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FRONTAL FILAMENT MORPHOGENESIS IN THE SALMON LOUSE *LEPEOPHTHEIRUS SALMONIS*

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Anatomy & Physiology

Faculty of Veterinary Medicine

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ABSTRACT

The objective of the present study was to understand how and when the frontal filament (FF) in the salmon louse *Lepeophtheirus salmonis* is produced by examining the sequence of morphological changes leading to FF production in the copepodid and early chalimus stages.

Atlantic salmon (*Salmo salar*) were heavily infested with 8 day post-hatch copepodids. Sea lice were sampled prior to infestation and at 1, 2, 3, 4, 5, 6, 7, 8, and 9 days post-infestation (dpi). FF morphogenesis from late copepodid to chalimus II (ie. through 2 moults) was studied using high resolution light microscopy of serial transverse and sagittal resin sections. Some histochemistry of the FF was determined using periodic acid-Schiff (PAS) and alcian blue staining. Previous studies by others have shown the FF to consist of a basal plate, stem and external lamina. Three groups of cells, identified as A, B and C, are thought to be involved in the production of the secretions (S1 and S2) which form the filament material. At 0 and 1 dpi, cell groups A and B were evident, but there was no evidence of external or internal FF material. A duct passed anteriorly through the ventral portion of the B-cell group extending to a small pore through the cuticle at the anterior margin in the vicinity of the rostrum. The S1 material was first observed at 2 dpi. B-cells had structures resembling microvilli in contact with the S1 material. The second type of internal filament material (S2) was first observed at 3 dpi in close association with the C-group by dark staining cytoplasmic extensions. The amount and shape of the S1 and S2 changed with development. At 4 dpi 25% of the copepodids had completed their moult to chalimus I stage. The FF had a pale staining basal plate and dark staining filamentous material in the stem. In these recently moulted lice there was no internal filament material. At 5 dpi new internal filament material began to be produced to form the next filament, which was extruded at the moult to chalimus II at 9 dpi.

The following scenario for FF developmental is proposed: the first secretion to form after the moult for both copepodid and chalimus stages is the S1 which is formed by B-group cells and becomes the basal plate of the external FF. C-group cells produce S2 during mid-intermoult to premoult stage. The S2 becomes the stem of the external FF. The S1 and S2 were contained within a cuticle lined invagination and had a form similar to that of the extruded filament. The axial duct present in both copepodid and chalimus originates from the A-group cells and probably carries a secretion used to attach the filament to the host. This study provides strong evidence that *L. salmonis* produces a new filament with each moult. This fact makes the possibility of using a sea lice control method based on interference with filament production more feasible.

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DEDICATION

I dedicate this work to my family, especially my wife Yémile, and to the memory of my father in law Manuel Melchor Flores Gómez, whose support has helped me from the start. Thank you Don Manuel.

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A	A-group of cells
A1	Lobe 1 of group-A cells
A2	Lobe 2 of group-A cells
AD	Axial duct
B	B-group of cells
B1	Major portion of group-B cells
B2	Minor portion of group-B cells
BP	Basal plate
C	C-group of cells
CD	Collecting ducts
°C	Degrees Celsius
cDNA	Complementary deoxyribonucleic acid
CL	Cuticle
d	Days
D	Duct
DD	Degree day
DDPI	Degree days post infestation
DOPA	Dihydroxyphenyl - L - alanine
dpi	Days post infestation
E	Eye
EC	External cuticle
EL	External lamina
EP	Eye pigment
FF	Frontal filament
FM	Fibrous material
FS	Fish scale
h	Hours
IMB	Institute for Marine Biosciences
IPN	Infectious pancreatic necrosis virus
ISA	Infectious salmon anemia virus
l	Litre
L	Lens
Lu	Lumen of the duct
MAX	Maximum temperature reached per day
MIN	Minimum temperature reached per day
min	Minutes
μm	Micrometres
N	Number of days post infestation
NC	New cuticle
N1	Nauplius 1
N2	Nauplius 2

NOF	Never-on-fish
n	Number of samples
OC	Old cuticle
P	Pigment
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PR	Proximal region
PO	Pore
ppt	Parts per thousand
R	Rostrum
S1	Probable secretion of group B cells
S2	Probable secretion of group C cells
SA	Second antenna
spp	Species
SD	Standard deviation
ST	Stem
T	Temperature
TEM	Transmission electron microscopy
T_{mean}	Mean temperature
UV	Ultraviolet
x	Times

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1. INTRODUCTION

1.1. Sea lice.

Sea lice is the name given to numerous species of marine ectoparasites belonging to the phylum Arthropoda, class Crustacea and to the order Copepoda. Of these species, members of the family Caligidae are the most important with respect to causing disease in aquaculture (1). Only in rare cases have epizootics of sea lice been reported in the wild (2).

The feeding and attachment activities of sea lice result in the formation of lesions. These lesions range in severity from minor skin discoloration to large open lesions that expose the musculature or the brain. Severe cases of host tissue damage in wild and farmed salmonid stocks have been reported, including lesions that consist of complete removal of the skin from the heads and dorsal surface and hemorrhages of the perianal region. The lesions can result in secondary fungal and bacterial infections as well as osmotic problems (3,4).

1.2. Sea lice species of importance to salmonid aquaculture.

Sea lice species belonging to the Caligidae family that have been reported from farm-reared fish in the Northern Hemisphere are *Caligus clemensi*, *C. elongatus*, *Lepeophtheirus salmonis* and *L. cuneifer* (5-11). Of these species *L. salmonis* is limited in its host range to salmonids, whereas the other species have broad host ranges (9). Of the salmonid species, Atlantic salmon (*Salmo salar)* is the most susceptible to be

infested by *L. salmonis* (12).

With respect to salmon disease, *L. salmonis* has had the greatest economic impact. This species is distributed throughout the Northern Hemisphere and has been reported to occur on most species within the genera *Salmo, Oncorhynchus* and Salvenius (9,13) (Table 1.1). Only rarely has this species been reported on non-salmonid fish (13,14). Another *Lepeophtheirus* species, *L. cuneifer,* is known to occasionally infect salmonids. It is known to parasitize seawater-reared rainbow trout *(Oncorhynchus mykiss)* and Atlantic salmon in the west coast of Canada and is not considered to be an economically important parasite in salmon farming (11,13,14). *Caligus elongatus* and *C. clemensi* have broader ranges with *C. elongatus* having been reported on approximately 80 fish species in Atlantic waters and *C. clemensi* on 37 species in Pacific waters (7,9). Table 1.1. summarizes the salmonid species parasitized by sea lice in Canada.

Sea lice infestations of *L. salmonis* became a problem on farmed salmon in Norway in the 1960's (7), followed by Scotland and Ireland in the late 1970's and on the west and east coasts of Canada in the mid to late 1980's (15). In the fall of 1994, disease caused by *L. salmonis* occurred on many farms in the Bay of Fundy region, resulting in $20 million in losses to the aquaculture industry in 1995 (12,16).

1.3. General life-cycle of *L. salmonis*.

The life cycle of caligid copepods described by Kabata (17) is comprised of five phases and ten stages. There are two free-swimming nauplius stages, one

Table 1.1. Salmonid species parasitized by sea lice on the west and east coasts of Canada (9).

Common name	Salmonid species	Lice species
Coastal cutthroat	*Oncorhynchus clarki*	*L. salmonis*
Pink salmon	*O. gorbuscha*	*L. salmonis, C. clemensi*
Chum salmon	*O. keta*	*L. salmonis, C. clemensi*
Coho salmon	*O. kisutch*	*L. salmonis, C. clemensi*
Rainbow trout	*O. mykiss*	*L. salmonis, C.elongatus, L. cuneifer*
Sockeye salmon	*O. nerka*	*L. salmonis, C. clemensi*
Chinook salmon	*O. tshawytscha*	*L. salmonis, C. clemensi*
Brook trout	*Salvelinus fontinalis*	*C. elongatus*
Brown trout	*Salmo trutta*	*C. elongatus*
Atlantic salmon	*S. salar*	*L. salmonis, C. elongatus, C. curtus, L. cuneifer*

free-swimming infective copepodid stage, four chalimus stages attached to the host by a frontal filament (FF), two pre-adult stages, and one adult stage.

The nauplius and some chalimus stages were described by Johannessen (18) and White (19), respectively. Johnson and Albright (20) provided the first detailed description of all of the developmental stages of *L. salmonis* from the Pacific coast. Further descriptions of *L. salmonis* from the Atlantic coast have been reported by Schram (21). The life cycle of *L. salmonis* is depicted in Figure 1.1.

During the four chalimus stages, copepods are attached to the host by means of a FF. These four stages take about 40 % of the development time from copepodid to adult (22). The FF is used as an attachment device during a major portion of the life cycle of *L. salmonis.*

Filament production also occurs during the preadult stage in *L. salmonis* and other species, including *L. pectoralis, L. hospitalis, C. spinosus* and *L. dissimulatus* (20). These filaments are believed to help the preadult stage remain on the host during the moulting process (20).

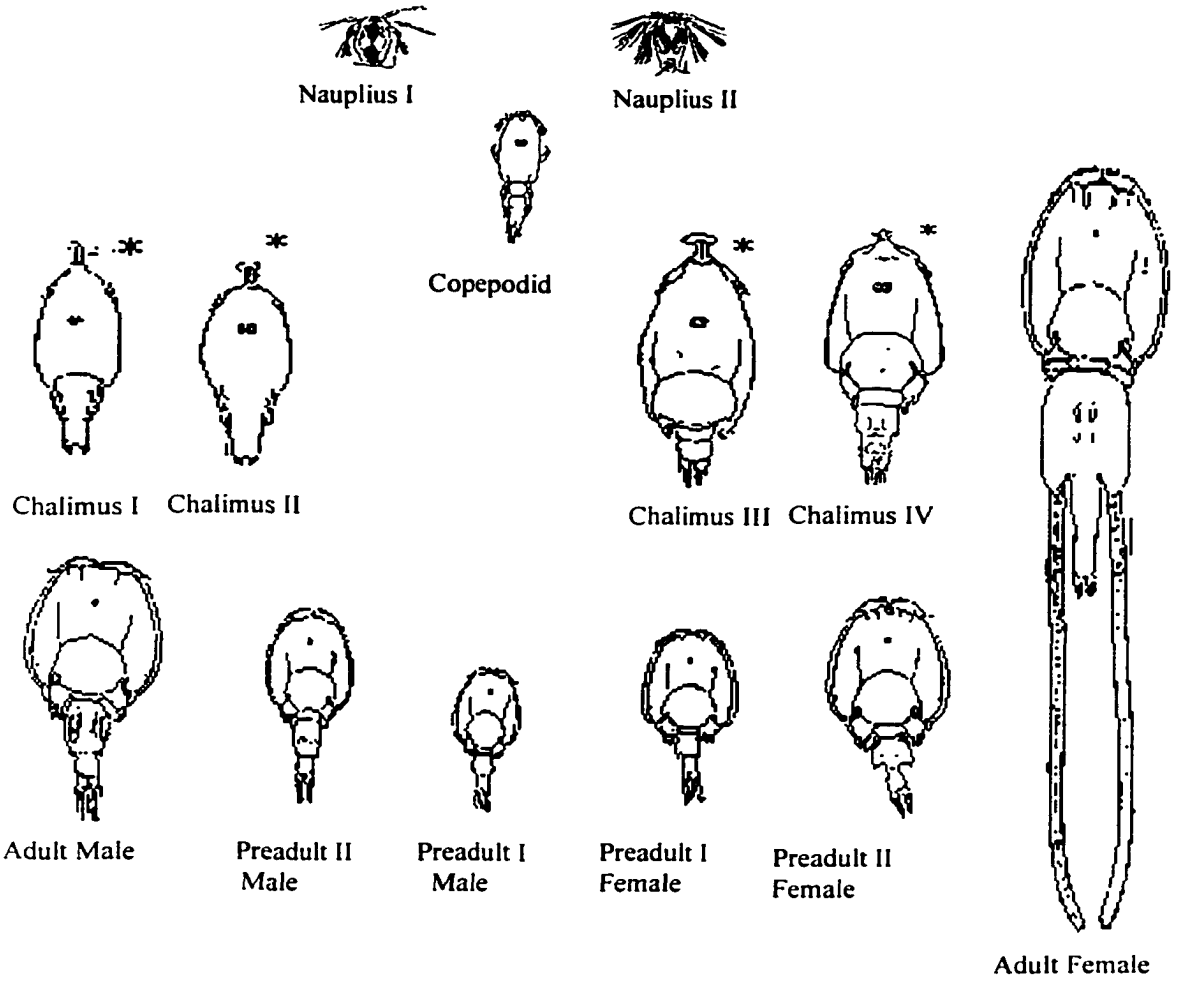


Fig 1.1. The life cycle of *Lepeophtheirus salmonis* (After Schram (21)). The chalimus stages use a FF (*) to attach to the host.

1.4. Development rates of *L. salmonis*.

Since the economic importance of *L. salmonis* infestations has been increasing in the last decade, interest in having more knowledge about the biology of the parasite and its relationship with the host has also been increasing. Preliminary studies have shown that development rates correlated with temperature can help to predict abundances of lice in salmonid fish farm sites (23).

The development rate of *L. salmonis* is affected by physical conditions such as temperature and salinity and aspects of the host's biology (22). Under laboratory conditions, the eggs of *L. salmonis* that developed and hatched at higher salinities (20, 25, and 30 ppt) produced active nauplii but those at 20 and 25 ppt died at the copepodid moult (22). Johannessen (18) demonstrated that the survival at 11.5 ppt salinity and temperatures of 5 - 12 °C is low. The development time of each stage of *L. salmonis* raised at 10 °C in flowing water of 28.5 ppt - 30.5 ppt salinity has been described by Johnson and Albright (22) (Table 1.2).

The time of eggs to hatch (first appearance) is 8.6 days (d) at 10 °C and 5.5 d at 15 °C, the moult to the second nauplius took 30.5 h at 10 °C and 9.2 h at 15 °C. Development from the nauplius to the copepodid stage took 87.4 h at 10 °C and 44.8 h at 15 °C. Johnson and Albright (22) reported that fifty percent of the population were chalimus I at 19 days (d), chalimus II at 22.5 d, chalimus III at 29.8 d, and chalimus IV at 32 d. The earliest adult male was reported at 40 d and the earliest adult female

Table 1.2. Development time of *L. salmonis* at 10°C, adapted from (22).

Stage	First appearance (days)	Last appearance (days)
Egg	-	-
Nauplius 1	9	-
Nauplius 2	10	-
Copepodid	12	22
Chalimus 1	19	24
Chalimus 2	22	27
Chalimus 3	23	32
Chalimus 4	29	35
Preadult 1 Male	32	40
Preadult 1 Female	32	43
Preadult 2 Male	36	45
Preadult 2 Female	40	52
Adult Male	40	-
Adult Female	52	-

at 52 d after egg hatching at 10 °C (22).

The duration of the first nauplius stage is shorter than that of the second nauplius stage (22). This is a characteristic of all caligid species with the exception of C. elongatus. The relatively long duration of the copepodid stage may be due to the need to recover energy lost during the non-feeding naupliar stages, or the requirement of additional energy for development of the FF and moulting (23). As Johnson and Albright (22) reported, the availability of information about sea lice development is limited to the Caligidae family. Descriptions for pennellid copepods (*Lernaeocera branchialis* and *L. sprattae*) and for lernaeopodid copepods (*Salminicola californiensis*) have been used to compare the development rates of *L. salmonis*. However, results showed that *L. salmonis* development is faster (22). Moreover, *L. salmonis* also grow faster on Atlantic salmon than on Chinook salmon (24). In brief, the generation time of *L. salmonis* is estimated to be 7.5 - 8 weeks at 10 °C. However other estimations of the generation time based on field measurements reported 6 weeks at 9 - 12 °C (7), and up to 13 weeks, depending on the water temperatures (10).

1.5. Sea lice infestation.

1.5.1. Pathology and conditions under which diseases can occur in aquaculture / wild fish.

Copepodid, preadult, and adult *L. salmonis* have the ability to move over the fish surface. Preadult and adult stages can also transfer from host to host (25).

Although damage to the host is caused by the feeding activities of all of the stages it is more severe once the copepods have attained the preadult and adult stages, due primarily to their larger size. The host-parasite relationship and the level of infestation depends on various host factors such as the host immunological system, stage of development, age, sex, nutrition, genetic history and migratory behaviour (24). These factors interact with the external environmental to play an important role in the level of susceptibility of salmonids to sea lice infection. Differences of susceptibility to *L. salmonis* between naive salmon species were demonstrated by Johnson and Albright (26). Coho salmon were the most resistant to infection, followed by Chinook salmon and then Atlantic salmon (26).

Feeding and attachment mechanisms of lice can be responsible for the development of severe tissue lesions which cause osmoregulatory failure, anemia, and stress, allowing secondary infections to occur in fish (3,27-29). Secondary infections more frequently reported associated with sea lice infestation, include *Aeromonas salmonicida*, *Vibrio anguillarum*, *V. salmonicida*, *Yersinia ruckeri*, infectious pancreatic necrosis (IPN) virus and infectious salmon anaemia (ISA) virus (29). It is believed that *L. salmonis* is not only a parasitic problem, but also a potential vector for spreading diseases among fish cultures. For example, Nylund *et al.* (30) demonstrated horizontal transmission of ISA virus via sea lice. Nylund's group found ISA virus in the gut of lice and suggested that sea lice act as a reservoir for ISA virus. (30).

1.5.2. Cost to the industry.

Severe infestation of Atlantic salmon with *L. salmonis* can result in heavy losses especially in pen-reared fisheries. In 1995, sea lice infestation on the Atlantic coast of Canada resulted in approximately $20 million in lost revenues as a result of decreased fish quality, deaths, and treatment costs (12,16).

1.6. Methods of sea lice control.

In many regions, sea lice are presently controlled using an integrated pest management approach which uses chemotherapeutants, biological control methods and site management strategies. This approach has reduced the incidence of sea lice. However, costs associated with treating for sea lice are still a major expenditure for the salmonid aquaculture industry.

1.6.1. Chemotherapeutants.

Successful health management in aquaculture depends on strategic and effective use of chemotherapeutants. However, compared to mammalian therapeutics, the use of drugs in aquaculture is limited because of the lack of approved chemotherapeutants and due to environmental considerations (31). Several chemotherapeutic methods for control of sea lice have been investigated in Canada and Europe including: 1) dip and bath-type drugs, such as the organophosphates, pyrethrins, pyrethroids, and hydrogen peroxide; and 2) drugs incorporated in feed, such as the neurotoxic avermectins and the chitin synthesis inhibitors (31). The chitin synthesis inhibitors are of particular interest

to this study because these compounds disrupt cuticle formation during the intermoulting period, causing death shortly after moulting (32). Significant reductions in preadults and larval stages of lice occur after treatment. However, the chitin inhibitors have less effect on adult sea lice that have already formed their final exoskeleton (31).

Although chitin synthesis inhibitors, particularly diflubenzuron and teflubenzuron, are relatively non-toxic to mammals they are extremely toxic to crustaceans, causing morphological deformities and death (32). However, preliminary studies have indicated that diflubenzuron used according to the recommendations for oral treatment against sea lice does not have detectable adverse effects on marine fauna (33).

1.6.2. Management strategies and biological methods.

Management strategies and biological control methods have been used to reduce the need for chemotherapeutant treatments. Examples include fallowing and the use of cleaner fish, light lures and fish pumps (16,34).

Resistance to infection with sea lice may have a heritable component (16), and the susceptibility of salmonids to infection with sea lice differs between different salmon species as well as between individuals (35). Therefore selection for natural resistance would reduce the need for using drugs to treat for sea lice infections. Thus, breeding programs to develop salmon resistant to sea lice should also be considered as a possible management strategy. Improved understanding of the salmon-louse relationship, including louse attachment, is necessary to develop breeding strategies so

that fewer larval lice will seek out, attach to, and develop on the salmon host.

Due to concern about the effects of the non-selective chemotherapeutants on marine environments, the costs associated with these treatments, and evidence that some sea lice populations may be developing resistance to some of the currently used pesticides (28), there is a great deal of interest in developing a vaccine against *L. salmonis* (36). To date, studies have been done to identify potential antigens from the salmon louse (37) and to screen and select monoclonal antibodies to the salmon louse in order to select monoclonal antibodies which may form the basis for experimental vaccines against the salmon louse (38). A current study based on the analysis of partial cDNA sequences is being utilized to identify *L. salmonis* genes (39). This may lead to the identification of targets for chemotherapy and vaccine development.

1.7. Attachment systems in parasitic copepods.

Sea lice maintain contact with their hosts by using a variety of attachment mechanisms. Attachment is a result of the evolution of parasitism among copepods and is defined as "the ability to maintain at least semipermanent contact with the host" (13). Kabata (13) suggested that these adaptations in most cases are cuticular outgrowths. The type of attachment depends on the species of copepod and the site of attachment on the host. The majority of Copepod parasites of fish use more than one type of attachment in the course of their life cycles (13).

Lepeophtheirus salmonis uses a variety of attachments during its life cycle. The infective copepodid stage uses hooks on its second antenna and maxillipeds to maintain

contact with the host (13,25). The chalimus stages of *L. salmonis* remain attached to the host using a FF. Johnson and Albright (20) reported that a small proportion of first preadult *L. salmonis* are attached by FF. Some authors believed that the FF is replaced at each moult of the four chalimus stages (26,40), whereas others suggest that the FF is formed only once at the moult to the first chalimus stage (27). It has been reported that *C. elongatus* extrudes the FF to attach permanently to the host at the late copepodid stage and subsequent chalimus stages inherit the same filament (41). A temporary FF is produced by *L. pectoralis* which acts as a holdfast at the moult from chalimus IV to the first preadult stage and at the moult to the second preadult and adult stages (42).

In the suborders Siphonostomatoida (to which *L. salmonis* belongs) and Poecilostomatoida, both the preadult and adult stages have the capability of moving over the surface of their host. In these stages, the cephalothorax is used as a sucker with supplementary anchoring provided by the second antennae during those times when the louse is immobile (13). To generate suction, the saucer-like cephalothorax must be pressed down onto the host, and suction is created within the enclosed space. This allows the louse to remain firmly attached to the surface of the host while still allowing free sliding movement over the surface of the host (13,43). To detach this grip, the suction must be released and the second antenna unflexed.

1.7.1. Frontal filament.

1.7.1.1. Function.

The attachment organ used during the chalimus stages of most siphonomastoid parasites of fish is the FF, previously known as 'the mediofrontal', 'median sucker', 'cordon frontal' (44) and 'attachment filament' (45). This structure has been described for various *Lepeophtheirus* and *Caligus* species.

The FF is a larval attachment structure produced in the frontal area of the copepod (13). It is present throughout the chalimus stage and as stated above, in a modified form in the preadult stage of *L. salmonis* (21) (29).

The function, development and composition of the FF in the family Caligidae have been studied since the last century and the similarity between various genera is evident (46). In 1858 Hesse (cited in (44)) observed "embryos of *Caligus rapax* follow their mothers like a small boat towed by a large ship". This led him to propose a nursery function for the FF (referred to by him as the cordon frontal) in *C. rapax*. This idea was later refuted, based on difficulties the larvae would have in finding their mothers and attaching to them (44). Wilson (44) concluded that this observation of larvae attached to adult females was purely coincidental. In 1966, Kabata (13) suggested that the FF in *Dissonus manteri* (Copepoda: Dissonidae) develops in the copepodid stage so that the chalimus stages can firmly attach to the host.

Anstensrud *et al.* (42) also noted that a temporary FF was used by *L. pectoralis* (Müller) to attach to the host during the process of moulting in preadult stages.

In preadult and adult stages the temporary FF was extruded from the region of the filament gland (also known as the frontal organ) during the moulting process (within 1-2 h). Then after the new exoskeleton was hardened (another 2-3 h), the newly moulted individual detached itself from the FF, becoming free to move on the body surface of the host (42). It was concluded that, this temporary FF prevents the moulting animal from falling off the host while the exoskeleton is still soft. Furthermore, Anstensrud (42) also suggested that the formation of a temporary or secondary FF during a moult in the preadult stages may be common to all members of the Caligidae family.

1.7.1.2. Origin.

The frontal organ is located in the center of the anterior margin of the dorsal shield. Kabata (43) suggested that the point at which the frontal organ is situated is the first to come in contact with the host when the host is located by the copepod and proposed a sensory function for this organ. The surface of the frontal organ consists of numerous villiform papillae, resembling the surface of the intestinal epithelium. Similar organs have been found in *C. curtus, C. clemensi* and *L. salmonis* and it is probable that all Caligidea possess them (43).

Oldewage and Van As (45) refuted the idea of a sensory function for this structure in *Caligus* spp. and first suggested its role in the attachment of the first chalimus stage to the host.

It is now generally accepted that the temporary frontal filament present in the preadult and at the moult to the adult stage is produced by the frontal organ which is

located in the center of the anterior margin of the dorsal shield (43).

Original observations by Wilson in 1905 suggested that in the family Caligidae, the FF material was secreted by a heart-shaped gland situated medially, just in front of the eyes (44). Bron *et al.* (25) provided a preliminary description of a set of glands they considered responsible for the production of the FF in *L. salmonis* (Fig. 1.2). Bron's diagram simply displayed the organization of the "glands" and "ducts" within the cephalothorax. A revised diagram incorporating information obtained in the present study is provided later in this thesis (see Fig. 3.28) They noted two lobes of a group of cells near the eye, which they called A1 and A2. B-cells were more anterior, adjoining a secretion they called S1. A third group of cells, designated C, was ventral to the B-cells, and was in contact with a second secretion, S2. A "collecting duct" appeared to originate in A, pass anteriorly through C, and exit anteriorly through an "axial duct". Similarly, Piasecki and Mackinnon (41) described the presence of two secretory pads (external cuticular elements) of the frontal organ as responsible for the formation of the FF in older copepodids of *C. elongatus*. Although the formation of the FF described for the *Caligidae* family by Wilson (44) suggest that the frontal organ starts to secrete material at the moult to the first chalimus stage, *C. clemensi* is described to have an internal FF in the copepodid stage (17).

The FF of *C. elongatus* originates from the extreme anteroventral surface of the chalimus body, corresponding to the frontal organ in adult lice (40). In *L. pectoralis*, the FF is extruded from a structure situated ventrally between the frontal plates called

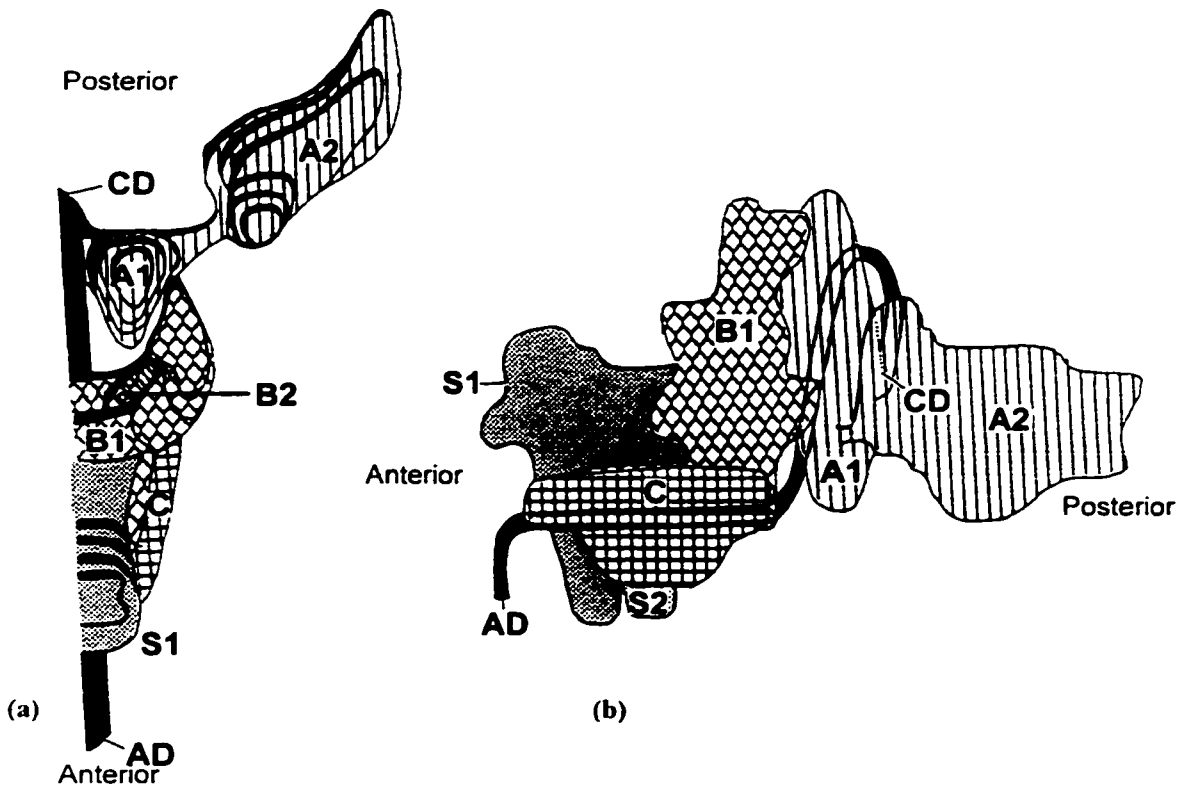


Figure 1.2. Diagrammatic representations of filament-producing apparatus and associated structures in chalimus larvae of *Lepeophtheirus salmonis*: (a) dorsal view; (b) left lateral view: A1, A2 lobes of A-group of cells ; AD, axial duct; B1, major portion of group B; B2, minor portion of B-group of cells; C, group C; CD, collecting duct (continuous with axial duct); S1, probable secretion of group B; S2, probable secretion of group C. After Bron *et al.* (25).

the filament gland (42). Similarities in these descriptions suggest different terminologies for the same structure. In conclusion, the FF is produced by a cellular structure named by different authors as: filament organ, frontal organ, filament gland, interantennary organ, and cement gland (45). This organ is also likely responsible for the production of the temporary FF of the preadult stage as well (42).

The FF production in *L. salmonis* is believed to begin in the copepodid stage prior to the moult to the first chalimus larva, i.e. similar to what has been observed in *D. manteri* and *C. clemensi* (17,46). The filament adheres to the host skin basement membrane under the epithelium by secreting a glue-like substance to form a basal plate (17,25).

A detailed study of the anteroventral surface of the preadult's body using scanning electron microscopy revealed that the origin of the FF for *C.elongatus* and *L. salmonis* is a structure which has a microvillar like-surface inside the external cuticle. This structure is similar for both species and corresponds in adult lice to the frontal organ, as described by Pike *et al.* (40) and Kabata (43)(40,43).

1.7.1.3. General shape.

Morphological descriptions of the FF and its attachment to the host differ slightly among *C. elongatus*, *L. pectoralis* and L. salmonis species. The filament of *C. elongatus* is described as long and slender like an 'umbilical cord' and the surface is smooth and straight and is fixed directly to the fish scale by a large basal plate (40). The temporary FF of *L. pectoralis* is thin and hyaline and may take the form of two

twisted strings (42). The filament of *L. salmonis* is short and stumpy and inserted onto the host epidermis by a basal plate (40).

1.7.2. Frontal filament of *L. salmonis*.

The FF of *L. salmonis* is considered to be an integral part of the body of the chalimus larvae (40). It is described as consisting of a basal plate and stem (25,26). Johnson and Albright (26) described the stem as being divided into an outer and an inner region. The outer region described by Johnson and Albright (26) is defined as the external lamina by Bron *et al.* (25), and the inner region reported by Johnson and Albright (26) is the stem according to Bron *et al.* (25). Both sets of authors provided identical descriptions of the basal plate. It is generally agreed that the structures described by Bron *et al.* (25) and Johnson and Albright (22) are identical.

Histological staining characteristics of the stem and basal plate of the FF give some indication of the materials used in its makeup. Johnson and Albright (26) reported that the basal plate stained darker than the outer and inner layers of the stem with Lee's stain (methylene blue and basic fuchsin). Based on the similar staining characteristics between the external FF and the internal FF material, these authors suggested that the FF is preformed internally before the moult (26). Bron *et al.* (25) reported that the basal plate gives a lightly positive reaction to the carbohydrate test using the periodic acid-Schiff (PAS) reaction and is also strongly eosinophilic. Based on this observation, it was proposed that the basal plate has polysaccharide properties. It also appears to have proteinaceous characteristics, as shown by bromophenol blue and Millon's (tyrosine)

tests (25).

The stem stains differently from the basal plate (25,26). Internally, it seems to be formed of densely-packed protein fibres and contains an axial duct. This axial duct reacts positively with PAS stain and runs through the center of the stem (25). Evidence for this was later confirmed by Johnson and Albright (26) and by Pike *et al.* (40). Covering the stem is the external lamina which does not reveal fibrosity when stained with PAS. This external lamina is continuous with the cuticle of the chalimus larvae and has similar staining properties (25,26,40).

The process of FF production in *L. salmonis* is poorly understood. The presence of both precursor and fully formed filaments within the copepodid stage have been described by Pike *et al.* (40). Bron *et al.* (25) reported that there was no sign of any preformed filaments in any sections of copepodid or chalimus stages, but speculated that reservoirs of secreted material seen in the copepodid were filament precursors. Later, Johnson and Albright (26) reported the presence of fully formed FF (stem and basal plate) in the anterior cephalothorax of two chalimus larvae that were in the process of moulting. The internal FF had similar staining characteristics to attached external filaments but they differed in the structure of their stems. This may be a characteristic unique to chalimus larvae immediately prior to the moult.

Bron *et al.* (25) described a polysaccharide-containing adhesive secretion conducted to the tip of the filament through an axial duct and then deposited on the host basement membrane to form the basal plate. The duct has been observed in TEM sections, but has not been traced to its origin (40). There is another description of the

release of the cement from the FF for *Salmonicolla californiensis* (47) that is comparable with that of *L. salmonis*.

The FF could be a potential weak link in the developmental cycle of *L. salmonis* at which control methods might be directed (40). It may be possible to effectively prevent the copepodid from establishing itself on the host if it is possible to interfere with the formation or attachment of the FF. Development of such a strategy would be helped greatly by an analysis of the chemistry of the FF and its development in the different stages of copepodid and chalimus. Studies of the morphology, composition, and mode of production of the FF in *L. salmonis* are an important first step in determining whether control of this parasite can be affected by this means.

1.8. Attachment systems of other invertebrates.

Similar to sea lice some other marine invertebrates require attachment to a surface at some stage of their life cycle. The blue mussel *Mytilus edulis* and zebra mussel *Dreissena polymorpha* secrete byssal beards to attach themselves opportunistically to hard surfaces. Zebra mussels are economically significant as a fouling pest (48). To discover specific measures against this pest by targeting the attachment mechanism, but also to gain a better understanding of this adaptation, several studies in the last four decades have been focused on byssus and foot gland ultrastructure, histochemistry and byssus composition. These studies have provided information about the morphology and biochemical composition of the three differentiated regions of the byssus. At the present time, new lines of investigation are

being developed toward the clear understanding of the structure-function relationship of byssal proteins (48,49).

The descriptions of byssus threads have some similarities with filament attachment in parasitic copepods (17,44). The organisms to keep themselves temporally attached to a host or surface, and are capable of reattaching in a short period of time by secreting adhesive material. However, in contrast to FF attachment, the information available on byssus and producing glands is extensive and increasing due to the interest in the hold fast material (50) and its potential biotechnological applications, including medical adhesives and coatings, dental adhesives and fillers, water resistant inks etc. (48). Research on FF attachment is still immature but developmental, morphological, histochemical, and biochemical studies similar to those done on byssus could be helpful for a better understanding of FF attachment of parasitic copepods.

1.8.1. Byssus morphology.

Byssus threads are defined as: "a series of adhesive plaques, each connected through a filament to the stem region at the base of the animal's foot" (49). The threads are produced by the foot of the mussel which can make a new thread in 5 min or less. The threads contain a graded distribution of molecular elements which make the byssus stronger than the Achilles tendon (51).

The byssus threads in *Mytilus* consist of three primary parts: a) a single main stem, b) multiple threads attached to the stem by means of rings consisting of thread material, and c) a distal attachment plaque at the end of each thread (50) secreted by

three glands: the white gland, the phenol gland, and the enzyme gland. Mucous glands which probably aid in the final attachment of the byssus plaque to the substratum are also present (52).

1.8.1.1. Bysssus histochemistry.

The threads are composed of two main proteins, a non-collagenous protein in the proximal elastic region and a collagenous protein that provides rigid support in the distal region. The plaque contains an acid sulfated mucopolysaccharide, protein and a collagenous substance that together mediate adhesion and cohesion (49). This substance has the ability to bind effectively to the surface under the water (49). Moreover, byssal threads can remain without shrinkage and melting under temperatures up to 90°C (53).

At least four distinct exocrine secretion mechanisms are generally assumed to be involved in the formation of the byssus threads in *Mytilus*. There are 5 types of byssus glands: 3 protein secreting glands, named collagen, enzyme and phenol glands, and 2 mucus-secreting glands, one proximal and the other distal (50,54). Byssal proteins are divided into three types depending on the function that they serve in byssal threads: *i*) fibrous proteins forming the core of the threads, which could be collagenous, silk-like, elastic or any combination of these, *ii*) cuticular proteins forming the protective coat around the core characterized by their high content of lysine and the exotic amino acid 3,4 -dihydroxyphenyl - L - alanine (DOPA), *iii*) adhesive proteins form the hold fast material, distinguished by their low molecular weight, containing DOPA, and forming

microcellular solids (foams) (48).

1.9. Objectives of and rationale for the research.

The objective of this research project was to understand how and when the frontal filament in the salmon louse *(L. salmonis)* is produced. High resolution light microscopy was used to examine the formation of the FF and the histological changes associated with the FF from copepodid to chalimus II, involving two moults. Improved knowledge about the morphological characteristics and mechanisms of sea lice FF formation and attachment will form the basis for future research into methods of preventing the attachment of the chalimus stages.

2. MATERIALS AND METHODS

2.1. Sea lice collection and culture in the laboratory.

One hundred and twelve ovigerous sea lice *L. salmonis* were collected from Atlantic salmon that were being harvested and processed for marketing at Connors Aquaculture Ltd., Eastport, ME. Sea lice were transported to the Institute for Marine Biosciences (IMB), Sandy Cove Research Station, National Research Council Canada, Halifax, NS, in a 2 l container filled with seawater from the collection site. During transport, the seawater was aerated using a portable air pump and an air-stone diffusor and the temperature was maintained at approximately 10°C using a cooler.

After transfer to the facilities at IMB, egg-strings were excised and placed in 45 l garbage containers. These containers were provided with flow-through, filtered seawater at a constant temperature (10 ± 1 °C). Circulation within the containers was maintained by gentle aeration. The container drains were covered with a 100 μm Nitex mesh screen to prevent larval loss.

Nauplii were hatched and reared to the infectious copepodid stage. The larval stage of development was determined by examining random samples under a dissecting microscope (Wild Leitz, Willowdale, ON) and comparing the larvae to the descriptions of the developmental stages provided by Johnson and Albright (20). At 9 days post-hatch, the majority of the population had attained the copepodid stage.

2.2. Fish infestation and sample collection.

Thirty-two post-smolt Atlantic salmon (100 to 150 g) were introduced, acclimatised and maintained in a 1500 l tank with flow-through, filtered, UV-treated seawater for two months prior to the onset of the experiment. Water temperature was set at 10°C and the actual temperature determined every 40 min using a temperature logger. The overall average temperature observed was 12.38 ± 0.50 °C, with a fluctuation range of 9.0 - 13.7°C (Table 2.1). The fish were all assessed to be in good health and fed twice daily with pelleted dry commercial salmon feed (Corey Feeds, Fredericton, NB).

Fish were infested by placing approximately 1120 newly molted copepodid larvae into the tank (production estimated from 112 ovigerous sea lice females with fully formed egg-strings), reducing the water flow and eliminating the light. Lice were sampled from three fish at the same time each day from day 1 to day 10 post-infestation. Fish were caught in a dipnet, withdrawn from the tank and killed by a single blow to the head. The body surfaces of the fish were examined under a dissecting microscope and at least 60 sea lice from each fish were removed by excising the integument of the fish to which they were attached.

For an accurate assessment of the development rate as a function of temperature-growth, degree day (DD) values were calculated for each day post infestation (dpi) by using the average temperature (T_{mean}) for each sampled day (Table 2.1) and the

Table 2.1. Mean ± SD seawater temperature (°C) and calculated degree days (DD) for the sea lice infestation. Never-on-fish (NOF), days post-infestation (dpi), minimum temperature reached per day (MIN); maximum temperature reached per day (MAX).

DAY	MEAN	SD	MIN	MAX	DD
NOF	10.7	0.1	10.6	10.9	0
1dpi	11.1	0.2	10.8	11.4	11.1
2dpi	11.2	0.4	10.9	12.2	22.3
3 dpi	12.6	0.6	11.5	13.4	34.8
4 dpi	12.7	0.8	11.1	13.7	47.5
5 dpi	10.8	0.7	10.1	12.6	58.4
6 dpi	10.3	0.6	9.5	11.7	68.7
7 dpi	10.3	0.8	9.1	11.7	78.9
8 dpi	11.5	0.5	10.8	12.3	90.4
9 dpi	11.2	0.1	11.0	11.3	101.6
10 dpi	11.7	0.4	11.2	12.4	113.2

following formulae: never on fish = 0 DD

$$1\text{dpi } T_{mean} = DD^{1dpi}$$

$$DD^{N-1\ dpi} + N\ \text{dpi}\ T_{mean} = DD^{N\ dpi}$$

N = number of days post infestation

2.3. Fixation, embedding and analysis of samples.

2.3.1. High resolution light microscopy, Spurr's plastic resin

For high resolution light microscopy, 45 sea lice per day were fixed at room temperature in a solution of 3% glutaraldehyde in Millonig's (55) phosphate buffer (pH 7.4) containing 4 % NaCl (1100 - 1200 mosmol) for 1 h, then transferred to 4°C overnight. This was followed by two rinses with fresh buffer for 10 min each time at room temperature. After rinsing, the samples were post-fixed in 2% osmium tetroxide in Millonig's phosphate buffer for 2 h at 4°C, washed twice with fresh phosphate buffer for 10 min each time followed by a 10 min wash in distilled water. Samples were dehydrated through a graded series of ethanols, infiltrated and embedded in Spurr's (56) low-viscosity embedding media (Appendix A).

The following numbers of sea lice were serially sectioned for analysis: NOF (n = 5), 1 dpi (n = 5), 2 dpi (n = 5), 3 dpi (n = 5), 4 dpi (n = 8), 5 dpi (n = 5), 6 dpi (n = 5), 7 dpi (n = 5), 8 dpi (n = 6), 9 dpi (n = 7), 10 dpi (n = 5). For each day sampled, sets of horizontal and sagittal sections were cut to a thickness of 0.5 μm using an ultramicrotome (Reichert-Jung Ultracut E, Vienna, Austria) and histoknife (Diatome Ltd. Bienne, Switzerland). Sections were collected and mounted in consecutive order

onto clean glass slides, giving approximately seven rows (each consisting of 16 sections) on each slide. The number of slides generated per sample varied depending on the size of the louse and the orientation of the section.

Sections were stained with 1 % toluidine blue in 1 % sodium borate on a warmer plate at 60 °C for about 10 minutes, then rinsed with double distilled water, dried on the warmer plate, and cover-slipped. A minimum of five sets of slides were examined for each day using an Olympus BH2 light microscope (Carsen Medical & Scientific Co. Ltd. ON). Light micrographs were generated using a Zeiss photo microscope III (Carl Zeiss Ltd. Jena, Germany) with Kodak TMax 100 ASA film for black-and-white prints.

The histological examination focused on the FF and the tissues within the anterior portion of the larvae. At least five individuals were examined for each sampling day. The number of samples examined was based upon time considerations and the fact that the level of development of the FF was very similar between individuals collected on the same day.

2.3.2 High resolution light microscopy, JB-4 embedding medium.

For high resolution light microscopy using JB-4 embedding medium. 20 sea lice per day were fixed and stored in 10% neutral buffered formalin, then dehydrated through a graded series of ethanols and infiltrated and embedded in glycol methacrylate JB-4 embedding medium (JB-4 Embedding Kit, Polysciences, Inc., Warrington. PA) (Appendix B). Serial sagittal sections were cut from 3, 4 and 10 dpi samples at a thickness of 3 or 5μm using an ultramicrotome and histoknife and mounted on clean

glass slides. Sections were stained with Lee's methylene blue-basic fuchsin as described in Bennett *et al.* (57) and examined using an Olympus BH2 light microscope.

2.4. Routine light microscopy.

Samples for routine light microscopy (paraffin embedding) were fixed and stored in 10% neutral buffered formalin, dehydrated through an ascending graded series of ethanols, cleared in xylene and embedded in Paraplast Plus ® (Appendix C). Ten individuals from 6 days post-infestation were horizontally sectioned at a thickness of 5μm using a Spencer "820" rotary microtome. Sections were mounted on glass slides which had been previously coated with a 2% silane-acetone solution. Tissue was then deparaffinized in xylene and hydrated through a series of ethanols to water, then stained with alcian blue pH 2.5 (for acid mucins), alcian blue / PAS (for neutral and acid mucins) (58,59), picro / ponceau (for collagen) (60), hematoxylin and eosin (for acidophilic and basophilic structures) (60), Verhoeff's (for elastin) (61), or Weigert's resorcin-fuchsin (for elastic and elaunin fibres) (61) (Table 2.2). Fish tissues were fixed and processed along with the lice samples and used as positive controls for the different stains. Sections were analysed using an Olympus BH2 light microscope.

Table 2.2. List of stains used for routine light microscopy, the *L. salmonis* developmental stages examined, the tissues used as controls and the staining times used.

Stain	Stage	Positive Control Tissue	Time of exposure to the stain
Hematoxylin and Eosin	*6 dpi*	Fish skin	5 min (each stain)
Picro/Ponceau	*6 dpi*	Fish skin	5 min
Verhoeff's	*6 dpi*	None	15 min
Weigert's	*6 dpi*	None	1 h
Alcian blue pH 2.5	*6 dpi*	Fish skin	5 min (each stain)
Alcian blue/PAS	*6 dpi*	Fish skin	5 min (each stain)

2.5. Chemicals and reagents.

Eosin, glutaraldehyde and Schiff reagent were obtained from Canemco (Quebec City, PQ). Alcian blue and ponceau where purchased from Sigma Chemical Co. (St. Louis, MO). Periodic acid and 10% neutral buffered formalin were from BDH Chemicals (Dartmouth, NS). Sodium borate, toluidine blue and xylene were from Fisher Scientific Ltd (Dartmouth, NS). Hematoxylin was from JT Baker Inc. (Phillipsburgh, NJ), and ethanols were from Commercial Alcohols Inc. (Brampton, ON).

3. RESULTS

3.1. Frontal filament morphogenesis.

Never on fish (0 DDPI) and 1 dpi (11.06 DDPI).

At 0 dpi and 1 dpi the two major cell groups believed to be involved with filament production were evident, but there was no evidence of an external FF or internal FF material. The A-group appeared to be larger than the B-group. The designation of these cell types is based on the description by Bron *et al.* (25). Cells of the A-group (A-cells) stained more intensely than those of the B-group (B-cells) (Fig. 3.1). In horizontal medial section, the A-group of cells was "H" shaped having two lateral lobes connected medially anterior to the eyes. Anteriorly the lateral lobes of A-group cells extended beneath group B (Fig. 3.2) and posteriorly the lateral lobes extended to the eyes. Some A-cells in the medial region of the group A contained densely stained granules (Fig. 3.1).

In horizontal medial sections the B-group was horseshoe-shaped and was partially surrounded by cuticular pigment cells. The left and right portions of the B-group were lateral to the anterior lobes of the A-group (Fig. 3.1). Ventrally the anterior medial portion of the B-group narrowed to a triangular shape. Nuclei in cells of the B-group appeared more abundant than those in group A and were organised in rows. No granulated material was present in B-group cells (Fig. 3.1).

A duct passed anteriorly through the ventral portion of the B-cell group. Cells that surrounded the duct were elongated and lay parallel to the long axis of the duct (Fig. 3.3). The anterior portion of the duct appeared as a lumen extending to a small

Figs. 3.1-3.3. Copepodid, (never on fish = 0 DDPI).

Fig. 3.1. Horizontal medial section of the anterior cephalothorax. Note the two cell groups (A and B) involved in FF production. A-group cells are dark staining and granulated (arrow); B-group cells are lighter staining and nuclei appear organized in rows (arrowheads). Scale bar = 5 μm. CL, cuticle; EP, eye pigment; P, pigment.

Fig. 3.2. Sagittal section of the anterior cephalothorax shows the A-group (A) extending anteriorly beneath the B-group (B) and ventral to the eye (E). Scale bar = 4 μm. EP, eye pigment; L, lens; P, pigment.

Fig. 3.3. Horizontal ventral section of the anterior cephalothorax. The lumen of a duct (arrow) which is surrounded by elongated cells (arrowheads) passes anteriorly through the B-group (B). Nuclei of the cells within the B-group are aligned along the long axis of this duct. This duct opens to the environment through a small pore (PO) in the cuticle. Scale bar = 4 μm. A, A-group of cells; P, pigment; SA, second antenna base, CL, cuticle, R, rostrum.

3.1

3.2

3.3

pore through the cuticle at the anterior margin of the cephalothorax in the vicinity of the rostrum (Fig. 3.3). The origin of this duct could not be determined.

2 dpi (22.21 DDPI).

Internal FF material (S1), based on the designation by Bron *et al.* (25), was first observed at 2 dpi (Fig. 3.4). Four out of the five copepodids examined contained small amounts of S1 material. The S1 was located within the anterior dorsal region of the B-group cells (Fig. 3.4). B-cells anterior to S1 formed a thin layer. B-cells posterior to S1 had structures resembling microvilli presented on their surface where they made contact with the S1 material.

The two cell groups A and B were well defined and appear similar in structure and staining characteristics as those seen at 0 and 1 dpi (Fig. 3.4). The duct described at 0 dpi was easier to resolve at 2 days. Cells that surround the duct were elongated and lay parallel to the long axis of the duct (Fig. 3.5) but the duct origin could still not be distinguished. The anterior portion of the duct appeared as a lumen (pore) extending through the cuticle (Figs. 3.6 a, 3.6 b). A portion of the external cuticle extended ventrally from the pore and passed posteriorly between the second antennae forming a cup-like structure at the midpoint of the body (Fig. 3.6 b).

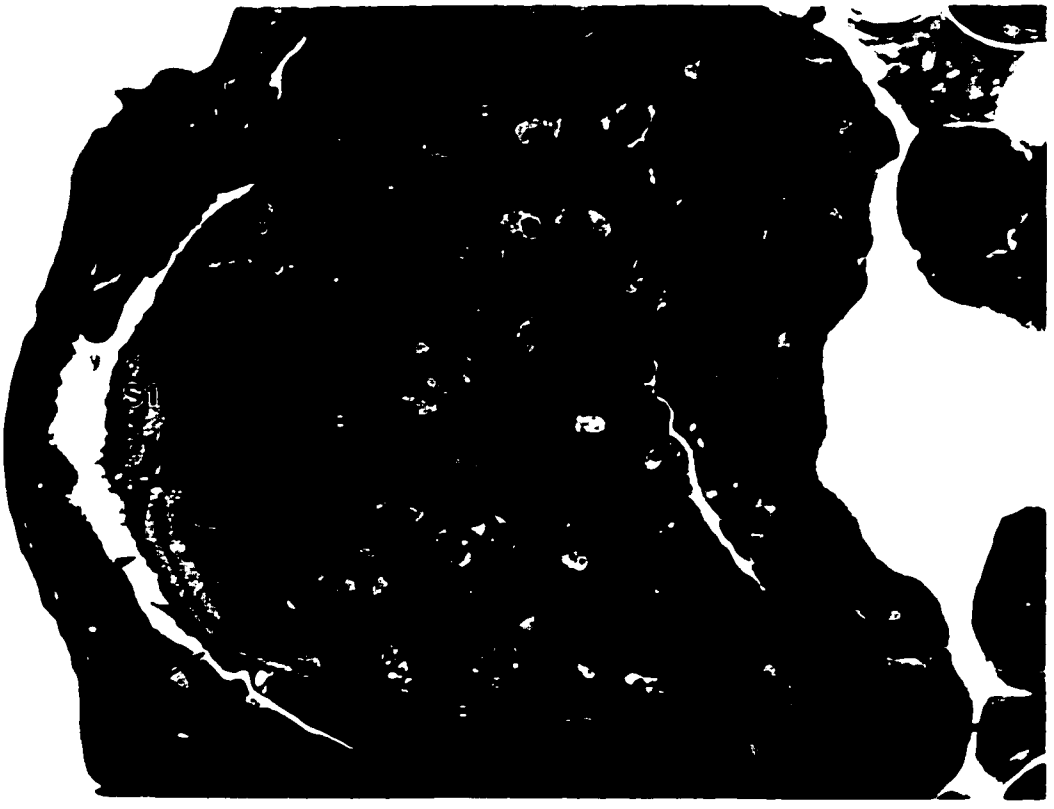
3 dpi (34.77 DDPI).

The S1 and a second type of internal filament material (S2), based on the designation of Bron *et al.* (25), were present in all five copepodids examined at 3 dpi

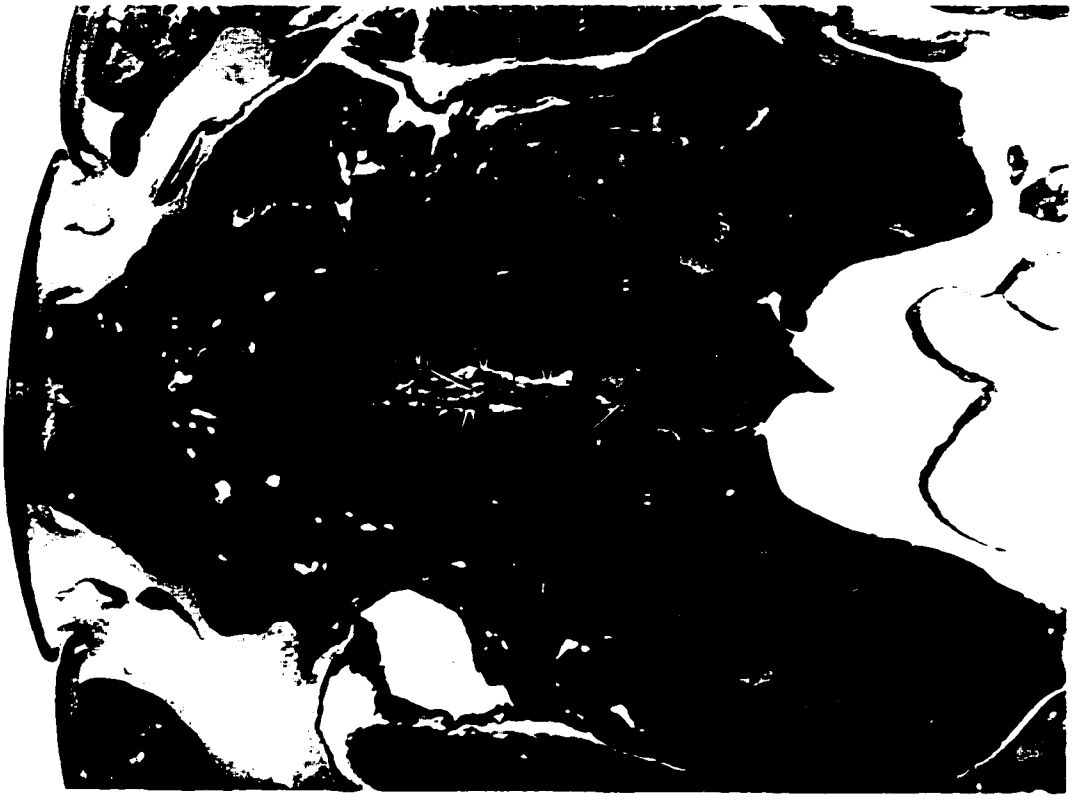
Figs. 3.4-3.5. Copepodid, 2 dpi (22.21 DDPI).

Fig. 3.4. Horizontal dorsal section of the anterior cephalothorax. Newly formed filament material (S1) is surrounded by group B cells (B). Anterior to the S1 the B cells are elongated (arrows), posteriorly the group B cells in contact with S1 have microvilli (arrowheads). Scale bar = 2.8 μm. A, A-group of cells;

Fig. 3.5. Horizontal ventral section of the anterior cephalothorax. Showing the duct lined with elongated cells (arrows) passing through B-group (B). Scale bar = 2.8 μm. Lu. lumen of duct; R, rostrum.

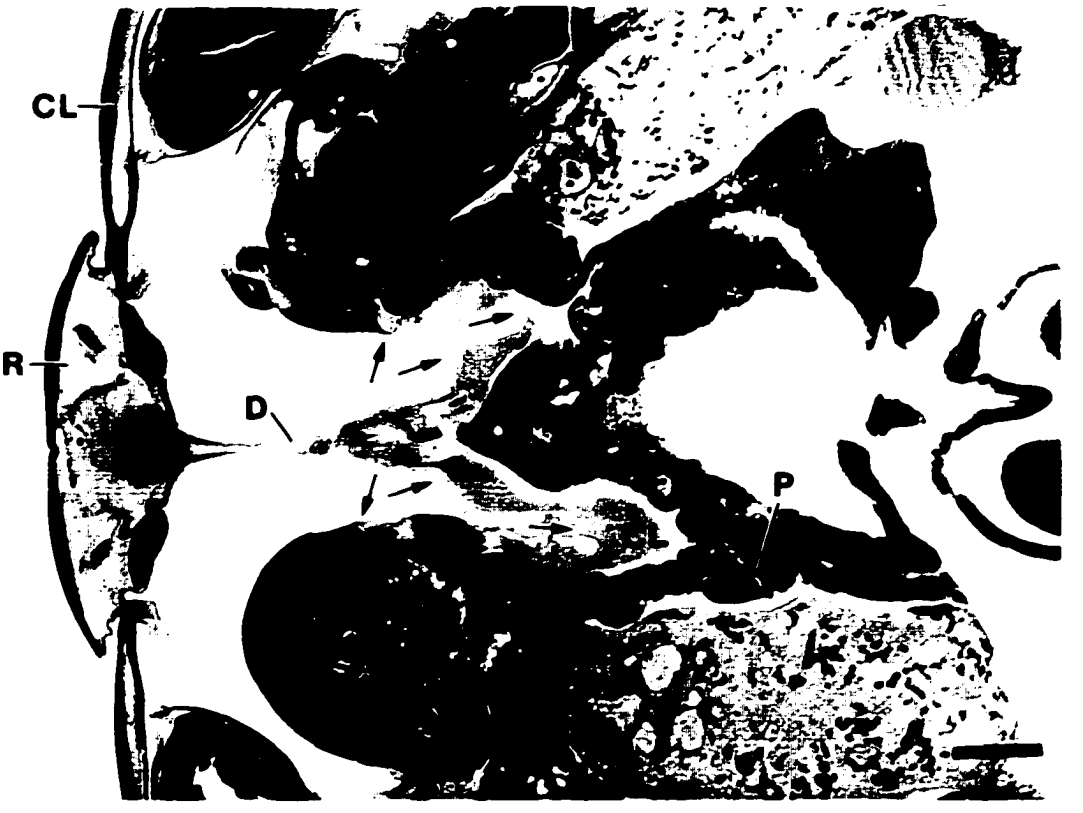


3.4



3.5

Fig. 3.6. Copepodid, 2 dpi (22.21 DDPI). Horizontal ventral sections of the anterior cephalothorax.
(a) Duct (D) surrounded by cuticle terminates at (b) a pore (PO) through the external cuticle. Scale bar = 2.8 μm.
Arrows indicate the cup-like structure; CL, cuticle; P, pigment; R, rostrum; SA, bases of second antennae.

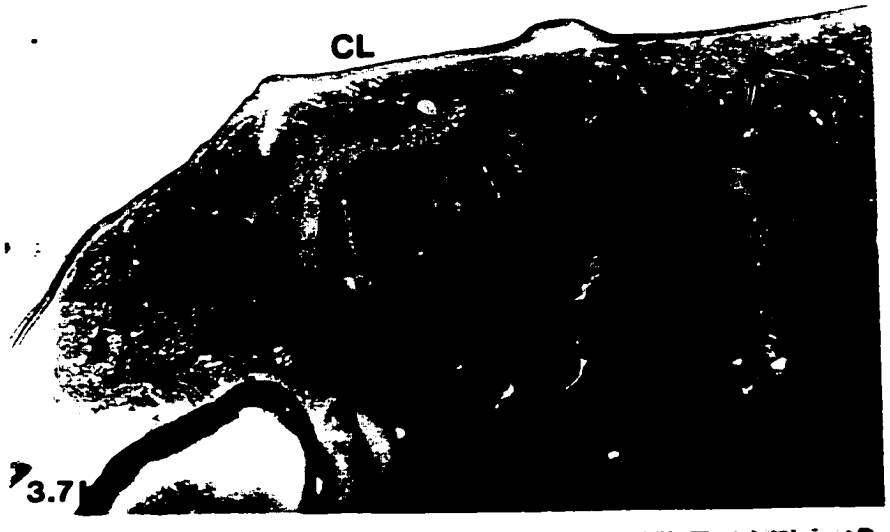


3.5 a

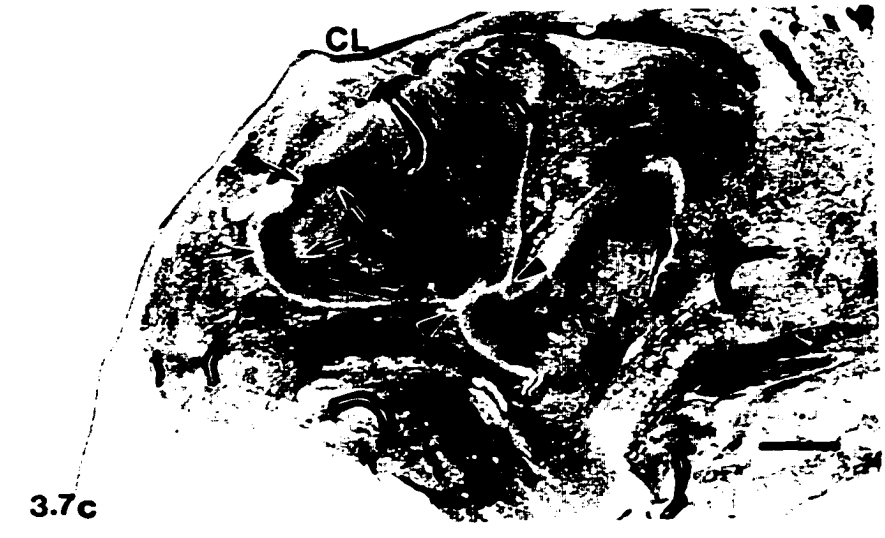
3.6 b

Fig. 3.7. Copepodid larva, 3 dpi (34.77 DDPI). Sagittal sections of the anterior cephalothorax. Note the variation in the amount and shape of the S1 and S2 material with development of the internal filament.
(a) The S1 material is crescent shaped with laminations. Note the convex portion of the B-group (B), with microvilli-like structures (arrowheads). Shape of the S1 material conforms to the convex surface of the B group. Densely stained cytoplasmic extensions (arrows) of C cells (C) contact the S2. Scale bar = 4 μm.
(b) The S1 material is now within a more cuboidal shaped area and the surface of B-group at the posterior end of the S1 is flat. Scale bar = 2.2 μm.
(c) The S1 and S2 are in contact with one another (arrowheads). The S1 material is in a triangular shaped area. Lamellae within S1 anteriorly are C-shaped (arrows); posteriorly the orientation of the lamellae are parallel to surface of the B-group. Scale bar = 4 μm. P, pigment; CL, external cuticle.

3.7



3.7



3.7c

(Fig. 3.7). There was variation in the amount and shape of the S1 and S2 material among the copepodid at 3 dpi. Three of the animals sampled had S1 and S2 separated from one another (Fig. 3.7a, b) and two animals had both S1 and S2 in contact with each other. The S1 and S2, when continuous, presented an interface medially between the two secretions (Fig. 3.7c). The S1 always appeared larger in volume than S2 and had an internal structure consisting of numerous laminations which varied in orientation with development. The S1 was surrounded by B-group cells, and both B-group and S1 changed shape as the S1 enlarged (Fig. 3.7). In lateral section S1 appeared crescent shaped and its laminations were also crescent shaped. The B-group cells immediately posterior to S1 conformed to the shape of the S1 and had microvilli-like structures where they contacted S1 (Figs. 3.7a, 3.8). As the S1 enlarged, its shape became more cuboidal (Fig. 3.7b) and then triangular (Fig. 3.7c). The B group cells on the flattened side of S1 showed a reduction in their microvillar structures (Fig. 3.7b, c). In this later stage of filament development laminations in the S1 material were crescent shaped anteriorly and were vertical and parallel to each other posteriorly (Fig. 3.7c).

A third group of cells, the C-group, could be defined in all 5 animals at 3 dpi (Figs. 3.7 - 3.10). The C-group of cells was the most ventral. However, unlike A-group and B-group, the boundaries between this group and surrounding cells were difficult to define (Figs. 3.7 - 3.10). The C-group was identified by its ventral location and close association with the S2 material (Figs. 3.7 - 3.10).

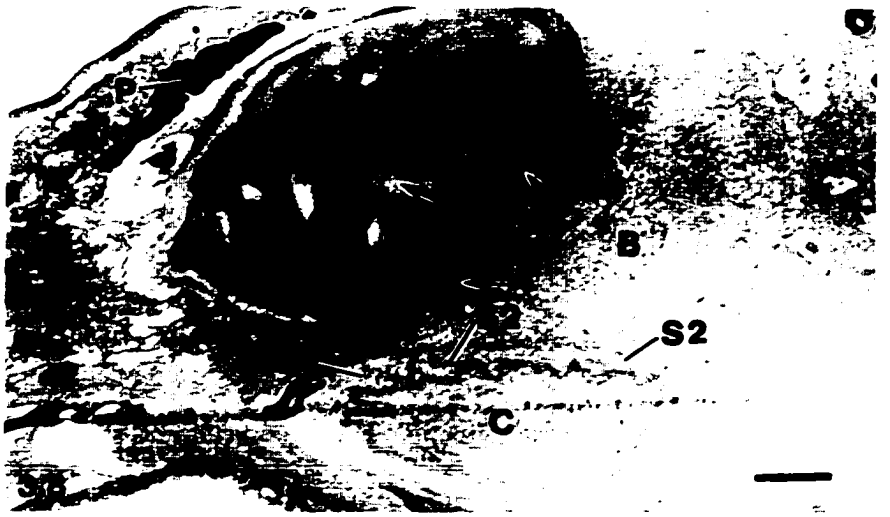
The S2 was covered dorsally by group B cells and was found in close association with the ventrally located cells of the C-group. C-cells showed dark staining

Figs. 3.8.- 3.10. Copepodid larva, 3 dpi (34.77 DDPI). Sagittal sections of the anterior cephalothorax.

Fig. 3.8. Note the long microvilli -like structures (arrows) from B-group cells (B). Scale bar = 2.2 μm. S1, secretion 1; S2, secretion 2; C, C-group of cells; P, pigment.

Fig. 3.9. The wall of the duct (D) passes between the S1 and S2 material. Note the reduction of microvilli-like extensions of B-cells (arrows). Scale bar = 2.2 μm. B, B-group cells; C, C-group cells; P, pigment.

Fig. 3.10. Note the close association of the C-group (C) with S2 by their cytoplasmic extensions (arrows). Scale bar = 2.2 μm. B, B-group cells S1; secretion 1; P, pigment.


B
S2
C


P
S1
B
B
S2


P
B
3.10

cytoplasmic extensions at their boundary with the S2 (Figs. 3.7 - 3.10).

The A-cells remained well defined and appeared similar in structure and staining characteristics to those seen at earlier times. The microvilli-like structures of the B-cells where they contacted the posterior surface of the S1 initially appeared more developed than those seen at 2 dpi (Fig. 3.9). However, as the amount of S1 material increased, a reduction in their microvilli-like structures was evident. The duct seen at earlier times could now be traced between the S1 and the S2, passing anteriorly through the ventral portion of the B-group and anterior through the cuticle (Fig. 3.9).

4 dpi (47.46 DDPI).

At this stage of infestation a proportion (25%) of the copepodids had completed their moult to the first chalimus stage. Because of this, 8 animals instead of 5 animals were examined at 4 dpi. Six out of eight individuals examined were at the late copepodid stage, identified by the lack of an external FF. Two of the individuals were chalimus I larvae as they each had an external FF. The shape of the recently moulted chalimus I larvae was more slender and elongated than at the late copepodid stage.

Late copepodid

All of the late stage copepodids had completely formed internal FF within their anterior cephalothorax and were apparently near the moult to the chalimus stage as evidenced by having two cuticles surrounding their bodies (Fig. 3.11). The duct and its associated pore appeared to be the same as described for earlier stages (Fig. 3.11). At

Figure 3.11. Late-copepodid, 4 dpi (47.46 DDPI). Horizontal medial section of the anterior cephalothorax. A completely formed internal FF (S1 and S2) is contained within a cuticle-lined hour glass-shaped pocket. Note the thick (*) and thin (**) regions of the new cuticle lining the pocket. Long cytoplasmic processes (arrows) extend from C-group cells (C) at the posterior end of the pocket into the S2. A lumen (Lu) passes through C-group and S2. Scale bar = 2.8 μm. Arrowheads indicate laminated pattern of S1; B, B-group; NC, new cuticle; OC, old cuticle.

3.11

this stage both the S1 and S2 were contained within an hour glass shaped pocket lined with new cuticle which varies in thickness (Fig. 3.11). The S2 was restricted by a narrowing of the cuticle lined pocket (Fig 3.11). C-cells were present at the posterior ventral region of the pocket and B group cells surrounded the pocket. Cuticle covered the C-cells except where they extended into the S2 material.

In horizontal section the S1 showed laminations which were arranged perpendicularly to the long axis of the body. The S2 appeared to contain densely stained fibrous material and long cytoplasmic extensions from the C-cells (Fig. 3.11).

The structure and staining characteristics of the A-group were similar to those seen in earlier stages. Cells of the B-group no longer contacted the S1 due to the presence of the new cuticle (Fig. 3.11).

Chalimus

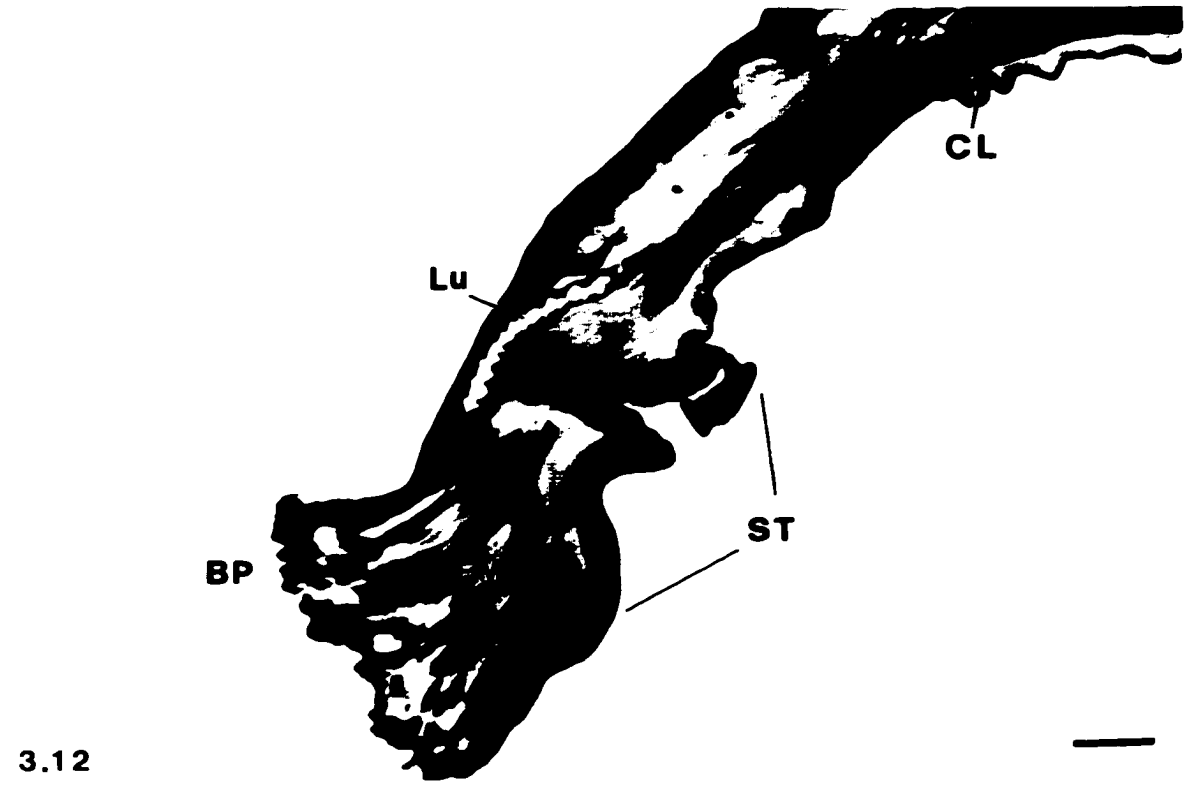
Examination of the two recently moulted chalimus I larvae revealed an extruded FF which consisted of two major regions: the stem and a distal basal plate (Fig. 3.12). The basal plate stained pale and adhered to the fish skin surface, adopting its irregular shape. The basal plate was attached to the stem, which consisted of two regions: a distal region containing dark staining filamentous material, and a proximal region which consisted of pale staining material (Fig. 3.12). Within the now extruded FF there was a lumen from the proximal region of the stem to the basal plate (Fig. 3.12).

The proximal portion of the stem was continuous with the inner wall of the external cuticle of the anterior cephalothorax (Figs. 3.12, 3.13). The external surface of

Figs. 3.12. - 3.13. Newly moulted chalimus larva FF, 4 days post-infection (47.46 DDPI).

Fig. 3.12. Sagittal section of the newly formed external FF. Note the light staining of the basal plate (BP) in relation to the stem (ST); the presence of fibrous material (FM) in the distal end of the stem; and the presence of a lumen (Lu) dorsal through the stem. Scale bar = 2.8 μm. C, C-group cells; CL, cuticle.

Fig. 3.13. Sagittal section of the proximal region of a newly formed external FF. The proximal region (PR) of the stem (ST) is pale staining and in contact with cytoplasmic process of C-group cells (C). The external surface of the stem is delineated by a external lamina (EL) continuous with the external cuticle layer (CL). Scale bar = 2.8 μm.



3.12

3.13

the stem was delineated by a layer of material that was similar in appearance to and in continuum with the external surface of the cuticle. This surface covering extended to the basal plate. The material at the proximal end of the stem was in contact with cells, presumably from the C-group (Figs. 3.12, 3.13).

In these recently moulted animals there was no second cuticle beneath the external cuticle and no evidence of S1 or S2 secretory material. B-cells surrounded a cavity. B-cells dorsal to the cavity appeared narrow and long. Ventral to the cavity, the B-cells were vacuolated and had microvilli at the surface of the cavity (Fig. 3.14).

The duct appeared similar to that seen in earlier stages. The origin of this duct could be traced to a region close to group A cells near the eyes (Fig. 3.15). From this region of origin the duct coursed anteriorly and ventral to the B-group, continuing ventrally towards the stem of the extruded filament (Figs. 3.14, 3.15). No pore was seen through the cuticle at the anterior margin of the cephalothorax.

5, 6, 7 and 8 dpi

All lice examined at five (n = 5), six (n = 5), seven (n = 5) and eight (n = 6) dpi showed evidence of an external FF and therefore had moulted. They were thus in at least the first chalimus stage. The newly moulted chalimus was more elongated and slender than intermoult and premoult chalimus larvae.

The cell groups and secretory material in all the samples at 5 dpi (58.35 DDPI) and 6 dpi (68.65 DDPI) appeared similar to those observed at 2 dpi. Neither C-group cells nor S2 material were distinguishable. In newly moulted chalimus a small amount

Figs. 3.14. - 3.15. Newly moulted chalimus I larva, 4 dpi (47.46 DDPI). Sagittal sections of anterior cephalothorax.

Fig. 3.14. The B-group cells appear vacuolized (*). Microvilli (arrow) are present on the surface of the B-group cells (B). Note the absence of filament material. Cells covering the duct (D) are passing under the B-group cells. Scale bar = 2.2 μm.

Fig. 3.15. The origin of the duct (D) is in close proximity to group A cells (A). The duct passes ventrally to the B-group (B) towards the anterior of the cephalothorax. Scale bar = 5 μm. Lu, lumen; E, eye.

3.15

of S1 material was found in contact with the anterior dorsal region of the B-group cells with structures resembling microvilli (Fig. 3.16). The two cell groups, A and B, were well defined. The B-group appeared to be partially surrounding the S1, forming a thin layer at the anterior end (Fig. 3.16).

The lice at 5 dpi differed from those at 2 dpi as follows: at 5 dpi the origin of the duct could be traced close to group A cells (Fig. 3.17); the pore observed in earlier stages extending through the cuticle at the anterior portion of the duct was not seen; cell groups A and B had large spaces between, and appeared more distant from each other (Fig. 3.18) and the external FF was present (not shown).

All the individuals sampled at 7 dpi (78.86 DDPI) showed similarities with copepodids in the intermoult stage at 3 dpi. All the groups of cells (A, B and C) and the internal filament material (S1 and S2) were seen at different stages of development. Two different degrees of S1 and S2 development were evident. Early in its development the S1 was not in contact with S2 material. Lamina of the S1 were evident and the B cells have long microvilli-like processes where they contact the S1 (Fig. 3.19). Later in its development the S1 was in contact with the S2 material. Lamination of the S1 was crescent-shaped anteriorly and had a parallel orientation posteriorly (Fig. 3.20). The microvilli-like processes of the B-group cells were completely lost (Fig. 3.20). At 7 dpi, four samples out of five had the S1 in contact with the S2.

Neither 3 dpi nor 7 dpi had a newly formed second cuticle. However the S1 and S2 were present within an hour glass-shaped pocket (Fig. 3.21).

The 7 dpi lice differed from those at 3 dpi in that: all the groups of cells and

Figs. 3.16-3.17. Chalimus I larva, 5 dpi (58.35 DDPI). Sagittal section of the anterior cephalothorax

Fig. 3.16. Note the early development of filament material (S1) partially surrounding the B-group. Microvilli (arrows) originating from cells of the B-group (B) extend into the S1 material. Scale bar = 4 μm. A, A-group.

Fig. 3.17. The origin of the duct (D) seen in earlier stages is among the A-group cells (A) close to the eyes (E). Scale bar = 4 μm. L, lens.

3.16

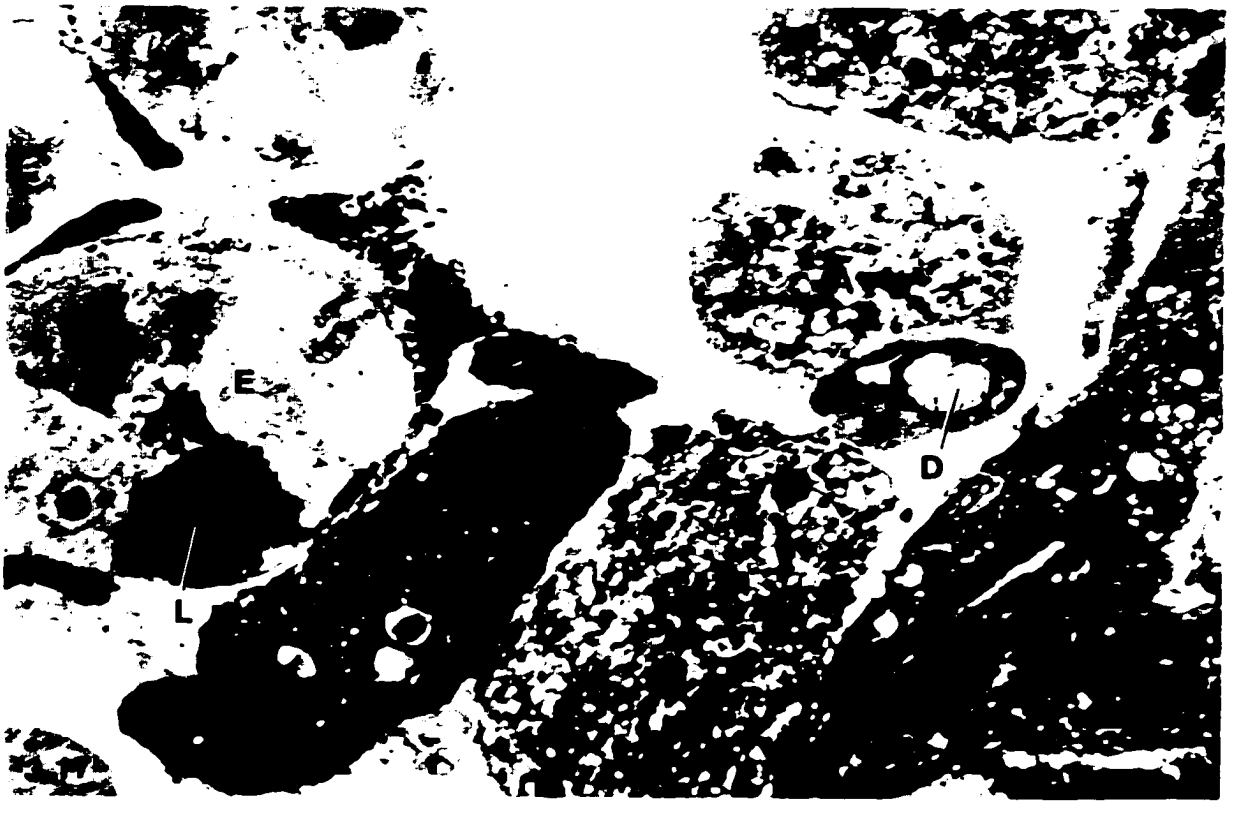


Fig. 3.18. Chalimus I larva, 5 dpi (58.35 DDPI). Horizontal section of the anterior cephalothorax. Large spaces (*) appear between the cell groups involved in the filament production. Scale bar = 3.3 μm. A, A-group of cells; B, B-group of cells; D, duct; E, eyes; EP, eye pigment; CL, cuticle layer.

3.18

Figs. 3.19-3.20. Chalimus I larvae, 7 dpi (78.86 DDPI). Sagittal section of the anterior cephalothorax.

Figure 3.19. The groups of cells (A, B and C), the internal material (S1 and S2) and external filament are present. The S1 material is crescent shaped and the B-group (B) posterior to the S1 conforms to the concave region of the S1 (arrowheads). The S1 and S2 are not in contact with one another. Scale bar = 6.6 μm. ST, stem.

Figure 3.20. The S1 material is within a more cuboidal shaped area. Lamina within S1 anteriorly are cresent shaped (*); posteriorly the orientation of the lamina (arrows) are parallel to surface of B-group (B). The S1 and S2 are now in contact with one another (arrowheads) and there is no evidence of microvilli-like structures on the surface of B group cells. Note the close association of the C-group (C) with S2 by their dark staining cytoplasmic extensions. Scale bar = 1.6 μm. C, C-group of cells.

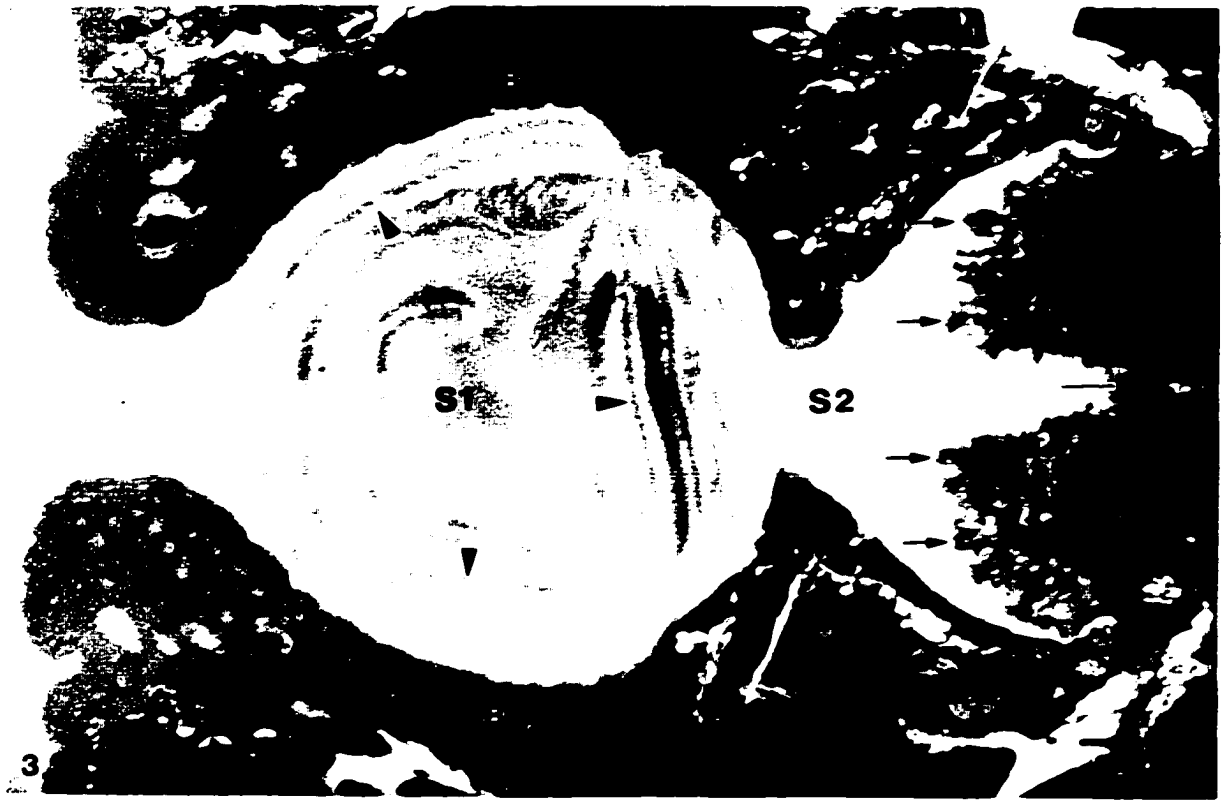
3.19

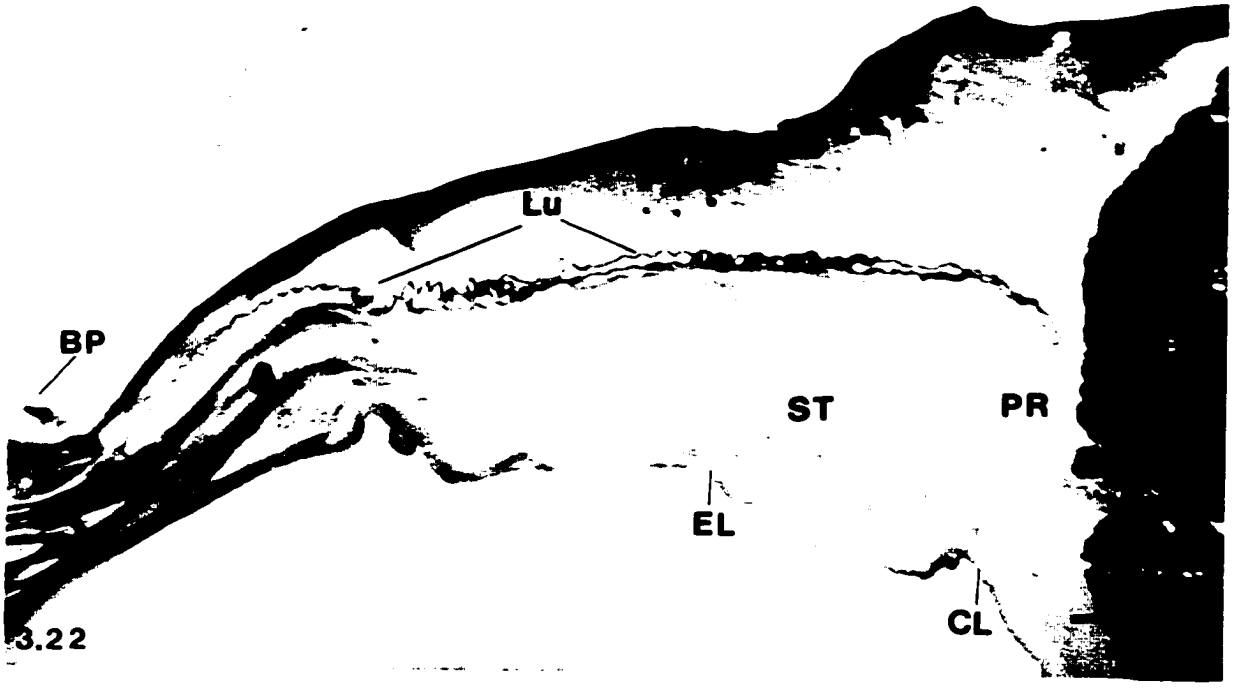
3.20

Figs 3.21- 3.22. Late-chalimus I, 7 dpi (78.86 DDPI).

Fig. 3.21. Horizontal medial section of the anterior cephalothorax. A completely formed internal FF (S1 and S2) is in an hour glass-shaped pocket without any cuticle lining. Note the long cytoplasmic extensions (arrows) from the C-group cells (C) into the S2. A lumen (Lu) passes through the C-group and S2. Scale bar = 2.8 μm. Arrowheads indicate the laminated pattern of S1; B, B-group.

Fig. 3.22. Sagittal section of the external FF. The proximal region (PR) of the stem (ST) is in contact with the B-group (B). The external lamina (EL) delineating the stem is in continuum with the cuticle (CL) and a lumen (Lu) extends through the stem. Scale bar = 2.8 μm. BP, basal plate.


S1
S2
3


Lu
BP
ST
PR
EL
CL
3.22

secretions associated with the FF production were more apparent, as were the duct and its origin (Figs. 3.21 - 3.22).

Five out of six chalimus I examined at 8 dpi (90.36 DDPI) showed similar characteristics to the late copepodid stage at 4 dpi. These individuals were near to moulting (premoult) as evidenced by the two surrounding layers of cuticle with the inner most cuticle layer (new cuticle) having abundant folds (Fig. 3.23). In these individuals the S1 and S2 were in full contact with each other forming a new FF. One individual examined was at an earlier stage in development having the S1 not in contact with the S2, similar to that seen in 7 dpi animals.

In the five premoult animals B-group cells surrounded the hour glass-shaped pocket but had lost most of their contact with other surrounding tissues (Fig. 3.23). A large portion of the S1 material projected out from the pocket and was in contact with the proximal portion of the stem of the external FF (Fig. 3.24). The origin of the duct within cells of the A-group was more distinctive (Fig. 3.25).

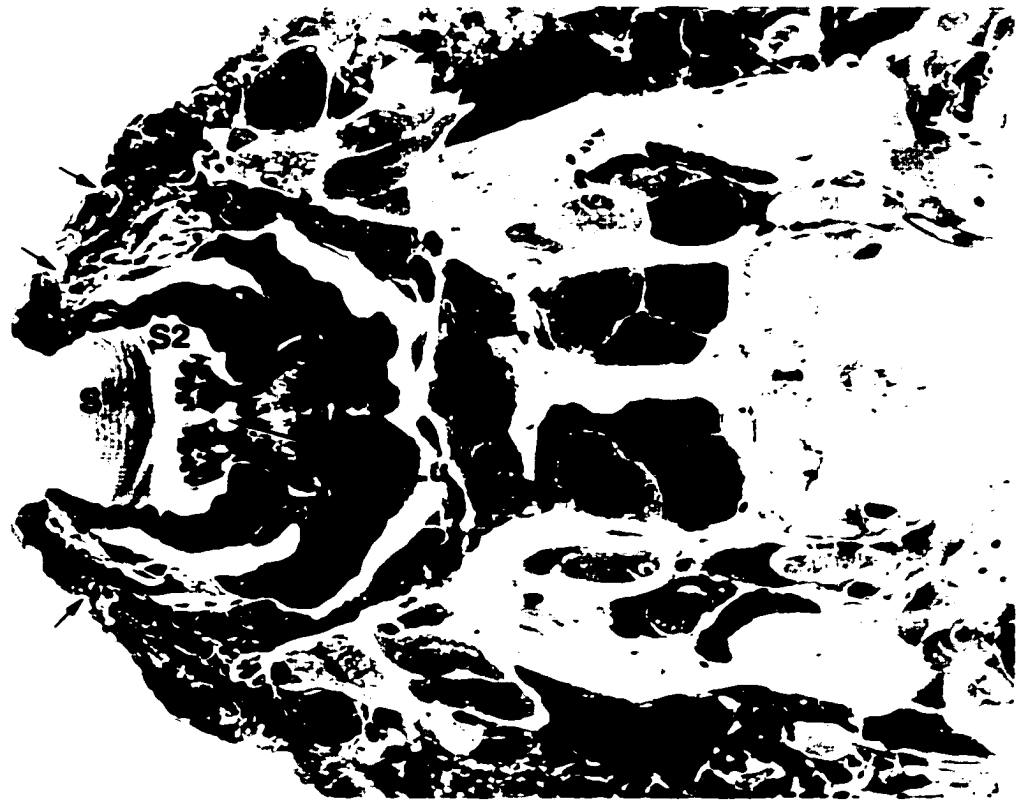
9 dpi (101.53 DDPI).

At this stage of infestation two out of seven of the chalimi examined were ready to moult (premoult) and showed characteristics similar to the late copepodid stage at 4 dpi and premoult chalimus at 8 dpi. The other five examined had completed moulting to the second chalimus stage. The shape of the anterior cephalothorax in the recently moulted louse was more slender, seeming more elongated than a premoult louse (Fig. 3.26).

Figs. 3.23-3.24. Late-chalimus I larva, 8 dpi (90.36 DDPI).

Fig. 3.23. Horizontal ventral section of the anterior cephalothorax. Note the presence of a completely formed FF (S1 and S2) contained within a cuticle lined pocket which narrows (*), resembling an hour glass. Abundant folds are present in the newly formed cuticle (arrows). The lumen (Lu) passes through S2. Scale bar = 10 μm. B, B-group; C, C-group; OC, old cuticle.

Fig. 3.24. Sagittal section of the anterior cephalothorax. All the groups of cells (A, B and C), the internal material (S1 and S2) and external frontal filament (FF) are present. A large proportion of the S1 material projects from the pocket and is in contact with the proximal portion of the stem (ST). Note the separation of B-cells (B) from adjacent tissue (arrow). Scale bar = 10 μm. CL, external cuticle layer; FF, external frontal filament, E, eye.



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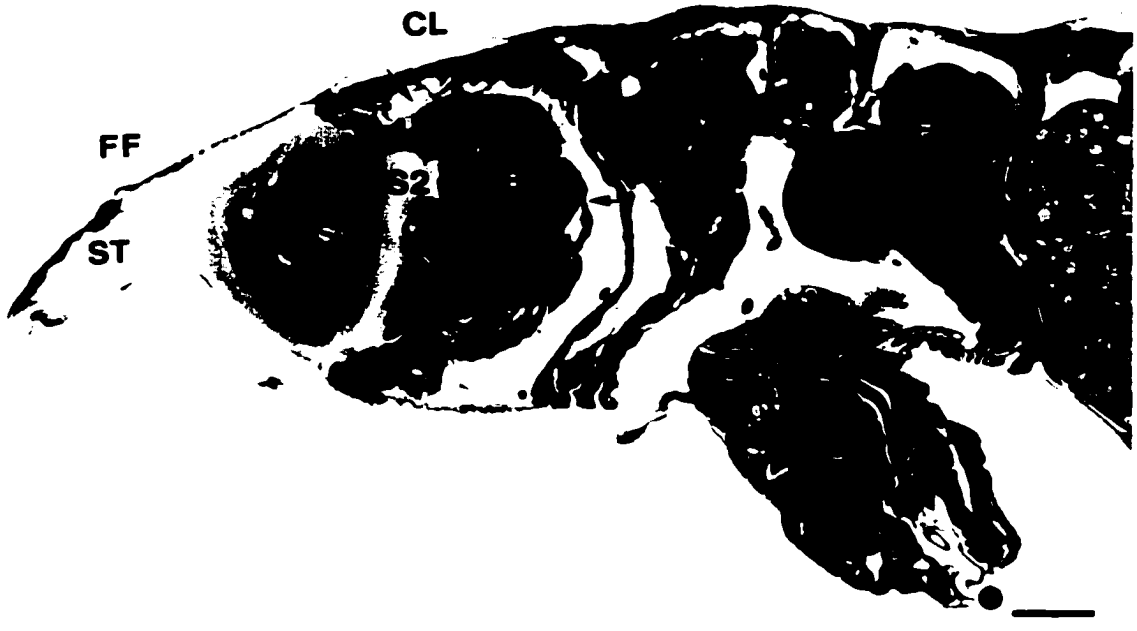


3.24

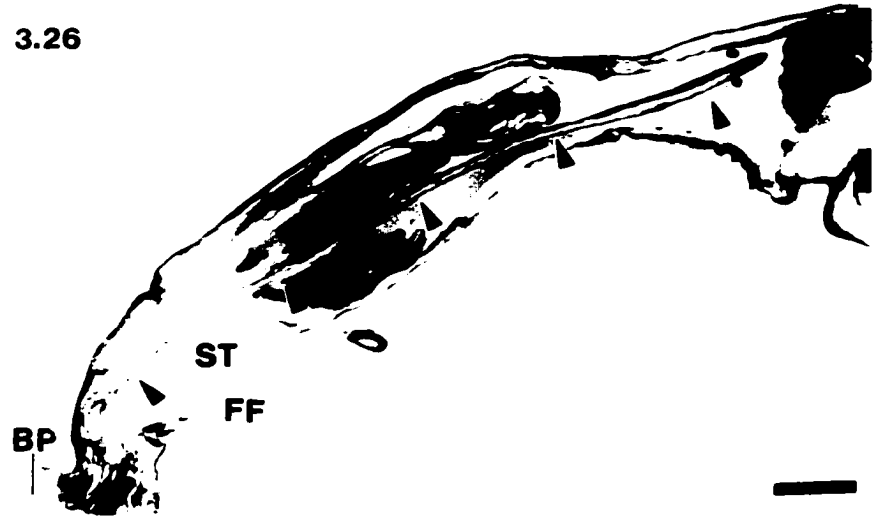
Fig. 3.25. Late-chalimus I larva, 8 dpi (90.36 DDPI). Sagittal section showing the origin of the duct (arrow) within A-group (A). Scale bar = 3.3 μm. D, duct.

Fig. 3.26. Newly moulted chalimus II larva, 9 dpi (101.53 DDPI). Sagittal section of the anterior cephalothorax. Note the elongated shape of the proximal region (PR) to the recently extruded frontal filament (FF) and the clearly traceable lumen (arrowheads) from the basal plate (BP) through the stem (ST). Scale bar = 10 μm.

Fig. 3.27. Late-chalimus I larva, 9 dpi (101.53). Sagittal section of the anterior cephalothorax. Note the thick cuticle lining the hour glass-shaped pocket, and the long cytoplasmic extensions (*) from the C-group cells (C) in contact with S2. Scale bar = 3.3 μm. B, B-group; D, duct; S1 and S2 Secretions.

3.25

3.26



3.27

The two premoult individuals had completely formed FF contained within cuticle lined hour glass-shaped pockets in the anterior cephalothorax. They could be identified as being near moulting as they had two layers of cuticle (Fig. 3.27).

The inner new cuticle demonstrated abundant folding (Fig. 3.27). The duct was evident and clearly traceable from the basal plate to the A-cells group and appeared to be the same as described for the earlier stages (Fig. 3.26). In the recently moulted animals there was no second cuticle beneath the external cuticle and no evidence of the S1 or S2. No old cuticle or FF was observed at this stage of development.

Figure 3.28 is a diagrammatic representation of the arrangement of groups of cells A, B and C, S1 and S2 material and duct in a chalimus.

3.2. Histochemistry of the FF.

The basal plate, stem, S1 and S2 were eosinophilic (acidophilic). The A, B and C groups and most other tissues showed basophilic staining. The internal filament material showed no picro-ponceau staining for collagen. Verhoeff's and Weigert's showed no differential staining, whether or not elastin or elaunin is present remains inconclusive. Two different recipes for alcian blue pH 2.5 and alcian blue/PAS showed no differential staining; the presence of neutral or acid mucins still inconclusive. Similar staining characteristics in the internal FF material with the extruded FF were observed in most of the stains used for routine light microscopy material.

No serial sections were obtained using JB-4 embedding medium for high resolution light microscopy. Most of the sections obtained were difficult to mount onto

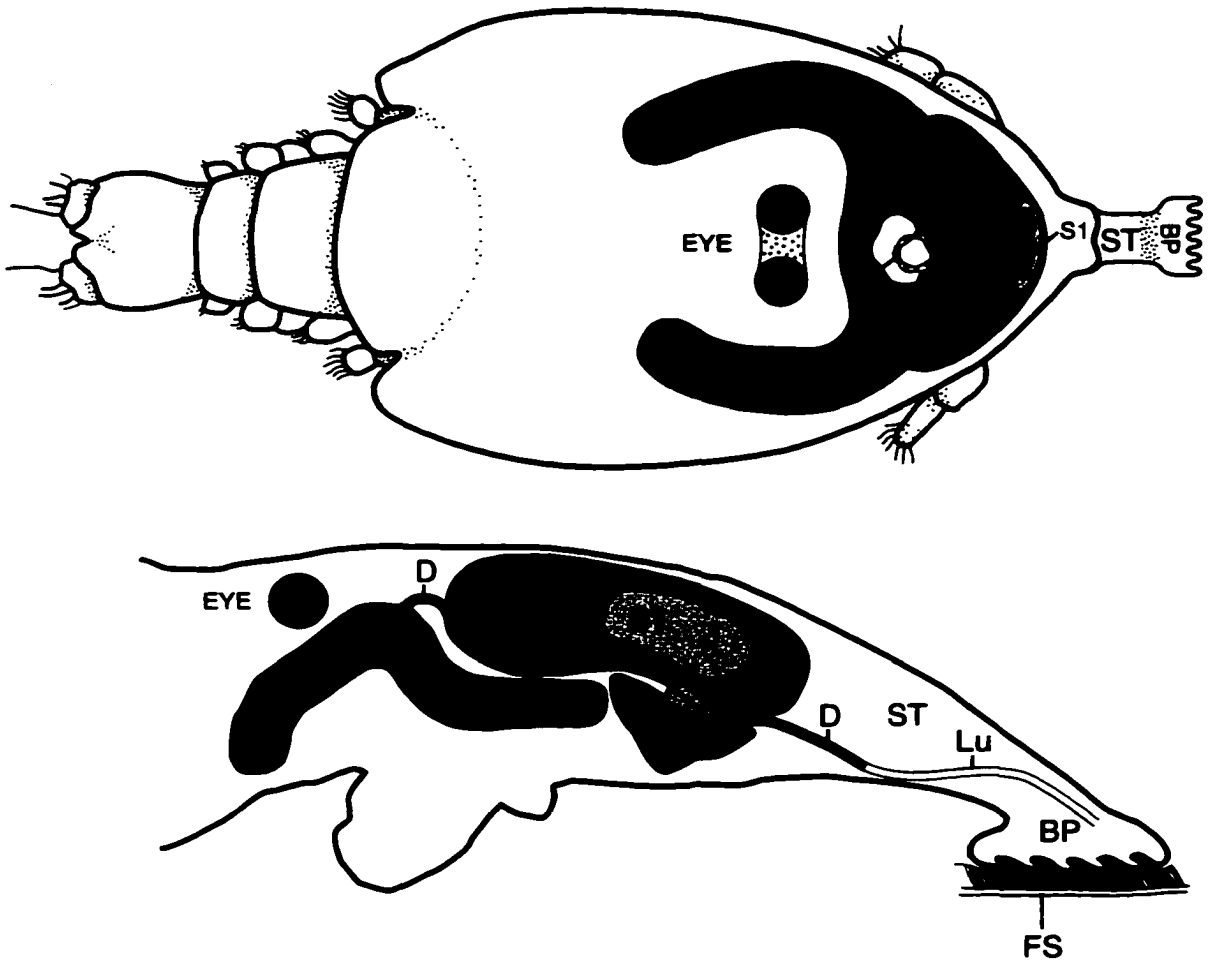


Fig. 3.28. Schematic representations showing the arrangement of cell groups A, B and C and internal filament material S1, S2 and duct (D) in chalimus at 7 dpi. (a) Dorsal view. (b) Sagittal view. BP, basal plate; ST, stem; Lu, lumen; FS, fish scale.

the glass slides and extensively folded. However, the following results were observed after Lee's staining of JB-4 embedded chalimi: 1) The basal plate stained pale pink and stained lighter than the fish scale to which it was attached, 2) The stem stained dark blue with a filamentous appearance, 3) The cell-groups stained dark blue, but showed no differential staining.

4. DISCUSSION

The frontal filament (FF) is the major attachment structure for the chalimus stages of parasitic copepods. For copepods within the family Caligidae several studies have described the structure of the FF and its mode of formation (25,26,40,41). However none of these studies provide sufficient detail for us to fully understand the timing and mechanisms of FF formation.

The objective of this study was to investigate both how and when the FF in the salmon louse *L. salmonis* is produced. High resolution light microscopy was used to examine the formation of the filament during development of the louse on Atlantic salmon. This study expands upon the work of Bron *et al.* (25) who initially described production of the FF in *L. salmonis* in limited detail. Improved knowledge about the morphological characteristics of the FF, its mode of production, and how the FF attaches to the host will provide the basis on which future research into methods which might prevent the attachment of the chalimus stages to the host can be conducted.

The present investigation describes the morphogenesis of FF during the copepodid, chalimus I and chalimus II stages of *L. salmonis*. These observations have been used to modify the diagrammatic representations of Bron *et al.* (25). Modifications were made with respect to the location of the groups of cells (A, B and C) involved in filament production, the positioning of the filament material (S1and S2) within the anterior cephalothorax, and the position of the axial "filament" duct. These modifications have resulted in a more detailed and precise description of the timing and mode of production of the FF of *L. salmonis*.

4.1. Frontal filament morphogenesis.

The results from the present study suggest that *L. salmonis* has a duct involved in FF extrusion and three cell groups responsible for FF production. Within the anterior cephalothorax two secreted materials (S1 and S2) are produced and stored until the moult. The extruded FF consist of a basal plate and a stem. The morphological evidence suggests that the basal plate is composed of S1 and the stem of S2.

4.1.1 Cell groups and secretions involved in FF production.

Several differences in morphology and histological characteristics of the A, B, and C cell groups as originally described by Bron *et al.* (25) were identified in this study.

In newly moulted copepodids, the location and the differentiation of staining properties of the cell groups A and B were similar to that reported in the copepodid and chalimus stages (25). However, in contrast to descriptions by Bron *et al.* (25), there was no evidence of any encapsulation of the cell groups by squamous cells.

Since boundaries between cells in the different cell groups (A and B) were indistinct, the different staining properties of the cell groups were used to define the boundaries and therefore the shapes of these individual groups. The A group was distinctly darker than the B group cells and intracellular granules were evident.

The A-group was observed in the present study to be the largest group through development to chalimus II and identified to be the origin of the axial duct, as first described by Bron *et al.* (25). However, the PAS-positive collecting ducts previously

reported from analysis of 5 μm thick paraffin sections by Bron *et al.* (25) were never observed in this study. It is possible that Bron *et al.* (25) misinterpreted the presence of this duct due to their use of paraffin sections with their limited degree of resolution.

Cells of the A-group may be responsible for the production of an adhesive which is carried via the axial duct and applied to the host to attach the FF (25). Although the origin of the axial duct within the A-group was traced and described in the present study, there was no evidence of any secretory material staining with toluidine blue or PAS inside the duct. However, there were secretory granules within the cells of the A-group.

The location and appearance of the B-group cells were similar to the description made by Bron *et al.* (25). Nuclei with pronounced nucleoli in cells of the B-group were more abundant than those in A and organized in rows. However, no granulated material was observed within the B-group cells.

Bron *et al.* (25) reported that the B cells divide ventrally, forming a commissure around the duct. The present study describes elongated B cells aligned parallel to the long axis of the duct which may function as support. Cells surrounding the duct appeared to be more than one layer thick, including a layer of cuticle and two types of tissue. There was no evidence that the cells surrounding the duct formed FF material but it is possible that they form cuticle.

Results from this investigation suggest that cells of the B-group produce the internal filament material S1 as discrete layers that accumulated with time to form a laminated pattern. The B-group cells had microvilli-like structures extending into the

S1 while S1 is being produced. The microvilli appeared to disappear prior to moult when secretion apparently concluded. A striated pattern in the internal filament has been reported for *C. elongatus* (41); however, the arrangement of layers are different from those observed in *L. salmonis.*

There was no evidence of S1 material early in the moult cycle. First production of this material in the copepodid appears to start within 22.21 DDPI hours of attachment to the host and at this time the surface of B cells had structures resembling microvilli where they contacted the S1. In newly moulted chalimus larvae the B cells have microvilli-like structures which extend into an empty reservoir into which the new S1 will be secreted. Since B cells in the copepodid stage contain no microvilli prior to the appearance of S1, it is possible that B-cells modify their cellular surface to form the microvilli-like structures when actively secreting S1 material.

Although many cells containing microvilli have an absorptive function, secretory surfaces of epithelial cells are also known to be characterized by microvilli (62). Microvilli-like structures have been reported to be associated with cuticle formation in crustaceans (63) and more specifically in some parasitic copepods (64).

In later stages of the moult cycle (premoult copepodids and chalimus larvae), the B cells were seen not to have the microvilli-like structures where they contact the S1. It is possible that when the B cells are no longer producing S1 material (ie. the material is fully formed and ready to be extruded), the microvillar surface of the cells of the B-group are lost. Electron microscopic studies are needed to further investigate the role of B-cells in S1 production.

The B-group changed in morphology as the S1 enlarged during the development, as reported in FF formation for larval parasitic copepods in general (65) and for *C. elongatus* in particular (41). In the present study, changes within the S1 laminated pattern were observed and the laminations followed changes in the shape of the B cells involved in production.

The third major group of cells (C-cells) were the most ventral and the most difficult to define. The C-group was identified both by its ventral location, as described by Bron *et al.* (25), as well as by the presence of dark staining cytoplasmic extensions where these cells contact the S2 (present study). In cells specialized for absorption and secretion, modifications associated with function are observed in the plasma membrane (66): either microvilli as presented by the B-cells or cytoplasmic extensions as presented by the C-cells. The C-group did not present any distinctive staining properties, contrary to the description provided by Bron *et al.* (25). Because of this, the C-group could not be distinguished early in the moult cycle of both the copepodid and chalimus stages in the present study. However, an unidentified group of cells was always observed anteroventral to the B-group in newly moulted individuals where the C-group is reported to be located (25).

The presence of secreted material (S2) beside the C-group has been described by Bron *et al.* (25). As discussed above there are long dark staining cytoplasmic extensions of C-cells into the S2 material. This characteristic suggests that the cells of the C-group are responsible for the production of S2 filament material. The presence of the cytoplasmic extensions may be a mechanism for increasing the secretory area of the

C-cells and/or a mechanism by which C-cells can align the S2 material to give it its characteristic filamentous looking structure. The differences in the appearance of the S1 and S2 in both internal and extruded filaments support the view that these two secretions are produced by different cell types, using different mechanisms of production.

The stem of the FF appeared to contain filamentous or membranous material near the tip. This may originate from the C cells. The C-group was observed to lose apical cells or portions of the cytoplasmic extensions with the extrusion of the FF. This suggests that the finger-like extensions of the C cells are torn from the cells and become part of the stem of the FF during extrusion. However, in contrast with the description provided by Johnson and Albright (26), there is no evidence of entire C cells within the S2 material or external FF since nuclei were not observed. After a period of recovery, the C cells started another secretory cycle.

The formation of the FF within the cephalothorax appears closely linked to the moult cycle of *L. salmonis* with a similar pattern of development seen in each of the copepodid and chalimus developmental stages studied. Early in the moult cycle, both the copepodid and chalimus stages were observed to have formed only S1. Later in the intermoult period both S1 and S2 were present in different degrees of development. Initially S1 and S2 are seen to be separated from each other. In the later stages of the intermoult period development has proceeded so that S1 and S2 are in contact with each other. At this stage S1 has lost contact with B-cells. Immediately preceeding the moult an internal FF formed by the fusion of S1 and S2 is present within a cuticle-lined

pocket.

The continued presence of fibrous material in contact with S2 suggested that these become the stem of the FF. The laminar nature, similar staining characteristics, and the proximal position of S1 (with respect to S2) suggest S1 becomes the basal plate as the external FF is formed. In contrast, Bron *et al.* (25) reported no relationship between S1 and S2. The present study indicates both secretions are contained within a cuticle-lined pocket forming the external filament, similar to an earlier description (26).

Whether the groups of cells involved in the FF production have another function besides those described in the present study remains unknown. It would be worthwhile to carry out a detailed study using TEM techniques to elucidate if these groups produce just one type of material or several different materials during development.

4.1.2. Duct.

In this study a duct which originated in the A-group and passed anteriorly through the ventral portion of the B-group was identified. In the copepodid stage this duct was seen to open to the outside via a small pore through the cuticle at the anterior margin of the cephalothorax in the vicinity of the rostrum. However in chalimus larvae there was no evidence of an external pore and the duct terminated near the proximal end of the FF stem. The pore was difficult to distinguish but presumably was present in the chalimus stages. It appeared that the stem of the FF covered this pore.

This duct is analogous to the main axial duct described by Bron *et al.* (25). It is theorized that this duct carries a proteinacious cement with a polysaccharide component

to form the basal plate (25).

Bron *et al.* (25) reported a structure at the tip of the rostrum in the copepodid stage which they called the filament duct. A pore was described at the end of this tubular structure. No filament duct was observed in the present study, but a pore in the cuticle was found near the rostrum. The fact that the duct emerged near to the rostrum as a pore suggested that the pore may function as an applicator for some filament associated material. There was evidence that the duct is coupled to this pore. The present study suggests that this pore allows a material produced by the A-group cells into the external environment. It is possible that this secretion is the glue-like material suggested by Johnson and Albright to bind the FF to the epidermis of the fish (26).

The present results suggest that the adhesive and the basal plate described by Pike *et al.* (40) are the basal plate and the distal stem, respectively. It is probable that either the basal plate attaches to the host integument by changing its properties or, more likely, by using a secretion carried by the axial duct to act directly as a glue.

Other researchers have previously described structures which appear to be similar to the pore observed in our study. Wilson (44) described a median sucker in *C. clemensi*. More recently, Anstensrud (42) observed a pore-like structure which he name the "filament gland" in *L. pectoralis*.

Piasecki *et al.* (41) reported a "T-shaped" slot located at the anterior margin of the cephalothorax of early copepodid of *C. elongatus*. They suggested that this slot was an opening where the filament is extruded to the exterior. Scanning electron micrographs of this "T-shaped" slot look morphologically analogous with the pore

observed in *L. salmonis* during early copepodid development. Although the function of this slot described for *C. elongatus* may be different than that described in the present study for *L. salmonis*, it is possible that both structures share more similarities than morphology.

Preadult and adult stages of caligid copepods have circular structures on the anteroventral margin of the cephalothorax which were originally described as sensory structures. These structures are now believed to be involved with the production of the temporary FF which are formed at the preadult moults (42). These structures have been referred to by several names including "frontal organ", "frontal gland", and "median sucker".

The presence of internal, closely packed villiform papillae in the frontal organ is described for both *C. elongatus* and *L. pectoralis* (41,42). These papillae were erroneously thought to be remnants of the cement gland and the FF in an earlier study using paraffin sections (67). Whether villiform papillae are present or not in the pore of *L. salmonis* in the copepodid stage remains unknown.

The pore seen in the copepodid stage of *L. salmonis* could be analogous to the frontal organ reported for the preadult stages of this species in previous studies (20). The frontal organ is reported to form a temporary FF in preadult and adult stages of *L. pectoralis* (42). Although nobody has reported the presence of a temporary FF in preadults of *L. salmonis*, it is possible that at these stages the A-group remains active to produce a temporary FF or material which may maintain the louse attached to the host while moulting occurs. The base of the proximal end of the FF is also where this organ

eventually forms, which also supports this hypothesis.

The pore is apparently cuticular in origin, since it stained similarly to the rest of the exoskeleton and was continuous with it, as previously described for *Caligus* spp. (45). No duct was associated with the structure described by Oldewage and Van (45), who suggested it was a cuticular ornamentation. Since only the duct was associated with the pore observed in our study, it is possible that the pore observed during the development of the copepodid stage is the same, or similar to, the cuticular ornamentation described by Oldewage and Van As for *Caligus* spp (45) and to the filament gland described by Anstensrud (42) for *L. pectoralis*, but in an earlier stage of louse development.

Within the extruded FF there was a PAS positively stained opening (lumen) from the proximal region of the stem to the basal plate (25). This lumen, previously described as carrying secretory material to form the basal plate (25), was not observed to run through the centre of the stem but tended to be orientated dorsally in most of the stem and then ventrally at the proximal end to join the indiscernible pore. This lumen had an irregular internal surface. This opening may not have any function and may be formed within the stem either by the pore or by the duct while the filament is extruded. While moulting, the louse is contracting its body to shed the old cuticle (42). Therefore, this lumen may be created in the stem at the same time the new FF is extruding.

The attachment mechanism suggested for *L. salmonis*, based on the present study, is similar to that described for *C. curtus* (67). Heegaard (67) described a drop of secretion emerging from the mouth of a gland which may be pressed out of a reservoir

where the secretion has been gradually amassed. The maxillipeds then carry the secretion from the mouth of the gland, pressing it down into a previously drilled lesion on the host to form a disk-like secretion. The rest of the secretion is then drawn out into a long thin thread to secure the attachment. Based on Heegaard's description (67) and the results from the currently study we are speculating that a similar process ocurs in *L. salmonis* when it is moulting: the attachment may begin with a drop of A-group secretion emerging from the pore and being applied to the host surface. The internal filament material (S1) would then be pressed down on this drop of secretion which is now firmly glued to the host. The larva then begins to back out and the S2 and the cuticle pocket are drawn away to form the stem of the filament which may also include some C-group cellular material. The stem may actually have stretched from the cephalothorax of the larvae, as suggested by the elongated shape of the anterior cephalothorax observed in newly moulted larvae. However, further studies on moulting behaviour would be useful to elucidate the moulting process in *L. salmonis* and its relation with FF formation.

The presence of the axial duct and the pore at the newly moulted copepodid stage prior to any other structure involved in the FF production suggests that other secretions are probably being applied through the duct and the pore from the A-group.

Bron *et al.* (25) concluded that the basal plate material is secreted via the axial duct from the A cells. Results from this study show no evidence that the basal plate is formed in this way. Since the pore is connected to the duct and the duct to the A-group cells, it is possible that the duct carries a glue-like secretion produced by the A-group to

the pore and this is applied to the host. This glue-like secretion would then fix the basal plate (S1) to the host.

4.1.3. External filament.

The first external FF was initially seen in newly moulted chalimus I stages at 47.46 DDPI. This FF consists of two major regions: the stem and a distal basal plate (25,26,40). Based on the observations made in the present study, I have confirmed that S1 and S2 material will become the FF and are extruded to the exterior to attach to the host, as was previously proposed by Johnson and Albright (26). In addition I propose that S1 becomes the basal plate and S2 the stem in the external FF.

The basal plate is a pale staining homogeneous substance which adheres to the fish skin surface adopting its irregular shape, as previously described (25,40). In this study the basal plate stained lightly with toluidine blue, and was strongly eosinophilic and stained with PAS, confirming the description by Bron *et al.* (25).

As reported by Bron *et al.* (25) and Johnson and Albright (26), the stem stained differently from the basal plate. Based on the observations from the present study, the stem consists of a distal and proximal region. The distal region contains dark staining filamentous material which may also include some C-group cellular material observed at the interface between S1 and S2. This filamentous material corresponds to that described in an SEM study of *C. elongatus* (40). The proximal region of the stem consists of pale staining amorphous material. Different mixtures of fibrous materials at the distal region may provide elasticity and flexibility to the stem to facilitate feeding

around the basal plate while maintaining permanent contact with the substrate (in this case the host fish) in similar ways as has been described for the byssus of *Mytilus edulis* (49). An elastic and a rigid region of the thread and a plaque adhering to the substrate has been described for *M. edulis* (49). The thread acts as a guy wire containing an elastic region that can stretch without breaking and absorb and transmit the turbulence-induced shocks to the rigid region which absorbs the shock and secures an anchor to the plaque.

In newly moulted larvae, C-cells were observed extending from the cephalothorax into the proximal region of the stem. This supports the suggestion that the filament material is pulled out as a mode of extrusion prior to hardening of the new cuticle (26). A large portion of the S1 material projected out from the cuticle pocket of the anterior cephalothorax of premoult (8 dpi) chalimus and was in contact with the proximal portion of the external stem, supporting the concept that the FF will be lost and replaced at the moult. Thus, the current study suggests that the FF is extruded at the moult to chalimus I and is replaced at the moult to chalimus II. Based on this it is possible that the FF in *L. salmonis* is replaced every moult during subsequent phases up to chalimus IV, as previously suggested by Johnson and Albright (26). Further studies on FF at chalimus III and IV are necessary.

Pike *et al.* (40) described the stem as being constrained by a sheath produced at the same time as the internal elements of the filament. The origin of the sheath was unclear. An external lamina continuous with the external cuticle covering the body of the louse was observed covering the stem, as described by Bron *et al.* (25).

The present study provides evidence that the hour-glass shaped, cuticle-lined pocket covering the S1 and S2 produced in premoult is extruded together with the secretions at the moult to form most of the external lamina of the stem of the FF after extrusion. Similar descriptions have been made for other Caligidae species (17,41,67). Moreover, the hour-glass shaped pocket cuticle varied in thickness similar to the thickness variations of the external lamina of the FF. I also observed that the external lamina is formed simultaneously with the external filament and not during formation of the stem, as described by Pike *et al.* (40).

As already mentioned, the components of the filament have a general morphological resemblance to the byssus threads of mussels (40). The structure and formation of the byssus attachment has been studied extensively. At least four different exocrine secretions are involved in the formation of the byssus threads (50,54). The byssus consist of three different regions that can be replaced rapidly (51). The flexible part of the thread is composed of collagenous and other fibrous proteins; the plaque contains an acid sulfated mucopolysaccharide protein having the ability to act as an adhesive so that the byssus binds effectively to wet surfaces (49). The byssus is surrounded by a protective lysine coat (49). However, in contrast with filament attachment, the information available on byssus and production glands is extensive and increasing due to the interest for the hold fast material and its potential as a strong waterproof adhesive for use in marine structures. Similar studies to those done on byssus could be helpful for a better understanding of filament attachment of parasitic

copepods.

4.2. Summary and Conclusions.

This study is the first to provide detailed information on the timing and cell groups involved in the formation of the FF of the salmon louse, *L. salmonis*. The FF is the major mechanism of attachment used at the chalimus stages in *L. salmonis* and other caligid copepods. The FF is a potential target for the development of new methods and/or therapies aimed at interfering with its formation or function, thereby negating the ability of chalimus larvae to attach to the salmon. This study of the FF of *L. salmonis* forms an important basis on which future studies can be built with the ultimate aim being to determine whether such methods of control are feasible. Future studies on *L. salmonis* FF will likely follow the same path as that followed by researchers looking to understand the mechanisms behind the attachment of mussels by byssus threads to substrates. Such studies will ultimately include: determination of structural composition and biochemistry of the internal and external FF materials and determination of the biochemistry of the cell-groups involved with filament production.

The production and extrusion of the FF is closely tied to the moult cycle of *L. salmonis* and may be under the control of the same hormones. In both the copepodid and chalimus stages the first type of secretion to form after the moult is the S1 which is apparently formed by B-group cells. The close apposition of B-cells and S1 and the morphological changes in the B-cells support this hypothesis. The similar staining characteristics of S1 and the basal plate suggest that S1 is the origin of the basal plate of

the external FF. C-group cells begin to produce a second type of secretion (S2) during the mid-intermoult period. Again, close apposition between C-group cells and S2 and morphological changes in the C cells were observed. The production of this material continued up to the premoult stage. Continued contact of the filamentous material, apparently originating from C-cells, with S2 suggests that S2 forms the stem of the FF. In the premoult stage S1 and S2 are contained within a cuticle-lined invagination of the anterior cephalothorax and have obtained a form similar to that of the extruded filament. The axial duct which is present in the copepodid and chalimus stages is postulated to carry a glue-like secretion formed in the A-group cells that is used to attach the filament to the host. This area of filament production warrants futher study both for the development of new sea lice control methods as well as the potential such substances have for use in medical and biotechnological applications. It would be interesting to see the effects of known moulting hormones or their inhibitors on FF formation.

The present study displayed a cyclic pattern in FF development between moults from copepodid to chalimus II (Table 4.1). Similar characteristics with respect to the presence and absence of internal FF material (S1 and S2) were observed at comparable points in the cycle. The newly moulted copepodid at 0 and 1 dpi had no S1 and S2. This lack of internal FF material is also characteristic of newly moulted chalimus I and chalimus II at 4 dpi and at 9 dpi respectively.

Once moulting is completed the copepod at 2 dpi forms the S1. This is a typical characteristic of this early intermoult stage which is also present in early intermoult chalimus I stage at 5 and 6 dpi.

Table 4.1. Summary of the FF development during copepodid, chalimus I and chalimus II. Days post-infestation (dpi), secretion 1 (S1), secretion 2 (S2), frontal filament (FF), newly-moulted (NM), intermoult (INT), pre-moulted (PRE), copepodid (Cop), chalimus (Cha), presence (✔), absent (✘).

dpi	S1	S2	FF	Stage
0+1	✘	✘	✘	NM, Cop.
2	✔	✘	✘	INT, Cop.
3	✔	✔	✘	INT, Cop.
4	✔ 75 %	✔ 75 %	✘	PRE 75 %, Cop.
4	✘ 25 %	✘ 25 %	✔	NM 25 %, Cha I.
5+6	✔	✘	✔	INT, Cha I.
7	✔	✔	✔	INT, Cha I.
8	✔	✔	✔	INT 17 %, Cha I.
8	✔	✔	✔	PRE 83 %, Cha I.
9	✔	✔	✔	PRE 29 %, Cha I.
9	✘	✘	✔	NM 71%, Cha II

The late intermoult copepodid at 3 dpi forms the S2. The S2, in combination with S1, gradually increases in amount and a single cuticle layer (exoskeleton) surrounding the body is observed at this stage. These characteristics are also distinctive in late intermoult chalimus I at 7 and 8 dpi.

Premoult copepodid at 4 dpi and premoult chalimus I at 8 and 9 dpi are characterized by presenting a second newly formed cuticle layer underneath the exoskeleton. The S1 and S2 material is surrounded by an extended portion of this cuticle layer, forming a cup-like shaped pocket. These characteristics precede an impending moult.

Although no observations were made on the third chalimus stage, it is likely that *L. salmonis* produces a new FF with each moult to subsequent chalimus stages. This is in contrast to *C. elongatus* which is reported to produce only one filament and to increase its length at each moult through the addition of new material. Whether this is actually the case is important as it has implications for any control mechanisms devised to interfere with filament production. The fact that *L. salmonis* does produce new filaments at each moult to the chalimus stage makes the possibility of using a control method based on interference with filament production more feasible.

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6. APPENDICES

6.1. Appendix A

Processing schedule for high resolution light microscopy, using Spurr's resin

1) - 3 % glutaraldehyde 1 h at room temperature

2) - 3 % glutaraldehyde overnight at 4°C

3) - Wash in buffer 2 x 10 min

4) - Post fix in 2 % OsO_4 x 2 h at 4°C

5) - Wash in buffer 2 x 10 min

6) - Wash in distilled water 1 x 10 min

7) - Dehydrate in 50 % ethanol 1 x 15 min

8) - Dehydrate in 70 % ethanol 2 x 15 min

9) - Dehydrate in 95 % ethanol 2 x 15 min

10)- Dehydrate in 100 % ethanol 2 x 30 min at 4 °C

11) - Infiltrate in 50:50 Spurr / 100 % ethanol 1 x 2 h

12) - Infiltrate in 75:25 Spurr / 100 % ethanol 1 x 2 h

13) - Infiltrate in 100 % Spurr overnight

14) - Embed in fresh 100% Spurr's resin

15) - Polymerize overnight at 70 °C

6.2. Appendix B

Processing schedule for high resolution light microscopy, using JB-4 embedding medium.

1)- Fixation in 10 % neutral buffered formalin

2) - Dehydrate in 90% ethanol 2 x 15 min

3) - Dehydrate in 100% ethanol 2 x 30 min

4) - Infiltrate in 100% resin and keep in cold (4 °C) 4 x 60 min

5) - Embed in JB-4 at room temperature in a plastic mould.

6) - Polymerize in a desicator.

6.3. Appendix C

Processing for routine light microscopy

1) - Formalin in 10% neutral buffered formalin

2) - Dehydrate in 70 % ethanol 2 x 1 h

3) - Dehydrate in 95 % ethanol 3 x 1 h

4) - Dehydrate in 100 % ethanol 2 x 1 h

5) - Cleared in xylene 2 x 1 h

6) - Infiltrated and embedded in Paraplast Plus ® (Camemco, Quebec, PQ)