy to remove using a formalin bath. The eggs, however, can be difficult to eradicate from tanks and there is concern that the parasite may become very significant in ongrowing of halibut. Svendsen and Haug (1991) assessed the *in vitro* efficacy of different chemicals and concentrations of saline as potential treatments for halibut infected with *E. hippoglossi*. The adult parasites were tolerant to 200ppm formalin, readily detached from a glass slide in 80ppm benzocaine and were effectively killed in hypersaline and freshwater treatments. The eggs of the parasite were also treated with freshwater at 50°C and 500ppm formalin at three stages during the incubation period (6, 14 and 35 days). Immersion in hot (50°C) freshwater for 5 minutes at each stage resulted in a <20% hatching success. Formalin treatment resulted in a <10% hatching rate if treated on day 6, but treatment on day 14 or 35 resulted in a higher hatch rate.

There has been very little published work on the biology of *E. hippoglossi*. Kearn (1974 a & b) describes the nocturnal hatching behaviour and the attachment mechanism of the oncomiracidia. Kearn and his co-workers (Kearn, 1962, 1963 a; b; c; 1964; 1967a; b; 1973; 1974a; b; c; 1975; 1982, 1984; 1985; 1988 a; b; 1990; Kearn & Whittington, 1991; Kearn *et al.*, 1993; Kearn *et al.*, 1996; Kearn *et al.*, 1999) have studied many aspects of the biology of *Entobdella solea* (van Beneden & Hesse, 1864), a parasite of the common sole (*Solea solea*) which form a useful basis for studies on *E. hippoglossi*, although the biology and ecology of the hosts are very different and it might therefore be expected that there will be corresponding differences in the biology and ecology of the two parasite species.

2.1.1. Growth and Longevity

The growth rate, longevity and time to sexual maturity of Monogenea varies greatly. For example Gallien (1935) showed experimentally that *Polystoma integerrigum* Froel takes three years to reach sexual maturity, while at the other extreme Bychowski (1957) found that it took only 6 days for the dactylogyrid *Dactylogyrus vastator* (Nybelin, 1924) to reach maturity on carp. Sproston (1945) suggests that growth may not stop when sexual maturity is reached, for example *Kuhnia (=Octostoma) scombri* (Kuhn, 1829) began ovarian and testicular activity at a length of 3.5 mm but grew up to 6 mm. Maturity was reached at a size of 7.0mm in *Heterobothrium okamotoi* and they continued to grow up to a length of 24.3mm (Ogawa & Inouye, 1997). This is also true for *Entobdella soleae* which matures at a length of 2.0 mm and continues to grow to 5.0 mm (Kearn, 1963a). Kearn (1990) found that *E. soleae* can survive for up to 6.5 months at 12±1°C on *Solea solea*. The oldest parasite measured 6.9mm with an anterior hamulus length of 695µm after 182 days (Kearn, 1990). *Anoplodiscus cirrusspiralis* on juvenile snapper (*Pagrus auratus*) can live for up to 6 months, with a median longevity of 1.5 months (West & Roubal, 1998a). However West and Roubal (1998b) found a maximum longevity of 20 weeks (5 months) for the same species on adult snapper in sea cages. The growth rate of *A. cirrusspiralis* to 40 days p.i. was described adequately by a linear regression. However, from about day 30 growth reached a plateau (West & Roubal, 1998a). Less than half (41.6%) of the *A. cirrusspiralis* survived for over one month after attachment, with the greatest losses (32.7%) occurring in the first week (West & Roubal, 1998a).

2.1.2. Egg Production

Entobdella soleae first produced eggs 85 days p.i. (Kearn, 1990) and adults laid eggs at an approximate rate of 2 eggs per hour (Kearn, 1963a), but this rate increased with parasite size (Kearn, 1985). The time taken to assemble an egg was constant for all sizes of the parasite at 4-6 minutes (Kearn, 1985). As the parasites grew larger they tended to lay larger eggs (Kearn, 1985). The stalked eggs carry a sticky droplet and became attached to the sediment in groups (Kearn, 1963b). The eggs of *E. hippoglossi* are similar in form to those of *E. soleae*. They are tetrahedrally shaped (each side approximately 175μm in length) and connected by a long, fine filament. Pale eggs are released from the genital pore singly and, as they age, they become darker presumably due to the tanning process (Kearn, 1985). Once a number of eggs are produced the strings tend to become entangled and appear as a mass, rather than a single string of eggs (Gil-Ha, 1998). The eggs of *E. hippoglossi* do not appear to have the sticky substances associated with them as described for *E. soleae* (Kearn, 1963b).

There are few other studies on egg production in relation to parasite age or size in monogeneans. West and Roubal (1998a) found that *Anoplodiscus cirrusspiralis* eggs are first produced in worms 1.2mm in length, at about 20 days post-infection . Forty three adult *A. cirrusspiralis* deposited an average of 17.6 eggs (range 2-45 eggs) over a 24-hour period. In this species no correlation was found between worm length and number of eggs produced. Worms in the 2-2.5mm size group laid most eggs, with a reduced fecundity for larger, presumably older worms (West & Roubal, 1998a).

Hatching and development in marine monogeneans can be prolonged. For example the eggs of *A. lobianchi* have fully developed larvae present after 15-20 days at 15°C (Kearn, 1967) whilst the eggs of *A. elegans* began to hatch after 20 days at 16°C (Kearn, 1967). The eggs of *E. hippoglossi* take 46 days to hatch if maintained at 4-5°C for three weeks followed by incubation at 7°C, but only 25 days if kept at 12-13°C (Kearn, 1974a). Hatching in *E. soleae* is brought about by a proteolytic hatching fluid which is produced by two pairs of ventral head glands of the oncomiracidium and is spread over the inner surface of the opercular joint by rotation of the larva around its longitudinal axis. There is no sign of these glands after hatching has been completed (Kearn, 1975). The oncomiracidia of *Entobdella soleae* is 250µm in length, swims at 5 $mmsec^{-1}$ and lives for 24+ hours (Kearn, 1967). Their survival is dependent on their finding and attaching themselves to a specific host. The early part of development of this parasite takes place on the dorsal surface of the fish and then migration to the lower, ventral surface occurs.

This chapter looks at each of the phases throughout the life cycle of *E. hippoglossi*, including egg production *in vitro* and *in vivo* and incubation times, growth, survival and longevity. This information has been used in order to compile a poster for halibut farmers, outlining an efficacious treatment strategy.

2.2. Materials and Methods

2.2.1. Halibut Maintenance

Halibut are valuable fish and throughout this study there has been a limited number of fish available for experimentation. This meant that each experiment involved only a relatively small number of fish and separate experiments could not be set up for each study. Occasionally data for a number of studies had to be collected from the same fish.

Tank Set Up

Considerable difficulties were experienced in maintaining the number of *Entobdella* on the host. One of the main problems appeared to be the retention of eggs within the tanks. The fish were held in $1m^2$ tanks with a relatively high flow through rate and it was assumed that the eggs were being flushed from the tanks. Gravel from Machrihanish beach was placed into the tanks to form a substrate for the halibut to rest on and also for the egg bundles to tangle amongst. Care was taken to ensure that the gravel was too big to block the holes of the outflow grill so that the tank did not overflow. When halibut were transferred from the $1m^2$ tanks into the 2m tank (see below) the gravel was transferred at the same time.

2.2.2. Growth and Longevity

Infection Procedure

To establish experimental infections *Entobdella* were obtained from commercial halibut farms and university research facilities. The parasites were collected by removing them gently from the fish with the curved edge of forceps. It was not possible to anaesthetise the fish and therefore only parasites on the dorsal surface of the fish could be collected. Parasites were placed into wide necked thermos flasks with clean seawater and transferred to the Institute of Aquaculture marine facility at Machrihanish, Argyll. They were then removed from the flask using the curved edge of forceps and placed on the dorsal surface of the test halibut. The halibut to be infected were anaesthetised in 2-phenoxyethanol (1:1500) and placed into a shallow tray with seawater. The parasites were always placed in a group on the dorsal surface, to reduce the stress to the host.

Sampling Procedure

To monitor the progress of infections the halibut were removed from their tank and anaesthetised in 2-phenoxyethanol (1:1500). They were then placed into a shallow tank with clean seawater. The halibut were placed on to their dorsal surface first and the ventral surface observed for the presence of parasites, then turned over and the dorsal surface observed. Any parasites dislodged during this process were placed into clean seawater and then placed back onto the dorsal surface of the halibut before it was returned to the holding tank.

At each examination the position of parasites was marked onto a fish map and the length of each parasite measured. The parasites were measured while still attached to the host using a ruler, to the nearest millimetre at their "average" length i.e. not fully elongated or contracted. *Entobdella* continually elongate and contract and these measurements are therefore an approximation.

Owing to the complex nature of the following experiments designated infection experiment 1,2 and 3, the details described below are summarised in Fig 2.1.

Infection Experiment 1

Experiment 1 was established to study the life-cycle, growth, longevity and egg production of *E. hippoglossi*. An adult male halibut was obtained from the Sea Fish Industry Authority, Ardtoe on 19th September 1997 and transported to Machrihanish. It was maintained in a 2m round tank with a constant through flow supply of seawater at ambient temperature and an air supply. The fish was allowed to acclimatise for one week before being infected with *Entobdella*.

25th September 1997

Sixty nine *Entobdella hippoglossi* were collected from the dorsal surface of one broodstock halibut held in a cage at the North Atlantic Fisheries College, Shetland. The *Entobdella* were gently removed from the fish with the curved edge of forceps, and placed into plastic containers filled with clean sea water. Parasites were transferred into a thermos flask filled with clean sea water and the flask was placed into an

insulated box filled with ice packs to ensure that a constant temperature was maintained during the 12 hour transportation period. Thirty-four of these parasites were then placed on to the dorsal surface of the experimental adult fish at Machrihanish. The parasites were removed from the thermos flask using the curved edge of forceps and placed on the anaesthetised halibut, ensuring that the haptor was in contact with the dorsal surface.

29th September 1997

Eighty seven *Entobdella* were collected and transported as above from Otterferry Salmon, a commercial fish farm where the broodstock halibut were held in 4m round tanks. A further 33 *Entobdella* were placed onto the dorsal surface of the same adult fish in Machrihanish.

The infected halibut was examined (see monitoring procedures above) approximately once per week for 27 weeks to observe the parasite population. The number and position of the parasites were recorded, including the details of first re-infection i.e. the appearance of juvenile *E. hippoglossi*.

8th January 1998

After approximately 4 months two uninfected immature halibut (1994 year class, obtained from SFIA, Ardtoe) (designated Juv 1 and Juv 2) were placed into the tank with the adult fish to ascertain whether they could become infected with oncomiracidia present within the tank system.

12th January1998

Due to the natural mortality of the parasites the same adult fish was re-infected with 79 more *Entobdella* from Otterferry Salmon.

1st April 1998

The infected adult fish died 189 days after the start of the experiment. Forty of the fifty four parasites remaining on the adult fish were removed and attached to Juv 1 and Juv 2, 21 and 19 parasites respectively using the method described above. The juvenile fish (Juv 1 and Juv 2) were placed into tank 1.4 along with the 3 fish from Infection Experiment 2 (see below) on 14th April 1998.

Infection Experiment 2.

Due to difficulties maintaining numbers of the parasite on the adult fish alone it was necessary to increase the number of fish infected thus creating a greater reservoir of parasites from which growth data could be obtained and that were available for egg laying experiments. Experimental Infection 2 was initiated to determine the rate at which juvenile *E. hippoglossi* first become visible on halibut previously infected with adult *E. hippoglossi* and then to obtain growth data for the newly attached parasites and also to monitor fish for pathology caused by the parasite. Three 1994 year class halibut (obtained from SFIA, Ardtoe) were placed into a $1m^2$ tank (tank 1.4) with a constant flow through supply of seawater at ambient temperature and an air supply. These fish were designated 1.4.1, 1.4.2 and 1.4.3.

12th January 1998

Each of these halibut had 25 *Entobdella* from Otterferry Salmon placed onto their dorsal surface. These fish were monitored weekly for the first 245 days and then on a monthly basis until monitoring ceased on day 875. The number, length and distribution of *Entobdella* was noted on a fish map at each sampling.

14th April 1998

After 3 months Juv 1 and Juv 2 were moved into this tank and the monitoring continued. The fish were readily distinguishable from each other by their natural markings. Juv 2 and 1.4.2 both died during the course of this study (10th December 1999 and 14th July 1999, respectively).

The fish in this tank were each re-infected with 15 and 18 individual *Entobdella* from Otterferry on 11th February 1999 and 10th December 1999 respectively. All surviving fish (1.4.1, 1.4.3, Juv 1) were moved to a 2m tank on 10th February 2000 due to changing tank requirements at Machrihanish.

Infection Experiment 3.

In order to obtain more data for analysis of the distribution of *Entobdella* on its host (see Chapter 5), and for growth data and re-infection rates a further experimental infection was initiated. Approximately 8 months (22nd September 1998) after infection experiment 2 was set up, four 1994 year class halibut from SFIA, Ardtoe were placed

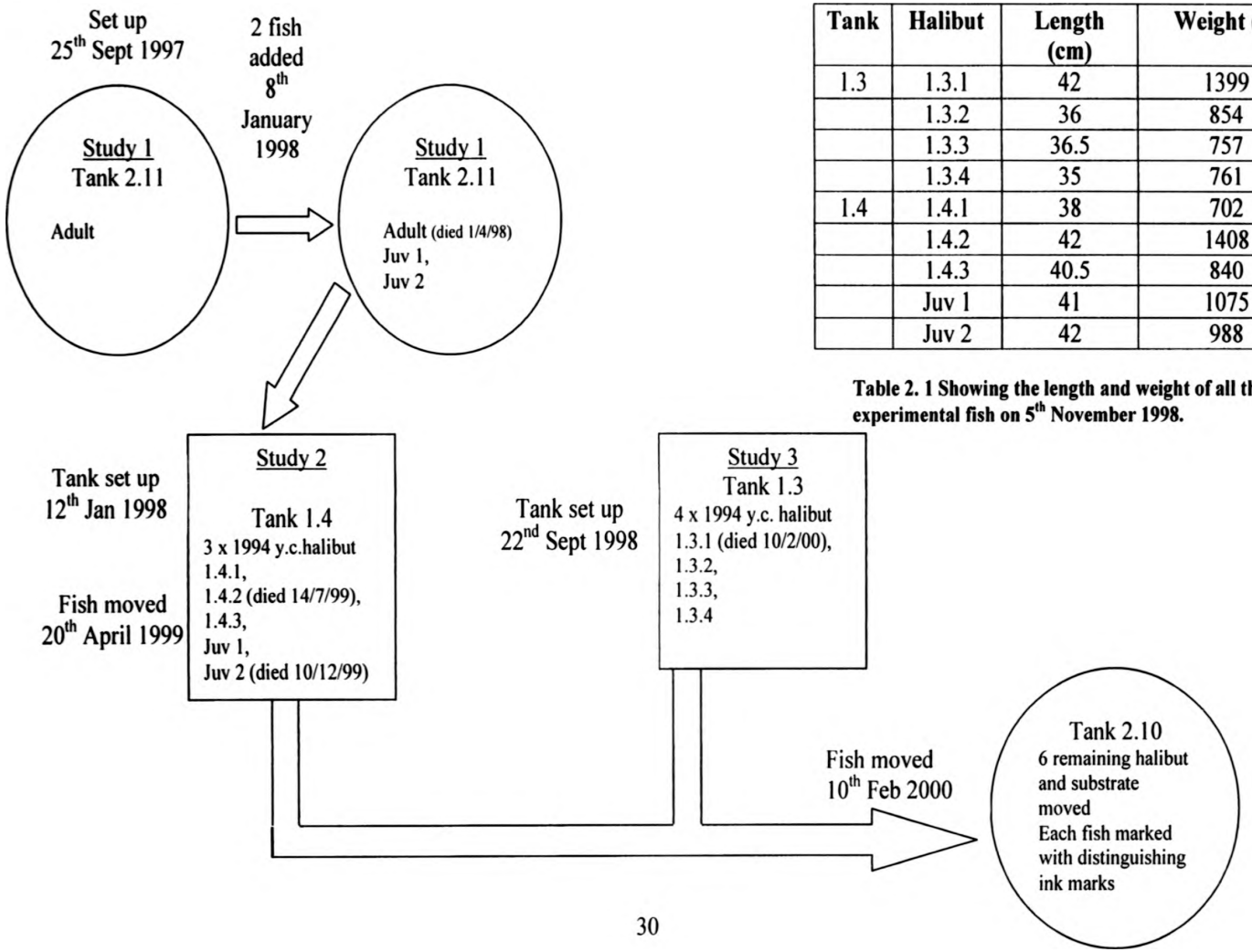
into a $1m^2$ tank (tank 1.3) with identical conditions to experimental infection 2. These halibut were designated 1.3.1, 1.3.2, 1.3.3 and 1.3.4. These fish were each infected with 25 *Entobdella* from Otterferry Salmon. These fish were monitored weekly until day 45 when only 9 of the original 100 parasites remained in this tank, however, the fish were retained in the tank. On day 307 post-infection it was noticed that the fish had large numbers of *Entobdella* (27-49 per fish). From this day the fish were examined on a monthly basis until the experiment was ceased. Fish 1.3.1 died on the 10th February 2000 during the study.

On 10th February 2000 the 6 remaining halibut were moved into a 2m round tank. Each of the halibut was marked individually using a panjet. The halibut continued to be examined over the next 5 months, at least once per month until 7th June 2000. Figure 2.1 summarises these movements and the sizes of the halibut used during these studies.

2.2.3. Egg Production

The number of eggs produced by *Entobdella* was ascertained for parasites *in vivo* (attached to the host) and *in vitro* (parasites removed from the host and placed into plastic six well plates with sea water).

Figure 2. 1. A summary of the movements of halibut infected with *E. hippoglossi* at Machrihanish during the experimental period (25th Sept 1997-7th June 2000).



Tank	Halibut	Length (cm)	Weight (g)
1.3	1.3.1	42	1399
	1.3.2	36	854
	1.3.3	36.5	757
	1.3.4	35	761
1.4	1.4.1	38	702
	1.4.2	42	1408
	1.4.3	40.5	840
	Juv 1	41	1075
	Juv 2	42	988

Table 2. 1 Showing the length and weight of all the experimental fish on 5th November 1998.

In vivo egg production

The number of eggs produced by *Entobdella in vivo* was determined by anaesthetising 2 halibut (1.4.1 and 1.4.3), counting the number of *Entobdella* present and measuring their lengths. The fish were then placed individually into sealed tanks (90x55cm, perspex tanks) with a flow through supply of seawater and a constant supply of air. The water from the out flow was passed through two mesh sizes (5mm and 50μm) in order to retain any eggs flushed from the tank. The fish were left in the tanks for 24 hours and then removed and put back into their holding tank. The tank and any tubes within were rinsed thoroughly to dislodge any remaining eggs. All eggs captured in the mesh were collected by examining the meshes using a dissecting microscope and gently picking the egg masses with a pair of fine forceps. The eggs were placed into clean seawater in three 500ml glass beakers and held in the dark at 6°C until they hatched. The water was changed every other day and observations of the developing embryos using a dissecting microscope were made at least once per week, daily towards the end of the development period, in order to ascertain when hatching commenced. A random selection of the eggs were temporarily placed on to a glass slide with a large drop of sea water, viewed under the microscope then returned to their glass beaker. Once hatching had ceased, i.e. no hatching occurred for at least two days, the eggs were fixed in 70% ethanol. The total number of eggs in each beaker and the number of hatched eggs, recognised by the loss of the operculum were, counted with the aid of a Zeiss (Kontron System 3000) image analysis package attached to an Olympus BH-2 binocular microscope. The egg masses were placed on a microscope slide, teased apart gently so that they formed a single layer and covered with a

coverslip. They were then observed at x20 using an Olympus BH-2 binocular microscope with a video camera attached. Once a group of eggs was isolated on the video monitor the image was saved and using the image analysis package the eggs were counted by marking each one, thus ensuring that they were not counted twice. This process was repeated with each bundle of eggs and the total number of eggs calculated.

In vitro Egg Production

Five *Entobdella* were removed from fish 1.4.1,1.4.2 and 1.4.3 and 10 *Entobdella* from the adult fish and placed individually into the wells of 6 well plates. Each of the parasites was measured. The plates were then incubated at 6°C. The number of eggs laid was observed every 3 hours over a 24-hour period. At each sampling the eggs present in each well were counted and removed. The sea water was replaced with clean seawater at 6°C. All eggs collected from parasites removed from fish 1.4.1, 1.4.2 and 1.4.3 were combined and those from the parasites from the adult fish were combined and put into separate 500ml beakers with clean seawater which was changed every other day. Incubation procedures were as for the *in vivo* experiments. After the 24-hour period the parasites were gently removed from the 6 well plates and put back onto the host.

Egg Production and Size of Entobdella

To determine how many eggs were produced by different sized *Entobdella in vitro* over a 24-hour period 50 parasites ranging in length from 5-17mm were removed from

the hosts (Adult, Juv1 and Juv2, 1.4.1,2,3), measured, placed individually into 6-well plates and kept in an incubator for 24 hours at 6°C as described above. The total number of eggs produced over the 24 hour period were then counted as described above. The parasites were replaced onto the host.

Egg Hatching and Temperature

The eggs collected from the size relationship experiment were collected and split randomly into three groups and incubated at three different temperatures (mean temperatures were 5.1°C, 9.9°C and 14.4°C). Each group of eggs was split into 5 replicates of approximately similar sizes. Splitting the eggs was done by naked eye as it was thought that counting the eggs under a microscope with high light levels, at this early stage of development, might damage the embryo. The eggs were kept in the dark, apart from during weekly microscope observations and while water changes took place. Incubation and counting procedures were as described above.

Egg Hatching in Relation to Habitat of *Entobdella*

Twelve *Entobdella* were removed from the ventral surface and 12 from the dorsal surface of the adult fish. These groups of parasites were placed separately into petri dishes filled with clean seawater and placed into an incubator at 6°C. After 24 hours, all the eggs produced from each group were collected and incubated separately until hatching occurred as described above. The hatching success of eggs produced by parasites living on different areas of the halibut was compared. The eggs were counted as described above.

2.3. Results

2.3.3. Growth and Longevity

Infection experiments 1 & 2 showed that the immature halibut were suitable hosts for attachment of the oncomiracidia of *Entobdella* and for their subsequent growth, egg laying and adult survival.

During infection experiment 1 the first natural infection of juvenile *Entobdella* on the adult fish was seen 83 days after the initial introduction of *Entobdella* to the tank (average temperature 11.4°C, range 8-14°C). Juvenile *Entobdella* were first seen on the immature fish 48 days after they were put into the tank with the adult fish (average temperature 8.3°C, range 7-10°C). The first juvenile parasites were observed 93 days after the original infection (average temperature 8.3°C range 7-10°C) during infection experiment 2. Juvenile *Entobdella* first became visible to the naked eye when they reached 2-3mm in length. It was not possible to ascertain the exact date of attachment or the growth rate from attachment to 2-3mm in length as it was not possible to examine these fish (1-1.5kg) under a microscope.

Table 2.2 shows the mean length±standard deviation of the parasites from the fish in infection experiment 2 at the start of this study and on the last sample date. The study started on the first day that newly attached juvenile parasites were observed on each fish and finished at the point when either all of the study parasites had died or when the

next generation had grown to the same size and therefore the study parasites could not be distinguished. Therefore the start and finish dates are slightly different for each fish studied. Also shown are the number of parasites sampled on the first and last sample day and the peak number of parasites present during the study period. The increase in length over the period of study is also shown. Each of the populations grew continually throughout the study period. The growth rate for each of the populations was approximately 0.1mm per day. The average temperature throughout the study period was 11.6°C (range 7-15°C).

The continual growth of the individual populations of parasites on 5 halibut (1.4.1, 1.4.2, 1.4.3, Juv 1 and Juv 2) are shown in Figure 2.2. Each of the populations was monitored over a slightly different period as the juvenile parasites became apparent at different dates. These fish had become re-infected naturally from eggs laid by the *Entobdella* originally introduced. The number of parasites present on the hosts fluctuated over the period as new parasites attached and then towards the end of the study period as they began to die off.

Fish	Mean length±S.D. (mm) and Number in Sample (n)				Peak population size	Mean Size increment over sample period	No. of days of study	Growth rate ($mmday^{-1}$)
	Initial sample		Final sample					
	mm	n	mm	n				
1.4.1	2.3±0.6	3	12.5±3.5	2	12 (Day 50)	10.2	80	0.12
1.4.2	4.5±0.7	2	14.0±1.0	3	7 (Day 55)	9.5	84	0.11
1.4.3	2.4±0.5	7	11.9±2.5	14	26 (Day 70)	9.5	100	0.10
Juv 1	4.2±1.7	6	10.6±2.6	5	10 (Day 34)	6.4	65	0.10
Juv 2	4.3±0.6	3	12.6±2.9	7	16 (Day 40)	8.3	70	0.12

Table 2. 2. The growth and number of individuals within the *Entobdella* populations from individual hosts. The length of time each of the populations was monitored is presented in days. Data collected from infection experiment 2.

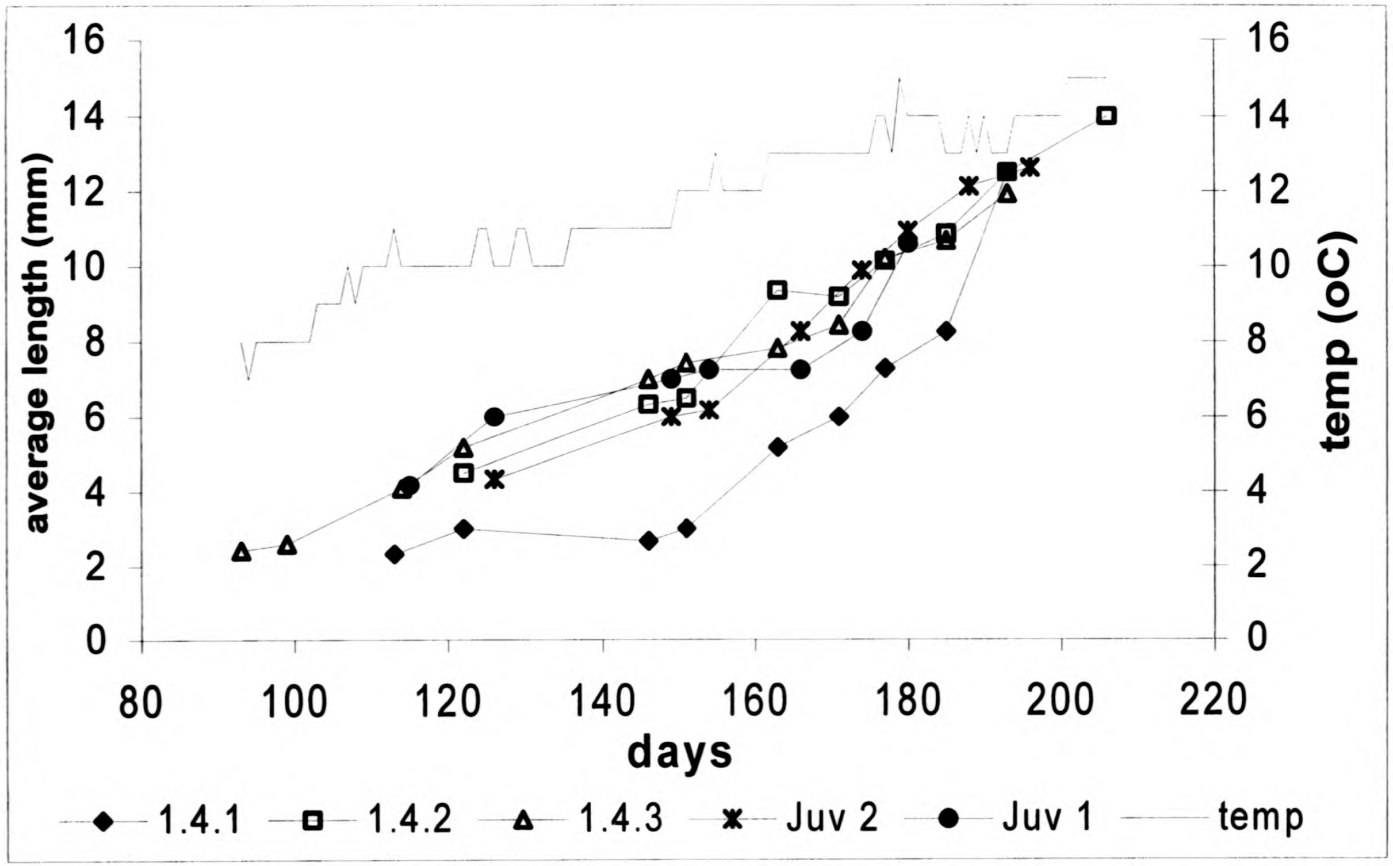


Figure 2. 2. The growth of the *Entobdella* populations on individual halibut with time in infection experiment 2. Temperature (no markers) is shown on the secondary Y axis.

The longevity of *Entobdella* was difficult to ascertain from these studies as there were mixed cohorts of parasites present on the halibut. Longevity of adult parasites was ascertained by monitoring the parasites used to infect the 3 halibut in infection experiment 2 over approximately 150 days. Within this study period new parasites became established on the hosts, however, as the older parasites were much larger in size it was clear how many of the original parasites remained. On the day of the infection the average length of the 75 parasites was 12.6mm. It is not known how old these parasites would have been at this point but they would be at least 100 days old assuming the growth rate of 0.1mm per day. Figure 2.3 shows the survival of *Entobdella* over the 21-week period. After 52 days 46.6% of the parasites remained and after 99 days 18.6% remained (Figure 2.3). On day 146 only one of the original parasites remained. It measured 21mm in length.

Table 2.3 shows a summary of the growth of the size of this population of *Entobdella* over time. Although the population was ageing and decreasing in number the surviving parasites were still growing and they were also observed to produce eggs as late as day 112.

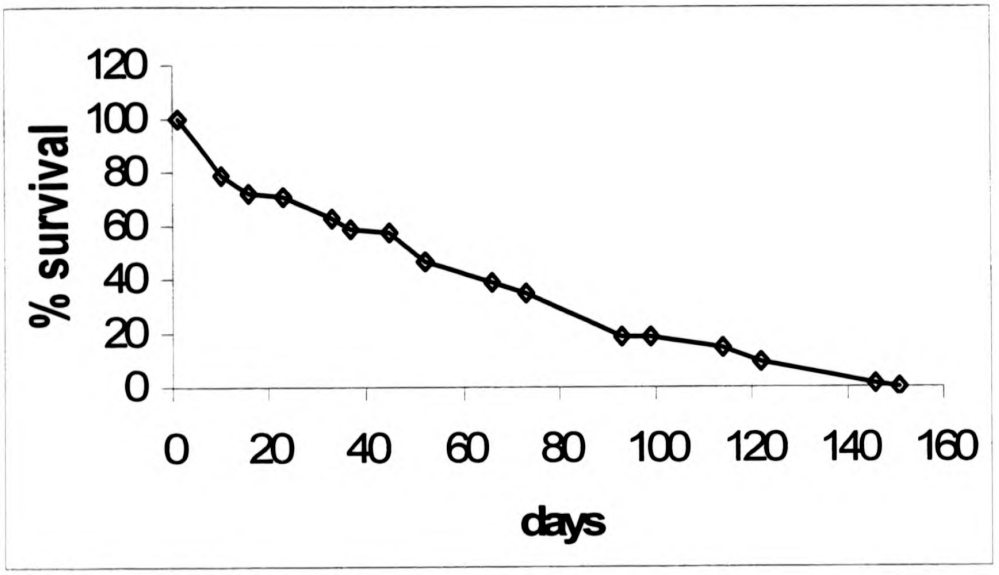


Figure 2. 3. The survival of a population of *Entobdella hippoglossi* from 3 halibut. These parasites had a mean length of 12.6mm on day 1 of this experiment (infection experiment 2).

Day	Number of parasites	Length range (mm)	Mean length±S.D. (mm)
1	75	10-15	12.6±2.6
73	26	15-19	16.5±1.3
99	14	16-20	17.6±1.4
146	1	21	21

Table 2. 3. A summary of the survival of *E. hippoglossi* over a 146 day period in infection experiment 2 and the growth data for the survivng parasites.

2.3.4. Egg Production

Table 2.4 shows the summarised results for the *in vitro* and *in vivo* egg laying experiments.

In vivo Egg Production

The mean number of eggs produced by *Entobdella in vivo* was 90.2 eggs from fish 1.4.1 (mean length of *Entobdella* 13.24mm; n=14) and 156.5 eggs from fish 1.4.3 (mean length of *Entobdella* 14.06mm; n=13) (Table 2.4).

In vitro Egg Production

There was a close relationship between the length of the *Entobdella* and the total number of eggs produced over the 24-hour period (see Fig. 2.5). Of the 10 parasites removed from the adult fish 5 were smaller (mean length 8.2mm) than those removed from fish 1.4.1,1.4.2 and 1.4.3 (mean length 12.1mm) and thus in order to compare the number of eggs produced from all 4 individual halibut only the egg production of the 5 largest parasites from the adult fish were used in the calculations.

Parasites *in vivo* produced fewer eggs over the 24-hour period than those removed from the host, i.e. a mean of 122.1 and 186.0 respectively. The total number of eggs produced by 5 parasites from adult halibut was 932.0 and is not significantly different from the number of eggs produced by 5 parasites from the 3 juvenile hosts, mean of 928.3±28.7 eggs (Student's t-test, d.f.=18; p=0.966). Figure 2.4 shows that the pattern of *in vitro* egg laying was very similar in parasites from all fish. The number of eggs

produced over each 3-hour period was relatively similar for the first 9 hours and then the numbers decrease throughout the remainder of the 24 hour period.

The eggs produced by *Entobdella in vivo* had a higher hatch success than those produced *in vitro*. Significantly more eggs hatched if produced in vivo than if they were produced in vitro (χ^2=1591.4; d.f.=3; p=0)

Egg Production and Size of *Entobdella*

Figure 2.5 shows the number of eggs produced over a 24-hour period by *Entobdella in vitro* plotted against parasite length and a linear trendline (calculated using the linear equation y=mx+b) added (R^2=0.714). It is clear that as the parasite grows in length it has the ability to produce more eggs and that this relationship is linear. *Entobdella* appear to be unable to produce eggs before they reach 9mm in length but can produce as many as 465 eggs at 17mm over 24 hours.

In vivo* or *in vitro	**Fish**	**No. of parasites**	**Mean parasite length±S.D. (mm)**	**Mean no. of eggs produced (24 hours)±S.D.**
In vitro	1.4.1	5	12.0±1.2	186.4±33.4
	1.4.2	5	11.8±0.8	179.6±37.8
	1.4.3	5	12.4±1.1	191.0±15.3
	Adult	5	12.4±1.1	186.4±45.2
Mean overall		**20**	**12.2±1.0**	**186.0±32.1**
In vivo	1.4.1	14	11.9±1.1	90.2
	1.4.3	13	12.2±1.3	156.5
Mean overall		**27**	**12.1±1.2**	**122.1**

Table 2. 4. Summary of mean number of eggs produced by *Entobdella hippoglossi in vitro* and *in vivo* over a 24-hour period.

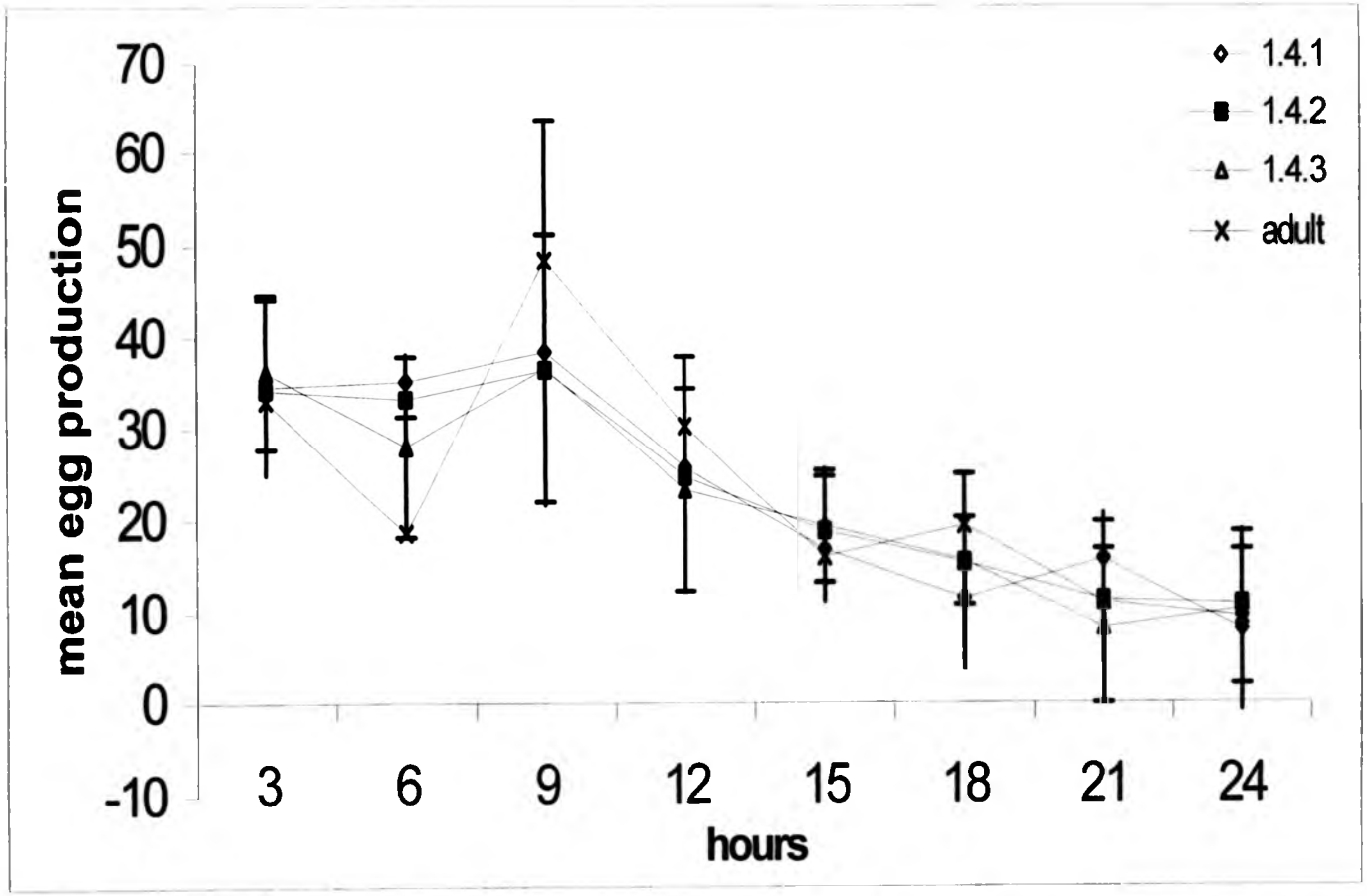


Figure 2. 4. Shows the mean±s.d. number of eggs produced at 3 hour intervals over a 24 hour period. Each point represents the mean number of eggs produced by 5 parasites.

Egg Hatching and Temperature

The time to first hatch for *Entobdella* eggs is temperature dependent. It took *Entobdella hippoglossi* eggs 51-54 days for hatching to complete at 5.1°C, 27-30 days at 12°C and 34 days to commence hatching at 9.9°C. Eggs incubated at an average temperature of 14.4°C failed to hatch.

Temperature (°C)	Time (days)
5.1	51-54 (complete hatch)
12.0	27-30 (complete hatch)
9.9	34 (commence hatching)
14.4	No hatch

Table 2. 5. Summary of hatching data for *Entobdella hippoglossi* eggs incubated at different temperatures.

Egg Hatching in Relation to Habitat of *Entobdella*

The mean length±S.D. of the two groups of parasites was calculated and were virtually identical, worms from the dorsal surface measured 14.67±1.6mm and those from the ventral surface measured 14.67±1.2mm.

The eggs taken from the dorsal and ventral surfaces of the halibut had a very similar hatch success. A total of 3841 eggs were laid by parasites from the ventral surface and 3426 were produced by parasites from the dorsal surface. There was no significant difference between the hatch success of the eggs from the eggs produced on either surface of the fish (χ^2 =3.519; d.f.=1; p=0.061)

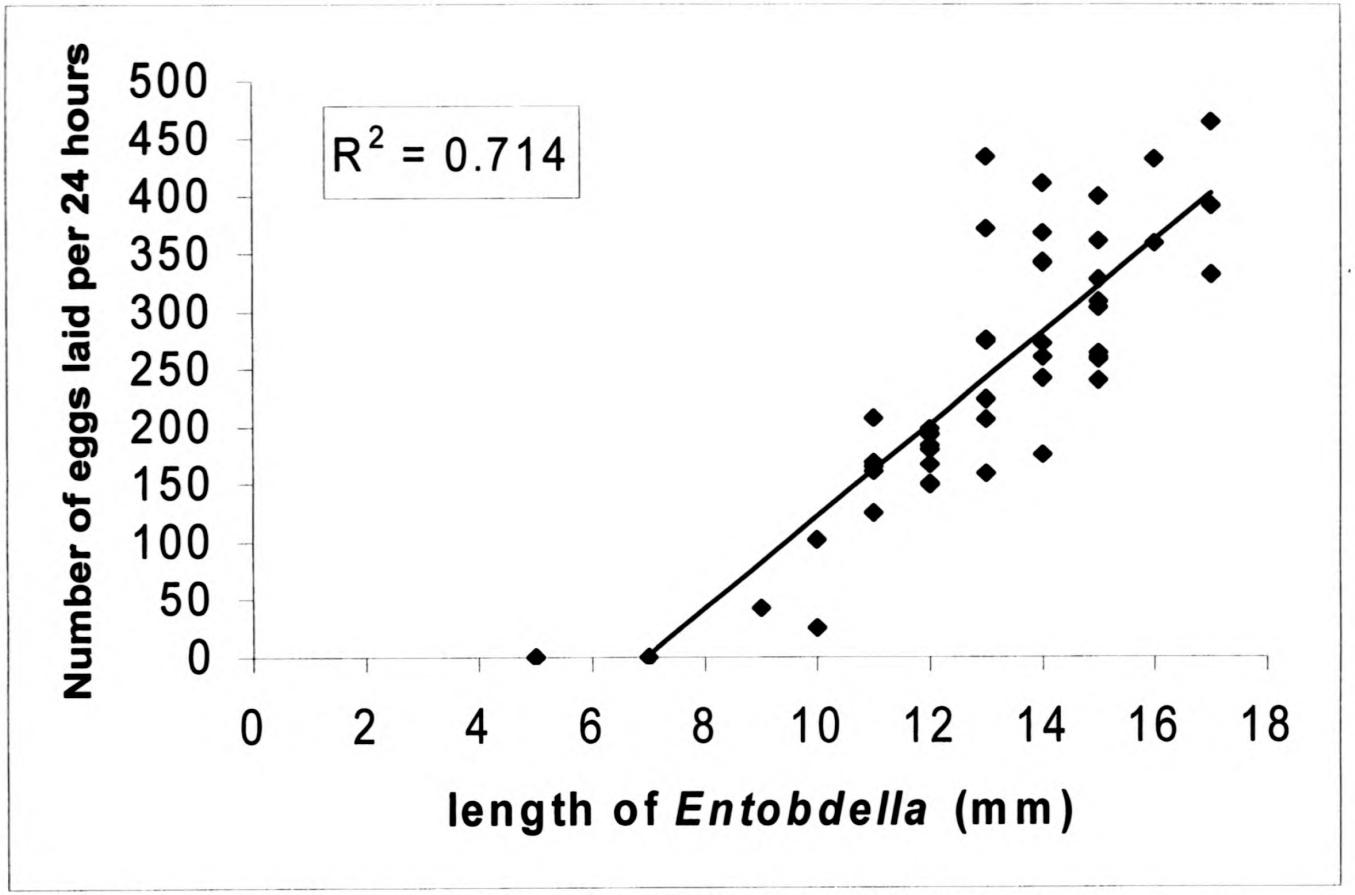


Figure 2. 5. Showing the number of eggs produced over a 24 hour period by *Entobdella* of different lengths. Trendline shows the mean number produced for each size group.

2.4. Discussion

2.4.3. Growth and Longevity

The time taken for the appearance of juvenile parasites after an initial infection with adult *E. hippoglossi* appears to be temperature dependent. At 8°C juveniles were first seen after 93 days and at 11°C after 83 days. This might be expected from the egg hatching data which showed that eggs took considerably longer to hatch at lower temperatures. Attached oncomiracidia may also grow more slowly at lower water temperatures. Unfortunately due to their small size it was not possible to determine the exact date of attachment or early growth rate of the parasites.

Adult *E. hippoglossi* were long-lived and survived for at least 146 days after they reached an average length of 12.6mm. It is unclear how long it takes from attachment to reach egg laying size (9-10mm) as juvenile parasites only became visible at 2-3mm, however, it took approximately 84 days to reach maturity from this size at 11.6°C. Thus the longevity of *E. hippoglossi* is at least 230 days at that temperature. The parasites continued to grow after they reached sexual maturity and the maximum length of any parasite on experimentally infected halibut was 21mm. Egg production began at 9mm in length and the number of eggs produced increased with size of the parasite.

Ogawa and Inouye (1997) found that *Heterobothrium okamotoi* on cultured tiger puffer (*Takifugu rubripes*) began producing eggs at around 7mm in length and continued to

grow to 24.3mm. They estimated the life span to be approximately 4 months at an average temperature of 21.1°C. *Anoplodiscus cirrusspiralis* first produces eggs at a length of 1.2mm and continues to grow to a length of 2.5mm, reaching 2mm in length in approximately 40 days (West & Roubal, 1998a). These authors suggest that *A. cirrusspiralis* on snapper (*Pagrus auratus*) can live up to 6 months with a median longevity of 1.5 months under experimental conditions, whereas in cage culture conditions the maximum longevity was estimated to be 5 months with a median longevity of 2.2 months. It was unclear whether these differences were caused by the different environmental conditions or due to a difference in the size of the host (West & Roubal, 1998b).

Kearn (1990) experimentally infected sole (*Solea solea*) with specimens of *Entobdella soleae* in order to determine their rate of development and longevity. He found that the parasite began to assemble eggs 85 days post-infection and may survive for six and a half months (182 days) at 12±1°C and reach a length of 6.9mm. The anterior hamuli continued to grow all through the life of the parasite but the growth rate decreased with age. *E. hippoglossi* also continued to grow throughout its life, although hamulus length was not examined. There does not appear to be a decrease in overall body growth rate with age. The largest *E. hippoglossi* collected from this experiment (21mm) were similar in size to those reported from wild halibut (20mm) (Schram & Haug, 1988). It should be noted however, that in captivity, the size of infection is usually higher (e.g. 641 parasites, Gil-Ha (1998)) than in the wild (e.g. up to 97 parasites, Schram and Haug

(1988)). More detailed analysis would therefore need to be carried out to determine whether growth rate and longevity are influenced by parasite density.

The longevity of *E. hippoglossi* can be attributed to its natural habitat of deep cold waters. Possibly due to low temperatures they do not reproduce or grow quickly, however they are longlived and can grow to be very large compared to most other Monogenea. Most examples mentioned in this chapter inhabit warmer water, e.g. *A. cirrusspiralis* experiments were undertaken at 20+°C at which they grow to 2.5mm in length and survive for up to 6 months (West & Roubal, 1998a). This parasite can produce 4 generations within one year, whereas it is quite possible that *E. hippoglossi* only produces one generation per year which coincides with the halibut spawning season in order to increase the chances of finding a host. In cage culture conditions halibut are maintained in ambient water temperatures which are considerably higher (~12°C) than those where halibut occur naturally (~6°C). These conditions may mean that this parasite will be able to reproduce more rapidly, therefore increasing the potential infection rates. Furthermore any eggs produced throughout the winter months will also develop (at a slower rate) and due to culture conditions there will always be a host present for the oncomiracidia to attach to, further increasing the risk of infection.

2.4.4. Egg Production

Larger *E. hippoglossi* produced more eggs over a 24-hour period than smaller specimens. This trend was also found in *E. soleae,* worms measuring 5mm in length produced 30 eggs per day while those measuring 6mm produced 60 eggs in the same

time period (Kearn, 1985). Whittington (1997) suggests that a similar trend exists across monogenean species, smaller species laying fewer eggs than larger ones, possibly due to smaller species having limited vitelline reservoirs or a slower rate of vitelline cell production (Kearn, 1985). This is apparently true for the two *Entobdella* species, *E. hippoglossi* reaching up to 21 mm and producing up to 465 eggs *in vitro* in 24 hours, whereas *E. soleae* measures up to 6mm in length and produces up to 60 eggs per 24 hour period *in vitro* (Kearn, 1985). This is probably due to the greater energy and vitelline reserves of the larger worm allowing for a greater rate of production.

West & Roubal (1998a) found that the number of eggs produced by *Anoplodiscus cirrusspiralis* decreased with size, after a critical length of 2.5mm. They suggest that this was possibly due to senescence of the larger worms. It would be interesting to monitor *E. hippoglossi* larger than 17mm (the largest observed in the egg laying study) to ascertain whether larger worms (up to 21mm) would produce fewer eggs. No *Entobdella* larger than 17mm were used in this experiment but, from incidental observations, parasites as large as 20mm were seen to produce eggs, however, it is not known whether senility affects the numbers of eggs produced. Kearn (1985) did not comment on whether egg production decreased once *E. soleae* reached a certain length. However he did note that some large adult parasites produced no eggs over a period of hours and suggests that egg production slows down or ceases at the end of the parasite lifespan.

This study showed differences in the number of eggs produced by parasites *in situ* on their host and those removed and laying eggs *in vitro*. The parasites produced on average 186 eggs *in vitro* and 122 eggs *in vivo* per worm per 24-hours, although the latter experiments were limited in scope. These experiments would need to be repeated with a larger number of fish and perhaps varying intensities of parasites to get a clearer picture of the real egg output of *Entobdella* under more natural conditions. Most other studies on egg production in monogeneans have been undertaken under *in vitro* conditions which may over-estimate egg production. *E. soleae* measuring 6mm produce 60 eggs in 24 hours (Kearn, 1985) and 17.6 eggs were produced per *A. cirrusspiralis* in 24 hours (West & Roubal, 1998a). Whittington (1997, Table 2) gives a summary of egg output across the spectrum of lifestyles in the platyhelminths *in vitro*. This author reported that the digenean liver fluke *Fasciola hepatica* measuring 15-30mm (similar to *E. hippoglossi*) produced 14 eggs per worm per minute, which would be over 20,000 eggs in 24 hours if this rate was maintained (from Tinsley, 1983). It is suggested that in these large digeneans there is a large capacity for the production of oocytes and vitelline cells and therefore the limiting factor for egg production may be the rate of nutrient uptake and assimilation. Parasites removed from the host may initially increase egg output as a response to adverse environmental conditions, however this rate can not be maintained if feeding is not resumed. To gain a more complete and realistic understanding and estimation of the number of eggs produced by flatworms, more studies should assess egg output *in vivo* as well as better understanding of the reproductive lifespan of the worm (Whittington, 1997).

This study on *E. hippoglossi* has shown that a higher number of eggs are produced *in vitro* over the first 9 hours after removal from the host and the number of eggs declines after this time, although egg production can continue up to 24 hours. The decline in egg numbers is presumably due to declining energy reserves of the worms. A sharp decline in the number of eggs produced by *Benedenia lutjani* was noted by Whittington (1997), such that after only 5 hours virtually no eggs were produced. These worms are much smaller (2.2mm) than *E. hippoglossi* and presumably have less capacity for egg production.

The hatching success of the eggs collected *in vivo* and *in vitro* differed. A successful hatch rate of 30-32% of the eggs laid *in vitro* was achieved whilst 50-85% of those laid *in vivo* hatched. This may be due to the parasites that had been removed from the host producing eggs of a lower quality due to diminished reserves. They also produced more eggs over the same period of time which may have been of reduced quality. However, there was a large variation in the successful hatch rate of the *in vivo* eggs and there may be a number of other factors such as light regime, movement and salinity which affect the hatching success. Kearn (1974a) found that *E. hippoglossi* eggs hatched within the first few hours of darkness when incubated in a 12:12 light:dark light regime. In this study the eggs were maintained in darkness apart from microscope examination of the eggs and it is possible that this environment was not beneficial to the hatching success of the eggs. However, all batches of eggs were treated in the same manner and hatching success of up to 85% was achieved. Kearn (1973) found that the oncomiracidia hatch from the eggs of *E. soleae* only at dawn. This is likely to have a

significant survival value on the larvae, as the sole are actively feeding during the night and at rest during the day so as the oncomiracidia hatch their host is stationary and, therefore, an easier target. This pattern is reversed in *E. hippoglossi* as hatching occurs in the first few hours of darkness when eggs are incubated at 7°C and with alternating 12 hours of dim blue light and darkness (Kearn, 1974a). Larvae do not emerge in significant numbers if the eggs are mechanically disturbed during the light or dark periods. Treating the eggs of *E. hippoglossi* with washings of halibut skin, sole skin or mucus from the halibut did not stimulate the hatching of the eggs (Kearn, 1974a). The free-swimming life of the oncomiracidia at 7°C is in excess of 24 hours, increasing to as much as 80 hours if the temperatures are low and within 4 hours of hatching they are able to attach to their host and shed their ciliated cells. This experimental evidence suggests that egg hatching corresponds with the halibut resting on the sea bed at night (Kearn, 1974a).

The length of time that the eggs are held in artificial conditions could also be a factor in hatching success. Incubating eggs appeared to have some fungal growth on them. The eggs of *H. okamotoi* took only 5-17 days to hatch at 15-25°C and hatch successes of 85.6-94.1% were achieved (Ogawa, 1997). Oncomiracidia were obtained from eggs of *E. soleae* after 27 days at 14.5°C (Kearn, 1985) and eggs of *A. cirrusspiralis* begin to hatch after 10 days at 20°C (West & Roubal, 1998a). The length of time taken for the *E. hippoglossi* eggs to hatch (up to 54 days at 5°C) gives opportunistic epibionts a chance to build up around the eggs, reducing the water quality and possibly directly affecting the health of the embryos. *E. hippoglossi* eggs are suitably adapted to their

natural habitat, they would be produced in cold water (~6°C) and presumably would take this length of time to develop in the wild. No parasite eggs hatched at 14°C which is towards the upper limit of the host's temperature tolerance and is presumably too high for the embryos to develop.

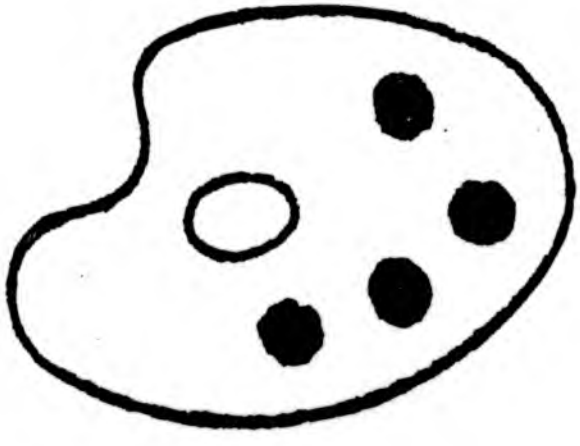
2.5. Conclusion

PLATE I shows the poster produced from data accumulated from these studies. This data has made it possible to design a control strategy for halibut farmers with *Entobdella* outbreaks in tanks. The industry uses formalin baths (1:2000 for 30-60 minutes) to control the attached stages of this parasite. This bath treatment does not appear to have an effect on the eggs and frequent re-infection occurs. These treatments are repeated when farmers observe high numbers of parasites on the halibut. Using the relevant data from this study it is possible to time these treatments more effectively, killing off the re-attached stages before they begin egg production.

Eggs take 27-54 days to hatch, depending on the temperature (5-12°C). The oncomiracidia have a short life and must attach to the host within the first 24-72 hours after hatching (Kearn, 1974a). It takes at least 84 days for the attached *Entobdella* to grow from 2mm to 10mm at 11.6°C and this period will be increased at lower temperatures. Further work is necessary to give more precise information on growth rates at lower temperatures. Sexual maturity and egg production commences when the parasite reaches approximately 9mm in length. At temperatures around 12°C it would be necessary to carry out a second formalin treatment around 100-110 days after the

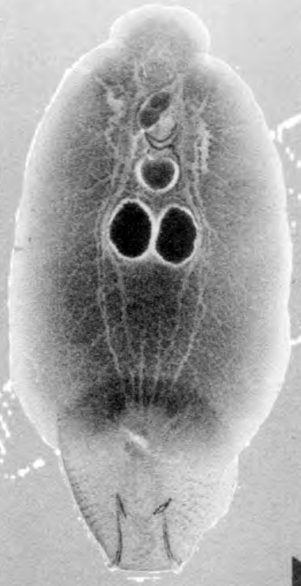
initial treatment in order to kill attached parasites before they begin egg production. At lower temperatures this period should be extended to around 150 days, allowing for the slower development rate. These controls allow the parasite to grow to a size that should be visible on the fish within a tank although not large enough to produce eggs. The farmers are thus able to determine whether parasites have been successfully removed by the treatment. If necessary a further formalin treatment can be carried out to ensure that all the attached stages are killed and no new eggs are produced. If this strategy is followed the number of eggs harboured within the tanks will be depleted over time and the threat of re-infection greatly reduced.

Original In Colour



Life cycle of *Entobdella hippoglossi*

SUMMARY OF TIMING	
egg ⇒ hatch	54 days @ 5°C 27-30 days @ 12°C
Oncomiracidium	24 hours without host
Attached *Entobdella*	approx. 200 days p.i.
First Eggs	approx. 84 days @ 11.6°C



Adult *Entobdella hippoglossi* grow up to 21mm in length.

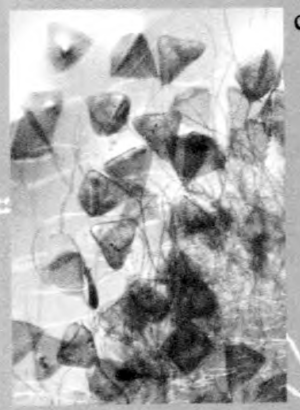
Longevity

Entobdella can live up to about 200 days (temperature dependent) once attached to the fish.

Egg production

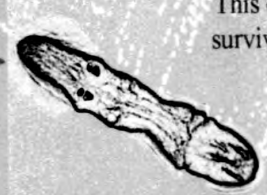
On average an adult *Entobdella* can produce 125 eggs in 24 hours (*in vivo*)

Clusters of tetrahedral eggs connected by a filament are laid and may accumulate on the substrate. Length of side approx. 175µm

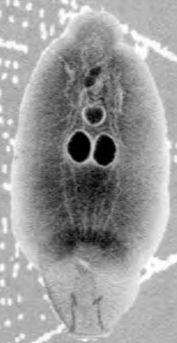


Eggs develop in sea water. Hatching can take c. 54 days @ 5°C, c. 27-30 days@ 12°C

Oncomiracidia emerge from the eggs. Approx. 500µm in length This ciliated, free swimming stage survives for up to 24 hours without the host.



The oncomiracidia finds and attaches to the host fish then loses its ciliated outer surface and uses its hooked posterior attachment organ.



When the *Entobdella* reaches 9mm in length it can begin to lay eggs

Continual Growth Period

It takes approx. 53 days @ 10°C (mean temp) for the attached oncomiracidia to grow to 11mm.

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PLATE I

Chapter 3

Developmental stages of *Lepeophtheirus hippoglossi* (Krøyer, 1837) (Copepoda, Caligidae)

3.1 Introduction

Lepeophtheirus is a common genus of caligid copepod, with up to 90 species found worldwide. They are well documented ectoparasites, predominantly found on marine teleost hosts (Kabata, 1979). Kabata (1979) describes the adult stages of the seven species of *Lepeophtheirus* found in UK waters and details their hosts and distribution. The main host of *Lepeophtheirus hippoglossi* (Krøyer, 1837) is the Atlantic halibut (*Hippoglossus hippoglossus* L.) and all parasitic stages are found predominantly on the ocular side of the fish ('parasitic on the backs of large halibut' (Scott & Scott, 1913). This chapter will describe the morphology of each of the life stages of *L. hippoglossi* and compare it to those of other North Atlantic caligid copepods, especially those known to cause damage to commercially valuable fish species. These include *Lepeophtheirus salmonis* (Krøyer, 1838), *Lepeophtheirus pectoralis* (Müller, 1776) and *Caligus elongatus* (von Nordmann, 1832). The *Lepeophtheirus* species tend to be relatively host specific, for example *L. salmonis* is found on Atlantic salmon (*Salmo salar* L.), brown trout (*Salmo trutta* L.), and *Oncorhynchus* species (Kabata, 1979), *L. pectoralis* is found predominantly on pleuronectid flatfish e.g. plaice (*Pleuronectes platessa* L.), dab (*Limanda limanda* L.) flounder (*Platichthys flesus* L.) and the Arctic flounder (*Liopsetta glacialis* (Pallas, 1776)). *Caligus elongatus* is less host specific and

has been identified from 80 different species of fish, including elasmobranchs and teleosts (Kabata, 1979). It is discussed here because it has been reported on Atlantic halibut in cages off the west coast of Scotland and is probably the most common parasitic copepod in British waters (Kabata, 1979).

3.1.1. Caligid Copepods

Figure 3.1 shows the genus *Lepeophtheirus* has a life cycle consisting of five phases and 10 stages, including 2 free-living nauplius stages, one infective copepodid stage, four permanently attached chalimus stages, two motile pre-adult stages and the adult stage, which is also motile (Kabata, 1972). In general the adult stages have a suborbicular or oval shaped cephalothorax with dorsal H-shaped sutures. In the female the genital segment varies in size and shape from suborbicular to elongate; in the male it is usually smaller and oval. The abdomen is usually cylindrical, consisting of up to 5 segments (Kabata, 1979). In the genus *Caligus* there is some discussion in the literature about how many stages exist in the life history. Piasecki (1996) suggests that *C. elongatus* has 8 life cycle stages with 2 nauplius, 1 copepodid, 4 chalimus stages and then an adult stage with no pre-adult stages. However, other authors have described early adult stages of *C. elongatus* as pre-adults (Hogans & Trudeau, 1989; Laverack & Hull, 1993). Other species of *Caligus* have been described as having one pre-adult preceding the adult stage, for example *C. clemensi* (see Kabata, 1972) or having 6 chalimus stages and one pre-adult stage, for example *C. epidemicus* Hewitt (see Lin & Ho, 1993). This may be due to the persistence of the filament into the pre-adult stage which sometimes is seen. *Caligus* species are distinguished by having two anterior lunules which are absent in *Lepeophtheirus*.

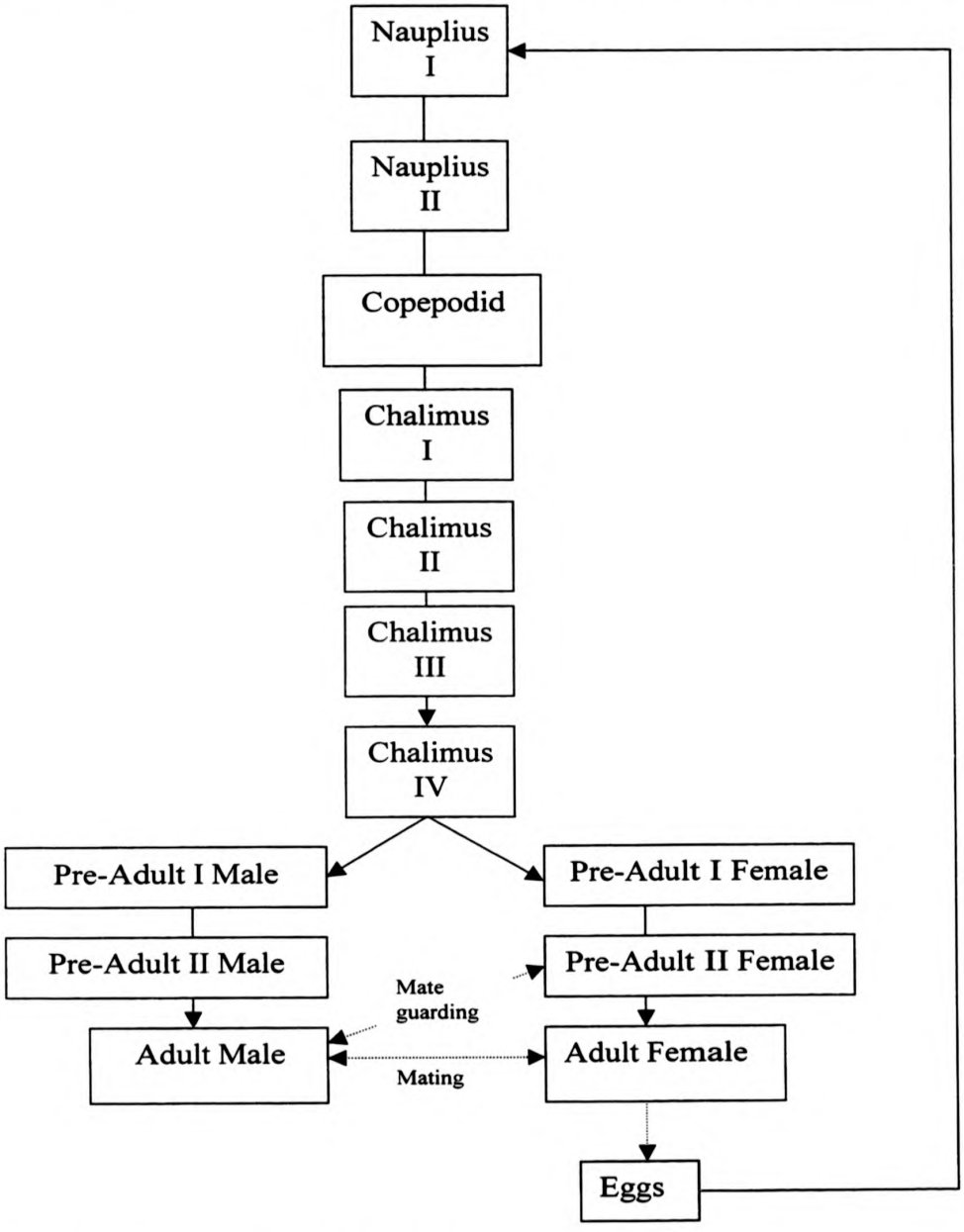


Figure 3. 1. Diagram showing the lifecycle of the genus *Lepeophtheirus*

3.1.2. *Lepeophtheirus hippoglossi*

Krøyer originally described *L. hippoglossi* in 1837 when he named it *Caligus hippoglossi*; Baird (who also reported it as *L. obscurus* in the same year) changed this to *Lepeophtheirus hippoglossi* in 1850. Drawings of the morphology of adult stages have been produced by Krøyer (1837), Wilson (1905), Scott & Scott (1913) and Kabata (1979). Kabata (1979) includes one drawing of the genital segment of the pre-adult female stage. However, there are no descriptions of all life stages of *L. hippoglossi*.

In 1913 Scott and Scott described the adult female *L. hippoglossi* as having a carapace longer than broad, comprising more than half of the total body length, about half an inch (c. 12mm), with frontal plates developed but no lunules. They described the free thoracic segment as small and the genital segment as being half the length and three quarters the width of the carapace, with lateral margins only slightly arcuate. The abdomen is short, with only one segment and a caudal ramus bearing a few apical setae. The male was described as having an orbicular carapace, as long as broad and equalling two-thirds of the total body length (6.5mm). The free thoracic segment and the genital segment were very small, with a pair of minute setiferous processes on each side at the postero-lateral corners (this may have refered to Leg 6). Wilson (1905) described the female as having an elliptical carapace, widest at the centre with distinct but narrow frontal plates. The genital segment was three fifths the length of the carapace, narrow and elliptical in outline and the abdomen one tenth the entire length, conical in shape and terminating in a pair of small anal lamellae armed with short plumose setae. Total length of the female was 12mm and the male 7.2mm. The male was again described as

having an orbicular carapace as wide as long, the genital segment was small and carrying two pairs of large papillae. The abdomen was described as more quadrilateral than the female with larger anal lamellae and longer setae (Wilson, 1905). Kabata (1979) described each of the appendages of the adult stages of *L. hippoglossi* in a little more detail. However, in general the three descriptions agree closely with one another. He described the female as having an orbicular cephalothorax with well developed frontal plates. The male had a similar cephalothorax and a suborbicular genital segment. The abdomen was one segmented, shorter than half the length of the genital segment and with a prominent uropod (Kabata, 1979).

Lepeophtheirus hippoglossi is unique among the European species of *Lepeophtheirus* in having a sternal furca with bifid tines (Kabata, 1979). This was described by Scott & Scott (1913) as the 'sternal fork with the basal part somewhat expanded, the rami short, tolerably broad and divergent, each ramus truncated at the end and split into two portions by a longitudinal sinus'. Due to this it has rarely been misidentified but there have been two such instances in the literature, one by (Baird 1850), misinterpreting the sexual dimorphism and describing the male and females as separate species and a second by Brian (1898; 1899) Kabata, 1979 gave no reason for this latter misidentification.

3.1.3. Other Caligid Species

All or most of the life cycle stages of six other species of *Lepeophtheirus* have been described in the literature. These include, *Lepeophtheirus dissimulatus* Wilson, 1905 (see Lewis, 1963), *L. hospitalis* Fraser, 1920 (see Voth, 1972), *L. pectoralis* (see Boxshall, 1974a), *L. salmonis* (see Johannessen, 1978; Johnson & Albright, 1991a), *L. tuberculatus* (see Il-Hoi, 1993) and *L. europaensis* (see Zeddam *et al.*, 1988). Additionally the life cycle stages of at least seven species of *Caligus* have also been described: *Caligus centrodonti* Baird, 1850 (see Gurney, 1934), *C. curtus* (see Heegaard, 1947), *C. orientalis* Gusev (see Hwa, 1965), *C. spinosus* Yamaguti, 1939 (see Izawa, 1969), *C. clemensi* (see Kabata, 1972), *C. pageti* Russel, 1925 (see Ben Hassine, 1983) and *C. elongatus* (see Piasecki, 1996).

Of these species, *L. dissimulatus* is a common parasite on Hawaiian acanthurid (surgeon) fish (Lewis, 1963), *L. hospitalis* is a parasite of the starry flounder, *Platichthys stellatus* (Pallas, 1787), on the Pacific coast of the USA (Voth, 1972), *L. europaensis* is found on brill (*Scophthalmus rhombus* L.) and flounder (*Platichthys flesus* L.) (Zeddam *et al.*, 1988) and *L. tuberculatus* is found on species of *Hexagrammos* (greenling) in Japan (Il Hoi, 1993). *C. spinosus* is found off both coasts of Japan (Izawa, 1969) on *Seriola* species (e.g. yellowtail), *C. clemensi* is found on pelagic fish such as the pink salmon (*Oncorhynchus gorbuscha* Walb.) off British Columbia (Parker & Margolis, 1964). *C. pageti* is found in the Mediterranean Sea (Ben Hassine, 1983). Thus some of these species are from hosts or geographical

locations widely disparate from that of *L. hippoglossi* and will not be discussed further here.

This chapter provides the first detailed descriptions of all the life-stages *of L. hippoglossi* and will enable their distinction from those other caligid species in the North Atlantic where halibut occur.

3.2. Materials and Methods

3.2.1. Collection of Material

Life stages were collected every two days following an experimental infection with copepodids of *L. hippoglossi* on Atlantic halibut (for full details of this procedure see Chapter 4, Section 4.2.3.1.). Sixty halibut weighing 49.6±20.3g (length 15.7±2.0cm) were held in two 1m square tanks with a flow through of water from a recirculation system and an air supply. The mean temperature over the period of the infection was 9.7±0.4°C. They were fed to satiation once per day. Copepodids were introduced to the fish and allowed to attach, every other day 2 fish were anaesthetised and all parasites present were removed. This process was continued until adult stages were present and samples of all life stages had been collected. Additional adult stages were collected from further experimental infections carried out at Machrihanish. In order to collect the free living stages ovisacs were collected from gravid females and incubated at 10°C (for full details see Chapter 4). Each of the three free-living stages were collected soon after hatching or after the moult from the previous stage.

3.2.2. Preparation of Material

The parasites were fixed and preserved in 10% formalin and then stored in 70% ethanol. Specimens were cleared using lactic acid, then temporarily mounted on glass slides and covered with a cover slip. The free-living stages tended to distort when fixed so they were drawn live. They were mounted in a well slide with glycerol to immobilise them and covered with a cover slip. Whole specimens and dissected appendages were examined under phase contrast using an Olympus BH-2 binocular research microscope. Drawings were made using a camera lucida and all measurements were made using a Zeiss Kontron System image analysis software. Images of whole animals were captured on the screen and using a specifically designed macro, measurements were collected.

As a guide to the following descriptions, Figure 3.2. shows both ventral and dorsal views of an adult male *L. hippoglossi* with the appendages annotated with the terms to be used in this chapter (from Huys & Boxshall, 1991). Further notes may be found on individual drawings in the descriptions of stages.

3.3 Descriptions of Developmental Stages

See following diagram.

3.3.1 Annotated Diagram of *Lepeophtheirus hippoglossi* (male)

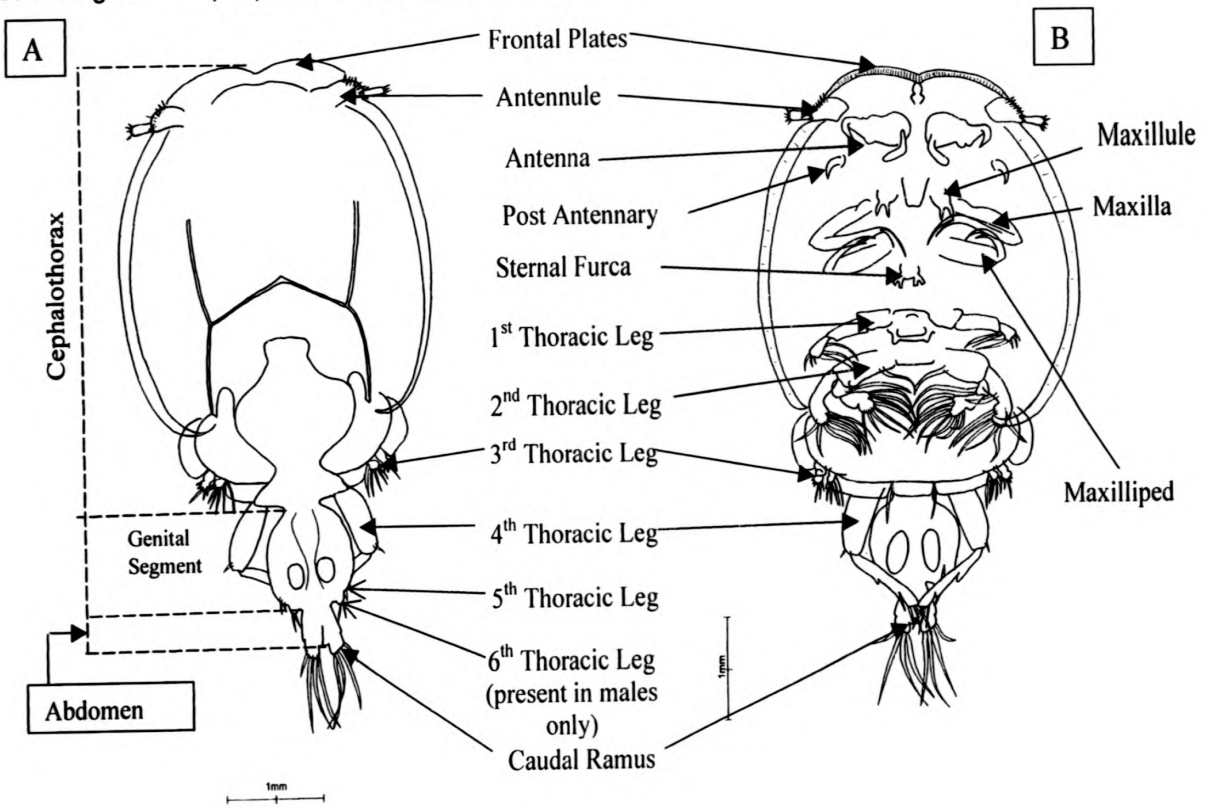


Figure 3. 2. A. dorsal and B. ventral view of an adult male *L. hippoglossi*, annotated with the names of each of the legs and other appendages (From Huys & Boxshall, 1991).

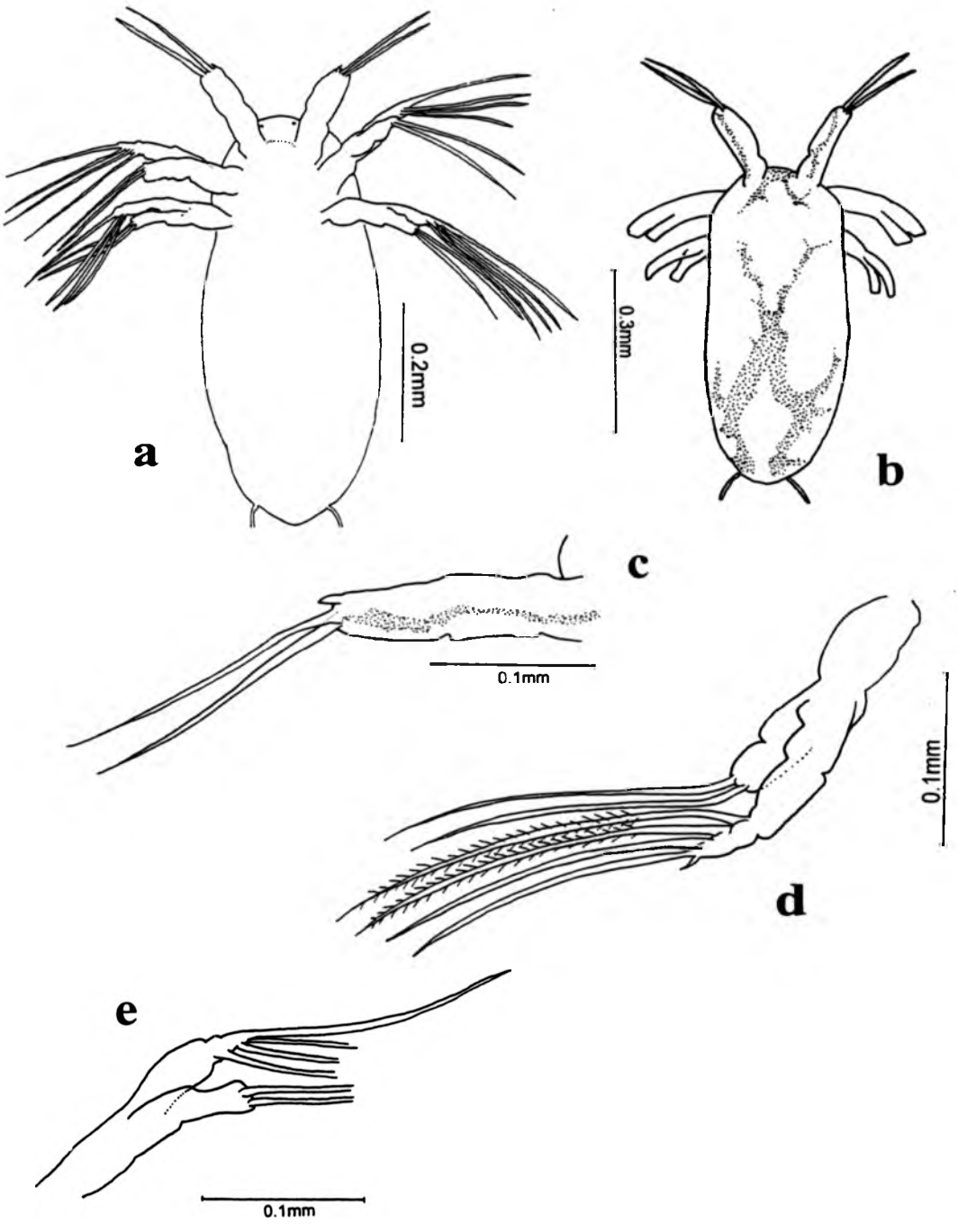


PLATE II. Figure 3.3. Nauplius 1. **a** ventral view; **b** dorsal view, including pigmentation pattern; **c** antennule, dorsal including pigmentation; **d** antenna, dorsal; **e** mandible, dorsal.

3.3.2. First Nauplius (NI)

(Figure 3.3 a-e) PLATE II

The first nauplius stage is 0.68±0.03mm in length and 0.24±0.01mm wide, based on 14 specimens. Free-swimming specimens (Fig. 3.3a) are cylindrical in shape, tapering posteriorly. The body has no external segmentation. There is a pair of unsegmented balancers on the lateral margins, at the posterior apex. The nauplii are virtually transparent and colourless over much of their body except for the deep pink/purple pigment spots forming a distinctive cross on the dorsal surface and along the antennules (Fig. 3.3b). This pattern is carried over to the second nauplius stage. There is a pair of eyes at the anterior end. All appendages originate at the anterior portion of the body. No mouth or anus is present.

Antennule (Fig. 3.3c) projects anteriorly, is uniramous and two segmented. The distal segment has 2 long unarmed setae, there is a short spine on the anterior tip. A line of pigment spots runs up the length of both segments.

Antenna (Fig. 3.3d) is biramous. The sympod is one segmented and unarmed. Endopod is shorter than exopod and indistinctly 2 segmented, proximal segment longer and unarmed, distal segment squat with 2 long setae. Exopod indistinctly 5 segmented, segments decreasing in size distally. Proximal segment with no armature, each of the other segments with one long seta originating from a distal process, distal segment with short spine at apex.

Mandible (Fig. 3.3e) is biramous, sympod indistinct from exopod. Endopod 2 segmented with 2 long setae from the distal segment. Exopod indistinctly 4 segmented with one long seta protruding from a distinct process on each of the three proximal segments. Segments decrease in size distally. Distal segment very small with one large seta.

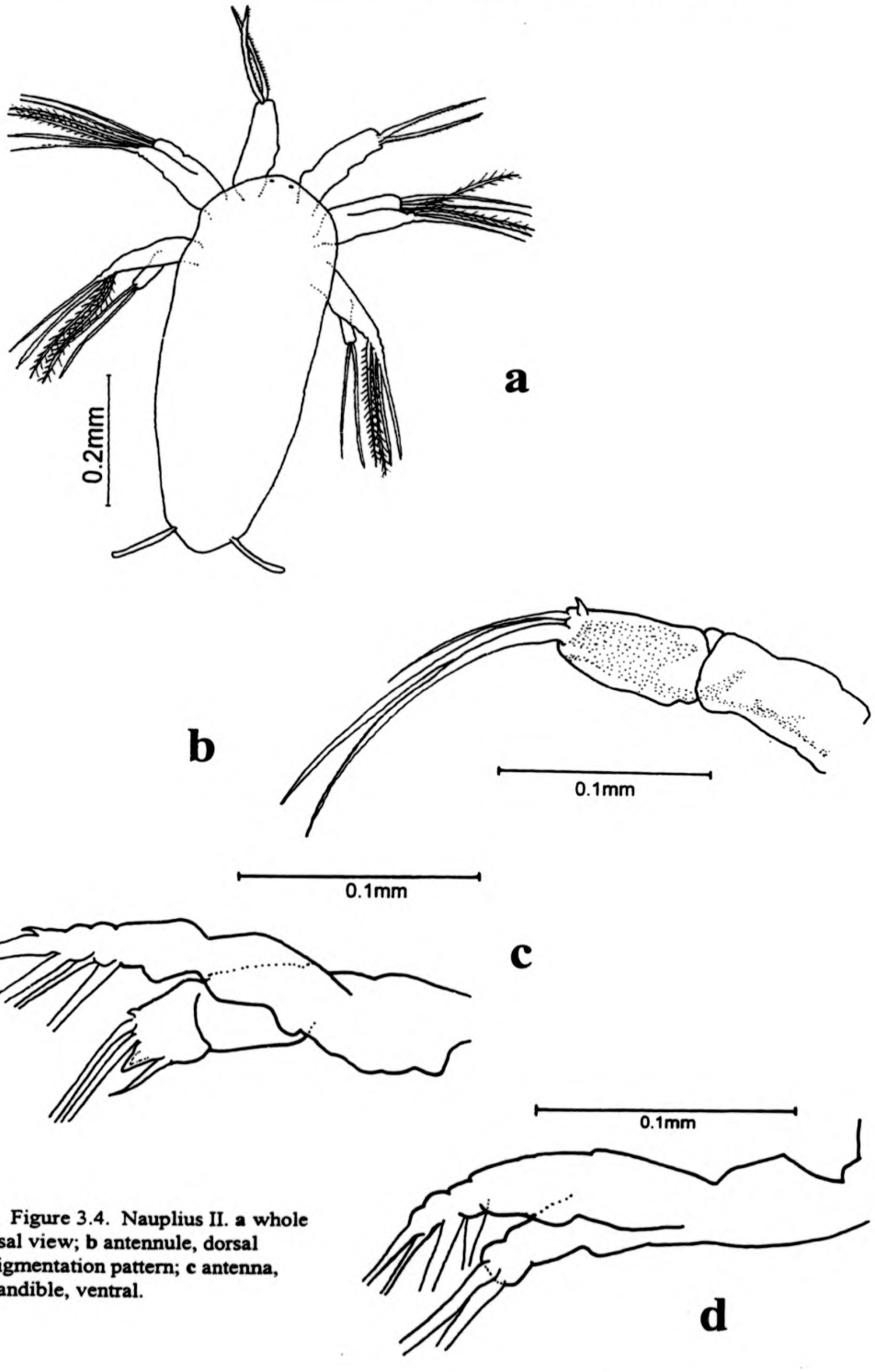


PLATE III. Figure 3.4. Nauplius II. **a** whole animal, dorsal view; **b** antennule, dorsal including pigmentation pattern; **c** antenna, dorsal; **d** mandible, ventral.

3.3.3. Second Nauplius (NII)

(Figures 3.4a-d) PLATE III

The second of the nauplius stages is longer but the same width as the first stage. They measure 0.73±0.02mm in length and 0.24±0.01mm in width, based on 13 specimens. They are very similar in shape (Fig. 3.4a) to the first stage. Each of the appendages is similar to the NI although the armature of the setae is more pronounced in the NII stage.

Antennule (Fig. 3.4b) distal segment with a further shorter seta situated between the longer ones.

Antenna (Fig. 3.4c) endopod, proximal segment longer and unarmed, distal segment squat with 2 long setae one short stout spine and one short seta, about one third the length of the other two. Exopod as previous stage, setae of most proximal segments pinnate.

Mandible (Fig. 3.4d) has pinnate setae on segments 3 and 4 of the exopod, all other features unchanged from NI.

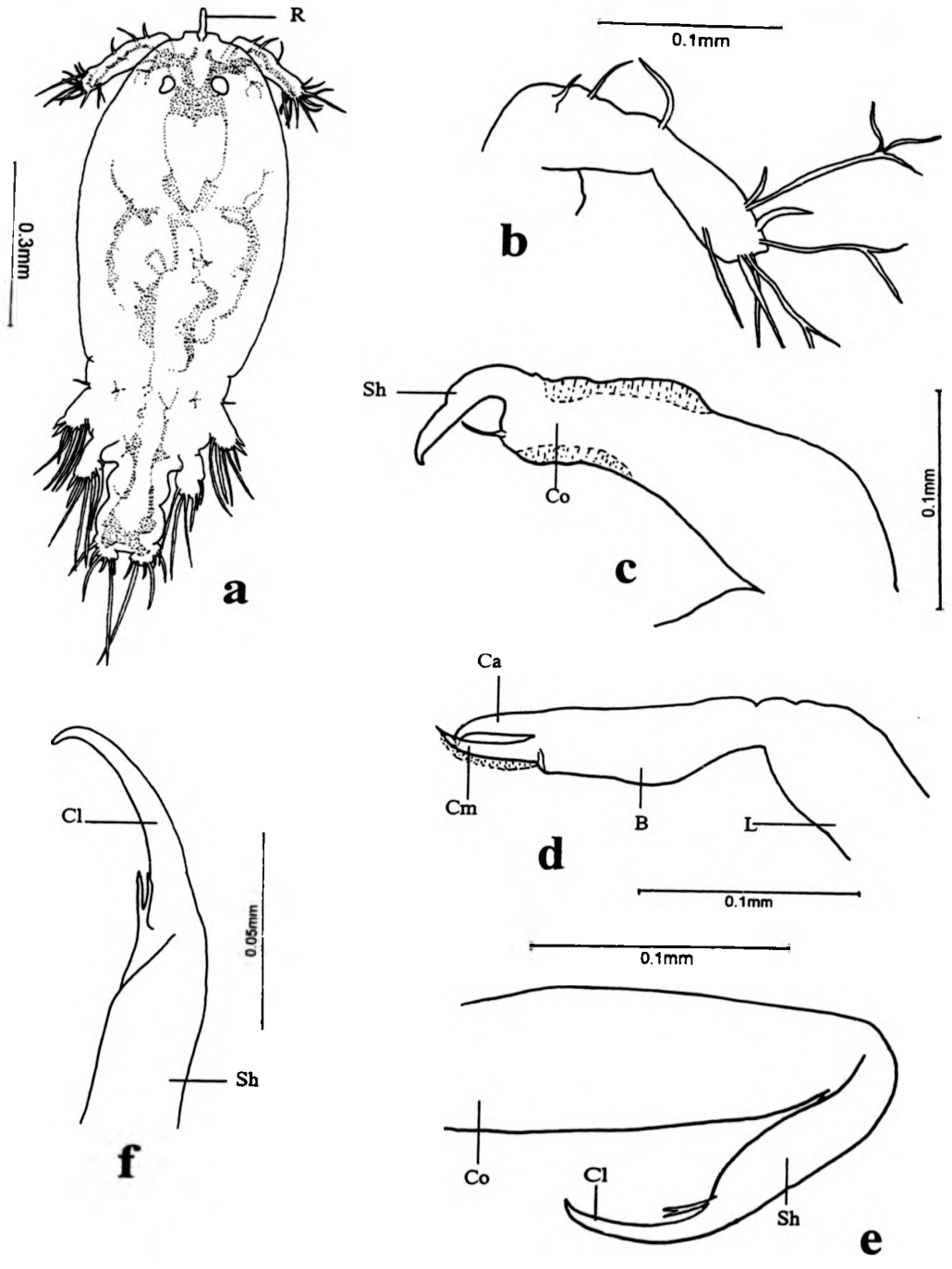


PLATE IV. Figure 3.5. Copepodid. **a** whole animal, dorsal including pigmentation; **b** antennule, dorsal; **c** antenna; **d** maxilla; **e** maxilliped; **f** close up of maxilliped shaft. R-rostrum; Co-corpus; Cl-claw; Sh-shaft; L-lacertus; B-brachium, Ca-canna; Cm-calamus.

3.3.4. Copepodid

(Figure 3.5a-m) PLATES IV & V

Copepodids (Fig. 3.5a) are longer and wider than nauplii, measuring 0.97±0.03mm in length and 0.4±0.04mm in width (based on 30 specimens). Body distinctly in two sections, the cephalothorax and the abdomen in 4 segments. The whole body is distinctly dorso-ventrally flattened compared to the nauplius stages. Patterns of deep pink/purple pigment spots run down entire length of dorsal surface. Rostrum projects anteriorly. Two large eyespots on dorsal surface.

Antennule (Fig. 3.5b) is two segmented and cylindrical. Proximal segment with three setae on anterior margin, distal segment with between 8 and 11 unarmed setae across the distal end, these setae sometimes with irregular branching tips (this is not uniform between individual specimens).

Antenna (Fig. 3.5c) is robust and cylindrical in cross section with three segments; distal segment in the form of a hook (terminal claw) and a small spine projecting ventrally during attachment. The proximal segment is small with no armature and the middle segment has two distinct ridges of cuticle.

Maxilla (Fig. 3.5d) lacertus and brachium about equal in length, brachium narrower. Canna and calamus about equal in length, calamus with distinct ridge of cuticle on lateral edge.

Maxilliped (Fig. 3.5e) subchelate appendage with elongate corpus and narrow sub chela divided into 2 segments. Corpus unarmed, shaft with double auxiliary spines (Fig. 3.5f), claw elongate and unarmed.

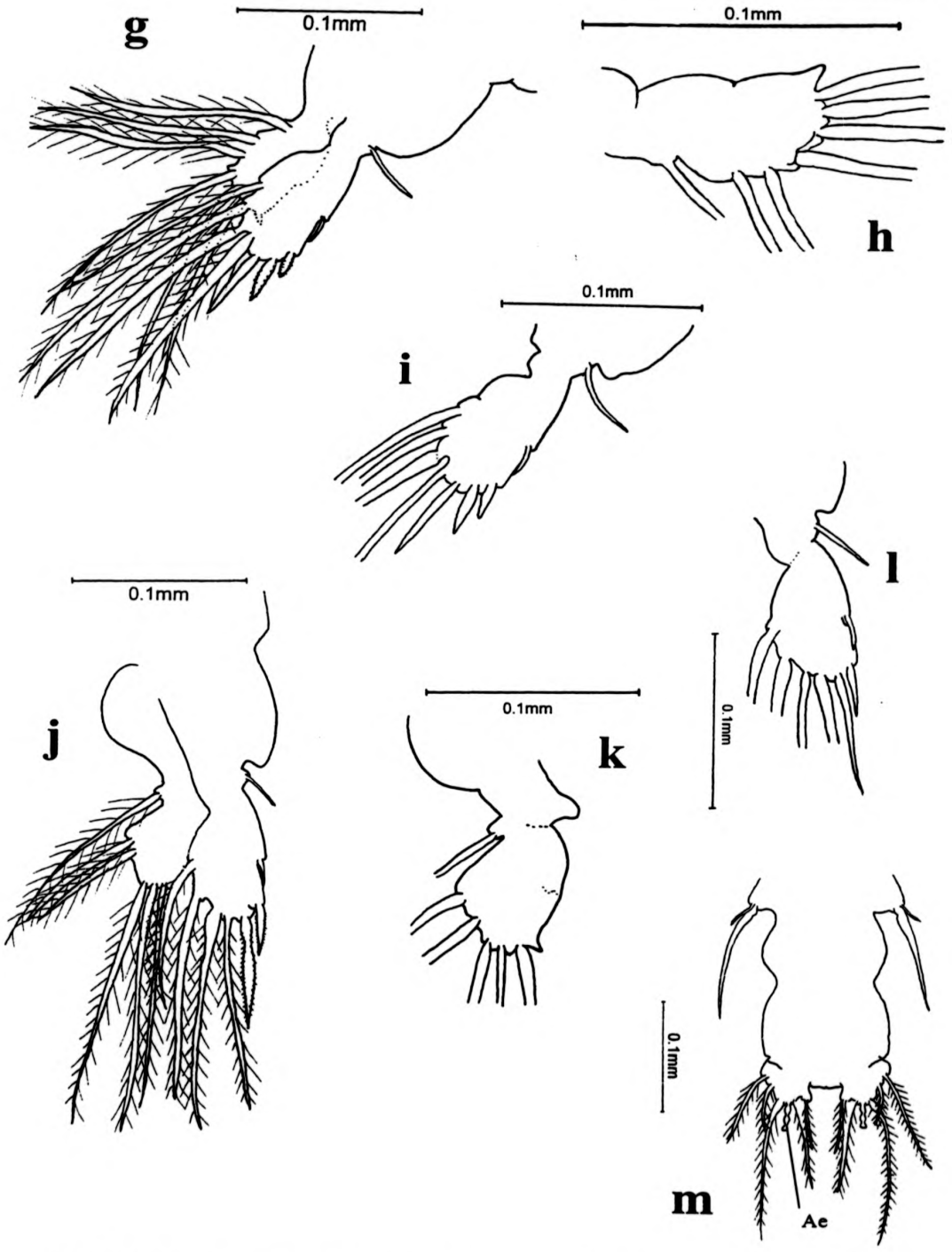


PLATE V. Figure 3.5 continued. Copepodid. **g** 1st thoracic leg; **h** 1st thoracic leg endopod; **i** 1st thoracic leg exopod; **j** 2nd thoracic leg; **k** 2nd thoracic leg endopod; **l** 2nd thoracic leg exopod; **m** caudal ramus. Ae-aesthete

1st Leg (Fig. 3.5g) biramous with indistinct 2-segmented sympod, distal segment with lateral projecting unarmed seta. Sub-rectangular endopod (Fig. 3.5h) with lateral margin stretched distally to form a terminal projection, with four pinnate setae on the distal margin and 3 pinnate setae on the medial margin. Exopod (Fig. 3.5i), flat and ovoid with small spine on lateral margin, 3 stout spiniform setae with fine membranes on distolateral margin and 4 long pinnate setae on distal margin.

2nd Leg (Fig 3.5j) biramous with subrectangular sympod, distal segment with unarmed lateral seta. Very similar to first leg, endopod (Fig. 3.5k) with 3 distal pinnate setae. Distolateral margin of exopod (Fig. 3.5l) with 2 spiniform setae.

3rd Leg (Fig. 3.5m) small swelling in cuticle on abdominal section with two unarmed, unequally sized setae.

Caudal ramus (Fig. 3.5m) with 2 pinnate setae on distolateral margin, one aesthete, 2 long pinnate setae and a shorter one sharing a base with the medial long seta on the distal margin.

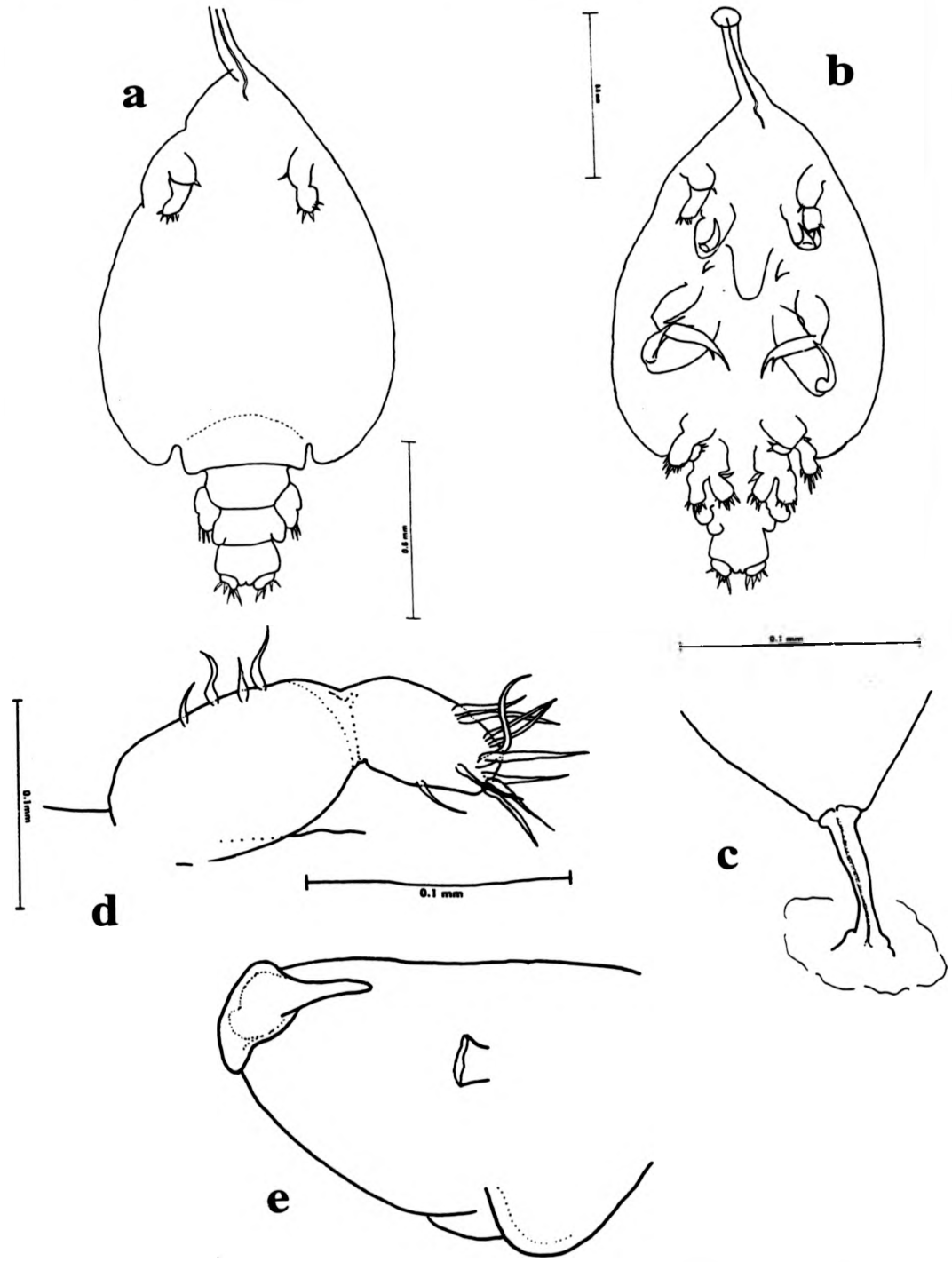


PLATE VI. Figure 3.6 Chalimus I. **a** whole animal dorsal view; **b** whole animal, ventral view; **c** frontal filament; **d** antennule; **e** antenna.

3.3.5. Chalimus I

(Figures 3.6a-n) PLATES VI, VIII & VIII

The chalimus I stage (Figs. 3.6a & b) is longer and broader than the copepodid measuring 1.29±0.06mm in length and 0.68±0.04mm in width based on 7 specimens. Cephalothorax elongate, wider at posterior end, includes third thoracic segment. Anterior end has filament (Fig. 3.6c) for attachment to host, posterior margin of cephalothorax has two posterior sinuses (Fig. 3.6a).

Antennule (Fig. 3.6d) distinctly two segmented; proximal segment with 4 unarmed setae on anterior margin; distal segment with 10 terminal unarmed setae and one half way down the posterior margin.

Antenna (Fig. 3.6e) terminal claw projecting anteriorly; proximal segment squat with a blunt process on ventral margin, lobed medially; terminal claw short with striated membrane.

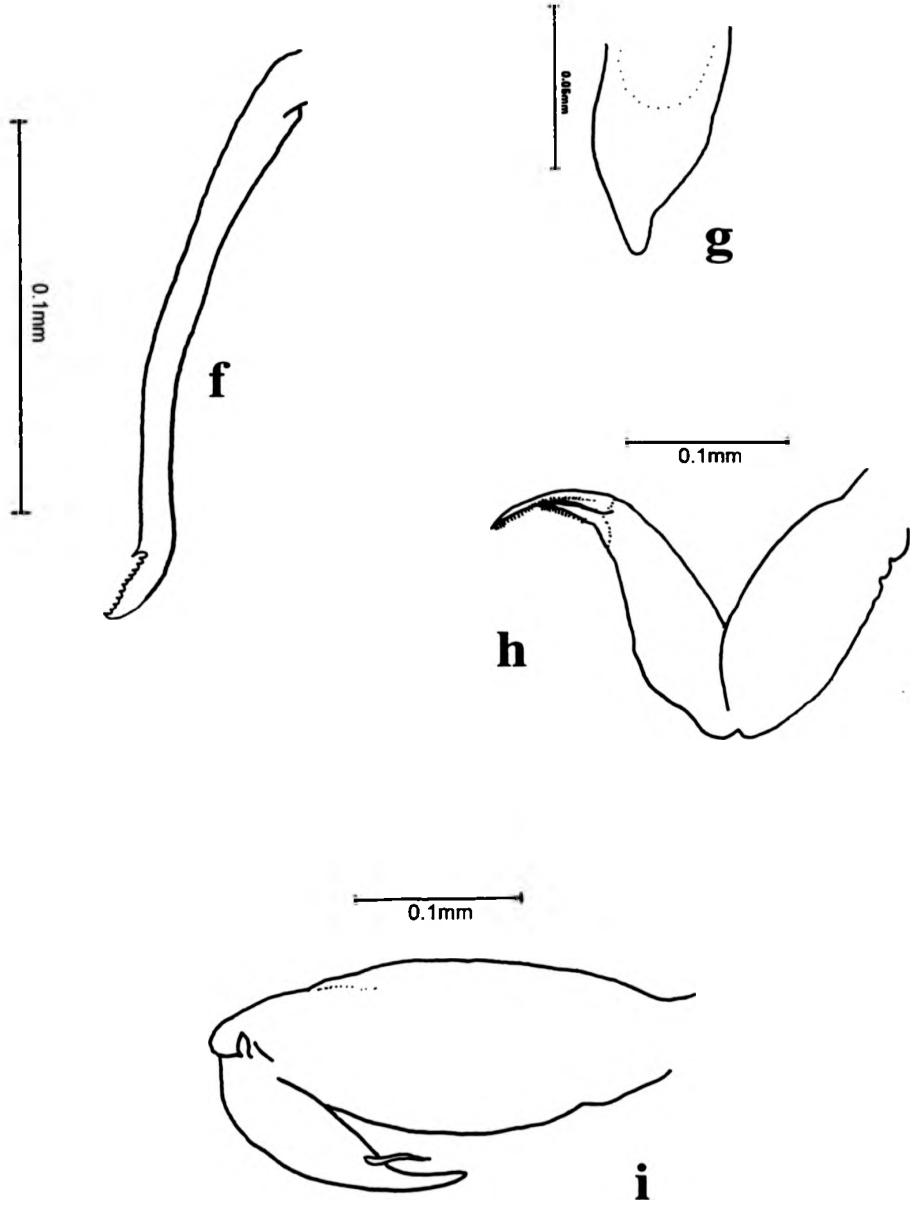


PLATE VII. Figure 3.6 continued. Chalimus I. **f** mandible; **g** maxillule; **h** maxilla; **i** maxilliped.

Mandible (Fig. 3.6f) as adult, ventral side with 11 uniform teeth.

Maxillule (Fig. 3.6g) posterior process; elongate process with simple, single tip.

Maxilla (Fig. 3.6h), lacertus unarmed, brachium unarmed, calamus and canna both curved, calamus nearly twice as long as canna, both with serrated edges.

Maxilliped (Fig. 3.6i) subchela unsegmented, bearing smooth and slightly sigmoid spine. Claw straighter than in copepodid.

No sternal furca present.

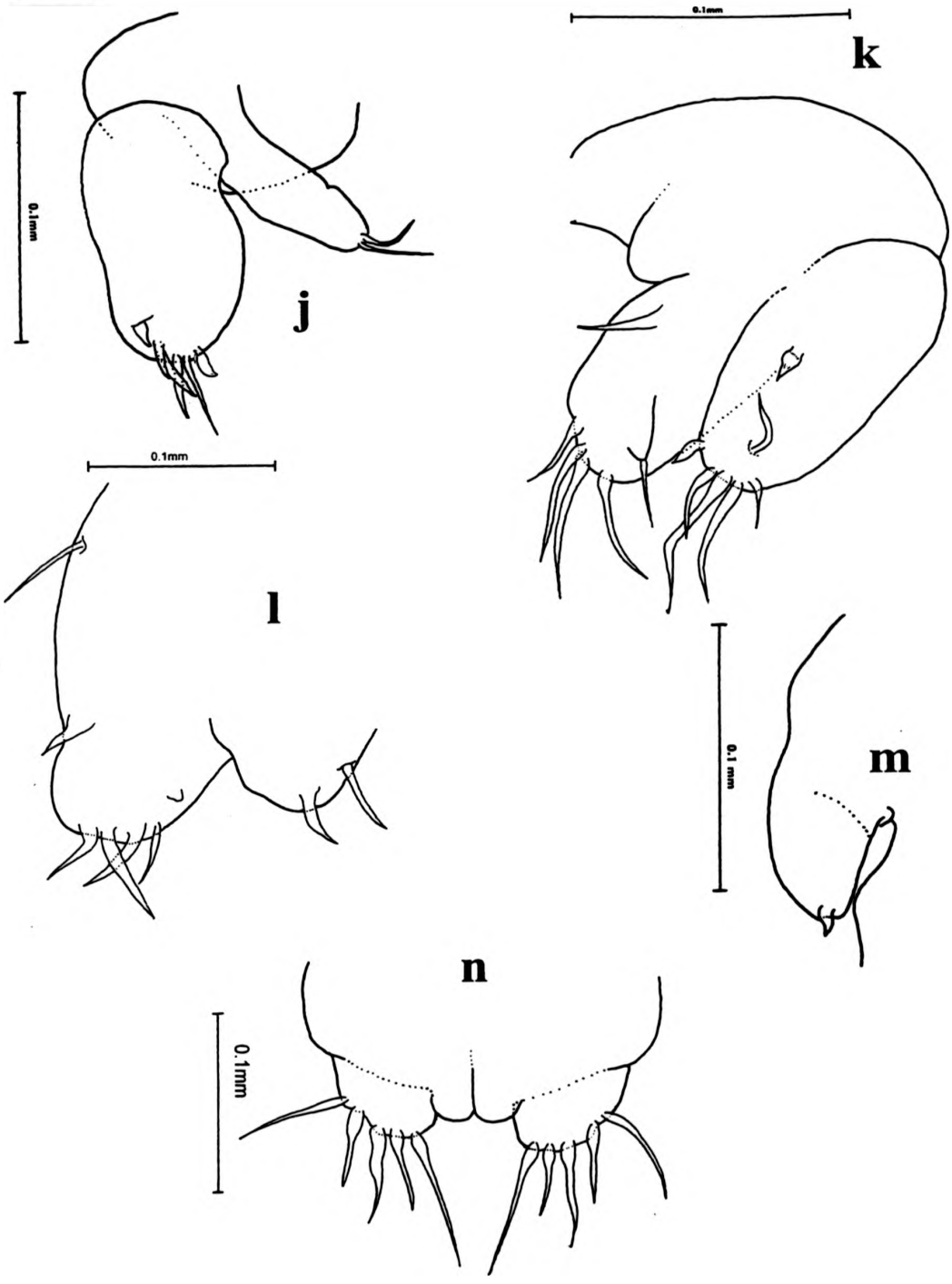


PLATE VIII. Figure 3.6 continued. Chalimus I. j 1st thoracic leg; k 2nd thoracic leg; l 3rd thoracic leg; m 4th thoracic leg; n caudal ramus.

1st Leg (Fig. 3.6j) sympod unarmed; exopod sub-rectangular equipped with 6-7 setae the lateral and medial ones short, scoop-like setae, central 4 elongate and unarmed; endopod thinner than exopod, 2 elongate unarmed setae at apex. Interpodal bar absent.

2nd Leg (Fig. 3.6k) sympod indistinctly segmented, unarmed similar to 1st leg but more extended medially; elongate, sub-rectangular exopod with one short spiniform and one longer seta on lateral margin, 4 setae of varying length and 1 short spine on medial margin; endopod with one lateral and 4 distal setae, all unarmed, one medial seta. Endopod with small medial palp towards proximal margin, 1 short spine, 3 long setae with swollen bases on distal end and 2 setae on lateral margin.

3rd Leg (Fig. 3.6l) sympod with seta on lateral margin, endopod with 2 short distal setae; exopod with one lateral spiniform seta, 4 elongate seta on distal margin and one short spiniform seta on the medial margin.

4th Leg (Fig. 3.6m) short bulbous outgrowth from body wall, with one small stout seta.

Caudal ramus (Fig. 3.6n) with 5 unarmed setae, all with basal swellings.

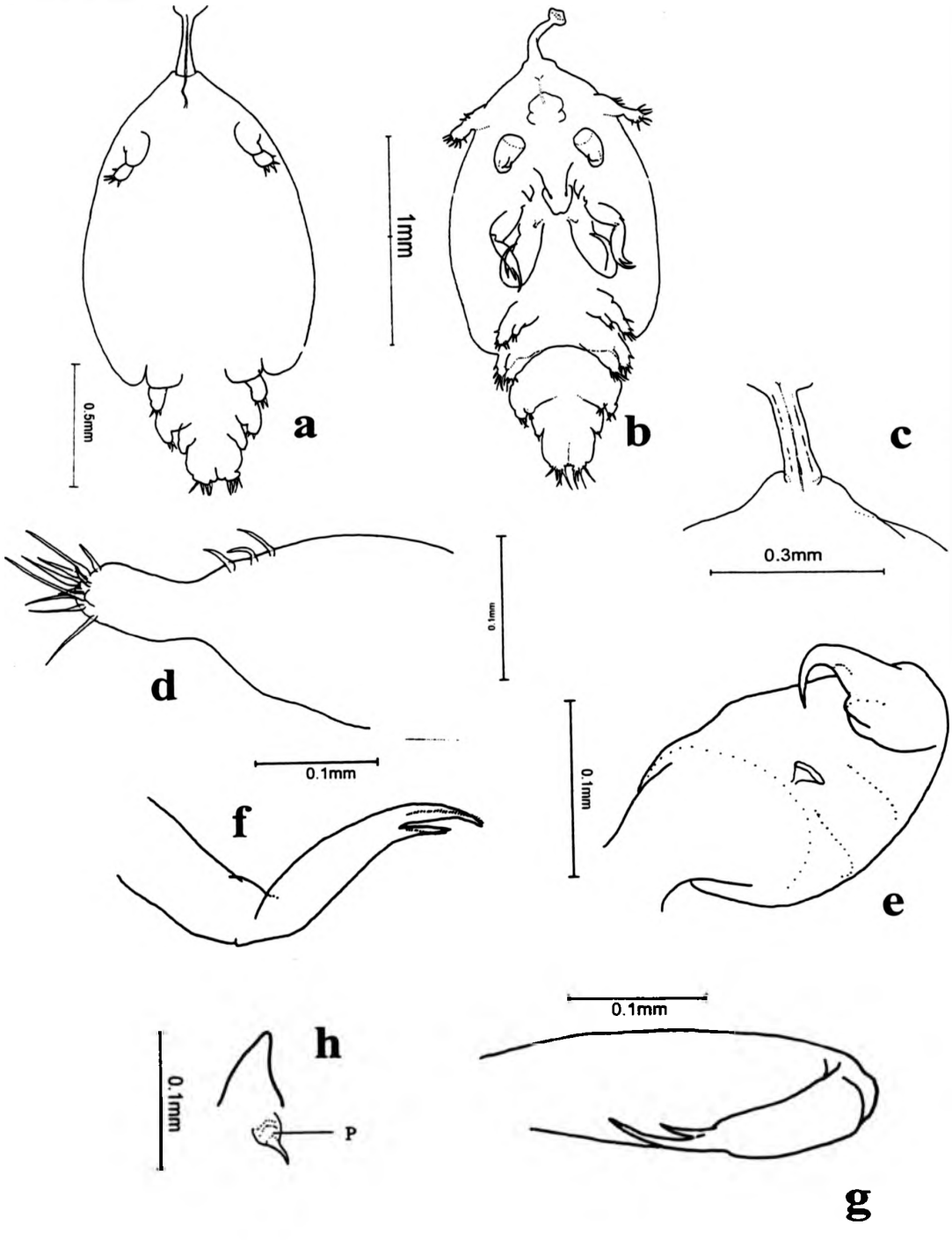


PLATE IX. Figure 3.7 Chalimus II. **a** whole animal, dorsal; **b** whole animal, ventral; **c** frontal filament; **d** antennule; **e** antenna; **f** maxilla; **g** maxilliped; **h** maxillule. P-palp.

3.3.6. Chalimus II

(Figures 3.7a-m) PLATES IX & X

Chalimus II (Figs. 3.7a & b) measures 1.52±0.06mm in length and 0.67±0.04mm in width, based on 8 specimens. Cephalothorax elongate with 2 posterior sinuses and filament at anterior end (Fig. 3.7c).

No major differences in antennule (Fig. 3.7d), antenna (Fig. 3.7e), maxilla (Fig. 3.7f) and maxilliped (Fig. 3.7g) between first and second chalimus stages.

Maxillule (Fig. 3.7h) posterior process elongate, simple palp present, arising from cuticle at base of process

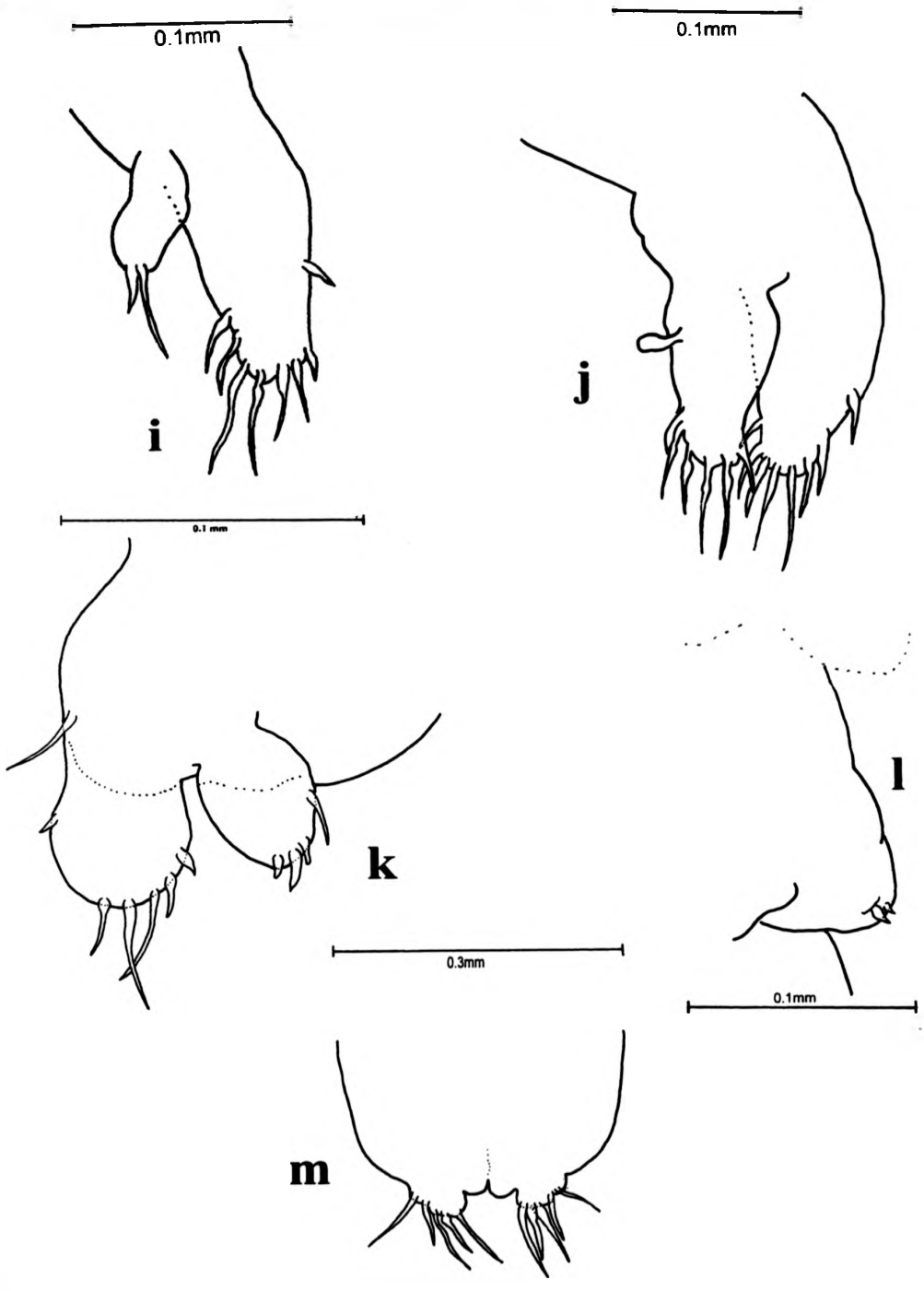


PLATE X. Figure 3.7 continued. Chalimus II. **i** 1st Leg; **j** 2nd Leg; **k** 3rd Leg; **l** 4th Leg; **m** caudal ramus.

1st Leg (Fig. 3.7i) exopod and endopod indistinct from sympod, endopod smaller and thin with 2 unarmed setae at distal end; exopod sub-rectangular with one lateral seta, 1 spiniform and 3 elongate setae on distal margin and 3 setae on medial margin. All setae unarmed.

2nd Leg (Fig. 3.7j) endopod and exopod indistinct from sympod, exopod with 1 lateral short seta, 2 short stout setae and 3 elongate setae on distal margin and 2 shorter setae on medial margin, all unarmed.

3rd Leg (Fig. 3.7k) biramous, single unarmed seta on lateral margin of unsegmented sympod. Exopod with one short lateral spine and 5 setae on distomedial margin of varying lengths; endopod with 3 short stout spines at distal margin and 1 on medial margin, some of the setae with basal swellings.

4th Leg (Fig. 3.7l) indistinctly 2 segmented, two terminal tiny unarmed setae.

Caudal ramus (Fig. 3.7m) with five elongate, unarmed setae on distal margin.

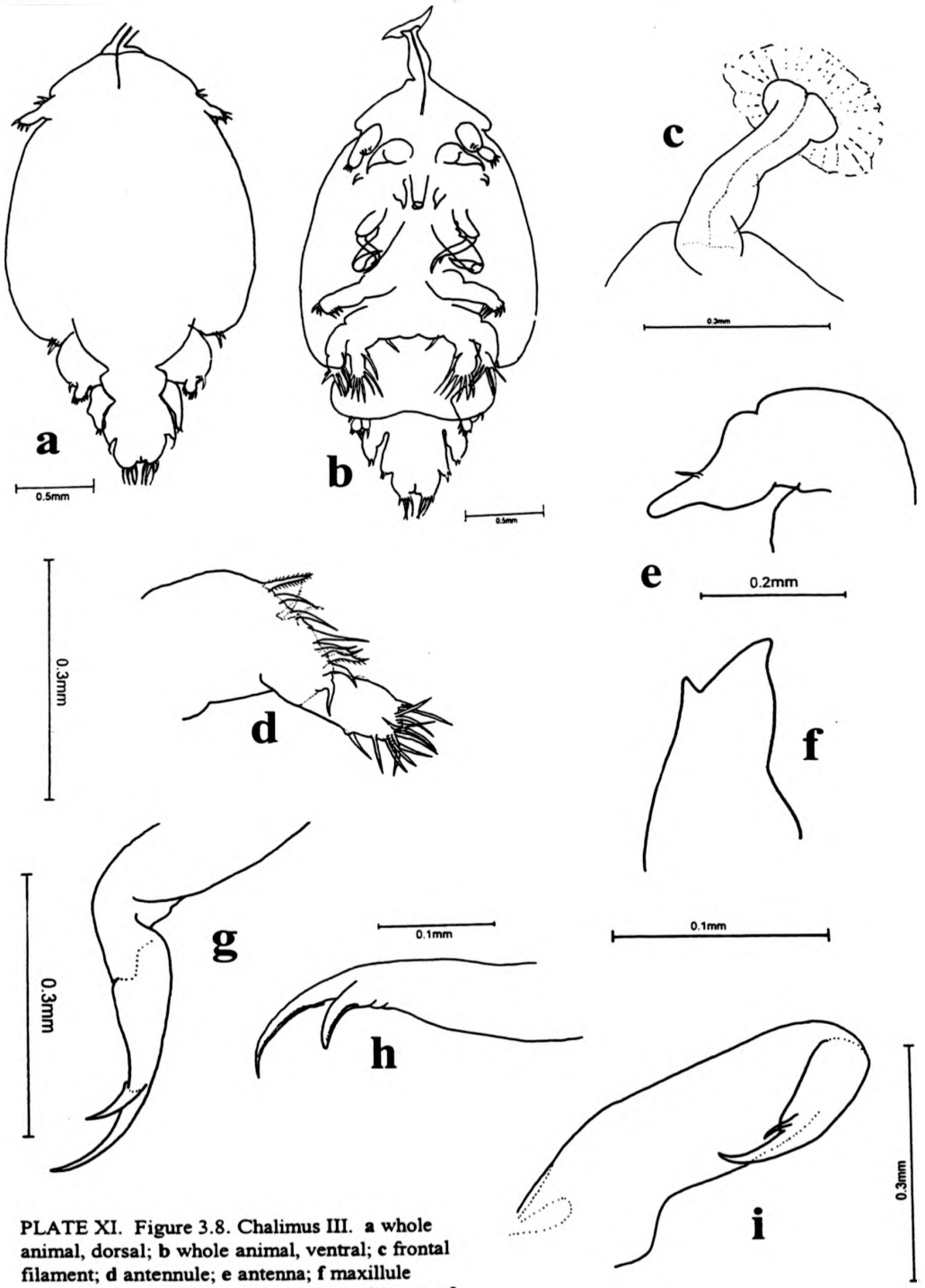


PLATE XI. Figure 3.8. Chalimus III. **a** whole animal, dorsal; **b** whole animal, ventral; **c** frontal filament; **d** antennule; **e** antenna; **f** maxillule (medial palp not shown); **g** maxilla;**h** close up of maxilla **i** maxilliped.

3.3.7. Chalimus III

(Figures 3.8a-o) PLATES XI & XII

Chalimus III (Figs. 3.8a & b) measures 2.74±0.25mm in length and 1.59±0.15mm in width, based on 18 specimens. Cephalothorax includes 4th thoracic segment, filament at anterior end (Fig. 3.8c).

Antennule (Fig. 3.8d) distinctly 2 segmented, proximal bigger than distal, both subcylindrical. Proximal segment with 9 ventral setae and a further 2 on the dorsal surface, some pinnate. Distal segment with 14 unarmed setae, one on posterior margin all others at distal margin.

Antenna (Fig. 3.8e) indistinctly 2 segmented with small spine on anterior margin at distal end.

Maxillule (Fig. 3.8f) medial palp present, posterior process with bifid tip, medial projection larger than lateral.

Maxilla (Fig. 3.8g) distinctly segmented; brachium cylindrical, elongate, calamus and canna slender and tapered, less curved than in previous stage, both with short spines running down their length (Fig. 3.8h).

Maxilliped (Fig. 3.8i) structure as previous stage.

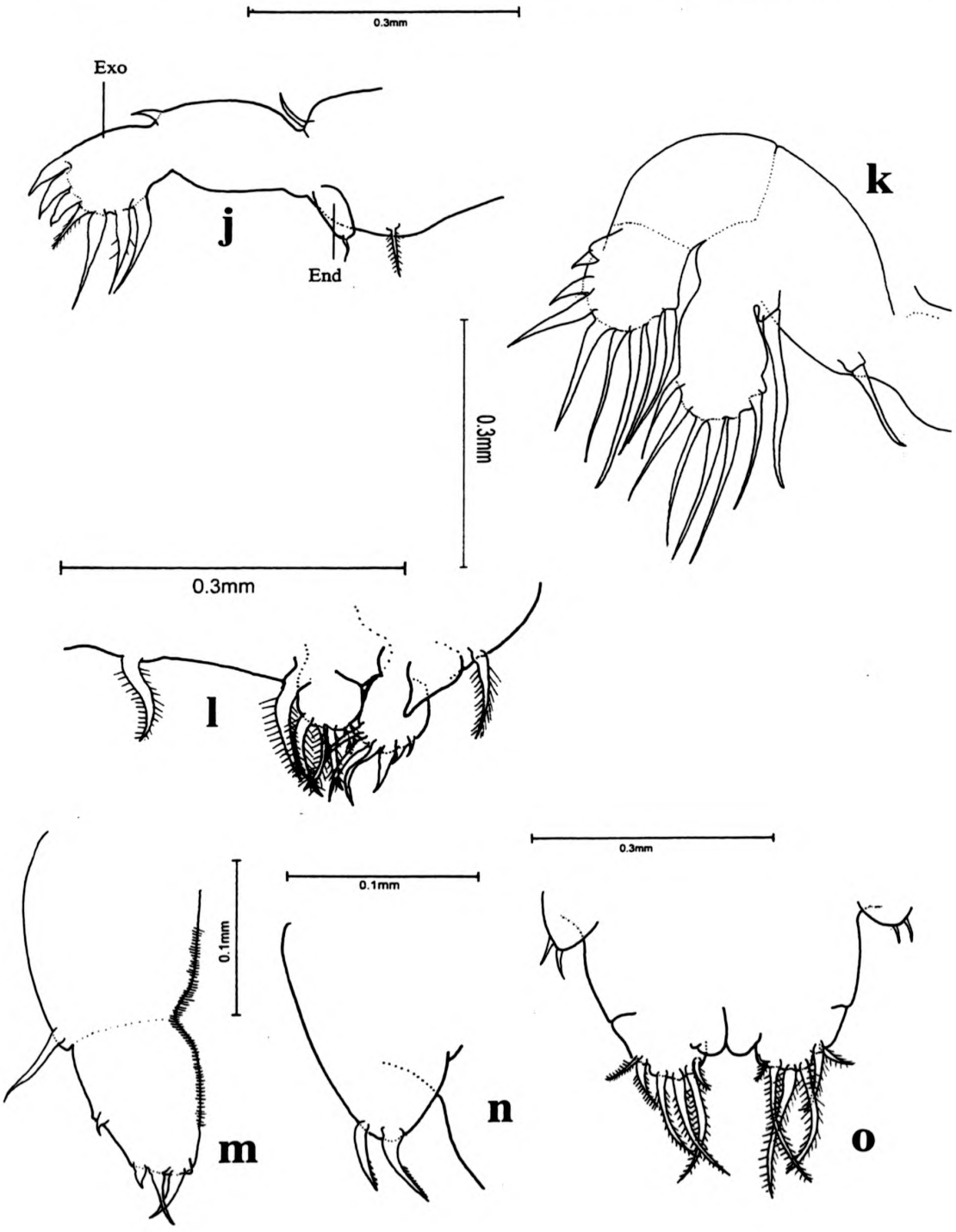


PLATE XII. Figure 3.8 continued. Chalimus III. **j** 1st leg; **k** 2nd leg; **l** 3rd leg; **m** 4th leg; **n** 5th leg; **o** caudal ramus. End-endopod, Exo-exopod.

1st Leg (Fig. 3.8j) biramous, sympod with one lateral seta and one pinnate seta on the medial margin. Endopod much reduced with two reduced unarmed seta at distal tip. Exopod 2 segmented, proximal segment longer with one short seta on distolateral margin, distal segment with 4 short setae on distal margin (one pinnate) and three longer, sparsely pinnate setae on the medial margin.

2nd Leg (Fig. 3.8k) broad interpodal bar, sympod sub-rectangular with one unarmed seta on medial margin, exopod indistinctly 2 segmented, proximal segment with one lateral seta; distal segment with 2 short spiniform setae on lateral margin and 4 elongate setae on distal margin, a further 2 elongate setae on the medial margin. Endopod indistinctly 2 segmented, proximal segment with one elongate seta on medial margin, distal segment with 6 elongate setae on distal margin. All setae unarmed.

3rd Leg (Fig. 3.8l) sympod unsegmented, fused to opposite member by broad interpodal bar, stout seta on medial margin and one on lateral margin, both pinnate. Exopod with stout seta on lateral margin and 6 unarmed setae on distal margin. Endopod with stout medial seta and 4 pinnate setae on the distal margin.

4th Leg (Fig. 3.8m) uniramous, sympod with one unarmed seta on distolateral margin, indistinct from exopod with one short spine on lateral margin and 3 longer setae at the distal margin. Medial margin of leg with membrane.

5th Leg (Fig. 3.8n & o) represented by small outgrowth of body wall with 2 short setae with serrated edges on distal end.

Caudal ramus (Fig. 3.8o) setae longer than in earlier stage and all setae armed.

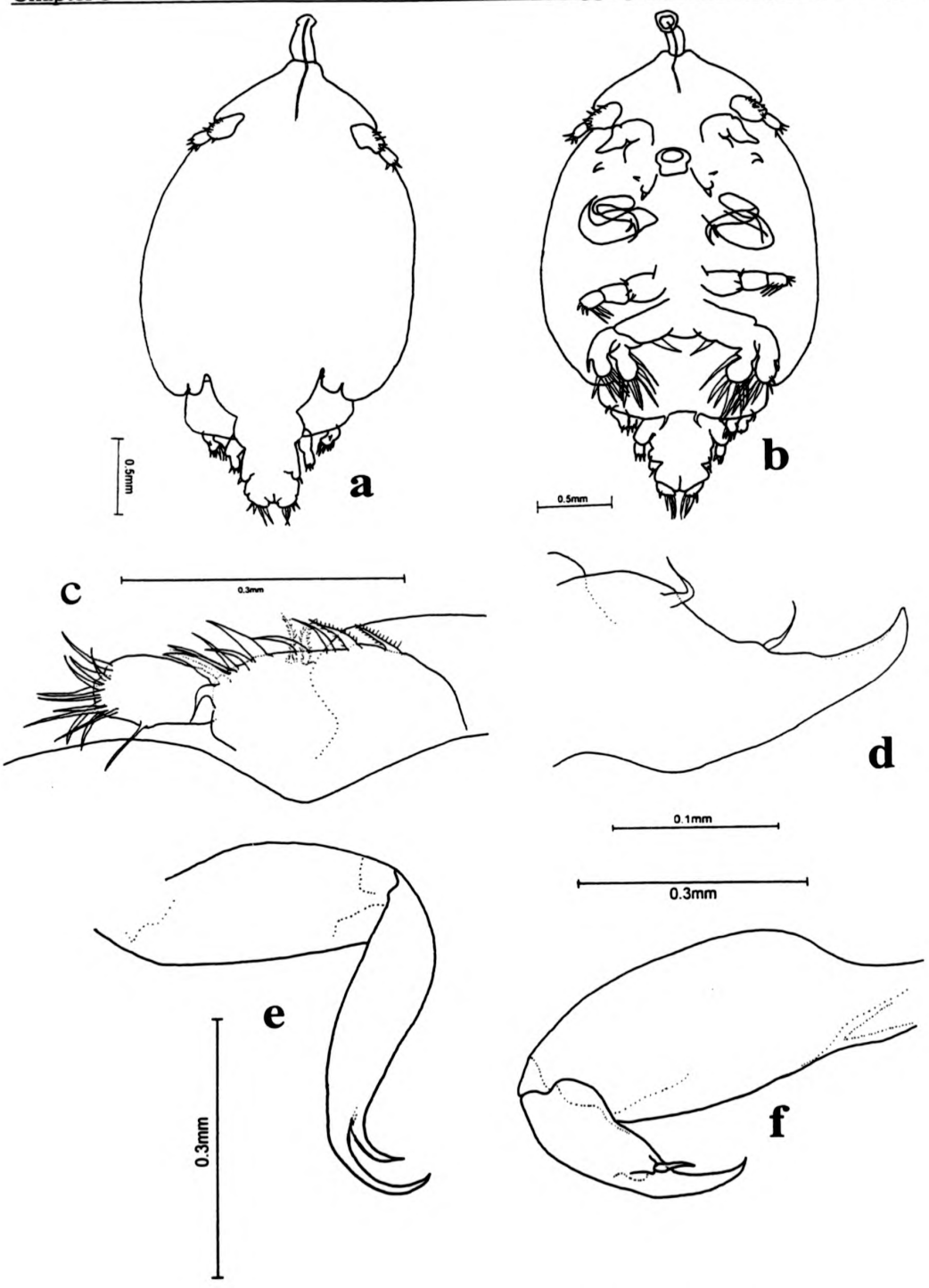


PLATE XIII. Figure 3.9. Chalimus IV. a whole animal, dorsal; b whole animal, ventral; c antennule; d antenna; e maxilla; f maxilliped.

3.3.8. Chalimus IV

(Figures 3.9a-k) PLATES XIII & XIV

Chalimus IV (Fig. 3.9a & b) measures 3.47±0.24mm in length and 1.91±0.13mm wide, based on 15 specimens. Similar in overall appearance to chalimus III stage, posterior sinus more distinct. Male and female indistinct.

Antennule (Fig. 3.9c) distinctly 2 segmented. Proximal segment larger, cylindrical with 9 anterior setae, one stout distal seta and 2 dorsal pinnate setae, distal segment with 11 distal setae and one on the posterior margin.

Antenna (Fig.3.9d) terminal claw highly sclerotized, claw tapering to point. With 2 unarmed setae.

Maxillule, maxilla (Fig. 3.9e) and maxilliped (Fig. 3.9f) as in chalimus III stage.

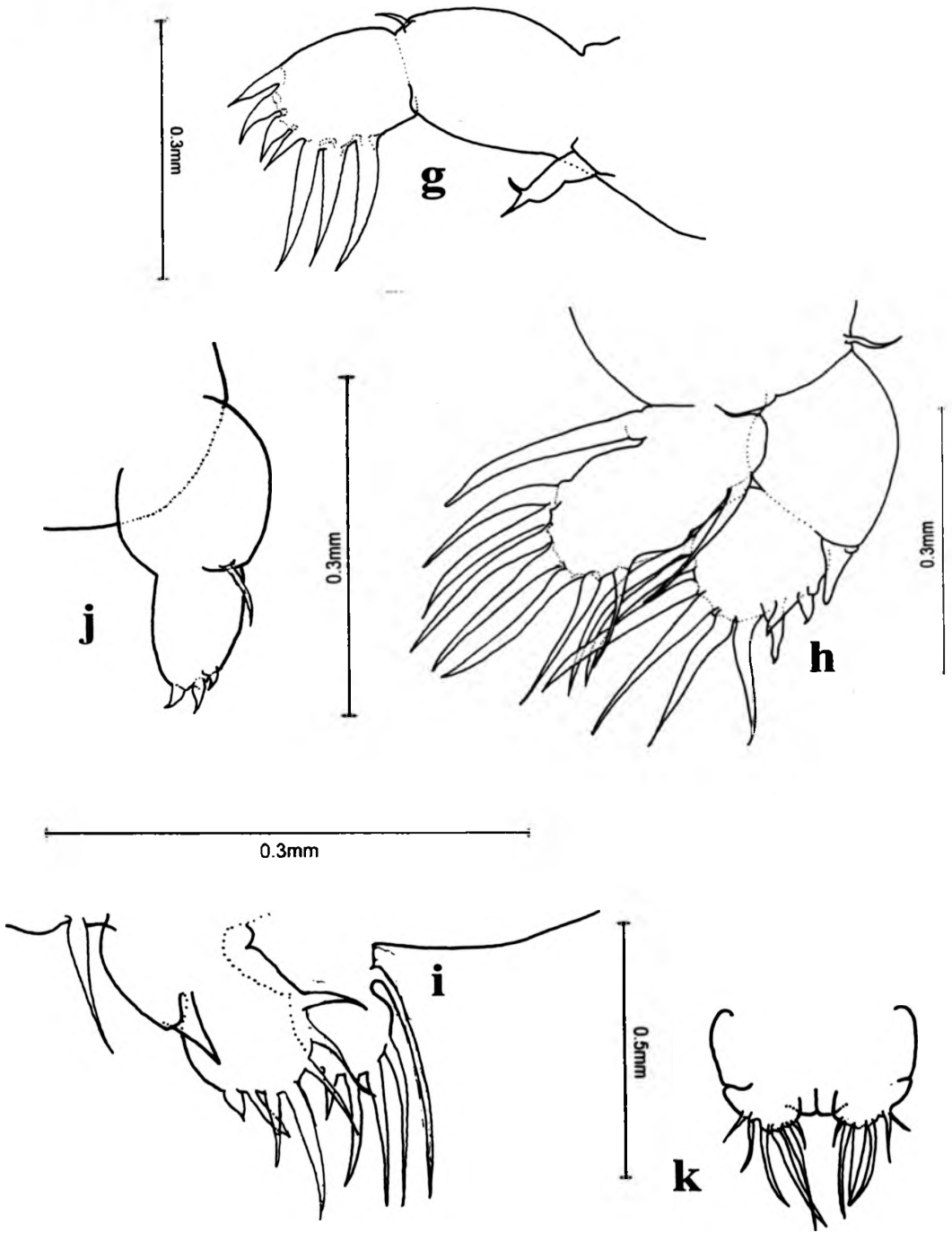


PLATE X1V. Figure 3.9 continued. Chalimus IV. g 1st leg; h 2nd leg; i 3rd leg; j 4th leg; k caudal ramus.

1st Leg (Fig. 3.9g) with interpodal bar, as chalimus III.

2nd Leg (Fig. 3.9h) very similar to previous stage; exopod with one extra short spine on lateral margin, endopod with one additional elongate seta on distal margin.

3rd Leg (Fig. 3.9i) both rami indistinctly 2 segmented, as previous stage.

4th Leg (Fig. 3.9j) sympod longer and wider than exopod, armature as previous stage.

5th Leg and caudal ramus (Fig. 3.9k) unchanged from previous stage.

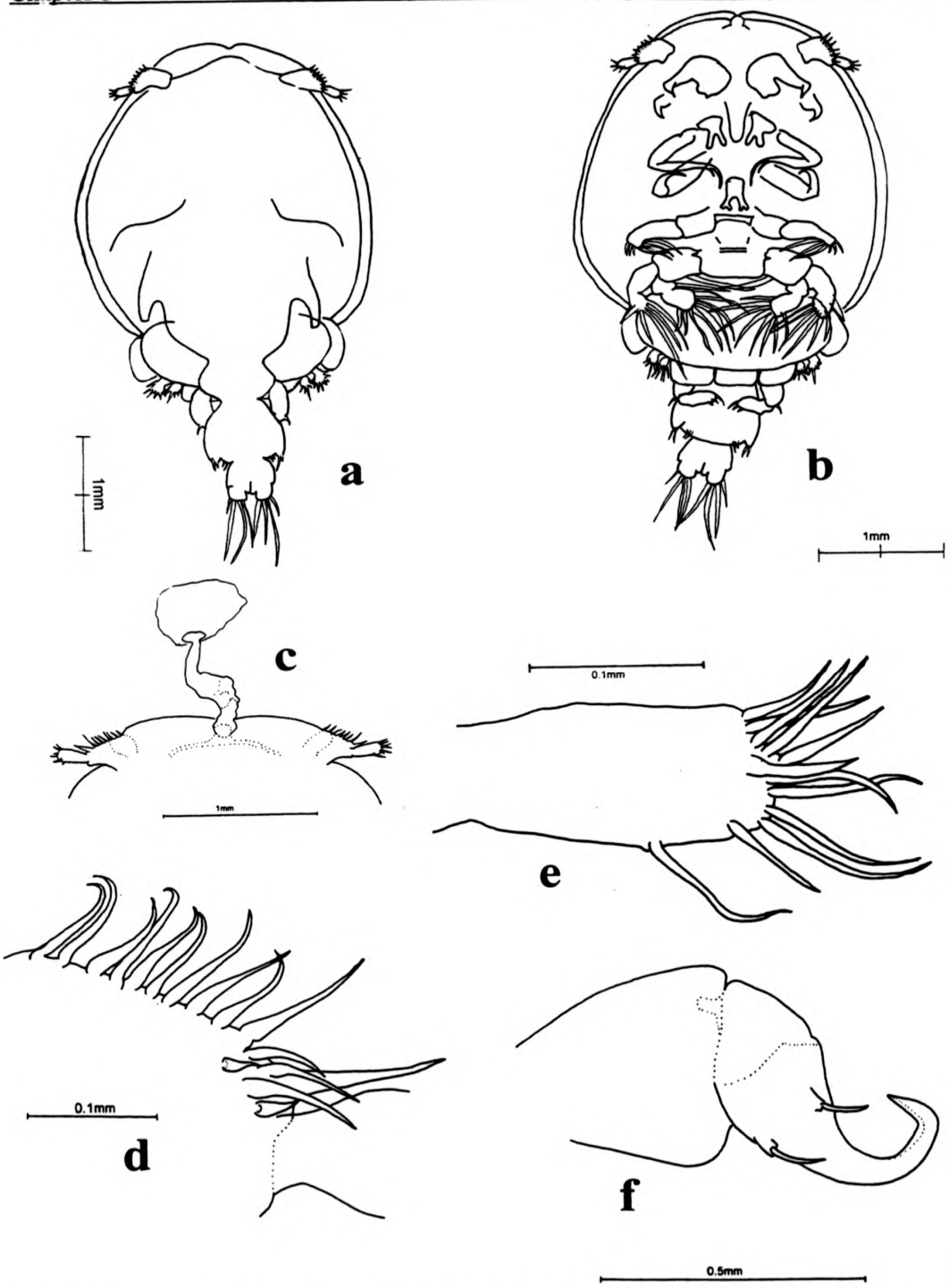


PLATE XV. Figure 3.10. First Pre Adult - Male. **a** whole animal, dorsal; **b** whole animal, ventral; **c** frontal filament; **d** proximal segment of antennule, ventral; **e** distal segment of antennule, ventral; **f** antenna.

3.3.9. First Pre-Adult

All of the pre-adult and adult stages range from a pinky brown to a sandy brown colour, sometimes with a hint of green. While attached to the host, the colour of the host skin may be seen through the cuticle, especially with the smaller, thinner males, thus making them well camouflaged.

3.3.9.1. Males

(Figures 3.10a-r) PLATES XV, XVI & XVII

The first of the pre-adult male (Figs.3.10a & b) stages measures 3.63±0.17mm in length and 2.37±0.12mm wide based on 10 specimens. They resemble the adult male in shape but are a little smaller. They are generally motile, however occasionally they may retain a frontal filament following the moult from chalimus. This filament (Fig. 3.10c) is weaker and will be severed soon after moulting, then the individual is fully motile. All the pre-adult stages are brown in colour. Cephalothorax with fully developed frontal plates and marginal membranes along the anterior and lateral margins, posterior sinuses on posterior margin. Genital segment ovoid and distinct from abdomen. Antennule (Fig. 3.10d & e) distinctly 2 segmented, proximal segment with 16 setae on the ventral surface with a further 2 on the dorsal surface, distal segment with 11 distal setae and one on the posterior margin.

Antenna (Fig. 3.10f) three segmented, basal segment short and wide, middle segment robust; terminal claw strongly curved, tapered to point, long and slender with two setae at its mid-length.

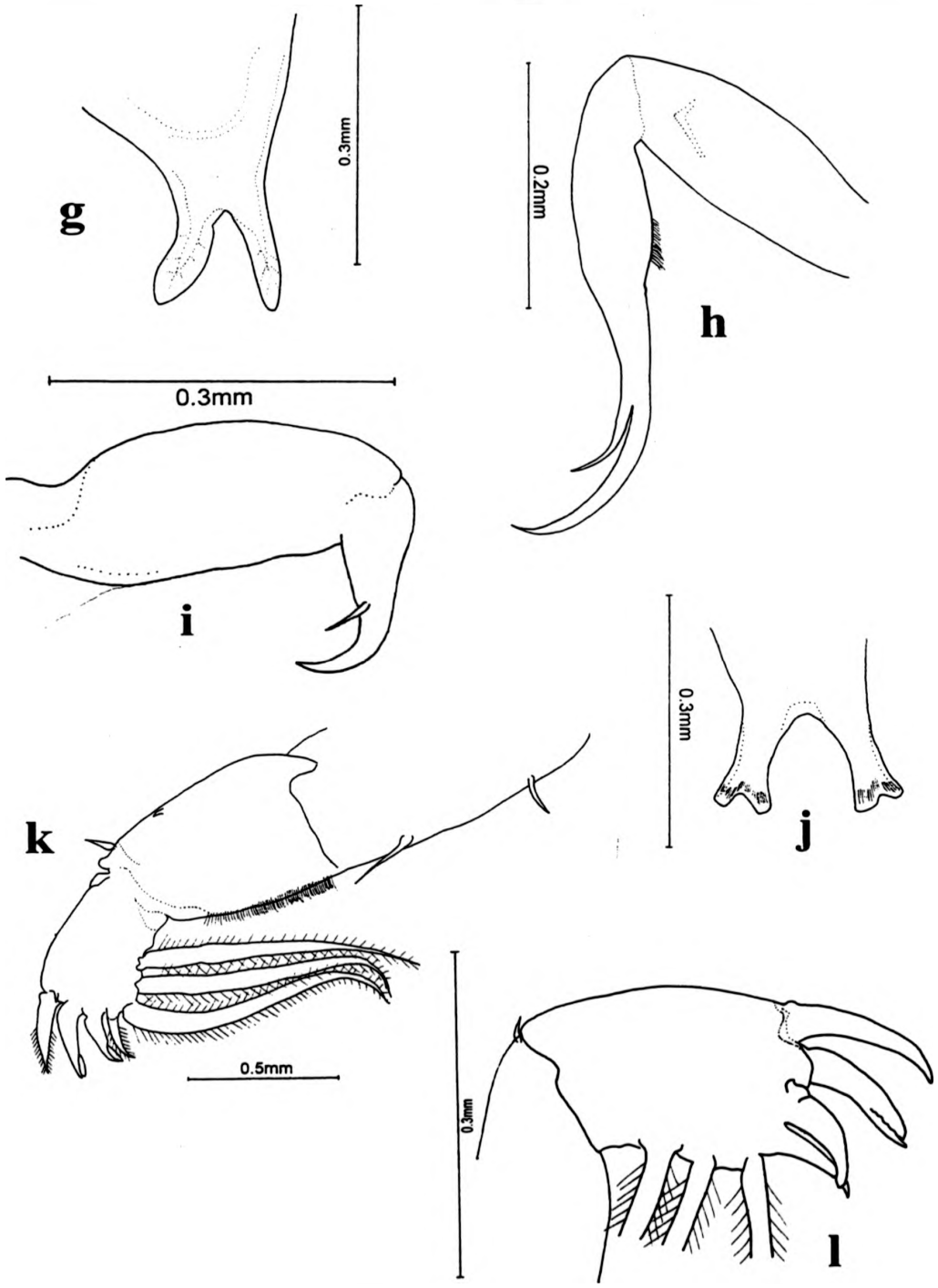


PLATE XVI. Figure 3.10 continued. First Pre-Adult - Male. **g** maxillule; **h** maxilla; **i** maxilliped; **j** sternal furca; **k** 1st leg; **l** distal portion of 1st leg.

Maxillule (Fig. 3.10g) longer, bifid tip developed into two equally sized tines more pronounced than in earlier stages, tines triangular in cross-section. Projecting medially.

Maxilla (Fig. 3.10h) very similar to previous stage, calanus and canna more curved and tapered to a point. Brachium with small area of setules.

Maxilliped (Fig. 3.10i) unchanged from previous stage.

Sternal furca (Fig. 3.10j) consisting of subquadrate box and two tines, each tine bifid in distal quarter. Apex of tines flattened.

1st Leg (Fig. 3.10k & l) sympod distinctly 2 segmented with 2 small setae on distal margin, exopod 2 segmented, separate from sympod, proximal segment with a small spine at distolateral margin. Distal segment, medial margin with three large pinnate setae, distomedial (Fig. 3.10k) margin with four short curving setae on apex. The central two setae armed with a short spine on the concave surface. Endopod not present.

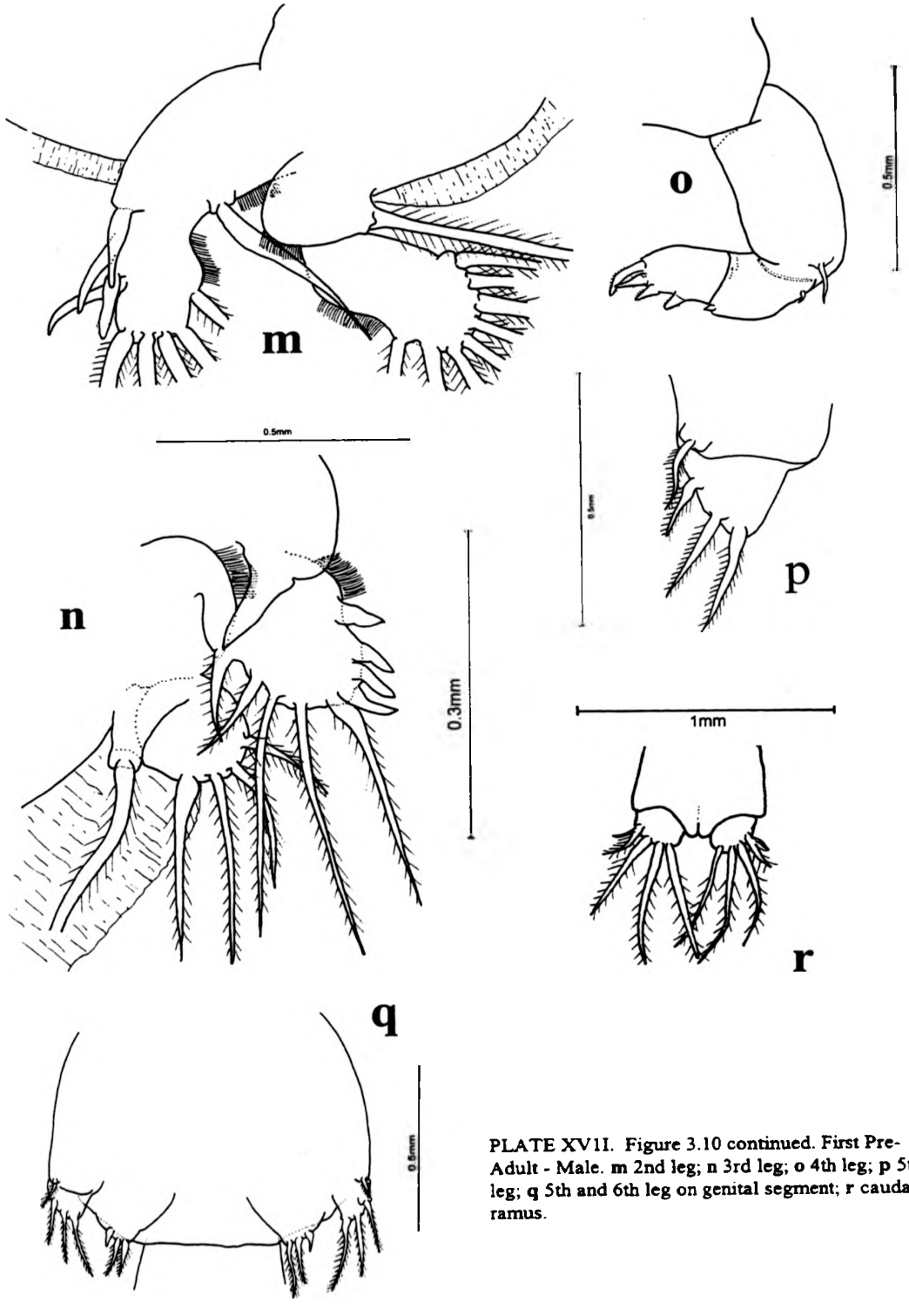


PLATE XVII. Figure 3.10 continued. First Pre-Adult - Male. **m** 2nd leg; **n** 3rd leg; **o** 4th leg; **p** 5th leg; **q** 5th and 6th leg on genital segment; **r** caudal ramus.

2nd Leg (Fig. 3.10m) connected to opposite member by a broad interpodal bar with broad membrane on distal margin. Sympod indistinct from rami. Endopod 2 segmented, proximal segment with one pinnate seta at distolateral margin, distal segment with 8 pinnate setae from medial margin through to distolateral margin. Exopod with 2 segments, proximal segment about twice the length of distal segment. Proximal segment with one short unarmed seta on distolateral margin and a longer unarmed seta on medial margin, distal segment with one short spine and 3 thinner short setae on lateral margin, 5-6 pinnate setae on apex through medial margin. Broad membrane on medial margin of proximal and part of distal segment of the exopod and lateral margins of the endopod.

3rd Leg (Fig. 3.10n) sympod well developed, unsegmented, fused to opposite member by a broad interpodal bar with broad membrane on posterolateral and posteromedial margins. Both rami distinctly 2 segmented; proximal segment of exopod with stout spine on distomedial corner, distal segment with 4 short spiniform setae on lateral margin and 5 pinnate setae ranging from apex up the medial margin. Proximal segment of the endopod with one long pinnate seta on distomedial corner, distal segment with four pinnate setae along distal margin. Row of setules along the lateral margin of the exopod.

4th Leg (Fig. 3.10o) sympod and exopod distinct, sympod with one pinnate seta on distolateral margin. Proximal segment of exopod with two small spines on lateral margin, distal segment with 4 short stout setae with fine pinnules on apical margin.

5th Leg (Fig. 3.10p) with two processes. Smaller, lateral process with one pinnate seta on lateral margin, larger medial process with 3 pinnate setae running down lateral margin to apex.

6th Leg (Fig.3.10q) bulbous outgrowth on posteroventral surface of the genital complex, with one unarmed seta on the lateral margin and two pinnate setae on the apex.

Caudal ramus (Fig. 3.10r) armed with 5 pinnate setae.

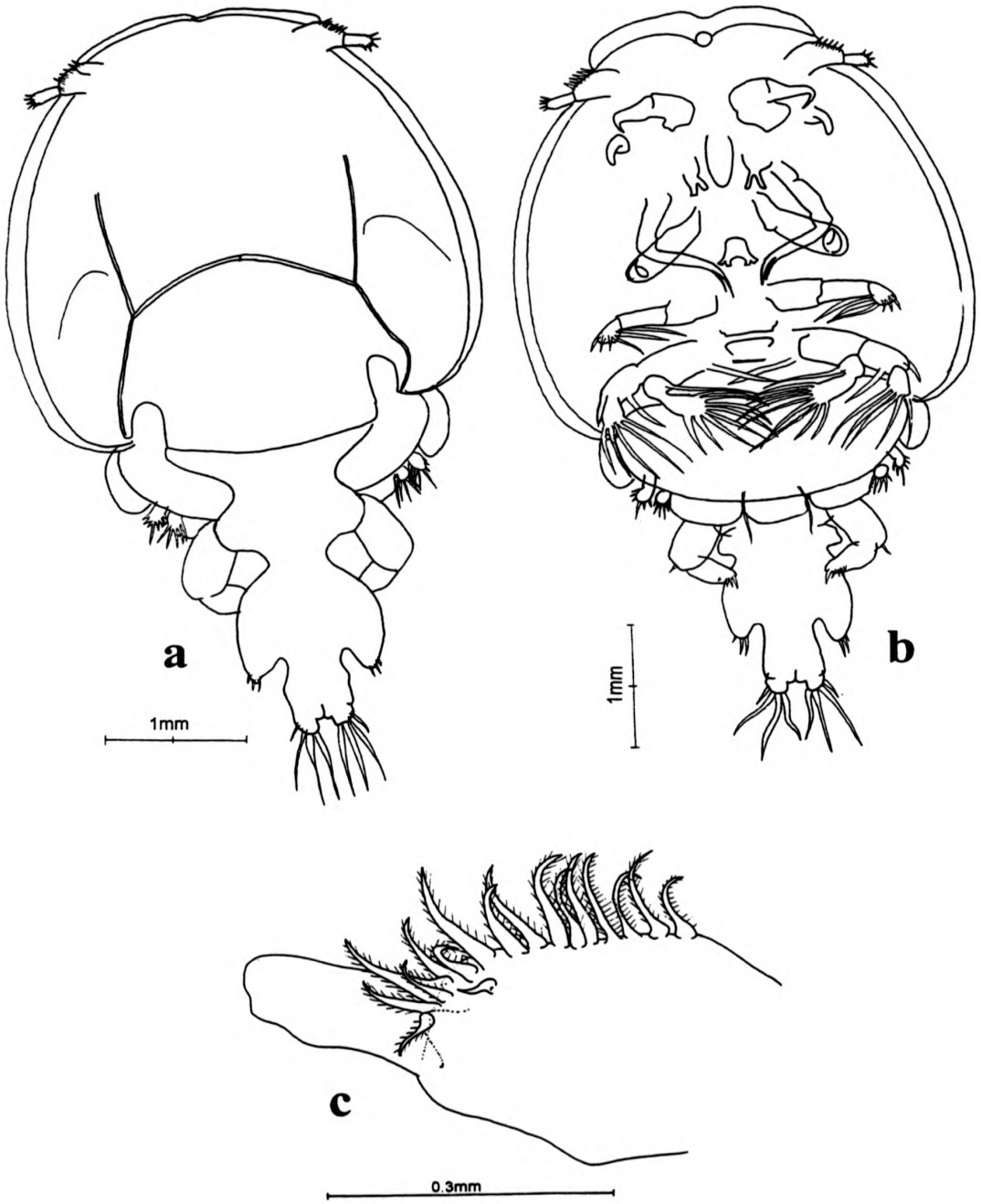


PLATE XVIII. Figure 3.11 First Pre Adult - Female. a whole animal, dorsal; b whole animal, ventral; c antennule.

3.3.9.2. Females

(Figures 3.11a-c) PLATE XVIII

The first of the pre-adult female (3.11a & b) stages measures 4.83±0.1mm in length and 3.03±0.08mm wide based on 10 specimens. The female cephalothorax is similar in shape to the corresponding male with distinct 'H' shaped sutures. The main difference between the sexes is the shape of the genital complex, the female having defined posterolateral lobes. Females do not have the 6th leg present. Antennule (Fig. 3.11c) with 17 ventral and 2 dorsal setae on proximal segment, most are sparsely pinnate.

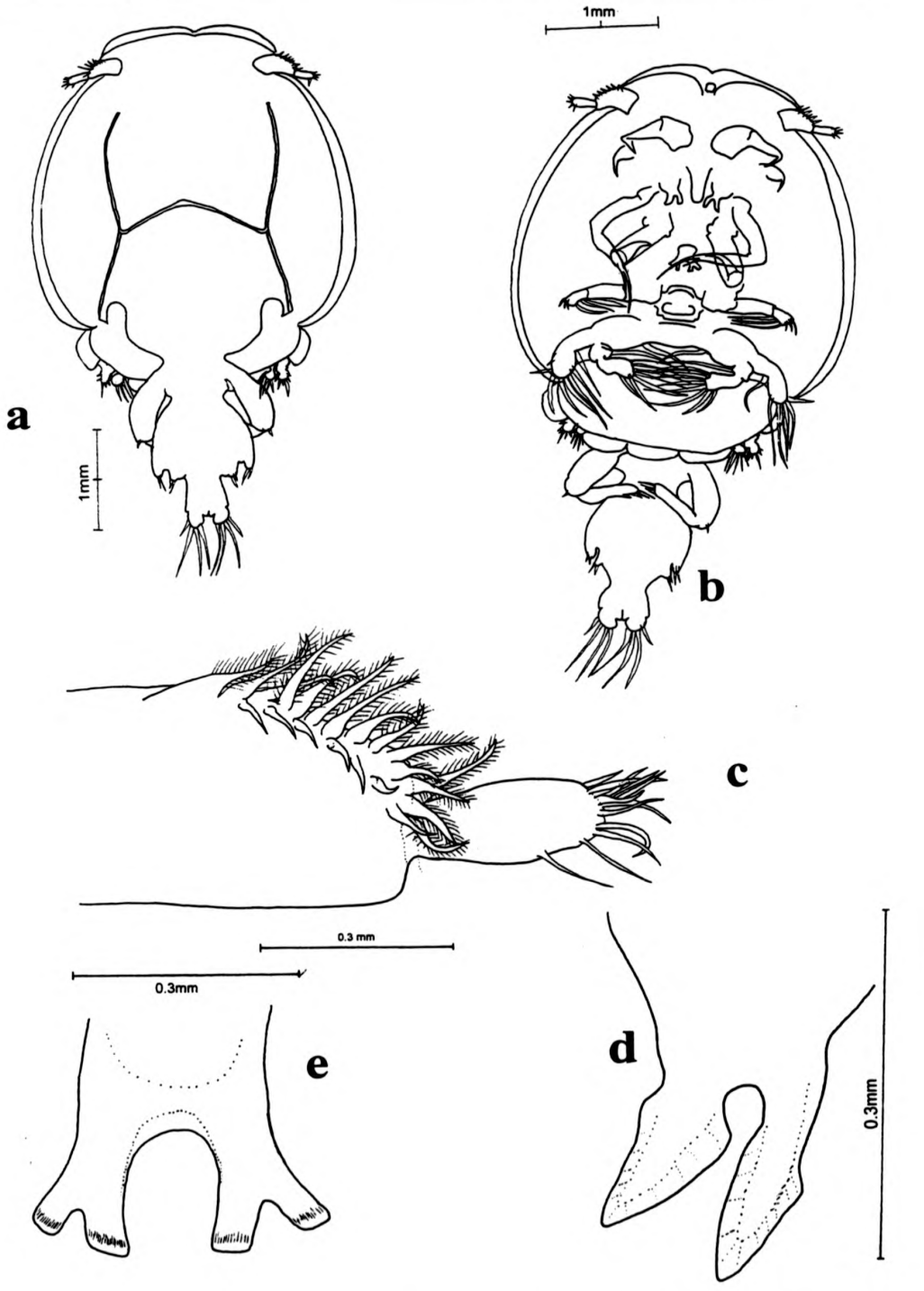


PLATE XIX. Figure 3.12 Second Pre Adult - Male. **a** whole animal, dorsal; **b** whole animal, ventral; **c** antennule, **d** maxillule; **e** sternal furca.

3.3.10. Second Pre-Adult

3.3.10.1. Male

(Figures 3.12a-i) PLATES XIX & XX

The second pre-adult male (3.12a & b) measures 4.28±0.17mm in length and is 2.68±0.1mm in width based on 10 specimens. General morphology very similar to that of the adult male, genital segment not so large but of similar shape.

Antennule (Fig.3.12c) as adult stages, proximal segment with 23 ventral and a further 2 dorsal setae, most pinnate, distal segment with 11 setae on apex and one on distal margin, all unarmed.

Antenna unchanged from previous stage.

Maxillule (Fig. 3.12d) elongate and more robust than previous stage, tines with broadened lobes along the midlength.

Maxilla, maxilliped and 1st leg unchanged from previous stage.

Sternal furca (Fig. 3.12e) similar to previous stage, split in tines more pronounced, cleft wider and further up tine.

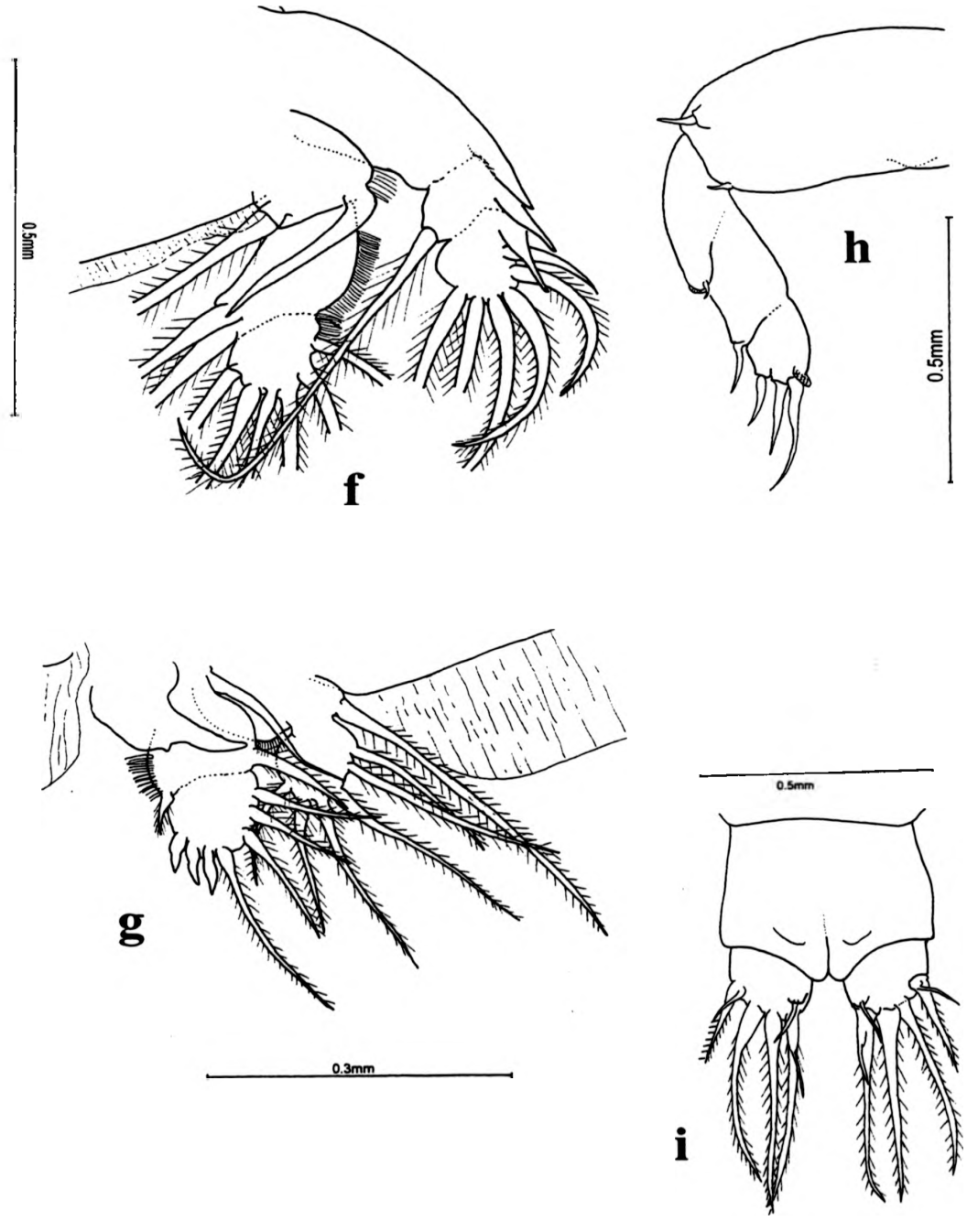


PLATE XX. Figure 3.12 Continued. Second Pre-Adult - Male. f 2nd leg; g 3rd leg; h 4th leg; i caudal ramus.

2nd Leg (Fig. 3.12f) as adult, each ramus three segmented. Exopod with stout spine at distolateral corner and long unarmed seta on medial margin of long proximal segment, squat mid segment with long pinnate seta on medial margin and short unarmed spine on distolateral corner, distal segment with 2 stout short setae on lateral margin and 5 pinnate seta covering the distal and medial margins. Endopod with short proximal segment with one unarmed seta on lateral margin and one pinnate seta on medial margin, mid segment with two pinnate seta from the medial margin and setules covering lateral margin, extended into slight lateral lobe. Distal segment with 6 pinnate setae from the distal and medial margins.

3rd Leg (Fig. 3.12g) as adult stage, exopod 3 segmented, proximal segment with stout spine and small projection at base of spine, mid segment with two pinnate setae on lateral margin and short pinnate spine on lateral margin, distal segment with three short unarmed spines on lateral margin and four long pinnate setae from the apex through medial margin. Endopod with 2 segments, proximal segment has one long pinnate seta on medial margin, distal segment with six long seta ranging from the distolateral to the distomedial margin.

4th Leg (Fig. 3.12h) sympod with pinnate seta on distolateral margin and one on distomedial margin, endopod indistinctly 3 segmented, proximal segment with stout curved spine with pecten at base on distolateral corner, mid segment with armed seta with pecten at base on lateral margin, distal segment apical margin with three armed setae, increasing in length toward the medial margin, longest with pecten at base.

5th and 6th Legs unchanged from previous stage.

Caudal ramus (Fig. 3.12i) with four long pinnate setae and a shorter unarmed seta on distomedial corner. Most medial long seta with secondary setae part way up shaft, lateral seta with short unarmed seta sharing base.

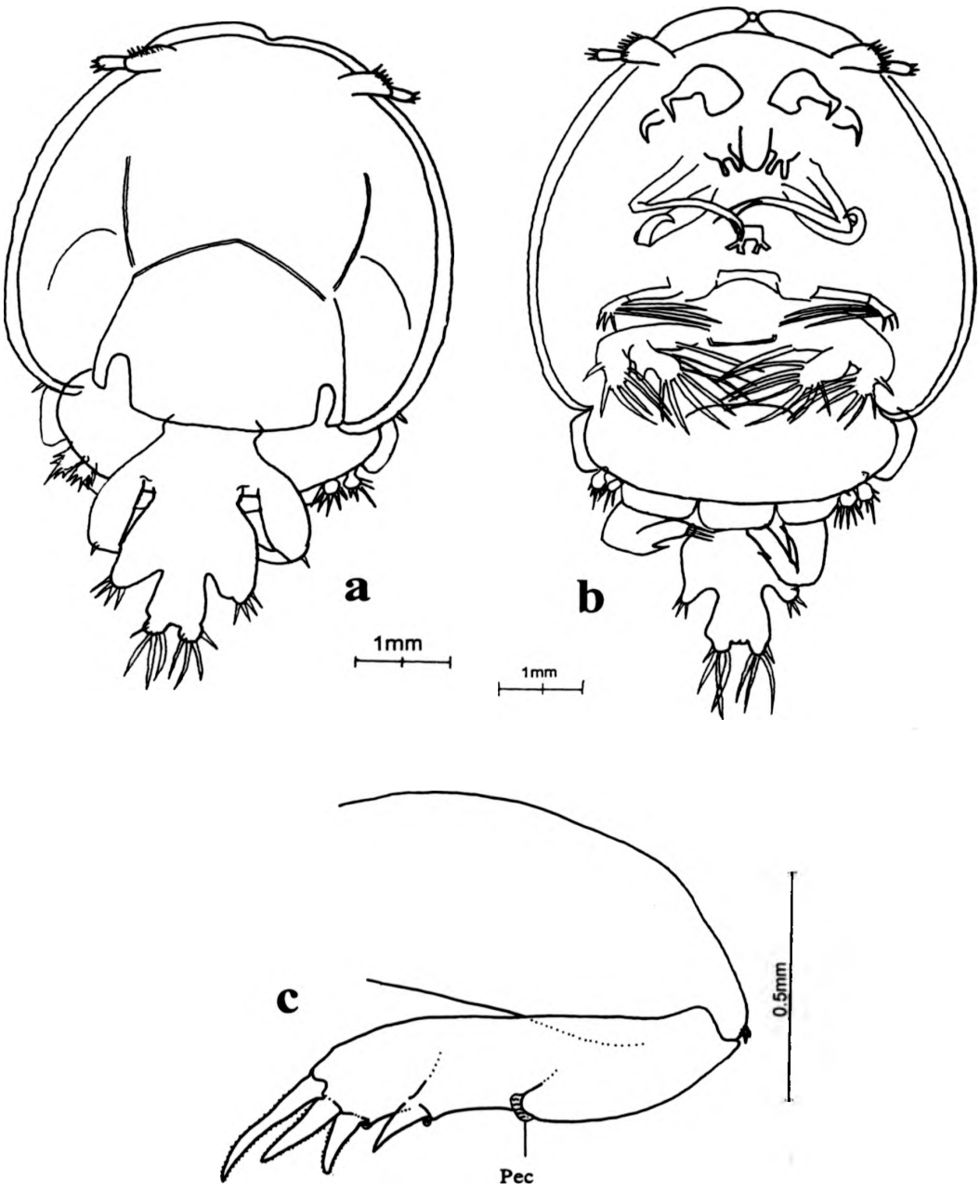


PLATE XXI. Figure 3.13. Second Pre Adult - Female. **a** whole animal, dorsal; **b** whole animal, ventral; **c** 4th leg. Pec-pecten

3.3.10.2. Female

(Figures 3.13a-c) PLATE XXI

The pre-adult 2 female (Figs. 3.13a & b) measures 6.57±0.4mm in length and is 4.09±0.13mm wide, based on 9 specimens. General appearance similar to adult female, genital complex reduced in size. Posterolateral lobes elongate compared to previous stage. Legs and appendages similar to those of the male except Leg 4 (3.13c) has no short spine on the proximal segment of the exopod, only the pecten is present. Fifth leg is as previous stage and no 6^{th} leg is present.

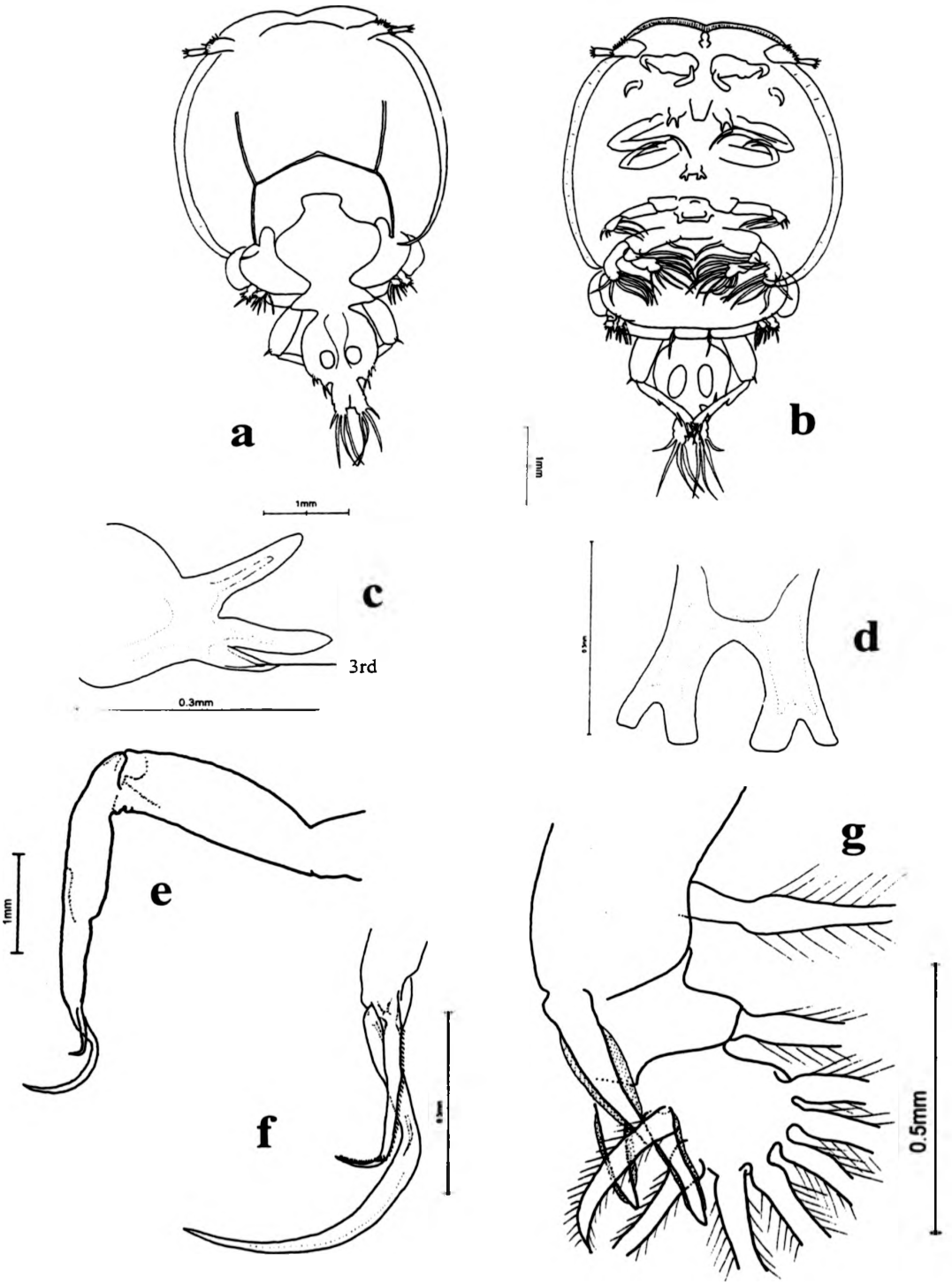


PLATE XXII. Figure 3.14. Adult - Male. **a** whole animal, dorsal; **b** whole animal, ventral; **c** maxillule; **d** sternal furca; **e** maxilla; **f** distal end of maxilla; **g** 2nd leg. 3rd-third tine of maxillule.

3.3.11. Adult

3.3.11.1. Male

(Figures 3.14a-m) PLATES XXII & XXIII

Additional descriptions of adult morphology are given in Scott & Scott (1913); Wilson (1905) and Kabata (1979). The adult male (Figs. 3.14a & b) stage measures 4.96±0.16mm in length and 2.99±0.15mm in width, based on 13 specimens.

Cephalothorax ovoid, posterior sinuses shallow. Genital segment ovoid, slightly wider than lateral margins of the fifth thoracic segment, with two posterolateral outgrowths, legs 5 and 6. Abdomen shorter than half the length of genital segment, only one segment.

Antennule and antenna unchanged from previous stage.

Maxillule (Fig. 3.14c) with third tine on medial margin, third tine about half the length of other two. Tines borne on base longer than length of tines.

Sternal furca (Fig. 3.14d) with more pronounced bifid ends to tines, lateral side of tines narrower than the medial sides.

Maxilla (Figs.3.14e & f) lacertus and brachium slender and longer than previous stage, brachium tapering toward distal end, Calanus and canna strongly curved, sharply tapered to fine point. Canna with fine denticles along its length. Setules on brachium no longer present.

Maxilliped unchanged from previous stage.

1st Leg unchanged from previous stage.

2nd Leg (Fig. 3.14g) as previous stage with modifications to stout spines on lateral margin- all three segments now with striated membranes. All other setae pinnate.

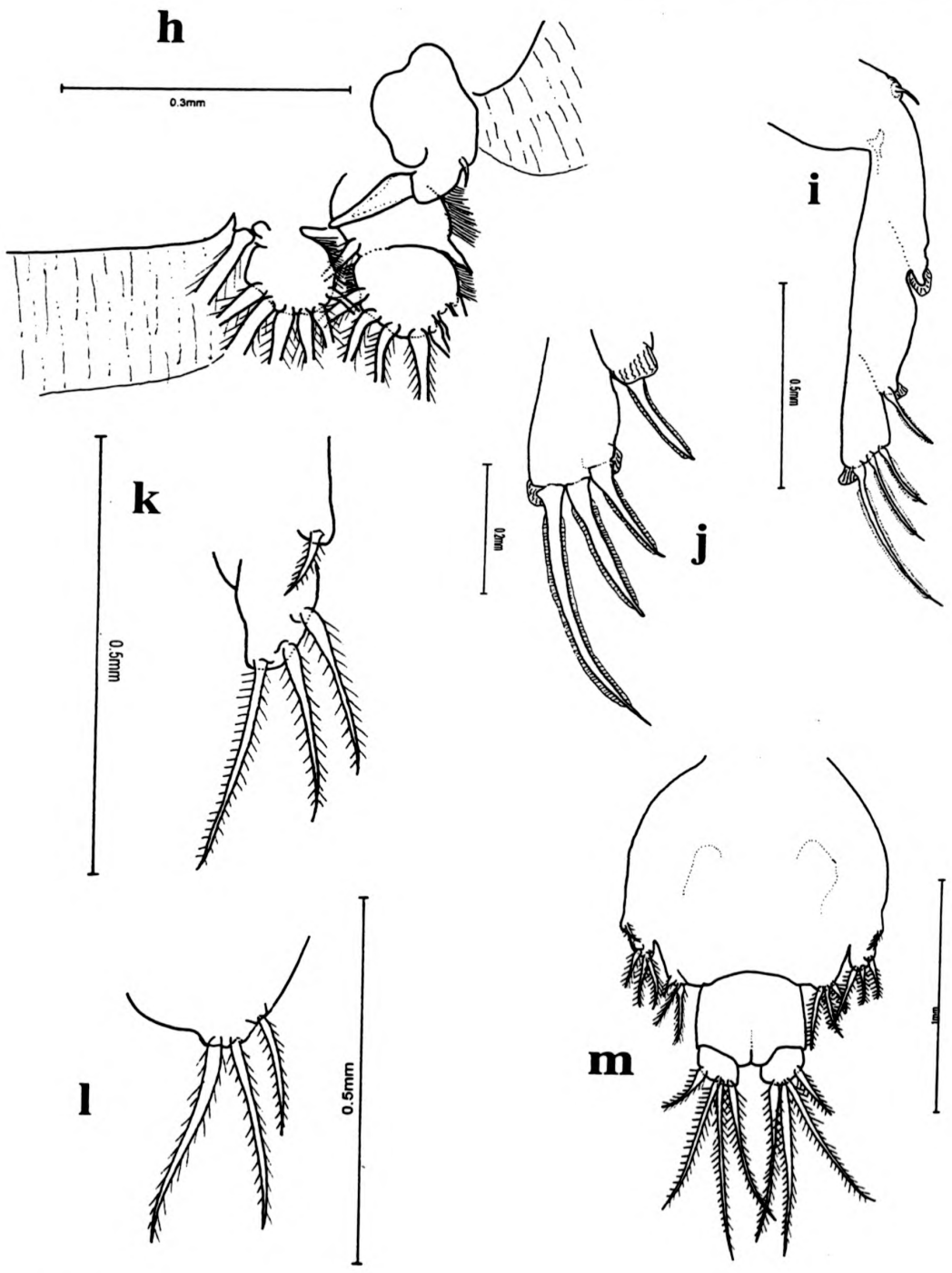


PLATE XXIII. Figure 3.14. continued. Adult - Male. **h** 3rd leg; **i** 4th leg; **j** distal portiol of 4th leg; **k** 5th leg; **l** 6th leg; **m genital segment, 5th and 6th leg and** caudal ramus.

3rd Leg (Fig.3.14h) as previous stage, with short spine on lateral edge of proximal segment of exopod.

4th Leg (Fig. 3.14i & j) longer and narrower than previous stage, sympod with short spine on distolateral corner. Exopod 3 segmented, mid segment longest. Proximal segment with pectenate membrane on distolateral projection, mid section with one long slender seta with striated membranes and pecten at base, distal segment with three long seta with striated membranes at apex, longest towards the medial margin, lateral and medial setae with pectenate membranes on base.

5th Leg (Fig. 3.14k) as previous stage, all setae pinnate and elongate.

6th Leg (Fig. 3.14l) all three setae pinnate and longer than previous stage.

Caudal ramus (Fig. 3.14m) with three long pinnate setae on distal tip and one shorter pinnate seta on distolateral corner.

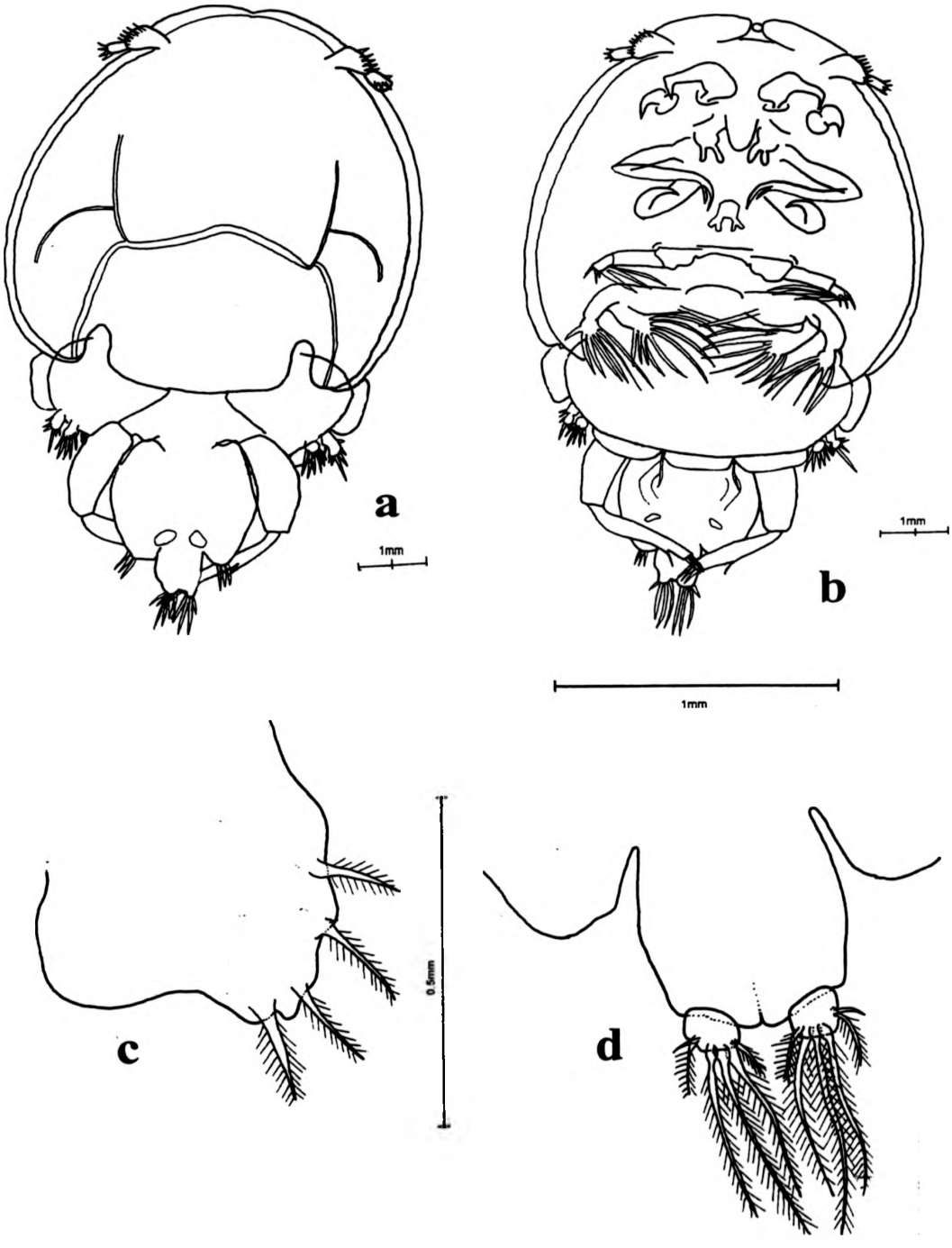


PLATE XXIV. Figure 3.15. Adult - Female. **a** whole animal, dorsal; **b** whole animal, ventral; **c** 5th leg; **d** caudal ramus.

3.3.11.2. Female

(Figures 3.15a-d) PLATE XXIV

Newly moulted adult females (Figs. 3.15a & b) measure 9.26+0.14mm in length and are 5.3±0.17mm wide, based on 9 specimens. As ovisacs develop within the genital segment, the size of the genital complex increases, maximum length of gravid females becomes 11.86±0.64mm at the time of egg production, based on 10 specimens. Genital complex of young females with folds in cuticle on anterolateral lobes, similar to second pre-adult stage. Postovigerous female genital segment swells posteriorly and laterally, becoming wider than the fifth thoracic segment, posterolateral lobes of genital complex reach half the length of abdomen. Abdomen, one segmented - very short, less than quarter the length of the genital segment, longer than its width, tapering slightly posteriorly.

Appendages and legs as male except 5th leg (Fig. 3.15c) found on posterolateral lobes of genital segment, two pinnate setae on lateral margin and two on distolateral corner. Caudal ramus (Fig. 3.15d) three long pinnate setae on distal apex with swollen bases, one shorter pinnate seta with short spine sharing base on lateral margin, one short pinnate seta on medial margin.

3.3.12. Summary Tables

A number of tables have been compiled in order to highlight the main differences between each of the stages. These tables are a summary of the detailed descriptions given above and should be used in conjunction with this information. Table 3.1 summarises the maximum length and width data and includes the range of lengths and widths found within each of the stages. Table 3.2 summarises the changes that occur with the overall body shape and segmentation of each of the stages of the life cycle. Table 3.3 gives a brief summary of the number and morphology of the legs present at each of the life stages and also the number and type of setae found on the distal segment of each leg.

Life stage	mean length±s.d. (mm)	mean width±s.d. (mm)	n	Range	
				Length (mm)	Width (mm)
nauplius 1	0.68±0.03	0.24±0.01	14	0.62-0.70	0.21-0.25
nauplius 2	0.73±0.02	0.24±0.01	13	0.70-0.76	0.21-0.26
copepodid	0.97±0.03	0.40±0.04	30	0.90-1.03	0.38-0.43
chalimus 1	1.29±0.06	0.68±0.04	7	1.23-1.39	0.62-0.68
chalimus 2	1.52±0.06	0.67±0.04	8	1.39-1.59	0.62-0.72
chalimus 3	2.74±0.25	1.59±0.15	18	2.18-3.14	1.39-1.80
chalimus 4	3.27±0.24	1.91±0.13	15	2.95-3.79	1.65-2.15
pre adult 1 male	3.63±0.17	2.37±0.12	10	3.35-3.94	2.22-2.65
pre adult 2 male	4.28±0.17	2.68±0.10	10	3.95-4.50	2.47-2.86
adult male	4.96±0.16	2.99±0.15	13	4.73-5.23	2.8-3.47
pre adult 1 female	4.83±0.10	3.03±0.08	10	4.72-5.03	2.93-3.10
pre adult 2 female	6.57±0.40	4.09±0.13	9	5.98-6.99	3.82-4.21
pre-gravid female	9.26±0.41	5.30±0.17	9	8.64-9.95	5.29-5.55
post gravid female	11.86±0.64	5.67±0.40	10	11.24-13.06	5.44-6.53

Table 3. 1. Summary of length and width data for each of the life stages of *L. hippoglossi*, including the range of lengths and widths within each life stage. n= number in sample.

Life Phase	Sections	Description
Nauplius I & II	• Unsegmented	• Cylindrical-tapering posteriorly
Copepodid	• Cephalothorax • Abdomen	• Abdomen-4 segmented • Whole body dorso-ventrally flattened.
Chalimus I - IV	• Cephalothorax • Abdomen • All stages with anterior filament	• Cephalothorax becomes more ovoid with each moult • CI – Cephalothorax including 3rd thoracic segment. • CIII – Cephalothorax including 4th thoracic segment.
Pre-Adult	• Cephalothorax • Small genital segment (females with small protruding lateral lobes) • Abdomen • May have anterior filament present after moult-short lived.	• Frontal plates developed • H shaped sutures on dorsal surface of cephalothorax. • Cephalothorax-ovoid with marginal membranes
Adult	• Cephalothorax • Fully developed genital segment • Abdomen	• Very similar in shape to pre-adult. • Genital segment: male-ovoid; female-lateral lobes. • Abdomen-1 segment, very small

Table 3. 2. Changes in overall body shape throughout the life cycle of *L. hippoglossi*

Life Stage	Legs Present	Brief description of Distal Segment
Nauplii	None	Swimming appendages only
Copepodid	3	1st Leg- Biramous: endo: 7 pinnate setae, exo: 3 stout, 4 long pinnate setae 2nd Leg-Biramous: endo:6 pinnate setae, exo: 2 stout, 5 long pinnate setae 3rd Leg-small swelling, 2 short setae
Chalimus I	4	1st Leg-Biramous: endo: 2 long setae, exo: 4 long, 2 stout setae 2nd Leg-Biramous: endo: 6 unarmed setae, exo: 7 setae 3rd Leg-Biramous: endo: 2 stout setae, exo: 1 stout, 4 long setae. 4th Leg- small protrusion with 1 small setae.
Chalimus II	4	1st Leg-Biramous: endo: 2 long setae, exo: 2 stout 6 long setae 2nd Leg-Biramous: endo: 6 setae, exo: 8 setae 3rd Leg-Biramous: endo: 4 setae, exo: 6 setae 4th Leg-2 segment, 2 short setae.
Chalimus III	5	1st Leg-Biramous: endo: much reduced, 2 unarmed setae, exo: 7 setae 2nd Leg-Interpodal Bar, Biramous: endo: 7 unarmed setae, exo: 2 stout, 6 long setae (unarmed) 3rd Leg-Interpodal Bar, Biramous: endo: 1 stout, 4 pinnate setae, exo: 1 stout , 6 unarmed setae. 4th Leg-Uniramous, 3 distal setae. 5th Leg-small swelling, 2 short setae
Chalimus IV	5	1st Leg-Interpodal Bar (as CIII) 2nd Leg- Biramous endo: 8 setae, exo: 3 stout, 6 long setae 3rd Leg-as CIII 4th Leg- sympod longer than CIII, armature as before. 5th Leg- as CIII
1st Pre-adults	M: 6 F: 5	1st Leg-endopod not present, exopod as before, long setae armed. 2nd Leg- Biramous, endo: 8 pinnate setae, exo: 3 stout, 5 long pinnate setae. 3rd Leg-Biramous, endo:4 pinnate setae, exo: 4 short, 5 pinnate setae 4th Leg- Uniramous, 4 stout setae 5th Leg- two processes, 3 pinnate setae 6th Leg- bulbous outgrowth, 1 short, 2 pinnate setae.
2nd Pre-adult	M: 6 F: 5	1st Leg-unchanged 2nd Leg- Biramous, endo: 6 pinnate setae, exo: 2 stout, 5 pinnate setae. 3rd Leg-endo: 6 pinnate setae, exo: 3 stout, 4 pinnate setae. 4th Leg-3 setae, 1 with pecten membrane. 5th & 6th Legs- unchanged.
Adult	M: 6 F: 5	1st Leg- unchanged 2nd Leg- 2 stout spines with membranes 3rd Leg- unchanged 4th Leg- longer and narrower, 3 long setae, all with membranes 5th Leg- unchanged, all setae pinnate 6th Leg- all setae longer and pinnate

Table 3. 3. A summary of the legs present at each life stage *of L. hippoglossi*. endo=endopod, exo=exopod, all numbers of setae are for the distal segments of each ramus only. M=Male, F=Female.

3.4. Discussion

The life stages of *Lepeophtheirus hippoglossi* are representative of the typical caligid life cycle with 2 nauplii, 1 copepodid, 4 chalimus, 2 pre-adult and 1 adult stage (Fig 3.1), as described for *L. salmonis* by Johnson & Albright (1991a) and *L. pectoralis* by Boxshall (1974a). However, *Caligus elongatus* has four chalimus stages with no pre-adult stages before adulthood (Piasecki, 1996). In *L. hippoglossi, L. salmonis* and *L. pectoralis* the sexually dimorphic characteristics do not become apparent until after moulting into the 1st pre-adult stage (Johnson & Albright, 1991a; Boxshall, 1974a). Sexual dimorphism becomes apparent in the 4th chalimus stage in *C. elongatus* (Piasecki, 1996) and as early as the 3rd chalimus stage in *C. clemensi* (Kabata, 1972).

Unless otherwise indicated for the remainder of the chapter any reference made to *L. salmonis* will be taken from Johnson and Albright (1991a), *L. pectoralis* from Boxhall, (1974a) and *C. elongates* from Piasecki (1996).

Lepeophtheirus hippoglossi, as with other *Lepeophtheirus* species (Johnson & Albright, 1991a; Boxshall, 1974a) undergoes the first major metamorphosis during the moult from nauplius II into copepodid. In older specimens of the nauplius II individuals, some of the features of the copepodid can be seen within the cuticle, as has also been reported in *C. elongatus*, *L. salmonis* (Johannessen, 1978) and *L. pectoralis*. The change from copepodid form through to the adult form is a more gradual one. There is a loss of segmentation in the body and appendages in the first chalimus compared with the copepodid but segmentation then increases through the chalimus stages to the adult.

At the same time the complexity and number of components within the appendages increases. The rate of this process of reduction and then development is very similar for *L. salmonis* and *L. hippoglossi*, but it occurs at a different rate in *L. pectoralis*. In general the development towards the adult form of the appendages in *Lepeophtheirus* species is slower than in *Caligus* species.

The adult female *L. hippoglossi* undergoes a further change in shape as described by Boxshall (1974a) for *L. pectoralis*. The change from being pre-gravid to gravid requires changes in shape and size of the genital segment after copulation but without a further moult, as has also been reported in *L. salmonis* and *L. pectoralis*. Johnson and Albright, (1991a) report folds of cuticle at the anterior end of the genital segment in newly moulted adult female *L. salmonis* which may act as a reservoir of cuticle in order for the growth to occur without a further moult.

The developmental pattern of the antennule in *L. hippoglossi* is slightly different from that of *L. salmonis* and *L. pectoralis*. In the latter two species the armature of the distal segment is attained at the third chalimus stage but in *L. hippoglossi* it is not attained until the fourth chalimus stage. In both *L. salmonis* and *L. pectoralis* there are 11 distal and 1 posterolateral setae but an additional 2 aesthetes are reported. These were not observed in *L. hippoglossi* although the number of setae was identical. Also the number of anteroventral setae on the proximal segment is different for each species, 23 in *L. hippoglossi*, 25 in *L. salmonis* and 29 in *L. pectoralis*, however the species all have two anterodorsal setae in all the pre-adult and adult stages. In *C. elongatus* the

adult antennule form is obtained by the chalimus IV stage, however the full complement of setae on the distal segment is reached by the chalimus II stage.

The two nauplius stages of *L. hippoglossi* can be distinguished mainly by the antenna. In the second stage a distinctive broad spine appears; this is described as an apical process by Johnson & Albright (1991a), which elongated between stages in *L. salmonis*. The antenna undergoes a marked change in the copepodid stage of *L. hippoglossi* and the terminal claw is at its most pronounced; in the following two stages it undergoes proximal broadening. At the chalimus three and four stages the terminal claw elongates and appears to be 'blunt'. With the moult to pre-adult the antenna takes on a more hook like appearance once again, with two stout setae. This resembles the antenna of the adult, with only some slight changes between the sexes.

In all *Lepeophtheirus* and *Caligus* species the mandible is more or less unchanged from chalimus I to the adult, although there may be more distinct segmentation (4 segments) in the latter stages (Johnson & Albright, 1991a). In *L. hippoglossi* the mandible has 11 crenulated teeth. The distal portion of the mandible of *L. pectoralis* bears 12 denticles on its medial margin and *L. salmonis* and *C. elongatus* are also armed with 12 teeth.

The pattern of development of the maxillule of *L. hippoglossi* is very similar to those of *L. salmonis* and *L. pectoralis*. However in *L. hippoglossi* the posterior process becomes bifid in the third chalimus stage, not in the fourth as the other two species.

There is a third tine in the adult male stage which is not present in the female, as in *L. salmonis* and *L. pectoralis*.

The maxilla of the copepodid of *L. hippoglossi* is distinct from all other stages due to the virtually equal size of the canna and calamus. In all other stages the calamus is much longer. The overall shape of the maxilla is conserved throughout the developmental stages, as in *L. salmonis*, with the main changes occurring in the curvature of the canna and calamus and the relative lengths of the brachium and lacertus.

The maxilliped of the copepodid stage of *L. hippoglossi* is similar to that of *C. elongatus* as it has double auxiliary spines on the shaft. *L. salmonis* has an auxiliary spine which forms a branching barb. *L. pectoralis* has a short pectinate spine present at the suture line. The maxilliped of *L. hippoglossi* undergoes its largest transformation in the moult to the chalimus I stage, as in other *Lepeophtheirus* and *Caligus* species (Johnson & Albright, 1991a).

In *L. salmonis* and other *Lepeophtheirus* and *Caligus* species the true sternal furca is present from the chalimus IV stage (Johnson & Albright, 1991a), however in the present study the sternal furca was not present until the first pre-adult stage of *L. hippoglossi*. In this stage of *L. hippoglossi* the bifid tines of the furca are of equal width and the suture is more shallow than in later stages. In the adult stage the medial tines are narrower than the lateral ones. The sternal furca of *L. hippoglossi* is different

from that of all other *Lepeophtheirus* species in European waters as it is the only one with bifid tines (Kabata, 1979).

The development of the 1st leg of *L. hippoglossi* is exactly the same as in *L. salmonis* and many other species of *Lepeophtheirus* and *Caligus*. *C. clemensi* differs by having only one seta on the endopod rather than two as in all other species (Parker & Margolis, 1964; Johnson & Albright, 1991a).

Except for the number of endopod and exopod setae, the development of the second leg of *L. hippoglossi* is virtually identical to that reported for *L. salmonis* and *L. pectoralis*. In all of the *Lepeophtheirus* species the broad marginal and surface membranes appear at the first pre-adult stage and the adult form of the leg is reached by the second pre-adult stage (Johnson & Albright, 1991a). In the adult stage of *L. salmonis* there is one extra exopodal seta compared to *L. hippoglossi*. The adult form of the second leg in *C. elongatus* is reached at the chalimus 4 stage, however, the armature and membranes are much heavier in the adult stage. In *L. hippoglossi* the marginal membranes first appear in the first pre-adult stage.

The development of the 3rd leg of *L. hippoglossi* is very similar to that of other *Lepeophtheirus* species. The setation on the adult form of this leg is more or less identical for *L. salmonis* and *C. elongatus*. The first chalimus stages of *C. elongatus* have a uniramous third leg which makes it different from the *Lepeophtheirus* species which all have biramous forms.

The fourth leg appears at the first chalimus stage of *L. hippoglossi*, as in other *Lepeophtheirus* species (Johnson & Albright, 1991a). In *Caligus elongatus* it does not appear until the second chalimus stage; this is consistent with other *Caligus* species. The *Lepeophtheirus* species have 3 elongate setae at the distal apex whereas *C. elongatus* only has 2 distal setae. by the fourth chalimus stage all species have a fourth leg resembling the adult form although these do elongate and the segmentation becomes more distinct with each later stage (Johnson & Albright, 1991a).

The fifth leg only appears in the third chalimus stage and in all stages remains small in *L. hippoglossi*. This is also true for *L. salmonis*, *L. pectoralis* and *C. elongatus*. In *C. clemensi* (see Kabata, 1972) and *C. spinosa* (see Izawa, 1969) the fifth leg first appears in the first pre-adult stage.

The sixth leg only appears in the males and first appears at the first pre-adult stage of *L. hippoglossi*, and all other species of *Lepeophtheirus* studied. The caudal ramus is slightly different between the sexes at the adult stage, In *L. hippoglossi* and *L. salmonis* the males have four main long pinnate setae and the females have an extra two small setae on the margins.

In summary the development of *L. hippoglossi* is very similar to that of *L. salmonis* and *L. pectoralis*, with only slight differences occurring in the number of setae in the 1st to third legs. The main and most obvious differences between the species discussed in

this chapter are the presence of the bifid tines of the sternal furca in *L. hippoglossi* and the lack of lunules in the *Lepeophtheirus* species. The most obvious difference between adult female stages of *L. hippoglossi* and *L. salmonis* is the length of the abdomen, which is much longer in relation to the genital segment in *L. salmonis*.

Chapter 4

Aspects of the biology of *Lepeophtheirus hippoglossi* (Copepoda, Caligidae).

4.1. Introduction

4.1.1. *Lepeophtheirus hippoglossi* on wild halibut

Lepeophtheirus hippoglossi is a commonly occuring parasite of wild halibut. Schram & Haug, (1988) found *L. hippoglossi* present at prevalence rates of 66 and 78%, with up to 38 individuals per fish examined from two areas in northern Norway. *L. hippoglossi* was found by Zubchenko (1980) on 10% of specimens examined from Newfoundland. During the present study, halibut caught on Baileys Bank in Faeroese waters (60° 36' N, 10° 10' W) showed a 100% infection rate with up to 9 parasites per fish, but this is likely to be an underestimate as excessive handling during capture and transport may have caused the loss of some parasites before examination. None of these accounts of *L. hippoglossi* have included reports of any pathology caused to their hosts, possibly due to the low number of individual parasites on large (10+ kg) fish or simply due to it not being recorded. This parasite has never been reported from halibut under culture conditions.

4.1.2. Life cycle

As previously described (Chapter 3), *L. hippoglossi* has a typical life cycle of the genus *Lepeophtheirus* with two nauplius, a copepodid, four chalimus, two pre-adult and an

adult stage. A number of copepod species, especially those harmful to commercial aquaculture, have been studied to determine the timing of their life cycle at specific temperatures, particularly those pertinent to the culture of their host. Such information is highly relevant to the development of control measures. Temperature has been shown to have an important impact on the timing of caligid life cycles. Johnson and Albright (1991b) reported the effect of temperature and salinity on the development, growth and survival of *L. salmonis* on its natural host the Atlantic salmon (*Salmo salar*). In their study copepodids were present 12 days after egg sac extrusion, chalimus IV stages first appeared on day 29 after egg sac extrusion and it took 40 and 52 days to reach the adult male and female stages, respectively, at 10°C. However, the predicted development of *L. salmonis* from egg to pre-gravid adult female can take between 23 and 120 days depending on the season (maximum summer water temperature13-17°C; minimum winter water temperature 5-8°C over an 11 year period) (Tully, 1992). These predicted figures correspond to the data of M^{c}Andrew *et al.*, (unpublished data) who reported that it took 24 days post infection to reach adult female *L. salmonis* at a mean temperature of 12.8°C. Development to adult and production of the first batch of eggs was four times as long at 7.2°C than at 12.2°C in *L. salmonis* (see Heuch *et al.*, 2000). Piasecki and MacKinnon (1995) described the life cycle of *Caligus elongatus* under laboratory conditions at 10°C and suggested that the generation time from egg to gravid female is 43.3 days at this temperature. A further study by Hogans and Trudeau (1989) studied the life cycle of *C. elongatus* and suggested that the generation time (from nauplius 1 to mature adult female) was 5 weeks (35 days) at 10°C. The two reports on the generation time for *C. elongatus* are

comparable as Hogans and Trudeau (1989) also report that the free living stages live for approximately 9.6 days, giving a generation time from egg to mature adult female of 44.6 days at 10°C.

4.1.3. Survival and Longevity

Heuch *et al.* (2000) reported that adult female *L. salmonis* survived for up to 191 days at 7.2°C. Piasecki and MacKinnon (1995) found that in experimental conditions the adult males of *C. elongatus* left the host after mating and suggest that in the wild they search for other females. However, Hogans and Trudeau (1989) found a similar trend in cage culture sites for the same species, particularly over the winter months when over 90% of the population were females. Thus adult caligid copepods can survive for a long period after reaching maturity.

There are no published accounts of the developmental time of free living or attached stages of *L. hippoglossi*. This study also investigates the longevity and survival rates of adult stages of *L. hippoglossi* under laboratory conditions.

4.1.4. Reproduction and Egg Production

Copulation in caligid copepods occurs only between adult stages (Boxshall, 1990). However mate guarding occurs between adult males and females of both pre-adult stages and the adult stage of *L. salmonis*. Over two thirds of mating pairs were made up of pre-adult II females and adult males (Jaworski & Holm, 1992).

Caligid copepods produce a number of pairs (batches) of egg strings. Piasecki and MacKinnon (1995) reported that the females of *C. elongatus* produce at least two batches of egg strings. Jackson and Minchin (1992) reported that *C. elongatus* from untreated farmed salmon produced between 108 and 178 eggs per egg string whilst Hogans and Trudeau, (1989) found that each string contained a mean of 89 eggs for the same species also in culture conditions. Heuch *et al.* (2000) reported that over a 191 day period at 7.2°C female *L. salmonis* can produce 11 pairs of egg strings. The egg strings varied from 3.5 to 41.0mm in length and contained between 55 and 704 eggs per string. Johnson and Albright (1991b) found that *L. salmonis* produced egg strings containing 100-500 eggs under laboratory conditions, whereas Jackson and Minchin (1992) suggested that the reproductive output of *L. salmonis* from wild salmon was 965±30.86 eggs per egg string. Temperature has a great effect on egg string length and number of eggs produced; female *L. salmonis* from winter generations were shown to produce longer egg strings with more but smaller eggs than those from summer generations (Ritchie *et al.*, 1993).

This chapter covers aspects of the biology of *L. hippoglossi*, including the timing of the life cycle at a range of temperatures, survival and egg production.

4.2. Materials and Methods

4.2.1. Parasite Source

As part of a British Halibut Association (BHA) broodstock recruitment effort a long liner was commissioned from Kirkwall, Orkney to fish Baileys Bank in Faroese waters (60° 36'N, 10° 10' W). Seventy six adult halibut (size range 5 to 100kg) were brought back alive to Orkney for a period of quarantine and routine health screening. The fish were held in tanks aboard the ship, then transported to the quarantine facility (Orkney Marine Hatcheries, Deerness) by road and thus underwent a long period of stressful conditions before being placed into large (10m diameter) indoor tanks. Forty of the halibut were required to be killed and screened for viral, bacterial and parasitic disease by government scientists. At this time approximately 60 adult female *Lepeophtheirus hippoglossi* were collected from these wild halibut. They were transported to the Institute of Aquaculture Research Laboratory, Machrihanish within plastic bags filled with clean seawater and with a constant air supply placed within an insulated carrying box. The transportation period was approximately 20 hours. At Machrihanish they were allowed to attach to cultured halibut. These provided a stock of parasites for experimental purposes. Once the parasites had acclimatised to their new host, the females produced egg strings. Collection of egg strings was achieved by removal of the females from the host using the flattened edge of curved forceps and placing them in a petri dish containing a small amount of clean seawater. They were then held with forceps around the body between the third and fourth legs and the egg strings were pulled off with a second pair of forceps. The females were then allowed to re-attach to the host. The egg strings were placed in a 10 litre plastic container filled with seawater

filtered through a 10μm mesh and held at ambient temperature and salinity with an air supply. The container was placed in a tank with flowing water also at ambient water temperature to maintain a constant temperature for egg development. The seawater was changed daily and, at this time, dead and moribund individuals were removed along with empty egg cases and other debris using a siphon. The egg strings were monitored for signs of development i.e. darkening in colour and hatching. Nauplii were kept under the same conditions as the egg strings, in the same containers and, once they had moulted into copepodids, they were used for experimental infections. The time taken to collect sufficient copepodids depended on the age (colour) of the egg strings at collection and the temperature, but was within 6-10 days after egg string removal.

4.2.2. Experimental Infections

The nauplius (Fig 4.1) and copepodid (Fig 4.2) stages were counted using a Bogorov chamber which held 10ml of liquid. This chamber facilitated counting since the channel cut into the Perspex could be examined under a dissecting microscope while the free swimming stages could not move very freely along the length of the chamber. The copepodids from three separate 10ml aliquots were counted, the mean number calculated and multiplied by the total volume of the bucket to give the total number of copepodids present. The mean number of copepodids per experimental fish could then be calculated. During infections the water level in the fish tank was lowered so that the halibut were covered by approximately 5cm of water but the air supply remained on. The copepodids were introduced to the tank and left for 3-6 hours. After this time the water supply was resumed and the tank allowed to flush. Temperature and salinity at

which eggs were incubated was the same as those at which fish were maintained in each experiment.

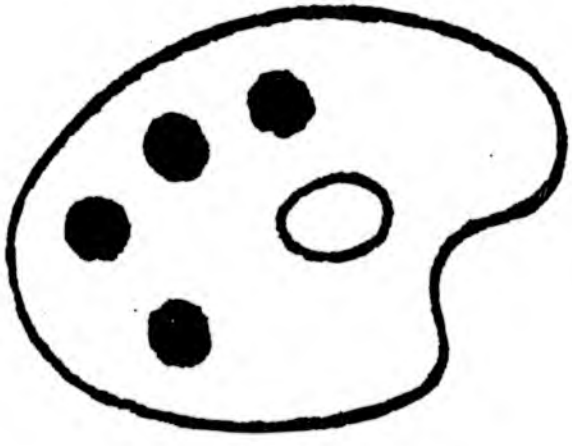
4.2.3. Life cycle

Three separate experimental infections were undertaken, one each at low (7.5°C), medium (9.7°C) and high (13.1°C) mean temperatures. It was not possible to run all experiments concurrently due to the utilisation of natural ambient temperatures at different times of the year. Differences in the procedures between experiments are described in paragraphs 4.2.3.1 and 4.2.3.2. Most of these arose from the necessity to use different sized groups of fish for each experiment due to the low numbers of fish available for each of the three experiments.

4.2.3.1. Medium Temperature Experiment

Sixty juvenile halibut (1998 year class, approximately 1 year post hatch) of average weight 49.6±20.3g, range 9.1-106.0g and average length 15.7±2.0cm (range 10.0-19.5cm)) were brought from a commercial site and transported to the aquarium at the University of Stirling. These fish were placed into two 1m square tanks, 30 fish in each and allowed to acclimatise for 10 days. The halibut were fed to satiation with 3.5mm salmon pellets once each day. Salinity and temperature were measured daily; mean temperature 9.7°C (range 9-10°C), mean salinity 34‰. The aquarium received water through a recirculation system.

Numerous Originals in Colour



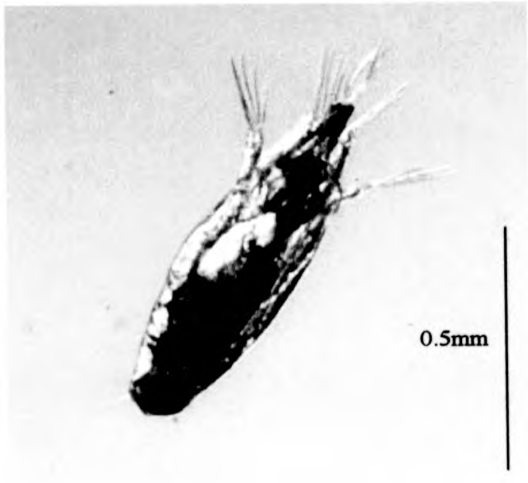


Figure 4. 1. *Lepeophtheirus hippoglossi* free-swimming nauplius I stage

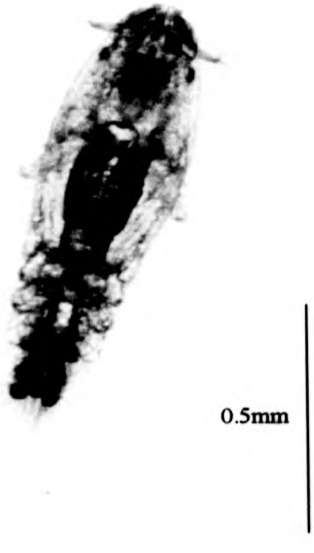


Figure 4. 2. *Lepeophtheirus hippoglossi* free-swimming copepodid stage

Egg strings from adult female *Lepeophtheirus hippoglossi* were collected from previously infected halibut in Machrihanish, transported back to IOA, Stirling and incubated at 10°C in aerated seawater in 5 litre containers. Once large numbers of copepodids were obtained they were counted (total=3876 copepodids, 64.4 per fish) and split into two groups.

Due to the nature of the re-circulation system being used in the aquarium (and the presence of other fish, including halibut), static tanks were used for the infection. Each group of 30 fish was removed from its tank and placed into a large (1m diameter) round plastic tank with approximately 50 litres of sea water and aeration. One group (half) of the copepodids was placed into each tank. A net was placed tightly over the top of the tanks to stop the fish jumping out. The fish were left in contact with the copepodids for 5 hours and 30 minutes. After this time the nets were removed, and the fish placed back into the 1m square tanks.

The infected halibut were then left for three days to allow the parasites to securely attach. After this time the sampling regime began.

On day 3 post infection two halibut (one from each tank) were collected from the aquarium and placed into separate 30cm square plastic containers, measured and weighed, then killed humanely and examined carefully under a dissecting microscope. Any parasites present were mapped onto a fish map (for example see Fig 5.1, Chapter 5 page 155), their life stage noted and then removed for further examination. The water

in the square containers was filtered through a 10μm mesh which was examined under a dissecting microscope for any parasites that may have become detached. This sampling regime was continued on alternate days and was expected to continue until the parasites reached the adult stage and the first egg strings were produced. Unfortunately due to high numbers of pre-adult and adult male stages on the fish at day 30 a number of fish mortalities occurred and it was necessary to reduce the number of parasites present. The fish were anaesthetised in 2-phenoxyethanol (1:1500) and excess numbers of parasites removed, leaving approximately ten individuals per fish. The fish were then returned to their tanks and the sampling regime was resumed. However, the number of parasites remaining rapidly decreased and by day 39 none remained.

4.2.3.2. Low and High Temperature Experiments

The two life cycle experiments investigating the effect of low and high temperatures on the development of *L. hippoglossi* were carried out at Machrihanish. These experiments were carried out at different times of the year using ambient sea temperature in a flow to waste system. The mean temperature for the low temperature experiment was 7.5°C (range 6-9°C), average salinity was 33‰ (range 32-33‰) and for the high temperature experiment 13.1°C (range11-15°C) and 33‰ (range 32-33‰). Five fish in each of two 2m round tanks were used in the low temperature experiment (weight range 1-1.5kg) and 6 halibut (0.9-1.6kg) in each of two 2m round tanks were used for the high temperature experiment.

Infections took place within the 2m tanks, the water level was lowered and the air supply left on (for details see section 4.2.2.). An average of 38.7 copepodids per fish

was used in the high temperature experiment and 102.4 copepodids per fish were used in the low temperature experiment. The differences between the numbers used in the infections is due to the difference in number of copepodids that could be obtained at each time. Fish were maintained in their tanks for 7 days in the low temperature and 4 days at the high temperature before the sampling regime commenced.

In each of these experiments the number of parasites and their developmental stage were monitored twice weekly over a 71 day period at the low temperature and a 63 day period for the high temperature experiment. On each sampling date, two fish (one from each tank) were randomly selected, anaesthetised in 2-phenoxyethanol and observed under a dissecting microscope (for early parasite stages) or with a hand lens (for larger parasite stages). The number and stage of parasites was noted. The anaesthetic bath was examined between each fish and any parasites which had detached were allowed to re-attach to the host by placing them back on the fish. The halibut were allowed to recover in a small tank of clean sea water which was also monitored for any detached parasites, which were also re-attached before the halibut was returned to its original 2m tank.

4.2.4. Longevity of Adult *L. hippoglossi*

Three tanks of halibut (tank 1.6, two fish; tank 1.7, three fish and tank 1.8, four fish) were infected with copepodids of *L. hippoglossi*. The main purpose of this infection was to provide a 'stock' supply of parasites which could be used for material for future experiments, therefore the number of copepodids used in the infection was not recorded and the parasites were allowed to develop without being closely monitored. However,

when the adult stage was reached, the number of adult parasites present was more closely monitored. The first sample was taken on day 36 post infection (when the adults were first observed on the host) then subsequently on days 43, 63 and either 104 or 133 days post infection. Tanks 1.7 and 1.8 were not sampled on day 133 and tank 1.6 was not sampled on day 104, on all other sample days all fish were anaesthetised and examined using a hand lens as described above (paragraph 4.2.3.2). This monitoring period was brought to an end when a new generation of parasites (the progeny of these adults) naturally re-infected the fish and it became impossible to separate the original adult parasites from the new infection. The mean temperature during this experiment was 13.2°C (range 9-15°C).

4.2.5. Egg Production

Egg strings were collected from the first batch of eggs produced by eighteen adult females from one halibut during the low temperature life cycle experiment (temperature 8°C at time of collections, mean temperature throughout development period: 7.5°C). The following week the second batch of eggs produced by the same group of parasites from the same fish were collected. The number of eggs per egg string was counted using a Zeiss Kontron image analysis system. Only eggs within one egg string from each of the eighteen pairs were counted on both occasions.

As the length of the egg strings were not measured in the experiment above a third group of ten egg strings were collected from parasites from one halibut and measured

using the Zeiss Kontron Image analysis system and the number of eggs were also counted.

4.3. Results

4.3.1. Life Cycle

The development of *L. hippoglossi* in each of the three temperature experiments is shown in Figures 4.3-4.5. Figure 4.3a shows the progression of the stages with time at high temperature (13.1°C). For clarity chalimus III through to the pre adult 2 male stages have been plotted on a separate graph (4.3b). Pre-adult II females and adult males first appeared on day 18, while adult females first appeared on day 20. Figure 4.4 shows that at the medium temperature (9.7°C) pre-adult II females appeared by day 24 and adult males were found by day 30 (no adult females were collected due to the early termination of this experiment). Figure 4.5 shows that at the lowest temperature (7.5°C) pre-adult II females and adult males were present on day 40, and adult females were present from day 48.

In Figures 4.6-4.17 the results of the three temperature experiments have been combined so that a comparison can be drawn between each of the life stages at each temperature. It is clear from Figures 4.6-4.17 that the temperature has an effect on the rate of development, the lower the temperature the slower the development for each of the stages of *L. hippoglossi*. This difference becomes more pronounced as the life stages progress.

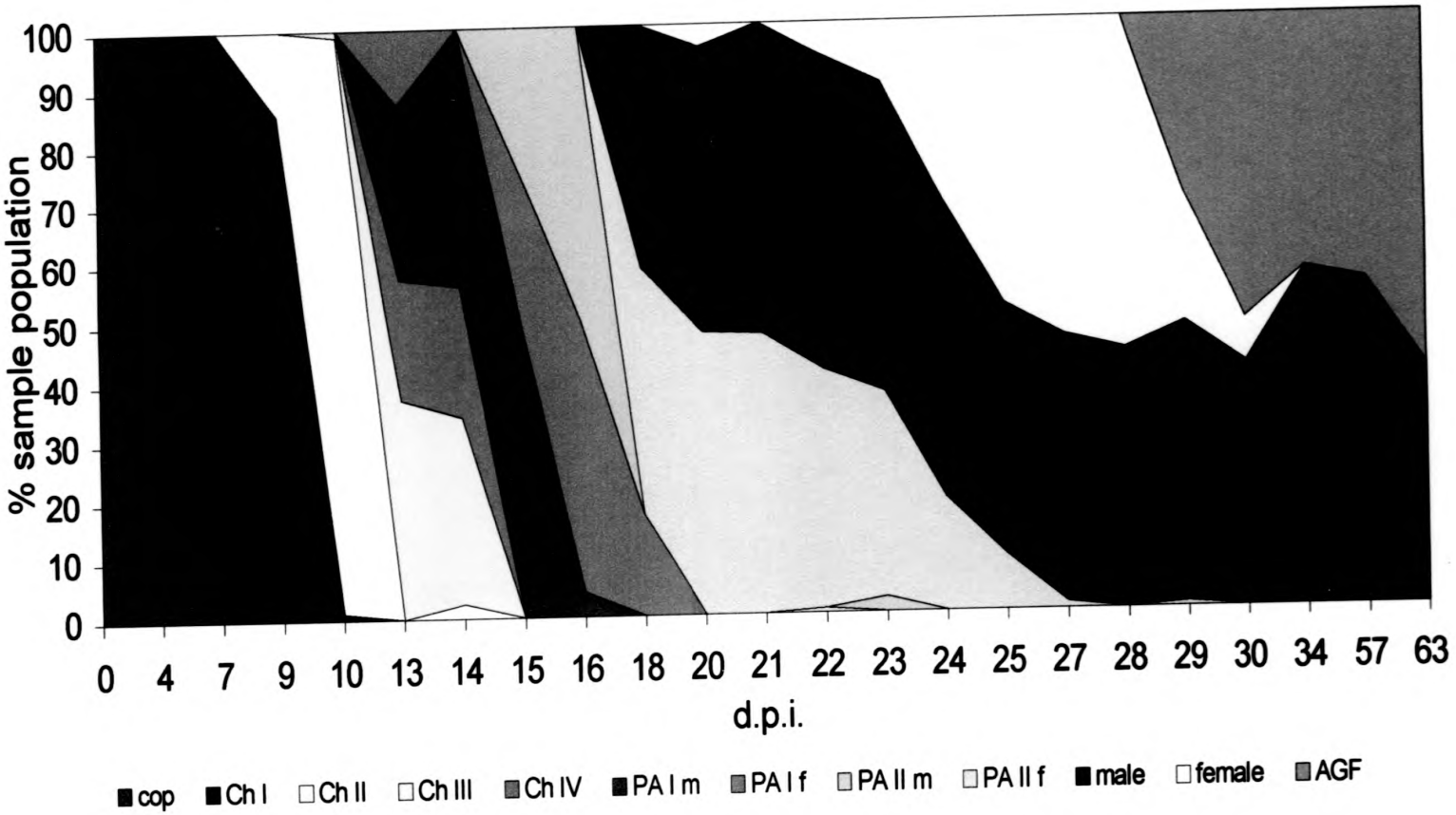


Figure 4. 3a. The progression of the life stages of *L. hippoglossi* with time at high temperature (13.1°C). cop-copepodid; Ch I-IV-chalimus I-IV; PA 1-first pre-adult; PA 2-second pre-adult; AGF-adult gravid female; m-male; f-female.

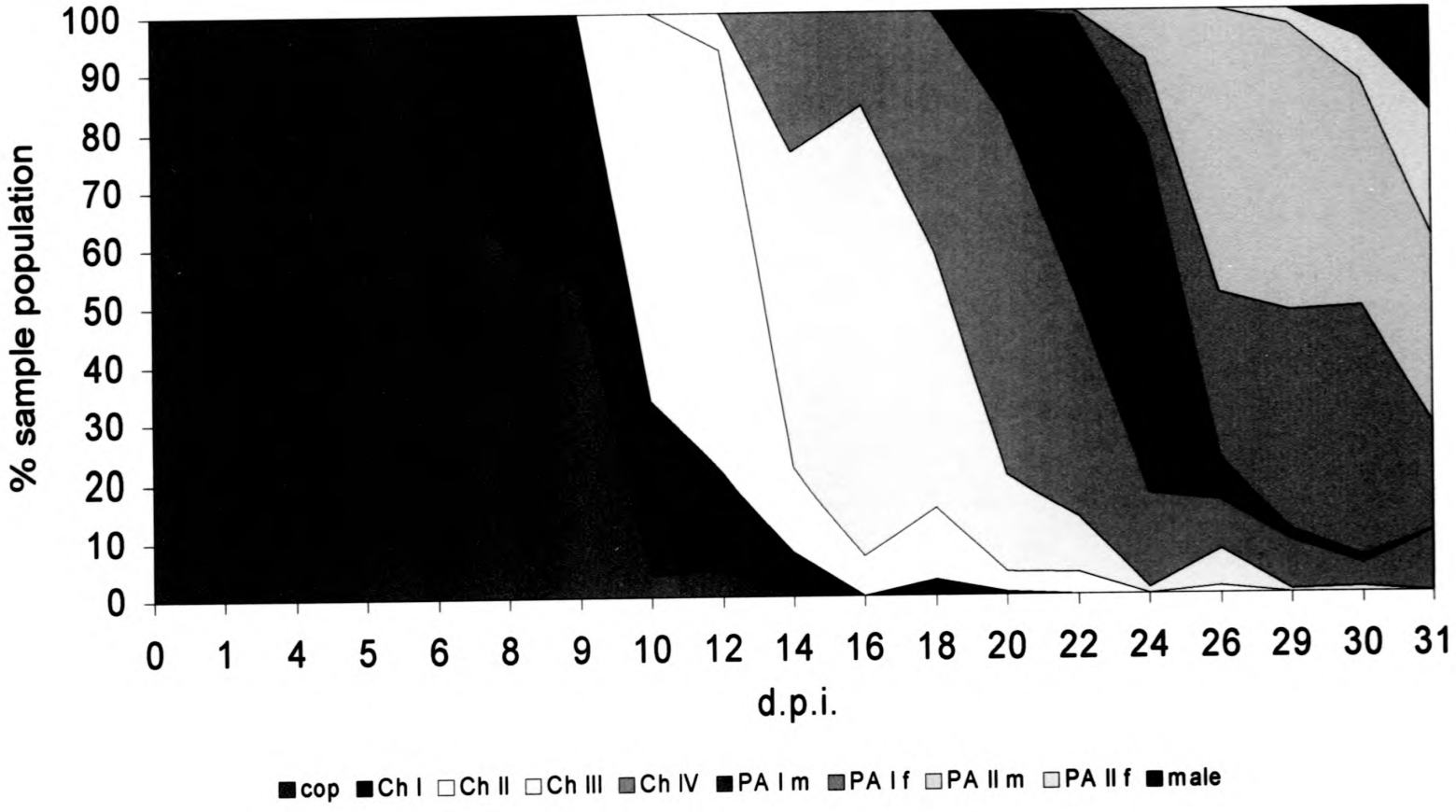


Figure 4. 4. The progression of the life stages of *L. hippoglossi* with time at the medium temperature 9.7°C. No adult female stages were recorded at this temperature due to the early termination of this experiment. cop-copepodid; Ch I-IV-chalimus 1-4; PA I-first pre-adult; PA II-second pre-adult; m-male; f-female.

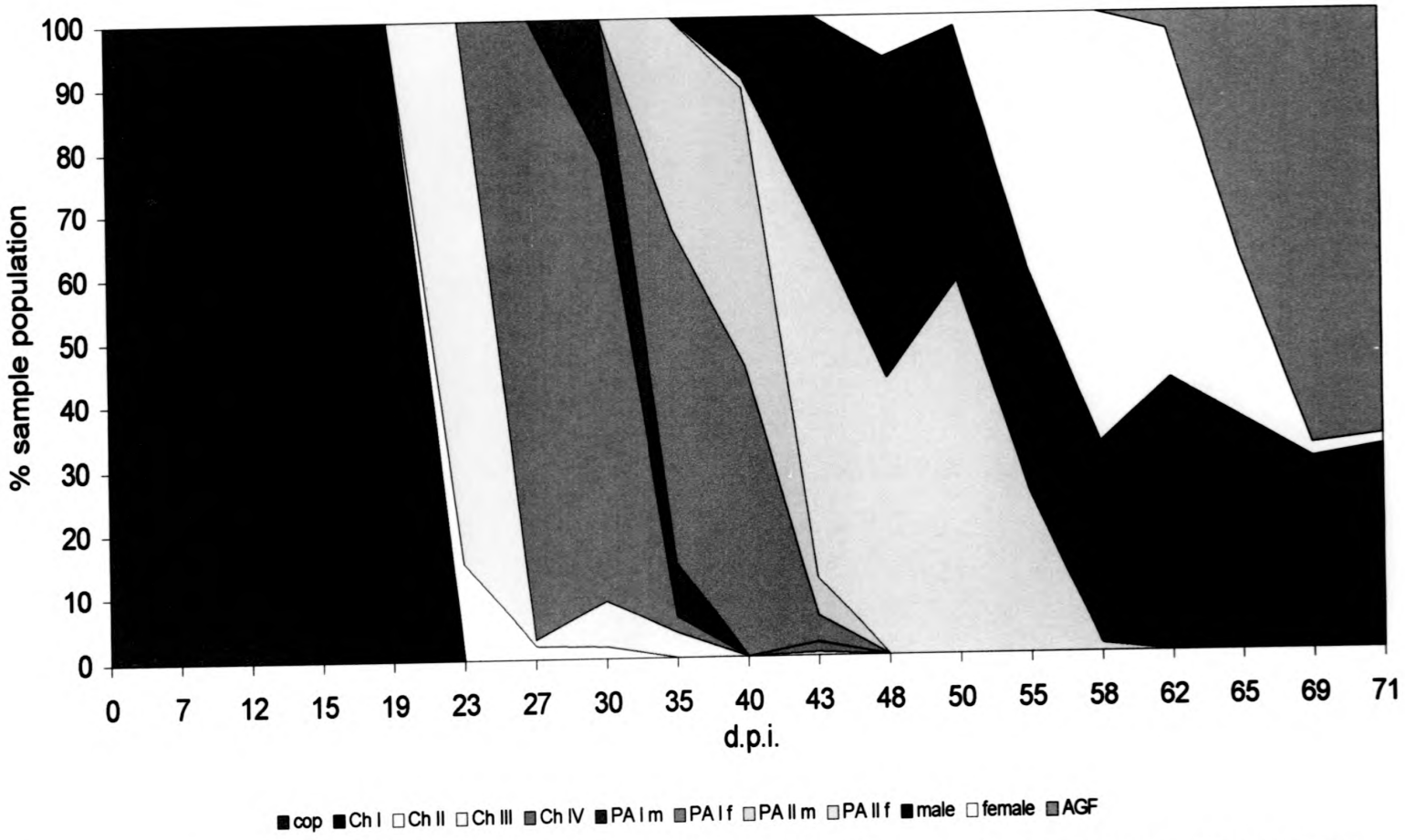


Figure 4. 5. The progression of life stages of *L. hippoglossi* at the low temperature 7.5°C. cop-copepodid; Ch I-IV-chalimus 1-4; PA 1-first pre-adult; PA 2-second pre-adult; AGF-adult gravid female; m-male; f-female.

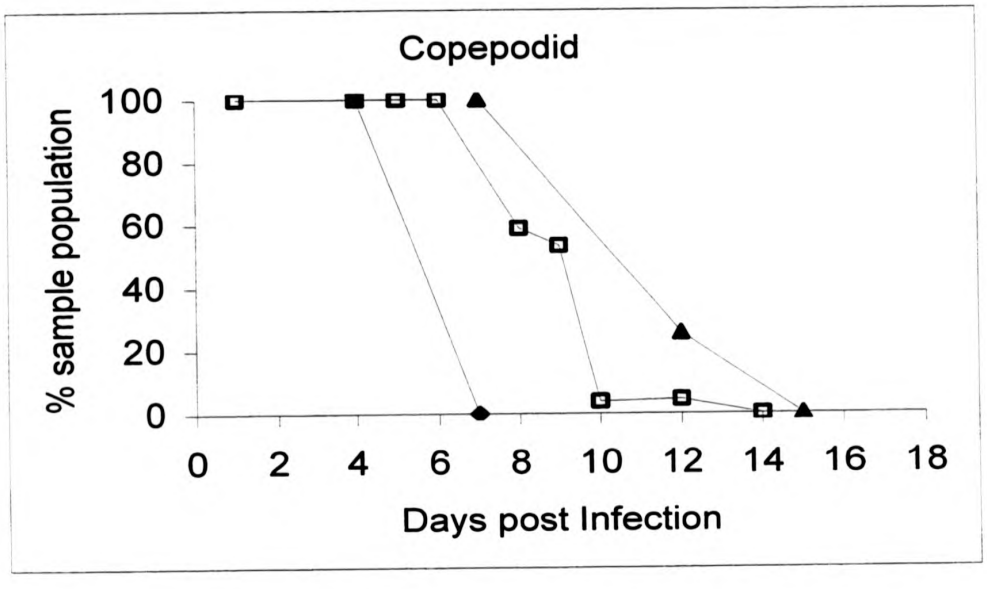


Figure 4. 6. The duration of the copepodid stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (♦).

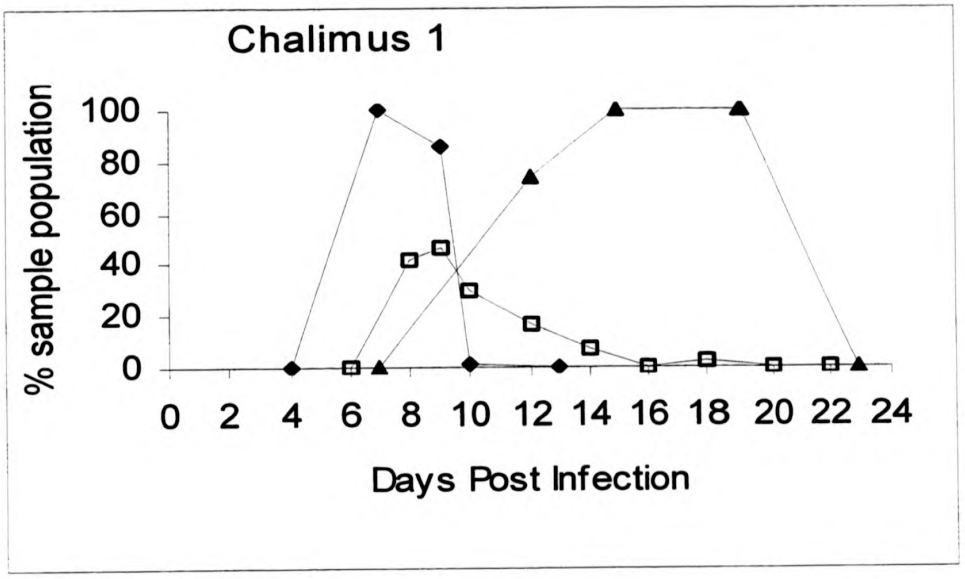


Figure 4. 7. The duration of the chalimus I stage at three different temperatures. high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

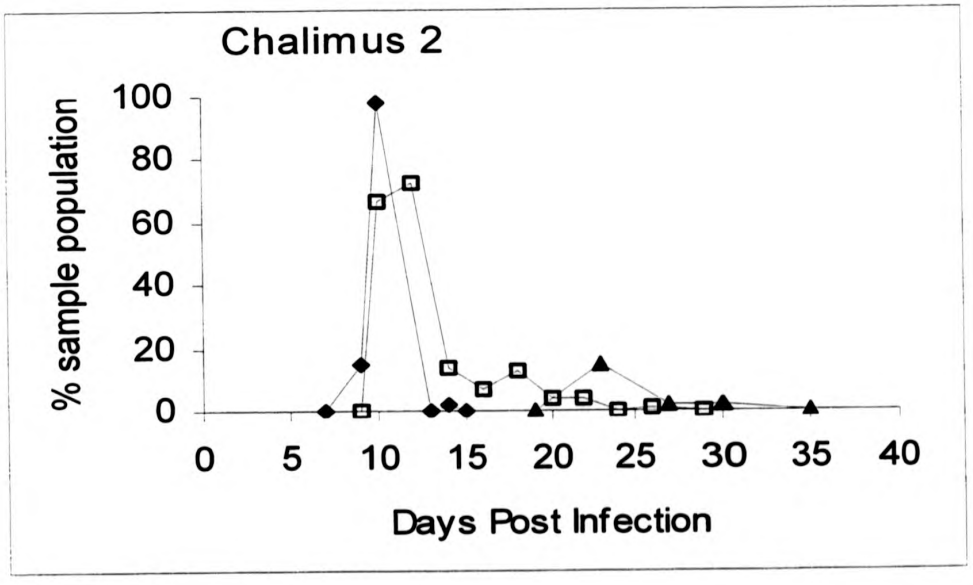


Figure 4. 8. The duration of the chalimus II stage at three different temperatures. high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

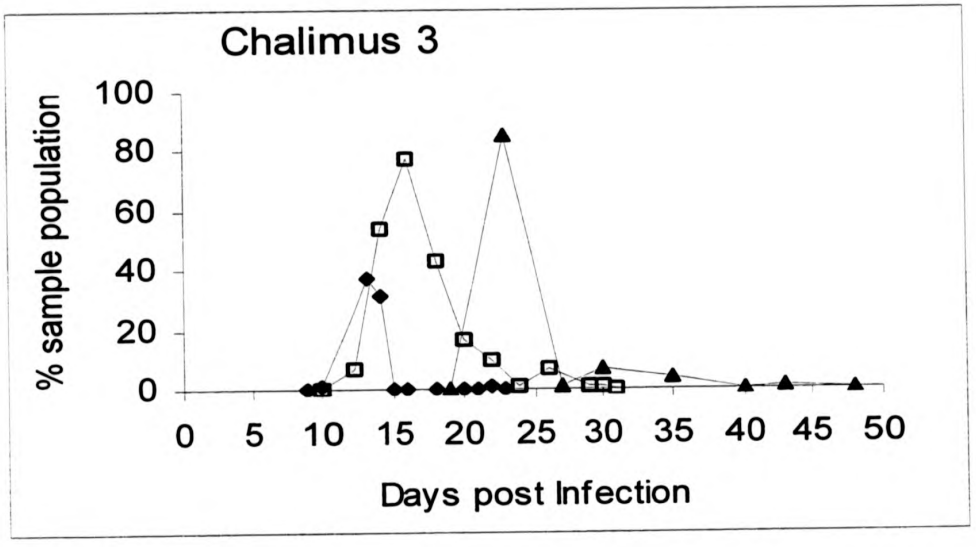


Figure 4. 9. The duration of the chalimus III stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

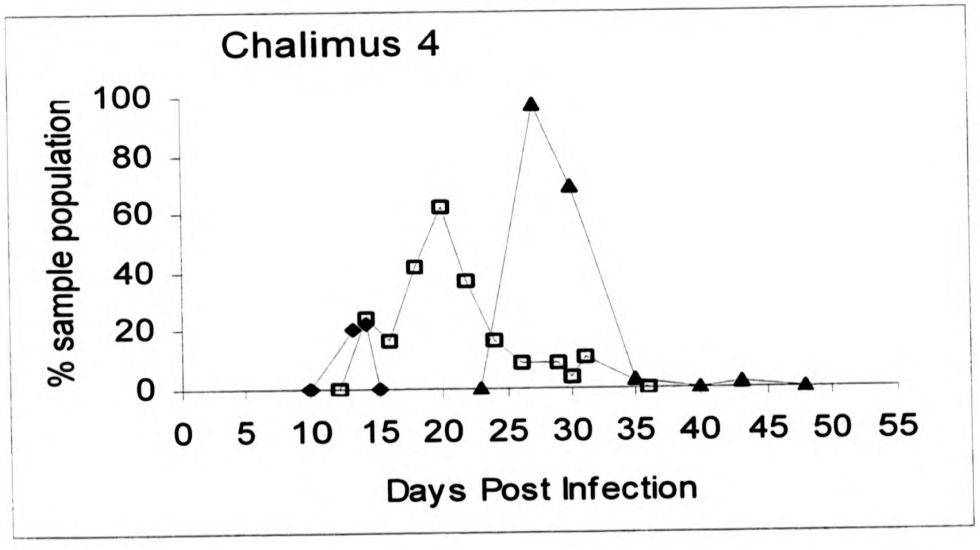


Figure 4. 10. The duration of the chalimus IV stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

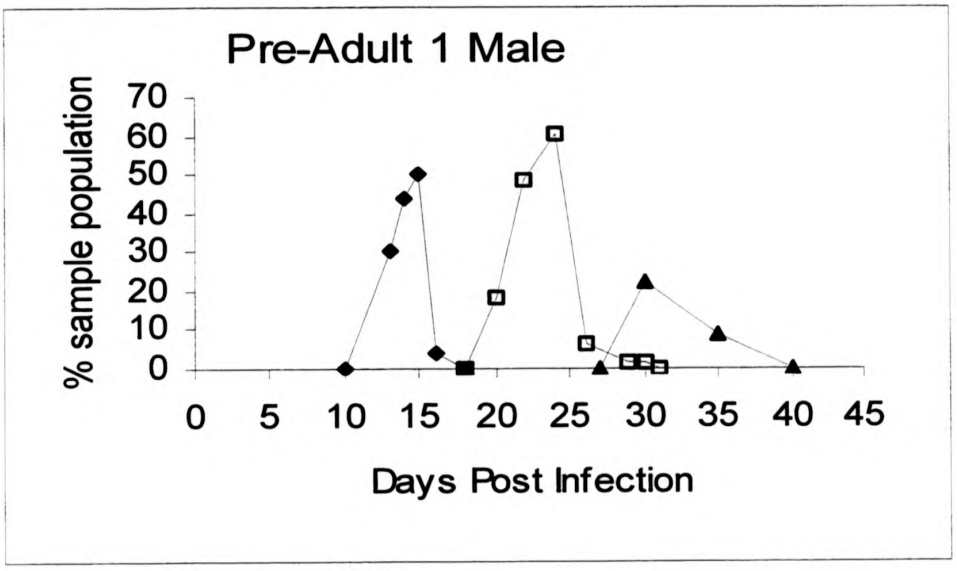


Figure 4. 11. The duration of the first pre-adult male stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

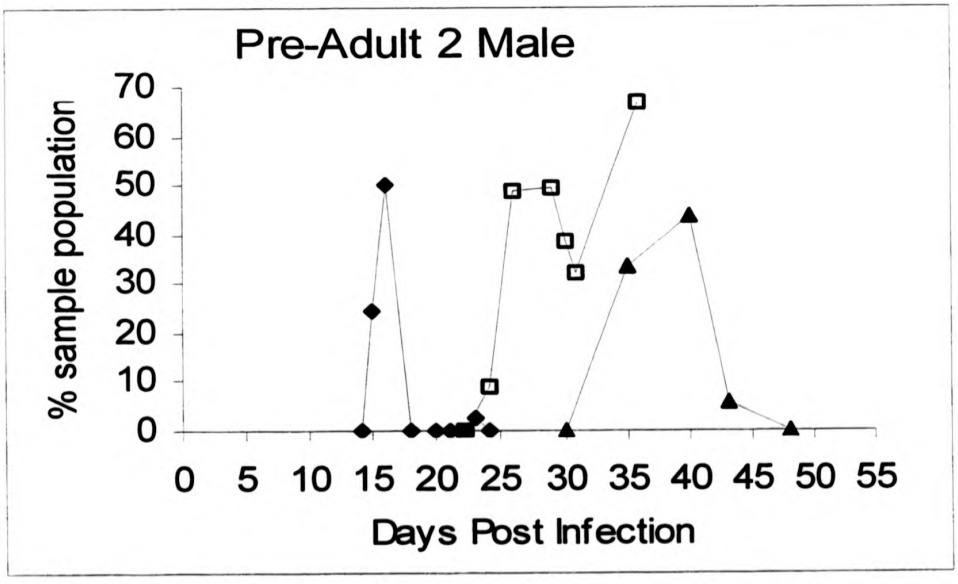


Figure 4. 12. The duration of the second pre-adult male stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

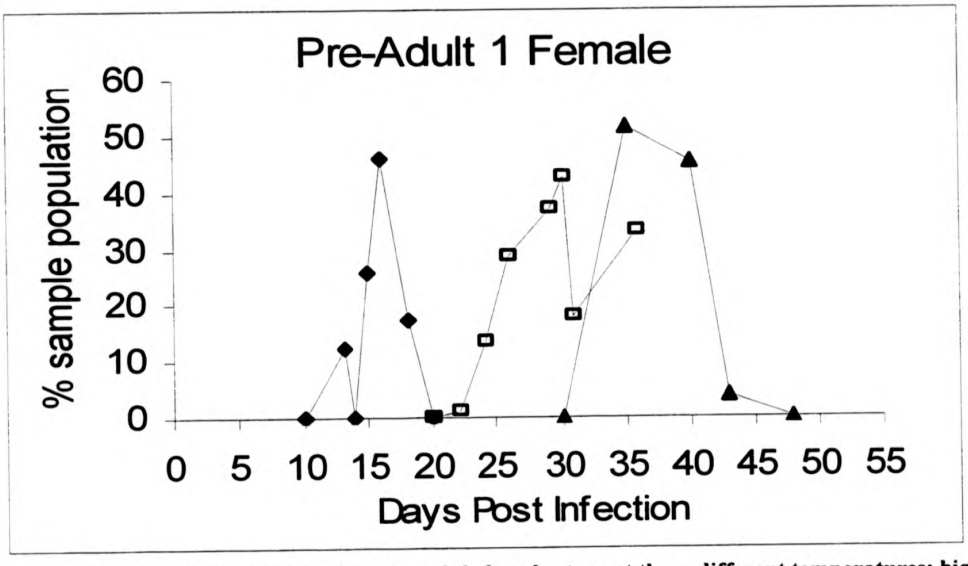


Figure 4. 13. The duration of the first pre-adult female stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

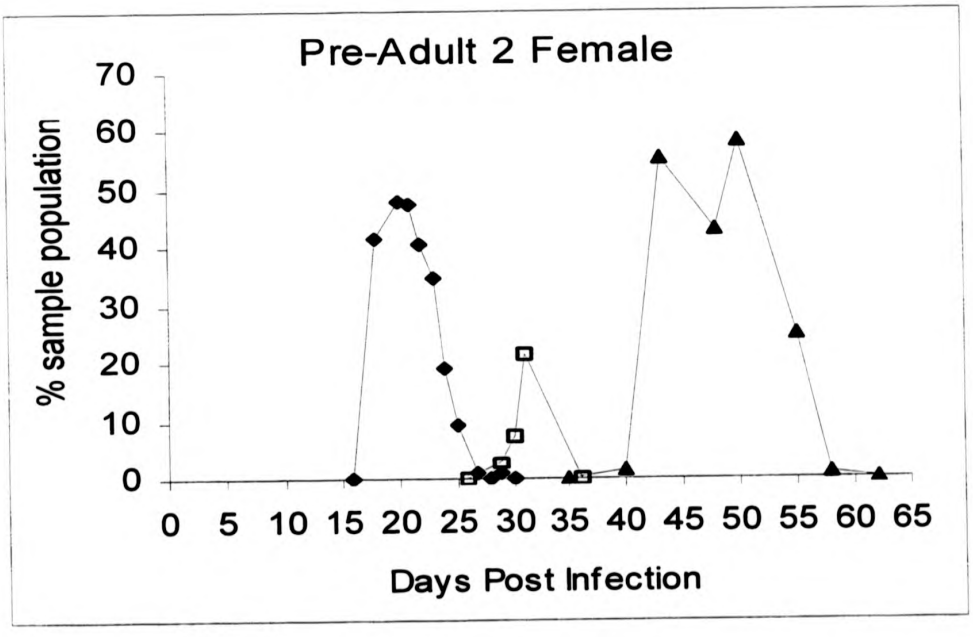


Figure 4. 14. The duration of the second pre-adult female stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (•).

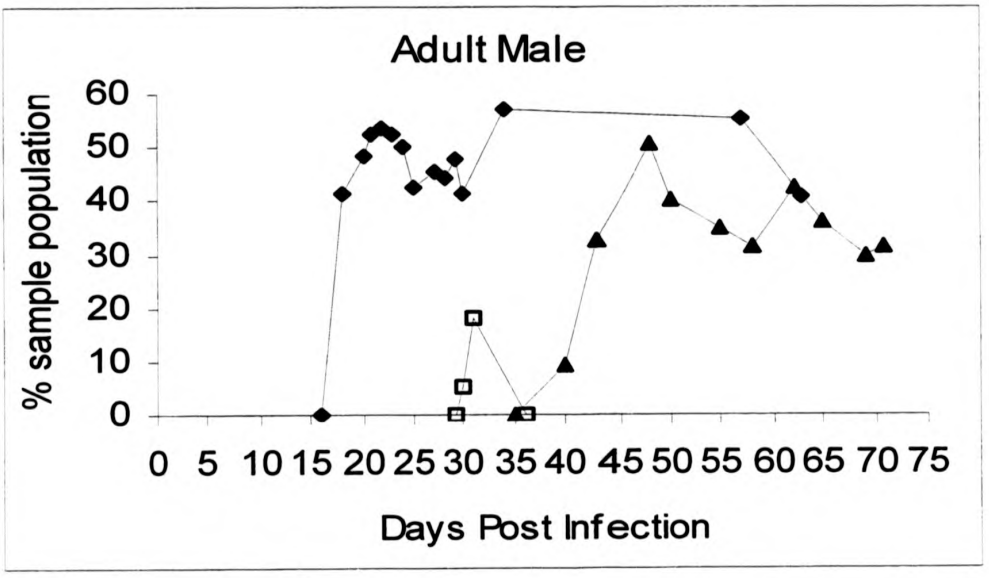


Figure 4. 15. The duration of the adult male stage at three different temperatures: high-13.1°C (♦), medium-9.7°C (□), low-7.5°C (♦).

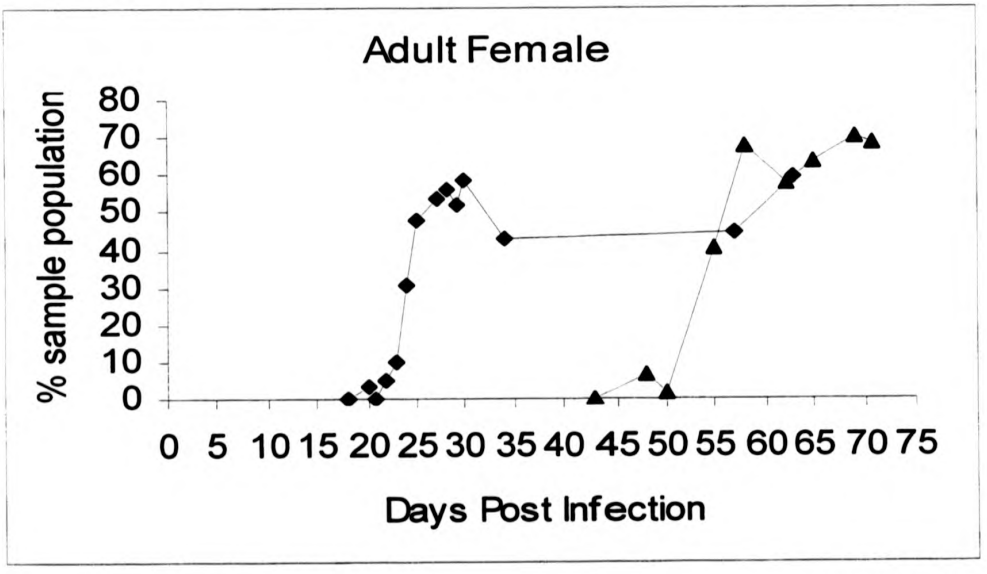


Figure 4. 16. The duration of the adult female stage at two different temperatures: high-13.1°C (♦), low-7.5°C (♦).

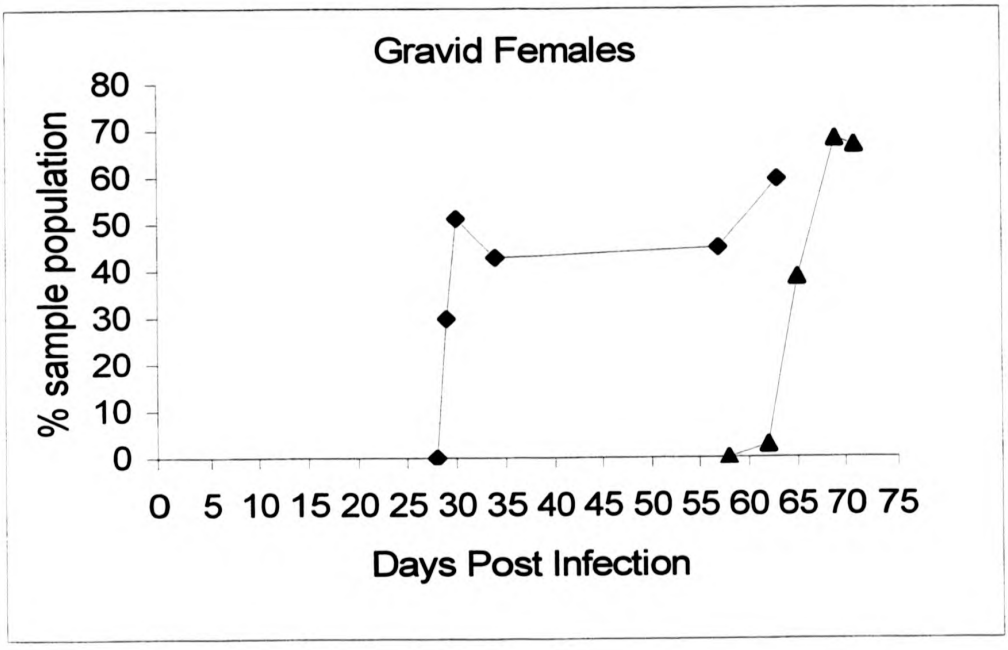


Figure 4. 17. The duration of the gravid female stage at two different temperatures: high-13.1°C (♦), low-7.5°C (•).

Figures 4.6-4.9 and Table 4.1 show that chalimus stages were reported on days 7 and 8 at the high and medium temperatures, respectively, but not until day 12 at the low temperature. At the high and medium temperature there was only one or two days separating the first appearance of most of the chalimus stages i.e. day 9 and day 10 for chalimus II, day 10 and day 12 for chalimus III and day 13 and day 14 for chalimus IV, respectively. However in the low temperature experiment the four chalimus stages appear first on days 12, 23, 23 and 27 respectively, which is approximately double the time taken for the medium and high temperature experiments (Table 4.1).

Table 4.1 also shows the duration of each of the stages at each of the three temperatures. From this table and from Figures 4.3-4.9 it can be seen that at the high temperature all chalimus stages (1-4) had a duration of 15 days, i.e. it took 15 days from the first chalimus I stage to appear until the last appearance of any chalimus stage (one chalimus III stage remained until day 22). At the medium temperature it took 22 days for the same developmental sequence and at the lowest temperature it took 23 days.

The difference in development rate became more pronounced in the pre-adult and adult stages (Figures 4.11-4.17, Table 4.1). At all temperatures the males developed quicker than the females. In the high temperature experiment the male stages first appeared on days 13, 15 and 18 for pre-adult I, pre-adult II and adult males, respectively. In the medium temperature experiment the same stages first appeared on days 20, 24 and 30, respectively, and at the low temperature the first appearance of each stage was on days 30, 35 and 40 respectively. There is approximately 10 days difference between the first

appearance of each of the three male stages at the high and medium temperatures and approximately 20 days between corresponding stages at high and low temperatures (Table 4.1).

The first female pre-adult I stages appeared on day13 (Figure 4.13) at the high temperature, succeeded by the second pre-adult stage on day 15 and adult females on day 20, and egg production began on day 29. At the low temperature the same stages appeared on days 35, 40, 48 and the first gravid females were seen on day 62, more than twice as long as at the high temperature. The medium temperature experiment was terminated before adult females appeared, however, the first pre-adult I stage appeared on day 22 and the first pre-adult II on day 29. No adult females were present on day 36 when this experiment ended.

Figures 4.10, 4.13, 4.14, 4.16 and 4.17 show a bimodal peak at some of the temperatures (particularly 13.1°C). Most of these Figures depict female stages and it could be due to some of the population developing more rapidly than the majority. It may also be due to mis-identification of the stages occurring at a particular sample date. The size difference between the early pre-adult stages is not as pronounced as the later stages and it may be that pre-adult II males have been identified as pre-adult I females giving a false peak earlier on in the development pattern. In the medium temperature experiment in Figures 4.12 and 4.13 there appears to be an increase in the population of pre-adult I females and pre-adult II males in the medium temperature experiment. However this is a sampling

error. Due to the enforced reduction of the population towards the end of this experiment the proportion of each stage in the population has been affected.

Figures 4.18-4.20 show the day when the peak (maximum) number of each of the life-stages occur at the three experimental temperatures. The peak is reached quicker at higher temperatures for all stages and represents a better indicator of population dynamics than the day of first or last appearance. The former may be skewed by early developers and the last appearance may be affected by late moulters or dead chalimus stages which remain attached and are still present long after the rest of the population have moulted. Information on the peak abundance is very important when devising timing for treatment strategies. Some treatments are most effective against certain life stages, e.g. hydrogen peroxide is only effective against motile stages, and it is important to be able to calculate when these stages are becoming dominant within the population so that treatment can be administered for maximum efficacy.

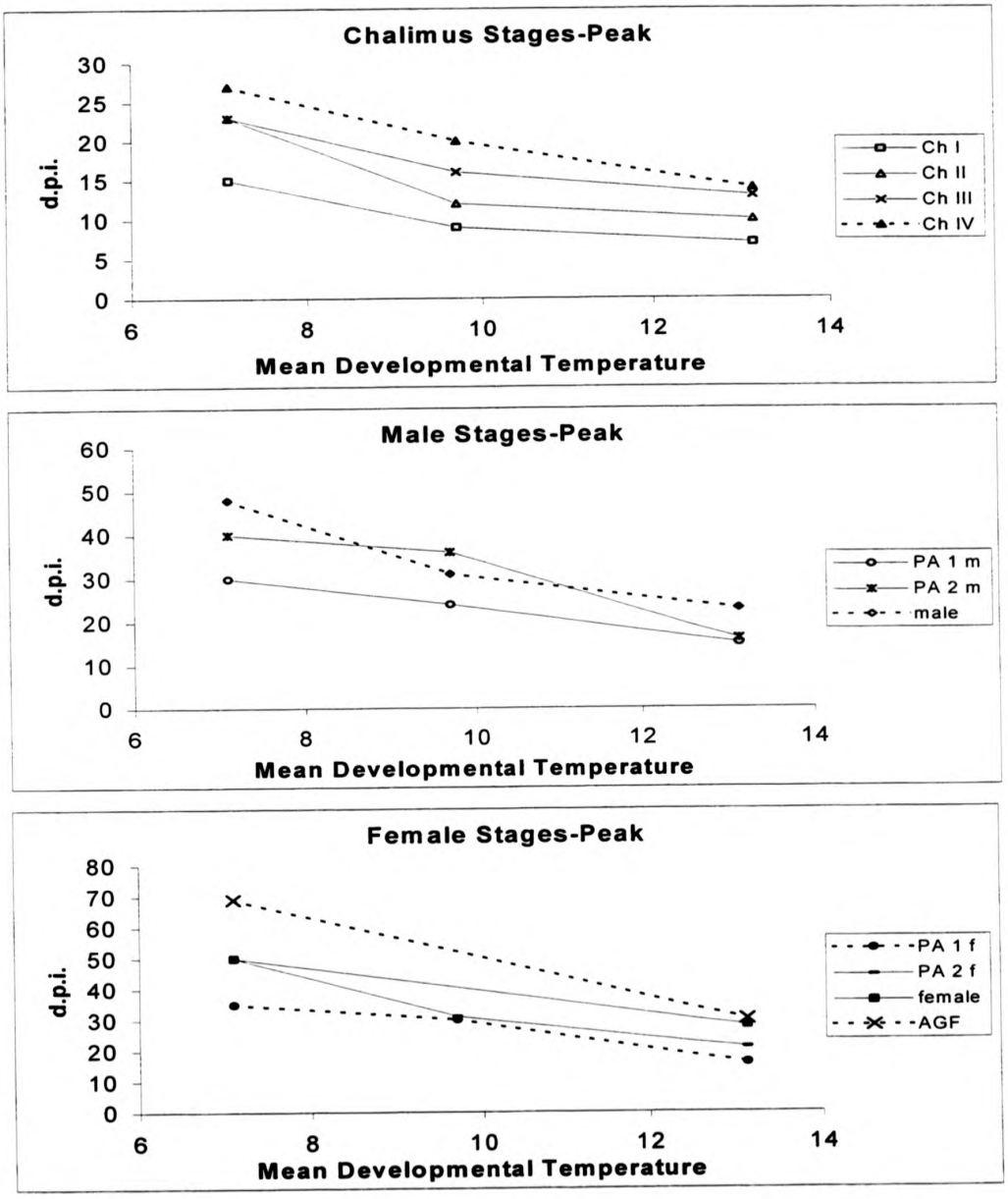
4.3.2. Survival and Longevity

Figure 4.21 shows that 26.7% of the adult female *L. hippoglossi* in tank 1.6 survived for at least 133 days post-infection (d.p.i.) and 25.5% survived in tanks 1.7 and 1.8 until day 104 d.p.i. Figure 4.22 shows that 14.0% of the adult males remained on day 104p.i. but in tank 1.6 at day 133p.i. no adult males remained. Thus females survive for considerably longer than male *L. hippoglossi*.

From this data it would appear that the number of parasites occurring on the 2 fish in tank 1.6 increased between sample points e.g. Figure 4.22, day 43 p.i., tank 1.6 – 133.3% survival (this represents an additional 4 individual parasites on day 43p.i. than were present on day 36p.i.). This is possibly due to parasites moving between fish during the sampling process and thus the same lice may be counted twice. Male *L. hippoglossi* are very well camouflaged against the host and it is possible that they were overlooked at the first sample point (36 d.p.i.).

D.P.I.	Temperature	cop	Ch I	Ch II	Ch III	Ch IV	PA I M	PA I F	PA II M	PA II F	male	female	AGF
1st Appear	High	0	7	9	10	13	13	13	15	18	18	20	29
	Medium	0	8	10	12	14	20	22	24	29	30	36+	36+
	Low	0	12	23	23	27	30	35	35	40	40	48	62
Last Appear	High	4	10	14	22	14	16	18	23	29	63+	63+	63+
	Medium	12	20	26	30	31	30	36+	36+	36+	36+		
	Low	12	19	30	43	43	35	43	43	58	71+	71+	71+
duration	High	4	3	5	12	1	3	5	8	11	45+		34+
	Medium	12	12	16	18	17	10				6+		
	Low	12	7	7	20	16	5	8	8	18	31+		9+
peak reached	High		7	10	13	14	15	16	16	21	23	28	30
	Medium		9	12	16	20	24	30	36	31	31		
	Low		15	23	23	27	30	35	40	50	48	50	69

Table 4. 1. Day post infection when each stage of *L. hippoglossi* first and last appeared on the host, the day the maximum number of each stage within the population (peak) occurred and the duration of each of the stages upon the host at each of the temperatures. D.P.I.=days post infection. +=the last sampling date, actual longevity unknown, experiments terminated at this time. Abbreviations: cop-copepodid; Ch I-IV-chalimus I-IV; PA 1-first pre-adult; PA 2-second pre-adult; AGF-adult gravid female; M-male; F-female. High Temperature (13.1°C); medium temperature (9.7°C); low temperature (7.5°C).



Figures 4. 18., 4. 19. & 4. 20. Days post infection when the maximum (peak) number of each life-stage of *L. hippoglossi* occurred within the population at the three experimental temperatures (7.5, 9.7, 13.1°C). Ch I-IV-chalimus stages 1-4; PA1-first pre-adult stage; PA2-second pre-adult stage; m-male; f-female; male-adult stage; female-adult tage; AGF-adult gravid female.

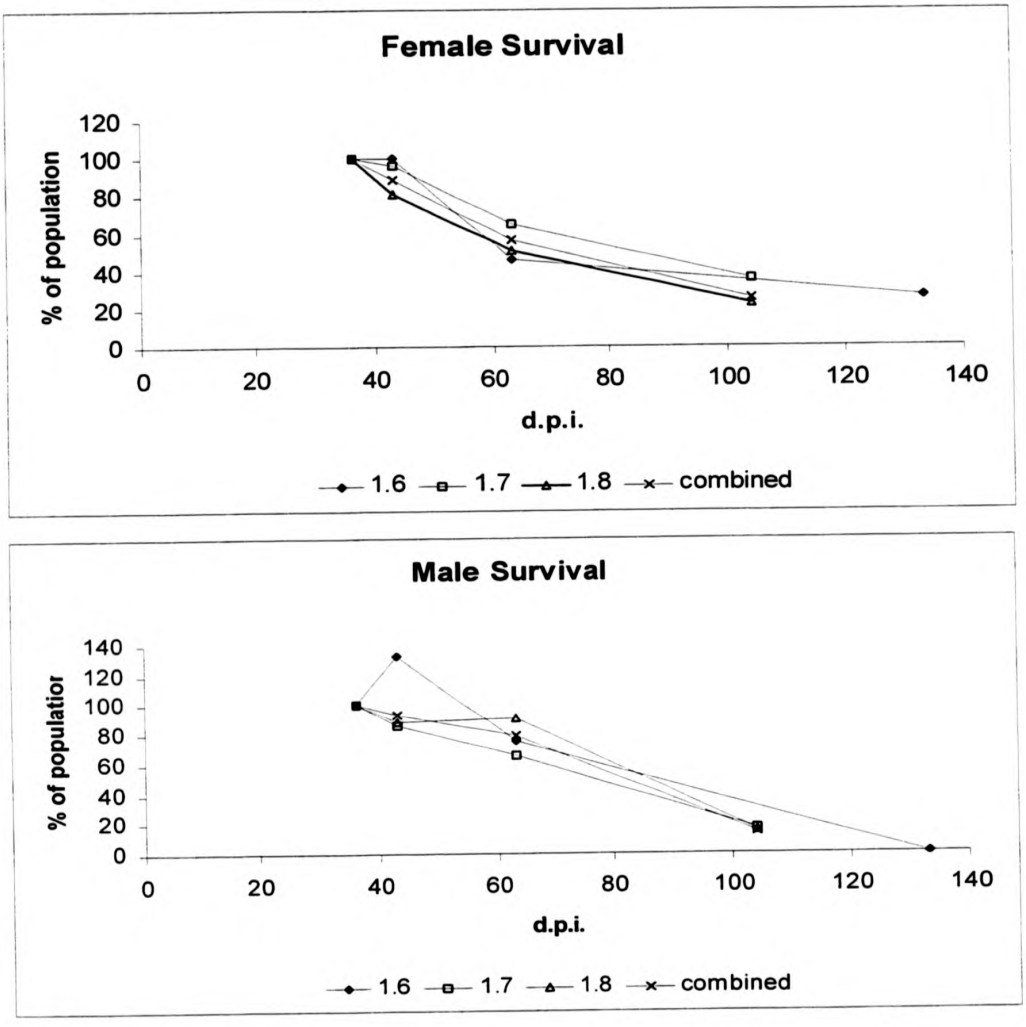


Figure 4. 21. & 4. 22. Percentage loss of adult females and adult male *L. hippoglossi* with time. Tank 1.6 has 2 halibut, Tank 1.7 has 3 halibut and Tank 1.8 has 4 halibut. The mean data for all three tanks is also shown (combined).

4.3.3. Egg Production

The number of eggs per egg string was counted from one egg string of the pair obtained from 18 individual parasites. The first pair of egg strings produced from parasites were collected and egg strings were then sampled from the same group of parasites one week later.

	1st Collection	2nd Collection
Mean±s.d.	94.6±14.4	195.1±29.6

Table 4. 2. The mean number±standard deviation of eggs per egg string. N=18 strings for each egg collection.

Table 4.2 shows the difference in the number of eggs produced in the first batch is less than half the number produced in second. The number of eggs per egg string ranged from 66-116 in the first batch and from 150-264 in the second batch.

The third group of ten egg strings were measured, the average length was 8.29±0.85cm and the average number of eggs was 131.9±15.03 per egg string.

4.3. Discussion

4.4.1. Life Cycle

Many studies of copepods have highlighted the importance of temperature on the development time. In general the higher the temperature the shorter the development period. Generation time (Nauplius 1-mature adult female) was estimated by Hogans & Trudeau (1989) to be 5 weeks at 10°C and 3 weeks at 12°C for *Caligus elongatus*. Development time from first nauplius to second pre-adult in *C. pageti* took 23.5 days at 16-18°C, 16 days at 20-22°C and 10.5-11.5 days at 24-26°C (Ben Hassine, 1983). The penellid copepod *Lernaeocera branchialis* (L., 1767) from flounder (El-Darsh & Whitfield, 1999) takes 25 days to reach adulthood at 10°C and only 11 days at 16°C. *L. salmonis* can produce eggs 32 days post infection at a temperature of 13-15°C but they will take up to 71 days to reach the same stage at temperatures of 6-8°C (M^{c}Andrew *et al.*, unpublished data). In *L. hippoglossi*, generation time (copepod attachment-adult female) also increased with lower temperatures, 20 days at 13.1°C and 48 days at 7.5°C. The halibut lives in deep waters and therefore the natural habitat for *L. hippoglossi* is cold. Thus the natural development time for this species will be longer than for those species naturally occurring in warmer waters. In halibut culture the host will be held in coastal waters, with a seasonal fluctuation in water temperature, so during the warmer summer months *L. hippoglossi* will be able to reproduce and develop at a much accelerated rate from that in its natural habitat, thus having the potential to cause damage to the fish very quickly.

The copepodid stage has been reported to have a long duration on the host for *L. salmonis* and *L. hospitalis* (see Johnson & Albright, 1991b; Voth, 1972). In this study the duration of the copepodid on the host was greatly increased between the highest and the medium temperatures, the copepodid being present for only 4 days at 13.1°C but remaining for 12 days at both 9.7 and 7.5°C. This is similar to *L. salmonis* copepodids which have a duration of 10 days at 10°C (Johnson & Albright, 1991b) on the host but apparently quicker than copepodids of *L. hospitalis* which moult after 6-8 days at 15°C (Voth, 1972).

It has been suggested that the long duration of the copepodid stage in *Lepeophtheirus* species may be due to the need to recover energy lost during the free swimming stages or the requirement of additional time or energy for completion of development before filament production and moulting (Johnson & Albright, 1991b). Free swimming copepodids of *L. salmonis* lose energy reserves markedly between days 1-2 and days 5-7 (Tucker *et al.*, 2000a). Tucker *et al.* (2000a) also showed that those copepodids unattached to a host after 7 days had a statistically significant decrease in the ability to attach to a host than those that attached within 2 days of moulting. However once the copepodids were attached the survival rate was not significantly different for the different age groups. In *L. hippoglossi* the time period to first chalimus stage was much shorter at the high temperature (4 days at 13.1°C) than at either of the other temperatures (12 days). It would be interesting to determine the highest and lowest temperature at which *L. hippoglossi* can survive and develop.

The development of the chalimus stage is also generally quicker at higher temperatures. There is an apparent anomaly in the data in that at 13.1°C the chalimus III stage appears to have a duration of 12 days, more than double the time for any of the other chalimus stages to develop at this temperature. This is due to a single chalimus III found on day 22, all other chalimus having moulted by day 14. This one remaining parasite may have been dead or unable to continue development but, due to its frontal filament, it still appeared to be attached to its host. This has also been reported in *L. salmonis* particularly during drug treatment trials (J. Stone, *pers. comm.*).

Comparing the data from the present study (9.7°C data) with that of Johnson and Albright (1991b) compiled at 10°C the developmental rate of *L. salmonis* and *L. hippoglossi* are comparable. Throughout development, the day post infection to first appearance is within 1-3 days for the two species. Chalimus I stages appear on day 7 for *L. salmonis* and day 8 in *L. hippoglossi*. The first pre-adult male stages of *L. salmonis* appeared on day 20, exactly the same as for *L. hippoglossi*. Adult males first appeared on day 28 in *L. salmonis* and day 30 for *L. hippoglossi*. There are no data for the timing of each development stage for *L. salmonis* at other temperatures. As the natural habitat of salmon is not the same as that of halibut it would be interesting to determine the developmental temperature range for the two parasitic species. It may be that *L. salmonis* has an optimum developmental temperature higher than that of *L. hippoglossi*.

Neither the pre-adult II female nor the adult female stages were able to be recorded in the medium temperature experiment. Possibly the anaesthetic used while reducing the numbers of lice had a detrimental effect on the parasites and either killed them or injured/weakened them sufficiently to allow them to be detached from the fish and washed out of the tanks between samples.

The first adult males of *L. hippoglossi* were obtained on day 30 at 9.7°C, which is very similar to the timing of males of *L. salmonis*, which first appear on day 28 at 10°C (Johnson & Albright, 1991b). It is unclear when the adult females of *L. hippoglossi* appear at 9.7°C but they were not present on day 36 when this experiment was terminated. Female *L. salmonis* first appeared on day 40 at 10°C (Johnson & Albright, 1991b). At 10-12°C and 30-31‰ salinity the males of *L. pectoralis* took 24-27 days and females 29-32 days to develop from the time of egg hatching (Anstensrud, 1990). The pennellid copepod *Lernaeocera branchialis* developed from copepodid to adult in a minimum of 25 days at 10°C (Whitfield *et al.*, 1988), but, it was suggested that at ambient summer sea temperatures for the south of England (16°C) it could take as little as 11 days to complete development. Both these species show more rapid development than the *L. hippoglossi* at ambient summer sea temperatures in Scotland (13.1°C) at which it took 20 days to reach adult females.

The time taken for first egg production in *L. hippoglossi* was 29 days at 13.1°C and 62 days at 7.5°C. McAndrew *et al.* (unpublished data) report that egg production commences at day 32 post infection at 13-15°C and 71 days at 6-8°C for *L. salmonis*.

Therefore *L. hippoglossi* can develop up to 9 days quicker than *L. salmonis* in UK winter ambient water temperatures. Piasecki and MacKinnon (1995) found that *C. elongatus* took 28.6 days to commence egg production at 10°C and, in contrast, Lin and Ho (1993) found that it took only 14 days to commence egg production in *C. epidemicus* parasitic on tilapia (*Oreochromis mossambicus*) at sub-tropical temperatures in brackish water.

Within a cohort of *L. hippoglossi* the males matured faster than the females, as also recorded for other caligids including *L. salmonis*, *L. pectoralis*, *C. elongatus*, the pennellid *Lernaeocera branchialis*, the lernaeopodid *Salmonicola californiensis* (Dana, 1852), and free living copepods (Johnson & Albright, 1991b; Piasecki & Mackinnon, 1995; Anstensrud, 1989, 1990; Kabata & Cousens, 1973; Landry, 1983). Mature males are found at the same time as the pre-adult II females in *L. salmonis,* and the males are able to guard the immature females until they moult to the adult female and then fertilise them immediately after the moult. Both pre-adult I and II females have been observed with adult males clasping onto the genital segment in *L. salmonis* (see Hull *et al.*, 1998) and in *L. pectoralis* (see Anstensrud, 1990). Occasionally males of *L. hippoglossi* were observed attached to both pre-adult I females and fully mature females. It is unclear whether this represents mate guarding. In *L. pectoralis* the first male to attain a pre-copulata (mate-guarding) position with an immature female may lose to another male while the female develops (Anstensrud, 1990). Both sexes of *C. elongatus* mature at the same rate, however, the males become motile (sever the frontal filament) sooner than the females and mating occurs between an attached female and a

motile male. Mate guarding occasionally occurs between a female chalimus stage and an adult male (Piasecki & Mackinnon, 1995). In the lernaeopodid *S. californiensis* fertilisation occurs before the female has matured and in one study 75% of female chalimus IV stages were found with spermatophores already attached (Kabata & Cousens, 1973).

In their natural habitat Atlantic halibut live in water with an ambient temperature of around 6°C. At 7.5°C *L. hippoglossi* reached maturity in 48-50 days and egg production commenced on day 69. At higher temperatures this rate is greatly increased and, at Scottish summer water temperatures (13-15°C), egg production could commence on day 30 post infection. In culture conditions halibut are held at ambient water temperatures and particularly through the summer months the threat of epizootics is greatly increased. However, it is known that the host in culture will lose appetite around 13°C and will become particularly stressed at 15°C (C. Mazorra, *pers. comm.*), so it would be logical to assume that these temperatures are also towards the upper limit for *L. hippoglossi*. During summer months the threat from this parasite may be further increased if the halibut are already stressed by their environmental conditions coupled with the shorter generation time.

4.4.2. Survival and longevity

The survival data presented here suggests that adult males are lost slightly quicker than females under the same conditions. However, adult males develop quicker than adult females. The temperature during this experiment was 13.2°C (range 9-15°C). After the

initial infection these fish were not closely monitored, however gravid females and adult males were observed from day 36 post infection. From the experimental development data presented earlier it can be seen that adult males and females appeared only 2 days apart at the high temperature (13.1°C). Therefore, it seems reasonable to assume that a similar period elapsed between male and female development during the survival experiment and thus the male and females observed in this experiment were assumed to be of the same age. After 133 days post infection 26.7% of the females still remained in one tank but none of the males survived for this length of time. Overall *L. hippoglossi* is long lived with 25.5% of the females and 14% of the males surviving for at least 104 days post infection. Similar results were found for *L. salmonis*; in two separate trials running simultaneously it was found that 58-81% of females and 39-44% of males survived for at least 89 days post infection (temperature range 6-9°C) (M^{c}Andrew *et al.*, unpublished data). The halibut and thus *L. hippoglossi* had been handled and anaesthetised (2-phenoxyethanol) at least 4 times within the experimental period, and it cannot be ruled out that the male parasites were more susceptible to the anaesthetic and therefore more easily lost from the host than the females.

This experiment was carried out at summer ambient temperature and the parasites survived for up to 133 days. At lower temperatures, development occurred at a reduced rate and it can be assumed that the parasites would also survive for longer. No data has been collected for longevity at winter temperatures and it would be interesting to see how long these animals can survive at temperatures closer to their natural habitat.

Development time doubled between 13. 1 and 7.5°C and longevity could be increased by a similar proportion, suggesting that they could live for 266 days post infection.

There are very little published data on the length of survival time of adult copepods. Heuch *et al.*, (2000) reported that adult females of *L. salmonis* can survive for up to 191 days at 7.2°C, but gave no data for males. The males of *C. elongatus* appear to die once mating has occurred (Piasecki & Mackinnon, 1995) after 600 degree days (approx. 60 days p.i.). Their study also showed that males are not present in natural conditions over the colder months of February-April in eastern Canada. In a similar study Hogans and Trudeau (1989) found only 10-35% of the total lice population was male over the winter months in cage culture conditions. It is possible that *L. hippoglossi* will be able to survive, develop and reproduce throughout UK winter months on farmed halibut. Their natural habitat is cold seawater (~6°C) and Scottish coastal waters during the winter months remains around this temperature. It is well within the assumed natural temperature range for both the fish and the parasite and development will be slowed but may not be halted. The potential threat to the health of the fish therefore may not be reduced throughout the winter months.

4.4.3. Egg Production

The mean number of eggs produced per female (2 egg strings) for *L. hippoglossi* was 189 for the first batch and 309 for the second batch at 8°C. This compares with 108 for *C. elongatus* in Ireland, but 178 in Canada, and for *L. salmonis* the number of eggs per female in Ireland was 754 but in Canada only 192. All data were collected from

untreated salmon farms (Jackson & Minchin, 1992; Hogans & Trudeau, 1989). Johnson and Albright (1991b) found that the numbers of eggs per egg string ranged between 251-423 (mean=344.6±79.8; n=16) for experimentally held *L. salmonis*. During the present study observations have shown that *L. hippoglossi* egg strings are shorter and broader than egg strings produced by *L. salmonis* in Scottish waters. Ritchie *et al.*, (1993) found a variation in egg string length and eggs per egg string with season. *L. salmonis* produced longer egg strings containing more eggs over the winter period than in other seasons and they concluded that temperature had a bigger affect than photoperiod on the length and number of eggs per egg string. Heuch *et al.* (2000) studied egg production in *L. salmonis* at 8.7 and 12.2°C and showed that egg string length and number of eggs produced was significantly greater at lower temperatures (individual eggs are longer at higher temperatures). Parasites held at 12.2°C were lowered to 7.2°C and in the next batch of eggs produced the individual egg length was reduced and the number of eggs per string increased after the change in temperature. The effect of temperature upon the production and development of *L. hippoglossi* eggs was not examined within this study although it might be assumed that similar temperature effects would be found. However, further studies are needed to ascertain the range of temperatures at which successful egg development will occur and the effect temperature has on egg production

Chapter 5

The Host/Parasite Interface: Studies on Parasite Distribution, Pathology and the Host Skin.

5.1. Introduction

5.1.1. *Entobdella hippoglossi*

5.1.1.1. Parasite Attachment and Distribution

The attachment of *Entobdella soleae* to the sole (*Solea solea*) has been described in detail by Kearn (1964). *E. soleae* attaches itself to the skin of the sole by hydraulic suction pressure generated between the cup shaped haptor and the skin of the fish; subsequently the hooks come into use to secure the parasite to its host. Based on observations of the anterior adhesive organs, Kearn (1974b) suggested that *E. hippoglossi, E. diadema* (Monticelli, 1902), and *E. squamula* (Heath, 1902) all have the same adhesive mechanism as described for *E. soleae*. *E. soleae* orientates with its haptor anterior relative to the fish with its body projecting towards the host's tail. If the parasites are placed round the wrong way they will readily re-orientate themselves in the absence of any water currents, by using the orientation of the fish scales as a reference. This orientation may be obligatory so that the parasites give least resistance to the movement of water flowing over the fish (Kearn, 1988a).

Kearn (1984) reported that, on wild caught sole, small specimens of *E. soleae* (average length 0.72mm, range 0.35-1.00mm) were found on the upper surfaces and larger specimens (average length 2.45mm, range 1.05-5.00mm) were found on the lower surfaces of the host. One sole held in captivity had very high numbers of *E. soleae* and the pattern was repeated, with parasites on the upper surfaces smaller [0.59 (0.14-2.29)mm in length] than those on the lower surfaces [1.63 (0.17-2.49)mm in length]. The author concluded that early development took place on the upper surfaces of the host and, at approximately 1mm in length, the parasites migrated to the lower surfaces (Kearn, 1963a).

Many other monogeneans settle on a particular part of the body surface and then migrate as they mature, presumably to areas where mates can be found or so that eggs are deposited into the correct habitat or because feeding or attachment conditions are better. The ciliated oncomiracidia of *E. soleae* (Kearn, 1963b) follow a similar migration pattern to *Anoplodiscus cirrusspiralis* Roubal, Armitage & Rohde, 1983, which settle on the head, skin and fins of snapper (*Pagrus auratus* Bloch & Schneider, 1801), but subsequently move to the fins as they mature (West & Roubal, 1998a). However, the whole of the life cycle of *Acanthocotyle lobianchi* Monticelli, 1888 appears to take place on the ventral surface of its elasmobranch host, *Raja montagui* Fowler, 1910. Kearn (1967) found that 94% of all parasites were present on the underside of the host. As the oncomiracidia are unciliated it would appear that they are unable to gain access to other areas of the host.

The oncomiracidia of *Benedenia hoshinai* Ogawa, 1984 attach to the body surface and fins of its host, the striped knifejaw (*Oplegnathus fasciatus* Krøyer, 1845) but the adults are more commonly found attached to the tail fins, dorsal soft fin rays and the posterodorsal side of the body (Ogawa, 1984). The adults of *Benedenia lutjani* Whittington & Kearn, 1993 also prefer attachment to the pelvic fins of its host *Lutjanus carponotatus* Richardson, 1842 from the Great Barrier Reef (Whittington & Kearn, 1993).

5.1.1.2. Pathology

There have been relatively few studies on the pathology of skin dwelling monogeneans; most studies have concentrated on the more common gill inhabiting forms. Histological sections showed that mechanical damage is caused as the opisthaptor of the microbothriid monogenean (*Dermophthirius carcharhini* MacCallum) depresses and erodes the epidermis of the Galapagos shark (*Carcharhinus galapgensis* Snodgrass & Heller), rupturing goblet cells at the base of scales (Rand *et al.*, 1986). In *E. soleae* the hooks of the opisthaptor pierce the epidermis and penetrate the dermis of the fish (Kearn, 1964). After removal from the skin, *E. soleae* leaves behind a 'dome shaped blister' surrounded by an imprint of the saucer shaped haptor (Kearn, 1964). Chemical erosion due to the feeding action also occurs. Proteolytic secretions are spread across the epidermis, and in the area of skin enclosed by the protruded feeding organ, the epidermal cells lose their ability to adhere to neighbouring cells and are ingested by the parasite (Kearn, 1963c). Adult *Anoplodiscus* species also erode the epidermis and attach to the basement membrane during feeding and attachment (Roubal & Whittington, 1990). Heavy infections of species of *Anoplodiscus* were associated with

wide areas of inflammation on the caudal and pectoral fins. Erosion of fin epidermis and nasal lamellae also occurred (Ogawa, 1994; Roubal *et al.*, 1992). Heavily infected fins are hyperaemic, detracting from the commercial value of the fish (Roubal & Whittington, 1990); (Roubal *et al.*, 1992); (Ogawa, 1994). No mortality has been attributed to this genus (Roubal *et al.*, 1992). *Benedenia seriolae* from *Seriola* species and *B. hoshinai* on striped knifejaw (*Oplegnathus fasciatus*) are the only species known to cause skin damage to cultured fish species in Japan. Recently the non-host specific *B. epinephelia* and *Neobenedenia girellae* have become a threat to many cultured and aquarium species. They have a wide distribution and high fecundity that frequently leads to mortalities (Bondad-Reantaso *et al.*, 1995). *B. monticelli* has been responsible for mass mortalities of cultured grey mullet species (Mugilidae) in Egypt (Paperna *et al.*, 1984). *Gyrodactylus salmonis* caused extensive fin damage and skin discolouration where its marginal hooks were lodged deep into the host epidermis (Cone & Odense, 1984). Epidermal thickness was reduced (Cusack & Cone, 1986) and mucous cell number increased in salmonids after *G. derjavini* infection (Buchmann & Uldal, 1997). Sterud *et al.* (1998) reported that *G. salaris* caused thinning of the epidermis, particularly on the pectoral fins and a reduced number of mucous cells in the epidermal layer of Atlantic salmon.

5.1.2. *Lepeophtheirus hippoglossi*

5.1.2.1. Parasite Attachment and Distribution

The initial attachment of *Lepeophtheirus* species to the host is by the copepodid stage, as described for *L. salmonis* by Bron *et al.* (1991) and *L. hippoglossi* (this study). Bron *et al.*, (1991) described a process divided into three stages: an initial searching phase when the copepodid searches the surface of the fish, probing with its anterior end and coming into close contact with its host. Once a suitable site is found the primary attachment phase commences where the maxillipeds grasp the host and the second antennae penetrate the host skin. The filament attachment phase is the final stage of anchoring the copepodid to the host, and was rapidly followed by the moult to the first chalimus stage (Bron *et al.*, 1991).

In *Caligus elongatus* the frontal filament is an acellular structure produced by a cement gland located inside the anterior part of the cephalothorax (Piasecki & Mackinnon, 1993). The filament of *C. elongatus* develops in older copepodids in a cuticular pocket in the cephalothorax. After attachment and the moult to chalimus the filament extrudes and attaches permanently to the host (Piasecki & Mackinnon, 1993). The filament of *C. elongatus* is composed of a wide cylindrical proximal region containing fibres within an outer sheath and a distal region that expands into a broad basal plate (Pike *et al.*, 1993). Bron *et al.* (1991) suggested that the filament of *L. salmonis* is secured by a glue-like secretion injected under the epithelium where it spreads to form a basal plate. The stem of the filament appears to be made of cuticular material with tightly packed protein fibres internally with an axial duct running down the centre through which the

basal plate secretion is passed (Bron *et al.*, 1991). The filament is present during all of the chalimus stages of caligid copepods.

In many copepod species the preferred area of settlement for copepodids is different from that where the adult stages are found. The preferred areas for settlement for *Lepeophtheirus salmonis* are the fins and to a lesser extent within the buccal cavity and on the gills (Bron *et al.*, 1991). However, Johnson and Albright (1991b) and Tucker *et al.* (2000a) found that the majority of copepodids of the same species attached to the gill filaments and the pelvic, pectoral and anal fins as well as the head. The buccal cavity and gill filaments have not been reported as a settlement site for *L. salmonis* in the wild and it is thought that gill attachment in experimental fish may result from slower current speeds through the gill chamber (Bron *et al.*, 1991). Todd *et al.* (2000) found that adult female *L. salmonis* predominated around the anal fin and along the posterior dorsal midline, however, males preferred the head and the dorsal midline between the head and the dorsal fin in wild caught salmon. This general pattern for pre-adult and adult stages of *L. salmonis* was shown to be similar in experimental infections of salmon by Dawson *et al.* (1997), however, they did not analyse the location of males and females separately. In the same study Dawson *et al.* (1997) also examined the pattern of attachment of *L. salmonis* to sea trout (*Salmo trutta* L.) and found that significantly more adult sea lice were found in the posterior ventral region of the trout and significantly more adult sea lice were found on the head and dorsal surfaces of the salmon. This may be explained by differences in the structure of the epidermis between the species (Pickering & Macey, 1977) or perhaps by differences in

body shape and size and water currents over the body surface (Jaworski & Holm, 1992). The preferred copepodid settlement area on both sea trout and salmon was the dorsal fin (Dawson *et al.*, 1997). Bron *et al.* (1991) suggest that the dorsal fin provides protection from water currents.

When fingerlings of the brook trout, (*Salvelinus fontinalis* Mitchill) were exposed to newly moulted copepodids of *Salmincola edwardsii* the gills, opercula and pectoral fins were the major sites of attachment (Sterud *et al.*, 1998). However, as the parasites grew, the opercula and pectoral fins became the preferred attachment sites (Conley & Curtis, 1994). Boxshall (1974b) found that the majority of gravid females of *L. pectoralis* were found on the inner surfaces of the pelvic or pectoral fins. The motile post chalimus stages were mostly found on the general body surface of the plaice (*Pleuronectes platessa*) and the chalimus stages were attached to all regions of the external body surface except the eyes (Boxshall, 1974b). Anstensrud and Schram (1988) found that copepodids of *Lernaeenicus sprattae* (Copepoda, Pennellidae) preferred to settle on the pectoral or dorsal fins of sprat and herring in both experimental infections and in the wild. The adults of this species were located in the eyes but the gravid females became scattered mainly across the body surface, possibly so that as the eggs hatched the free-swimming nauplius stages were dispersed into the water.

In general in parasitic copepods the relatively small copepodids attach to areas where water currents are slower and therefore attachment is made easier e.g. gills and fins.

The motile and larger adult stages gather in areas where food is plentiful and perhaps where water currents move faster to aid in their respiration, as they have more powerful attachment mechanisms than the copepodids there is less chance of the adult stages being washed away. Congregation of the motile stages into certain areas will also aid in the search for a mate.

5.1.2.2. Pathology

Lepeophtheirus hippoglossi is commonly found on wild halibut in low numbers and, in this situation, the pathology caused by the parasite has not been reported to be severe. Similarly *L. salmonis* is a common parasite on wild Atlantic salmon and does not cause extensive damage to the fish (Johnson & Albright, 1992). However, salmon held under cage culture conditions (i.e. relatively high densities) are extremely susceptible to major epidermal damage and scale loss, leading to death if not treated. The gross and histopathological damage caused by adult *L. salmonis* has been well documented (White, 1940; Wootten *et al.*, 1982; Egidius, 1985; Jonsdottir *et al.*, 1992; Nolan *et al.*, 1999). Oedema, hyperplasia, sloughing of cells and cellular inflammation were observed from the salmon at, and around, the point of feeding and attachment of motile stages (Jonsdottir *et al.*, 1992). With low numbers (<10) of adult stages infecting post-smolt salmon, shedding and swelling of epithelial cells occurred and, at sites of feeding, gross damage was visible with disrupted and swollen pavement cells (Nolan *et al.*, 1999). The effect of the chalimus stages has been described by Jones *et al.* (1990) and Nolan *et al.* (2000). Second antennal attachment, filament attachment, maxilliped and feeding activity caused skin changes including mechanical disruption followed by epidermal hyperplasia to the skin of salmon (Jones *et al.*, 1990). Nolan *et al.* (2000)

found that the number of acidophilic mucous cells decreased in *Onchorynchus mykiss* and the effect of a subsequent stressor was affected by prior exposure to *L. salmonis*. Jones *et al.* (1990) found no evidence of a host response to living attached chalimus stages in Atlantic salmon. However, Johnson & Albright (1992) reported that coho salmon (*Oncorhynchus kisutch*) showed a well developed epithelial hyperplasia and inflammatory response to the presence of *L. salmonis* and in contrast only minor gill and fin tissue responses to this parasite were observed in Atlantic salmon (Johnson & Albright, 1992).

Boxshall (1976) studied the mechanical and feeding damage caused by attached *L. pectoralis* on flounder. Gravid females attached to the pectoral fins and caused deep lesions by feeding with epidermal hyperplasia apparent around the periphery of the feeding sites. Chalimus three and four stages were found to cause deep lesions extending into the dermis around the point of filament attachment.

5.1.3. Host Skin

Fish skin consists of three main layers, the hypodermis, dermis (where the scales and pigment cells are located) and the epidermis. There is an additional layer of mucus and squamous elements known as the cuticle, which varies considerably in thickness depending on the fish species, the location on the body surface and the state of maturity and health of the fish (Whitear, 1970). The cuticle is a complex of cell protoplasm, sloughed cells and goblet cell mucus released onto the surface (Bullock & Roberts, 1975). It may also contain specific immunoglobulins, C-reactive proteins and

lysozyme, indicating both a specific and broader non-specific defensive capacity (Baldo & Fletcher, 1975). The epidermis is alive and capable of cell division throughout its depth and consists mainly of malpighian cells and also apocrine mucous cells, club cells, a variety of granule containing cells and specialist cells for taste and touch (Bullock & Roberts, 1975). The cellular structures are similar throughout the epidermis and the characteristic sequence of layers present in mammals does not occur (Bullock & Roberts, 1975). The epidermal thickness varies with species, age, position on body, and stage in the reproductive cycle (Bullock & Roberts, 1975). In most pelagic fish, the dorsal epidermis is thicker but in some benthic species the ventral epidermal layer is thicker (Bullock & Roberts, 1975). Mucous cells appear first in the middle layers of the epidermis and grow in size as they migrate to the surface layers (Bullock & Roberts, 1975). Fish mucus is made mostly of glycoproteins (Bullock & Roberts, 1975). Pickering (1974) found that the concentration of mucous cells of the skin varied in salmonids, most being found in the anterior regions and fewest on the fins. The surface of teleost fish is thus made up of the epithelial surface with an overlying layer of mucoid material, which is continually being sloughed into the water and is replaced from beneath (Roberts & Bullock, 1980).

Studies on halibut skin have not been published, however, the skin of plaice (*Pleuronectes platessa*), also a pleuronectid, has been examined (Roberts *et al.*, 1971). In plaice the epidermis varies between 4-12 cells deep and is a non keratinised mucous membrane on a regular basement membrane. There are three major cellular components, firstly, the malpighian cells found in all teleosts, which constitute the

majority of cells present in the epithelium. Secondly, mucous cells characteristic of the outer layers of epidermis. Thirdly a number of round or ovoid cells packed with highly eosinophilic granules found in the basal layers of the epidermis. It was concluded that this type of cell was a dendritic secretory cell, which had a function in the mucosa of the fish (Roberts *et al.*, 1971). Macrophages containing melanin are also prominent within the epithelium of the dorsal surface. The melanin is held in characteristic granular form, and each granule appears to be within a separate vesicle (Roberts *et al.*, 1971).

This study will discuss briefly the frontal filament of the chalimus stages of *L. hippoglossi* and also monitor the spatial distribution of this parasite and *Entobdella hippoglossi* on its host and how this changes with age. It also describes the gross pathology caused by *E. hippoglossi* and *L. hippoglossi* under culture conditions. The level of *L. hippoglossi* infection necessary to cause such damage to the halibut is also considered. Both *Entobdella hippoglossi* and *Lepeophtheirus hippoglossi* survive throughout the majority of their life-cycles on the skin of Atlantic halibut and therefore the structure of the skin must be important in their relationship with the host. The common areas of the body affected by the two parasites are discussed in conjunction with the parasite distribution and in association with the host skin habitat.

5.2. Materials and Methods

5.2.1. *Entobdella hippoglossi*

5.2.1.1. Parasite Attachment and Distribution

Data collected from 9 fish (50-80 cm in length) experimentally infected with *Entobdella* were used to analyse the distribution of the parasite on the host. Data from two separate sampling dates for each of the 9 fish were compiled. The sampling dates were chosen because of high parasite numbers, in order to provide as large a sample size as possible. The two sampling dates selected for a particular fish were chosen so that they covered different parasite generations; therefore each parasite was counted only once.

For the purpose of data collection, the body surface of the halibut was divided into 10 sections (Figure 5.1); head, anterior, mid, tail and fins (not including the tail fin) on both the dorsal and ventral surfaces. These areas were measured using the Zeiss kontron image analysis system and the percentage of the whole body calculated for each of the regions. All parasites present on each fish were marked onto a fish map and the parasites were measured (see Chapter 2, section 2.2.2. Sampling Procedures, for details of measurement technique). The distributions of juvenile (<10mm) and gravid (≥10mm) parasites were recorded separately as well as total numbers of parasites. The number of parasites in each region was divided by the percentage total body area for that region and statistical tests carried out to determine whether the distribution of

parasites differed significantly from random. Statistics were carried out using SigmaStat 2.0 software.

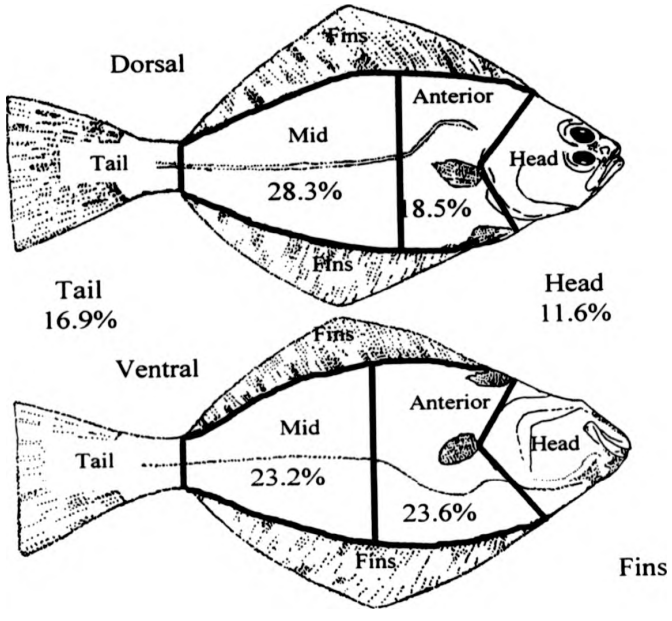


Figure 5. 1. Fish map showing the divisions of the halibut body surface for mapping of *E. hippoglossi* distribution and attachment data. Fins includes anal and pectoral fins but not tail fin.

5.2.1.2. Pathology

Gross observations were made of infected halibut during the experiments and the extent and nature of any lesions noted.

5.2.2. *Lepeophtheirus hippoglossi*

5.2.2.1. Parasite Attachment and Distribution

A 1994 year class halibut (approx. 60cm in length) was infected with copepodids (see Chapter 4, paragraph 4.2.3.1 for full infection details). Once the infection had progressed to chalimus stages (mainly II and III) the fish was sacrificed. In order to investigate the mechanism of attachment of the chalimus small squares of skin were removed around the frontal filament of attached chalimus stages. All blocks were taken from dorsal surface skin, mainly towards the anterior region of the fish. The blocks of skin with attached parasites were fixed in 10% buffered formalin for at least 24 hours and then processed for histology and stained with H&E (Table 5.1).

The distribution of *L. hippoglossi* on the surface of the host was determined by dividing the surface area of the halibut into 10 sections (head, body, tail, fins and gill cavity) on the dorsal surface and ventral surface (Figure 5.2). Each of these areas was measured using Zeiss Kontron image analysis software, the total body size was also measured and thus the percentage of the total body area was calculated for each of the regions.

The parasites were divided into 3 groups for analysis; i) copepodid and chalimus stages (attached), ii) pre-adult stages and iii) adult stages. On each examination of a halibut a fish-map was completed, marking the site of attachment and life stage of the parasites (Fig. 5.2). The data from these fish maps was used to collate the distribution patterns of *L. hippoglossi* on the host. The number of *L. hippoglossi* in each section of the host body surface were expressed as a percentage of the total number of parasites mapped.

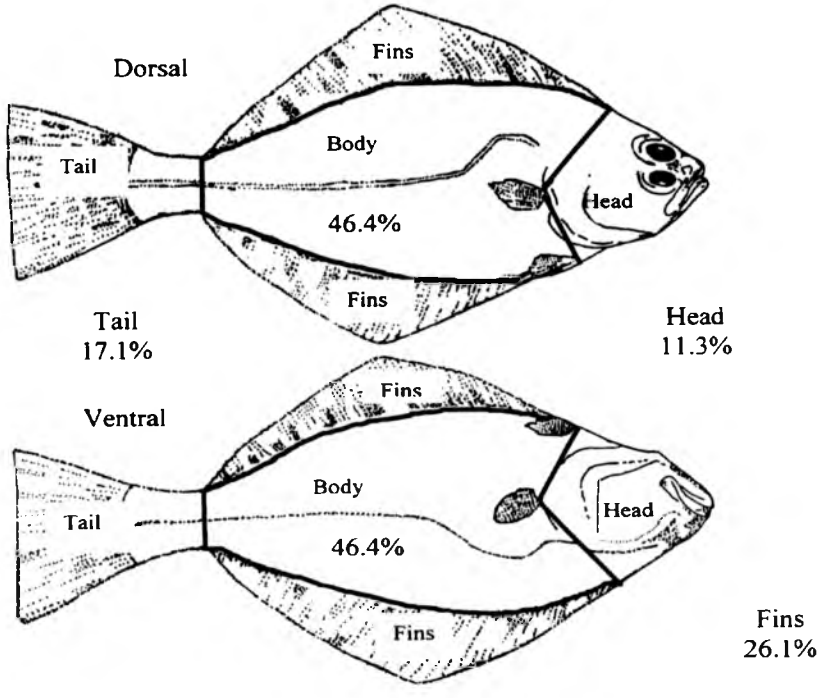


Figure 5. 2. Fish map showing the divisions of the halibut body surface for mapping the distribution of *Lepeophtheirus hippoglossi*. The gill cavity was also examined for the presence of parasites. Fins includes the anal and pectoral fins but not the tail fin.

The data used for the attached and pre-adult stages were taken from juvenile halibut (1998 year class, mean 15.7±2.0cm) infected with copepodids and maintained at 9.6°C throughout the parasite life-cycle (for further details see medium temperature life-cycle experiment, Chapter 4, Section 4.2.3.1). Twenty-five fish were sampled for attached stages and 878 parasites from the dorsal surface and 401 from the ventral surface plotted. Fifteen fish were sampled for the pre-adult stages with a total of 406 parasites from the dorsal surface and 73 from the ventral surface mapped.

The adult stage distribution data was collected from 1996 year class halibut (50-80cm in length) maintained at ambient temperature (range 6-15°C) in Machrihanish. Fish with high parasite numbers were selected from all infected halibut throughout the infection period. Fifteen fish were sampled for adult stage distribution data; 377 parasites from the dorsal surface and 10 from the ventral surfaces were mapped.

The same data were used to identify whether specific areas of the body surface, excluding the fins, were preferred by each of the three groups of parasites. The body surface was divided into anterior, mid and sides for both dorsal and ventral surfaces (Figure 5.3) for this analysis. The number of parasites in each region was divided by the percentage total body area for that region and statistical tests carried out to determine whether a random distribution pattern had occurred. Statistics were carried out using SigmaStat 2.0 software.

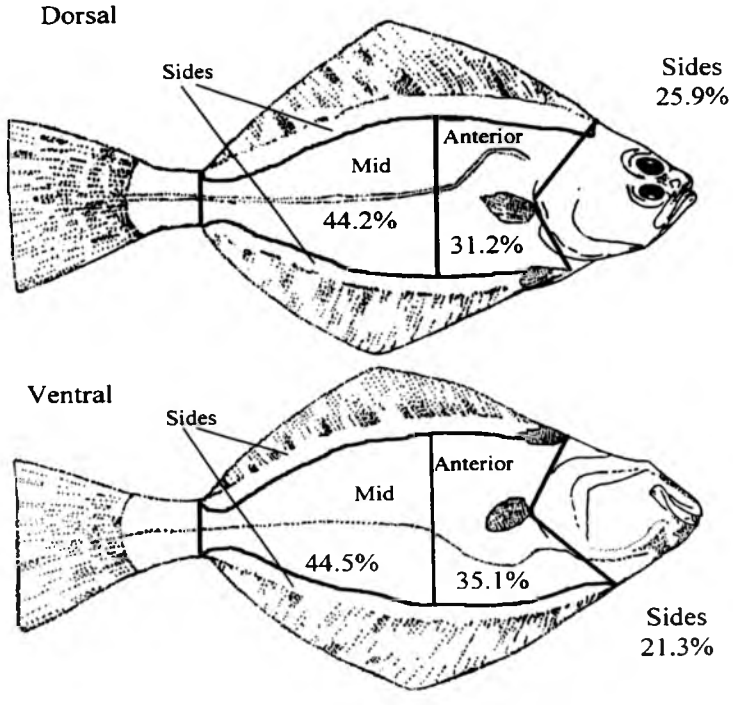


Figure 5. 3. Divisions of the body surface area (excluding fins) of halibut used to calculate the distribution pattern of *L. hippoglossi*.

5.2.2.2. Pathology

To determine the number of *L. hippoglossi* necessary to cause clinical signs of disease in halibut adult parasites were removed from infected fish and placed directly onto the skin of anaesthetised (2-phenoxyethanol 1:1500) halibut, which had not been previously exposed to *L. hippoglossi*. Three 1994 year class (approx. 4 years post-hatch, 50-80 cm; 1kg+) fish were infected with 10 adult gravid female *L. hippoglossi* and a further three fish of comparable size were infected with 25 gravid female parasites. The two groups of halibut were kept in separate 2m tanks and monitored

weekly by anaesthetising the fish with 2-phenoxyethanol (1:1500) and examining the skin with a hand lens for signs of damage. The mean temperature was 8.1°C and the mean salinity was 33‰. Fish were monitored for 20 days.

5.2.3. Host Skin

5.2.3.1. Fish Sampled

Samples of skin were collected from four different size groups of halibut in order to ascertain differences in epidermal thickness (Figure 5.4) with age. The smallest halibut used were approximately 15g in weight and from the 1998 year class (approximately 8 months post-hatch) from Otterferry Salmon. These fish were being held under hatchery conditions, in indoor tanks. Due to the small size of the fish only skin from the mid-region was collected. Dorsal and ventral skin samples were collected from each fish and fixed separately in 10% buffered formalin.

A second group of halibut were collected from a cage site near Oban, three months post-stocking and weighing approximately 100g (originally from Otterferry Salmon, 1999 y.c. approximately 12 months post hatch). Samples of dorsal and ventral skin were collected from the mid-region of the fish and fixed in 10% buffered formalin. Skin was also studied from broodstock halibut held at the Seafish Authority, Ardtoe. These fish had originally been caught in the wild but had been held in captivity for more than ten years, and weighed approximately 15kg. Two fish were killed as part of a separate experiment, however, dorsal and ventral skin samples were also collected from the mid-region of the fish.

For a comparative study, the skin from three 3kg Atlantic salmon (*Salmo salar*) from a Scottish cage site was also collected from the anterior area across the spine of the fish.

Two 1996 y.c. halibut (approximately 2+ years old, approx. 60cm in length, 1.5kg in weight) were also sampled. These fish were originally from the Seafish Industry Authority, Ardtoe, but had been held at Machrihanish for up to one year in a 1m square tank. Skin was taken from 12 areas of the body; 6 ventral and 6 dorsal skin (Figure 5.4) samples were collected and fixed separately in 10% buffered formalin.

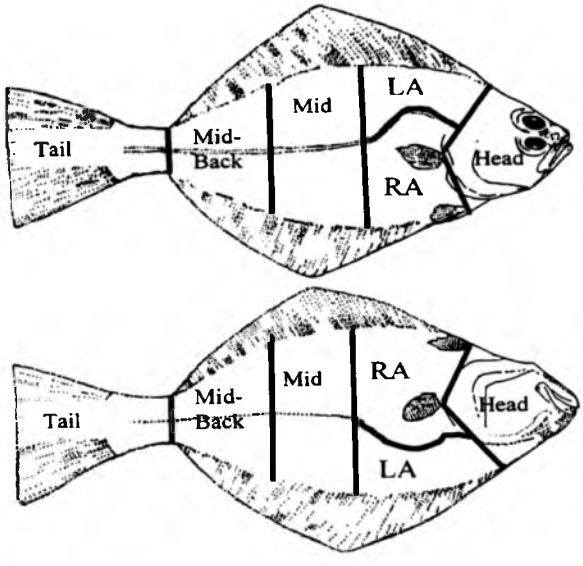


Figure 5. 4. Fish map detailing the areas of skin collected for epidermal thickness and mucus cell number counts from two 1996 year class halibut. R.A –right anterior area; L.A. –left anterior area

The skin from all fish was treated in the same manner. Skin removed from the fish was immediately placed into 50ml plastic fix pots containing 10% buffered formalin and allowed to fix for at least 24 hours. Each of the pots was labelled clearly to allow for identification of the area of the fish from which the skin had been removed. Skin was wax embedded, sectioned and stained using haematoxylin and eosin (H&E) (Appendix 1).

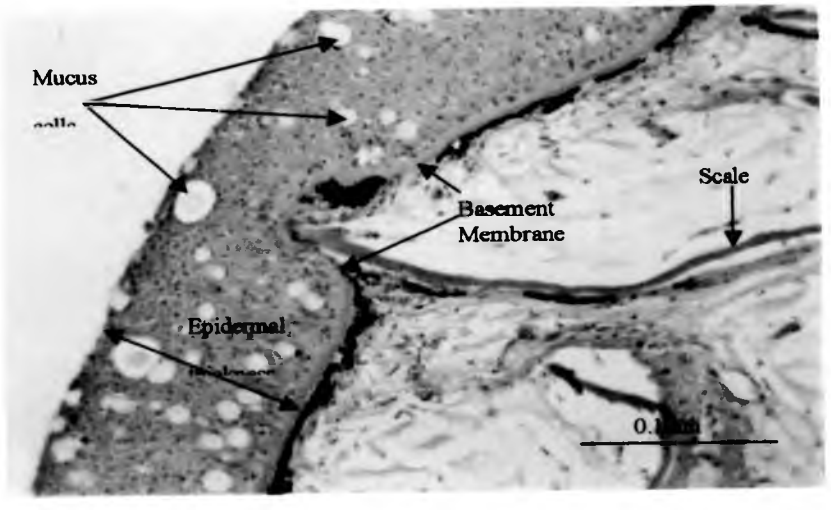


Figure 5. 5. A section of halibut skin stained with H&E. The epidermis is made up of tightly packed, eosinophilic (pink) malpighian cells and larger unstained mucous cells. The basement membrane separates the epidermis from the dermis where the scales are situated. The epidermal thickness measurements were taken as shown by the arrows. M-mucous cells; BM-basement membrane; S-scale; epidermal thickness is indicated by the arrowed line.

The thickness of the epidermal layer, illustrated in Figure 5.5, was measured using a calliper function on Zeiss Kontron image analysis software. This function allows measurement of the epidermis at right angles to the basement layer below, ensuring that the same measurements were taken for all areas of skin. Thirty measurements were taken randomly for each area of skin, for each halibut sampled. Measurements were only taken from areas of skin that were intact. The mean epidermal thickness was calculated for each of the size groups and areas of skin.

Mucous cells (see Figure 5.5) were also counted using the image analysis system. Sections of skin samples from 12 areas of the 1996 year class halibut (Figure 5.4) were mounted on microscope slides and stained using the periodic acid Schiff (PAS) technique (Appendix 2). These sections were orientated so that the skin lined up horizontally on the slide (and thus the computer screen), and viewed at x20 magnification, the number of mucous cells present in one screen width of the image analysis system were counted 25 times for each area of skin. The screen width was constant and all the skin samples were orientated the same way so that the counts of mucous cell numbers were directly comparable.

5.3. Results

All statistics tests were carried out using the statistical software package SigmaStat 2.0.

5.3.1. *Entobdella hippoglossi*

5.3.1.1. Parasite Attachment and Distribution

The Friedman repeated measures analysis of varience on ranks showed that there was a statistically significant difference between the number of *Entobdella* found on the areas of the body on both surfaces. All multiple comparisons were conducted using Student-Newman-Keuls Method, with a significance level of $p<0.05$. The distribution of the parasites was significantly different from random, for the dorsal surface ($\chi^2=37.75$, d.f.=4, $p<0.001$) and for the venral surface ($\chi^2=38.42$, d.f.=4, $p<0.001$). On the dorsal surface statistically significant different numbers of parasites were found between the head and all other areas (anterior, mid, tail and fins), between the anterior area and the

fins and mid areas and also between the tail and fins and mid areas. There was no statistically significant differences in parasite numbers on the anterior and tail regions or the mid region and the fins. On the ventral surface. The head had statistically signficantly more *Entobdella* present than the fins, tail and the mid region, but there was no significant difference with the number of *Entobdella* present on the anterior region. The anterior region had significantly more parasites present than each of the fins, tail and mid regions. The number of parasites present on the mid region was statistically significantly different from both the tail and fins, there was also a significant difference betweent the number of parasites present on the tail and fins.

When the data is split into juvenile and gravid *Entobdella* the Friedman repeated measures Analysis of Variance on ranks shows that there was a statistically significant difference between areas on both the dorsal (χ^2 =40.17, d.f.= 9, $p<0.001$) and ventral (χ^2 =39.62, d.f.=9, $p<0.001$) surfaces. When comparing the number of juvenile and adult parasites in corresponding regions on the dorsal surface there were statistically significant differences found on the tail, head and mid regions, although there was no significant difference in the anterior or fins regions. When looking at the ventral surface there were statistically significant differences between the number of juveniles and adults present in all of the areas.

There was a significant difference from a random distribution pattern of juvenile *Entobdella* on the dorsal surface (χ^2 =25.59, d.f.= 4, $p<0.001$) and on the ventral surface (χ^2 =20.75, d.f.=4, $p<0.001$). The number of juveniles found on the tail of the dorsal

surface differed significantly from all other areas, except the head. The number of juveniles found on the dorsal surface of the head also differed significantly from all other areas, except the tail. There were also significant differences between the number of juvenile *Entobdella* present on the dorsal surface of the anterior and fins, anterior and mid region, and mid region and fins.

The number of juveniles found on the ventral surface of the head and tail differed significantly from all other areas of the body except with each other. There were also significant differences between the anterior region and the fins, and between the mid region and the fins.

There was a significant difference from a normal random distribution pattern of adult *Entobdella* on the dorsal surface (χ^2 =9.75, d.f.= 4, p=0.045) and on the ventral surface (χ^2 =19.93, d.f.=4, p<0.001). The number of adult *Entobdella* found on the dorsal surface of the anterior region did not significantly differ from the fins, however all other regions of the dorsal surface showed significant differences in the number of parasites present. On the ventral surface the number of adult *Entobdella* present was not significantly different between the head and anterior region but all other areas did have significantly different numbers of parasites present.

5.3.1.2. Pathology

Occasional small, discrete areas of erosion appeared associated with congregations of *Entobdella* (Figure 5.6). Once the parasites were removed these healed within a few

weeks. Occasionally, petechial haemorrhages were observed around the eroded areas and very occasionally diffuse haemorrhage extended across large areas of the epidermis (Figure 5.7). These would always first appear on the head and anterior ventral surface, areas where parasites were congregated becoming more extensive as infection continued. Lesions healed within three weeks once the parasites were removed. All areas of erosion were superficial and did not appear to extend into the dermal layer.

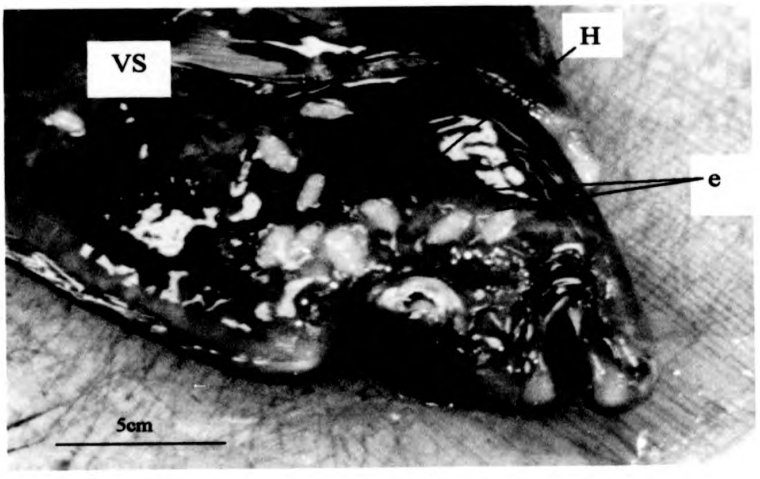


Figure 5. 6. Ventral surface of malpigmented halibut, showing extensive skin haemorrhage caused by *Entobdella hippoglossi*. e-*Entobdella hippoglossi;* H-haemorragic area; VS-ventral surface.

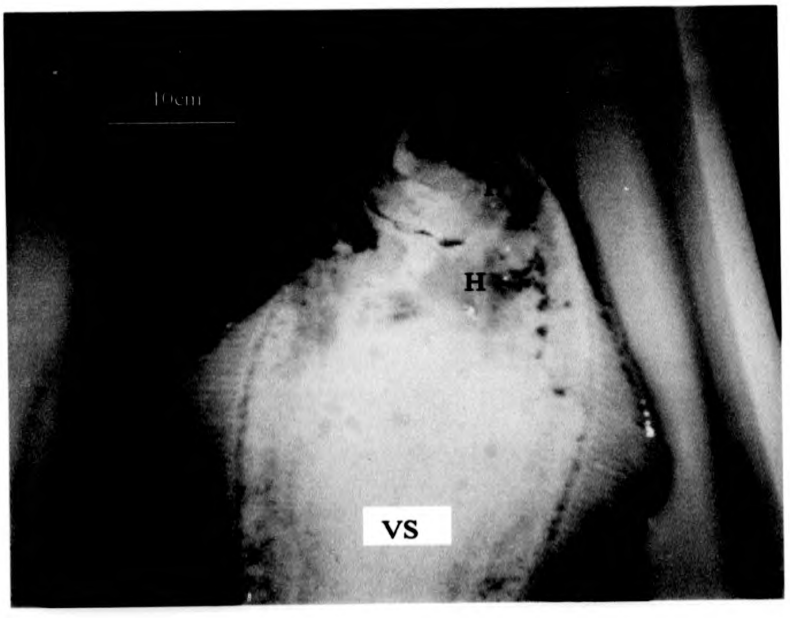


Figure 5. 7. Halibut ventral surface, showing the extent of haemarrhagic lesions caused by *Entobdella hippoglossi* infection. VS-ventral surface; H-haemorrhaged skin.

5.3.2. *Lepeophtheirus hippoglossi*

5.3.2.1. Parasite Attachment

Figures 5.8 & 5.9 show that the frontal filament of the chalimus stage pierced the epidermal layer and a broad basal plate fixed the parasite to the basement membrane of the host skin. The basal plate stained homogeneously and was eosinophilic and recognisably different from the main stem of the filament. The staining of the stem of the filament was indistinguishable from the exoskeleton of the parasite suggesting that it may be cuticular in origin. There appears to be a narrow channel or tube, the axial duct, running through the centre of the stem, which may be for the secretion of the basal plate material. The stem appeared to be fibrous in nature.

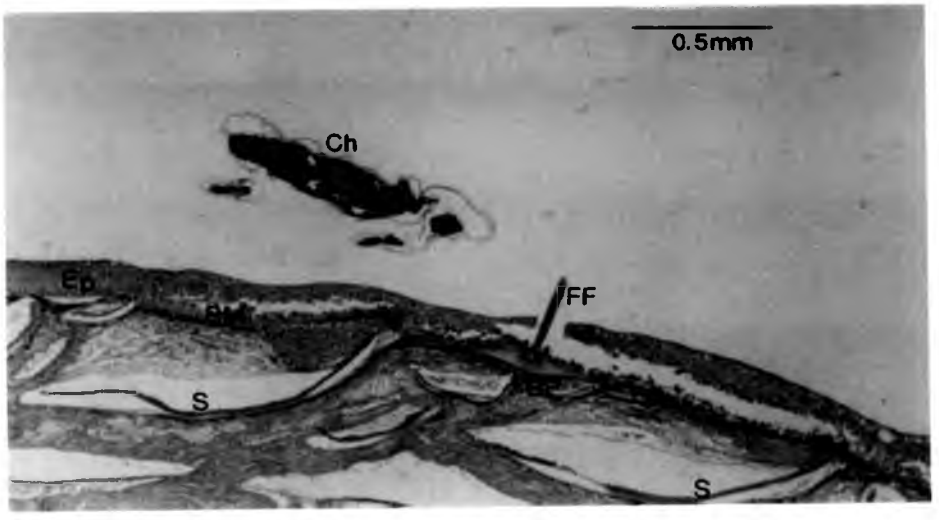


Figure 5. 8. A *Lepeophtheirus hippoglossi* chalimus stage II attached to the epidermis of a halibut. Ch-Chalimus; FF-frontal filament; BP-basal plate; BM-basement membrane; Ep-epidermis; S-scale. Stained with H&E.

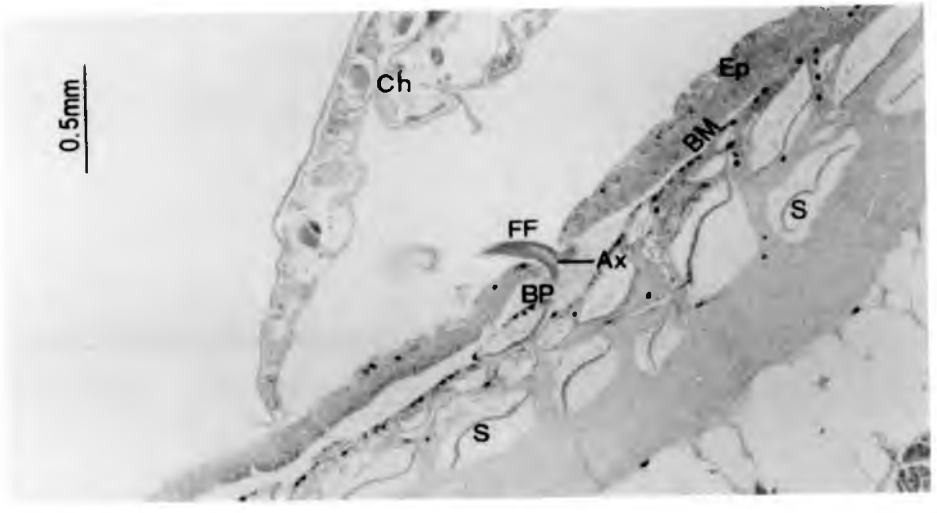


Figure 5. 9. A *Lepeophtheirus hippoglossi* chalimus stage III attached to the epidermis of a halibut. Ch-chalimus; FF-frontal filament; BP-basal plate; Ax-axial duct; BM-basement membrane; Ep-epidermis; S-scale. Stained with H&E.

5.3.2.2. Distribution of *L. hippoglossi* on fish surface

The Friedman Repeated Measures Analysis of variance on ranks shows that the distribution pattern differed significantly from random on the whole of the fish and for all stages of *L. hippoglossi*; adult stages χ^2 =55.14, d.f.= 5, p=0.001, pre-adult stages χ^2 =44.36, d.f.= 7, p=0.001, attached stages χ^2 =91.10, d.f.= 7, p=0.001. All multiple comparisons were conducted using Student-Newman-Keuls Method, with a significance level of $p<0.05$. The number of adult *L. hippoglossi* found on the dorsal head and dorsal body regions were statistically significantly different to all other areas of the fish, except with each other. The highest numbers of parasites were found in these regions (dorsal head and body) and no adult parasites were found on the ventral tail or fins. The number of adult parasites found on the dorsal tail surface and the ventral head were not significantly different, however. the number of adult parasites found on the ventral body surface was significantly different to the number found on the dorsal tail and fins and the ventral head. The pattern of distribution for the pre-adult stages was similar to that of the adult stages with most being found on the dorsal body and head areas and no statistically significant differences between them, but statisically significant differences between body and head and all other areas of the body. Neither were there any significant differences between the number of pre-adult stages between the fins of the dorsal and ventral surface nor betweeen the number found on the ventral body and tail surfaces. There were statistically significant differences between the number of pre-adult *L. hippoglossi* on all other areas of the fish.

The attached stages had a different distribution pattern from the pre-adult and adult stages. There were statistically significant differences between all areas of the fish except for the dorsal head and fins, and the ventral head and fins. A greater portion of the attached stages were found on the fins than the motile stages.

When the body region of the halibut was split into 3 areas the distribution was significantly different from random all stages of *L. hippoglossi*; adult stages χ^2 =60.67, d.f.= 5, p=<0.001, pre-adult stages χ^2 =46.16, d.f.= 5, p=<0.001, attached stages χ^2 =64.14, d.f.= 5, p=<0.001. There were statistically significant differences between the number of adult parasites found in all areas of the all body except between the ventral front and sides. There were statistically significant differences between the number of pre-adult parasites present on the dorsal sides and all other areas of the body except for the dorsal mid region. The number of parasites present on the dorsal front differed significantly from all other areas of the body. There was not a significant difference between the number of parasites present on the ventral front and mid regions although there was a statistically significant difference between all other regions. There was not a statistically significant difference between the number of attached parasites present on the ventral and dorsal side regions but there was a statistically significant difference between all other regions.

5.3.2.3. Pathology

Those fish infected with only 10 individual gravid female parasites showed no signs of pathology. Figure 5.10a shows the damage caused by 25 adult gravid female *L.*

hippoglossi after 7 days on one of the fish in this experiment. Figure 5.10b shows a second fish after 13 days of infection with the same number of female lice. The lesions became extensive and severe haemorrhage occurred. The third fish did not show signs of severe injury. All fish were killed after 20 days by which time the lesions had become severe, covering most of the anterior dorsal surface of the fish.

Figures 5.11a & b and 5.12a & b show other halibut infected with a mixture of motile stages. Figures 5.11a and 5.12a were taken of the same fish but 7 days earlier than 5.11b and 5.12b and show how lesions can change and become more extensive within a short time period. Earlier stages of damage consisted of thickened mucus with a darker grey appearance of the skin (Figure 5.11a) around the area of the anal and pectoral fins and petechial haemorrhaging and changes (lightening) in the pigmentation of the surrounding skin (Figure 5.12a). After 7 days, deep, open lesions covering up to 15% of the dorsal body surface had developed, extending over the abdominal region of the body (Figures 5.11b and 5.12b). Ulceration and erosion as well as scale loss can also be seen (Fig 5.11b). The lesions caused by *L. hippoglossi* were either focal (small localised lesions), patch (over a substantial area of the fish, e.g. extending over the skin covering the abdominal cavity) or extensive (across most of the anterior dorsal surface of the fish, not including the head). The area of the lesion showed increased hyperaemia with reddening to increased darkening of the skin pigment, erosion and ulceration. Parasites were seen feeding at the margins of the lesions. Scale loss and haemorrhages were common (Figures 5.10a & b; 5.11a & b; 5.12a & b).

Figure 5. 10a. Dorsal surface of one halibut showing lesion caused by 25 adult gravid female *Lepeophtheirus hippoglossi* after 7 days. L-lesion.

Figure 5. 10b . Dorsal surface of a second halibut, showing the lesion caused by 25 adult gravid female *Lepeophtheirus hippoglossi* after 13 days. L-area of Lesion; gf-gravid female *L. hippoglossi*.

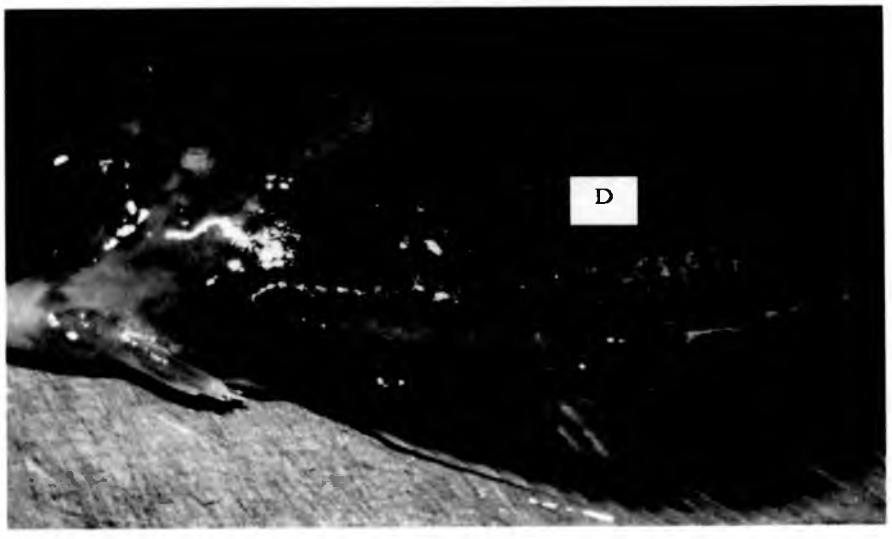


Figure 5. 11a. Skin damage caused by *Lepeophtheirus hippoglossi* to the skin on the dorsal surface between the anal and pectoral fins of a halibut. D-darkening of the skin pigmentation, M-thickened mucus.

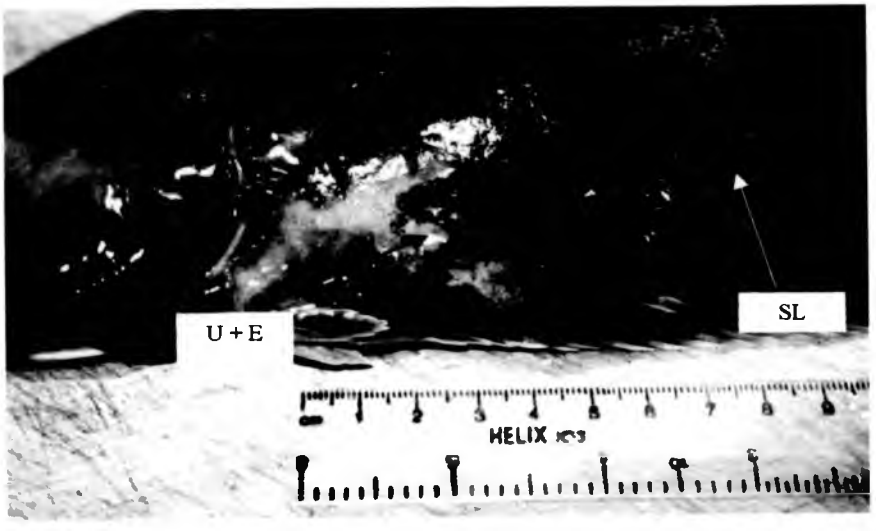


Figure 5.11b . Extent of lesions caused by *L. hippoglossi* 7 days later. Ulceration and erosion is evident and scale loss has occurred over an extensive area. U+E-ulceration and erosion, SL Scale loss.

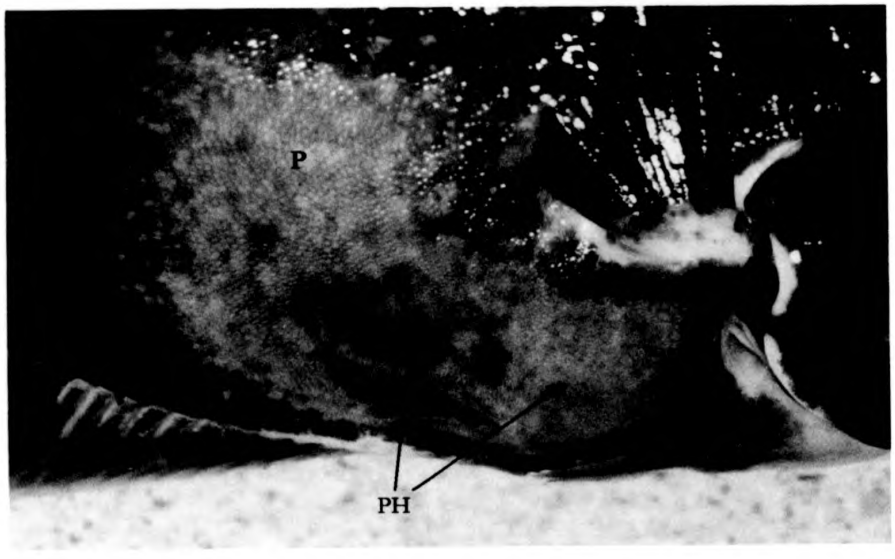


Figure 5. 12a. Skin damage caused by *Lepeophtheirus hippoglossi* to the skin over the dorsal abdominal region of a halibut. PH-petechial haemorrhages, P-changes in skin pigmentation

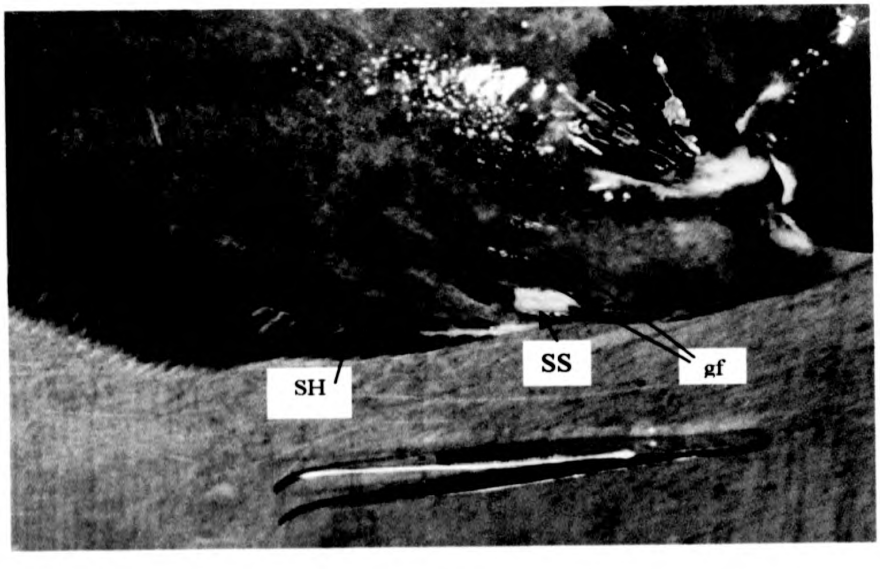


Figure 5.12b. Skin damage caused 7 days later by *L. hippoglossi*. SH-severe Haemorrhages, gf-gravid females, SS-sloughed skin.

5.3.3. Host Skin

Figure 5.13 shows a graphical representation of the epidermal thickness from the dorsal and ventral mid-region of all age groups of halibut and the salmon. The dorsal epidermal layer was significantly thicker in 1,500g and 10kg halibut compared with 15g and 100g fish (Kruskall-Wallis (KW), df=3, H=21.991, $p<0.001$). On the ventral surface the skin of 15g and 100g fish were significantly thinner than that of a 10kg halibut (KW, df=3, H=23.387, $p<0.001$).

There were significant differences between the thickness of the dorsal and ventral skin of the 100g and 1500g halibut. The ventral skin was significantly thicker than the dorsal skin (pairwise t-test, df=7, t=2.739, p=0.03) in the 100g fish. However the dorsal skin was significantly thicker than the ventral skin (pairwise t-test, df=7, t=4.605, p=0.002) in the 1500g halibut.

The epidermis from 3kg salmon was much thinner than in halibut of 1500g to 10kg and corresponded closely to that from 15g halibut. There was no significant difference in the thickness of salmon epidermis and 15g halibut (dorsal and ventral) and dorsal epidermis of 100g halibut, however, the ventral skin of a 100g halibut, was significantly thicker than the salmon (K.W. $p<0.001$).

Figure 5.14 shows that in the 1996 y.c. the dorsal epidermis appeared to be thicker than the ventral in all areas, however, the dorsal epidermis was only significantly thicker in the right anterior area (K.W. $p<0.001$) (Figure 5.14).

There were a higher number of mucous cells in the dorsal epidermis than in the ventral epidermis in all body areas of the 1996 y.c. halibut. However, this difference was only statistically significant for the right anterior area (Tukey-Kramer test $p<0.01$) (Figure 5.15).

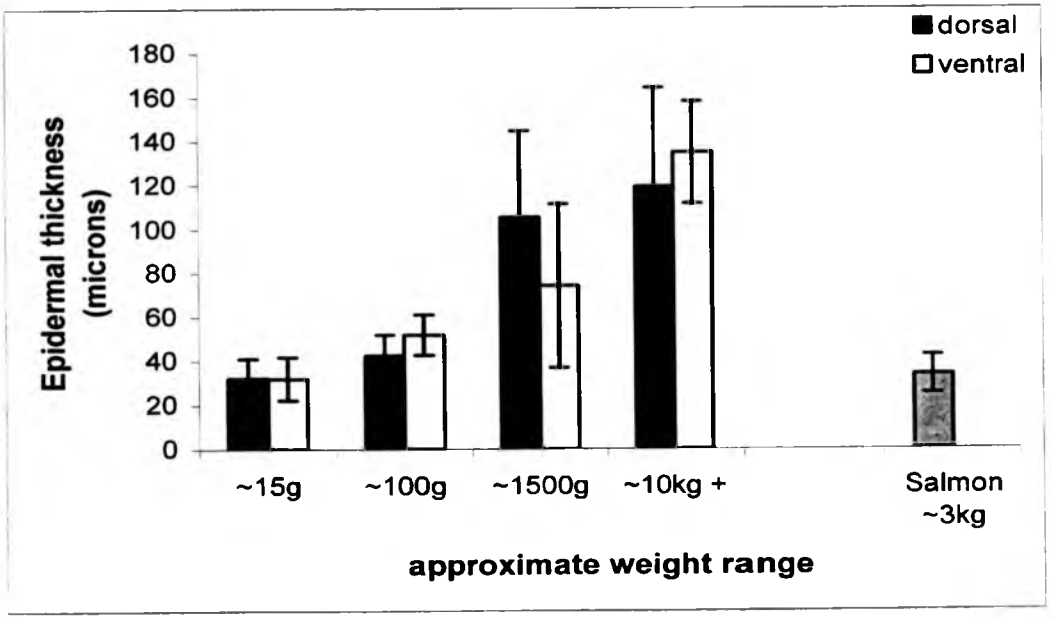


Figure 5. 13. The mean (±S.D.) epidermal thickness of dorsal and ventral skin from four size groups of halibut and from 3kg salmon

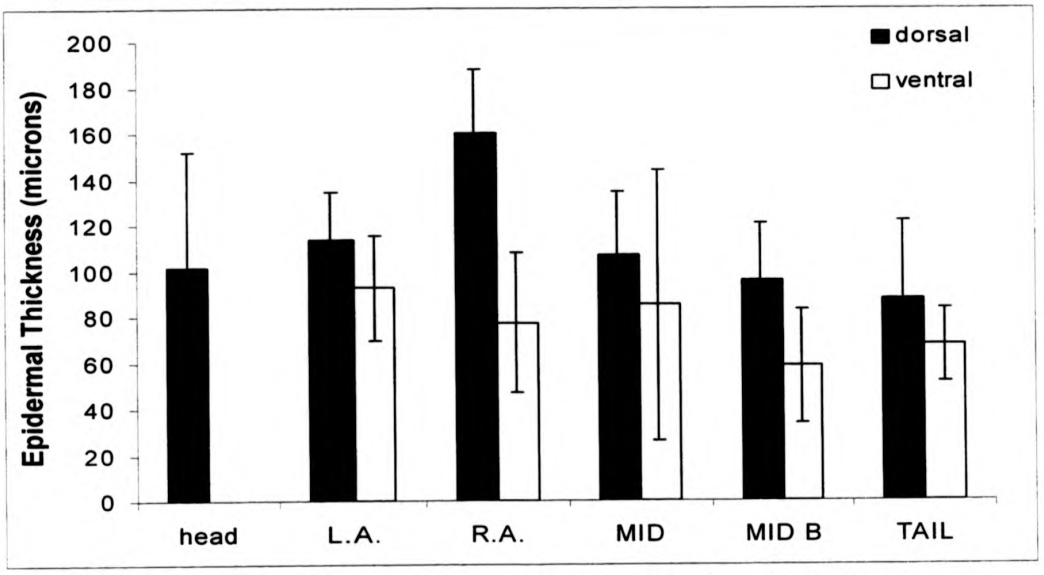


Figure 5. 14. The mean ±S.D. epidermal thickness for 6 areas of halibut skin (1996 y.c.), comparing the dorsal and ventral surfaces.

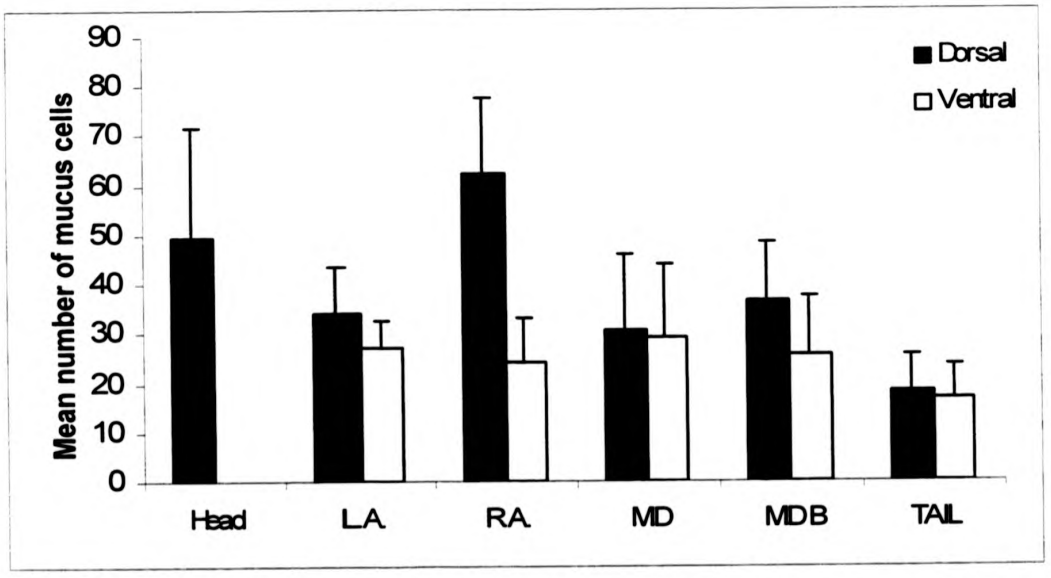


Figure 5. 15. The mean ±S.D. number of mucus cells present in 6 areas of halibut skin (1996 y.c.), comparing the dorsal and ventral surfaces.

5.4. Discussion

5.4.1. *Entobdella hippoglossi*

5.4.1.1. Parasite Attachment and Distribution

This study has shown that juvenile *E. hippoglossi* are found on the dorsal surface of the host, in particular on the tail region. The skin on the tail peduncle is thinner than on the body surface and presumably it is easier for the small haptor of the parasite to attach firmly. There is also less mucus present on fins, including the tail fin (Pickering, 1974) and therefore the small oncomiracidia will have a thinner mucoid layer to pass through before reaching the site of attachment. As the parasites grow and mature they are found underneath the host, on the body and head of the ventral surface. These findings are similar to Kearn (1963) who reported that *E. soleae* migrates to the ventral surface of its host as it reaches sexual maturity (approx. 1-2mm in length) and the adults are almost exclusively found on the under side of the sole. As the attachment organ of the parasite grows it may be better able to adhere to the thicker skin of the body, which might also provide a greater source of food for the parasites. Halibut are believed to rest on the seabed for extended periods of time. Adult parasites on the ventral surface will thus be in contact with the sediment where eggs settle and incubate and are available to re-infect other halibut. The concentration of adults on the ventral surface increases the likelihood of successful mating. Kearn *et al.* (1993) studied seven *E. soleae* kept in isolation to determine whether self-fertilisation occurs. No self-fertilisation occurred after many hours of observation of the isolated specimens and it was suggested that it was also unlikely that it would occur in the wild (Kearn *et al.*,

1993), therefore migration to a common area of the host must increase the chances of mate location. In halibut the main propulsion when swimming comes from movement in the posterior part of the body and tail, this movement may be detrimental to the attachment success of larger parasites, as they offer more resistance to water currents.

5.4.1.2. Pathology

The lesions that appeared on halibut during *Entobdella* infection were usually localised to areas with high numbers of parasites. Skin erosion and an increase in mucus production caused a grey appearance to the skin and occasionally small haemorrhagic lesions occurred. When death occurred in fish with very high parasite numbers over a large area of the ventral surface, osmoregulatory problems were probably the main cause of death. The damage caused by *E. hippoglossi* is probably caused primarily by erosion, possibly chemical, due to the feeding action and secondarily by the attachment organ as in *E. soleae* (Kearn, 1963c; 1964). The feeding mechanism is not mechanical and only the top layer of cells, enclosed by the protruding feeding organ will be affected by the proteases secreted (Kearn, 1964). Therefore the damage is not caused to deeper layers of the skin tissue, except during prolonged feeding periods or when a number of the parasites congregate in one area. All epidermal cells are capable of mitosis (Bullock & Roberts, 1975) and therefore have a short regeneration period, however, when the skin is being removed faster than it can be regenerated the deeper layers of epidermis, down to the dermal layer, are affected. The dermis has a much slower regeneration rate (Kearn, 1963c) and if this is breached then the healing process is prolonged. Only when high (20+) numbers of adult *Entobdella* congregated into a

relatively small area were haemorrhages observed, i.e. damage caused to the dermis. Histologically the epidermis of Atlantic salmon infected with *Gyrodactylus salaris* shows a decreased number of mucous cells suggesting exhaustion of mucus production and the epidermal thickness is reduced in infected fish (Sterud *et al.*, 1998). Histological sections of eroded rainbow trout (*O. mykiss*) dorsal fins caused by *G. salmonis*, a skin inhabiting monogenean, showed epidermal hyperplasia with zones of degeneration and necrosis (Cone & Odense, 1984), similar to the damage caused by high numbers of *E. hippoglossi.*

5.4.2. *Lepeophtheirus hippoglossi*

5.4.2.1. Parasite Attachment and Distribution

The frontal filament of the chalimus stages of *L. salmonis* and *L. hippoglossi* are very similar. The filament of *L. hippoglossi* was longer than that of corresponding stages of *L. salmonis* possibly due to the differences in the depth of the epidermal layer between the host fish. However, both species have a frontal filament with an external lamina similar to the cuticular substance. Internally there is a fibrous material described as proteinaceous in *L. salmonis* (Bron *et al.*, 1991), although the substance was not identified in this study. In *C. elongatus* the internal structure of the filament is more complex and the fibres are arranged to form helicoids (Pike *et al.*, 1993). Piasecki and Mackinnon (1993) describe the structures of the frontal filaments of *Lepeophtheirus* species as quite different from those of *C. elongatus*, whose proximal end can be used to aid in chalimus stage identification. Both *Lepeophtheirus* species have a tube, the

axial duct running down the centre of the filament. Bron *et al.* (1991), suggested that a glue like secretion is injected down this tube, under the epithelium, to form the basal plate. A basal plate is also present in *L. hippoglossi* and stains differently from the 'stem' of the filament, suggesting that it too may be made up of a different substance. The basal plate in both *Lepeophtheirus* species was eosinophilic and it seems likely that it has a similar composition. In this study and that of Bron *et al.* (1991) the basal plate appears to attached to be the basement membrane, however Paperna (1980) noted that *Caligus minimus* only attached to bony or dental material. The filament of *C. elongatus* is also attached directly to a fish scale and the basal plate is more fibrous than that of either of the *Lepeophtheirus* species. A filament duct has been described from *C. elongatus* but it may not have the same purpose as in *L. salmonis* as no secretions were identified (Pike *et al.*, 1993).

The most favoured area for *L. hippoglossi* copepodid settlement is the fins on the dorsal surface of the host. The thinner epidermis and thinner mucus layer in these areas presenting less of a barrier for the frontal filament to pierce before the basal plate can be secreted. The fins, particularly the pectoral and dorsal, were also found to be a preferred area of attachment for *L. salmonis* (see Bron *et al.*, 1991), *L. pectoralis* (see Boxshall, 1976), *Salmincola salmoneus* (see Kabata & Cousens, 1973), *S. edwardsii* (see Conley & Curtis, 1994), and *Lernaeenicus sprattae* (see Anstensrud & Schram, 1988). Kabata and Cousens (1973) explained the disproportionate number of *S. salmoneus* settling on fins and the operculum as due to currents from fin and respiratory movements attracting the copepodids. Anstensrud and Schram (1988) found that the

copepodids of *Lernaeenicus sprattae* were initially randomly distributed across the body but within 24 hours they were mainly (80%) attached to the fins. They suggested a positive response to water turbulence as part of the stimulus for host location and attachment. Bron *et al.* (1991) also suggested that there may be some 'shelter' from water currents between the fin rays, which may make these areas attractive to the small copepodid stages. Although no data were collected, observations in this study showed that another area favoured by attached stages of *L. hippoglossi* were empty eye sockets, where the halibut eye had been lost, a common occurrence in farmed halibut. Occasionally high densities of chalimus stages could be seen in these hollows that were presumably also sheltered from currents.

There was a large scale movement of *L. hippoglossi* from areas of settlement to those where the motile stages were found. The latter were most commonly found on the head and body of the dorsal surface, especially around the pectoral fin area. This may be due to the thickness of the skin in this area, which might constitute a better area for feeding for the larger motile parasites and an area where attachment will be more secure than on the relatively fragile fins. A similar movement was also seen in *Lernaeenicus sprattae* where the copepodids settle mainly on the fins but the adults are found in the eyes of the sprat (*Sprattus sprattus* L.) and herring (*Clupea harengus* L.). The gravid females move onto the surface of the fish so that free-living larval stages emerging from eggs are shed into the water (Anstensrud & Schram, 1988). Changes in distribution are also noted in *L. salmonis* where the adult females prefer the epidermis adjacent to and posterior to the insertion of the anal fin and along the posterior dorsal midline between

the dorsal and caudal fins (Todd *et al.*, 2000). These authors also suggest that the larger adult females prefer to be attached to areas of skin that are relatively flat in order to attain suction attachment and will aggressively oust smaller males and pre-adult females. They also suggest that in farmed fish, females attached behind fins are protected from abrasion. Males predominated on the head and along the dorsal midline between the head and the dorsal fin (Todd *et al.*, 2000). Motile stages of *L. hippoglossi* also predominate on the flattest parts of the halibut, across the anterior body region, suggesting that these areas are most suitable for suction attachment for the large adult stages. However, it seems unlikely that this is the most important factor for site preference. There are large areas of both salmon (flanks) and halibut (most of the body surface) that is 'flat', however, these areas are not favoured by the parasites. Food availability and shelter from adverse currents to maintain a hold to the host may be more significant factors in site selection.

It should be noted that the data collected from this study was taken only from heavily infected host fish. No data was collected from those fish with less than 10 individual parasites. The distribution patterns of the parasite at different stages of its life cycle may be affected by the density of the parasite population and possibly the presence of previous generations that may remain on the host. It would be interesting to see if the distribution of newly attached copepodids was affected by the presence of adult stages or if heavily infected hosts with only chalimus stages present would be such an attractive host for adult stages without a host.

5.4.2.2. Pathology

The feeding mechanism of caligid copepods is mechanical and utilises multiple (approximately 100) fine and sharp teeth (Kabata, 1974). Multiple layers of epidermal cells can be removed during feeding. When abundant, *L. salmonis* causes serious pathology characterised by extensive areas of skin erosion and haemorrhaging on the head and dorsal surfaces, and a distinct area of erosion and sub-epidermal haemorrhages in the perianal region (Johnson & Albright, 1992). Gross lesions were present on salmon infected with *L. salmonis* on dorsal surfaces of the head, between the dorsal and adipose fins and ventrally behind the anal fin (Jonsdottir *et al.*, 1992). These lesions had grey edges and haemorrhagic centres, with epithelium and scale loss (Jonsdottir *et al.*, 1992). These lesions are very similar to those seen in *L. hippoglossi* infections but they appear in different areas of the host body. The most common areas in which *L. hippoglossi* caused lesions were over the abdominal cavity and adjacent to the anterior dorsal fin, although when the lesion was extensive it would occasionally cover the entire anterior dorsal surface of the fish.

When infection levels were high (25 adult females on a 1.5kg halibut) lesions developed quickly and severe damage occurred in those areas where parasites congregated. Halibut are stocked into cages at approximately 200-300g, much smaller than 1.5kg, therefore it can be assumed that far fewer adult parasites would be needed to cause severe damage to this size of fish. Only small numbers of *L. salmonis* are needed to cause severe damage to salmon smolts (Wootten *et al.*, 1982) and it is likely

that similar levels of infection will severely affect juvenile halibut newly introduced into cages.

5.4.3. Host Skin

The epidermis of Atlantic halibut becomes thicker, on both the dorsal and ventral surfaces, as the animal grows. When considering specific areas of the body surface, the only area where the dorsal epidermis is significantly thicker than the ventral epidermis was in the right anterior area, corresponding to the area over the abdominal cavity. In this area the dorsal epidermis was also thicker than any of the other areas of dorsal epidermis. This may improve protection for the internal organs as there is only a very thin muscle layer over the cavity. The epidermis on the ventral surface of the halibut does not change in thickness across the whole animal. This may indicate that the weight of the fish when at rest on the substrate is distributed evenly across the ventral surface and no one area is particularly stressed due to weight pressure. However, broodstock held in tanks on halibut farms have been reported to suffer from ventral lesions, particularly during the spawning season (P. Smith, pers. comm.). When the ovaries and testes swell, the entire abdominal cavity is distended and the ventral skin is abraded on the tank bottom. At this time of the year the weight distribution may not be so evenly spread over the whole body and therefore the protruding area becomes damaged. In the wild, halibut may rest on softer substrates such as sand or mud, allowing for partial burial, relieving the pressure in specific areas of the body.

The only area of the halibut where there is a significant difference in the number of mucous cells is also in the right anterior area, around the abdominal cavity. The dorsal epidermis contains a larger number of mucous cells in this area than in any other dorsal area and a much greater number than in the corresponding ventral epidermis. Pickering (1974) reported that fins had fewer mucous cells than the body and that, in general, mucous cells were more frequent on the anterior regions of brown trout, *Salmo trutta* L., and char, *Salvelinus alpinus* L. It was suggested that this may ensure an even layer of mucus over the entire animal, as when the fish moves forward the mucus is moved posteriorly (Pickering, 1974). The two areas with highest numbers of mucous cells in halibut were the head and the dorsal right anterior area, corresponding with the pattern reported by Pickering. The number of mucous cells in the 6 areas of ventral skin does not vary as much as in the dorsal. This may be because the ventral surface is in contact with the substrate and an even layer is required across the entire surface to aid in protection against abrasions.

The epidermis of the salmon was much thinner than that of the halibut of a similar age and size. Salmon have large scales and the epidermis will cover and follow the line of the scales (Bullock & Roberts, 1975) whereas halibut have very small scales that may not provide the level of protection acquired by salmon scales and may therefore be at least partly why epidermal thickness is increased. The thickness of the halibut epidermis may be the reason why low numbers (10 individuals) of *L. hippoglossi* and quite high numbers (40+ individuals) of *E. hippoglossi* do not cause severe damage to the host. As discussed earlier it is highly likely that *E. hippoglossi* feed in a similar

manner to *E. soleae*, and partially digest the epidermis before ingestion. This digestion will presumably only affect the top layers of the epidermis and therefore, as the halibut has a very thick epidermal layer it would require a high concentration of *Entobdella* to cause damage to lower layers of the skin.

As few as five adult *L. salmonis* can cause very severe damage to a salmon smolt (Wootten *et al.*, 1982). Kabata (1974) considered that the main cause of damage from caligid copepods is from feeding; the attachment and movement over the host contributing little to the damage caused. The strigil scrapes regular strips of the skin from the surface that are taken into the mouth by the mandibles (Andrade-Salas, 1997). Adult female *L. hippoglossi* have been observed with dark coloured matter within their alimentary canals, this may have been pigmented cells taken up with other epidermal and dermal cells but it is more likely that it was blood. Brandal *et al.* (1976) report that blood is also an important food for *L. salmonis*, particularly for gravid females and to a lesser extent adult males. The feeding mechanism of *L. hippoglossi* and their relatively large sized motile stages will destroy skin very rapidly and thus will lead to the extensive lesions seen in this study.

This study has shown that the health of cultured halibut is at risk from these ectoparasites. Adult *Entobdella hippoglossi* are predominantly on the ventral surface, making it difficult to determine the extent of the infection. Although they have a long generation cycle, they also live for a long time and congregate into small areas as they become sexually mature, increasing the risk of deeper epidermal and dermal lesions.

Lepeophtheirus hippoglossi is visually more evident being present on the dorsal surfaces and therefore slightly easier to diagnose, however, extensive damage can occur relatively quickly if the chalimus stages are allowed to mature. If management practises and novel therapeutic treatments are to be implemented in the control of these parasites it is extremely important that the biology, behaviour and microhabitat of the attached stages of the different parasites be known.

Chapter 6

The study of the efficacy of emamectin benzoate as an oral treatment of *Lepeophtheirus hippoglossi* (Krøyer, 1837), an ectoparasite of Atlantic halibut (*Hippoglossus hippoglossus* L.).

6.1 Introduction

The copepod parasite *Lepeophtheirus salmonis* has caused major economic losses to the salmon farming industry worldwide and has caused damage recently estimated at £30 million in Scotland (G. Rae, pers. comm.). This parasite feeds on the epidermis especially around the head and fins of the salmon and causes lesions and death if found in high enough numbers (Wootten *et al.*, 1982; Jonsdottir *et al.*, 1992). The problem became apparent in the late 1960's in Norway (Brandal *et al.*, 1976) and in the 1970's in Scotland (Wootten *et al.*, 1982). Intensive efforts have been made to find suitable control methods *for L. salmonis*. As a result of the world value of the Atlantic salmon industry, pharmaceutical companies have been encouraged to invest in the search for newer chemotheraputants, which were reviewed by Costello (1993), Roth *et al.* (1993) and Roth (2000).

Bath treatments which contain organophosphates such as dichlorvos (Aquaguard®, Novartis) or azamethiphos (Salmosan®, Novartis), hydrogen peroxide (Salartect®,

Brenntag, Paramove®, Solvay-Interox), synthetic pyrethroids such as cypermethrin (Excis®, Vericore) or deltamethrin (Alphamax®, Alpharma) (Stone *et al.*, 1999) have been developed in recent years.

Organophosphates inhibit the acetyl-choline esterase activity of the cholinergic nervous system and are selective for arthropods (Wootten *et al.*, 1982). Early attempts to treat salmon with sea lice with an oral organophosphate treatment (Grave *et al.*, 1991) were associated with side effects such as blindness (Brandal & Egidius, 1977). These compounds, e.g. Neguvon were later used more successfully as bath treatments (Brandal & Egidius, 1977). Trichlorfon and Dichlorvos became widely used treatments world wide, however only Dichlorvos was licensed in the UK, and had been used for approximately 10 years before toxicity and environmental effects were studied (Costello, 1993). Dichlorvos will degrade to non-toxic products within 4-5 days in well aerated water (Samuelsen, 1987) and does not bioaccumulate (World Health Organisation, 1989). The effect on marine organisms of this chemical is limited to within 25m of the cage site (Murison *et al.*, 1990; Wells *et al.*, 1990; Dobson & Tack, 1991). Trichlorfon is used at high concentrations and degrades to Dichlorvos; in sunny conditions it will degrade at an accelerated rate, resulting in mass mortalities of salmon (Salte *et al.*, 1987; Horseberg *et al.*, 1989) and significant effects on marine fauna (Egidius & Moster, 1987). The use of organophosphates has been restricted in large measure by the public perception of its potential to harm humans and concerns over the environment.

In his review in 1993, Costello stated that synthetic pyrethroids are known to affect the nerve membranes of the lice and have a greater affect on chalimus stages than organophosphates. They have a fast degradation rate and an alternative mode of toxicity to organophosphates, which may be a useful alternative in lice control. Hydrogen peroxide is a powerful oxidising agent and causes bubbles to form within the haemolymph of lice, causing motile stages to float off the host (Thomassen, 1993; Bruno & Raynard, 1994). It has been shown to have some effect on the development of chalimus stages (McAndrew *et al.*, 1998). Because of the high degradation rate it is unlikely to have an effect on marine life out with the cage, however due to its caustic and inflammable properties it is difficult to handle and transport (Costello, 1993). At high temperatures treatment with hydrogen peroxide may cause damage to the gills of fish (Johnson *et al.*, 1993; Kiemer & Black, 1997) and at summer temperatures significant mortalities may occur (Bruno & Raynard, 1994). There is some evidence that *L. salmonis* on farms which repeatedly treat with H_2O_2 are becoming resistant, with only 15-16% reduction of adult females occurring compared to 87-90% reduction in adult females in previously unexposed lice populations (Treasurer *et al.*, 2000).

Bath treatments are stressful to the fish as well as being highly labour intensive and expensive and may not be feasible in adverse weather conditions or on exposed sites (Stone *et al.*, 1999). Furthermore reduced susceptibility has been identified to organophosphates (Jones *et al.*, 1992) and hydrogen peroxide (Treasurer *et al.*, 2000). With the exception of the pyrethroids these bath treatments are only effective against the motile pre-adult and adult stages of the parasite. The chalimus stages are left

behind to mature and continue the infection. This means that the treatments need to be repeated regularly in order to regain control of the parasite (Stone *et al.*, 2000a).

Oral treatments administered in feed have several advantages. Ivermectin (22. 23-dihydroavermectin B_1) was the first oral treatment for sea lice (Davies & Rodger, 2000) but has a low margin of toxicity. It builds up in the salmon muscle and can only be used for fish which will not be marketed for several months e.g. broodstock or newly introduced smolts (Davies & Rodger, 2000). There are also environmental concerns particularly for the benthic environment surrounding fish farms. Ivermectin is not very soluble in water (Halley *et al.*, 1989) but attaches to the sediment and filter feeding organisms e.g. mussels (*Mytilus edulis* L.) bioaccumulate the chemical more effectively than fish species (Davies & Rodger, 2000). Davies *et al.* (1998) concluded that there may be a significant risk to infaunal polychaetes in the sediment below and around fish cages from sediment-associated Ivermectin. Ivermectin has never been the subject of a licence application in Scotland and its use is very strictly regulated.

Diflubenzuron (Lepsidon®) and teflubenzuron (Calicide®) are benzoylephenyl ureas, insect growth inhibitors (Roth, 2000) and have recently been licensed for oral administration to salmonids. These chemicals are chitin synthesis inhibitors and stop the lice moulting successfully (Branson *et al.*, 2000). Teflubenzuron is administered as an in-feed treatment for salmon infected with *L. salmonis* at a rate of 10mg/kg/day for a 7 day period. It has greatest effect upon chalimus and pre-adult stages but does not kill adult stages, therefore it is recommended that treatment is undertaken before the

population matures to adult stages or in conjunction with a treatment effective against these stages (Branson *et al.*, 2000).

Other, non-synthetic techniques have been tried such as light traps, garlic as a feed supplement and onions placed within the cages, however none of these methods have proved to be very successful (Costello, 1993). Using cleaner fish (wrasse) to remove the lice from salmon in cages has had mixed results. In an Irish study Deady *et al.*, (1995) found that corkwing (*Crenilabrus melops* L.) and goldsinny (*Ctenolabrus rupestris* L.) maintained infection levels at below 5 lice per salmon in commercial cages. However, Tully *et al.*, (1996) report that two species of wrasse (*Centrolabrus exoletus* L. and *Ctenolabrus rupestris*) failed to prevent a rapid increase in the number of *C. elongatus* present on salmon in cages although the cleaner fish were feeding on the lice. Wrasse can consume up to 26-46 *L. salmonis* per day from salmon in cages at 10-12°C (Treasurer, 1994).

A novel avermectin, emamectin benzoate (the benzoate salt of 4"-deoxy-4"-epi-(methylamino) avermectin B_1) has been developed for treatment of sea lice of farmed salmonids (Stone *et al.*, 1999; 2000b). It is administered with food as a 0.2% premix (Slice® Aquaculture Premix, Schering-Plough Animal Health) at a dose of 50μgkg^{-1} body weight over a 7 day period, which is effective against immature and mature stages of *L. salmonis* in tank conditions (Stone *et al.*, 1999; 2000b). It is a semi-synthetic avermectin insecticide, developed for pest control in edible plant crops (Lasota & Dybas, 1991). It acts by binding to the glutamate chloride channels, increasing neurone

permeability to chloride ions at invertebrate inhibitory synapses, resulting in paraslysis and death (Roy *et al.*, 2000). Toxicity of emamectin benzoate in fish has been shown to be much lower than to ivermectin (Roy *et al.*, 2000). In marine species, the sheepshead minnow (*Cyprinodon variegatus* Lacepéde, 1803) exposed to emamectin benzoate had a LC_{50} value of 1340μgl^{-1}. In comparison the LC_{50} values for rainbow trout (*Oncorhynchus mykiss*) and bluegill sunfish (*Lepomis macrochirus* Rafinesque, 1819) exposed to ivermectin were 3.0 and 4.8μgl^{-1} respectively (Halley *et al.*, 1989).

Lepeophtheirus hippoglossus has not been reported from farmed Atlantic halibut, however, this study has shown that it can cause haemorrhagic lesions and death to the host (See Chapter 5). The new in feed treatment, SLICE® (0.2% emamectin benzoate) for the salmon louse has recently become available for use in fish against *L. salmonis* and *C. elongatus* on Atlantic salmon and rainbow trout. Since *L. salmonis* and *L. hippoglossi* are very closely related, it was considered to be potentially effective against *L. hippoglossi* on Atlantic halibut. A recommended dose of 50μgkg-1 for salmonids was ascertained by Stone *et al.* (1999) and this dose was used in this study as a preliminary efficacy study.

6.2 Materials and Methods

6.2.1. Source of *Lepeophtheirus hippoglossi.*

Lepeophtheirus hippoglossi were obtained from stock fish held at Machrihanish. The number of parasites was increased to ensure sufficient numbers for infection of trial fish

by infecting ten 1kg+ Atlantic halibut with copepodids. Egg strings were collected from the established population held at Machrihanish. Incubation of the eggs and infection procedures were undertaken in the same way as described in Chapter 4 (paragraph 4.2.1). The parasite population on these halibut was monitored until adult stages were present. Egg strings were harvested from these parasites once 90-100% of the females had produced eggs. The eggs were incubated in plastic containers filled with ambient temperature sea water with an air supply. The development of the eggs was monitored until the copepodid stage was reached.

6.2.2. Treatment Trial

The experimental design is summarised in Figure 6.1.

6.2.2.1. Source and Maintenance of Halibut

Atlantic halibut were obtained from Orkney Marine Hatchery ex-stock from Sea fish Industry Authority, Ardtoe. Eighty four mixed sex halibut, average length 257.1±24.5cm, were acclimated in a 2m round tank with an air supply and constant flow of seawater at ambient temperature and salinity. During the acclimation period, daily ration, fish behaviour and mortalities were recorded. The experiment was not started until normal food intake was resumed. The halibut were starved for a 24 hour period before any procedure was undertaken (i.e. anaesthetising, examining, copepodid challenges). Four of these fish were marked with a single pan-jet mark on the dorsal surface for easy recognition. These fish were used as marker fish that were examined after the infection to monitor the number of copepodids present and were not used for any data recording.

6.2.2.2. Infections

All 84 halibut were held in a single 2m round tank and were infected with copepodids of *L. hippoglossi* by introducing them to the water simultaneously. The water level was reduced, the current was turned off, but the air supply remained on. An average of 59 copepodids per fish were then poured into the water and left for 4 hours before the water current was resumed and the tank allowed to flush. The average water temperature throughout the experimental period was10.6°C (range 9-12°C) and salinity was 33‰.

The following day, the 4 marker fish were anaesthetised using 2-phenoxyethanol (1:1500ml) and examined thoroughly under a microscope to determine the level of infection. A mean of 13.5± 7.1 copepodids was present on these four fish.

This infection process was repeated after 14 days to ensure a range of chalimus and pre-adult stages present on the fish at the time of treatment. Seventy two copepodids per fish were introduced to the water for the second infection resulting in a mean of 16±6.7 copepodids and 5.7±5.9 chalimus stages (mainly chalimus III and IV) present on the marker fish.

6.2.3. Treatment

Twenty one days after the first infection, when there was a range of parasite stages on the fish, the treatment period commenced.

Tank set up

One marker fish was placed into each of four 2m tanks and the 80 remaining fish were divided randomly between these tanks. Small mesh plates were fitted over the outflow pipe in order to retain uneaten food pellets.

Diets

The experimental diet consisted of Trouw UK, marine halibut 5mm pellets (formula code 6793, expiry date 4/7/00) mixed with 0.2% emamectin benzoate premix (batch no. 012949, expiry date 3/01) at a rate of 50μg kg^{-1} then top coated with cod liver oil at a rate of 10ml kg^{-1}. The control diet consisted of pellets from the same batch, top coated with cod liver oil but with no emamectin benzoate present.

Tanks 2.10 and 2.14 were fed the experimental diet and tanks 2.11 and 2.13 were fed the control diet.

Feeding Regime

The halibut were fed for a 9 day period (days 0-8) with either the medicated or the control food. The fish were fed at a rate of 0.5% body weight per day (1.46g per fish per day) and on day 0 the food was given as one feed. Any pellets remaining in the tank after 6 hours were removed, using a siphon. The fish in all 4 tanks fed poorly, possibly due to the lack of an acclimatisation period after the re-distribution of the fish. For the following 5 days the daily ration was split into two equal portions and fed in two feeds to encourage a greater consumption. Any uneaten pellets were collected

twice per day. On days 6,7 and 8 the food was administered in two feeds, uneaten pellets counted and then the number of uneaten pellets was calculated and this number of new pellets was given to the fish as a third feed. This was to try to increase the overall food consumption of the fish in order to ensure that the required dose of emamectin benzoate was consumed.

The dose rate was determined by calculating the number of pellets consumed. After the treatment period all four tanks of fish were returned to standard unmedicated diets fed at 1% of body weight per day.

6.2.4. Evaluation

On days 9,16 and 23 post commencement of treatment fifteen fish were randomly selected from each tank and were examined under a microscope for the presence of lice. On day 30 all remaining fish from each tank were examined in the same manner. The fish were anaesthetised individually and the number of each stage of *L. hippoglossi* recorded. Any lice that fell off the fish in the anaesthetic were also counted and allowed to re-attach to the fish. The fish were allowed to recover from the anaesthetic and returned to their tanks for subsequent sample points, except on day 30 when all fish were culled. On day 30 the fish were re-weighed and measured.

6.2.5. Medication rate

The mean weight of the halibut was calculated to be 292.8g at day 0. The daily ration was calculated to be 30.74g per tank of 21 fish at a feeding rate of 0.5% body weight per day. Eight hundred and thirty grams of finished treated feed were required to ensure that there was a plentiful supply (plus excess) throughout the treatment period. A total of 813.5g of food were treated with 4.1g of premix 0.2% emamectin benzoate powder and 12.4g of cod liver oil to give the correct concentration of the drug - 10ppm of active drug. The control feed was treated with an equal proportion of oil.

A portion of the treated feed was frozen and sent away for chemical analysis to ascertain the exact concentration of drug present. The result of the chemical analysis was 8.86$\mu g\ g^{-1}$ of food, close to the target dose of 10$\mu g\ g^{-1}$.

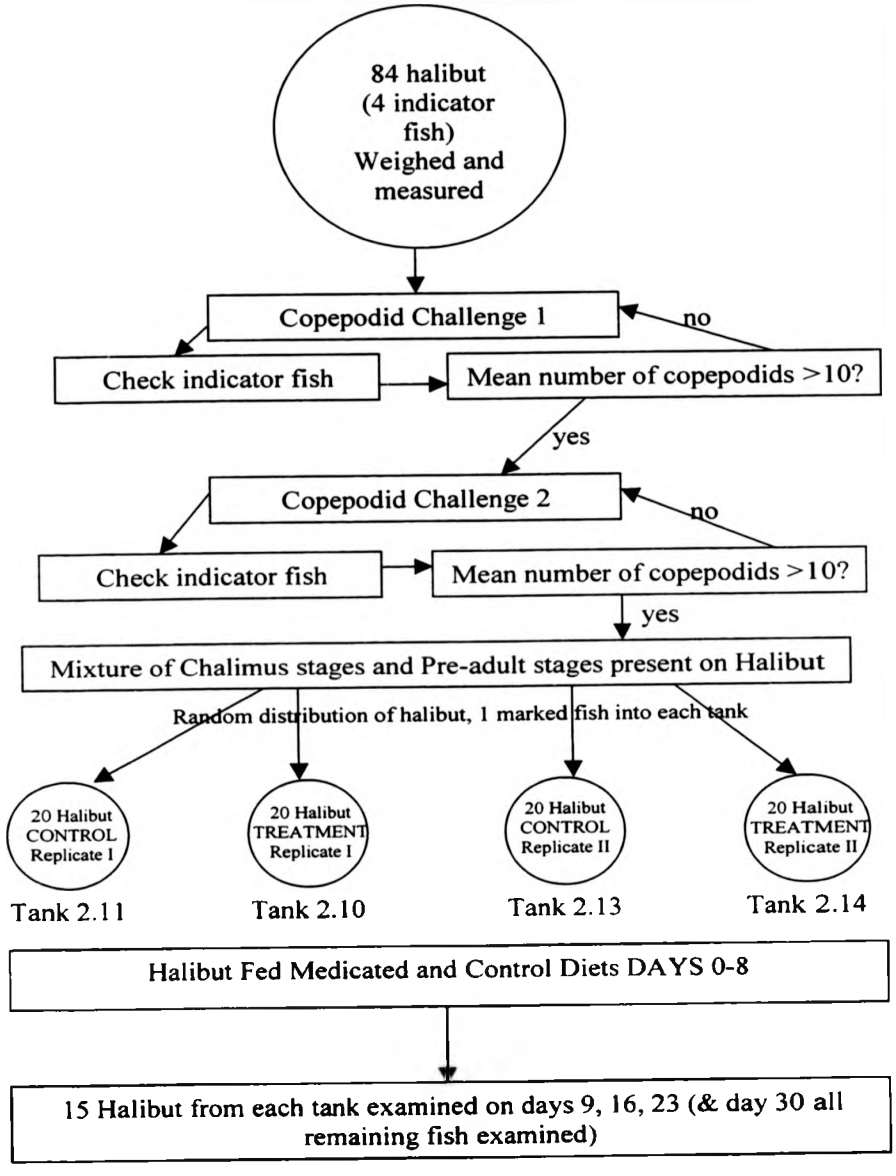


Figure 6. 1. The experimental design for the emamectin benzoate treatment of *Lepeophtheirus hippoglossi* trial.

6.3 Results

6.3.1. Food consumption

Table 6.1 shows that feed consumption ranged from 66.9-75.0% in the control tanks and 55.1-71.5% in the treatment tanks. The actual mean doses, based on the chemical analysis of emamectin benzoate in the food consumed, were 31.7 and 24.4μgkg^{-1} in tanks 2.10 and 2.14 respectively. The nominal dose was 50μgkg^{-1} for each tank.

Tank 2.14 was mistakenly fed with their usual ration of 1% body weight unmedicated food on day 1. This food was removed as soon as the mistake was noticed (less than 1 hour) and the medicated ration was given. It is assumed that the halibut had already fed and were no longer hungry as they ate none of the medicated food on day 1 and a reduced ration on day 2 which reduced the overall consumption rate. This was also the main reason why the medication period was extended by 2 days (recommended treatment period 7 days (Stone *et al.*, 1999)) in order for the drug consumption to be increased.

6.3.2. Body Weight

On day 30 the average fish weight was 290.0±77.1g. There were no significant differences (Mann-Whitney U test: $p=0.4$) in weight between the individual tanks of fish. There were only 2 mortalities throughout the experimental period, both were attributable to high lice numbers. The mortalities occurred in tanks 2.11 and 2.14.

Table 6. 1. Food consumption rate for halibut infected with *L. hippoglossi* throughout the treatment period with emamectin benzoate. *=this tank was fed its usual ration before receiving its medicated ration, therefore the consumption of medicated feed was zero on this day.

Wt of 1 pellet (C) = 0.15g T= treatment C = control
Wt of 1 pellet (T) = 0.16g
No. of pellets per feed (30.74g per tank)(C) = 204.9 per tank
No. of pellets per feed (30.74g per tank)(T) = 188.7 per tank

Day	Tank	Daily		Overall		
		Uneaten pellets	% consumed	Uneaten pellets	% uneaten	% eaten
0	2.11 C	115	43.88			
	2.13 C	103	49.74			
	2.10 T	73	61.31			
	2.14 T	120	36.41			
1	2.11 C	125	39.00			
	2.13 C	98	52.18			
	2.10 T	129	31.64			
	2.14 T *	201	-6.52			
2	2.11 C	81	60.47			
	2.13 C	60	70.72			
	2.10 T	96	49.13			
	2.14 T	124	34.29			
3	2.11 C	82	59.99			
	2.13 C	51	75.11			
	2.10 T	61	67.67			
	2.14 T	80	57.60			
4	2.11 C	61	70.23			
	2.13 C	48	76.58			
	2.10 T	28	85.16			
	2.14 T	61	67.67			
5	2.11 C	61	70.23			
	2.13 C	45	78.04			
	2.10 T	20	89.40			
	2.14 T	63	66.61			
6	2.11 C	37	81.95	562	39.18	60.82
	2.13 C	14	93.17	419	29.21	70.79
	2.10 T	20	89.40	427	29.77	70.23
	2.14 T	52	72.44	701	48.87	51.13
7	2.11 C	21	89.75			
	2.13 C	23	88.78			
	2.10 T	1	99.47			
	2.14 T	24	87.28			
8	2.11 C	27	86.82	610	33.07	66.93
	2.13 C	19	90.73	461	24.99	75.01
	2.10 T	56	70.32	484	28.50	71.50
	2.14 T	38	79.86	763	44.93	55.07

Table 6. 2. Showing the total number, chalimus and motile stages of *L. hippoglossi* with time after a 9 day (day 0-8) treatment period with emamectin benzoate. Data pooled for 2 tanks per dose. Day 9, 16 and 23 n= 30 for day 30 n= 41 per treatment.

Day	Dose (μgkg^{-1})	Mean total lice±S.D.	Mean Chalimus±S.D.	Mean Motile±S.D.	% reduction relative to control (total lice)
9	50	26.27±20.08	17.87±11.78	8.4±14.98	
	0	28.87±24.81	14.9±8.13	13.97±19.9	
					9.0
16	50	16.47±13.18	8.63±8.73	7.83±12.39	
	0	30.4±25.98	0.2±0.4	30.4±26.0	
					45.8
23	50	2.8±2.8	0.9±1.4	2.0±2.7	
	0	27.7±23.9	0	27.7±23.9	
					89.8
30	50	2.3±8.6	0	2.3±8.6	
	0	20.7±27.8	0	20.7±27.8	
					88.9

Table 6.2 shows that on the first sample days there were no significant differences between the total number of parasites found on the treatment and control groups (Mann-Whitney U test, p=0.82 and 0.13 on days 9 and 16 respectively). However, if the data is analysed separately for chalimus and motile stages on day 9 there were significantly fewer motile stages present in the treated group ($p<0.05$) but there was no significant difference between the number of chalimus stages (p=0.45). On day 16 the difference in both the number of chalimus and motile stages was significant ($p<0.0001$ and p=0.0002 respectively), there being a reduced number of parasites on treated fish. On days 23 and 30 there were significantly fewer total lice on the treated fish than on the control fish ($p<0.0001$ for both). On day 23 there were no chalimus stages remaining on the control fish and by day 30 there were no chalimus stages on any of the halibut.

6.2.3. Efficacy of emamectin benzoate

On day 9 post commencement of treatment there was a 9.0% reduction in the total number of lice on the treated fish, but in only 7 days this had risen to a 45.8% reduction on day 16. After 30 days there was a reduction of 88.9% of total lice on the treated fish compared with the control fish.

From the difference in efficacy between day 23 and day 30 (89.9-88.9%) it would appear that the efficacy of the drug was starting to decline by day 30. However this may not be the case. On day 30 there were fewer parasites on the control fish (20.7±27.8 parasites per fish) than there had been on day 23 (27.7±23.9 parasites per fish). This was probably due to natural loss of the existing population and no re-infection as this is not possible due to the tank conditions. There were also fewer parasites present on day 30 (2.3±8.6) than on day 23 (2.8±2.8) in the treatment tanks showing that either the drug was still having an effect up to day 30 or that there was also a natural loss in the treatment tanks. Due to the lower reduction rate of total numbers on day 30 in the treatment tanks (Table 6.2) the raw data was re-examined. It showed that in treatment tank 2.14 there were two individual fish which had much higher numbers of parasites (14 and 54 parasites) than the other halibut in the tank (average on remainder =0.8 parasites). On day 23 there was also one halibut which had 14 parasites present, the mean number on the other 29 fish was 2.4 parasites. It seems likely that these fish had not fed well during the treatment period and therefore had not consumed an effective dose of the emamectin benzoate. The graphs in Figure 6.2 show the number of the fish with actual numbers of parasites in both the control and

treatment tanks. If the fish with more than 10 parasites (1 fish on day 23 and 2 fish from day 30) are removed from the data for the treatment tanks and the percentage reduction in relation to the control fish is re-calculated, then the efficacy of emamectin benzoate is 91.4% on day 23 and 96.5% on day 30 for the pooled data.

In the control tanks the number of motile stages increased throughout the sampling time due to the chalimus stages developing (Table 6.2). However, in the treatment tanks the numbers of motile lice decreased to as low as 0.9 per fish on day 30 because the chalimus stages were being killed and not developing into motile stages. The number of total chalimus stages decreased very rapidly from 447 to just 6 over the seven day period between sample days 9 and 16 in the control tanks. However in the treatment tanks the number declined more slowly from 536 to 26 between days 9 and 23, presumably due to a reduced development rate caused by the drug.

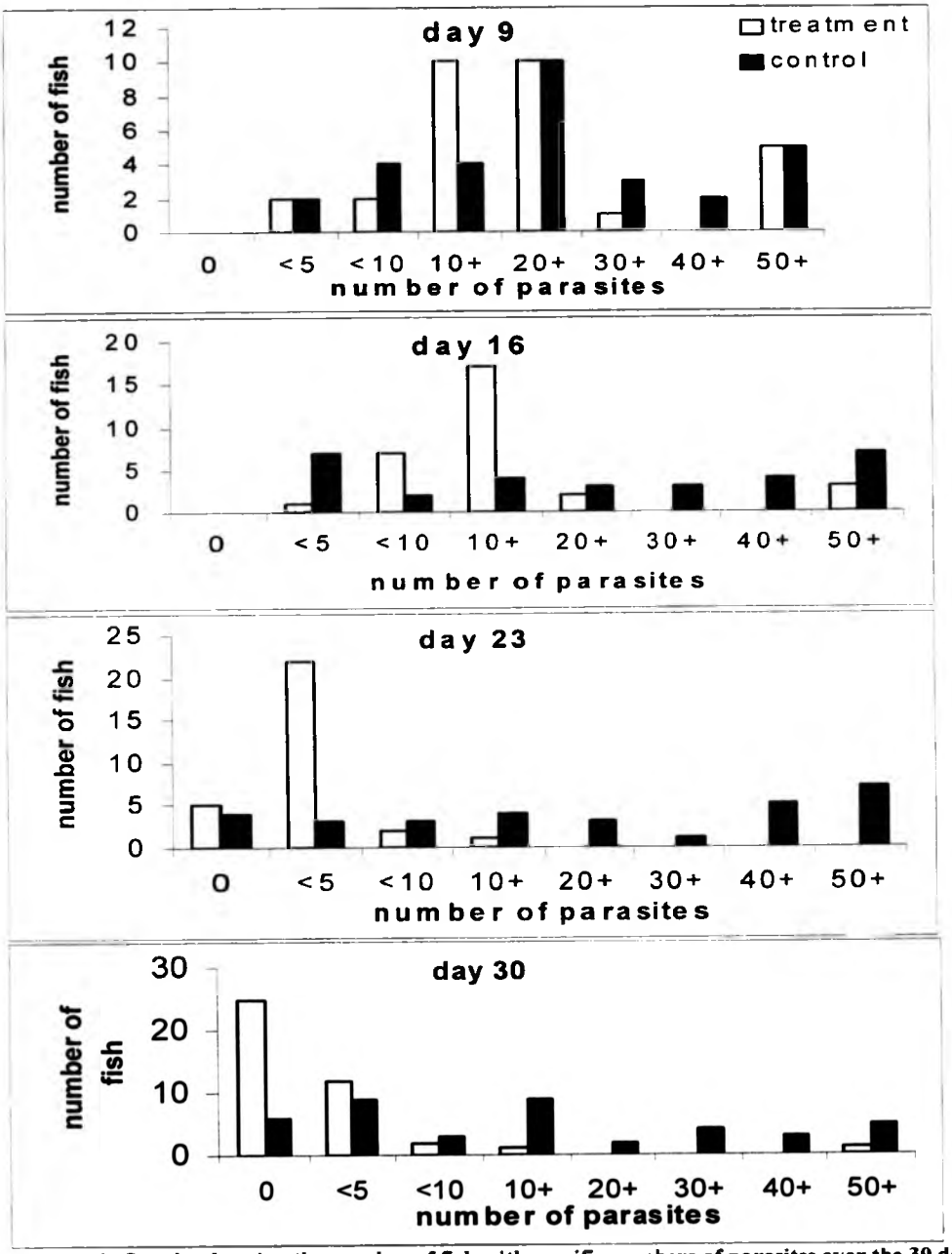


Figure 6. 2. Graphs showing the number of fish with specific numbers of parasites over the 30 day sampling period. Days 9, 16 and 23 n=30 and day 30 n=41 for each treatment group.

6.4 Discussion

In all published trials using emamectin benzoate as a treatment for fish, the feed has been administered for a period of 7 days (Stone *et al.*, 1999; 2000 a; b; c; Roy *et al.*, 2000; Armstrong *et al.*, 2000). In this study it was decided that the treatment period should be extended due to the low feeding rate of the halibut (51.1-70.2% on day 6) compared to those of the salmonid studies e.g. 81-92% (Stone *et al.*, 1999). Therefore in the present study the treatment period was extended for two more days and the feed regime was changed slightly to increase the chances of an efficacious dose being administered. On day 8 (final treatment day) the halibut in the treatment tanks had increased their intake slightly to 71.5 and 55.1% of their feed. The fish in tank 2.10 consumed 31.7μgkg^{-1} and in tank 2.14 they consumed 24.4μgkg^{-1} of the 50μgkg^{-1} nominal dose.

Considering this comparatively low consumption rate the oral administration of emamectin benzoate for the treatment of *L. hippoglossi* on Atlantic halibut was highly effective with up to 96.5% reduction in total lice numbers. This is comparable to the 94.6% reduction of numbers of *L. salmonis* on salmon (Stone *et al.*, 1999) with the same nominal dose rate of emamectin benzoate (50μgkg^{-1}). The salmon in their study had a dose consumption of 91.4%, which is much higher than that of the halibut which only consumed a mean of 63.3% (actual mean consumption was only 56.1% when calculated using the actual concentration of emamectin benzoate (8.86$\mu g\ g^{-1}$) on the feed). This suggests that halibut may need to consume a lower dose of emamectin benzoate than salmon to have a similar effect upon their copepod parasites. In this

study the optimum therapeutic dose for salmon was used (50μgkg^{-1}) (Stone *et al.*, 1999), however the most effective dose for halibut is unknown. Future dose ranging studies could be designed to ascertain the most efficacious dose for the treatment of *L. hippoglossi* on Atlantic halibut.

Chalimus stages were present for a longer period in the treated tanks (up to day 23), this is due to the emamectin benzoate reducing their ability to develop further. Similar patterns were also found in trials for *L. salmonis* (Stone *et al.*, 1999). This leads to a marked decrease in the number of the more damaging motile stages becoming present on the fish. Even on day 9 post treatment there was a significantly lower number of motile stages present on the halibut in the treated tanks. This reduction in the development of the motile stages reduces the chances of the fish becoming damaged by the parasite and therefore reduces stress to the fish. One of the other most beneficial points of the application of emamectin benzoate, as an in-feed treatment, is that there is none of the stress caused to the fish associated with bath treatments, thus further reducing the stressful effects of lice infection.

In a tolerance test, halibut were fed 0, 100 and 250μgkg^{-1} (0, x2 and x5 recommended dose for salmonids (Stone *et al.*, 1999)). Feed consumption was high in all groups (73.5-82.8%) and no differences in behaviour, co-ordination or feeding patterns were observed throughout the 7 day feeding period (Roy, unpublished data). In a similar tolerance study in salmon (*Salmo salar*), fish in the high dose group (500μgkg^{-1})

became lethargic, darker in colour and after 8 days went off their feed (Roy *et al.*, 2000).

After the tolerance test feeding period, the halibut were killed and then examined; no differences were observed in the number of halibut with food in the gut between the control and the treated fish (Roy, unpublished data). This would suggest that there are no palatability problems with the drug, when administered in this fashion, presuming that the halibut are feeding normally at the outset of the treatment period. The fish with high numbers of *L. hippoglossi* in the treatment tanks in the present study may not have been feeding normally before the trial therefore were not representative of the whole population.

Chapter 7

Summary and Discussion

This study has provided new information about two important ectoparasites which pose a potential threat to halibut farming. *Entobdella hippoglossi* is already present in a number of farms in Scotland, Norway and Iceland, mainly on wild caught halibut, presumably infected with the parasite when brought into the farms as broodstock. This study has shown that *E. hippoglossi* has a long life span of at least 146 days as an adult. It can reach up to 21mm in length and can continue to grow once sexual maturity is reached, at 9-10mm in length, and as it grows the number of eggs produced increases. This has also been shown to be the case for a number of other monogenean parasites such as *E. soleae* (see Kearn, 1985) and *Anoplodiscus cirrusspiralis* (see West and Roubal, 1998a). It is unclear whether the number of eggs produced begins to decline with senescence, as was shown for *A. cirrusspiralis* (see West and Roubal, 1998a). Infection parameters for oncomiracidia were not elucidated in this study and more work is necessary to understand the behaviour of the larval stage. Young *Entobdella* were observed 85-93 days (temperature dependent at 11 and 8°C, respectively) after the fish were infected with adult parasites. This period allows for eggs to be laid, develop, hatch, attachment to take place and the young *Entobdella* to reach 2-3mm in length. The exact timing for each of the individual stages was not elucidated. However as this study has shown that the adults live for at least 146 days, the full lifespan of an *Entobdella hippolossi* could be approximately 250 days.

Adult parasites produced a mean of 186 eggs over a 24 hour period *in vitro*, however initial studies suggest that fewer are laid when the parasites remain attached to the host. Eggs produced *in vitro* hatched after 27 days at 12°C and up to 54 days at 5°C, corresponding to the findings of Kearn (1974a) and indicating that this species has a long egg development period which could be a target for future control methods. More studies need to be undertaken to understand the intricacies of egg production and egg viability. For example, it is unknown whether eggs would settle or become entangled within the cage or on the seabed to develop, or perhaps both. If the eggs are to be targets for control measures this information is important. It is relatively easy to count how many eggs are produced by *E. hippoglossi in vitro* but this does not give an accurate estimate of reproductive output *in vivo*. It would also be helpful to know if there are triggers for hatching and if these could be manipulated in order to reduce re-infection rates. Kearn (1974a) suggested that when eggs are incubated at 7°C in 12:12 hours of dim blue light: darkness, hatching occurs within the first few hours of darkness. It is unclear whether high levels of daylight would be harmful to the eggs, however it can be assumed that within a cage there would be higher levels of light than in their natural habitat. Very little is known about the host finding behaviour of the oncomiracidia which would also be of interest to study. In their natural environment the light levels are very low and it seems unlikely that shadow response or other visual signals would be their main stimulus. Chemical signals may be given off by the fish mucus and detected by the oncomiracidia or they may be sensitive to localised water currents caused by halibut swimming near by.

Although *E. hippoglossi* does not cause such deep penetrative and severe lesions as *Lepeophtheirus hippoglossi* it does cause significant skin haemorrhaging, particularly on the ventral surface. Such skin damage may cause osmoregulatory problems and also leaves the host open to potential secondary bacterial infections, which may be severe. It is important for farmers to be aware of the early signs of infection and to have a quick and effective treatment plan. At present the most common treatment method for *Entobdella* in tanks is a formalin bath. This appears to be quite effective against the attached stages of the parasite but does not appear to have much effect on the eggs remaining in the tanks (Svendsen & Haug, 1991). The treatment regime devised using life-cycle information generated in this study (see chapter 2) will help to reduce the number of eggs over time within the tank thus reducing the re-infection rate. This is a simple yet effective way for the farmers to use their existing control method more effectively. *Entobdella* eggs are very well protected by their tanned shell; any method of rupturing the shell or increasing the permeability of the shell may be an effective control method. A different approach may be necessary for control of *Entobdella* in cage sites. Formalin baths are very labour intensive and dangerous to administer in cages, and formalin is toxic to the environment. More efficient and environmentally friendly applications need to be developed in order to control *Entobdella* effectively in such situations. Vaccines or in-feed treatments may be a more effective way to treat large numbers of halibut in sea cages.

Only the adult stages of *Lepeophtheirus hippoglossi* have been described in the literature (Kabata, 1979) and it has been known as a common parasite on wild halibut

(Schram and Haug, 1988). Although it has not been found in halibut farms to date, as it is so closely related to the salmon louse *L. salmonis* it was thought to be a considerable potential threat to the industry and thus worthy of study to gain as much information about its biology as possible. The life cycle and the morphology of the immature stages of this parasite were unknown prior to this study. This work has demonstrated that it has the typical life stages of the genus *Lepeophtheirus*, two nauplius stages, one infective copepodid stage, four attached chalimus stages, two motile, sexually dimorphic pre-adult stages followed by the adult. The morphology was similar to that of *L. salmonis* (Johnson & Albright, 1991a), however the most obvious differences were the bifid tines on the sternal furca of *L. hippoglossi* and the shortness of the adult female abdomen. In general all stages of *L. hippoglossi* were slightly larger, than *L. salmonis* and the free living stages had reddish pigmentation. The female stages developed at a slower rate than the male at all experimental temperatures, following the pattern of *L. salmonis* (see Johnson & Albright, 1991b) and *L. pectoralis* (see Boxshall, 1974c). Eggs were produced as paired egg strings and fewer eggs were produced in the first batch (189 eggs) than in later batches (309 eggs). Fewer eggs were produced per batch than *L. salmonis* with 282.18 in the first batch and 432.8 in the second batch (Gravil, 1996). Infective copepodid stages were produced 7-10 days (temperature dependent) after eggs were removed from the female lice. The effect of temperature and salinity on hatch success and larval behaviour was not studied, but these factors will be important in successful transmission of the parasite. It is known that *L. salmonis* nauplii and copepodids have a positive photoresponse and a very strong shadow response (Gravil, 1996). However this has been untested for *L. hippoglossi* and

due to the difference in the ecology of the two host fish species in the wild, it may be that the free-swimming stages of *L. hippoglossi* have different behavioural responses from those of *L. salmonis*.

The present studies on the *L. hippoglossi* life cycle have been undertaken at ambient Scottish water temperatures (range 6-15°C) and salinities (range 30-34‰). No work has been done to establish the maximum and minimum temperatures at which development can occur or what effect changed salinity would have on development, fecundity or behaviour. These factors would be important in determining the transmission success of *L. hippoglossi* in cages. Many environmental parameters, in addition to temperature and salinity, and including UV and light levels and water movement would show much greater variation than the parasite would encounter under natural conditions. These factors alone may be enough to prevent *L. hippoglossi* outbreaks, with their greatest effect probably upon egg development and hatching. For *L. salmonis,* salinity and temperature have a great effect on the hatch success of the eggs (Gravil, 1996). In summer conditions in northern Europe coastal water temperatures may become too high for successful egg development for *L. hippoglossi*, given the cold water habitat of their host and thus the parasite may be a greater threat to halibut in cages over the winter months when temperatures are closer to those found in the halibut's natural environment. However the results presented here were collected under conditions comparable to those in Scottish cage and land-based farm sites and are therefore particularly relevant to the British halibut industry.

There have been no reports to date of skin lesions caused by *L. hippoglossi* in wild fish. This study has shown that relatively low numbers (25 individuals) of the adult and pre-adult stages of *L. hippoglossi* have the potential to cause severe damage to relatively small halibut (1-1.5kg, within the on-growing size range), occasionally leading to death. Young halibut are put out into cages at around 200-300g, so such fish will be delicate and very susceptible to damage even by small numbers of copepods. Wootten *et al.* (1982) reported that as few as 5 adult *L. salmonis* caused severe pathology to salmon smolts newly introduced to sea water. Halibut as small as 50g were infected with *L. hippoglossi* in this study and full development to the adult stage occurred. Schram and Haug (1988) reported than no juvenile halibut were infected with this parasite, however they suggested that this was due to the solitary habits of juvenile halibut and that they only become infected when they congregate at the spawning grounds.

Initial attachment of both parasites occurred in areas other than those occupied by the larger, adult stages. Juvenile stages of *E. hippoglossi* attached mainly to the tail and the dorsal surfaces and *L. hippoglossi* copepodids attached to the fins and skin adjacent to the fins. The adult stages of both *L. hippoglossi* and *E. hippoglossi* preferred the anterior part of the body and head of the host, whilst *Entobdella* preferred the ventral surface and *Lepeophtheirus* preferred the dorsal surface. The smaller stages tended to be found on areas where the skin was thinner, particularly the fins and tail where there were fewer mucous cells. Adult stages preferred those areas with thick epidermal layers and higher densities of mucous cells. As both parasites feed on epidermal tissue

and mucus there was a greater food supply for the adults in these areas. There may also be some shelter from water currents in the areas where the smaller stages occur thus reducing their risk of being washed away during the initial stages of attachment. Kearn (1988a) found a similar migration pattern occurred in *E. soleae* and suggested that the adults congregated on the ventral surfaces to increase the chances of finding a mate and to deposit eggs into the sandy substrate where the host rested. Copepodids of *L. salmonis* preferred to attach to dorsal and pectoral fins whereas the adults were found most often around the head and mid-line adjacent to fins (Bron *et al.*, 1991). This migration pattern may provide better attachment sites for motile stages as well as an improved food source (Todd *et al.*, 2000).

It is likely that a chemotherapeutant will be needed for *L. hippoglossi* in halibut cage sites. This study tested the efficacy of emamectin benzoate (SLICE) at a dose of 50mgkg^{-1} against *L. hippoglossi* on halibut held in tank conditions. SLICE is currently licensed for use in salmonid farming and, as an in-feed treatment, it has advantages over the more traditional chemicals, most of which are administered as bath treatments. Dosing and administration were simple, and on a farm scale the workload would be much reduced, compared to labour intensive bath treatments. The main benefit of SLICE is that it acts against all stages of the parasite and can be employed in the early stages of an infection thus greatly reducing the likelihood that the larger and more damaging motile stages will develop. The main potential problem associated with SLICE, or any other in-feed treatment is that it is difficult to ensure that all the fish in a cage receive the correct dose of feed. Any fish that are not medicated sufficiently will

act as reservoirs of the parasite and re-infection may occur on a wide scale. Nonetheless, SLICE would seem to be a most suitable therapeutant for *L. hippoglossi*.

Parasite problems are not yet severe in halibut farming mainly because a) very few halibut farms exist and b) most of the culture procedure is undertaken on land in tanks. Fish that are on-grown in tanks are less likely to come into contact with the infective stages of the parasites as their water supply is filtered. As the halibut farming industry grows and more fish are produced and put to sea in cages the likelihood of parasitic infections will increase. Fish in cages are susceptible to any pathogens that may be present in the water column. In the inshore waters surrounding the west coast of Scotland there are few wild halibut, therefore the threat from halibut specific parasites such as *E. hippoglossi* and *L. hippoglossi* is probably low unless they are introduced, e.g. from wild caught halibut being recruited as broodstock or perhaps from escapees from neighbouring cage sites. However, non-host specific parasites, such as *Trichodina* species and *Caligus elongatu,s* may be a threat to halibut culture. Both have been reported on halibut and are known to cause damage to other fish species under culture conditions. At high intensities *C. elongatus* has resulted in death of salmon smolts and has been associated with outbreaks of vibriosis in Scottish sea cage sites (Wootten *et al.*, 1982). *Trichodina hippoglossi* has been described from a Norwegian halibut farm (Nilsen, 1995) and reported to cause a greyish skin colour and skin haemorrhages. This parasite was only found to proliferate after a period of high water temperatures (18°C). Trichodinid infections have also been reported in other marine aquaculture species, such as turbot and cod (Nilsen, 1995).

In other areas where halibut culture may be established such as around Shetland and Orkney, Iceland, Canada and Norway extensive wild halibut populations are present and therefore halibut specific parasites such as *Entobdella hippoglossi* and *Lepeophtheirus hippoglossi* may become a real threat to halibut on-growing sites.

For both *Entobdella* and *Lepeophtheirus* a long-term strategy for control may be a vaccine. Development of a vaccine for *L. salmonis* has met with little success, however as techniques advance and more is known about fish immunology the chances of success will improve. It is believed that halibut have a much more highly developed immune system than salmonids (Bricknell *et al.*, 1999; 2000) and it may be that vaccines will be easier to develop for this species. An oral vaccine would be ideal since it would reduce the handling stress caused when immunising large numbers of fish (Ellis, 1988). As concerns grow about the environmental implications of many chemical treatments and as resistance of parasites develops, a vaccine is potentially the most efficacious long-term route to reducing parasite burdens. Prototype vaccines against *L. salmonis* targeted intrinsic function proteins or the structural apparatus of the gut of the louse (Jenkins *et al.*, 1993). Other areas that may be useful targets could be the reproductive organs, reducing egg or spermatophore production or against the larval filament, thus preventing successful attachment.

This study has shown that there is significant potential for parasitic disease in Atlantic halibut farms. Farmers need to be aware of the potential dangers and need the means to

control outbreaks as and when they occur. With the co-operation of the halibut industry, this project has increased the awareness of *Entobdella hippoglossi* and *Lepeophtheirus hippoglossi* that could have an economic effect on the industry. It has provided farmers and fish health workers with detailed morphological descriptions of *L. hippoglossi* at each developmental stage so that identification can be rapid and precise. Life cycles, egg production, longevity, pathology have been studied for both parasites and potential control methods have been discussed. Emamectin benzoate has been evaluated as a treatment and it has been shown that it is equally effective against *L. hippoglossi* as against *L. salmonis*. Posters giving details of egg production and development, life-span and timing of the life-cycle of *E. hippoglossi* have been produced and circulated to the industry to enable them to time treatment strategies for the maximum effect.

This study has provided extensive data on two parasites with the potential to cause significant problems in commercial halibut culture. Such data will assist in the development of control strategies should the need arise.

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Stage of Staining Technique	Time
Xylene (dewaxing)	5 min
Absolute Alcohol I	2 min
Methylated spirit	1 1/2 min
Wash in tap water	
Mayers Haematoxylin	5 min
Wash in tap water	
1% Acid alcohol	3 quick dips
Wash in tap water	
Scott's tap water substitute	1 min
Wash in water	
Eosin	5 min
Wash in tap water	
Methylated spirit	30 sec
Absolute alcohol II	2 min
Absolute alcohol I	1 1/2 min
Xylene (clearing)	5 min
Xylene (coverslip)	

Appendix 1. The haematoxylin and eosin (H&E) staining procedure for wax embedded sections.

Staining Technique Process	Time
Xylene	5 min
100% Ethanol	2 min
Methylated Spirit	1.5 min
Rinse in Tap Water	
Oxidise in 1% periodic acid	5 min
Wash in running tap water	2 min
Place in Schiff's reagent	10-20 min
Wash in running tap water	2 min
Counterstain with Mayers haematoxylin	5 min
Rinse in tap water	
Differentiate in 1% acid alcohol	2-3 quick dips
Rinse in tap water	
Place in Scotts tap water substitute	1 min
Rinse in tap water	
Dehydrate rapidly through 100% ethanol	
Xylene (Clear)	5 min
Xylene (mount)	
Coverslip using pertex	

Appendix 2. The PAS staining technique for mucins. PAS positive shows as red or magenta, nuclei stain blue.