FEEDING AND DIGESTION IN THE ECTOPARASITIC COPEPOD

LEPEOPHTHEIRUS SALMONIS (KRØYER, 1837)

A thesis submitted to the University of Stirling

for the degree of

Doctor of Philosophy

By

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1997

DECLARATION

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

Obdulio Andrade-Salas

TO MY WIFE REBECA

AND OUR KIDS:

OBDULIO, ESTEFANIA AND IVONNE

TO MY MOTHER, MY FATHER AND MY SISTER

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ACKNOWLEDGEMENTS

First, my thanks must go to my supervisor, Dr. Christina Sommerville for her guidance and support throughout this study. Also I must thank Dr. G.A. Boxshall from the Natural History Museum and Dr. R. Wootten, my external and internal examiners respectively, for agreeing to read through this thesis at such a short notice.

This PhD would not have been possible without the help of many people. I thank Marguerite Kobs, technician in the EM Unit of the Institute for her constant help and guidance in the use of the electron microscopes and her help with the processing of samples, and most of all, for her friendship and concern. I thank also Maureen Menzies, technician in charge of the Parasitology Laboratory for her help and support during all these years in her lab.

I thank all the staff in the fish farms, particularly those of the SSGA and Marine Harvest for allowing the collection of biological material .

Thanks also go to James, Helen and Carl for their friendship and help to get sea lice material during their field trips. Also thanks to Janet, my "temporary boss", I'll never forget those air-filled rolls!

Thanks to all the other PhD students of the Parasitology lab, who made my stay in the Institute so memorable: Andy S., Jim, Andy B., Dave, Angela, Will, Vinobaba, Sultana, Eva, Zafar, Emine, Carl, Yoon and Ahmet.

I must express my very special thanks to those friends whose help was invaluable during the writing stages: Luis Perez-Carrasco and Jimmy & Carol Turnbull, who gave me shelter during those homeless months; to Joaquin Vargas for his continuous supply of material from the library, to Rodolfo and Mónica del Río for their help with typing, Richard Smullen for the lifts to the Uni (and the occasional chili-beans!) and to all of them for their friendship.

I also must thank Dr. Lindsay Ross from the Institute, Dr. Carlos Martínez and Dr. Cristina Chávez from the CIAD Mazatlán, México and Dr. Guillermo Salgado from the IB, UNAM, México for their support in obtaining the scholarship to finish my studies.

I also thank my cousin Ramon Salas for being a valuable "electronic" link between both families (and with the bank!).

Lastly, but not least I must express my deepest gratitude to my dearest wife Rebeca and our loveable kids Obdulio, Estefanía and Ivonne. This PhD simply would not have been possible without their love, understanding, constant support and endurance, specially during those long months that we were separated.

My studies were financed through a scholarship from the Consejo Nacional de Ciencia y Tecnología (CONACyT)

ABSTRACT

A study has been carried out to investigate some aspects of the feeding and digestion of the parasitic copepod *Lepeophtheirus salmonis* (Krøyer, 1837), a serious pathogen of wild and farmed marine salmonids.

The alimentary canal consists of a cuticularised foregut and hindgut and a midgut, the latter comprising most of the length of the alimentary canal. It consisted of an anterior diverticulum, and anterior midgut, mid midgut and posterior midgut. All the midgut is lined in the luminal side with a monolayer of digestive epithelium. The latter is supported by a porous basal membrane. Mesenteries suspend the gut in the haemocoel and attach to the basal membrane by means of extensions of their cytoplasm that interlock with the pores of the basal membrane and are intimately associated with the cellular basal membrane of the enterocytes.

Four types of cells could be recognised ultrastructurally and were named according a widely accepted nomenclature for the types of digestive epithelial cells found in decapod crustaceans. R-cells were the most abundant type and were believed to be involved in the absorption of nutrients, storage of lipids and probably excretion of waste material. Once the R-cells have finished their useful life, it is proposed that they finally die after passing through a stage, called A-cell stage, comparable to apoptosis or programmed cell death, after which the A-cell is finally sloughed off the epithelium and discarded in the faeces. F-cells constituted the second cell type, in charge probably of the secretion of digestive enzymes. Once this stage is finished, F-cells are believed to transform into the third type of cells, the B-cells, which engage in intracellular digestion of nutrients. At the end of their useful life they are sloughed off the epithelium. The last cell type, the E-cells, are believed to be embryonic cells that differentiate either into R- or F-cells.

A revised mode of feeding is proposed, in which the louse scoops up strips of host skin epithelium of relatively constant size and shape, by means of the structures associated with the mouth cone. Analysis of the gut contents and the faecal pellets revealed that the main food item was host skin epithelium. The feeding activity usually reaches the dermal layer, suggested by the common

occurrence of host melanin in the gut and in the faecal pellets. Two types of faecal pellets were found. The first type was a cylindrical one which was made of the ingested host's epithelial cells with little signs of having being digested. Cylindrical pellets could be clear or dark depending on the amount of host melanin present in them. The second type of pellet was a tape- or ribbon-like gelatinous pellet produced mainly when the lice were not eating (as during starvation) or sometimes associated to the end of the cylindrical pellets. Pellets were always surrounded by a delicate peritrophic envelope composed of several peritrophic membranes. Blood was believed to be another important food item. Blood feeding was commonly associated with the production of lipids in the R-cells and it is proposed (but not confirmed) that this alternative food item could be associated with some physiological process that requires lipogenesis, like vitellogenesis.

A method for manually feeding the lice was devised. Salmon blood and milk were fed in this way. No sign of digestion of these food items was observed suggesting that possibly an endogenous factor different from the presence of food in the gut is necessary to trigger the digestive process.

An analysis of type of gut contents, peritrophic membranes and cell type occurrences during a digestive period was carried out. The results suggested that some digestive processes take place preferentially in one or another gut region. Mid and posterior midgut are probably associated with the production of faecal pellets, production of digestive enzymes, intracellular digestion and excretion of waste materials judging by the higher occurrence of peritrophic membranes, B- and A-cells in these regions. Absorption of nutrients probably takes place throughout the midgut.

The morphological changes in the topography shown by the midgut epithelium suggest that it is a very active and plastic tissue.

The results of this study were discussed and compared with similar studies of other crustaceans, including other parasitic and free living copepod species. Suggestions were made of the implications of the present findings in the search for a control method for this parasite.

1. INTRODUCTION

1.1 BACKGROUND.

The salmon louse *Lepeophtheirus salmonis* (Krøyer, 1837) is a common ectoparasitic copepod of wild and cultured salmonids in the Northern Hemisphere (Wootten, Smith & Needham 1982). Even though this parasite had been known since at least the end of the 18th century before Krøyer described it in 1837 (Berland & Margolis 1983), it was not until 1991 that Johnson & Albright (1991b) published a complete description of all its developmental stages.

Although restricted to salmonid fish, *L. salmonis* has an ample range of hosts including *Oncorhynchus clarki* Richardson, *Oncorhynchus gorbuscha* (Walbaum), *Oncorhynchus keta* (Walbaum), *Oncorhynchus kisutch* (Walbaum), *Oncorhynchus masou* (Brevort), *Oncorhynchus mykiss* (Walbaum), *Oncorhynchus nerka* (Walbaum), *Oncorhynchus tshawytscha* (Walbaum), *Salmo salar* L. and *Salvelinus fontinalis* (Mitchill) (Kabata 1979, Nagasawa 1987,Kabata 1988).

The commercial salmon farming industry started to develop in the 1960s in Norway and in the 1970s in Scotland, and Ireland, and today this parasite is the most commercially restraining in salmonid culture in northern Europe (see Costello 1993).

Infestations of this parasite can kill or weaken the fish, which makes them more susceptible to secondary infections and it may also be a vector of microbial pathogens (Nylund, Wallace & Hovland 1993). Most damage is caused by the larger mobile adults, although sessile chalimus stages can also injure fish (Jones, Sommerville & Bron 1990).

1.1.1 Biology

The life-cycle of *L. salmonis* is direct. An individual passes through 10 stages separated by moults in 5 phases: 2 planktonic nauplii, 1 infecting swimming copepodite, 4 feeding chalimus stages sessile on the host skin, 2 preadults mobile over the host skin and the final mobile feeding adult stage (Johnson & Albright 1991b). At 10 °C eggs hatch after about 8 days of incubation. The nauplius last 4 d, copepodid 10 d, chalimus 25 d, and preadults 17 d (males) to 22 d (females)

(Johnson and Albright, *op. cit.*). The longevity of the an adult louse is not known, but preliminary results show that the females can live up to 30 days (Tucker pers. Com.) or 35 days (Bron pers com.).

In *L. pectoralis*, the male deposits a pair of spermatophores over the genital apertures of the female after the female's final moult into the adult. The spermatophores provide enough sperm for the fertilisation of several clutches of eggs. (Anstensrud 1990a, Anstensrud 1990b). Ritchie, Mordue, Pike & Rae (1993) mention that the latter reproductive behaviour also occurs in *L. salmonis*. It is not known how many clutches a female can produce but observations in experiments in the Institute of Aquaculture (University of Stirling) have recorded up to ten, but two, three or four are common in experimental systems. In related species where the reproductive output has been studied, like *Lepeophtheirus europaensis* Zeddam, Berrebi, Renaud, Raibaut and Gabrion 1988 and *Lepeophtheirus thompsoni* Baird 1850, the maximum number of clutches that a female can produce is 10 and the time between the production of clutches is 6 to 8 days (De Meeüs, Raibaut & Renaud 1993a).

L. salmonis infections occur throughout the year, with gravid females present at any time. There are winter and summer generations which show seasonal variations related mainly with reproduction. Females from winter generations are larger, produce longer egg strings and more eggs, although smaller, than those of summer generations. Temperature seems to have the greater effect as compared to photoperiod (Ritchie *et al.* 1993).

1.1.2 Pathology

The damage caused by *L. salmonis* to its host is thought to be mainly due to its feeding activity (see below) on the host skin (Wootten *et al.* 1982, Egidius 1985). White (1942) suggested that *L. salmonis* feeds on skin, mucus and blood and Brandal, Egidius & Romslo (1976) demonstrated spectrophotometrically that the red pigment observed frequently inside the gut of adult females was salmon blood. There seems to be a certain preference for certain parts of the body, particularly the head and operculum of small salmon and post anal area in large salmon (Jaworsky & Holm 1992). Jónsdóttir, Bron, Wootten & Turnbull (1992) suggest that this distribution on the

host is probably the result of lice preferring areas where they are less likely to be displaced by the water flow.

Early lesions are recognised as grey patches on the places where the parasites have been feeding. More severe damage is recognised by sub-epidermal haemorrhages, more commonly seen in the perianal region and on the head the abrasion can be very severe, to the point of exposing the skull bone (Wootten *et al.* 1982). More recently, Jónsdóttir *et al.* (1992) described the histopathology caused by *L. salmonis* pre-adults and adults. Oedema, hyperplasia, sloughing of cells and general inflammation were associated with the areas where the parasites had fed and these changes could also be associated with the activity of the louse mouth tube, marginal membrane, second antenna, maxilliped and sternal furca.

1.2 CONTROL

Most attempts to control sea lice infections have resorted to the use of chemicals. At the present time only one pesticide has been licensed for use in salmon culture in the UK. This is the organophosphate dichlorvos (Aquagard). The chemicals and their toxicity and use have been reviewed in detail by Roth, Richards & Sommerville (1993). As an alternative to organophosphates, hydrogen peroxide has been in use recently with acceptable results. It degrades quickly to water and oxygen and the hazards to marine life are probably negligible (Costello 1993, Thomassen 1993).

Environmental concerns have obviously arisen over the use of pesticides. Biological control of *L. salmonis* has been considered by the use of wrasse, native cleaner fish (Labridae). These cleaner fish can remove sea lice from salmon but there are still several obstacles that have to be overcome before this practice becomes more widely used. Availability, acclimation to the cages, mortality and escapement are some reasons for apparent failures to use wrasse to control lice but the practice seems promising, although it may not be a complete answer. Successful commercial culture of these cleaner fish may help to allow a wider use of them (Costello 1993).

1.3 VACCINES

Non-chemical methods are the ideal alternative to the problem of lice control. Among these, the development of a vaccine has been considered as another alternative. Stone (1989) demonstrated a serum antibody response to experimental anti-*L. salmonis* vaccination and Grayson, Jenkins, Wrathmell & Harris (1991) recorded a naturally produced serum antibody response in salmon exposed to heavy chronic infestation. This instigated several researcher groups to search for the antigen or antigens that could give a protective immune response in the salmon.

In Australia, the use of "concealed" antigens, antigens that are normally not available to the host, was used to find an appropriate target for antibodies which resulted in the development of a vaccine to successfully control the cattle tick *Boophilus microplus* Canestrini (Willadsen 1987, Willadsen & Kemp 1988, Willadsen, McKenna & Riding 1988). The protective antigen of the successful vaccine was a structural protein situated on the luminal side of the apical membrane of the gut enterocytes (Opdebeeck, Wong, Jackson & Dobson 1988, Kemp, Pearson, Gough & Willadsen 1989, Wong & Opdebeeck 1989). Ticks feeding on vaccinated cattle showed extensive damage to the gut and leakage of lumenal contents into the haemolymph. This apparently contributed to damage to other tissues (Agbede & Kemp 1986). Stimulated with this success, several efforts have been made to develop a sea lice vaccine (Reilly & Mulcahy 1992, Jenkins, Grayson, Hone, Wrathmell, Gilpin, Harris & Munn 1993, Andrade-Salas, Sommerville, Wootten, Turnbull, Melvin, Amezaga & Labus 1993).

Since the main target for a successful vaccine would be the digestive system of the sea lice, an understanding of its morphology and physiology is essential. This was the main motivation for the present study. A host response, specific to sea lice, would be better transmitted in the blood but the feeding habits of *L. salmonis* are little known. Brandal *et al.* (1976) suggested that blood feeding could be more common in females and this fact has also been observed in other species of *Lepeophtheirus* (Zeddam, Berrebi, Renaud, Raibaut & Gabrion 1988). Therefore, the present study focused on adult females. Their size was convenient and the different possible diets (mucus, skin and/or blood) anticipated a range of physiological conditions that needed to be investigated.

1.4 ALIMENTARY CANAL STUDIES.

The function of the crustacean midgut is only partly known. Most work has been done on adult specimens of decapods. The hepatopancreas, for example, has been well studied in shrimps, lobsters, crayfishes and crabs. In Table 1.1 are grouped some selected works on the microscopic anatomy of the digestive system of some crustaceans other than copepods. The list is by no means exhaustive. There is a wealth of information about the digestive system of crustaceans, but the table groups some of the most cited works. Recent reviews that deal with the entire gut include Dall & Moriarty (1983) McLaughlin (1983) Icely & Nott 1992 and Brunet, Arnaud & Mazza (1994).

In decapods, the hepatopancreatic tubules are the sites of digestion and therefore most of the knowledge of the digestive processes in crustaceans comes from research on this part of the digestive system. The current nomenclature for cells in the hepatopancreas of decapods is based on the classification of Jacobs (1928) and Hirsch and Jacobs, (1928, 1930) [cited in Brunet *et al.* 1994]. Accounts on the structure of these cells are reviewed by Gibson & Barker (1979) and Icely & Nott (1992).

According to this classification, there are four basic cell types: E-(embryonic), R-(resorptive), F-(fibrillar) and B-(blister-like) cells. In some decapods, a fifth cell has been described, termed M-(midget) cell.

E-cells are undifferentiated and their function is to replenish the cell population of the epithelium. Cell division is confined to these cells.

F-cells have a well developed rough endoplasmic reticulum (RER) with numerous free and bound ribosomes and mitochondria evenly distributed throughout the cell. Golgi complexes are common and produce the enzymes needed for the extracellular digestion of nutrients.

R-cells are the most numerous. The smooth endoplasmic reticulum (SER) is well developed and the RER is scarce in comparison to that of the F-cells. Ribosomes are sparse but numerous mitochondria are present in the apical cytoplasm. Golgi bodies are active and associated with the SER in the basal region of the cell. Multivesicular bodies are common and can store copper and

other metals (Al-Mohanna & Nott 1989). R-cells are in charge of most of the absorption of nutrients. They store lipids and glycogen.

B-cells are dominated by a large vacuole. The apical cytoplasm contains numerous pinocytotic vesicles, which enlarge by fusion to form digestive bodies. The function of this cell seems to be primarily intracellular digestion.

There has been much debate about the affiliation of digestive cell types and at least four different views exist. One of them (Gibson & Barker 1979, Dall & Moriarty 1983, Icely & Nott 1992) holds that E-cells differentiate into either R- or F-cells. R- cells continue their role of absorption and storage, while F-cells synthesise and store digestive enzymes in supranuclear vacuoles. When this cell is mature, it transforms gradually into a B-cell which then secretes the enzymes accumulated during the F-cell stage.

The second hypothesis was modified from the one described above and is based primarily on observations in the shrimp *Penaeus semisulcatus* (Al-Mohanna, Nott & Lane 1985a, Al-Mohanna & Nott 1989). In it, F-cells have two roles. They synthesise digestive enzymes and secrete them into the lumen. After this has been achieved they start to uptake partially digested material from the lumen by pinocytosis transforming then into B-cells which will intracellularly digest and assimilate those nutrients and finally eliminate the waste products by extrusion of the cell into the lumen. R-cells in this theory absorb soluble nutrients and store lipid and glycogen.

A third hypothesis from studies on the freshwater crayfish *Astacus astacus* L. (Vogt 1994) states that all three mature cells, R-, F- and B-, originate independently from E cells. The role of F-cells of synthesising digestive enzymes has been demonstrated clearly by immunohistochemical methods (Vogt, Stöcker, Storch & Zwilling 1989) and no intermediate stages have been observed between F- and B- cells that support an affiliation between them. The function of B-cells in this hypothesis is to degrade exhausted digestive enzymes and any other waste products which remain in the hepatopancreatic tubules after absorption of nutrients.

The last hypothesis (Biesiot & McDowell 1995) sustains that F- cells transform into B-cells and then they secrete digestive enzymes. No uptake of material from the lumen is attributed to B-cells, which does not explain then their clear pinocytotic activity .

At present it appears that, at least in decapods, the main patterns of digestion occur according to the scheme proposed for *Penaeus semisulcatus* (Al-Mohanna & Nott 1987b) and summarised by Brunet *et al.* (1994).

In copepods, the main digestive function is taken over by the midgut in the absence of specialised digestive glands as in the Malacostraca. The basic structure of the digestive system is relatively well known. Basically it consists of a cuticle-lined foregut, a endoderm-originated midgut lined by a unicellular layer of epithelial cells and a cuticle-lined hindgut. Recent concise reviews include those by Blades-Eckelbarger (1984) and Boxshall (1992). Recently, Brunet *et al.* (1994) reviewed the cellular digestive processes in this and other groups of crustaceans. In regard to digestion, calanoid copepods are probably the better studied group, mainly because this is one of the most important groups of zooplankton. Table 1.2 assembles most of the studies on the microscopic anatomy of the digestive system in copepods.

Several different cells have been described from the epithelium of the midgut in copepods. In studies restricted to light microscopy most authors have recognised two types of cells. The basic difference between the two is the presence or absence of vacuoles. These cells have been named "vacuolated" and "non-vacuolated" (Park 1966, Raymont, Krishnaswamy, Woodhouse & Griffin 1974, Defaye, Such & Dussart 1985, Perkins 1994), "gland cell" and "columnar cell" (Rigby & Tunnell 1971), "secretory" and "adipose" (Changeux 1960), "amoeboid" and "columnar" (Briggs 1977), "vacuolar" and "microvillar" (Bron, Sommerville & Rae 1993) or with less descriptive names such as "type 4" and "type 3" (Sullivan & Bisalputra 1980).

Under TEM, several cells have been recognised and have been compared to those of decapods and so termed according to the same nomenclature for Malacostraca. Arnaud, Brunet & Mazza (1978) pioneered the use of this nomenclature and, thanks to this, certain systematisation in the descriptions of the alimentary canal has been achieved, largely for free living copepods (*e.g.* Arnaud *et al.* 1978, Arnaud, Brunet & Mazza 1980, Hallberg & Hirche 1980, Nott, Corner, Mavin & O'hara 1985). Unfortunately this is not the case for parasitic copepods, in which only Nishida, Oh &

Nemoto (1991) and Gresty (1992) have adopted this nomenclature. Arnaud *et al.* (1978) Added another cell type, the D-cell but could not establish a function for it. They considered this dense microvillar cell as a possible involutionary stage of other cell categories, mainly of R-cells.

Some cytochemical studies of the midgut of copepods, largely on calanoids, have been done to clarify the role of each cell type. R-cells have ultrastructural features of absorptive and storage cells (Arnaud *et al.* 1978, Arnaud *et al.* 1980). Contrary to what is observed in decapods, these R-cells have secretory granules in the cytoplasm which may suggest that they are involved in enzyme synthesis and secretion, although there is no cytochemical confirmation of this. Also, contrary to what is accepted for decapods, there has been suggested a possible affiliation of R- and F-cells, largely due to the combined set of intermediate morphologies that are commonly seen (Arnaud *et al.* 1980, Brunet *et al.* 1994).

B-cells in calanoids are very regionalised, occupying the posterior part of the anterior midgut where they are the dominant cell type. After several ultrastructural and cytochemical studies, their function appears to be well understood and their role and morphological evolution (Arnaud, Brunet & Mazza 1982, Arnaud, Brunet & Mazza 1984a, Arnaud, Brunet & Mazza 1984b, Nott *et al.* 1985) seems comparable with that described for decapods (Al-Mohanna & Nott 1986, Al-Mohanna & Nott 1987b): absorption of nutrients from the lumen of the gut by endocytosis, intracellular digestion of them and elimination of residues at the end of the phase when the cell is extruded from the epithelium. But, in calanoid copepods, B-cells seem to have no direct association with F-cells and probably arise independently from E-cells (Brunet *et al.* 1994).

Few studies deal with other free living copepods, like harpacticoids and cyclopoids. Although the terminology of cell types of calanoids was not used, the studies in cyclopoids reveal that similar cell types are present with slight variations in morphology (Musko 1986, Yoshikoshi & Ko 1991a). Also, the distribution of the cells is similar, with B-cells occupying a distinct area (Musko 1983). In harpacticoids this does not happen and the different cells seem to be distributed evenly along the midgut (Fahrenbach 1962, Yoshikoshi 1975, Sullivan & Bisalputra 1980).

Table 1.1 Selected studies on the alimentary canal of crustaceans other than copepods.

GROUP & SPECIES	STUDY	SOURCE
CEPHALOCARIDA		
Hutchinsoniella macracantha Sanders	Anatomy, histology	Elofsson, Hessler & Hessler 1992
BRANCHIOPODA		
Artemia salina L.	Gut physiology (osmotic regulation)	Croghan 1958, Foster & Wolfe 1986
Daphnia magna Straus	Ultrastructure of starvation alterations Ultrastructure of midgut	Elendt & Storch 1990 Quaglia, Sabelli & Villani 1976, Elendt 1989
Daphnia obtusa Kurz	Ultrastructure of midgut	Quaglia *et al.* 1976
Daphnia pulex Leydig	Fine structure of digestive system	Schultz & Kennedy 1976
Alona affinis L.	Ultrastructure of post. gut & caecum	Guenzl 1991
MAXILLOPODA		
BRANCHIURA		
Dolops ranarum (Stuhlmann 1891)	Anatomy, histology	Avenant-Oldewage & Van As 1990
Chonopeltis australis Boxshall	Anatomy, histology	Avenant-Oldewage, Swanepoel & Knight 1994
CIRRIPEDIA		
Tetraclita squamosa (Bruguiere)	Anatomy, histology	Johnston, Alexander & Yellowless 1993
MALACOSTRACA		
MYSIDACEA		
Spelacomysis longipes (Pilai & Mariamma)	Anatomy and histology	Nath & Krishna-Pillai 1971
AMPHIPODA		
Corophium volutator Pallas	Gut morphology & fine structure Feeding and digestion Physiology of digestion	Icely & Nott 1984 Icely & Nott 1985 Agrawal 1963b
Orchestia gammarela (Pallas)	Midgut posterior caeca fine structure	Graf & Michaut 1980
Talitrus saltator Montagu	Nutritional stress effect in hepatopancreas.	Storch & Burkhardt 1984
Niphargus virei Chevreux	Unusual basement layer of midgut	Francois & Graf 1988
Gammarus minus Say	Anatomy of gut musculature	Carlton & Schmitz 1989
Marinogammarus marinus Leach	Physiology of digestion	Agrawal 1963a
Caprella equilibra Say *Cyamus boopis* Lütken	Anatomy, Physiology (comparative study of digestive tracts)	Keith 1974

Cont...

Table 1.1 cont. Selected studies on the alimentary canal of other crustaceans.

GROUP & SPECIES	STUDY	SOURCE
ISOPODA		
Ligia oceanica (L.)	Starvation alteration in hepatopancreas	Storch & Lehnert-Moritz 1980
Ligia italica Fabricius	Midgut gland ultrastructure (under different nutritional conditions) Ultrastrucure of midgut cells	Strus, Burkhardt & Storch 1985 Strus & Drasla 1988
Cyathura carinata (Krøyer)	Anatomy, histochemistry, ultrastructure	Wagele, Welsh & Muller 1981
DECAPODA		
Penaeus semisulcatus De Haas	Hepatopancreas cell function	Al-Mohanna *et al.* 1985a Al-Mohanna & Nott 1986, Al-Mohanna & Nott 1987b Al-Mohanna & Nott 1987a, Al-Mohanna & Nott 1989
Penaeus aztecus Ives	Midgut ultrastructure	Talbot, Clark & Lawrence 1972
Penaeus setiferus (L.)	Enzyme distribution in midgut	Lovett & Felder 1990
Penaeus monodon Fabricius	Ultrastructure of anterior midgut diverticulae (larvae) Morphology, ultrastructure, physiology	Abubakr & Jones 1992 Vogt 1993 and own references within
Penaeus vannamei Boone	Ultrastructure of hepatopancreas	Caceci, Neck. D.H., Lewis & Sis 1988
Metapenaeus bennetae Racek & Dall	Hepatopancreas functional anatomy	Dall 1967
Homarus gammarus (L.)	Morphology & physiology	Barker & Gibson 1977, Mykles 1979
Homarus americanus Milne	Ultrastructure	Mykles 1979, Anger, Storch, Anger & Capuzzo 1985, Biesiot & McDowell 1995
Astacus astacus	Life cycle of enterocytes Enzyme localisation & biosinthesis	Vogt 1994 Vogt *et al.* 1989
Palaemon serratus Pennant	Starvation alterations in the hepatopancreas Immunohistochem. of hepatopancreas	Papathanassiou & King 1984 Malcoste, van Wormhoudt & Bellon-Humbert 1983
Procambarus clarkii Girard	Morphology, function of hepatopancreas Lipolitic sites and ultrastructure	Loizzi 1971 Loizzi & Peterson 1971
Procambarus belandingii (Harlan)	Growth & cell differenciation in heppancreas	Davis & Burnett 1964
Orconectes virilis Hagen	Morphology, function of hepatopancreas Isolation of digestive enzymes	Loizzi 1971. Devillez 1965
Carcinus maenas L.	Digestive cycle in hepatopancreas	Hopkin & Nott 1980
Cancer magister Dana	Ultrastructure	Mykles 1979
Scylla serrata Forskål	Anatomy and histology, physiology	Barker & Gibson 1978
Birgus latro L.	Starvation alterations in hepatopancreas	Storch, Janssen & Cases 1982
Hyas araneus L.	Structure under starvation and feeding (larvae)	Storch & Anger 1983
Atya spinipes L.	Secretion, restitution, resorption in hepatopancreas	Van Well 1955
Orconectes rusticus Girard	Enzymatic activity in B cells	Devillez & Fyler 1986

From Table 1.2 it is evident that half of the studies on the alimentary canal of copepods have dealt with parasitic forms, perhaps reflecting the importance of this group. In most cases though, the studies are at light microscope level and again most authors have proposed their own terminology of cell types adding confusion to the already diverse set of morphologies observed.

Ultrastructural studies are available only for 9 species of parasitic copepods (Poquet 1980, Yoshikoshi & Ko 1991a, Gresty 1992, Nylund *et al.* 1992). Among those, 7 are described as a "group" (Yoshikoshi & Ko 1991a) and therefore there is little individual detail of the cell types. The remaining 2 correspond to the studies of Gresty (1992) on *Mytilicola intestinalis* in which she could assign the cell types to R- F- B- cell types and Nylund *et al.* (1992) who, studying *L. salmonis*, found some correspondence to these cell types (type "I" and "II" to R- and F-cells and type "III" to B-cells) but did not adopt the nomenclature.

The latter is the only ultrastructural study of *L. salmonis* available. Before, Bron (1993) and Bron *et al.* (1993) studied the gut morphology of *L. salmonis* larval stages by light microscopy and recognised 3 types of cells and correlated them to those of Arnaud *et al.* (1978): vesicular (B-cell), microvillous (R- and/or F- cell) and basal (D cell). These three last works (Nylund *et al.* 1992, Bron 1993 and Bron *et al.* 1993) represent all that is known about the gut epithelium morphology and function of *L. salmonis*.

1.5 FEEDING HABITS

Little is known about the behaviour or the feeding habits of *L. salmonis*. Adult males and preadults of both sexes are reported to be more active on the host than adult females (Johannessen, 1975 [cited in Bron 1993]) and brief observations of the behaviour of the louse while on its host have been mentioned by Kabata & Hewitt (1971).

Table 1.2 Studies of the alimentary canal of copepods. F: free-living, P: parasitic. P/C: parasitic or commensal.

SPECIES	MODE OF LIFE	STUDY	SOURCE
CALANOIDA			
Calanus finmarchicus Gunnerus	F	Anatomy and histology	Dakin 1908, Lowe 1935, Raymont *et al.* 1974, Hallberg & Hirche 1980, Marshall & Orr 1955, Hirche 1989
Calanus hyperboreus Krøyer	F	Digestive enzyme activity	Hirche 1989
Calanus helgolandicus (Brodsky)	F	Anatomy, histology, histochemistry Anatomy, histology, ultrastructure	Ong & Lake 1969, Arnaud *et al.* 1980, Hallberg & Hirche 1980, Nott *et al.* 1985 Arnaud *et al.* 1980
Calanus pacificus Brodsky	F	Digestive enzyme activity with different diets & starvation	Hassett & Landry 1990
Lophothrix frontalis Giesbretch *Scottocalanus securifrons* (T. Scott)	F	Anatomy, ultrastructure	Nishida *et al.* 1991
Centropages typicus Krøyer	F	Anatomy, histology and ultrastructure Enzyme cytochemistry	Arnaud *et al.* 1978, Arnaud *et al.* 1980, Arnaud, Brunet & Mazza 1987 Arnaud, Brunet & Mazza 1983, Kabata 1979, Arnaud *et al.* 1984a
Epilabdocera amphitntes McMurrich 1916	F	Anatomy and histology	Park 1966
Nannocalanus minor (Claus) *Temora stylifera* Dana *Labidocera wollastoni* Lubbock *Acartia clausi* Giesbretch *Candacia armata* (L.)	F F F F F	Anatomy, histology histochemistry and ultrastructure	Arnaud *et al.* 1980
Euaugaptilus placitus (Scott)	F	Anatomy	Boxshall 1985a, Boxshall 1985b
Eudiaptomus gracilis Sars	F	Anatomy and histology	Musko 1983, Musko 1988
Acartia tonsa Dana	F	Histology diel cycle, enzyme activity	Hassett & Blades-Eckelbarger 1995
Hemidiaptomus ingens (L.)	F	Histochemistry, ultrastructure	Arnaud, Brunet & Mazza 1991
HARPACTICOIDA			
Diarthrodes nobilis (Baird)	F	Feeding behaviour	Hicks & Grahame 1979
Diarthrodes cystaceus Fahrenbach	F	Anatomy and histology	Fahrenbach 1962
Tigriopus japonicus Mori	F	Anatomy and histology Morphology of absorption Ultrastructure Peritrophic membranes structure. & function Cellular renewal in midgut	Yoshikoshi 1975 Yoshikoshi 1988b Yoshikoshi 1988a Yoshikoshi & Ko 1988 Yoshikoshi 1980
Tigriopus californicus (Baker)	F	Anatomy and histology	Sullivan & Bisalputra 1980
CYCLOPOIDA			
Cyclops strenuus Fisher	F	Mineral concretions, ultrastructure	Durfort 1981, Durfort 1979
Macrocyclops albidus (Jurine)	F	Anatomy and histology	Defaye *et al.* 1985
Cyclops vicinus vicinus Ulianine	F	Anatomy and histology Ultrastructure	Musko 1983 Musko 1986

Cont...

Table 1.2 Cont. Studies of the alimentary canal of copepods.

SPECIES	MODE OF LIFE	STUDY	SOURCE
POECILOSTOMATOIDA			
Ergasilus sieboldi Nordmann	P	Anatomy and histology	Einzporn 1965a
Ergasilus orientalis Yamaguti	P	Histochemistry, digestive enzimes Peritrophic membranes structure	Yoshikoshi & Ko 1991b Yoshikoshi & Ko 1988
Neoergasilus japonicus Harada	P	Histochem. localization of digest. enzimes. Anatomy, ultrastructure	Yoshikoshi & Ko 1991b Yoshikoshi & Ko 1991a
Hyalopontius typicus Sars	F	Anatomy	Boxshall 1985a
Conchilurus quintus (L.) *Modiolicola bifidus* (L.) *Ostrincola koe* Tanaka *Panietis yamaguti* (Yin)	C/P C/P C/P P	Ultrastructure Histochemistry, digestive enzymes Peritrophic membranes structure & function	Yoshikoshi & Ko 1991a Yoshikoshi & Ko 1991b Yoshikoshi & Ko 1988
Mytilicola intestinalis Steuer	P	Histology, ultrastructure Ultrastructure, cycling of cells Ultrastructure Histochem. localization of digestive enzymes	Durfort 1975 Durfort 1977 Gresty 1992 Moore, Lowe & Gee 1978
Pseudomyicola spinosus (Raffaele & Monticelli)	P	Anatomy, ultrastructure.	Yoshikoshi & Ko 1991a
Sapphirina angusta Dana	C/P	Anatomy and histology	Marino & Onesto 1970
Paranthessius anemoniae Claus	P	Anatomy and histology	Briggs 1977
SIPHONOSTOMATOIDA			
Lepeophtheirus salmonis	P	Anatomy, histology (larvae) Anatomy, histology, ultrastructure (adult)	Bron *et al.* 1993 Nylund, Økland & Bjørknes 1992
Lepeophtheirus pectoralis Müller	P	Anatomy and histology	Scott 1901
Caligus minimus Otto	P	Ultrastructure	Poquet 1980
Caligus savala Gnanamuthu	P	Anatomy, physiology	Gnanamuthu 1948
Lernaea cyprinacea L.	P	Peritrophic membranes Ultrastructure	Yoshikoshi & Ko 1988 Yoshikoshi & Ko 1991a
Lernaeocera branchialis L	P	Anatomy and histology	Scott 1901, Capart 1948
Lernanthropus kroyeri Van Beneden	P	Ultrastructure	Poquet 1982, Poquet 1986
Lernaeolophus acertatus Ho & Honma 1983	P	Anatomy, histology, feeding	Ho & Honma 1983, Honma & Ho 1988
Lernaeenicus sayori Yamaguti 1939	P	Anatomy, histology	Honma & Ho 1988
Cardiodectes medusaseus (Wilson)	P	Ultrastructure of attachment organ	Perkins 1985
Peroderma cylindricum Heller 1865	P	Anatomy and histology	Monterosso 1930
Allantogynus delamarei (Sars)	P	Anatomy and histology	Changeux 1960
Melinnacheres steenstrupi (Bresciani & Lutzen)	P	Anatomy and histology	Bresciani & Lützen 1961
Pseudocharopinus dentatus (Wilson)	P	Anatomy and histology	Rigby & Tunnell 1971
Melinnacheres ergasiloides (Claus)	P	Anatomy and histology	Bresciani & Lützen 1975
Pseudocynus armatus Basset-Smith	P	Anatomy and histology	John & Nair 1975
Phrixocephalus cincinnatus Wilson	P	Ultrastructure	Perkins 1994
MISOPHRIOIDA			
Benthomisophria palliata Sars	F	Anatomy	Boxshall 1985a
MORMONILLOIDA			
Mormonilla phasma Giesbretch	F	Anatomy	Boxshall 1985a, Boxshall 1985b
NOT GROUPED			
25 species	F,C & P	Structure of peritrophic membranes	Yoshikoshi & Ko 1988

The precise composition of the diet of *L. salmonis* is unclear because it has not been demonstrated with hard evidence. Exceptions could be the study of Brandal *et al.* 1976 who demonstrated spectrophotometrically that the red contents of the digestive tracts commonly observed corresponded to the host blood ingested by the louse, and the study of Bron (1993) who observed large pieces of epidermis inside the oral cone of *L. salmonis* larvae, but not in the lumen of the midgut. He proposed that these fragments might be subjected to preliminary digestion by labral or other glandular secretions.

Other authors believe that *L. salmonis* feeds on mucus and epithelium and/or blood (White 1942, Hastein & Berjsgo 1976, Wootten *et al.* 1982, Egidius 1985, Johnson & Albright 1992). In other species of *Lepeophtheirus* the situation is similar, in the sense that the nature of the food that the parasites are ingesting is deduced indirectly. For example, *L. thompsoni* and *L. europaensis* are thought to feed on mucus. Once the females mate, they migrate from the host body surface to the branchial cavity and there *L. thompsoni* hangs onto the branchial filaments where it is said to become haematophagous while *L. europaensis* is believed to remain mucophagous on the walls of the branchial cavity, feeding only occasionally on blood (Zeddam *et al.* 1988).

As with other siphonostomatoids, *L. salmonis* is characterised by the presence of an oral cone which is comprised of a labium and a labrum. Other structures characteristic of caligids are present in the oral cone that help in the uptake of food. The mode of feeding of caligids has been described indirectly by Kabata 1974 by analysing the oral cone structure with the SEM. In this model, the fish skin is scraped off by movements of the strigil, a sclerotised dentiferous structure in the distal part of the labium, and this fragmented material is drawn back into the oral cavity with the aid of the mandibles. Some details of the mode of feeding of *L. pectoralis* have also been described (Boxshall 1977, Boxshall 1985a, Boxshall 1990) which also agree with this model of feeding.

1.6 STUDY OBJECTIVES

Although the general structure of the alimentary canal of *L. salmonis* is known there are still many aspects that have not been studied or that remain obscure. A better knowledge is badly needed as it has great implications in the developing of control methods, particularly vaccines.

The purpose of the present study has consequently been to conduct a series of approaches to broaden the knowledge of the function of the digestive system of *L. salmonis* with the intention that the knowledge gained helps in the improvement of existing methods of control or the development of new ones, and also to serve as a point of reference for further, more elaborate studies.

The present study has been divided in four main areas:

- General morphology of the alimentary canal.
- Feeding habits.
- Physiology.
- Digestive sequence.

1.6.1 General morphology of the alimentary canal.

The work in this section was undertaken with the combined use of morphological and histochemical studies to extend the present knowledge of the structure of the alimentary canal. Attention was given also to other structures generally not considered in similar studies but which can give valuable information about the physiology, such as the muscle network, the basement membrane and the mesenteries associated with the digestive tract. Also, with the observation of individuals with apparently different diets (blood and/or skin epithelium) it was expected to find a broader set of cell morphologies that would help to expand in their morphological characteristics, their function and how they may interact.

1.6.2 Feeding habits.

The main purpose in this chapter was to investigate how *L. salmonis* obtains its food and to determine more precisely the nature of the diet of the parasite. The structure of the oral cone was studied closely to try to associate its structure with its function. Attention was also given to the gut contents and the faecal pellets. It was deemed important also to pay special attention to any behavioural pattern that could be associated with the feeding activity.

1.6.3 Physiology.

Having established the structure of the alimentary canal and the nature of what is entering into it, the next step was to shed some light on what happens once the food is ingested. Again, answers to this issue were sought using visual, morphological and histochemical techniques to try to define what digestive processes might be taking place.

1.6.4 Digestive sequence.

It was hoped that once we had a better idea of how the digestive system was functioning we could put this knowledge to a test on a time scale basis to try to define the presence or absence of a pattern in the digestive processes. To do this the production of faecal pellets and the monitoring of the possible sequential changes in the gut morphology in time were investigated supported by the knowledge gained in previous sections.

2. GENERAL MATERIALS AND METHODS.

This chapter describes the materials and methods that are common to methodologies carried out throughout the present study. Methodologies particular to specific chapters will be given under the relevant chapter section.

The study was concentrated on adult females of *L. salmonis.* Their larger size was more suited to carry out the observations planned. In addition, they represent the main targets in any control programme that aims to break the life cycle of the parasite. A better knowledge of their biology would be advantageous in this respect.

2.1 SOURCE OF MATERIAL.

2.1.1 Parasite supply and maintenance.

Adult *L. salmonis* females were collected from infested fish at several salmonid farms on the west coast of Scotland. The host species was the Atlantic salmon *Salmo salar* L. since this species is the main marine-farmed species in Scotland. The lice were gently removed from the fish using curved forceps and placed in plastic bags filled with sea water from the site. The bags were then transported back to the laboratory in an insulated cool-box containing crushed ice. In the laboratory the lice were transferred to beakers or small aquaria filled with fresh sea water and fitted with an air supply.

2.1.2 Host supply and maintenance.

Salmon smolts were obtained from the facility at the Marine Station of the Institute of Aquaculture at Machrihanish. Two or three fish were maintained in a constant temperature (10°C) room in circular plastic bins of about 70 cm. diameter filled with sea water (33-34 ‰) and fitted with two Eheim filters and constant aeration. The fish were fed commercial salmon feed once a day.

2.2 MICROSCOPY.

2.2.1 Fixation

2.2.1.1 Fixatives selection and osmolarities.

Several preliminary tests were carried out to select a range of fixatives adequate for different protocols and to select a general fixative that was compatible with the majority of the histochemical techniques used, mainly those for the assessment of the presence of enzymes.

The tests consisted of processing and embedding adult female lice using a range of fixatives and two embedding media. Their suitability was assessed by observing the morphology of the tissue in sections stained with either Toluidine blue for plastic embedded material or Casson's stain for paraffin embedded material. Karnovsky type (glutaraldehyde and paraformaldehyde mixtures) fixatives with low concentrations of aldehydes (less than 2%) were tested for their possible suitability to preserve enzymatic activity. Other higher concentrations were also tested as well, as the main fixative used by Eisenman & Alfert 1982 (based on 4% glutaraldehyde) which was reported to give good results for marine invertebrates. Here it will be referred to as MIF (marine invertebrates fixative). The fixatives and their composition are outlined in Table 2.1 . After the initial tests it was observed that all the fixatives except MIF did not give good morphological preservation. The main fixation artefacts were swelling of the cells with the consequent budding of the apical surface, blebbing, fragmentation and vacuolation of the cytoplasm, loss of staining properties, frothy appearance of the cells, variable sloughing of cells from the basal membrane, from several cells to the complete loss of digestive epithelium. Some of these artefacts are illustrated in Plates 2.1 to 2.5 .

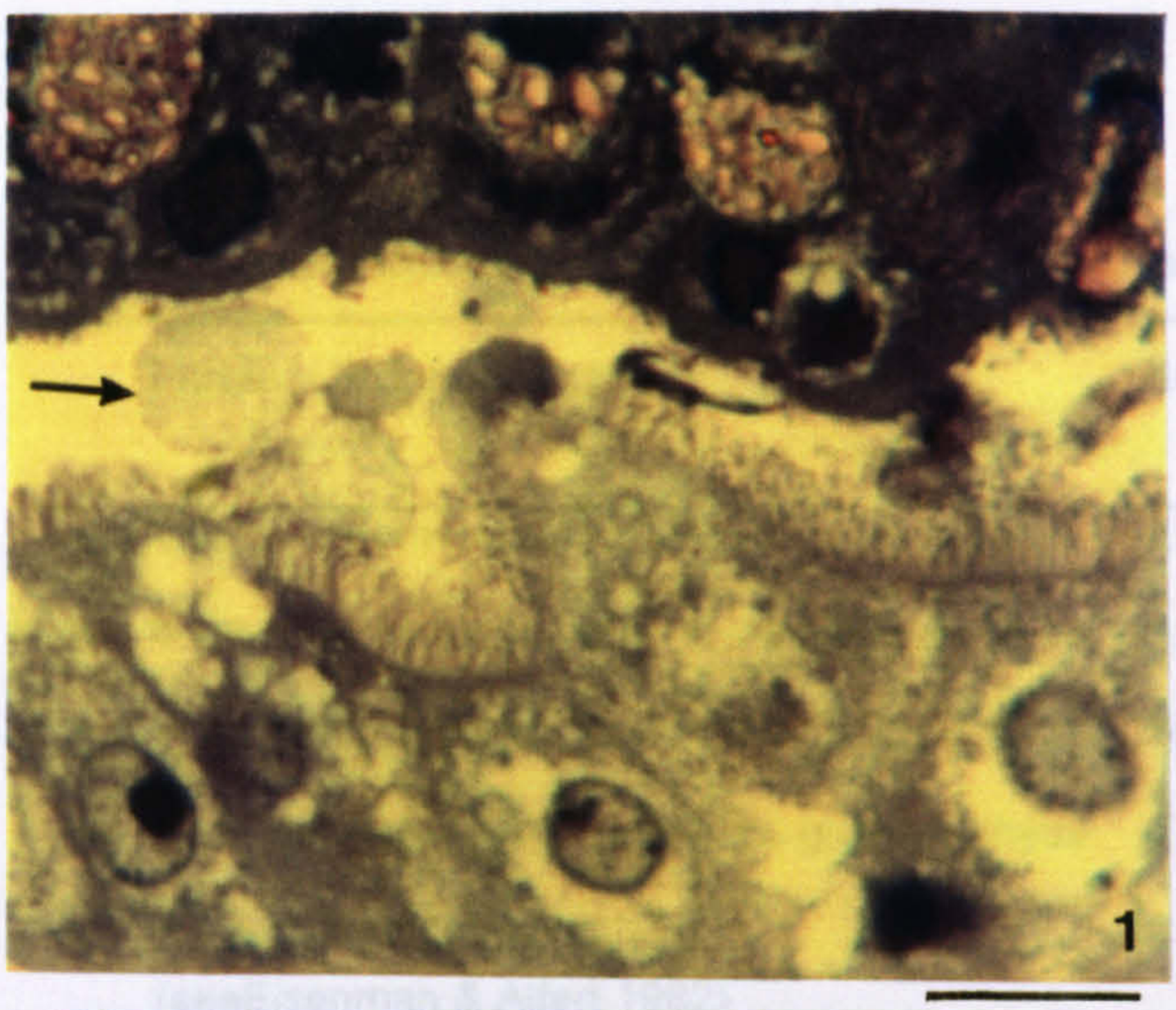
Plate 2.1 Extensive cell vacuolation with fragmentation of the cytoplasm and loss of organelles. Blebbing is also present (arrow). The cells at the top are from salmon skin epithelium ingested by the louse. Note that the latter is even better preserved than the louse's cells. TS through the anterior midgut. 1.6% paraformaldehyde + 1.3% glutaraldehyde in seawater. LMR. Scale bar 10 μm.

Plate 2.2 Various degrees of preservation of cells. Apical budding (arrows) and cell fragmentation (small arrow) are common. Some cells are relatively well preserved (arrow-heads). TS through the posterior midgut. 1.6% paraformaldehyde + 1.3% glutaraldehyde in 0.2 M cacodylate buffer. LMR. Scale bar 20 μm.

Plate 2.3 Complete loss of cell integrity. Many cells have burst leaving the basal membrane bare (arrow head). The remaining cells are barely seen due to the very low cytoplasmic density and almost complete lack of organelles. Some nuclei are still recognised. Compare with the cuticular epithelium cells at the top left corner which are better preserved. 2.5 glutaraldehyde + 1% paraformaldehyde in 0.1 M cacodylate buffer. LMR. Scale bar 20 μm

Plate 2.4 Cell fragmentation. The epithelium is completely disorganised. Sloughing of cells is extensive. The basal membrane is left exposed in some areas (arrow heads). 1.6% paraformaldehyde + 1.3% glutaraldehyde in 0.1 M cacodylate buffer. LMR. Scale bar 20 μm.

Plate 2.5 Apparently good preservation of cells. Note some with frothy cytoplasm and/or slight cytoplasm fragmentation (arrow). These cells under TEM would have a completely unacceptable morphology. LMR. Scale bar 20 μm.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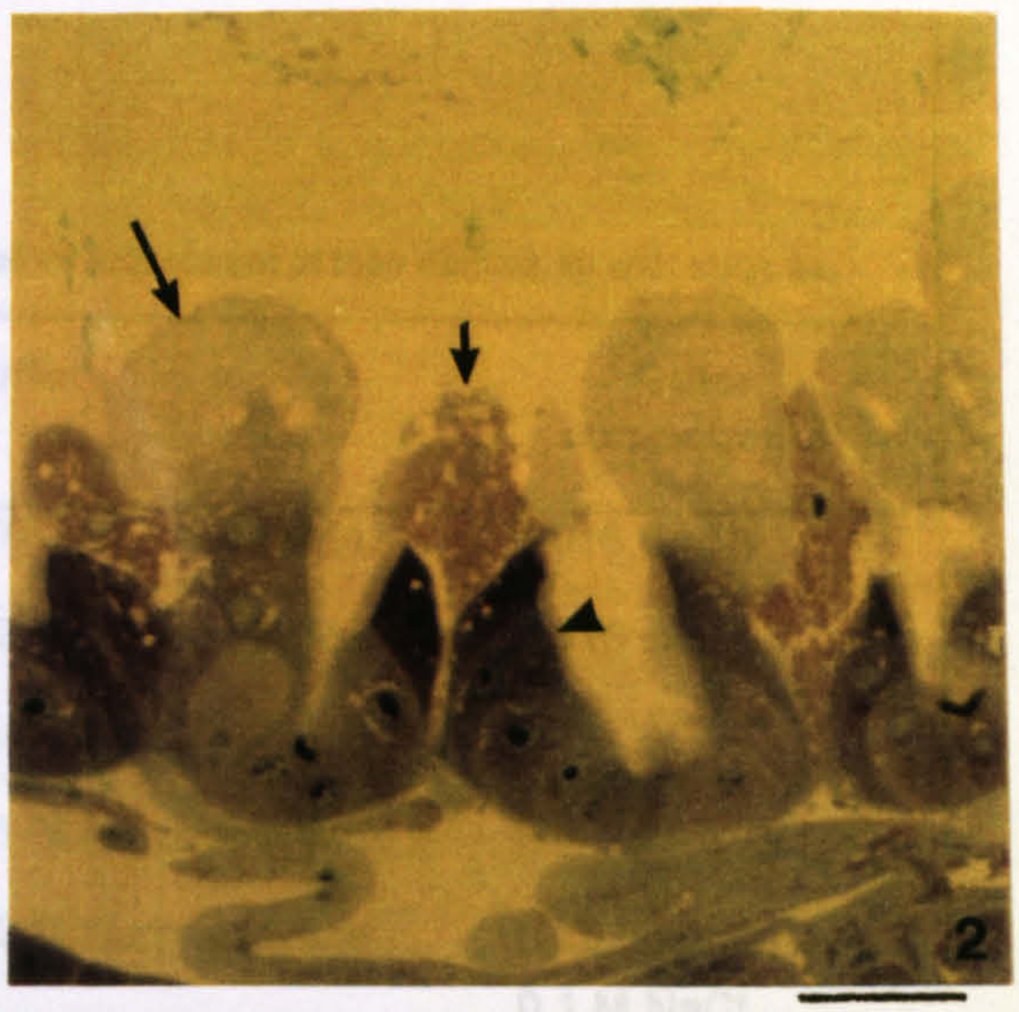
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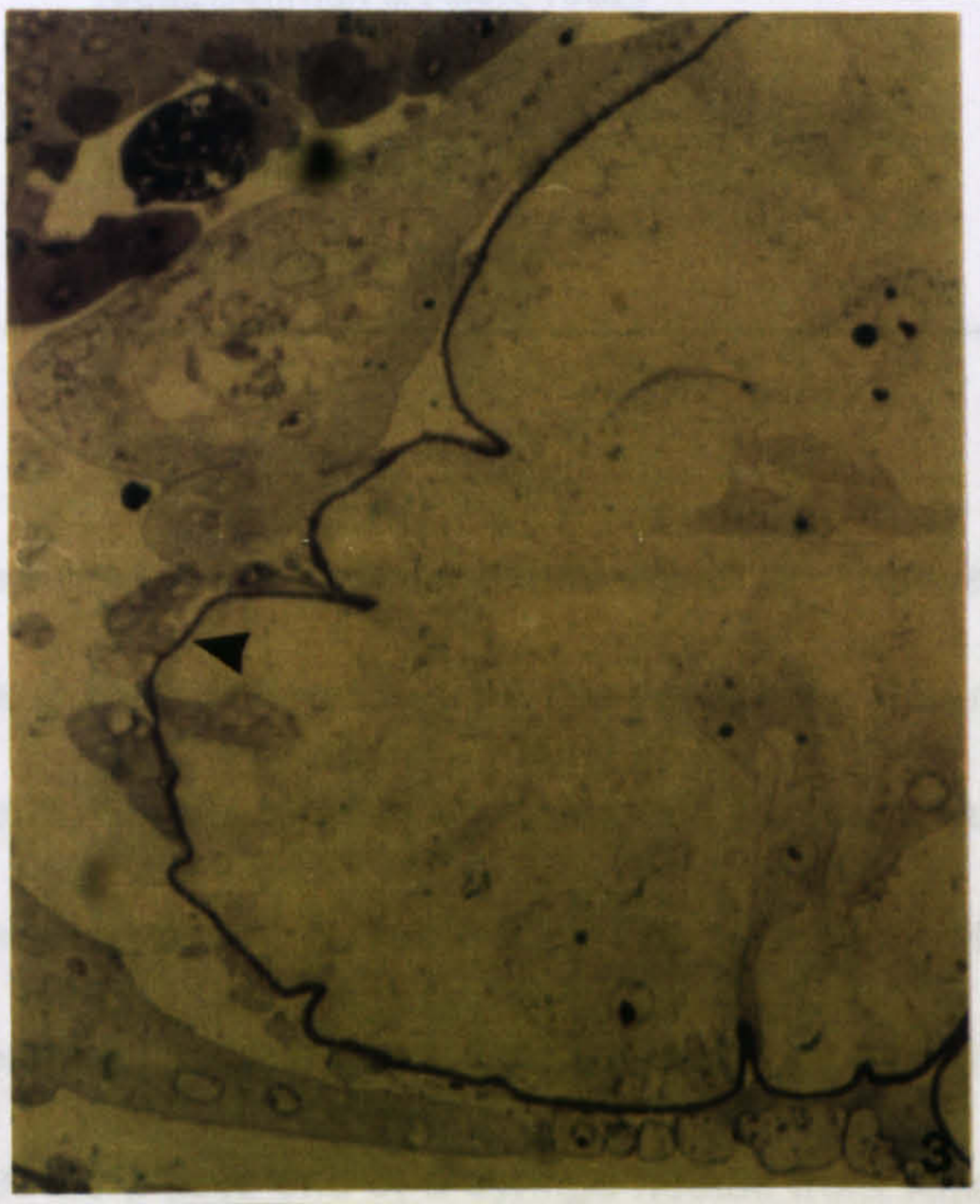
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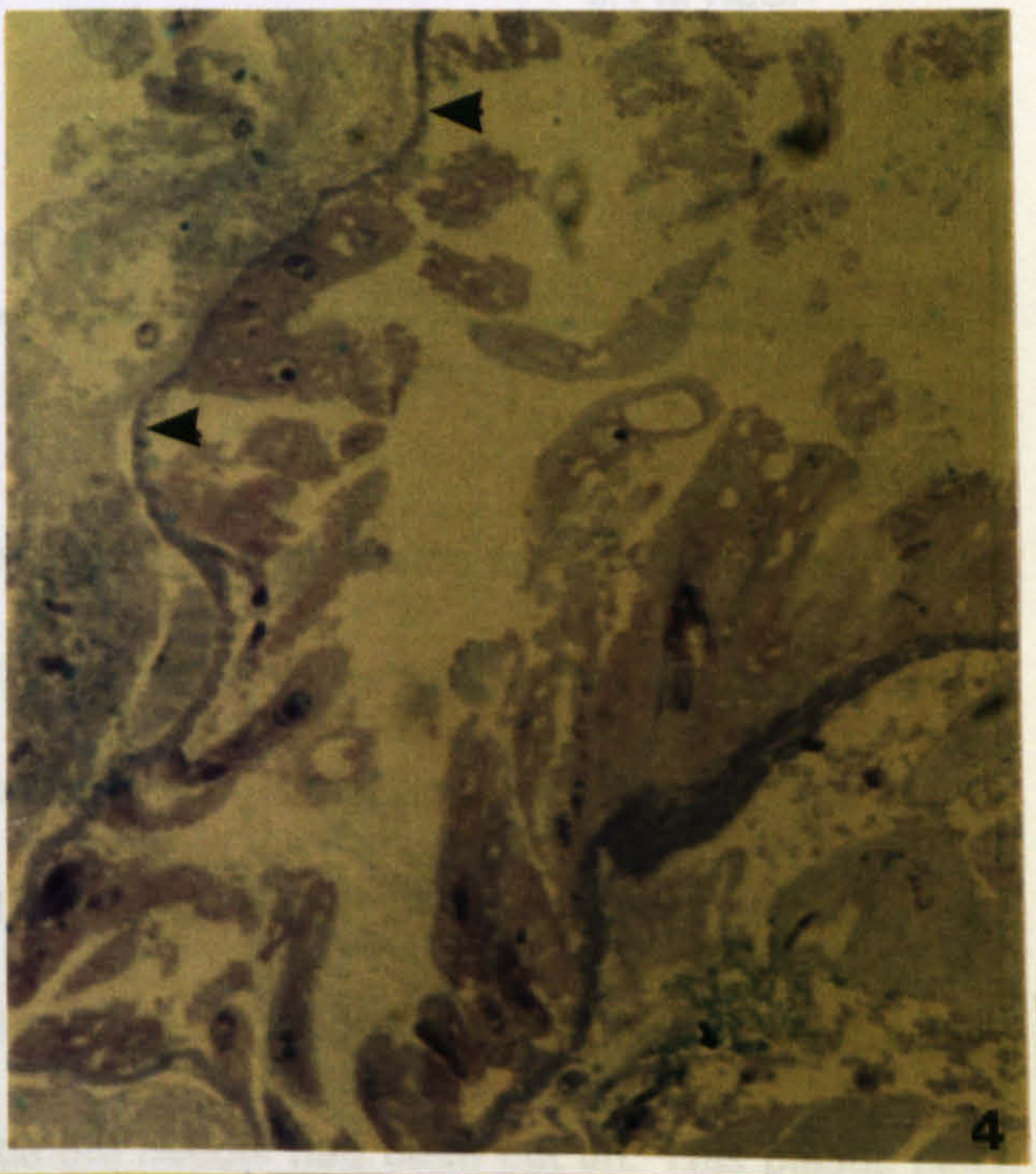
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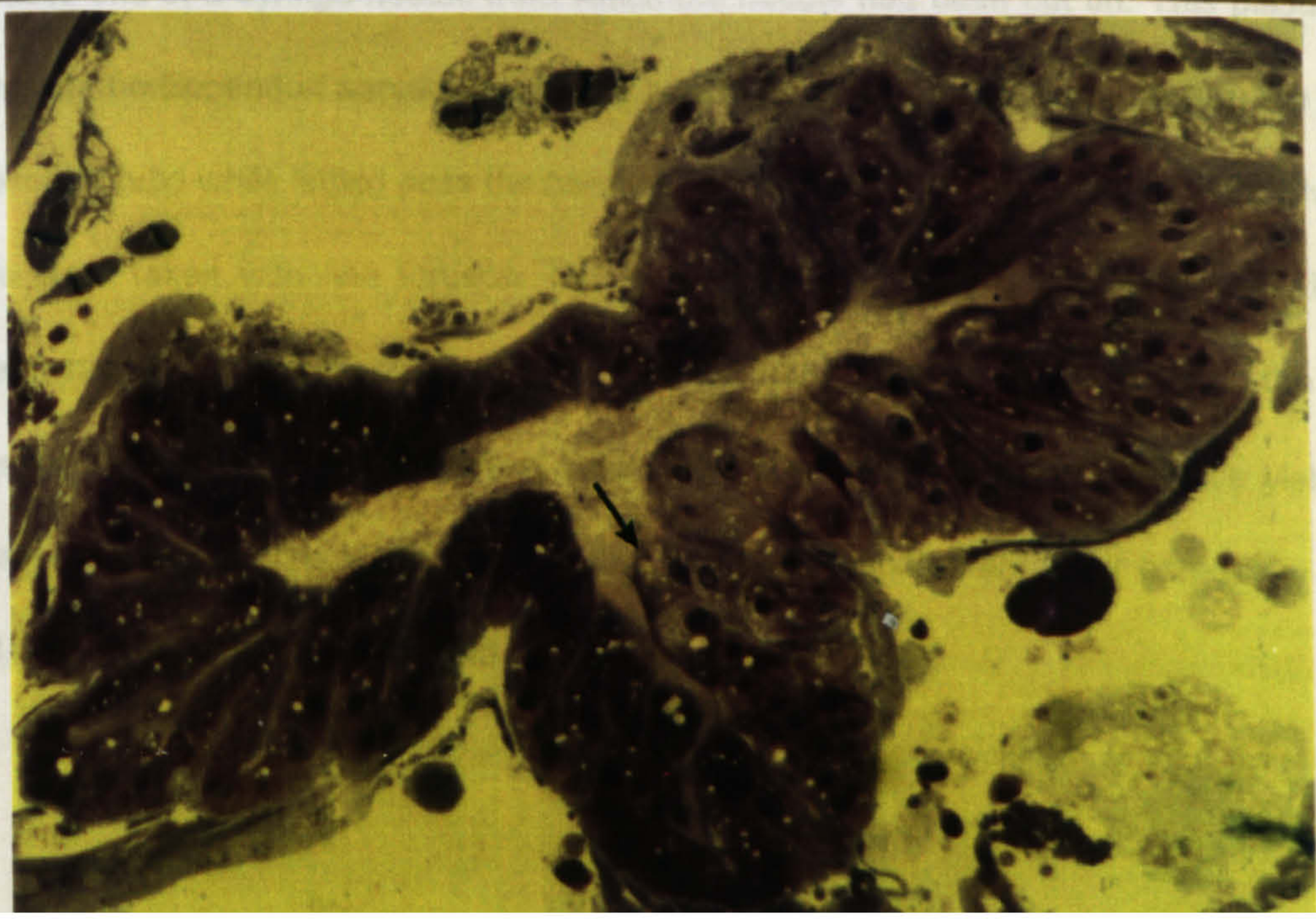


Table 2.1 . Composition of the fixatives tested in the present study before adjustment of their osmolarity with sucrose.

	Fixative & concentration	Buffer	Other components
1	1.6% paraformaldehyde + 1.3% glutaraldehyde	0.1 M Na cacodylate	0.016 $CaCl_2$
2	1.6% paraformaldehyde + 1.3% glutaraldehyde	0.2 M Na cacodylate	0.016 $CaCl_2$
3	1.6% paraformaldehyde + 1.3% glutaraldehyde	seawater	
4	1.0% paraformaldehyde + 2.5% glutaraldehyde	0.1 M Na cacodylate	
5	4.0% glutaraldehyde (MIF) (seeEisenman & Alfert 1982)	0.2 M Na cacodylate	0.35 sucrose 0.1 M NaCl
6	3% formaldehyde	0.2 M Na cacodylate	
7	10% formaldehyde	0.08 M phosphate buffer	
8	3% paraformaldehyde	0.2 M Na cacodylate	

It was suspected that the osmolarity of the fixatives was the main reason for the poor preservation. It was then decided to vary the osmolarity of some of the fixatives with sucrose to assess which was the more suitable concentration for each one. This was a trial and error test and the results are summarised in Table 2.2 . The osmolarity of the haemolymph of the adult females was also measured for reference. This was done by centrifugation using a 1 ml plastic Eppendorf tube fitted with the plastic end of a syringe needle from which the needle had been cut off. This plastic piece when put inside the Eppendorf served as a holder which did not allow the lice to pass to the bottom of the Eppendorf tube while letting pass the haemolymph extracted during centrifugation. Five adult female lice were taken with fine forceps. The anterior part of the cephalothorax was then cut transversally and the louse was put into the Eppendorf tube, the anterior part towards the bottom of the tube and the lid was closed. Several tubes were prepared in this way. Five lice yield about 20-30 µl of haemolymph. The samples of several tubes were pooled and their osmolarity and pH measured. The pH was between 7.1 and 7.2.

Table 2.2 Osmolarity (mosm) of selected fixatives after the addition of sucrose. The figures are the mean of three values read in an osmometer. Underlined values indicate that the best possible morphology was achieved with the corresponding concentration of sucrose added to the respective fixative solution. The number of the fixatives correspond to those of Table 2.1 .

	Molarity of sucrose.									
Fixative No.	**0.0**	**0.1**	**0.2**	**0.3**	**0.35**	**0.4**	**0.55**	**0.65**	**0.7**	**0.8**
1	437	-	-	720	-	900	1060	-	1290	1903
2	1075	1157	1242	-	-	1460	1600	1693	1750	
4	961	1070	-	1240	-	1430	1590	-		-
5	-	-	-	-	1295	-	-	-		-
6	890	-	-	1172	-	1277	-	-		-
7	1651	-	1870	1983	-	2120	-	-		-
8	1310	-	-	1728	-	1818	1938	-		-
Lice haemolymph	878									
Seawater	1150									

As it can be appreciated from Table 2.2 the osmolarity to achieve proper fixation (underlined values) was very variable and fixative dependent. A fixative had to be highly hypertonic compared to the osmolarity of *L. salmonis* haemolymph. At osmolarities lower than the ideal, the fixation was variable and probably dependent on the physiological state of the gut. The gut epithelium of lice containing a recent meal seemed more susceptible to osmotic damage than guts of lice starved for a few hours. But once the correct osmolarity for a fixative was defined, these different tissues were always properly fixed.

The rule for selecting the appropriate osmolarity was straight forward: At increasing osmolarities, there appeared to be an increase in tissue preservation, but a point was reached when the fixative became too hypertonic to the cells and a gradual shrinkage of them became evident. This was evident mainly at the apical part of the cells, which had a "jagged" appearance. When this point was reached, then the previous osmolarity was selected. If this previous osmolarity still showed some signs of cell damage or shrinkage, a fine tuning of the osmolarity was then needed. This

happened for example with the fixative No. 2 (see Table 2.2) with which, at 0.5 M of sucrose, the preservation was still not perfect but at 0.7 M there was some cell shrinkage. Intermediate concentrations of sucrose were then tried until the correct fixation was achieved at 0.65 M.

It was found to be important to include tissues of different physiological condition for the same fixative solution to be tested to be sure that even the most fragile tissues were well preserved. It is recommended that this trial and error test is assessed with resin embedded material because the subtle changes around the ideal sucrose concentration would be very difficult to appreciate in paraffin embedded material. The osmolarity of the fixative may also be changed by adding electrolytes such as NaCl or $CaCl_2$ (Hayat 1989) instead or along with sucrose. In which case, a new trial and error test should be done if the concentration of any of the components of the fixative is altered.

After fixation, tissues were washed for 18 h with two changes of the same buffer that served as vehicle for the fixative used.

In selected cases, after the buffer wash some specimens were posfixed in 2% aqueous osmium tetroxide for 1 h and washed again in buffer.

2.2.1.2 Preparation of the specimens for fixation.

2.2.1.2.1 Ensuring the permanence of the gut contents.

It was observed that the gut contents of the lice were expelled during fixation, mainly when weak fixatives were used as the lice took longer to die. Retention of the gut contents was very important in the present study. To ensure that they were preserved *in situ* the anus of the lice was close using a ligature made from a loop of hair before fixation. A number of hair loops were prepared in advance. Pieces of human hair of about six mm long were used, leaving a loop of around 3 mm wide. Working under the dissecting microscope, a louse was put at the corner of a glass plate with the abdomen protruding out of it. The loop was then placed at the level of the hindgut and slowly closed and fastened tightly. The excess hair at each side was then cut with scissors. This knot of

hair was also useful as an individual tag. Up to 7 different knot patterns could be used. This allowed several lice that had to be recognised individually to be placed in the same container for processing with the resultant economy in time and reagents and ensuring standardisation of treatment.

When this operation was carried out in the field the use of microscope was not possible. In this case a louse was placed at the tip of the left mid finger in such a way that the abdomen protruded freely from the finger and towards the person. The loop of hair was then handled with the index and thumb of both hands.

2.2.1.2.2 Dissection.

To allow for rapid penetration of the fixatives, the lice were cut with scissors at the sides of the cephalothorax and at the sides of the genital segment immediately before fixation. This was done after tying the anus with hair as described above.

In some instances the cuticle of the lice gave problems during sectioning, mainly with the use of hard resins such as LR White. An almost complete dissection of the louse gut was then performed leaving only the dorsal cuticle. This was done under the dissecting microscope and with the louse immersed in the fixative on a small plastic Petri dish. Two pairs of very fine pointed forceps and a opthalmological pair of scissors were used. The cutting edge of these scissors was 0.5 cm.

Working with the louse on its dorsum the limbs were pulled away with the forceps, holding the base of each one while pulling away the limb with the other pair of forceps. Four longitudinal cuts at the sides and close to the gut were then made with the scissors at the cephalothorax and at the genital segment, holding the louse with the forceps. Another cut was then made, passing the lower blade of the scissors between the ventral gut wall and the ventral cuticle of the genital segment, close to the 4th segment. This left a flap of cuticle that was then lifted towards the posterior part of the louse with the forceps whilst pinning down the louse with the other pair of forceps. At the junction with the abdominal segment, this flap of cuticle cannot be lifted. Two other fine cuts were needed at the sides of the junction of the genital-abdominal segments. Once this was done, the flap of

cuticle could be lifted and the entire genital and abdominal portions of the gut exposed. The final operation was to lift the floor of the cephalothorax. A first cut was needed just before the 4th segment, again passing the lower blade of the scissors between the ventral cuticle and the ventral gut wall. The free edge of cuticle was then held with the forceps while keeping the louse pinned down with the other pair. The cuticle was then carefully lifted towards the anterior part. Finally, the excess cuticle at the sides of the gut was trimmed.

2.2.2 Embedding.

To assess if there were differences (morphological or histochemical) between different sections of the alimentary canal, three portions of the gut were always considered. Anterior, corresponding to the cephalothorax; mid, comprising the genital segment and posterior corresponding to the abdomen. Lice were then cut with a razor blade at two points: at the level of the 4th segment and at the level of the genital-abdominal junction. To minimise the cutting time, the three portions were placed together, when possible, in the same block. A careful alignment of the pieces of louse before hardening of the embedding media was critical. Later, careful trimming of the blocks was needed to allow transverse sections to be cut at the same time through the three segments and at a relatively constant level of each gut portion (see Figure 2.1). Two embedding media types were used: paraffin wax and resin. In the photographs presented in this study, the embedding media used is indicated as LMPW (light microscopy, paraffin wax) or LMR (light microscopy, resin).

2.2.2.1 Paraffin wax

Fixed material for wax embedding was dehydrated through a series of methanol ethanol and chloroform in a Reichert-Jung Histokinette 2000 automatic processor. Working in a tissue embedding station, the infiltrated specimens were cut as described above and each piece was carefully oriented to give transverse sections. Again, alignment of the pieces was critical. Up to 12 lice (36 gut sections) could be accommodated in a large paraffin block, saving to a great extent the section cutting labour.

Sections were cut between four and five µm on a manual Leitz Wetzlar 1212 microtome using disposable metal knives and were picked up from a warmed waterbath at 45°C. The sections were left to dry for at least 6 h on a hot plate at 35-40 °C after which they were ready for dewaxing, rehydration and staining.

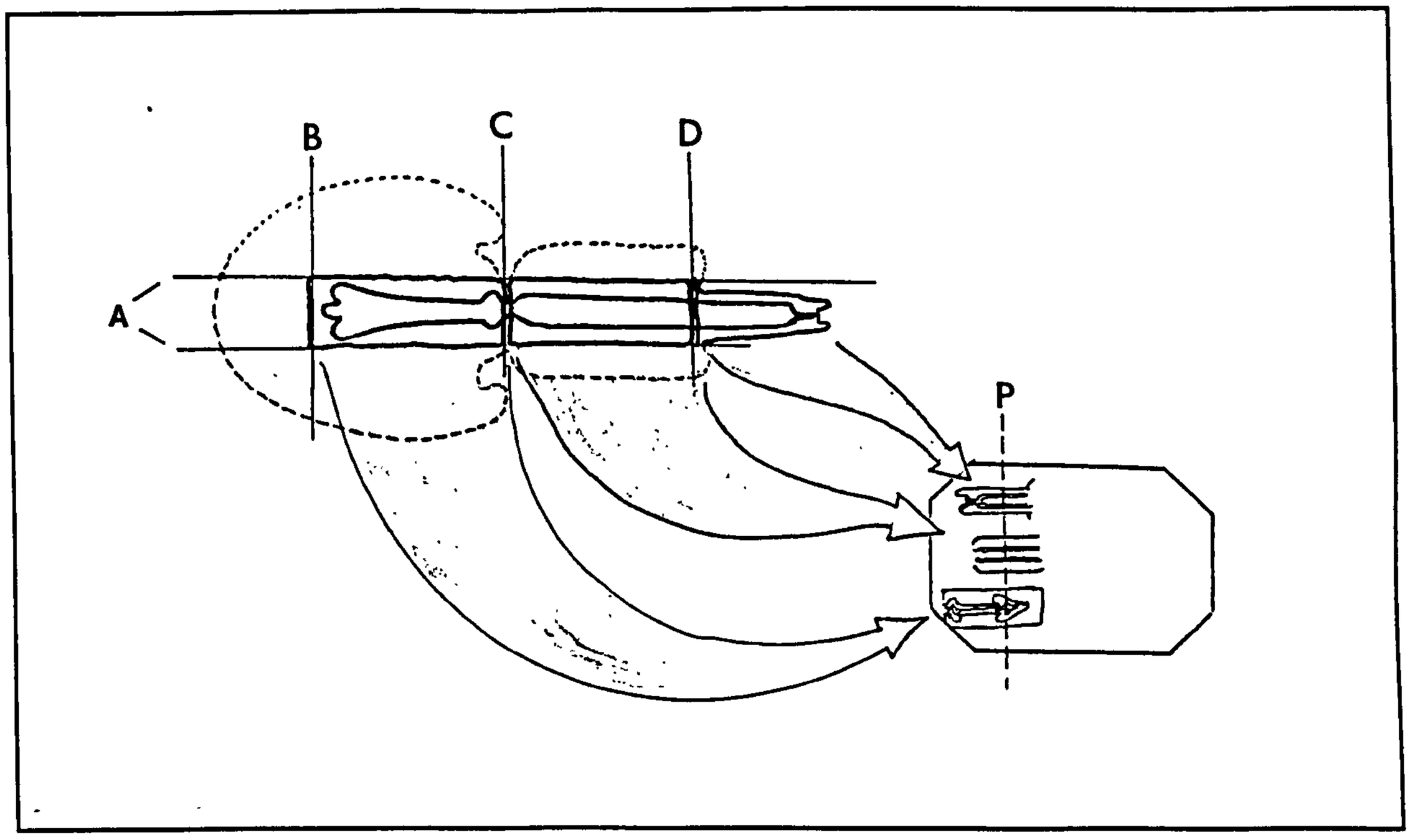


Figure 2.1 Diagram illustrating the procedure for dividing a louse in three portions and the alignment of these portions to give transverse sections of the gut at constant levels. Before fixation the louse was dissected as described before by cutting along the A and B axes. After processing and infiltration, the louse was then cut at points C and D. The three pieces were then carefully aligned inside the mould as shown. The final cutting plane is depicted by a dotted line (P). Careful trimming of the block was also required to achieve this. For resin blocks a line was drawn at the required position while observing the block under the dissecting microscope. This line served as a guide while trimming. For paraffin blocks the procedure was similar but as flat embedding was not possible due to the design of the blocking system the three pieces had to be inserted vertically into the paraffin. Carefully monitoring the level of the trimming every 20 µm was carried out until the desired level was reached.

2.2.2.2 Resin embedding.

Several methacrylate based embedding media were tested for their suitability. The main aim was to have an embedding medium compatible with most of the staining methods selected for this study, including those for enzyme histochemistry. It was also necessary to find a medium which allowed the examination of adjacent tissue, or sections, for electron microscopy. A great louse to louse variability of gut morphology was known to occur from previous experience of the author with

this parasite, therefore, the correlation of morphologies and histochemical tests had to be done preferentially on the same individual. Four media were tested: JB4, Historesin Plus, Unicryl and LR White.

2.2.2.2.1 JB4

JB4 (Polysciences Ltd.) is a glycol methacrylate based resin. It is water soluble and the tissues can be infiltrated at low temperatures, which is advantageous for retaining enzyme activity. The method followed was according to the manufacturers instructions with some modifications made by Hand (1987). After thoroughly washing the tissues in the appropriate buffer (the buffer used as a vehicle for the fixative), the following procedure was carried out:

All the processing and embedding solutions are pre-chilled.

1. Three changes of JB4 monomer at -20°C each for 30 min.

2. Infiltration with monomer and catalyst (0.4%) at -20 °C for 30 min.

3. Embed in catalysed monomer (0.4%) + accelerator (40:1) at 4 °C.

After infiltration the lice were cut and oriented carefully as shown in Figure 2.1 with the moulds already on a bed of crushed ice in a dessicator. As oxygen inhibits polymerisation, the dessicator was filled with nitrogen, taking special care not to move the dessicator as the slightest vibration would take the material out of alignment. After polymerisation, sections were cut at 1-2 µm using a glass knife on a Reichert-Jung Autocut. Sections were floated in a water bath at room temperature and mounted on slides coated with 0.1% poly L-lysine. They were allowed to air dry for at least 1 hour before staining.

2.2.2.2.2 JB4-TEM.

To be able to correlate light microscope morphology with ultrastructure each of the three pieces used normally as above was further divided in two, one for JB4 embedding and another for embedding in Unicryl or LR White (see below).

2.2.2.2.3 Historesin Plus

Historesin Plus is a hydrophilic glycol methacrylate-based resin for light microscopy use. It accepts partially dehydrated tissues which can be embedded after the 70% ethanol dehydration step. Because the polymerisation is chemical by means of a hardener that is added just before the final embedding, it was suitable for flat embedding and alignment of the three lice gut portions. Also it had been reported to give good results for a range of histochemical tests similar to those to be used in the present study, including enzyme histochemistry (e.g. Agyei, Runham & Blackstock 1992).

Dehydration and infiltration was carried out following the instructions on the product. The samples were left overnight for the last infiltration step. The plastic moulds for embedding were put on a large Petri dish filled with chilled water to disperse excess heat generated by the polymerisation reaction. The lice samples then were cut and carefully aligned as in Figure 2.1 . A piece of acetate foil was placed over each mould to keep the reaction air-tight as oxygen inhibits the polymerisation of this resin.

Sections were cut as described in section 2.2.2.2.1.

2.2.2.2.4 Unicryl.

This is a hydrophilic methacrylate based resin developed by Manara, Preda, Pasquinelli, Ferrari, Panfilis & Scala (1993). It has excellent cutting and staining properties and can be used for light and electron microscopy. It comes ready to use with no mixing of any reagents necessary. Polymerisation is achieved by the use of UV light for 72 h. at 4°C.

After washes, fixed lice were quickly dehydrated in graded ethanol and then infiltrated with Unicryl 3 X 3 h on a rotator inside the fridge, at 4 °C.

Flat embedding was not possible with the use of this resin. The flat embedding moulds compatible with UV light polymerisation were too small to accommodate the three pieces of tissue as required. Also, the samples had to be transferred first to a special case that held the UV lamp which in turn had to be transferred to the fridge with the samples loaded. With all these movements it was

impossible to keep the material aligned. The three portions of gut had to be embedded individually. For this purpose, hyperbolic Beem capsules were used, which kept each sample in vertical position during polymerisation.

For bigger tissues, such as the control tissues used for some histochemical tests, clear 0.5 -1 ml Eppendorf tubes were used instead of the Beem capsules.

Sections 1-3 μm thick were cut in a LKB Ultracut E ultramicrotome using glass knives. Ultrathin sections were cut as described in section 2.5.

2.2.2.2.5 LR White

This is another methacrylate based hydrophilic resin which can be used for light and electron microscopy. The polymerisation can be chemical with the addition of a hardener or by heat keeping the blocks at a constant temperature of 50°C for 48 h. It is of low viscosity and therefore has good penetration. Samples were dehydrated in graded ethanol changes of 30 min each and infiltrated in the resin with three changes of 60 min each, in a rotator inside the fridge. The samples were processed after the infiltration as for Historesin Plus except that the blocks did not need to be covered with acetate foil. The chemical polymerisation is not compatible with osmium-fixed material. For this, heat polymerisation was used. The moulds to be polymerised were put on a square piece of acrylic of about 30 X 30 X 0.5 cm. The samples then carefully orientated on each well of the moulds (each mould has 6 wells) and then they were put in the oven taking extreme care not to move the samples excessively as this would lose the alignment. The acrylic helped to control better any coarse movement during the transfer of the moulds to the oven.

Sections were cut for light microscopy as described for JB4 (section 2.2.2.2.1). When a correlation with the ultrastructure had to be made, each portion of the gut was cut out from the block with a saw, clamping the resin block in a vice. The block was trimmed to a suitable size to be accepted by the block holder of the ultramicrotome. Sections for electron microscopy were cut as described in section 2.5.

2.2.2.2.6 Selection of the routine resin.

Each resin had its advantages. JB4 and Unicryl gave good results with enzyme histochemical techniques. But correlating JB4 sections with electron microscopy was too laborious. Unicryl had the disadvantage that every single gut portion had to be cut individually and for large samples this was very time consuming. With Historesin there was limited success with enzyme histochemistry, the results varying from batch to batch.

LR White was the better choice, although a compromise had to be made with enzyme histochemistry, as the results also varied from batch to batch. It allowed flat embedding, therefore the screening of large samples by light microscopy was done quicker as the three portions of the gut could be cut in one section saving a lot of time and effort in cutting and staining. Also, ultrathin sections were easy to cut and performed very well under the beam of the electron microscope.

For small samples, Unicryl was a better choice. The cutting properties were better and the stains were very crisp. As with the other resins though, the results with enzyme detection were very variable.

2.3 HISTOCHEMISTRY.

The histological stains used in this study are shown in Table 2.3 . All were used according to the source except the Mallory-Heidenhain Cason's trichrome + Alcian green 2.5. The latter method consisted of first staining the sections with alcian green pH 2.5 for five min as detailed in Drury & Wallington (1980) and then the sections were stained with Cason's (Cason 1950) solution (1g phosphotungstic acid, 2 g Orange G, 1 g aniline blue and 3 g acid fuchsin are added to 200 ml of distilled water, one by one dissolving the mixture with each addition) for 15 minutes, washed briefly in running water, dehydrated, cleared in xylene and mounted in Pertex.

For enzyme histochemistry, tissue-free JB4 sections were placed on top of the histological sections as a way of forming a semi-permeable membrane that has been observed to enhance the staining (Gerrits, Horobin & Hardonk 1989). The method of the later authors was followed but using JB4 resin, which was equivalent to the resin mixture that they used:

After mounting and drying, the paraffin wax or resin embedded tissue sections were covered with 2 µm sections prepared from tissue-free JB4 resin blocks. To do this, the tissue-free sections were stretched by floating them in water and then placing them over the tissue sections. Paraffin wax sections were dewaxed and hydrated to water before placing the semipermeable membrane on them. The slides were then dried at room temperature for at least 6 h before use.

2.4 SCANNING ELECTRON MICROSCOPY (SEM).

2.4.1 General method

Specimens fixed in MIF or Karnovsky (fixative #2, see section 2.2.1.1) were dehydrated through graded ethanol series. Drying of specimens was done using the fluorocarbon Peldri II according to the method of Kennedy, Williams & Gray ((1989). This method was later discontinued as new regulations prohibited the use of fluorocarbon compounds. The Peldri II method was substituted with the use of HMDS (hexamethyldisilazane) adhering to the method of Nation (1983).

Dried specimens were mounted on aluminium stubs using double sided stickers or Araldite rapide. The stubs with the specimens were gold sputter-coated with an Edwards S150B sputter coater using a specimen distance of 3 cm, a current of 40 mA and a voltage of 0.8 KV.

2.4.2 Specific methods

2.4.2.1 OTOTO protocol

Usually the surfaces of the specimens to be looked at under the SEM are sputtered with gold but in complex topographies, such as the subject of this study, the gold coating cannot reach all surfaces. Also, the structures lying under the primary surface are not coated, and when exposed when the tissue is fractured due to further dissection of the specimen while trying to uncover the desired structures, charging artefacts occur on these uncoated surfaces. This makes a second or a third sputtering procedure necessary. But every sputtering covers more details of the receiving surface reducing the resolving power (Reiss & Reale 1989). The OTOTO method is a ligand-mediated-osmification that deposits a heavy metal coat in all the tissue and further fracturing is unlimited as

every newly exposed surface has a metal deposit on it. This method proved to be very useful as it allowed sequential dissections of the material. The method was performed according to Reiss & Reale (1989).

2.4.2.2 Paraffin carving.

Paraffin carving is a means of revealing the internal structures of an embedded specimen in a controlled fashion. The method was described by Oshel (1985) and consists of embedding the specimen in paraffin wax as for routine microtoming. The excess of paraffin is removed on all sides of the specimens with a razor blade and unwanted portions of the specimen are carved away with fine dissecting needles under the dissecting microscope. Cool illumination is advised to avoid softening of the paraffin. After carving the specimen is cut free from the block and de-embedded reversing the embedding routine. When the specimens are in absolute ethanol they can be processed as usual for drying, mounting and coating for SEM as described above. With delicate specimens, a variation of the method was carried out. The excess paraffin was removed by placing the carved specimen on the hot plate of the embedding centre for a few seconds. The specimen was allowed to solidify and was fixed on an aluminium stub in the desired orientation with Araldite rapide. Special care has to be taken to "wrap" the specimen with the Araldite usually at the edges so that the specimen could be held in place by the Araldite once the paraffin was removed. Once the Araldite has hardened (usually 5-6 h, 12 h recommended), the aluminium stub with the specimen was processed for SEM as described above.

Another modification was carried out by using the microtome to carve through the specimen until a full longitudinal section of the gut was achieved, keeping the last 10-15 sections for histomorphological study using general purpose stains. The remaining block was then dewaxed in xylene and processed for routine SEM. This allowed a better assessment of the gut morphology and correlation of histological and SEM pictures.

2.4.2.3 Dry fracturing.

This technique (Toda, Suh & Nemoto 1989) was employed usually in combination with the OTOTO technique described above. It consisted of attaching the dried specimen to an aluminium stub by means of a double sided sticker. With a dissecting needle, further pressure was applied to some areas of the specimen in contact with the sticker to ensure proper adherence. Then, another sticker was placed on the surface of the specimen, again sticking the sticker to the surface of the specimen with the dissecting needle. While holding the stub, the sticker on top of the louse was pulled away fracturing the specimen in two parts. Since the actual position of the fracture cannot be controlled with the method, several spare specimens are needed for repetition of fracturing.

2.4.2.4 Dry dissection.

This method was also used preferentially after the OTOTO technique. Dried specimens were first fixed to an aluminium stub, preferably with Araldite. Care was taken to distribute the Araldite so that most of the surface of louse in contact with the stub was coated with Araldite and therefore providing a firm specimen to work on. Any wobbling of the specimen made the dissection more difficult.

A special dissecting needle was made. The needle had to be strong as it was going to be used to pull apart the cuticular parts of the louse and at the same time very fine to be able to reach small places and get a better dissection. The needle was made of the blown half of an electron microscope filament, which is made of tungsten. When the filament blows, it usually leaves a small hook-like edge which was very useful for easier dissections. The filament was taken off the bakelite base and fixed with Araldite to the end of a standard dissecting needle. With a pair of these needles, successful dissections of the louse could be made by gradually tearing apart the unwanted structures to expose the desired ones. Sometimes, a combination of the dry fracturing technique or the paraffin carving with this technique worked well.

Once the desired structures have been exposed the specimen is ready to be observed if it was treated before with the OTOTO technique. If not, one or two sputterings with gold were carried out, depending on how intricate the exposed surface was.

2.4.2.5 Stereo photography

The technique to produce stereo pairs is called the "tilting" method (Boyde 1973, Howell 1975, Goldstein, Newbury, Echlin, Joy, Fiori & Lifshin 1981). The first photograph was recorded in the usual way and the position of a prominent feature, preferentially around the middle of the field was marked on the visual CRT screen. For the second photograph the stage was tilted several degrees. Once the desired number of degrees had been reached the prominent feature previously selected was aligned with the mark on the screen using the X and Y stage controls. The final focusing was done with the Z stage control and not with the focusing knob, as the latter would alter the lens current with the consequent change in magnification between the two members of the stereo pair.

The choice of tilt angle difference varied between 4° and 20°. The value that gave best results was between 6° - 8° but at low magnifications it was better to increase it to 10° - 15° to get a better depth effect. At high magnifications above 2500, a smaller tilt difference was chosen (4° - 5°).

The tilt axis of the microscope used in the present study lies parallel to the bottom of the CRT screen. To make this coincide with the interocular plane, the photographs had to be rotated 90° to the left before mounting. The lower, less tilted micrograph was placed for viewing by the left eye and the more tilted one for the right eye.

For viewing the size of the printed photographs should be no larger than the observer's interocular distance which is usually between 65 and 70 mm (Howell 1975). The camera attached to the microscope used in the present study had a large format film and the size of the final picture in the negatives was 73 x 57 cm. Therefore, no magnification was necessary and the final photographs were printed by direct contact print of the negatives.

The stereo pair was viewed with the aid of a stereo viewer (purchased from Agar), which is an inexpensive plastic spectacle with an arrangement of lenses that allows the presentation of only one micrograph image to each eye. The integrated image from the two photographs gives the appearance of depth through the brain's interpretation of the parallax effects observed.

In the absence of the stereo viewer, the same effect could be easily accomplished by cutting an A4 sheet or cardboard in half and placing it between the photo pairs with the shorter side on the surface of the document. The observer then places the tip of his/her nose on the free short edge of the card and relaxes his/her view. The two images then "fuse" to get the desired 3D effect. Slight adjustments tilting the head sideways may be necessary to ensure that the two eyes are parallel to the tilt axis.

2.5 TRANSMISSION ELECTRON MICROSCOPY (TEM).

Specimens were fixed either with MIF or Karnovsky #2 (see section 2.2.1.1). Fixed specimens were embedded in Unicryl or LR White as detailed in section 2.2.2. Trimmed blocks were sectioned using a glass or diamond knife on a KLB ultracut E microtome. Semithin sections were put on a small drop on a glass slide coated with poly-L lysine and air dried. Ultrathin sections were picked up on uncoated copper grids and stained with 2% aqueous uranyl acetate for 2 min, washed and further stained with lead citrate for 2 min according to the methods of Hayat (1989). Grids were observed using a Philips 301 TEM.

Table 2.3 Histological stains used to characterise *L. salmonis* adult female tissue components.

Histochemical technique	Purpose	Source.
General stains		
Haematoxylin & Eosin	General stain	Drury & Wallington 1980, Drury & Wallington 1980
Hubschman's modification of Gomori's for crustaceans	General stain	Cantwell 1981
Toluidine blue	General stain	Drury & Wallington 1980
Mallory-Heidenhain Cason's trichrome + Alcian green 2.5	General stain	This study. See text for details.
Himes & Moriber's (H & M) Triple stain	General stain (DNA / polysaccharides / proteins)	Himes & Moriber 1956
Unicryl polychrome stain		Scala, Preda, Cenacchi, Martinelli, Manara & Pasquinelli 1993and Unicryl staining kit manual (British Bio Cell International)
Specific stains		
Mercuric Bromophenol blue	Proteins	Chapman 1975
Millon's	Proteins	Stevens & Bancroft 1993
Periodic-Acid-Schiffs reagent.	Polysaccharides and mucins	Drury & Wallington 1980
PAS + diastase control	Glycogen	Drury & Wallington 1980
Aldehyde-fuchsin	Sulphated sialomucins	Drury & Wallington 1980
Phenilendiamine. Sudan Black	Lipids	Boyles 1984, Boshier, Holloway & Kitchin 1984 Drury & Wallington 1980
Alcian blue (or green) pH 1.0	Sulphated mucosubstances	Drury & Wallington 1980
Alcian blue (or green) pH 2.5	Acid mucosubstances	Drury & Wallington 1980
Combined Alcian green 2.5-PAS	Neutral and acid mucosubstances	Drury & Wallington 1980
Azure A + cationic surfactant 214	RNA	Benniom, Horobin & Murgatroyd 1975
Azure A + PAS	General stain (RNA+Polysaccharides)	as above
Methyl green - Thionine	RNA / DNA	Roque, Jafarey & Coulter 1965
Amidoblack 10B	Haemoglobin	Puchtler & Sweat 1962
Perl's Prussian blue	Ferric iron (haemosiderin)	Drury & Wallington 1980
Tirmann-Schmeltzer method	Ferrous and ferric iron	Stevens 1993
Stains for enzymes		
Simultaneous coupling method with Naphthol AS-BI.	Alkaline phosphatase	Borgers, Firth, Stoward & Verheyen 1991
Simultaneous coupling method with Naphthol AS-BI.	Acid phosphatase	Borgers *et al.* 1991
Simultaneous coupling method with L-alanyl-4-methoxy-2-naphthylamide.	Microsomal aminopeptidase (unsuccessful)	Lojda, Gossrau & Stoward 1991
Simultaneous coupling method with Naphthol AS-D acetate.	Esterases	Oliver, Lewis & Stoward 1991
Hopsu-Haru medium	Arylsulphatase (unsuccessful)	Arnaud *et al.* 1984a

3. GENERAL MORPHOLOGY OF THE ALIMENTARY CANAL.

3.1 MATERIALS AND METHODS.

3.1.1 Source of material and processing.

Adult females were collected as detailed in Chapter 2. The organisms were fixed immediately after removal from the fish or soon after arrival in the laboratory. In addition, material from experiments and observations from other chapters of this work were used. Fixation procedures, embedding for light microscopy and TEM, staining of sections, and procedures for SEM were carried out according to the techniques outlined in Chapter 2.

3.2 RESULTS.

3.2.1 Gross morphology of the alimentary canal.

The alimentary canal of *L. salmonis* is divided into three main regions: The foregut, which comprises the tubular oesophagus; the midgut, which constitutes the major part of the digestive tract and a short hindgut. A diagram of the general plan of the alimentary canal of *L. salmonis* is depicted in Figure 3.1 The structure of the oral cone and the cuticularised hindgut will be dealt elsewhere in this work (Chapters 4 and 5 respectively).

The cuticularised oesophagus lies centrally in the anterior quarter of the cephalothorax. From the posterior part of the oral cavity, it runs posteriorly, passing through the anterior part of the nervous system and then opens in the floor of the midgut in the form of a protruding cuticularised conical papilla (Plate 3.1). It has a thin, highly folded cuticular lining with flattened epidermal cells. The folds allow for extension of the lumen to accommodate the ingested food passing through it. Bands of smooth circular muscle surround the oesophagus along its length probably aiding in the ingestion of food by peristaltic movements.

The midgut constitutes the major part of the digestive tract. It is lined by a monolayer of epithelial cells which bear on their luminal surfaces a brush border consisting of numerous microvilli with a variable height of 1-4 μm. Beneath the brush border a thin layer stains darkly with Toluidine blue, corresponding to the apical cell membrane. The enterocytes rest on a thin basal membrane.

Two distinct regions can readily be recognised when the midgut is observed through the dissecting microscope, according to the part of the louse they occupy: Thoracic midgut and genito-abdominal midgut.

The thoracic or anterior midgut, occupies the central part of the cephalothorax and it has a triangular cross section for most of its length.

On closer observation the anterior midgut has 5 small lobes. Three in the anterior part, constituted by the anterior diverticulum (often referred to by other authors as anterior caecum) and two ventrolateral lobes. Another two smaller lobes are situated laterally, just before the gut enters into the free thoracic segment (also referred as the fourth leg-bearing segment). These lobes are barely recognisable when the gut is empty but are more conspicuous when the gut is full and contracting actively.

The anterior diverticulum is a globular protrusion from the anterior margin of the midgut of about 100-120 μm in diameter, overlying the cerebrum (see Plate 3.2 and 3.3)

The anterior lateral lobes are wing-like, directed ventrolaterally giving this part of the gut a characteristic triangular cross section. The ovaries lie dorsally to these extensions which are more conspicuous when this part of the gut is relaxed (see Plate 3.3) than when it is contracted (Plate 3.2)

The anterior midgut is separated from the genito-abdominal midgut by a vertical constriction situated in the free thoracic segment which seems to function as a valve (see Figure 3.1). The vertical walls of this constriction are kept close together, limiting the flux of gut contents to either side. This valve can be very active at times, opening and closing continuously, but the mechanism involved has not been determined (see Chapter 5 for more details. See also Plate 3.53)

The genito-abdominal midgut is a wide tube of circular cross section that ends in the posterior part of the abdomen, leading to the cuticularised hindgut. Although structurally there seem to be no clear-cut divisions, for practical purposes it will be divided in two portions, the genital and the abdominal, from here on referred to as mid-midgut and posterior midgut respectively. Later we will see that these divisions can in fact have a physiological significance (Chapters 4-6). Before describing the cellular components of the midgut epithelium, three important components of the digestive tract will be examined.

3.2.2 Associated structures to gut epithelium.

3.2.2.1 Basement membrane.

The epithelium of the midgut is separated from the haemocoelic cavity by an amorphous, flexible, acellular basement membrane which ensheaths the midgut epithelium completely (Plate 3.4).

This basement membrane (or basal lamina or lamina densa) consists of an extracellular sheet with a thickness of 0.5-1.2 μm.

Under the TEM it consists of a finely granulated matrix with some extremely fine fibrils. The matrix is interrupted by clear spaces which represent pores (Plate 3.5).

The pores are scattered in the basal membrane without forming any pattern.

The reaction for proteins is strongly positive. It gives negative results for alcian green 2.5 or 1.0 pH indicating the absence of acid or sulphated mucopolysaccharides. With toluidine blue there is no methachromatic reaction, staining with a faintly pale blue, and indicating the absence of carboxylated and sulphated compounds. It stains strongly with PAS, indicating that is mainly constituted of neutral glycoproteins.

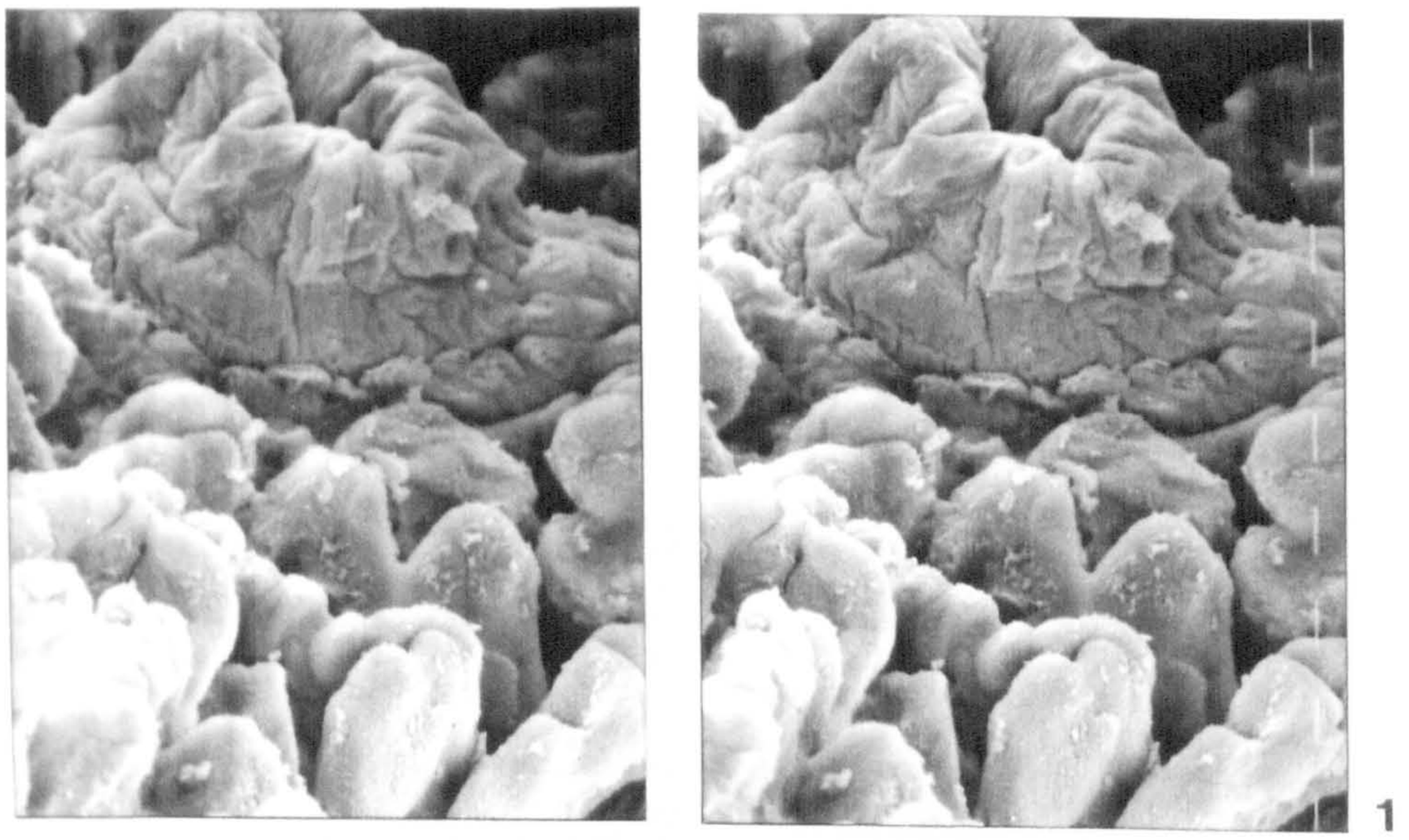
3.2.2.2 Muscle network.

Partial rings of circular muscle are present encircling the midgut along its whole length. The main body of each ring is not continuous around the midgut. It is open ventrally (Plate 3.6) although small ramifications that project from the edges of the muscle ring on both sides keep them connected.

Plate 3.1 Stereo micrograph of the oesophageal opening into the midgut of the louse. The cuticularised papilla (at the top of the photograph) protrudes among the enterocytes. SEM 640X.

Plate 3.2 . . Ventral view of the anterior midgut. Note the anterior diverticulum (ad), lateral lobes (lo), dorsal mesentery (arrows), attachments of lateral mesentery (arrow heads), circular muscles (stars). The oesophagus is not shown but its attachment to the ventral wall of the anterior midgut is at the level of the asterisk. The lateral lobes show some contraction (compare with 3.3 SEM. 160X

Plate 3.3 . Stereo micrograph showing a dorso-antero-lateral view of the anterior midgut. Note the three circular muscles surrounding the anterior diverticulum (arrow pointing to the second one); the dorsal mesenteric attachments (star) and the circular muscles. One of the posterior lateral lobes is also visible (arrow head). SEM 80X..



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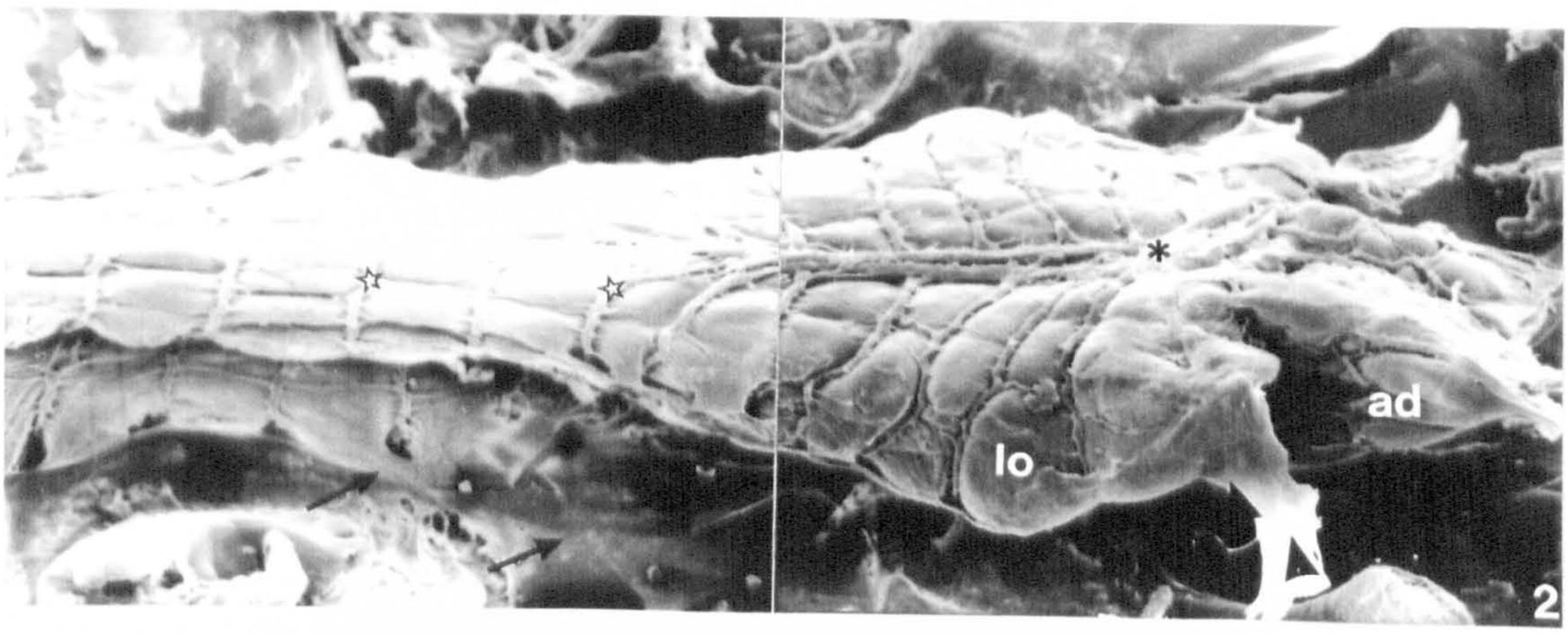

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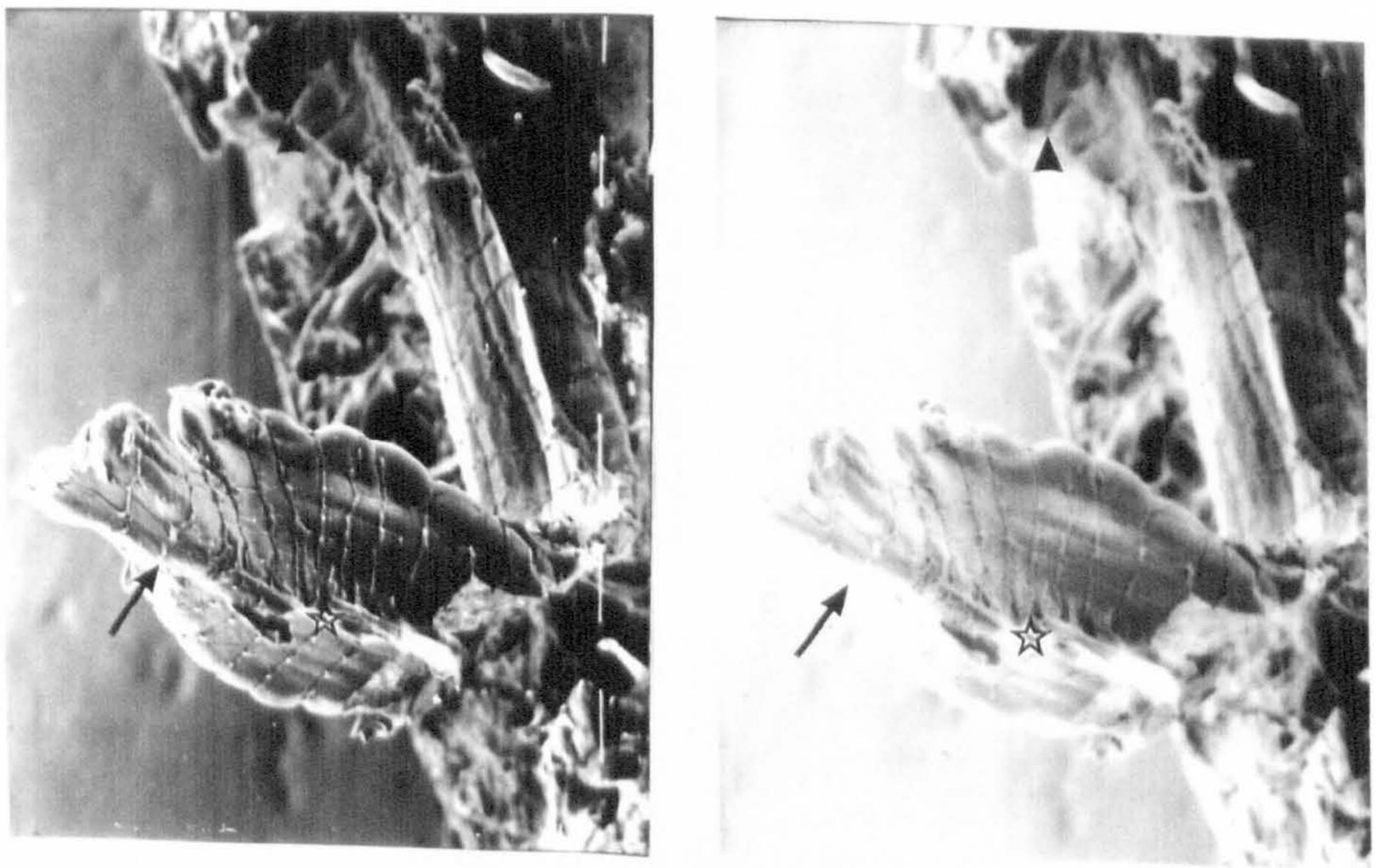

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Plate 3.4 . Scanning electron micrograph of a midgut sectioned longitudinally. Note the porous basal membrane (bm), the circular muscle (cm) with its lateral projections for attachment and the midgut epithelium (me). Microtome paraffin carving. SEM. 1250X.

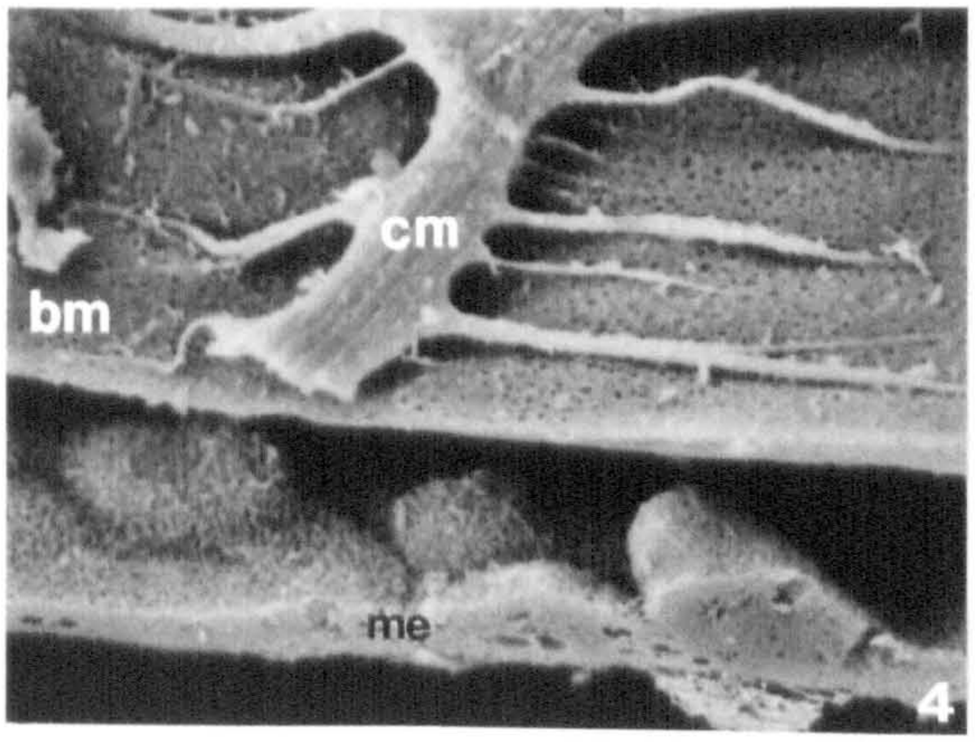
Plate 3.5 Basal portion of an enterocyte (en) showing the ultrastructure of the basal membrane (bm) which supports it. The open spaces in the basal membrane correspond to the pores that pass through it TEM 22,000X

Plate 3.6 Ventral view of a portion of the anterior midgut showing a gap in the main body of the ring of circular muscle. The two portions are connected instead by small ramifications (white dots) originating at the edges (small arrows) of the muscle. SEM 160X..

Plate 3.7 Two circular muscles (c) joined by lateral projections. Note the smaller projections (arrow) that attach to the basal membrane and the attachment of the dorsal mesentery (asterisk) to the basal membrane SEM 1250X..

Plate 3.8 Thinner lateral ramifications of the circular muscles in close association to the basal membrane. Note the pores and the continuity of the basal membrane with the sheath of the ramifications (arrows). SEM 1250X..

Plate 3.9 Dorso lateral view of the open genital segment revealing the midgut (mi) covered in its lateral part by the mesenteric membrane (mm). Note the point of attachment of the membranes to the midgut (arrow). The broken membrane labelled with an asterisk separates two haemolymph sinuses, a lateral one (star) where the oviducts are located and a dorsal one (open star) where the haemolymph circulates posterior-anteriorly. SEM 160X..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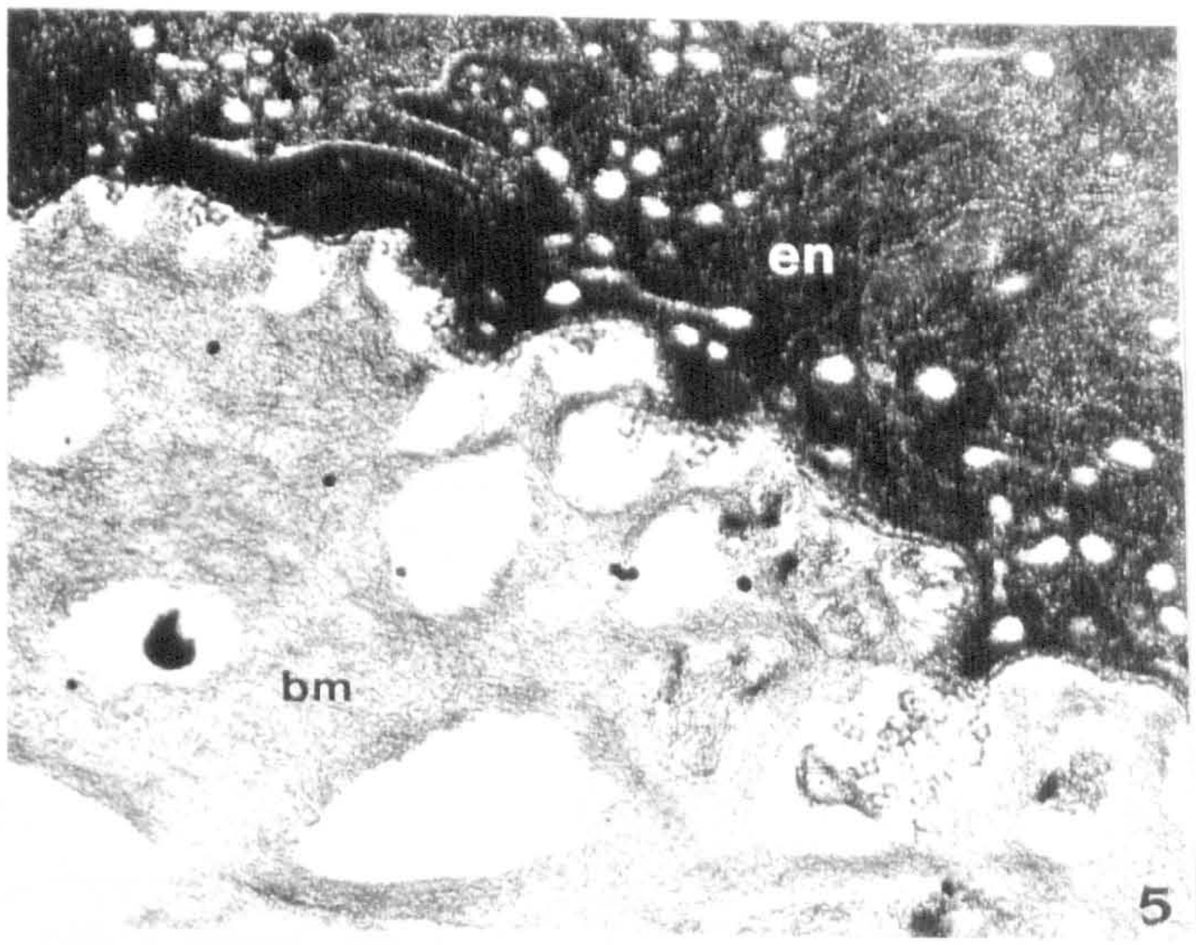
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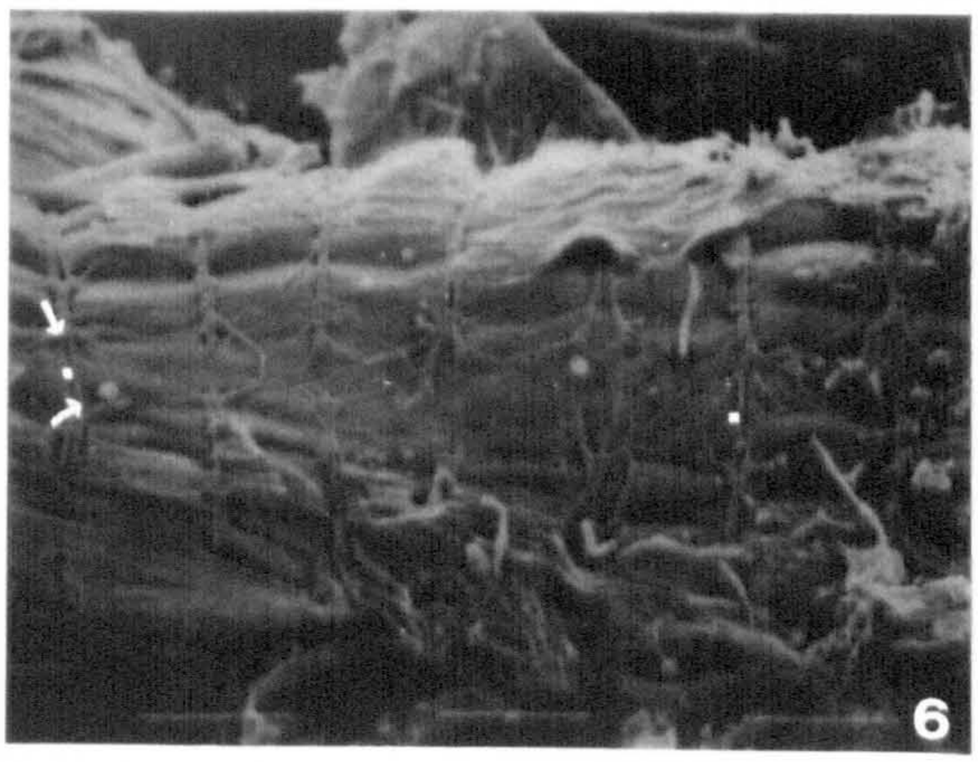
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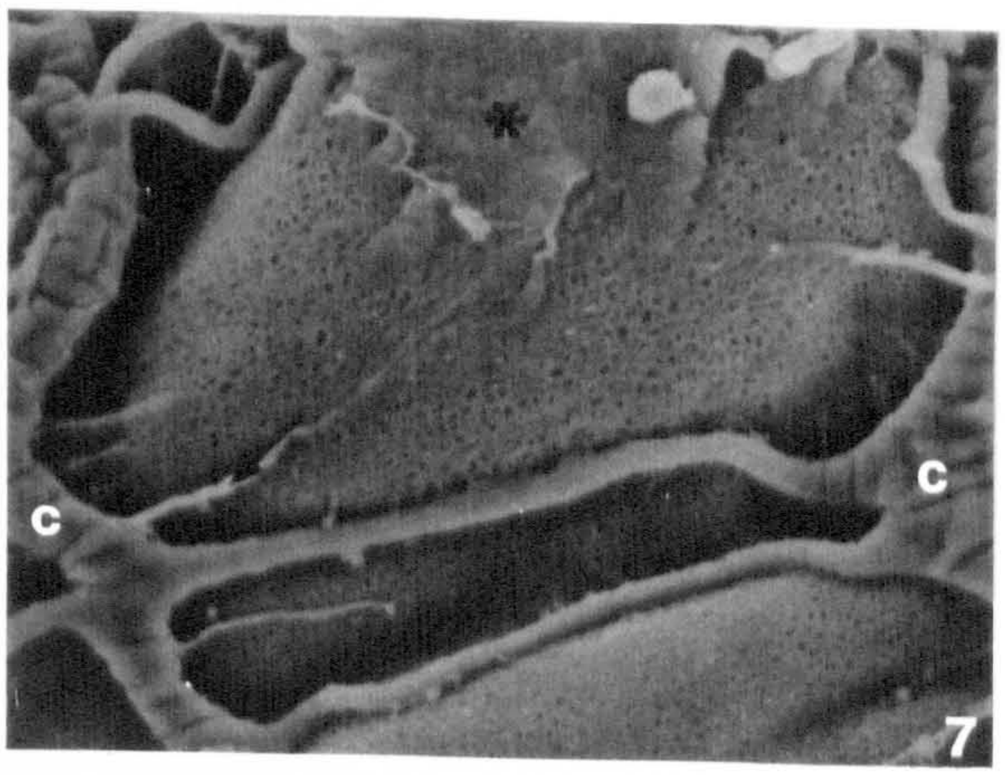
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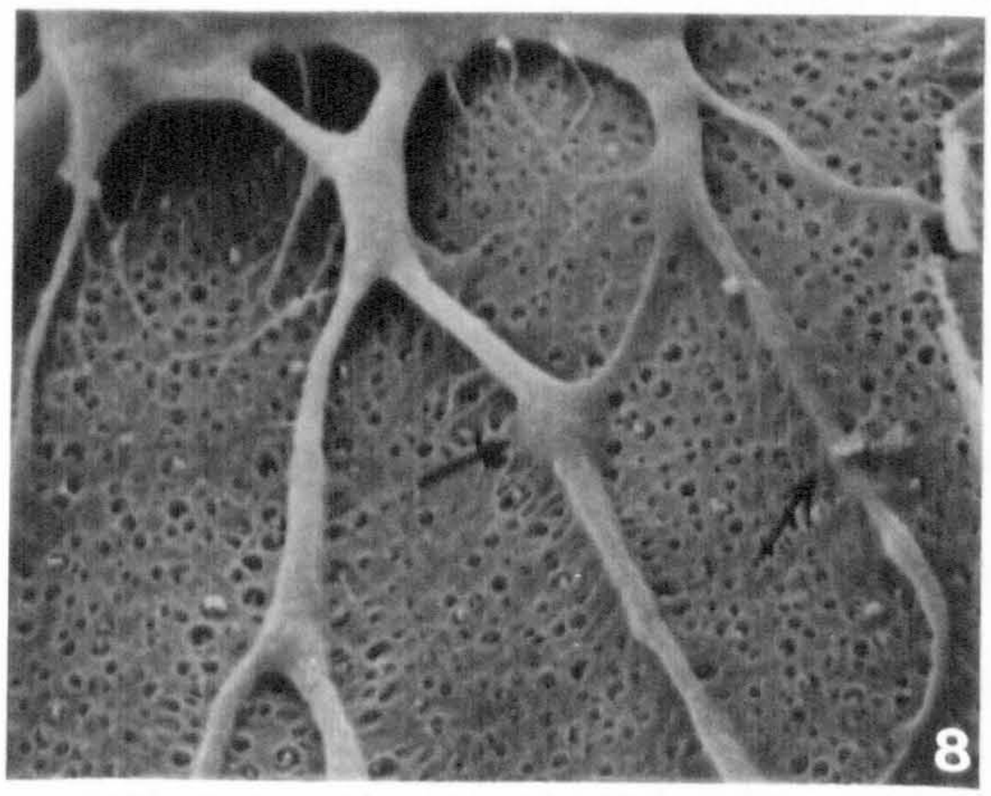
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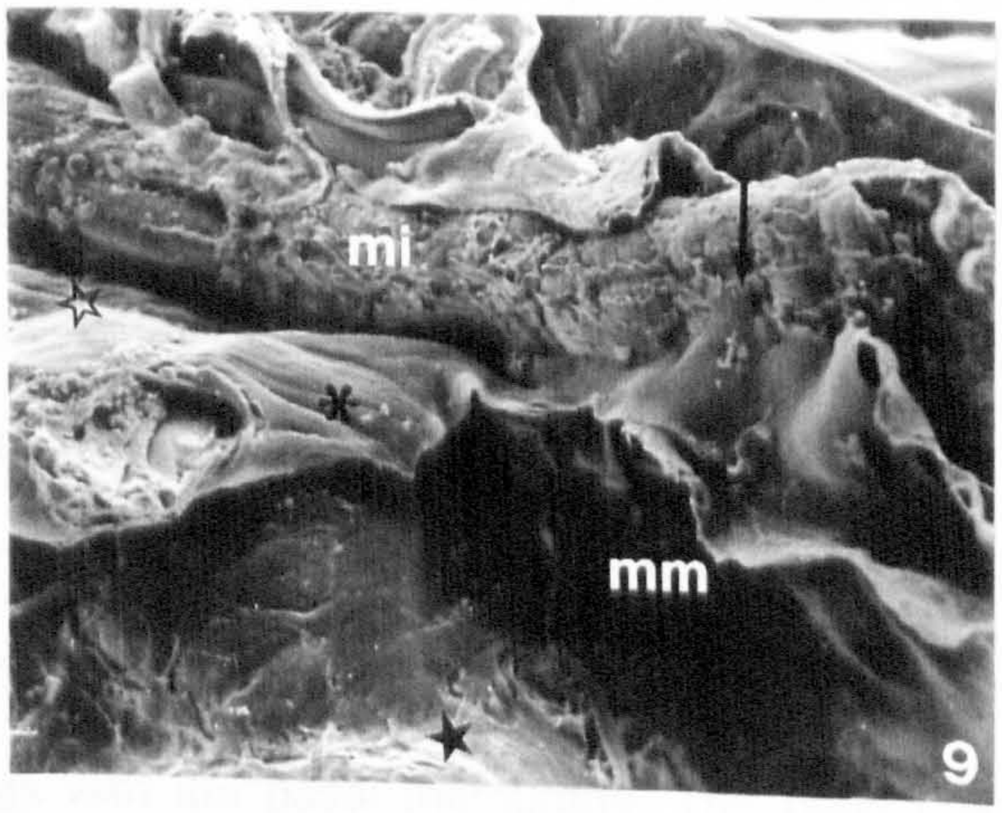
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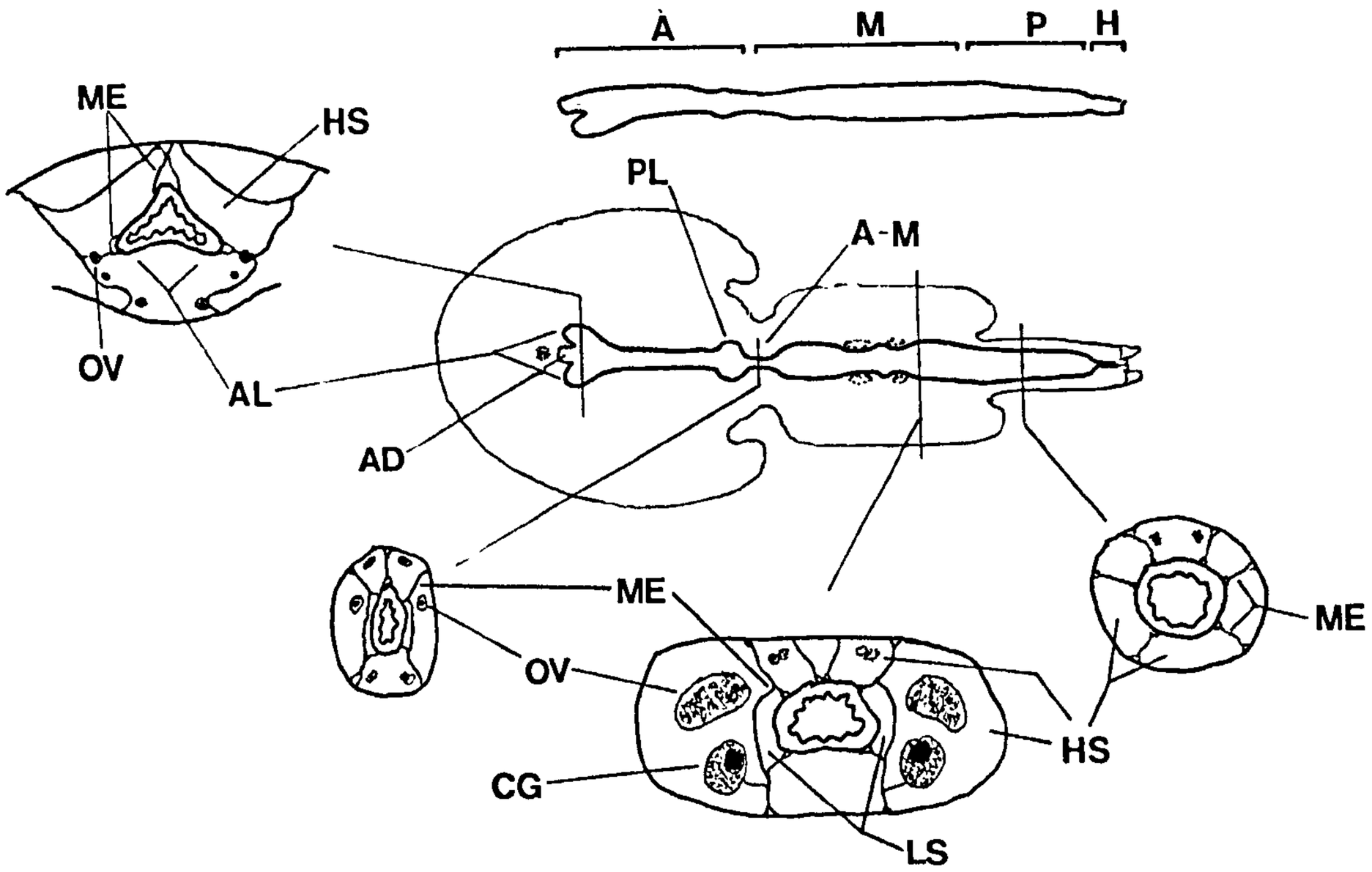


Figure 3 1 Schematic drawing showing a dorsal view of the outline of the gut, and the outline of the body of an adult female *L salmonis* . with sectional planes pointing to a representation of a cross section of the area. The cross sections at ANT, MID and POST are illustrated with the mesenteric membranes associated with the gut. AD: anterior diverticulum; A: anterior midgut; A-M. Constriction between the anterior midgut and the mid-midgut; CG: cement gland, H hindgut; HS: haemocoel sinuses; AL: anterior lateral lobes; LS: lateral sinuses; ME: mesenteries; M: mid midgut, OV oviducts; P: posterior midgut; PL: posterior lateral lobes. .

Each ring is probably formed of a single myofibril surrounded by a sheath of connective tissue similar to that of the mesenteries which in turn is surrounded by another layer of connective tissue similar to that of the basal membrane. The main body of the ring is separated from the basal membrane (see Plate 3.4) but it is kept in place by small lateral ramifications (0.8-2.0 μm in diameter) of the double-layered connective tissue sheath which attach firmly to the basal membrane providing anchorage for the muscles (Plate 3.8). This attachment is similar to the attachment of the mesenteries to the basal membrane (see below, Plate 3.8 and Plate 3.31) with interdigitations of the mesenteric connective tissue passing through the pores of the basal membrane while the second outer layer blends with the basal membrane. The rings possess another set of lateral ramifications which connect the rings between each other forming a myoepithelial network (Plate 3.7), but these ramifications are thicker and are actually ramifications of the circular muscle itself. They can be easily mistaken for "longitudinal" muscle in histological

sections, but under SEM their origin from the circular muscle ring is clear. The distance between these rings is about 40-60 μm.

3.2.2.3 Mesenteries.

The midgut is supported by mesenteric tissue. The mesenteric membranes are arranged longitudinally along the length of the louse, so that in transverse section they appear as thin structures. Close to the midgut external wall, these membranes send processes which in turn branch into projections that attach firmly to the basement membrane, passing through the pores of the latter and interlocking with it (Plate 3.10). On the other side they attach to the cuticular epithelium or to striated muscle bundles, surrounding them. The processes and projections that attach to the basal membrane have an homogeneous cytoplasm and bundles of fine filaments arranged parallel to the process (Plate 3.10). Figure 3.1 shows cross sections of the louse illustrating the pattern that the mesenteries form at 4 different levels of the body. As mentioned before, the mesenteric tissue is covered by a layer of connective tissue similar to that of the basal membrane, a feature that becomes more evident in the interlocking attachment previously noted (see also Plate 3.12). This layer is, as the basal membrane, also PAS positive .

In the cephalothorax, the midgut is supported by 3 groups of mesenteries. One dorsal and two ventrolateral, which together with the anterior midgut and its lateral lobes, give the triangular cross section to this part of the midgut.

The dorsal group is made up of two mesenteric membranes that arise separately from the dorsal body wall and attach to the dorsolateral part of the midgut (Figure 3.1). This forms a dorsal triangular network in the anterior part of the cephalothorax. This network is not continuous, as there are gaps where the circular muscles of the midgut pass through.

During some experiments designed to study the feeding behaviour (see Chapter 4), lice were left on a fish whose skin had been stained with neutral red. Those lice which had remained a long time showed areas inside the cephalothorax that retained the dye for long periods, up to several days.

Those areas retaining the stain were very discrete and corresponded to the lateral mesenteries that attach to the antero-lateral and postero-lateral lobes of the anterior midgut.

The mesenteries in the genital segment are continuous on the lateral side of the midgut, forming a sheath that separates the midgut and the oviducts (Plate 3.9) and contains the latter in a big sinus that bathes them with the antero-posterior flow of haemolymph which passes through. Here in the genital segment is the only place in the midgut where there is a continuous sheet of mesentery immediately below the basal membrane. Nevertheless, the basal membrane and this mesenteric layer are still separated by another small lateral sinus (labelled "ls" in Figure 3.1) and it is only at the level of the divisions between the cavities where the mesenteries attach to the basal membrane as described above.

In the abdominal segment, the number of mesenteries supporting the midgut increases, forming 6 distinct sinuses inside which the haemolymph circulates

Inside these "channels" the haemolymph flows in a very distinct pattern forming an open "circulatory system". The basic circulation pattern involves an anterior flow in the dorsal sinuses and cavities and a posterior flow in the ventral and probably the ventrolateral ones. At the level of the junction between midgut and hindgut, the posterior flow passes round to be collected by the dorsal sinuses to become the anterior dorsal flow.

When the anterior flow reaches the cephalothorax, it disperses antero-laterally and then ventrally through the spaces or sinuses of the cephalothorax, bathing the tissues. It has at least one "collection" point at the anterior portion of the cephalothorax, at the level of the eyes, just anterior to them, where it becomes an antero-posterior flow that eventually will join the posterior ventral flow. In this position there is a valve, a very delicate membrane ensuring a unidirectional flow from anterior to posterior. This valve is very small and thin and is not visible with the dissecting microscope. A restrained louse (see Chapter 5 for the description of the chamber used to restrain a louse) under the compound microscope was used to observe the flow of the haemolymph and it was then that this valve was detected and its activity recorded on video. At least further 2 valves exist in the free thoracic segment. These valves apparently prevent a backflow of haemolymph

when it passes from the cephalothorax to the genital segment. The flow of haemolymph is apparently maintained by the contraction of the dorsoventral muscles of the genital segment, which, probably in combination with the mentioned valves, ensure a one-way flow.

3.2.3 Morphology of the epithelial cells of the midgut.

3.2.3.1 Electron microscopy.

The ultrastructural differences observed between the cells in the midgut make it possible to discern several cell categories or types. These types were categorised to enable them to be assigned to the types of midgut cells in common use for the Malacostraceans (E, R, F and B-cells) and are based on the descriptions of these cells outlined in Gibson & Barker (1979).

3.2.3.1.1 Classification of midgut epithelium cell types.

3.2.3.1.1.1 E-cells.

These are short cuboidal cells 6-7 μm long with moderately dense cytoplasm, few organelles and a microvillar apical surface (Plate 3.11). The endoplasmic reticulum is scarce. The nucleus is round to oval and occupies most of the cell volume but is situated basally. When mitochondria are present, they are oval, and are situated to the sides or above the nucleus. A possible previous stage to the one described is illustrated in Plate 3.12 . It is a small basal cell, typically 2-3 μm high by 4-5 μm wide, which does not come in contact with the lumen. The cytoplasm is dense and has only a few mitochondria, which are oval or elongated and sometimes bifid. The nucleus is small, with oval to irregular borders. The nucleoplasm contains several perichromatin granules and the heterochromatin condensed into distinct separate clumps. Often with no nucleolus evident.

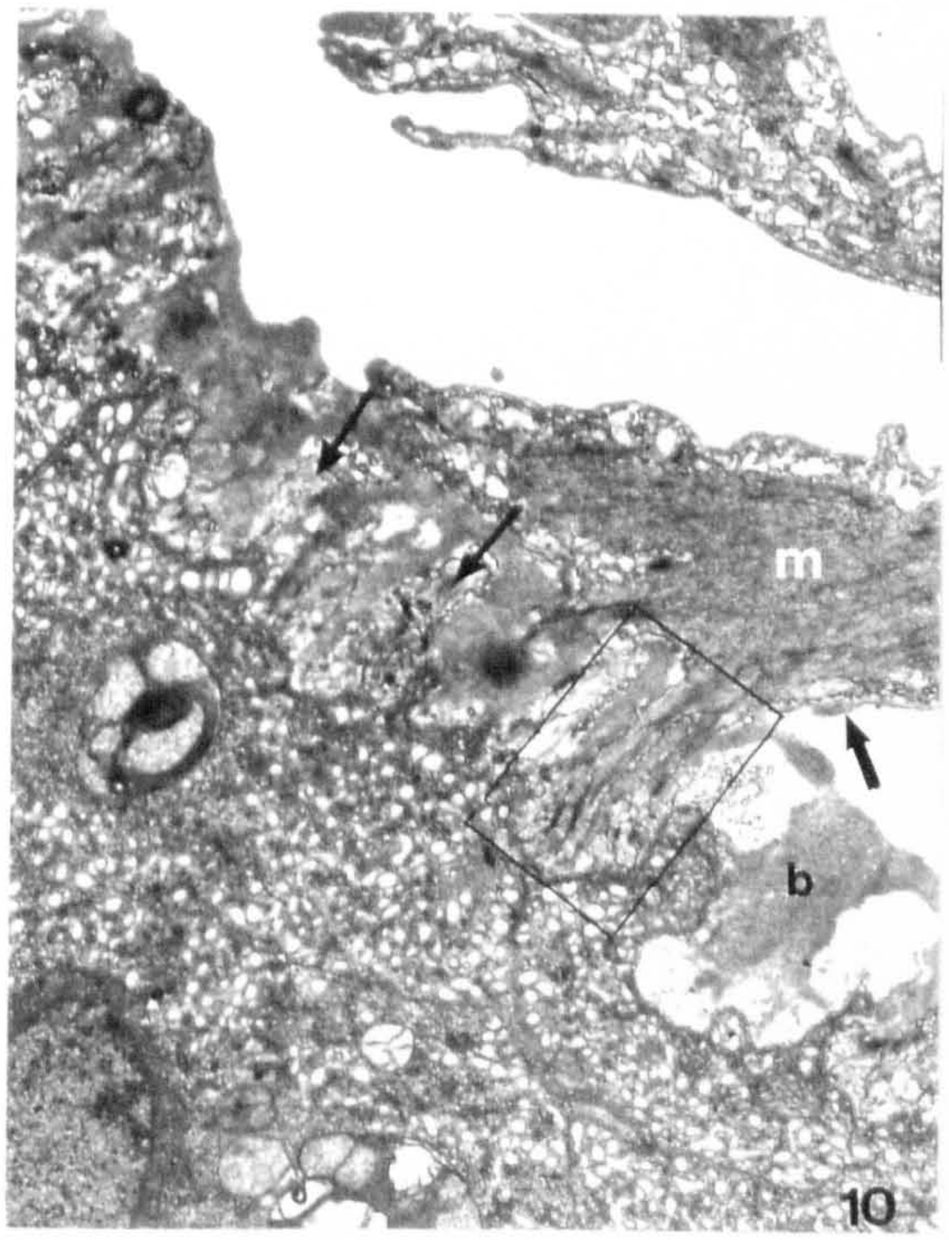
3.2.3.1.1.2 R-cells.

R-cells are the dominant cell type in the midgut of *L. salmonis*. They show a variety of morphologies dependent on their physiological state.

Plate 3.10 Mesentery (m) closely attached to the basal membrane (b). Note the processes passing through the pores of the basal membrane (arrows). The square shows the magnified area at right of one of the processes. The processes have fine filament bundles within them (big arrow). Note the close contact of the mesentery with the basal cell membrane of the enterocyte which at one point terminates in an inpocketing into the epithelial basal cell membrane (arrow heads). Note also how the layer that covers the mesenteric tissue is continuous with the basal membrane (thick arrow). TEM 9,800X and 43,000X.

Plate 3.11 A group of three E-cells probably starting to differentiate into R or F-cells. E-cells have few organelles and a small nucleus cytoplasm ratio. m: microvilli in the luminal side; b: basal membrane .TEM 4,150X.

Plate 3.12 Young E-cell (E) with no contact with the gut lumen. Note also a strand of mesenteric tissue, the external envelope of which is of similar material to the basal membrane and at two points is blended with it (arrows). TEM 7,800X..



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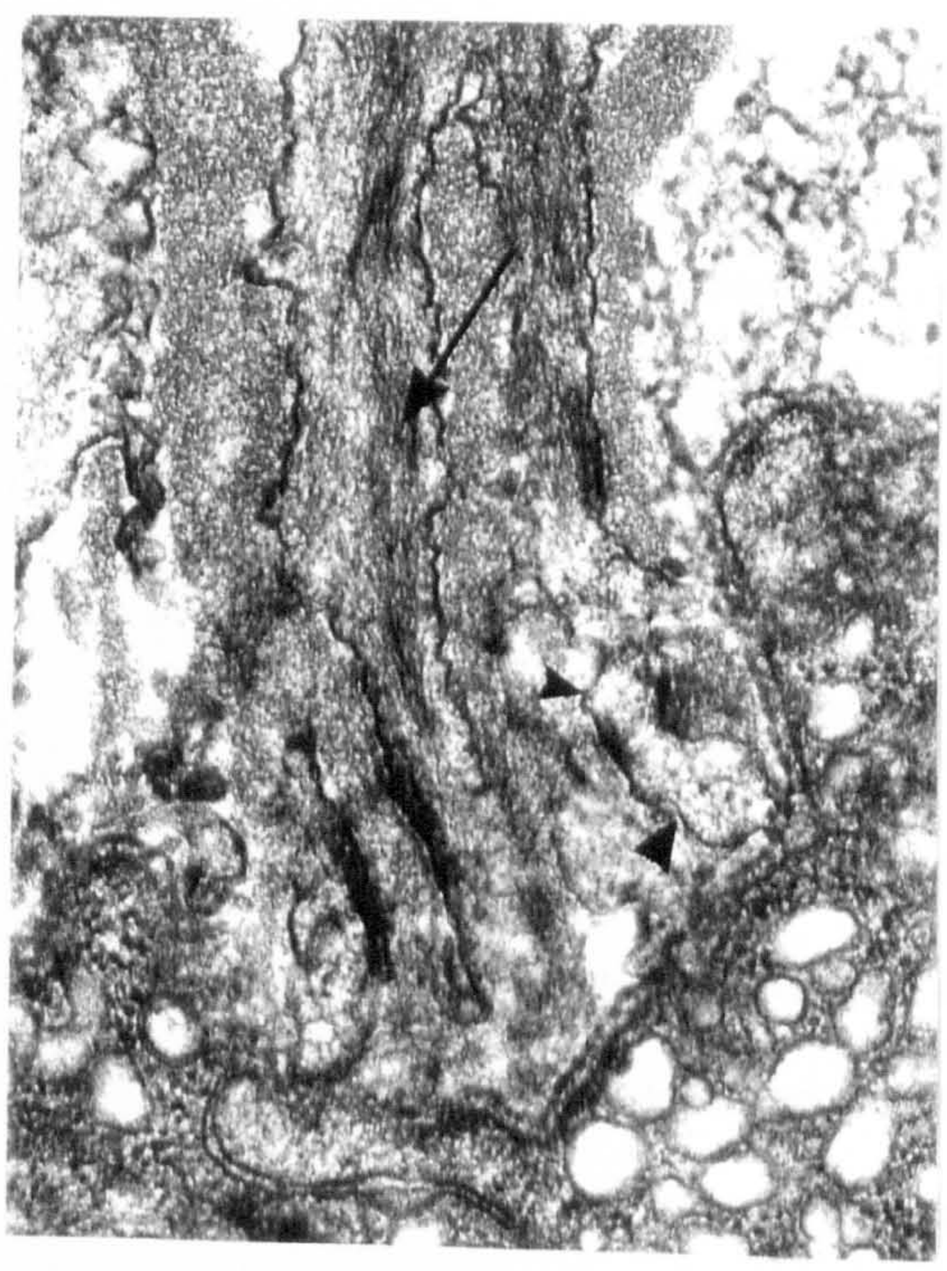

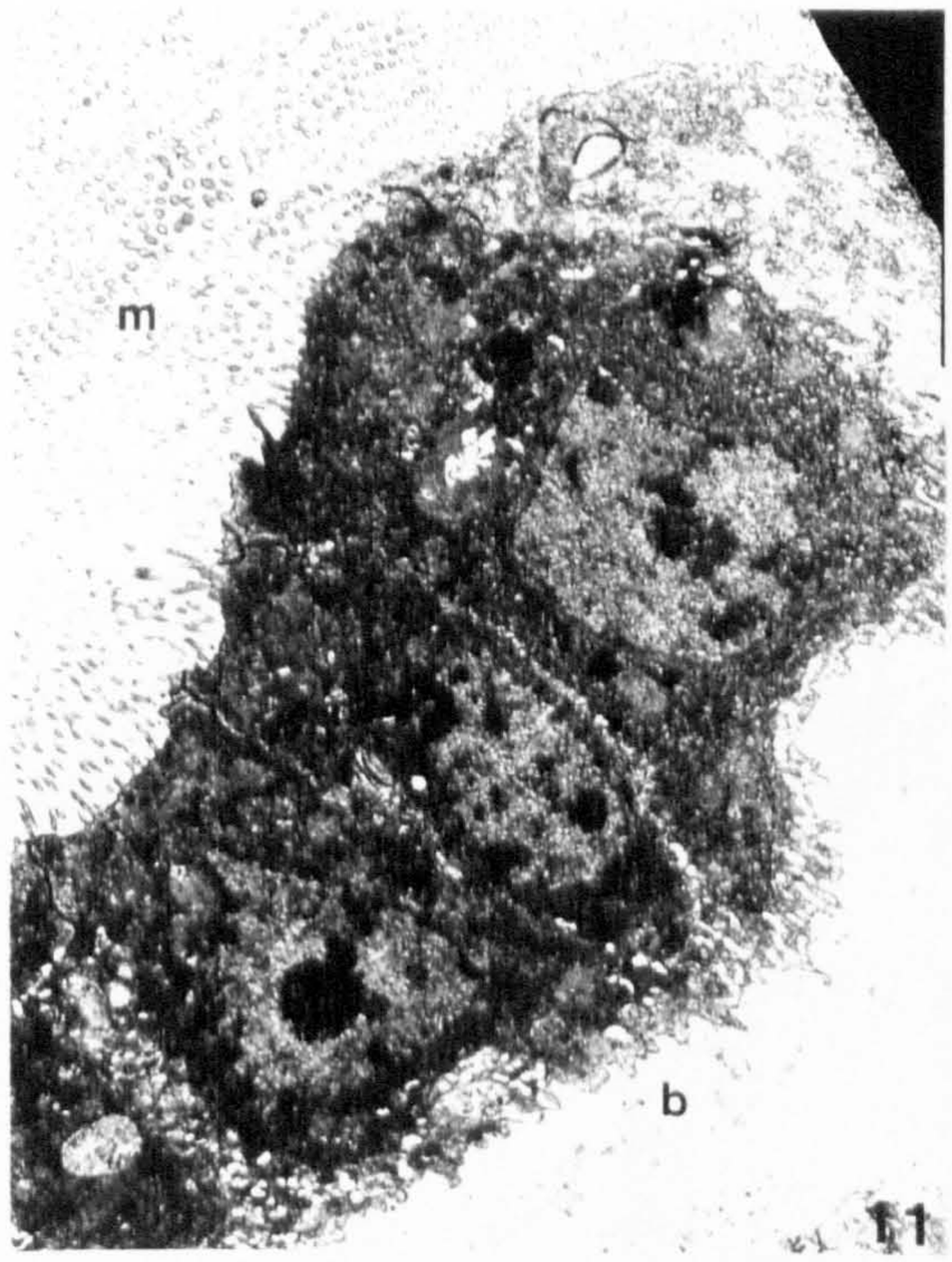

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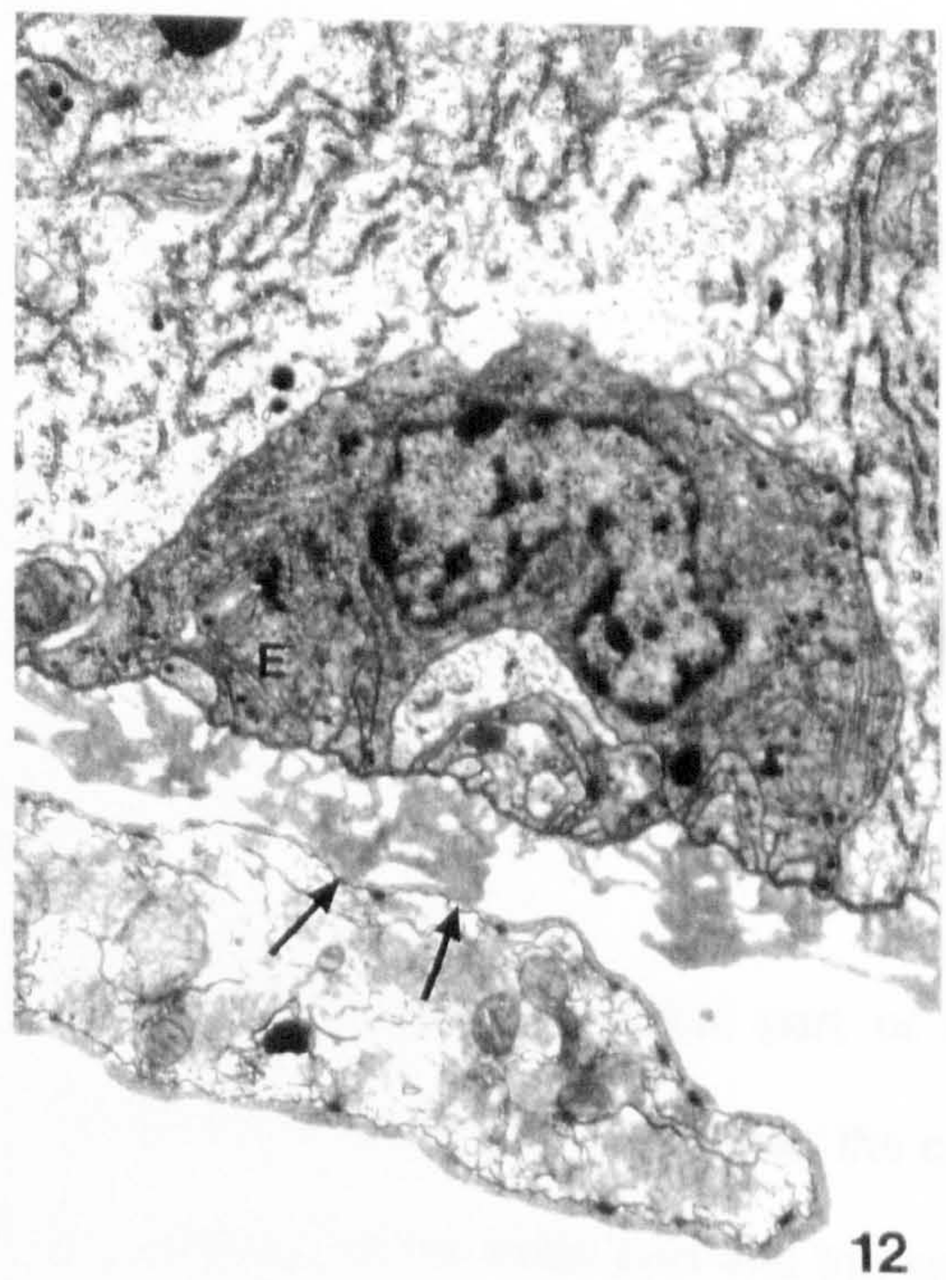

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A mature R-cell possesses numerous long microvilli at the apex, projecting towards the gut lumen. Their length is very variable, ranging from 1.5 to 4.0 µm, and they appear to contain parallel fibrils along their length but which do not project into the cytoplasm (Plate 3.13). No external coating was found covering the microvilli. The cell is cuboidal to columnar. It has moderately dense cytoplasm, profuse smooth endoplasmic reticulum (SER) which is richer at the boundaries with other cells and with the basal membrane taking the form of anastomosing tubules which is here termed the extracellular network. Mitochondria are circular or ovoid with parallel cristae and have a clear but flocculent matrix. They are numerous and can be found in any part of the cytoplasm, but tend to aggregate apically, just below the plasma membrane. Strands of rough endoplasmic reticulum (RER) are also present around the nucleus or below it. Golgi systems are associated with the SER. Dense inclusions with a limiting membrane occur in different parts of the cytoplasm. The nucleus is ovoid, the nucleoplasm has numerous perichromatin granules. Lipid droplets of various sizes are a common feature in lice that had fed on blood (see Chapter 5).

The epithelial cells are held together by tight junctions near the microvillous border.

Below the basal plasma membrane there is a well developed, porous, basement membrane, which was described previously in section 3.2.2.1.

R-cells seem to pass through a series of sequential changes along their life. The early stage, or R1, probably derives from cuboidal E-cells in which the mitochondria start a probably rapid period of divisions, as there is a high occurrence of long forms oriented along the axis of the R1 cell (Plate 3.14). The endoplasmic reticulum is of the rough type.

In the next stage, R2, mitochondria start to congregate towards the apical part of the cell, producing a bulge in the cell margin (Plate 3.15), which becomes more prominent as the cell starts to develop profuse SER in the form of anastomosing tubules at the basal part and the sides, and which pushes the cytoplasm and the nucleus apically, accentuating the bulge of the cell (Plate 3.16).

Stage R3 usually involves several R-cells in the same stage which together form elongations or extensions of the epithelium into the lumen (see Plate 3.1) which will form the "pseudovilli" characteristic of rough guts or "crypted" guts (see section 3.2.4). The cells at this stage are probably in the peak of activity. Many mitochondria crammed into the apical part of the cell among SER make the bulges very conspicuous. If the louse had fed on blood, lipid droplets will be a common feature. (Plate 3.17 , see also Chapter 5). At low EM magnifications or under light microscopy, the profuse vesicular system in the boundaries of the cell and from the adjacent neighbours give the appearance of clear channels running from the apical part towards the basal membrane. The "neck" of the pseudovillus complex is almost completely filled with the multi vesicular system of the cells involved. Autophagic vacuoles, myelin figures and multivesicular bodies start to appear in low numbers. Golgi occur among these latter. Most SER and RER tend to be of the vesicular type with clear contents.

Stage R4 marks the start of the degenerating process of the R cells. At the apex part numerous mitochondria are still functioning but, just below this layer of functional mitochondria, numerous residual and multivesicular bodies have started to develop (Plate 3.18). The cell has probably commenced to lose contact with the basal membrane and the neighbouring cells have started to wedge below it. As a result, the nucleus is pushed further towards the apex part. The long microvilli start to become coarser, are less uniform, and are apparently less numerous than in the previous stages. Bits of microvilli can be seen in the lumen, indicating that shedding of the microvilli is taking place. At later stages, the apical bulge is occupied almost completely by residual bodies (Plate 3.19 cell on the left) and, later on, the cell starts to pinch off from the neighbouring cells (cell at the top, same plate). This stage is probably the start of the next cell form that, for practical purposes, has been considered as a separate type, the A-cell.

SEM

Under SEM, R cells cannot be recognised individually. They form a continuous layer of microvilli interrupted only by A-cells or B-cells. This layer of R-cells shows a varied surface morphology: flat,

undulated, ridged or with deep folds and crypts according to the gut types (see classification of gut types in section 3.2.4).

3.2.3.1.1.3 A-cells.

These are considered to be exhausted R-cells that are in the process of being voided by pinching off in the apical epithelium to form a kind of blister that protrudes in between R cells. They are ovoid in shape; their size is variable, between 6-10 µm at the widest. The main characteristic of these cells is a large, usually electron lucent vacuole that pushes the elongated nucleus towards one side of the cell. This vacuole sometimes can have flocculate material inside (Plate 3.20). Often the large vacuole can be seen coalescing with smaller vacuoles possessing either clear or flocculate contents.

In addition to the large vacuole, there are numerous residual bodies, multivesicular bodies and electron lucent vacuoles. Apically, there can be some mitochondria. The RER and most of the SER have disappeared The microvilli of these cells are long (2.5-3.0 µm), somewhat coarser than R-cell microvilli and are eventually shed leaving the A-cell bare. The microvilli can be shed completely before the cell has pinched off (Plate 3.21) or the cell can still have some microvilli even after it has been sloughed (Plate 3.22)

When they are extruded into the lumen, they leave no scar in the epithelium and they still preserve the characteristics of an R-cell being readily recognised from their microvilli and ultrastructural characteristics of the cell organelles. The microvilli are irregular in arrangement and length compared to the intact R-cells of the epithelium. The cytoplasm, rich in vesicles, is very condensed. The nucleus is also present, with clear outline and usually with its chromatin condensed at the periphery (Plate 3.23). They are named A-cells as their morphology is indicative of cells undergoing programmed cell death or "apoptosis".

SEM

Under SEM, the A-cells can easily be recognised due to the "fluffy" appearance of their microvilli, in those that still have them, or from an apical membrane partially or totally devoid of microvilli (Plate 3.24 and 3.25). Another characteristic that is sometimes seen is a small notch in the lateral

surface of the A-cells which may be due to the collapse of the cell membrane towards the large vacuole that lies below (as shown in Plate 3.20).

3.2.3.1.1.4 F-cells.

The F-cell is cuboidal to columnar. Its main characteristic is the extensive RER and numerous Golgi bodies that can be found throughout the cell, but more commonly at the apical part (Plate 3.26). They have dense contents and apparently they form secretion vesicles of similar density (Plate 3.27). These secretion granules are also characteristic of these cells and are situated apically. The mitochondria usually have an electron dense matrix, are oval to elongated and are associated closely to the RER. The length of the microvilli is similar to that of the R-cells. The nucleus is round to ovoid and tends to be situated centrally; it possesses a prominent nucleolus.

Lipid droplets were not seen in these cells. The endoplasmic reticulum is granular. This, and the numerous free ribosomes, give the appearance of physical density, which is variable among F-cells (Plate 3.28) probably depending on their maturity. It is considered that denser F-cells are younger. Mature F-cells have a moderate number of dense mitochondria, a clearer cytoplasm and more vesiculated endoplasmic reticulum, but never reaching the rich vesiculation characteristic of R-cells. After maturation has been reached, the number of secretion granules diminishes and the morphological changes of the transformation into B-cells begin.

The transition from F-cell to B-cell starts by the profuse vesiculation of the microvilli (Plate 3.29), which eventually shed from the apical membrane of the F-cell. This is also the onset of the pinocytic activity characteristic of B-cells. The apical membrane develops invaginations which eventually form channels and small pinocytic vesicles which extend inside the cell (Plate 3.30) and eventually will coalesce with the dense bodies which have started to form Golgi bodies. The RER is ultimately reduced to a few strands scattered throughout the cytoplasm. Autophagic vacuoles and myelin figures are present in low numbers, compared to the late stages of the R-cells.

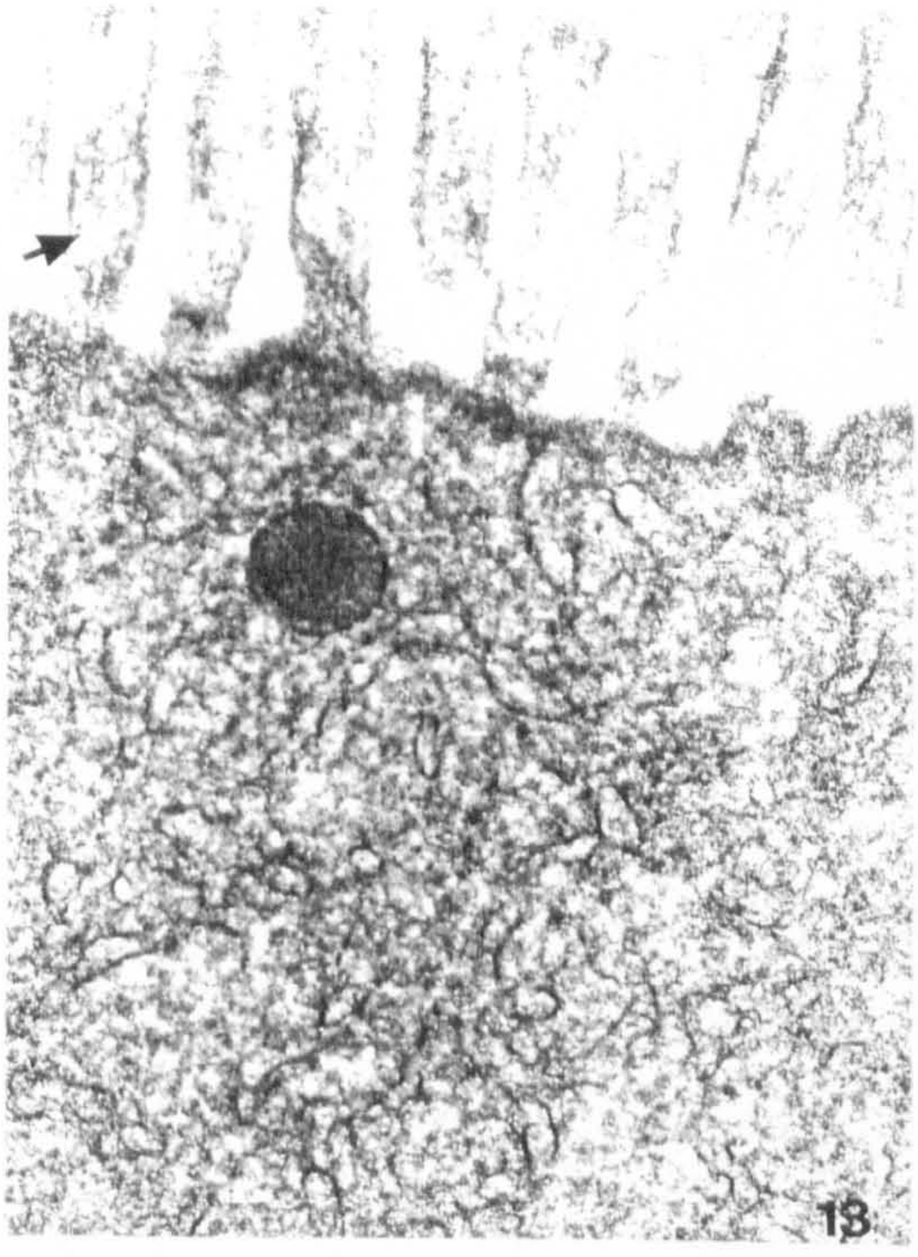
Under SEM, F-cells cannot be differentiated from R-cells.

Plate 3.13 Distal part of an R-cell. The apical microvilli have fibrils (arrows) along its length; there is a small dense droplet with limiting membrane in the cytoplasm surrounded by SER. TEM 59,000X.

Plate 3.14 R1-cell (R). Note the dense cytoplasm, the well developed RER and the long mitochondria. Some dense inclusions are scattered in the cytoplasm. A mature E-cell (E) is at the left but it cannot be appreciated yet which type of cell it will become. TEM 5,900X..

Plate 3.15 R2 cell. The mitochondria (arrows) begin to concentrate towards the apical part. The endoplasmic reticulum also starts to polarise. RER is more abundant in the basal part of the cell while the SER occurs mainly in the apical part. TEM 5,900X..

Plate 3.16 R2 cell with profuse basal and lateral anastomosing tubules. The organelles of the cell tend to concentrate at the apical part forming a characteristic bulge Note that also the nucleus (N) forms part of the bulge in this case. TEM 5,900X. .



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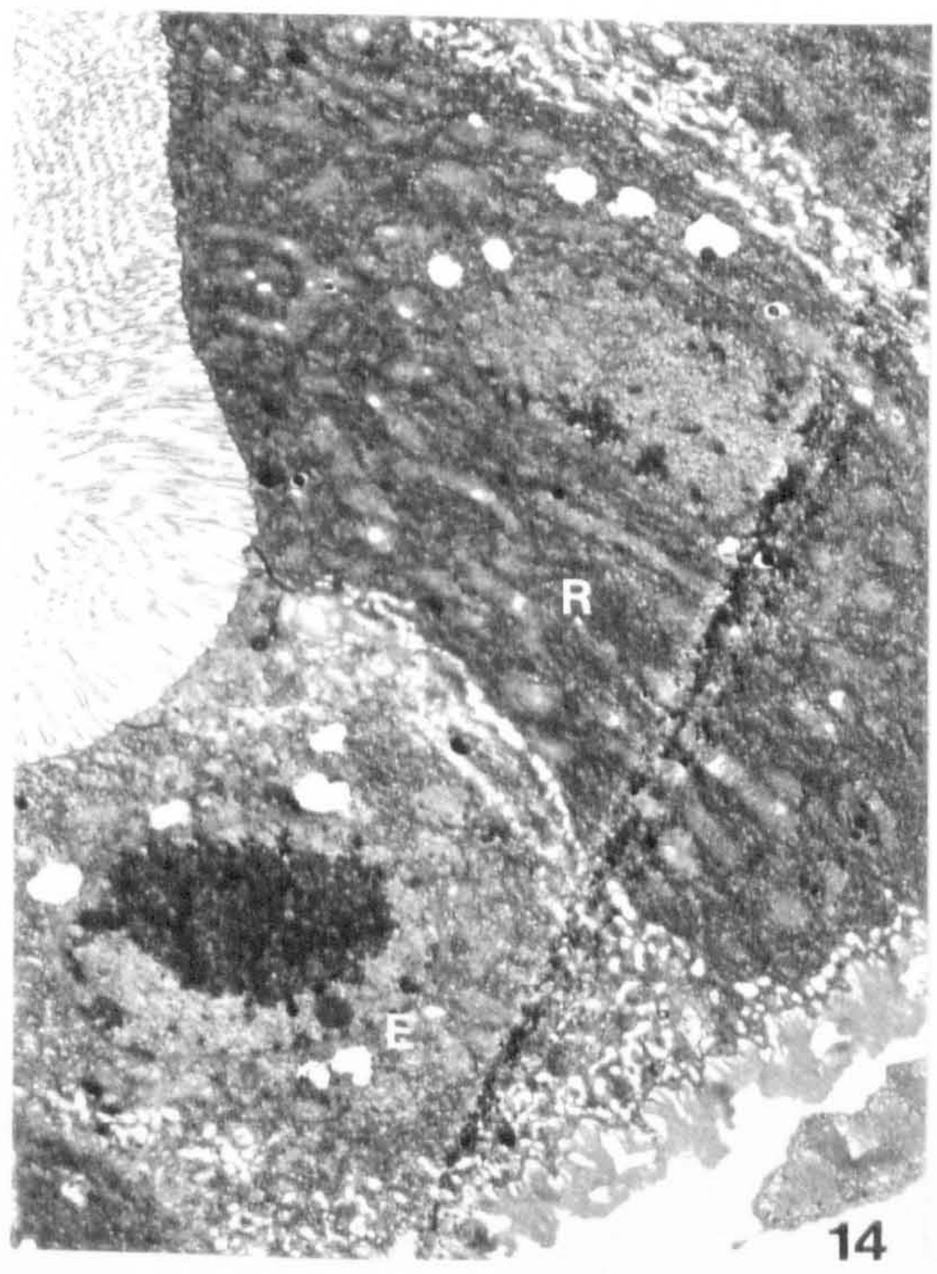

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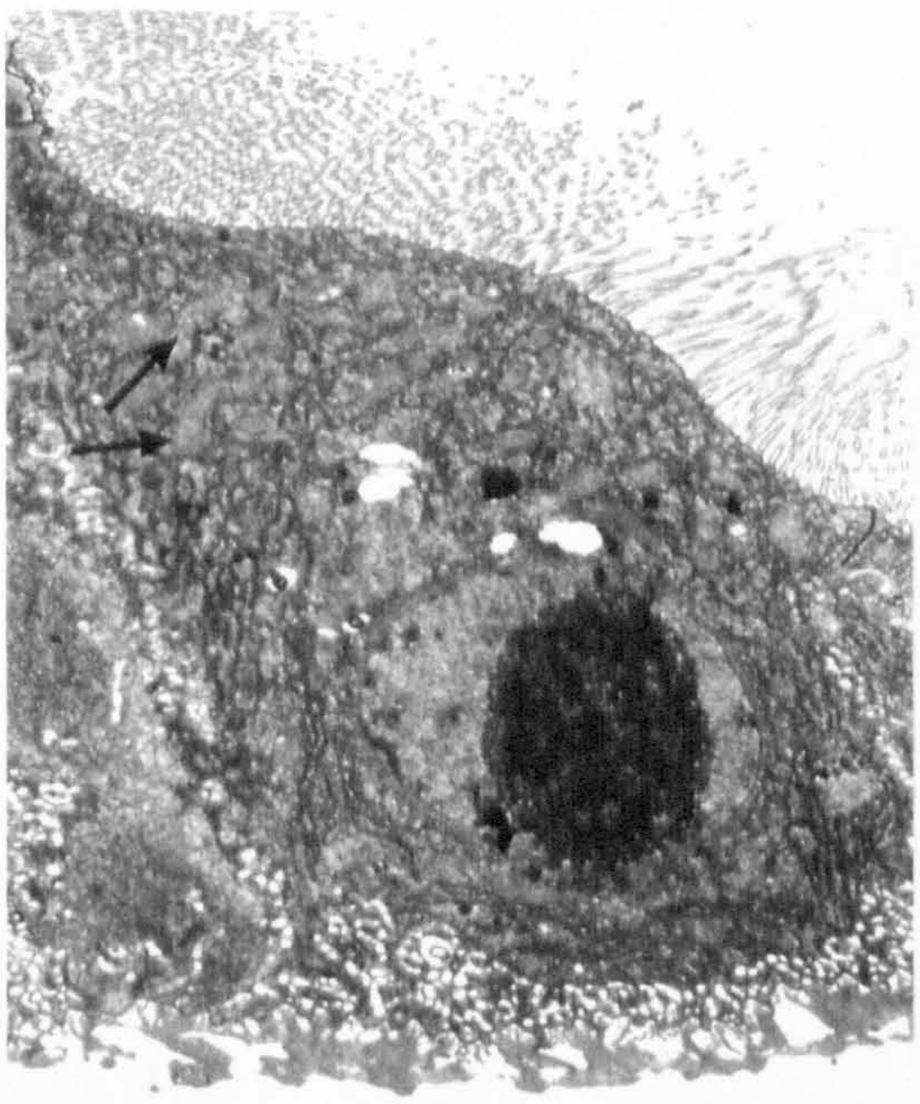

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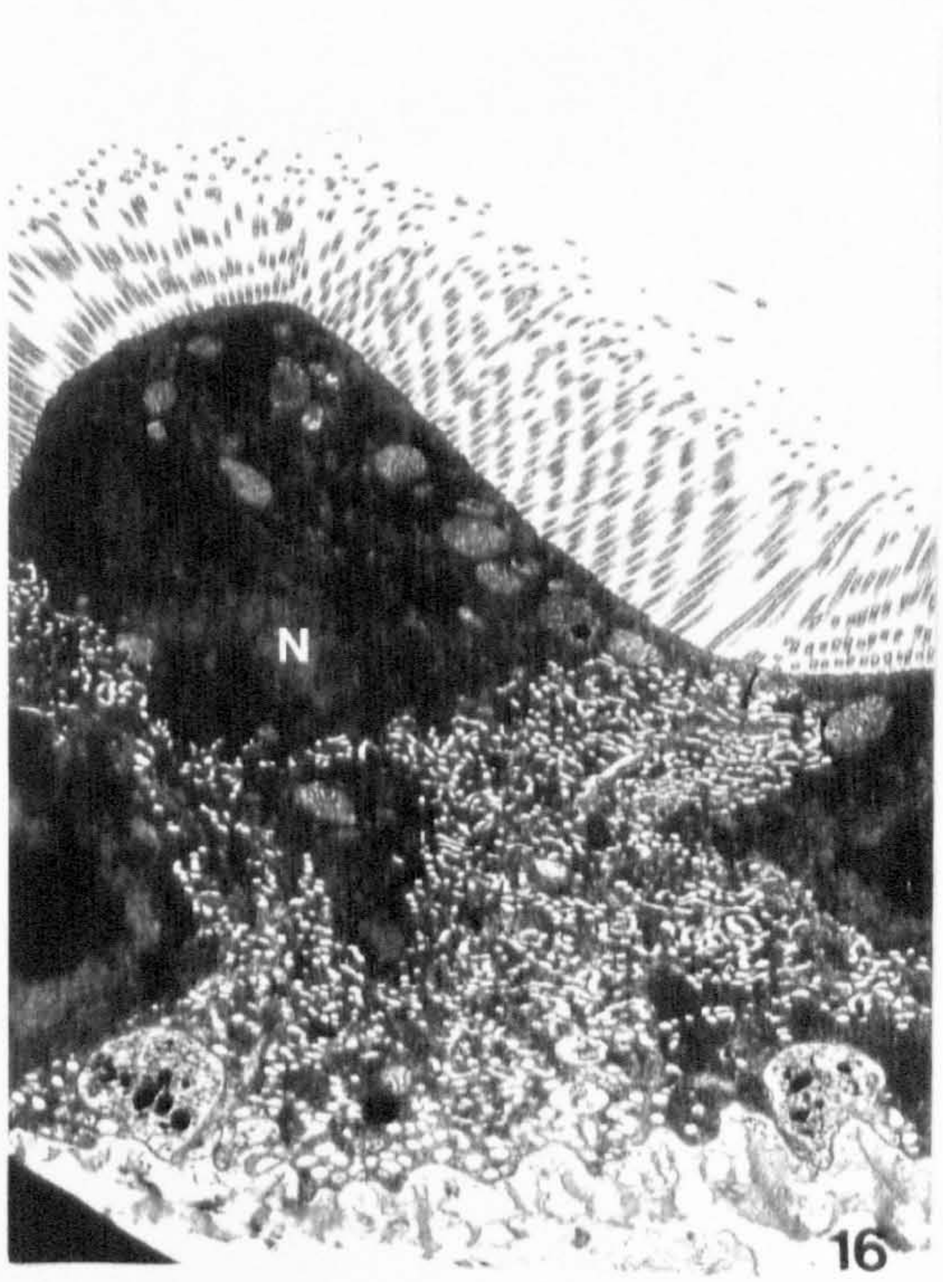

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Plate 3.17 A group of three R3 cells forming a pseudovillus. This finger-like projection is similar to those of the cells depicted in Plate 3.1 . The nuclei (N) of two of the cells are visible. The third cell is to the left (note the tight junction (arrow) separating it from the top cell. Another tight junction can be seen below the labelled lipid (L) droplet (L). Note the concentration of mitochondria in the apical part of the cells and the profuse small vesicles that form the clear "channel" seen at lower magnifications (as in the inset, 2800X) or with the light microscope (or as in Plates 3.40 or 5.2). TEM 9,800X.

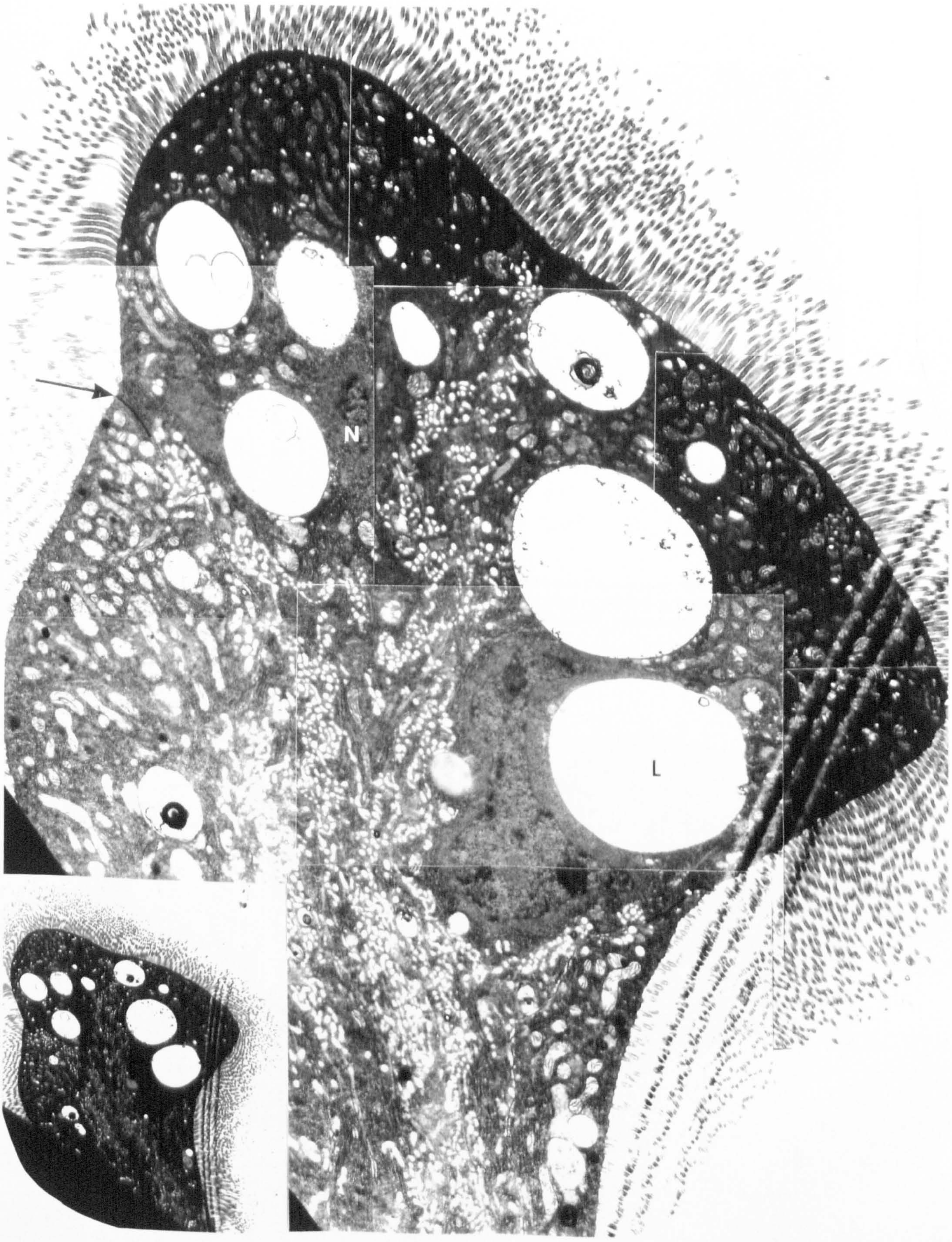
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Plate 3.18 R4 cell. This stage marks the start of the condensation process that will lead to the formation of an apoptotic cell. Autophagic vacuoles are abundant and usually concentrated above the nucleus (N) as in this micrograph. Note the mitochondria crammed at the apex of the cell and the change of microvilli appearance. TEM 5,900X..

Plate 3.19 . R4 or Early A-cell at the apex of a pseudovillus. The condensation process has almost finished and the R-cell has been separated from the basal membrane so it can be extruded. The cell boundaries are indicated by the arrows and two tight junctions at the apex. TEM 5,900X.

Plate 3.20 A-Cell. Note the cell membrane at the left beginning to collapse towards the large vacuole leaving a notch that is sometimes seen in these cells with the SEM (see Plate 3.24 and 3.25). Note that the microvilli are now scarce .. TEM 5,900X.

Plate 3.21 A-cell completely void of microvilli and about to be sloughed into the lumen. Most organelles have disappeared except for just a few mitochondria. Note the acentric nucleus which shows some signs of chromatin fragmentation and on the clear vacuole and another small vacuole with flocculate contents just below the nucleus. An R-cell with its microvilli is to the left. 5900X.

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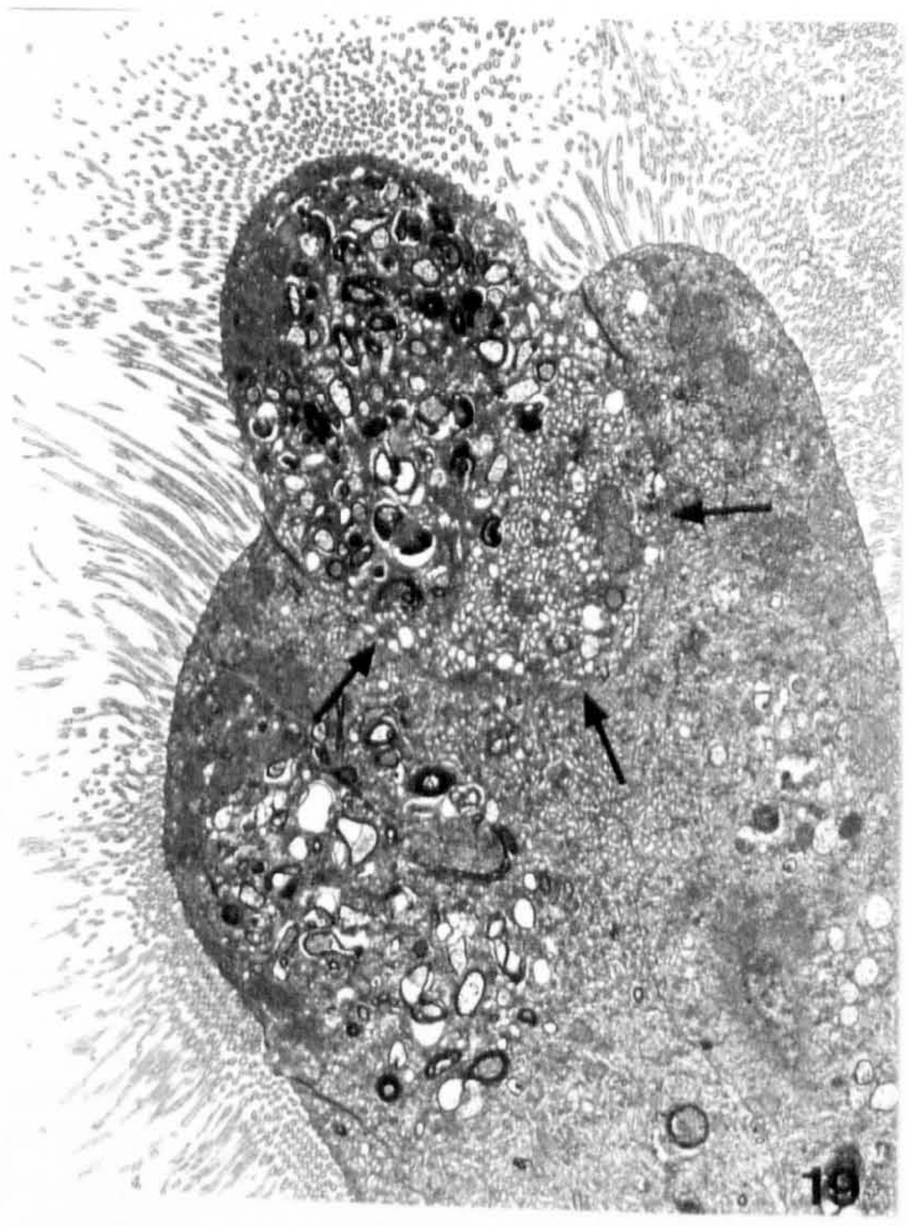

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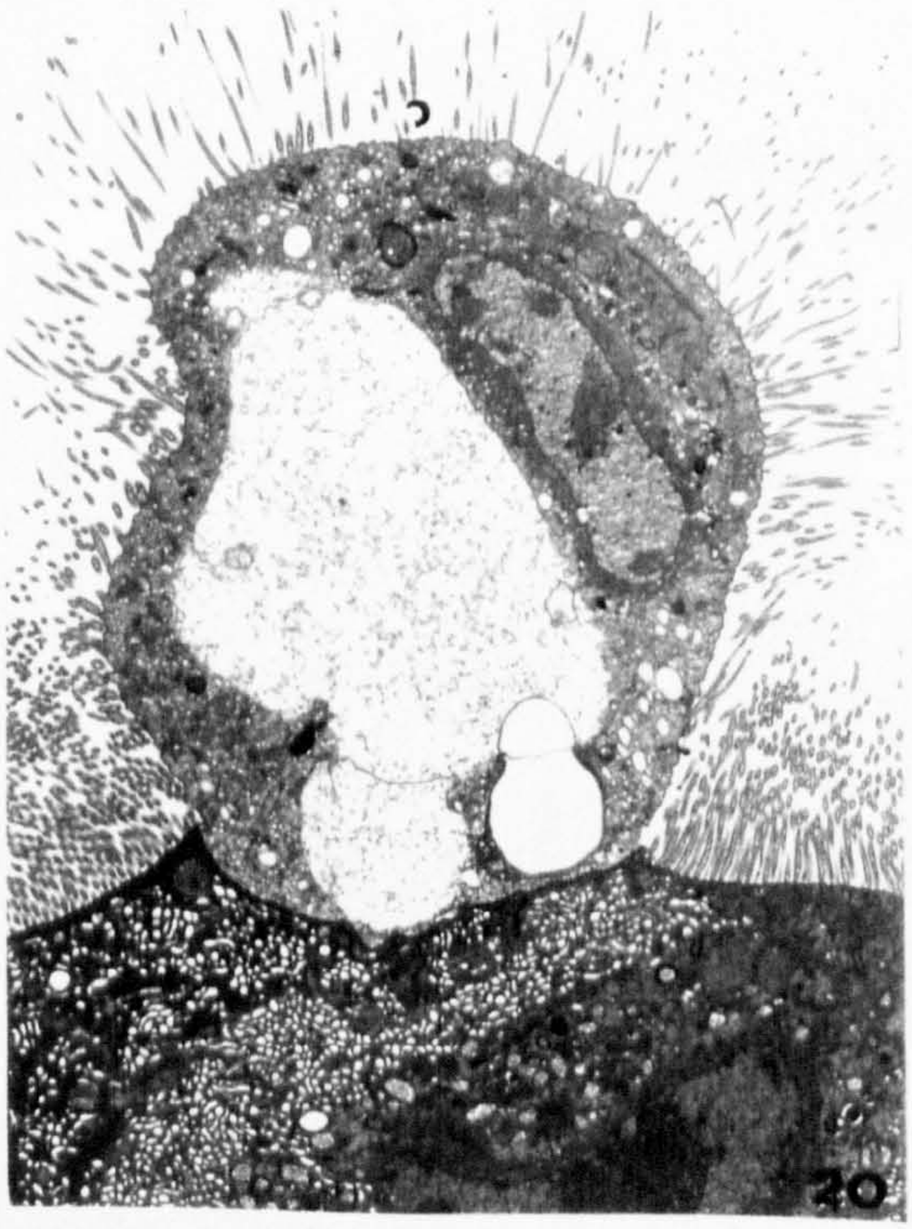

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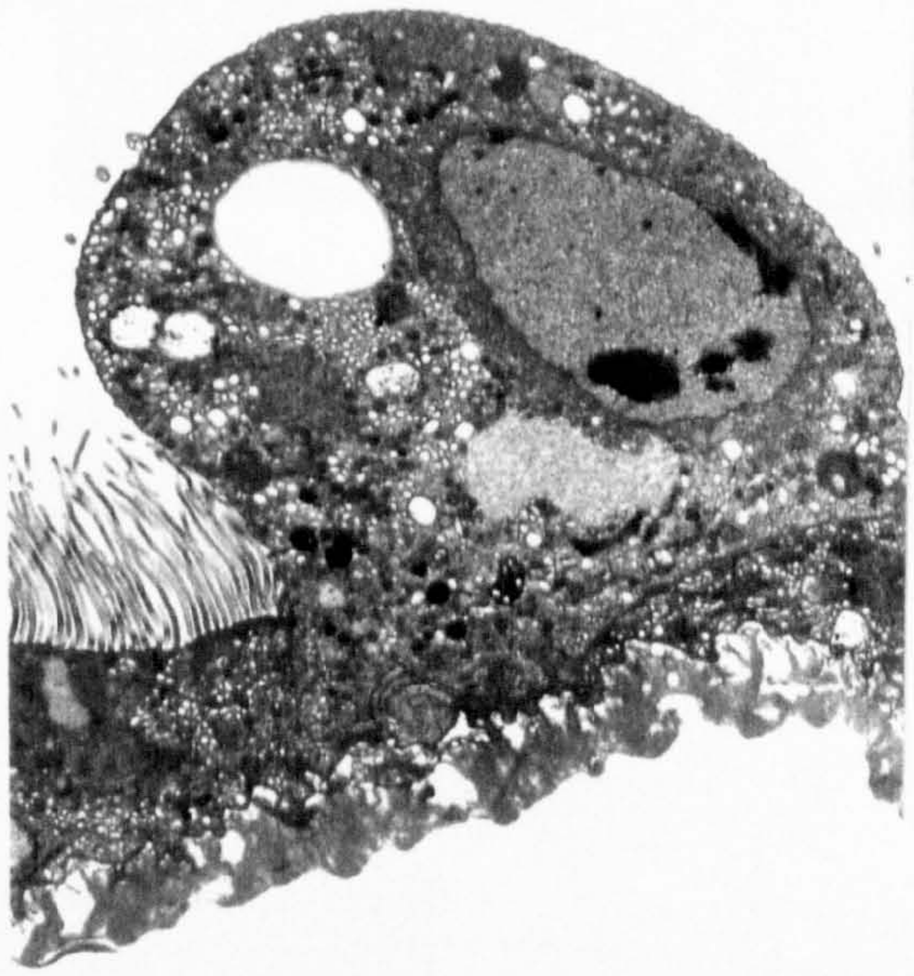

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Plate 3.22 Three A-cells, two of them sloughed into the lumen of the gut. Note the large clear vacuole in which the outer membrane has collapsed inwards. There are smaller clear vacuoles, multivesicular bodies and mitochondria, the latter still retaining a good condition. A small section of the nucleus can also be appreciated(n).. TEM 4,300X.

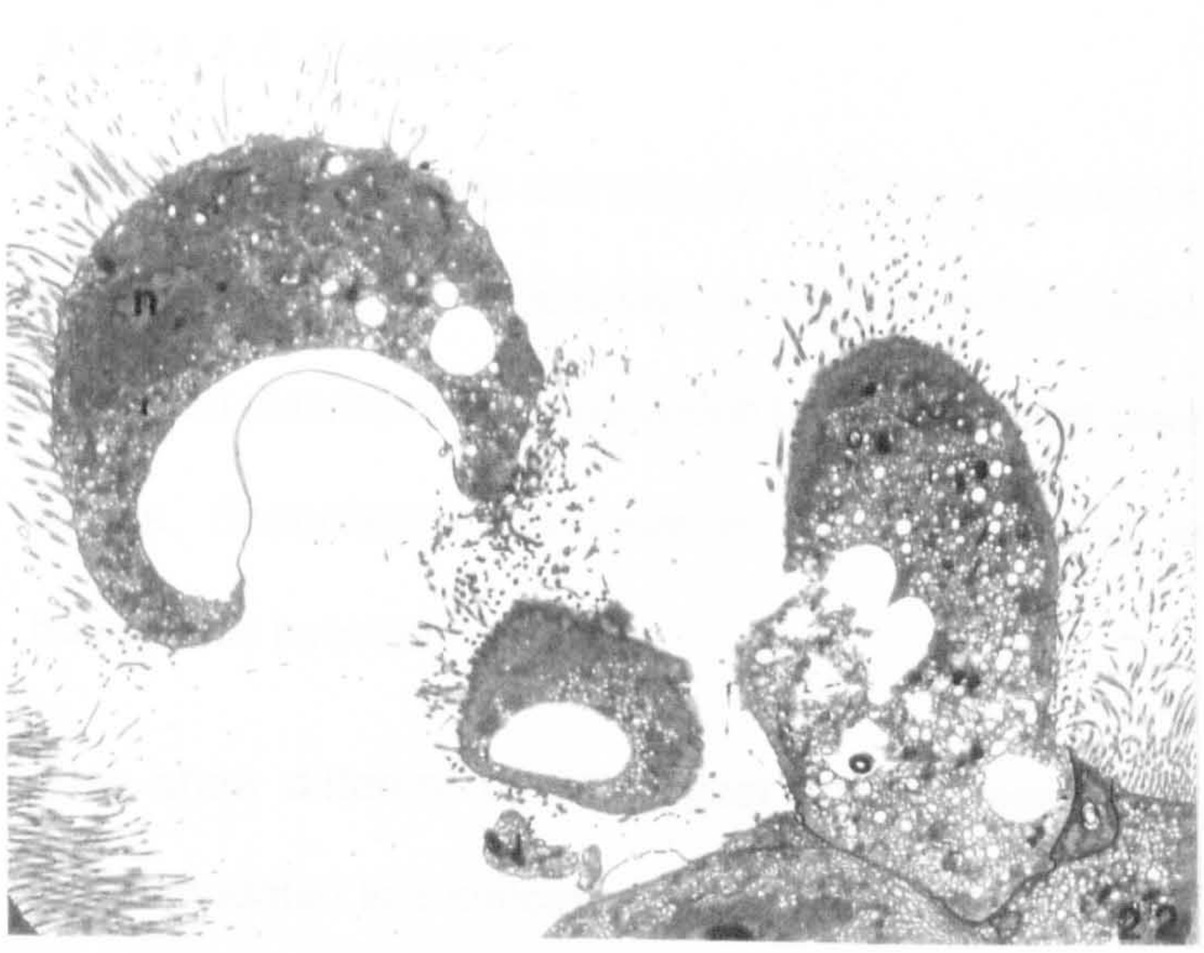
Plate 3.23 Two A-cells in the process of being extruded from the underlying epithelium. Note the chromatin condensed to the periphery of the nucleus in one of the A-cells (arrow). The cells are cleanly extruded, leaving no scar in the epithelium.. Note also the attachment of a projection of a circular muscle to the basal membrane (big arrow). TEM 1,800X

Plate 3.24 Group of A-cells protruding among R-cells. The two at the top left still have most of their microvilli but note the fluffy appearance compared to the R-cells. The two cells below have partially lost their microvilli. The A-cell on the lower right has completely lost its microvilli. Note the notch in their surface where the cell membrane has begun to collapse. 2500X.

Plate 3.25 Two A-cells showing the big notch in their surface corresponding to the clear vacuole that exists below it (compare with Plate 3.22). A sheet of peritrophic membrane (p) lies over the microvilli of R-cells near the A-cell on the left. SEM 2500X

Plate 3.26 Apical region of a mature F-cell. Mitochondria of various shapes and with dense matrix are scattered through the RER .Several Golgi bodies, also with dense contents, can be seen (arrows). Secretion granules are also a common feature (arrow heads). TEM 7,500X..

Plate 3.27 Apical region of a mature F-cell. A Golgi body (G) with dense contents is associated with several dense secretion granules (arrows). Note also the abundant RER (arrow head) and free ribosomes. TEM 22,000X..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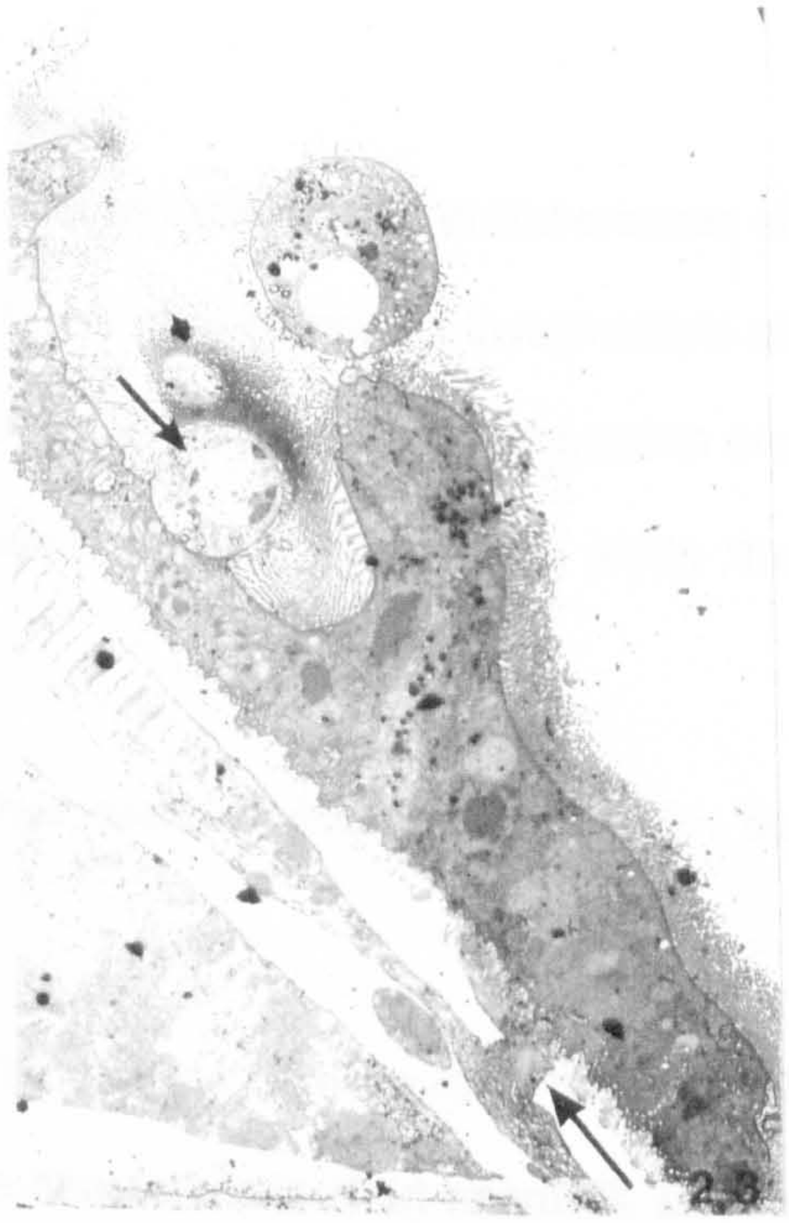
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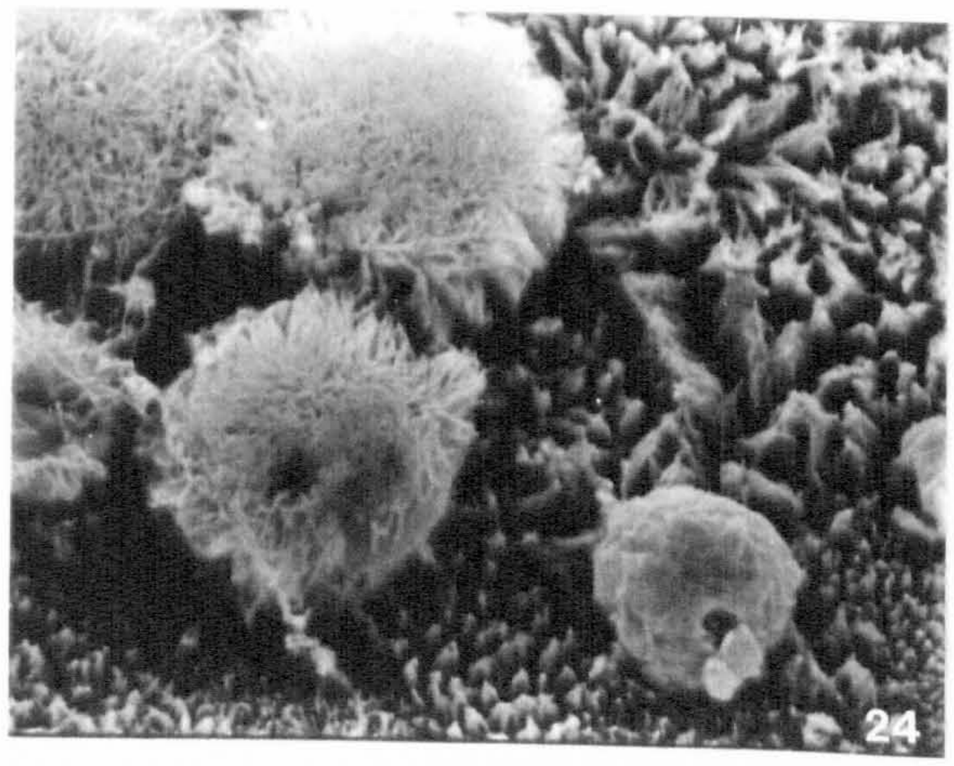
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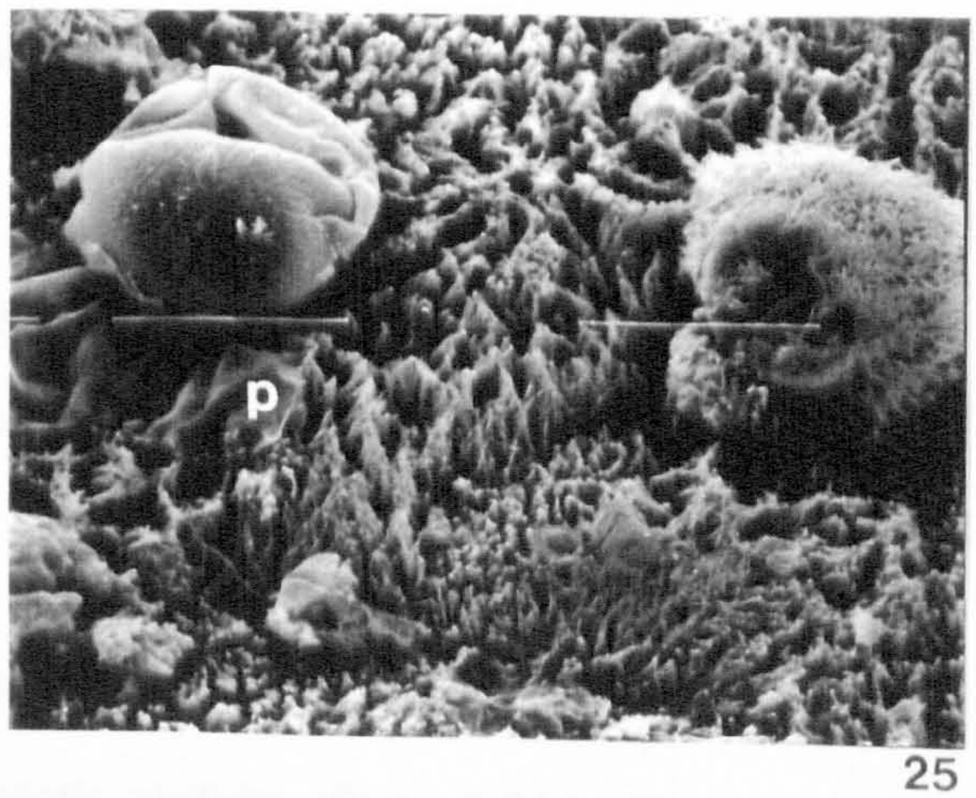
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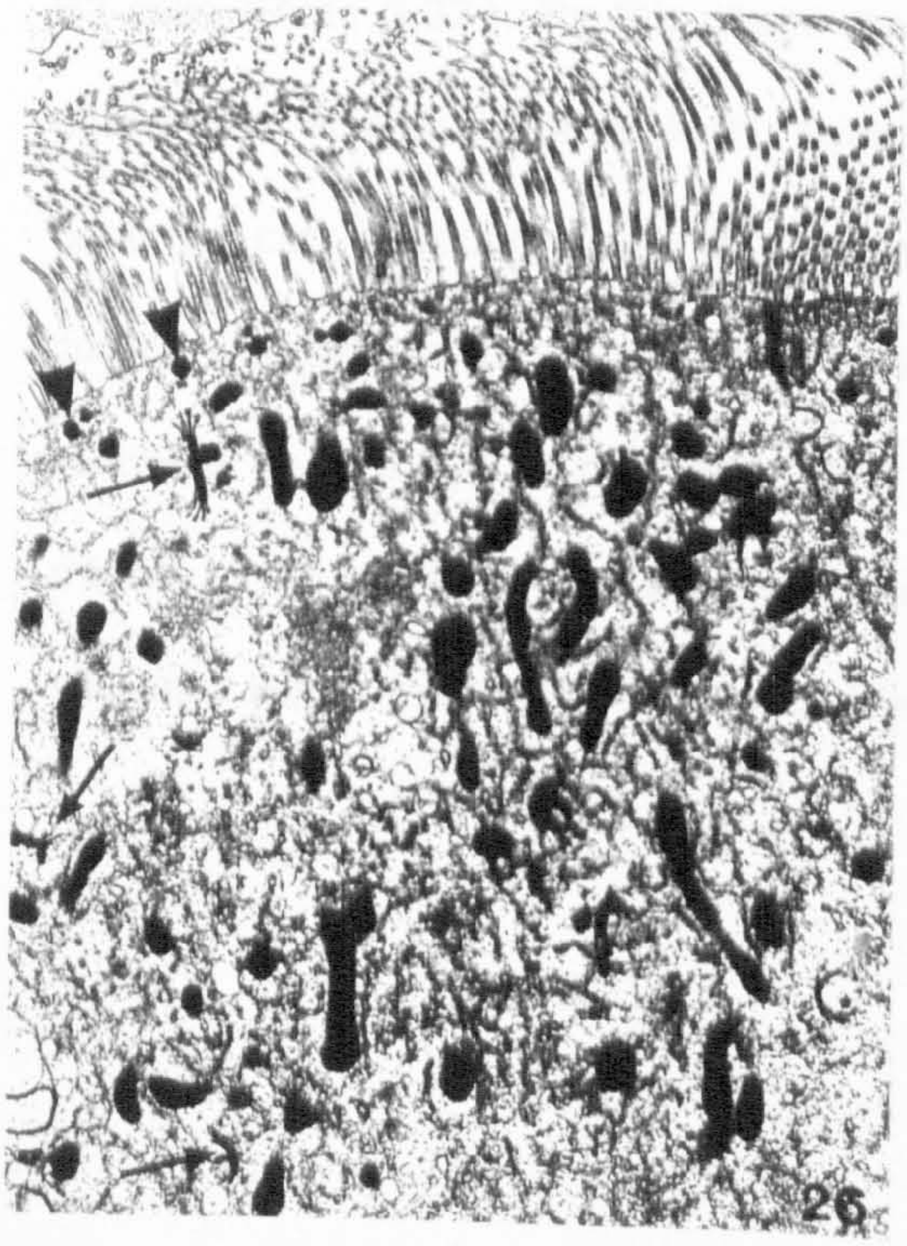
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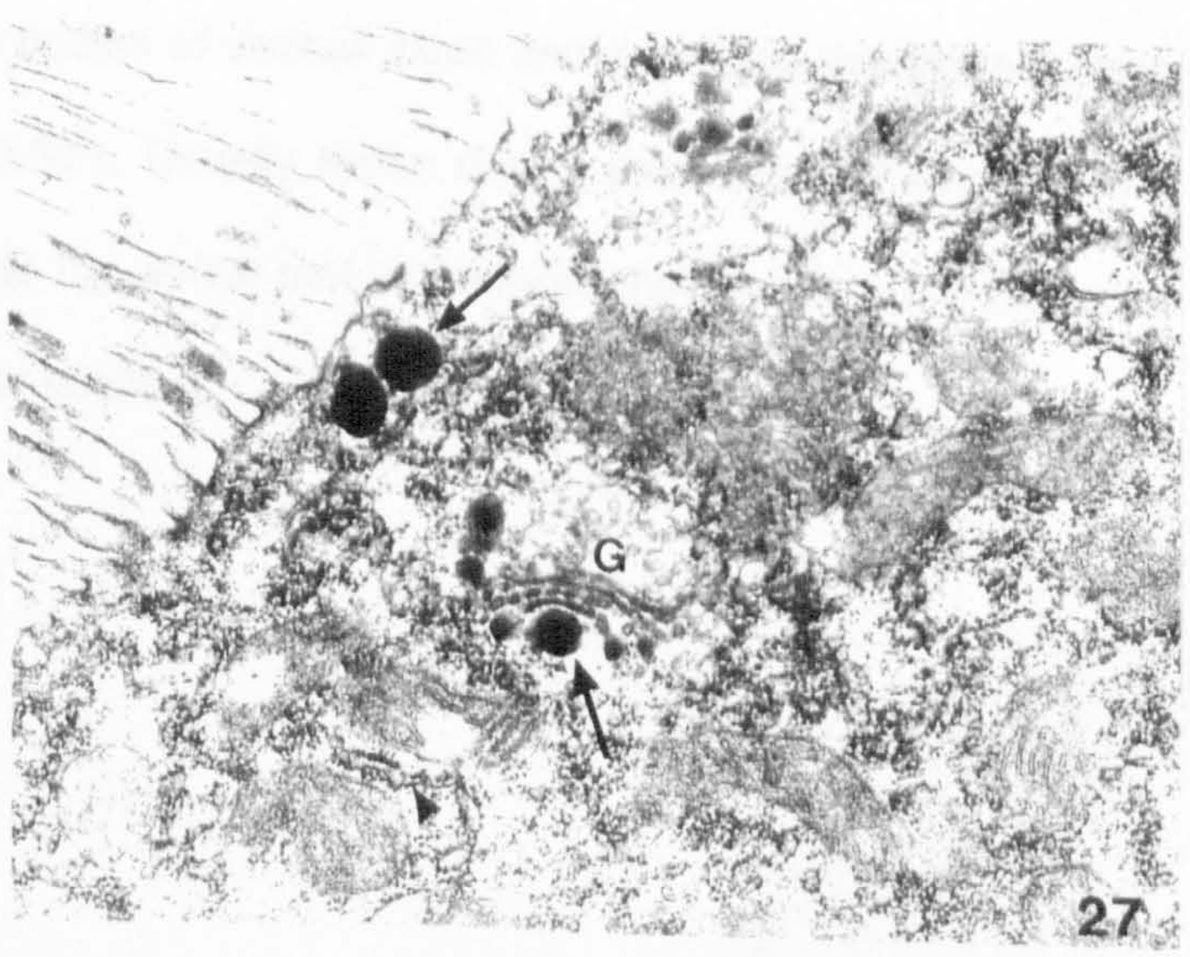
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3.2.3.1.1.5 B-cells.

The microvilli of B-cells are very short, 0.3-0.4 μm, or sometimes reduced to little protuberances of the apical membrane. Extensive pinocytotic activity exists in the form of numerous invaginations of the apical plasma membrane which then form small clear vesicles of varying size. Mitochondria are scarce. Endoplasmic reticulum is generally lacking and when present it is limited along the boundaries between cells.

They show different morphological features according to their state of evolution which can be accommodated in 5 successive stages, B1-B5.

B1-cells (Plate 3.31) are attached to the basal membrane. The remains of microvilli of the last F/B stage have disappeared and the apical membrane shows the characteristic short microvilli. Dense bodies are small but some have begun to coalesce into bigger ones.

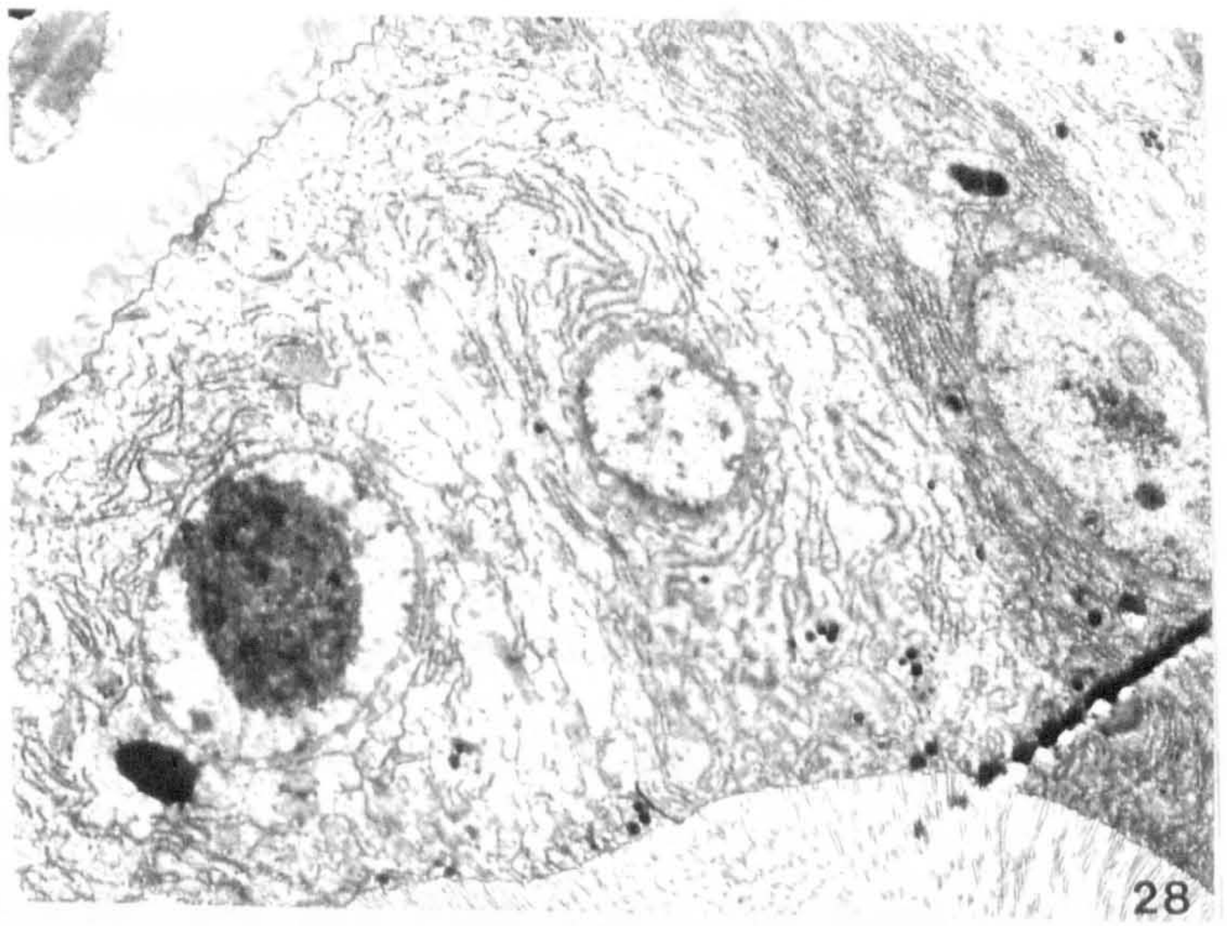
In B2-cells (Plate 3.32) the dense bodies are now very conspicuous. Clear vacuoles, probably secondary lysosomes, start to appear. Anastomosing tubules start to form at the basal and lateral cell membranes and the cell acquires an inverted cone shape as it narrows towards the base.

B3-cells (Plate 3.33). By this stage the B-cell has lost contact with the basal membrane. The pinocytotic activity is quite high, several early endosomes with electron lucent or flocculate contents can be appreciated just below the pinocytotic surface (Plate 3.34). The sub apical complex is dominated by numerous dense bodies of various sizes and densities, the biggest ones reaching up to 2 μm in diameter (Plate 3.35). Usually these dense vacuoles are larger in the middle of the complex and smaller towards the apical part or towards the nucleus. These dense vacuoles were never seen in an infranuclear position.

Plate 3.28 A group of three F-cells showing different degrees of density of the cytoplasm. Note a few dense secretion granules apically in the two clearer cells. TEM 4,300X.

Plate 3.29 Apical portion of an F/B cell showing heavy vesiculation of the microvilli and detachment of the microvilli from the apical cell membrane. Pinocytosis is also starting to develop. TEM 18,000X..

Plate 3.30 F/B cells. Sloughing of the microvilli is in a more advanced stage than in Plate 3.29 . The cell on the right has already started to produce the dense bodies of lysosomal origin characteristic of B-cells (arrows). They are still small and are associated with the pinocytotic vesicles that also have started to take up material from the lumen of the gut. Autophagic vacuoles (big arrow) are more common at this stage and the RER is reduced to some groups of strands. The F-cell on the right still has some secretion granules in the apical part. Note though that the endoplasmic reticulum is mostly vacuolar. Dense mitochondria can still be appreciated (m). TEM 9,800X..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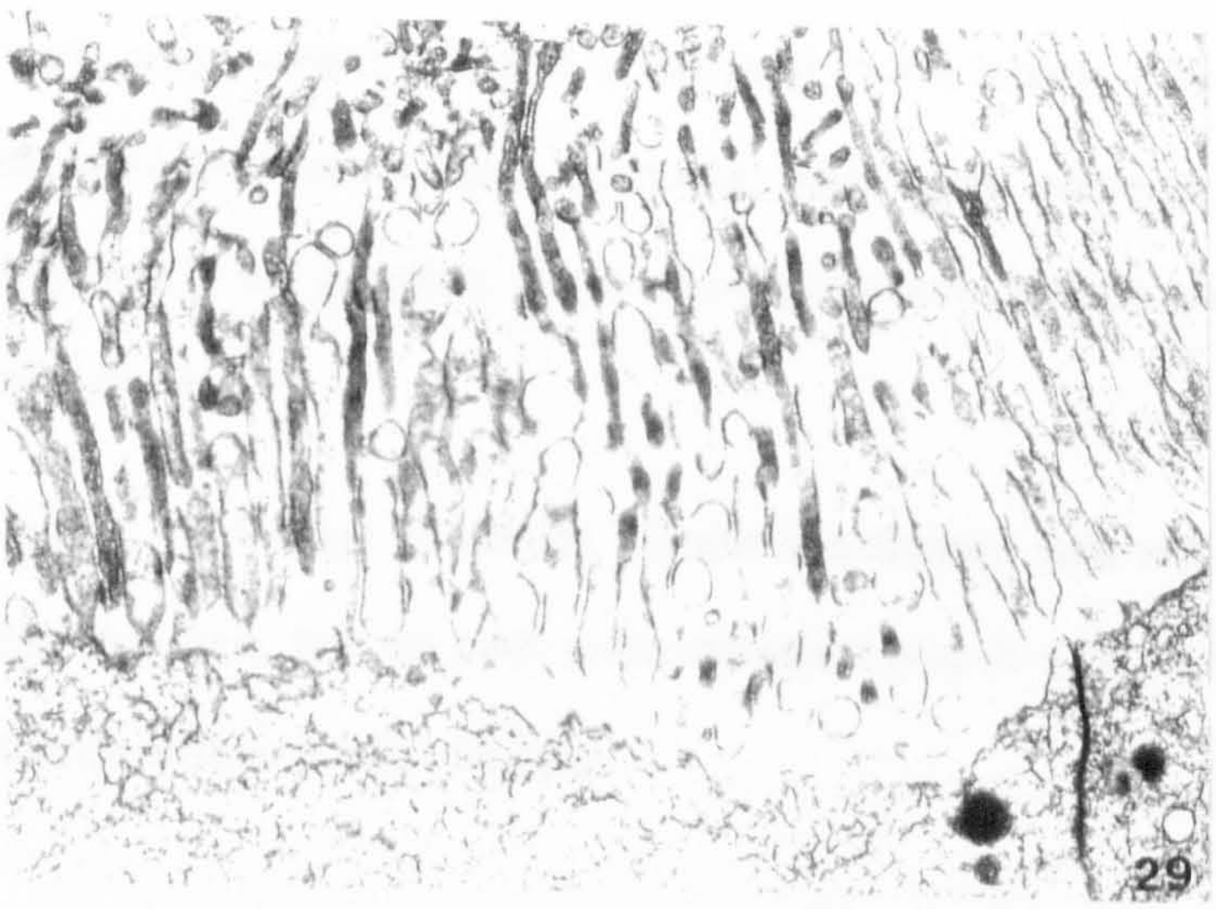
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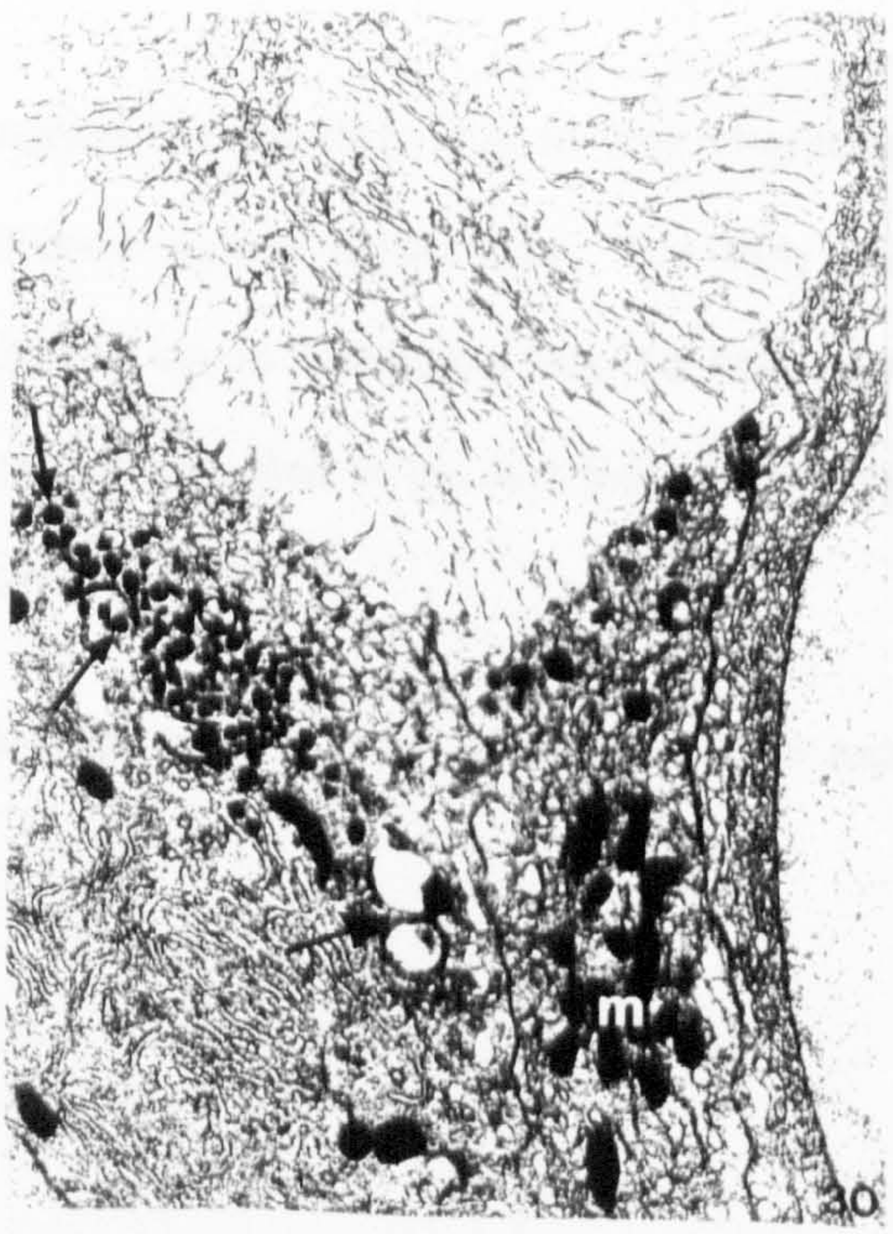
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Below the dense vacuoles and above to the nucleus there are smaller clear vacuoles, multivesicular bodies and autophagic vacuoles.

The Golgi bodies are common, measuring 0.3 X 0.1 µm.

A common feature was a labyrinth of SER at the base or the side of the cell, close to or below the basally situated nucleus, probably a remnant of the invaginations of the basal cell membrane and the basal anastomosing tubules and vesicles when it was attached to the basal membrane.

B4-cells (Plate 3.36 , see also Plate 3.38). The B-cell has started to protrude from the neighbouring R or F-cells; almost half of it is exposed to the lumen. The dense bodies are more numerous and show darkinclusions inside its matrix; there is still some pinocytotic activity, but in a lesser extent compared to the previous stage. More occurrence of autophagic bodies is evident in between the dense bodies

B5-cells (Plate 3.39). This stage of almost complete extrusion was not found in sections under TEM. Under SEM only very few B5-cells were detected and in one or two cases in semithin or paraffin sections (see section 3.2.3.1 above).

SEM

B cells are easily recognised under SEM due to their characteristic spherical surface with very short microvilli, giving them a rugose appearance . The early stages (Plate 3.37) can be easy to miss because the tip of their apical membrane may be obscured among the long microvilli of the surrounding R or F-cells and also due to the fact that usually they are on the sides of the rugose folds of the gut epithelium

More advanced B cells have most of their apical membrane exposed and are the most common figure (Plate 3.38)

Later stages are less common to see, the cells being almost completely exposed (Plate 3.39).This may suggest that the extrusion is a very quick process. These B-cells can be distinguished from the A-Cells by the rugosity of their exposed membrane and because there are no remnants of long microvilli. The exposed membrane of A-Cells is smooth and remains of microvilli are a common sight.

3.2.3.2 Light microscopy.

3.2.3.2.1 E-cells.

E-cells are small, cuboidal and possess a dense cytoplasm (Plate 3.40 . See also Plate 5.2). Their nucleus occupies most of the cytoplasm. Two or three small nucleoli are commonly seen. It is common to find E-cells without contact with the lumen, covered with R or F-cells. The apical surface, when exposed, possesses microvilli. They stain well with Toluidine blue.

3.2.3.2.2 R cells and F-cells.

These cells are the main type of enterocytes lining the midgut. The differentiation between F-cells and R-cells is very difficult under light microscopy. Only by comparison between the EM observations with adjacent semithin sections stained with toluidine blue it was possible in some cases to distinguish between the two, . Therefore, for practical purposes at least, under light microscopy it is safer to call R and F-cells together "microvillar cells", as Bron *et al.* (1993) did in the larval stages of *L. salmonis* .

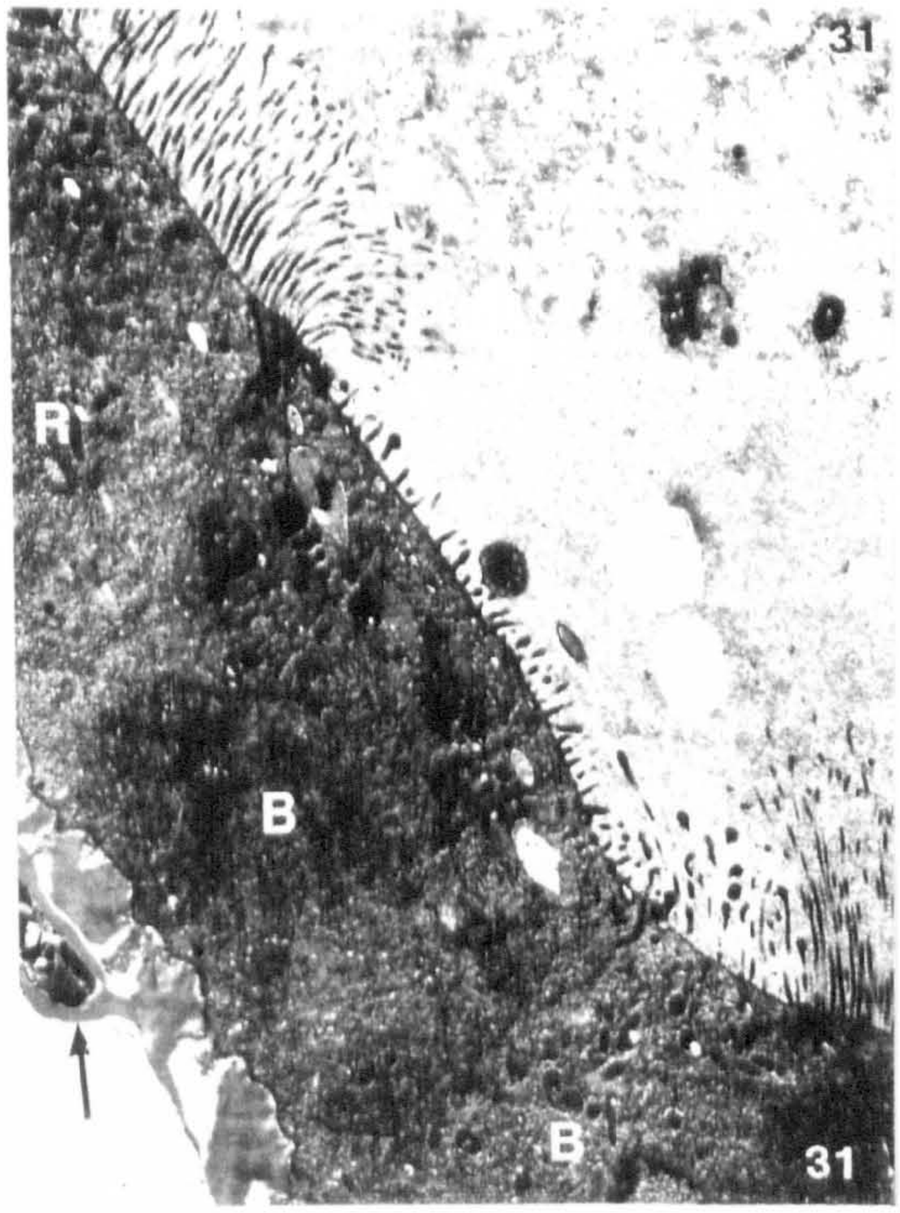
When the R-cells start to mature and apparently be more active they develop a bulge in the apical part which is packed with mitochondria. It is from these cells that the following description of R-cells is given. They are generally club shaped, although cuboidal, or cylindrical types can also be found.

Plate 3.31 B1-cell (B). The cell is attached to the basal membrane; the remnants of the microvilli characteristic of F/B cells no longer exist. Dense bodies are still small. A young R-cell (R) is at the left and another B1 cell at the right. Note at the bottom right of the photograph a lateral projection of a circular muscle surrounded by a layer of basal membrane material (arrow). TEM 5,900X.

Plate 3.32 B2-cell. The primary lysosomes (dense vacuoles) are now conspicuous. Note the inevrted cone shape, the short microvilli at the apex and the multivesicular network at the sides of the cell. 18,000X..

Plate 3.33 B3-cell. The cell is now separated from the basal membrane. Labyrinthic forms of endoplasmic reticulum are common feature (arrow). Note the subapical complex formed of large secondary lysosomes and smaller ones just above the nucleus (n). Note a group of autophagic vacuoles also above the nucleus. TEM 5,900X..

Plate 3.34 High magnification of the apical membrane of the B3-cell of Plate 3.33 showing pynocytotic vesicles (short arrows), early endosomes (long arrows) with flocculate material inside them and dense bodies below. TEM 36,000X..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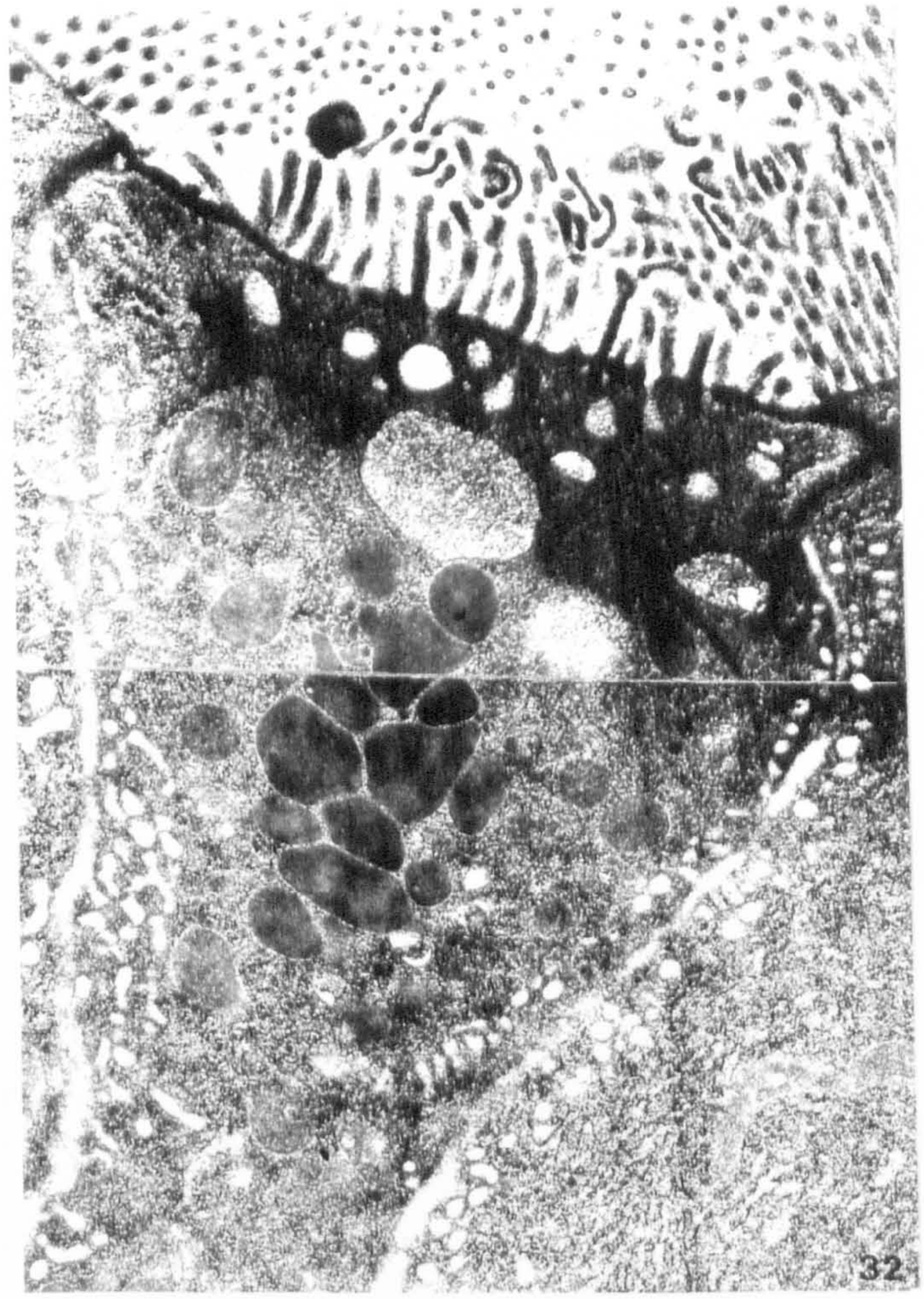
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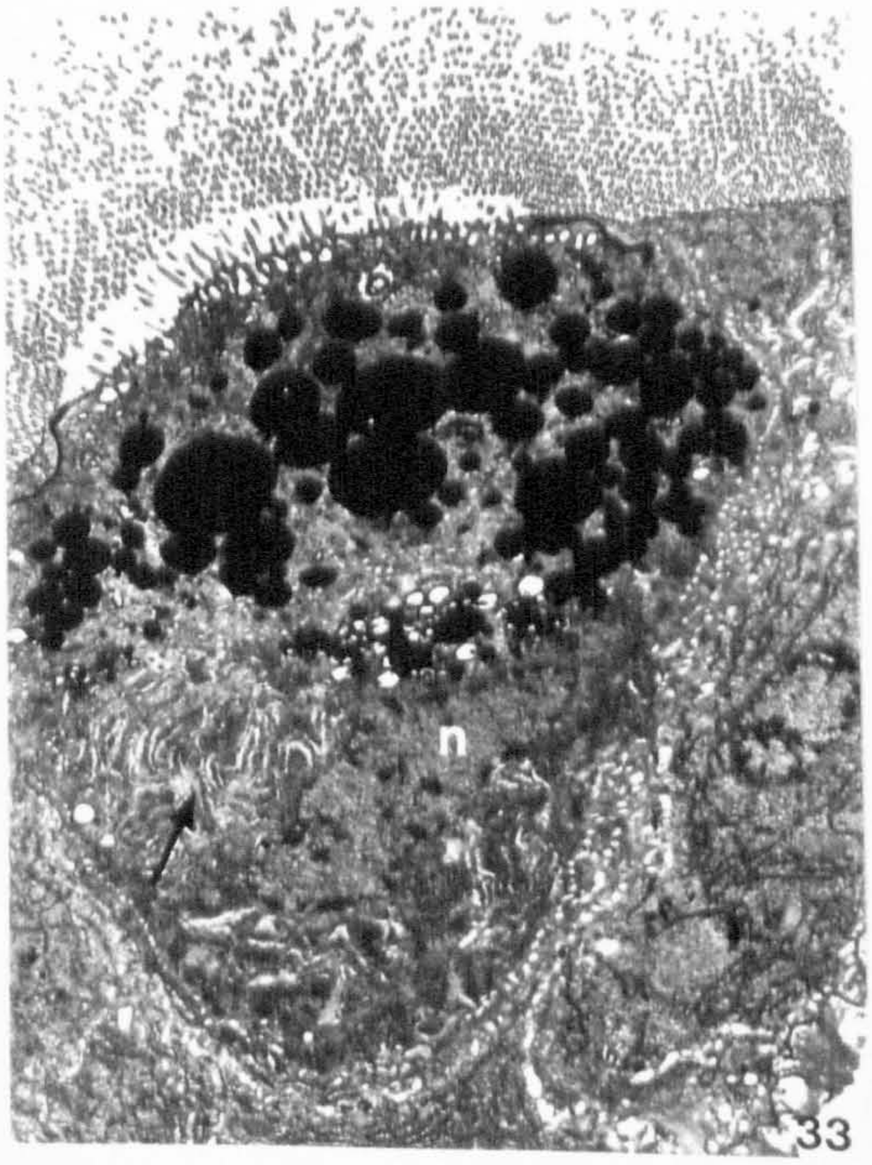
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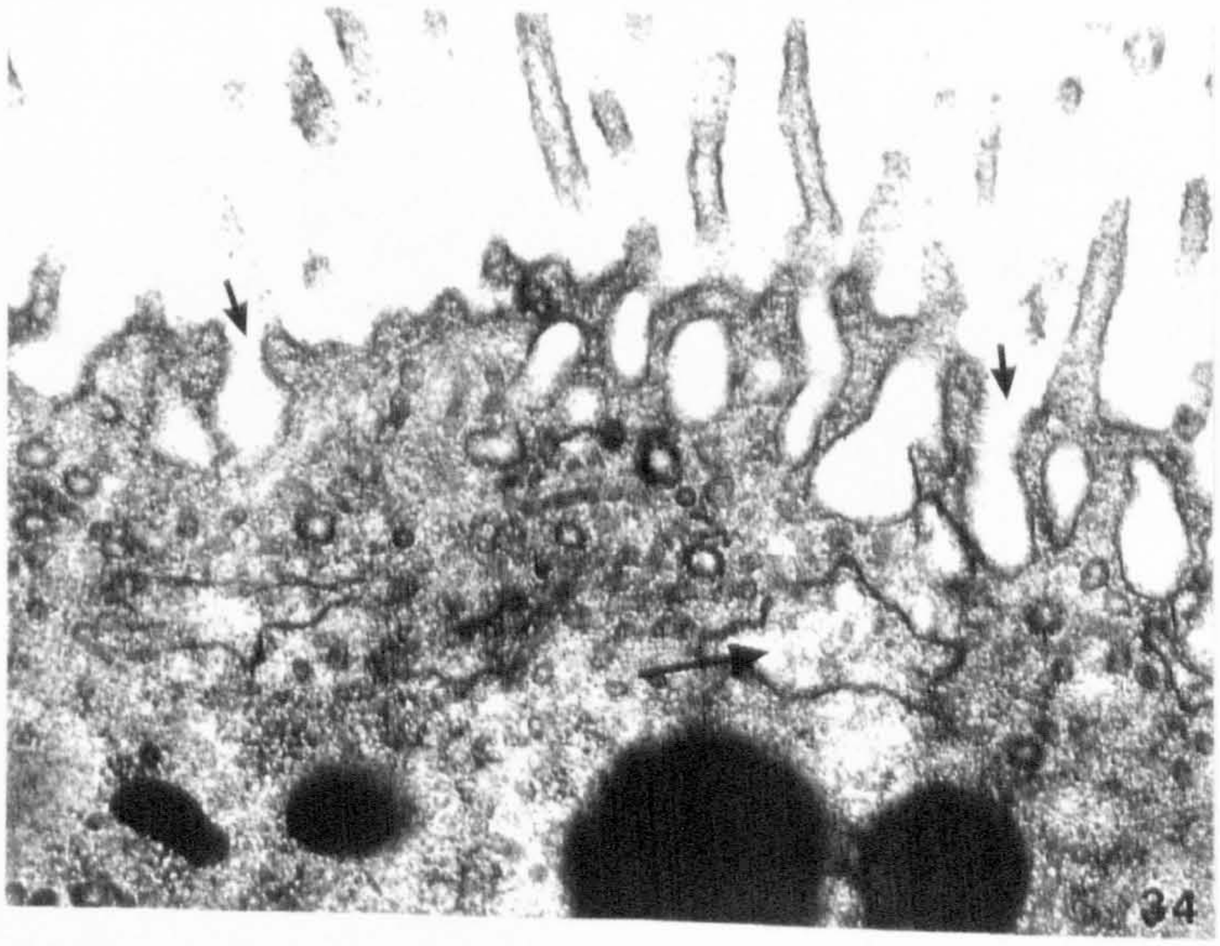
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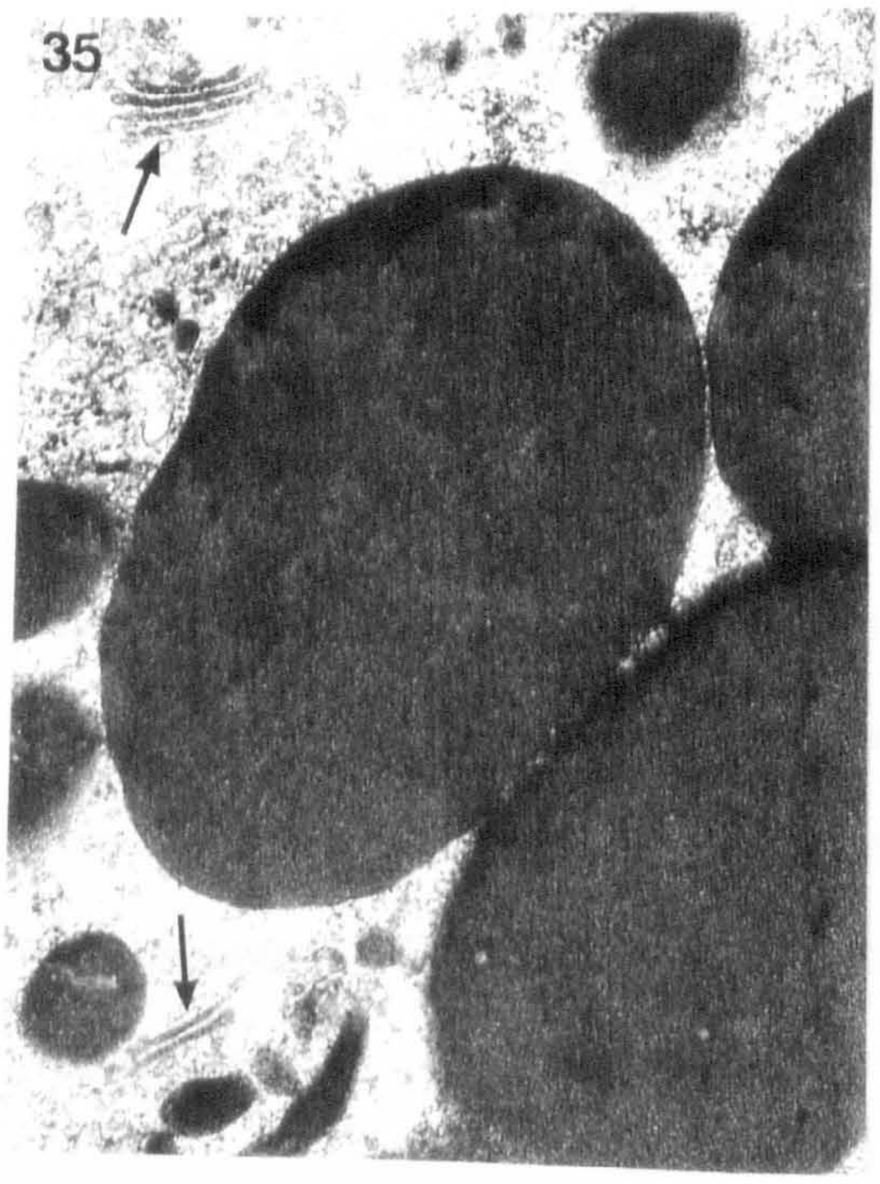
Plate 3.35 High magnification of the subapical complex of the B3 cell depicted in Plate 3.33 . Note the Golgi bodies (arrows) and the homogeneous contents of the primary lysosomes. 36,000X

Plate 3.36 B4-cell. Almost half of the cell is now protruding from the surrounding epithelium. The nucleus is not shown in this section. TEM 5,900X..

Plate 3.37 An early B-cell (arrow head) with its characteristic apical membrane with short microvilli starting to make its way between the surrounding R-cells. An A-cell can be seen in between a fold of epithelium (arrow). SEM 2500X.

Plate 3.38 B4-cell, with most of its apical membrane now exposed. SEM. 5000X.

Plate 3.39 Two B-cells in their latest B5 stage. SEM 2500X..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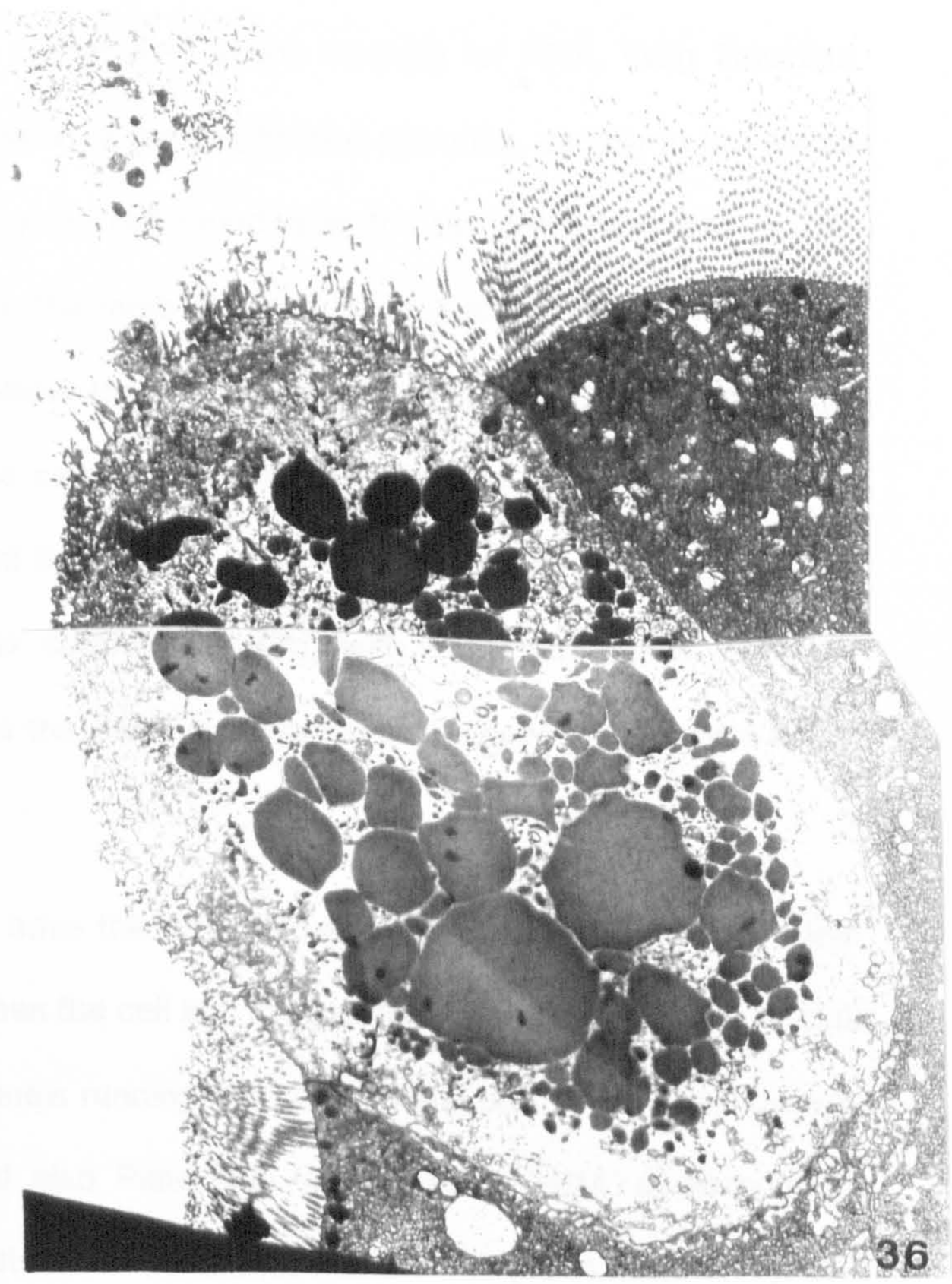
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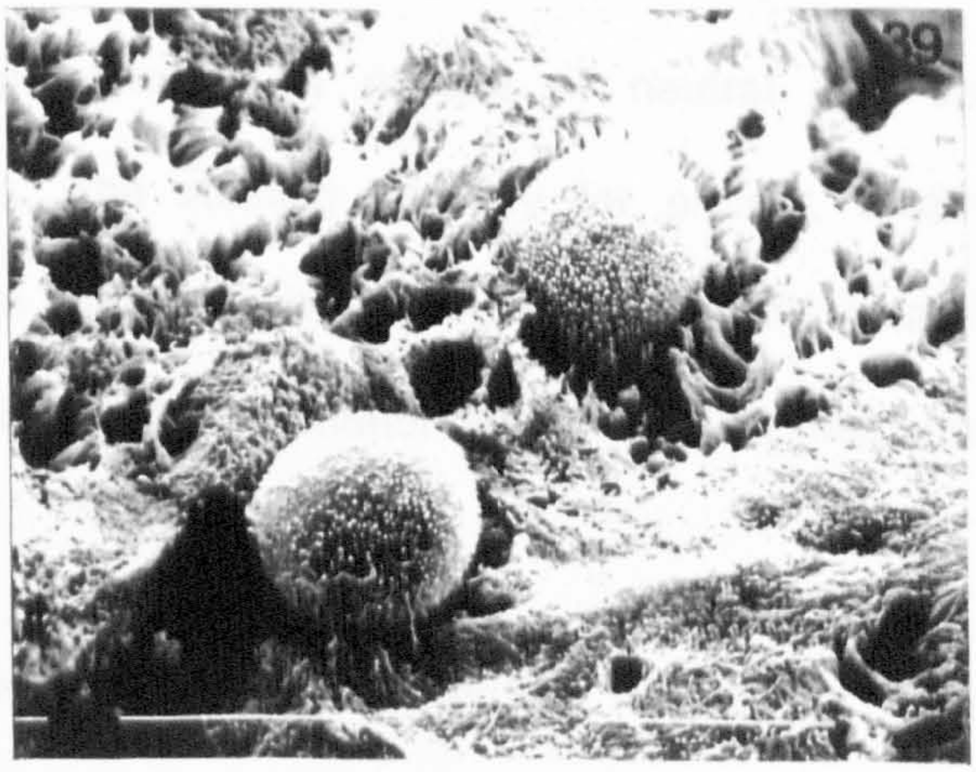
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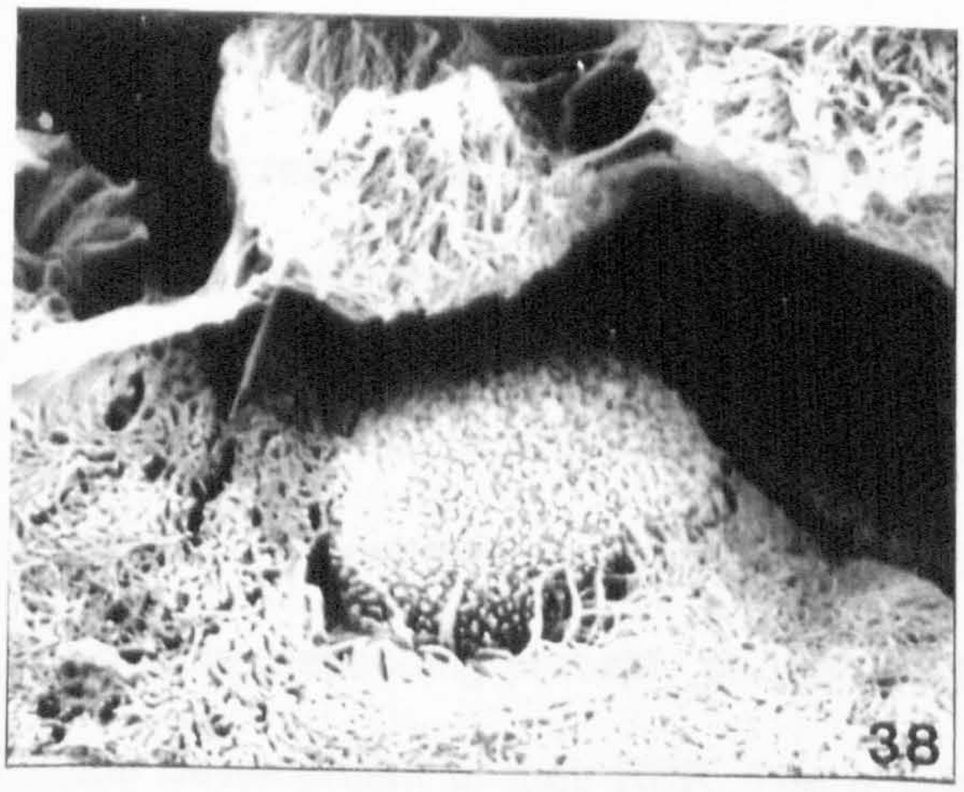
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They have a cytoplasm of variable density. Generally it stains strongly for RNA. With Toluidine blue, the mitochondria can be differentiated as very small light blue granules, usually in the apical region, forming a characteristic bulge making the cell rounded at the tip (see Plate 3.41). The more mitochondria present in the apical part, the more rounded the cell looks. Sometimes, small dark granules can be scattered around and above the nucleus, identified under TEM as autophagic vacuoles and/or multivesicular bodies. These cells can also have large lipid droplets in the apical region (Plate 3.40), which are more frequent if the louse has fed on blood (See Chapter 5). The nucleus in cuboidal or columnar R-cells is centrally located, with one, sometimes two very conspicuous nucleoli. In club shaped R-cells the nucleus is displaced apically towards the apical bulge.

The cell borders are sharply defined. They have the appearance of possessing an area of clear material between cells and sometimes between the cell and the basal membrane. In groups of club R-cells, this clear material appears like channels running from the luminal part of the cells towards the basal membrane (see Plate 3.40 and also Plate 5.2) which, under TEM, correspond to sections of the profuse vesiculation that exists at the boundaries of the cell (as in Plate 3.17). The apical part of the cells is elaborated into tightly packed microvilli. The microvilli usually stain strongly for protein, alkaline phosphatase and alcian green pH 2.5. They are also PAS (Plate 3.42) and aldehyde-fuchsin positive, indicating the presence of a mixture of neutral and acid mucopolysaccharides and sulphated sialomucins. The staining intensity for protein, alkaline phosphatase, PAS and aldehyde-fuchsin is variable amongst cells or groups of cells and usually a stronger reaction is associated with long microvilli. With Bromophenol blue, the apical membrane stains stronger than the microvilli.

As already noted, it is difficult to differentiate F-cells from R-cells under light microscopy. F-cells can also be cuboidal or columnar. Their density, when stained for RNA or with Toluidine blue is variable, depending probably on the quantity of RNA present, the quantity of free ribosomes and the development of their RER. The boundaries between cells can also be differentiated as clear

lines, just as in R-cells, but this is probably due to the extracellular network of the adjacent R-cells, as in F-cells this network is generally absent. F-cells do not develop the bulges characteristic of mature R-cells and apparently they do not store lipids.

Both R- and F-cells were always negative to esterases, and both showed a strong positive reaction to ALP in the brush border. Also, the brush border was PAS and alcian green 2.5 positive, and showed light to moderate staining for proteins. In plastic sections the staining of the brush border with Alcian green 2.5 was very weak and sometimes negative while, at the same time, the goblet cells of the fish skin stained intensely. It is probable that there is less material available to stain in the brush border in these thin sections, compared to the paraffin wax sections. In the latter, the staining with alcian green was sharp and intense in most cases, and although occasionally there were lice that showed a weak staining, they were never negative.

3.2.3.2.3 A-cells

A-Cells possess a pale cytoplasm and sometimes clear vacuoles of different sizes can be seen or one large vacuole. Sometimes A-cells also display dark granules in their cytoplasm which correspond to the multivesicular bodies and autophagic vacuoles seen under TEM. The nucleus is located to one side; generally they have no microvilli or very few. They are found in the process of being pinching off from the apical part of R-cells (Plate 3.41).

3.2.3.2.4 B cells

B cells stand out clearly among the surrounding R or F-cells, first because of the very short microvilli at their apical surface which, under the light microscope, can appear as if they do not have microvilli at all, and second, because of the characteristic staining of their vacuoles (Plate 3.40). These vacuoles react strongly for PAS (Plate 3.42) and for protein indicating that the contents are complexes of protein and polysaccharides. These same vacuoles stain positive for non-specific esterases (Plate 3.43). The cytoplasm stains positive for this enzyme as well, although weakly, and this is probably due to diffusion of the reaction product.

The length of the B-cells is less than the surrounding R or F cells and they appear to be wedged between adjacent cells, which accordingly curve around beneath them.

Although they give the impression of protruding and eventually pinching off, this picture was seldom seen, except for one or two cases (Plate 3.44).

Also, except for very few cases, they were not seen extruded into the lumen of the midgut.

3.2.4 Classification of midgut types.

3.2.4.1 Low magnification level.

At dissecting microscope level, three types of gut could be differentiated according to their appearance: Rough, Smooth, and Opaque.

The assessment was done at 4X with the louse lying on its dorsal surface so the gut could be seen through the cuticle of the ventral side, which usually has less pigmentation. On a heavily pigmented louse the assessment was more difficult.

Rough.

The gut looked as if it was creased (Plate 3.46) and usually only the ventral creases could be seen. It appeared as if the epithelium was not as "transparent" as that of the smooth type (see below). Under SEM (Plate 3.60) this gut showed that the creases were in fact groups of enterocytes forming lumps and crypts of variable size. This gut would correspond to a Medium Crypted type (see the classification at light microscope level below, section 3.2.4.2).

Smooth.

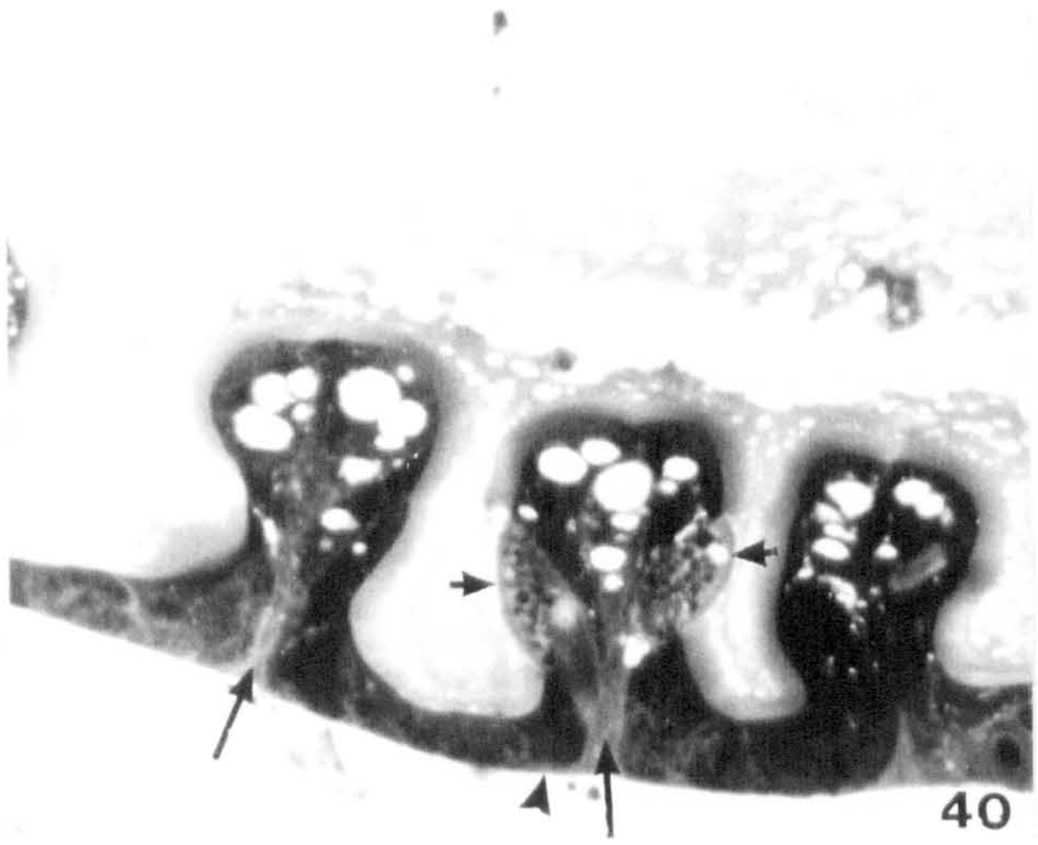
There was no sign of creases in this gut. The dorsal and ventral part of the epithelium could be seen, *i.e.* the epithelium was relatively transparent (Plate 3.47). Under SEM (Plate 3.51), almost all its surface was flat, occasionally interrupted by A-cells. The lumps and crypts were rare. This gut would correspond to a Thin or Shallow-Crypted type.

Plate 3.40 TS through the posterior midgut. Two B cells (short arrows) situated at the sides of a pseudovillus. Note the dense granules characteristic of these cells and the absence of microvilli. Note that each pseudovillus is formed of several club shaped R-cells. In this case. Most R-cells have lipid droplets in their apical cytoplasm. Note the clear "channel" (long arrows) formed by the multivesicular complexes of the cells. E-cells (arrow head) are usually at the base of the pseudovilli. LMR. Toluidine blue. Scale bar 20 μm.

Plate 3.41 TS through the posterior midgut. An A-cell (arrow) is about to pinch off from the epithelium. Note the absence of microvilli in the surface of the A-cell and the nucleus (arrow head) without nucleolus. At the right of the A-cell note a pseudovillus starting to develope. The R-cells that form it have developed apical bulges in which mitochondria are crammed together. Note that the nuclei of these R-cells (short arrow) are also displaced towards the apical part of the cell. LMR. Scale bar 10 μm.

Plate 3.42 TS through the posterior midgut stained with H&M Triple stain. B cells (arrows), brush border (arrow head) and basement membrane (small arrow head) are PAS positive. The PAS positive reaction in the B-cells is given by the granules (long arrow) which correspond to the primary lysosomes characteristic of these cells. LMPW. Scale bar 20 μm.

Plate 3.43 TS through the mid midgut stained for non-specific esterases. Only B-cells are positive with this stain. LMPW. Neutral red counterstain. Scale bar 50 μm..



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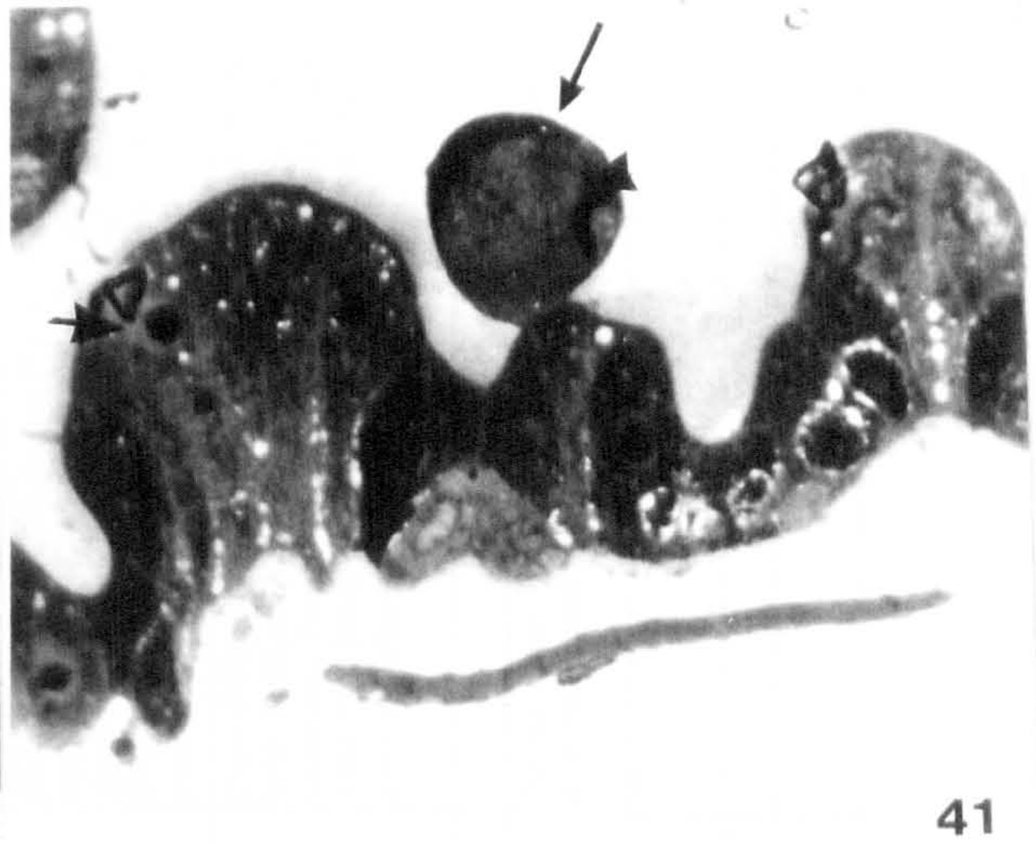

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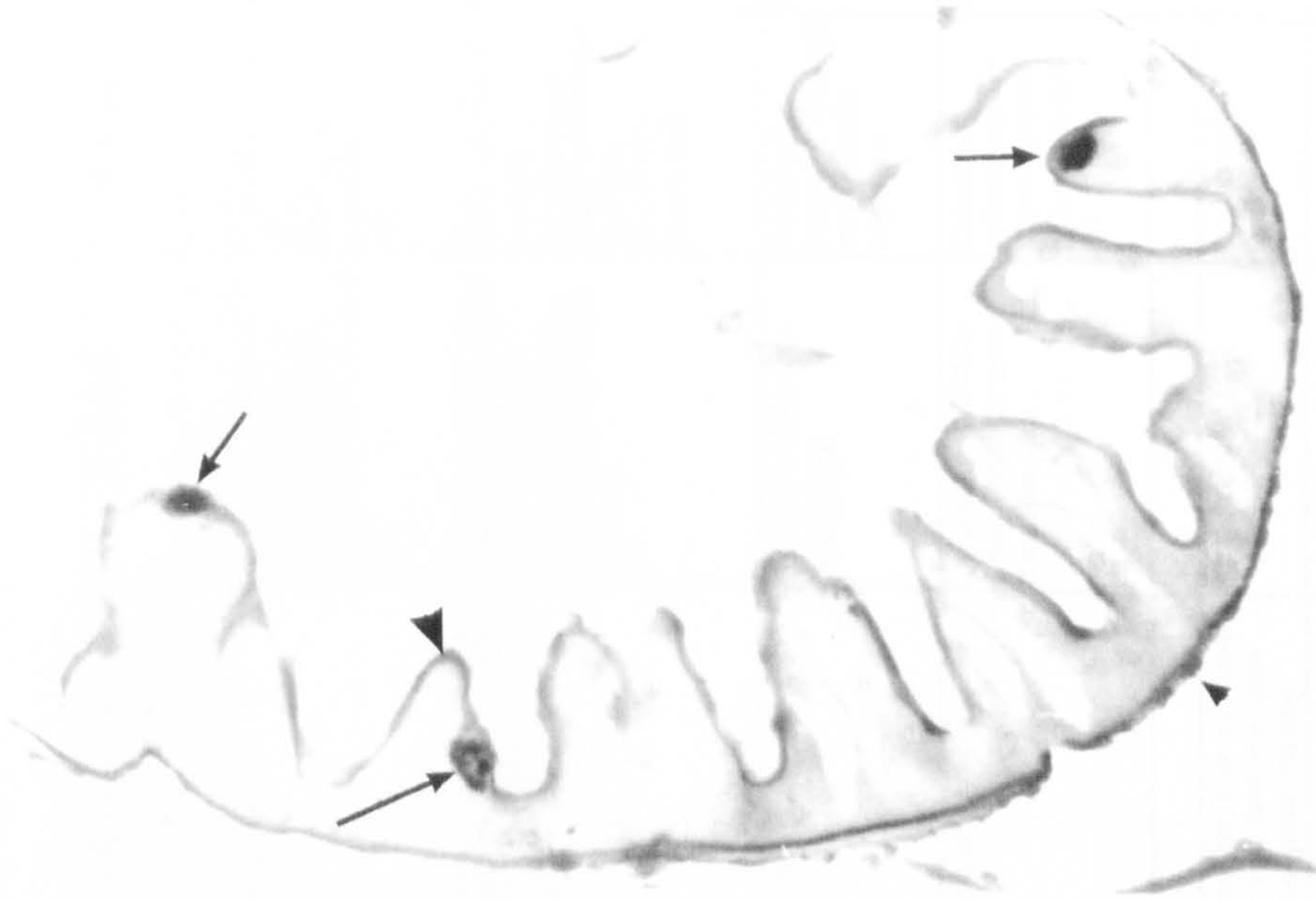

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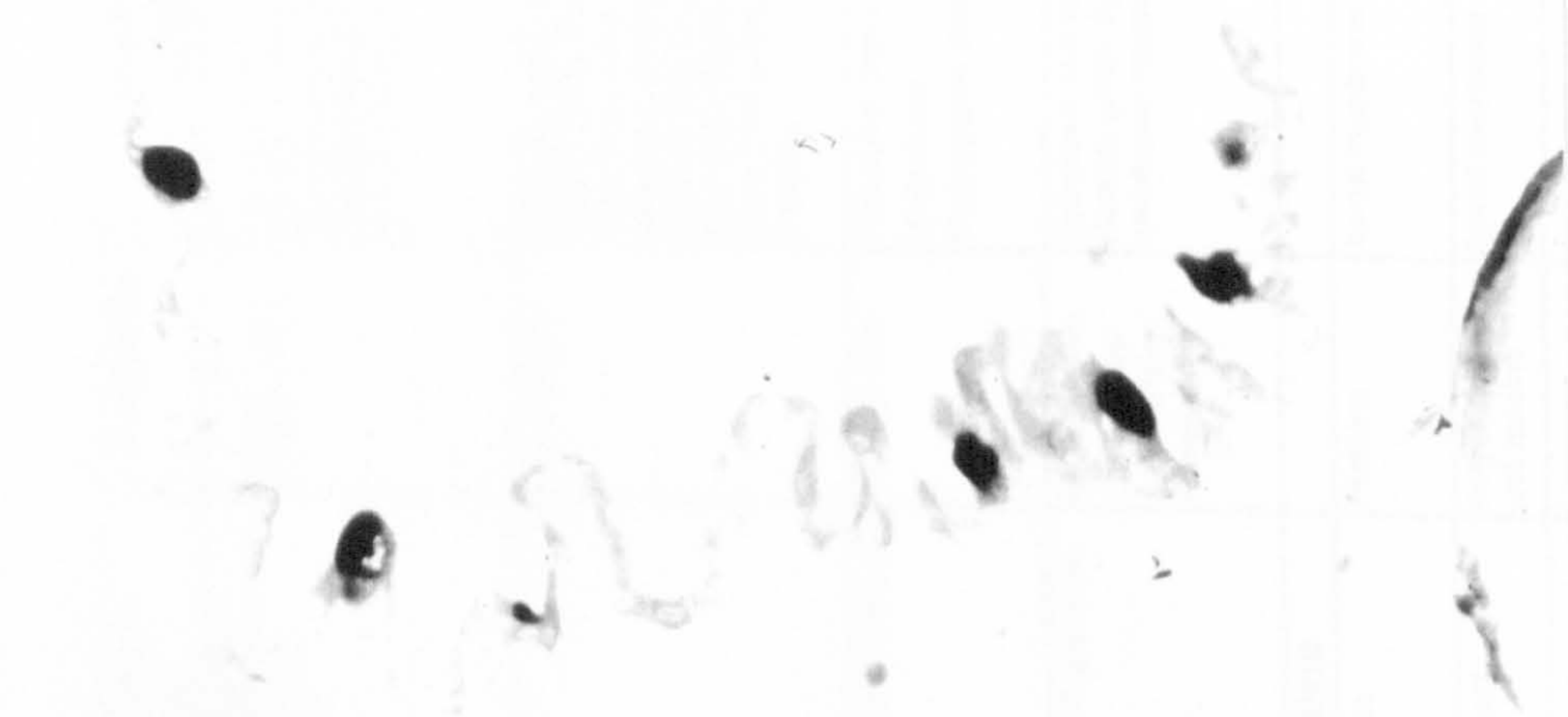

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Table 3.1 Histochemical techniques used to characterise different components of the midgut epithelium.

Histochemical technique	Purpose	R-or-F cells	Brush border of R- or F-cells	A-cells	B-cells	BM	NOTES
General stains							
Hubschman's modification of Gomori's for crustaceans	General stain	Grey-blue cytoplasm. Sometimes the 'multi-vacuolar complex' stains red. Nucleoli red	Bluish grey	Cytoplasm bluer than R cells. Sometimes red stripes in cytoplasm (dense bodies?). Nucleoli red	Cytoplasm grey blue Nucleoli and vacuoles bright red.	Bluish grey	This could be an excellent general stain if it was not for its unpredictability (probably due to the instability of the Azocarmine G stain). When it works, It differentiates well the B cells with its vacuoles stained bright red. A-cells can sometimes be differentiated as their cytoplasm would show darker than R cells. As with Cason's, it does not work well in plastic sections.
Toluidine blue	General stain	Orthochromatic cytoplasm, mainly due to RNA and metachromatic granules and vacuoles. Nucleoplasm mitochondria and stain light blue. Mitochondria can sometimes appear as dark granules.	Variable, from no staining or very faint to intense blue staining	Metachromatic granules; dark blue granules (multivesicular bodies), clear or methachromatic vacuoles	Clear vacuoles (primary lysosomes) and bluish to dark blue vacuoles (secondary lysosomes)	Faint blue	The intensity of orthochromatic staining (blue) seems to be related to the presence of RNA, as both stains, this one and Azure A + cationic surfactant (se below) stain quite similarly in adjacent sections. In the case of brush border, the intensity of stain seems to be related to the activity of the apical part of the cell, being more intense in cells with long microvilli . Possibly related to the activity of enzymes, particularly ALP. The apical membrane stains strongly as well in active cells in a similar way as the stain for protein..
Mallory-Heidenhain Cason's trichrome + Alcian green 2.5	General stain	Cytoplasm magenta. Nucleoli red Nucleoplasm light blue	Bright blue-green	Cytoplasm magenta. Nucleoli red	Cytoplasm magenta. Nucleoli and vacuoles bright red.	Blue	Clear differentiation between R or F-cells and B cells, the latter distinguishable due to the bright staining of the vacuolar apparatus. Brush border contrasting nicely when "active". Does not distinguish well the A-cells. Does not work well in plastic sections.
Himes & Moriber's (H&M) Triple stain	General stain (DNA / poly-saccharides / proteins)	Cytoplasm yellowish-green. Nucleus green. Apical membrane magenta.	Red or magenta	Cytoplasm yellowish-green. Nucleus green. Vacuoles clear	Cytoplasm yellowish-green. Nucleus green. Vacuoles bright red.	Bright red	Clear differentiation between R or F-cells and B cells, the latter distinguishable due to the bright staining of the vacuolar apparatus. Faecal pellet matrix and membrane magenta (H94174). Basal membrane clearly outlined in bright red. Not good cytoplasmic definition
Specific stains							
Mercuric Bromophenol blue	Proteins	Apical membrane	Variable, from no staining or very faint to intense blue-green staining	some granules	+ in vacuoles	+	The vacuoles of the B-cells stain well, although not as sharp as in the control tissue (pancreas).
Periodic-Acid-Schiffs reagent.	Polysaccharides and mucins	Apical membrane	+	-	+ in vacuoles	+	
PAS + diastase control	Glycogen	-	-	-	-	-	
Aldehyde-fuchsin	Sulphated sialomucins	apical membrane	+	-	-	-	

Table 3.1 (Cont) Histochemical techniques used to characterise different components of the midgut epithelium.

Histochemical technique	Purpose	R-or-F cells	Brush border of R- or F-cells	A-cells	B-cells	BM	NOTES
Phenilendiamine.	Lipids	in vacuoles after rich meal (e.g. blood)	-	-	-	-	
Alcian green pH 1.0	Sulphated mucosubstances	-	-	-	-	-	
Alcian green pH 2.5	Acid mucosubstances	Brush border	+	-	-	-	
Combined Alcian green 2.5-PAS	Neutral and acid mucosubstances	Brush border	+PAS +AG resultant colour is blue	-	vacuoles +PAS	+PAS -AG	Clear differentiation of B cells and brush border. Not good cytoplasmic detail
Azure A + cationic surfactant 214	RNA	+	-	+	+	-	
Azure A + PAS	General stain (RNA+Polysaccharides)	RNA+ Apical membrane PAS +	PAS +	PAS -	PAS +	PAS +	Clear differentiation between R-cells (RNA +ve) and B-cells (PAS +ve vacuoles)
Amidoblack 10B	Haemoglobin	-	-	-	-	-	Works well in paraffin sections but not in plastic. In controls (fish gills, spleen) red blood cells stain deep blue on a yellow background. Sections of lice that had fed on blood were always negative. Some areas of the muscles were sometimes positive (maybe due to presence of a kind of myoglobin?... or artefact?)
Perl's Prussian blue	Ferric iron (haemosiderin)	-	-	-	-	-	see below
Tirmann-Schmeltzer method (Bancroft p259)	Ferrous and ferric iron	-	-	-	-	-	Techniques to detect iron were negative, even in lice that had had a blood meal.
Stains for enzymes							
Simultaneous coupling method with Naphthol AS-BI.	Alkaline phosphatase	Apical membrane	+ Variable intensities. Generally stronger in club R-cells.	Apical membrane	-	-	The staining pattern was similar to the one of Toluidine blue or protein in adjacent sections.
Simultaneous coupling method with Naphthol AS-BI.	Acid phosphatase	-	-	-	-	-	Besides a faint stain in the brush border of one block, in the rest of the cases the staining was negative. Positive staining in the controls was batch-dependent, therefore the negative results shown here are not reliable.
Simultaneous coupling method with Naphthol AS-D acetate.	Esterases	-	-	+	-	-	Only B cells stain for esterases. The stain is diffuse but is sharper in the vacuoles. Probably some kind of diffusion takes place during fixation or incubation. Probably more reliable than PAS or Cason's as it can detect small sections of B-cells in which the granules are not conspicuous. Positive as well in kidney and spleen controls showing similar diffusion. Incubation time has to be controlled carefully to get a sharper definition.

Not infrequently there was a mixture of rugose and smooth types in a single louse. The posterior midgut could have a rough appearance while the mid or anterior midgut were smooth and vice versa.

Opaque.

The aspect of this gut was cloudy (Plate 3.48). This cloudiness could be seen either in certain areas or in the whole digestive tract. A microscopic examination of a pale gut revealed a massive invasion of rod-shaped bacteria in the enterocytes with an overall necrosis of the epithelium (Plate 3.45).

The occurrence of this type of gut was rare but more frequently observed in lice from winter samples where the temperature and salinity of the seawater was low (4-5°C and 22-25 ppt respectively). In healthy lice, bacteria are not present within the midgut epithelium, so it is likely that lice with the pale gut are either senile, moribund or both.

Lice with this type of gut did not survive for more than 2 days.

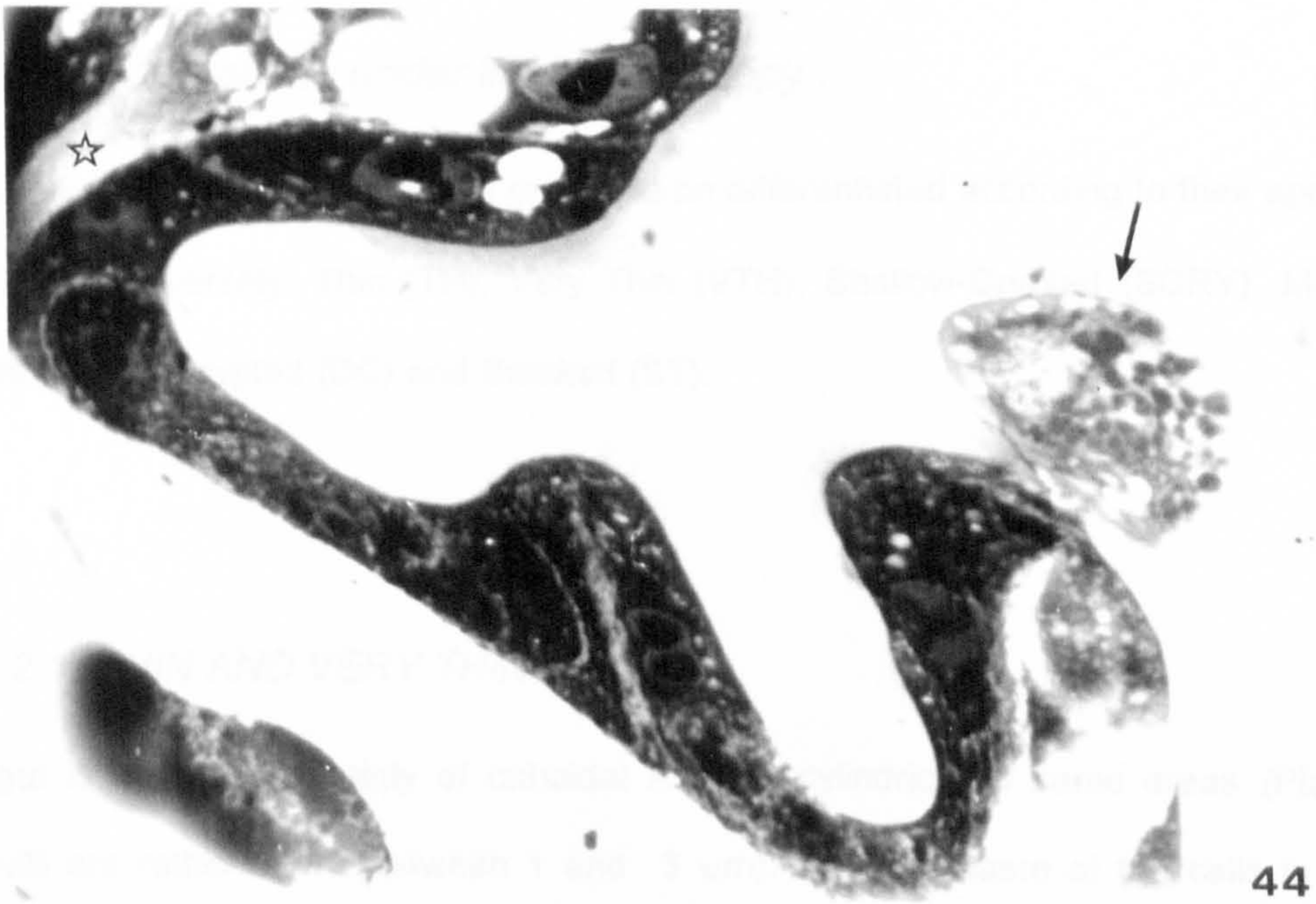
Table 3.2 Occurrence of Rough and Smooth gut types in different samples of *L. salmonis* adult females. Chi square analysis demonstrated significant differences between the proportions of the two gut types ($p<<0.001$).The mean and standard deviation (SD) of the percentages were calculated after transforming them with the Arcsine transformation as recommended by Zar (1996)

SAMPLE	ROUGH	SMOOTH
1	171 (74)	59 (26)
2	175 (67)	79 (33)
3	45 (72)	18 (28)
4	219 (70)	95 (30)
5	154 (69)	73 (31)
TOTAL	764	324
Mean of % ±SD	70.4±0.09	29.5±0.09

Table 3.2 shows the occurrence of both rough and smooth guts in several samples. A relation close to 2:1 was found between rough and smooth types, which was quite consistent between samples.

Plate 3.44 Last stage of a B-cell at the moment of pinching off from the epithelium (arrow). Note the clear "channel" (open star) of a well developed pseudovillus. LMR. Toluidine blue. Scale bar 10 μm. .

Plate 3.45 . Transverse section through a Pale gut. Note the massive invasion of rod-shaped bacteria in the epithelial cells and lumen of the gut. Most of the enterocytes have disintegrated leaving lots of cell debris. Note that the bacteria are confined to the gut. LMR. Toluidine blue. Scale bar 20 μm. .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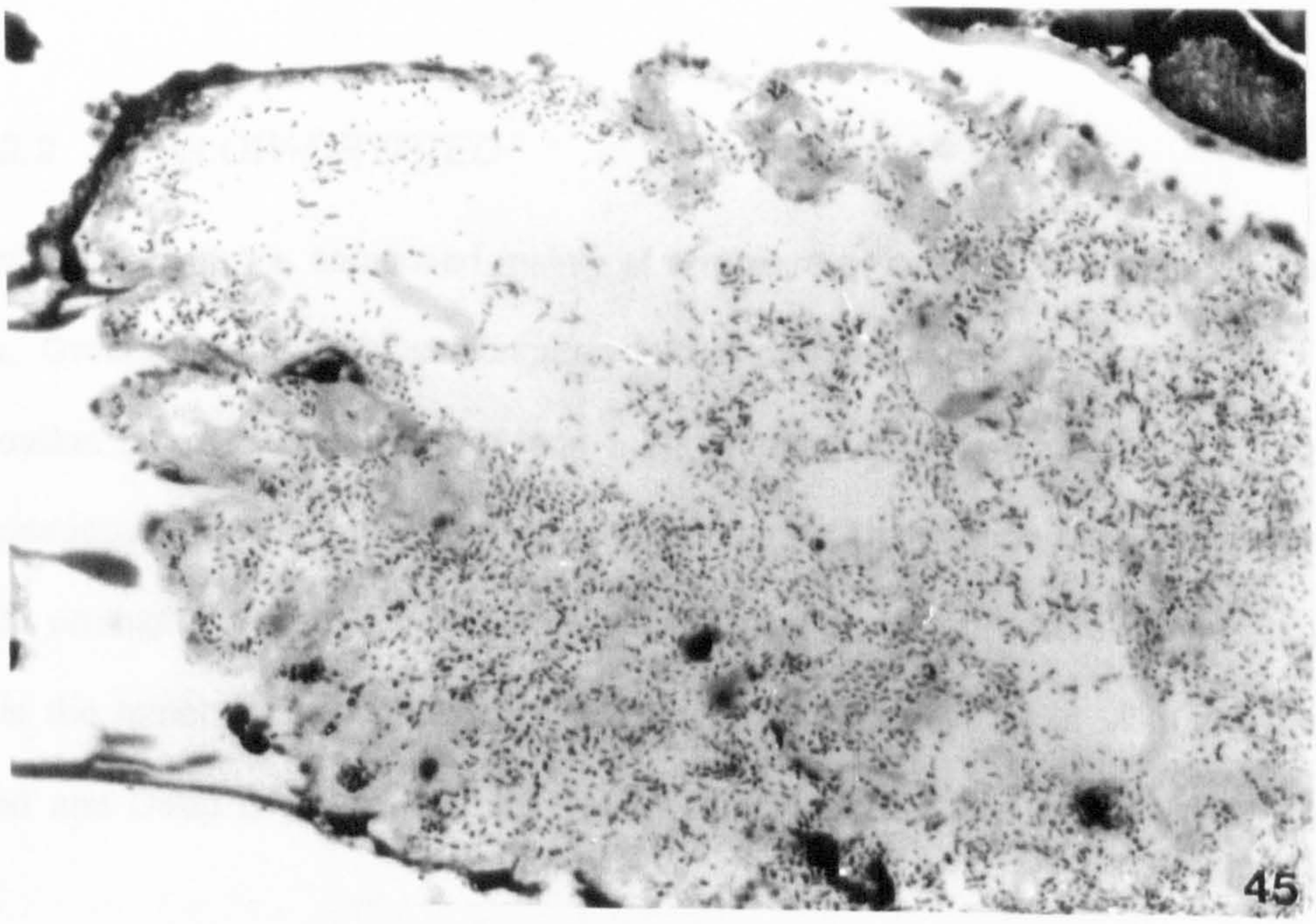
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3.2.4.2 Observations under light microscopy..

At light microscope level, 6 types of gut could be differentiated according to their appearance when sectioned transversely: Thin (TH), Very Thin (VTH), Shallow-Crypted (SCRY), Medium Crypted (MC) and Deep Crypted (DC) and Stacked (ST).

3.2.4.2.1 THIN AND VERY THIN .

This gut is composed mainly of cuboidal R cells, cylindrical in some areas (Plate 3.49). The microvilli are rather short (between 1 and 3 μm). The cytoplasm of the cells is usually dense, reacting strongly for RNA. Vacuoles are rare. A-cells are also present but to a lesser extent. B cells are rarely seen and when present they are also cuboidal, smaller and the electron dense vacuoles are very small or absent. Under SEM most of the surface is smooth, *i.e.* with almost no creases or ridges (Plate 3.51 , see also Plate 3.4).

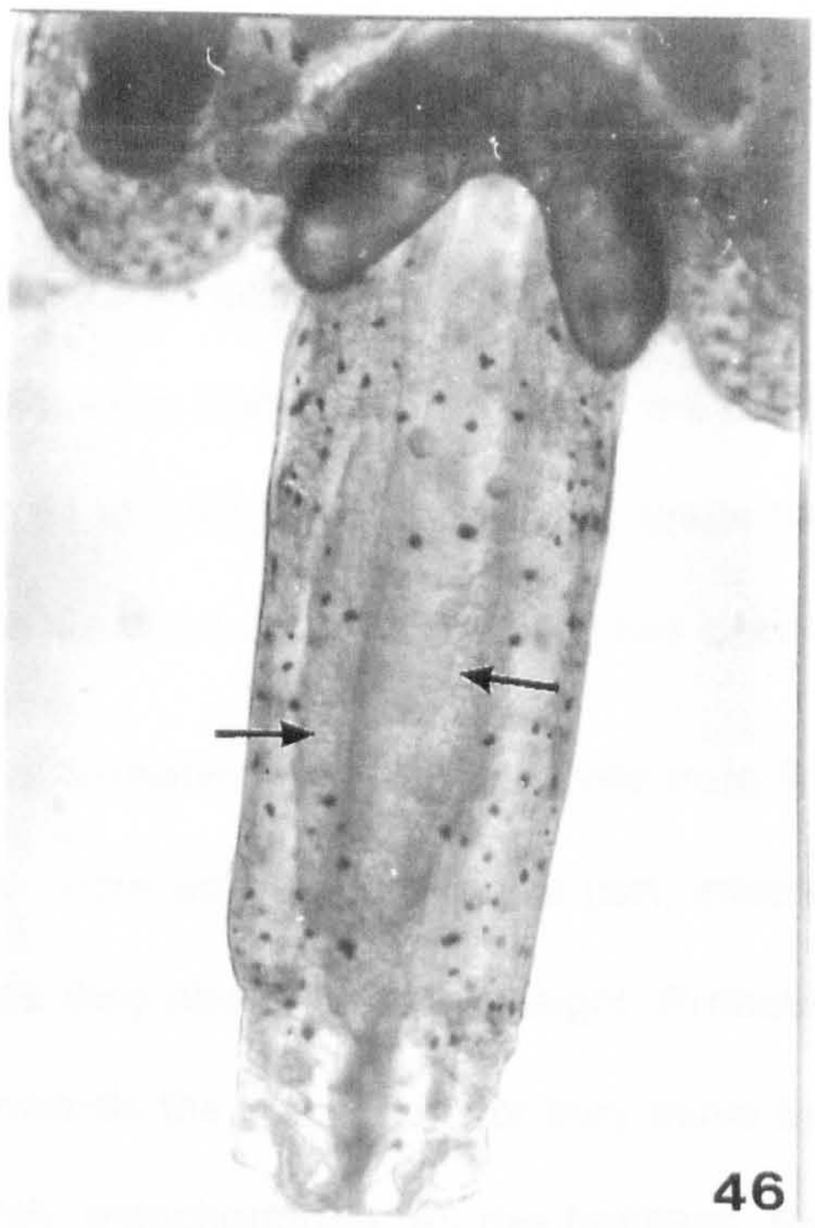
3.2.4.2.2 SHALLOW-CRYPTED

This gut (Plate 3.50) is composed mainly of a mixture of cuboidal and cylindrical or club shaped R-cells. Groups of 3-6 R-cells elongated into a cylindrical or club-shaped form to produce a pseudovillus (see Plates 3.40, 3.41 and 5.2). Alternation between areas of thin epithelium and these pseudovilli give the gut a undulating appearance . The pseudovillus in this type of gut can be up to 30 μm high. In contrast with a true villus of a vertebrate gut where the basal membrane folds towards the lumen, along with a core of connective tissue, in these villi (and those of Medium Crypted and Deep Crypted) the basal membrane does not fold inwards (Plate 3.50 , 3.52 and 3.53).

Plate 3.46 View of the abdominal segment of a *L salmonis* adult female as seen through the dissecting microscope showing the appearance of a Rough gut. Note the small creases in its surface (arrow). Scale bar 0.5 mm.

Plate 3.47 View of the genital segment of a *L salmonis* adult female as seen through the dissecting microscope showing the appearance of a Smooth gut. Note the transparency of the gut and the absence of creases. Scale bar 0.5 mm.

Plate 3.48 View of the genital segment of a *L salmonis* adult female as seen through the dissecting microscope. Note that the gut is very opaque (Compare with Plate 3.47). Scale bar 0.5 mm.



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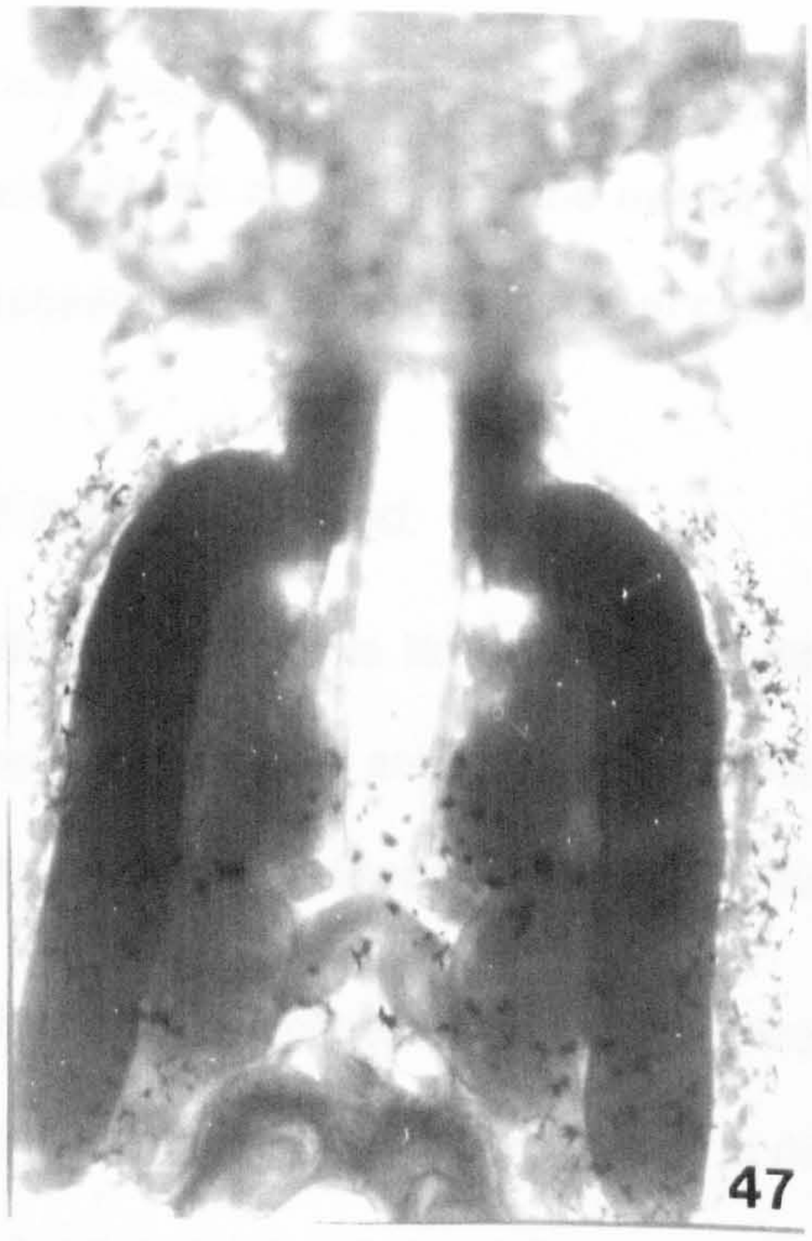

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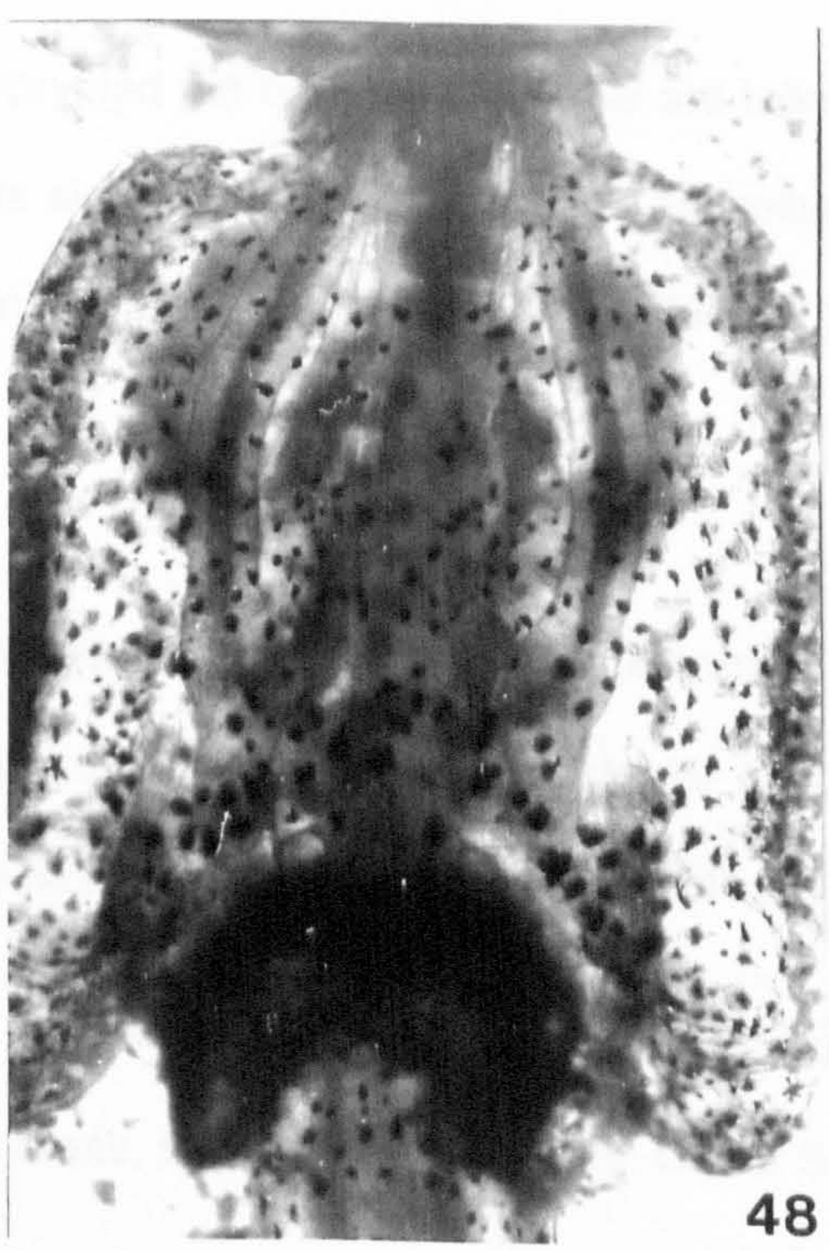

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3.2.4.2.3 MEDIUM AND DEEP CRYPTS.

Medium Crypted. Similar to Shallow-Crypted but the bulges are more pronounced, composed mainly of club shaped R-cells that are attached to the basal membrane forming a narrow neck (Plate 3.1 and Plates 3.54 to 3.60). This neck looks, under the light microscope, like a "channel" of clear cytoplasm, whose structure and ultrastructure has been described in previous sections.

The possible process of formation of these club cells from R-cells might be as follows: Normal R-cells probably become more active in the apical part, increasing the number of mitochondria. As the cells grow in volume they also increase in height. Probably the pressure of adjacent cells push the cellular contents towards the apical part (or they move there because this is the principal area of demand [e.g. for RNA, mitochondria]). As this happens, the vesiculated SER at the sides of the cell is brought together forming the so called "clear channel".

Deep Crypted . This is similar to the Medium Crypted gut type but club cells are taller, alternating with shorter cells, so that the overall picture is similar to a pseudostratified epithelium where the appearance is of several layers of cells when in fact there is only one (Plate 3.58 and 3.57).

3.2.4.2.4 Stacked.

The appearance is like that of a stratified epithelium (Plate 3.61), with apparently 2-4 layers of cells. These cells are small and cuboidal and show various stages of nucleolar fusion, i.e. single double, triple or dissociated nucleoli. The layer facing the lumen is composed of R or F cells.

This type of epithelium was always found in combination with the other types of gut, mainly the crypted ones and was present only in discrete areas, forming distinct bulges. Under SEM, this type of gut cannot be distinguished.

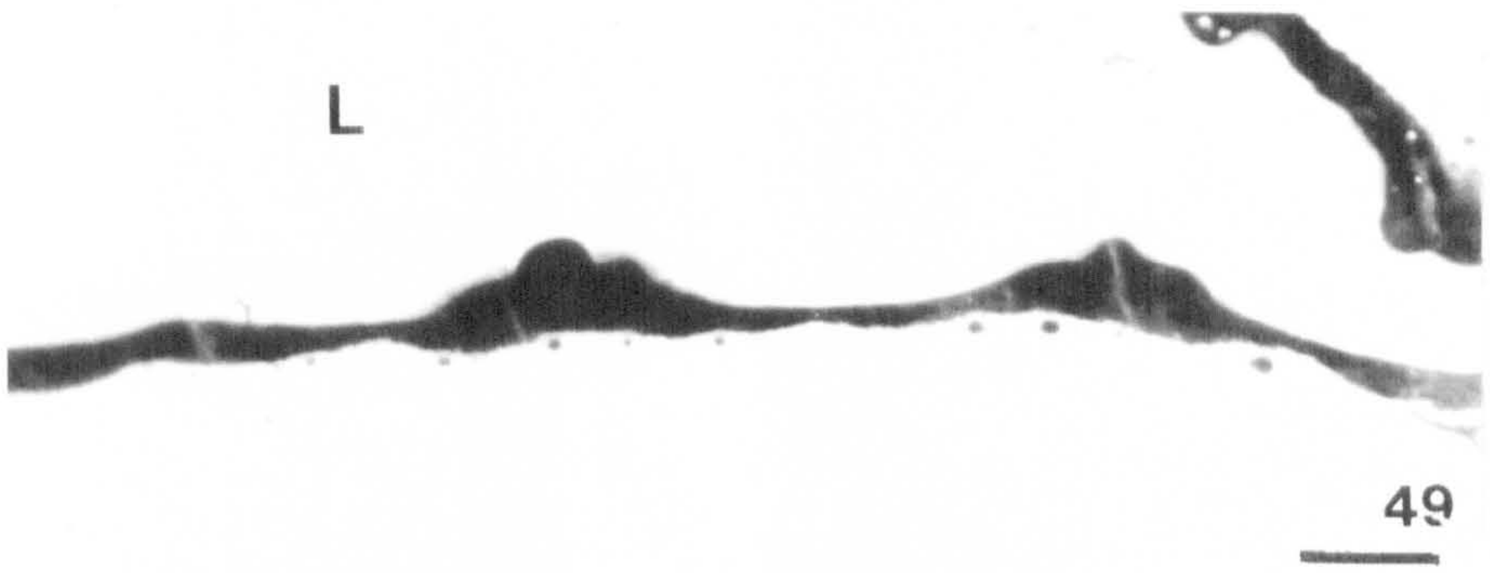
There was no homogeneity of any gut type. Usually a combination of morphologies was present. When classifying gut types, the dominant morphology was used to characterise the gut.

Plate 3.49 Transverse section of Thin and VeryThin guts. Mid midgut. L: lumen. Scale bar 20 μm..

Plate 3.50 Transverse section of a Shallow-Crypted gut. Posterior midgut. L: lumen. LMR. Scale bar 100 μm..

Plate 3.51 Mid midgut cut open to show a Thin type gut, which also corresponds to the Smooth type when the louse is observed live with the dissecting microscope. Dry dissection. Many A-cells (arrow) are scattered throughout the epithelium. SEM. Dry dissection. 160X.

Plate 3.52 Posterior midgut cut open to show a Shallow-Crypted gut. Note that the pseudovillus that is seen in a transverse section with the light microscope can be in reality a longitudinal ridge of the epithelium. The gut in this plate displays a combination of Thin and Crypted. SEM. Dry dissection. 320X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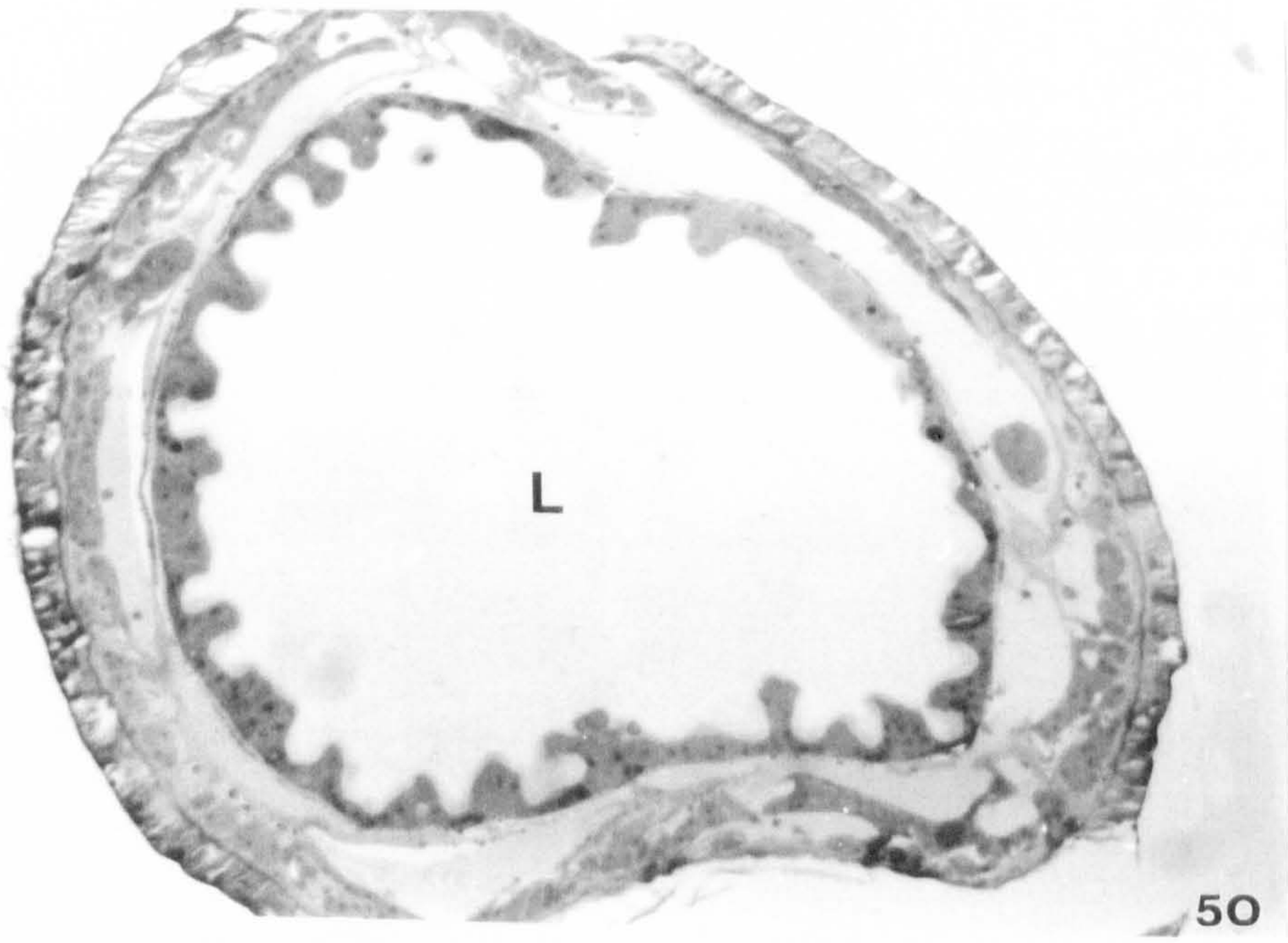
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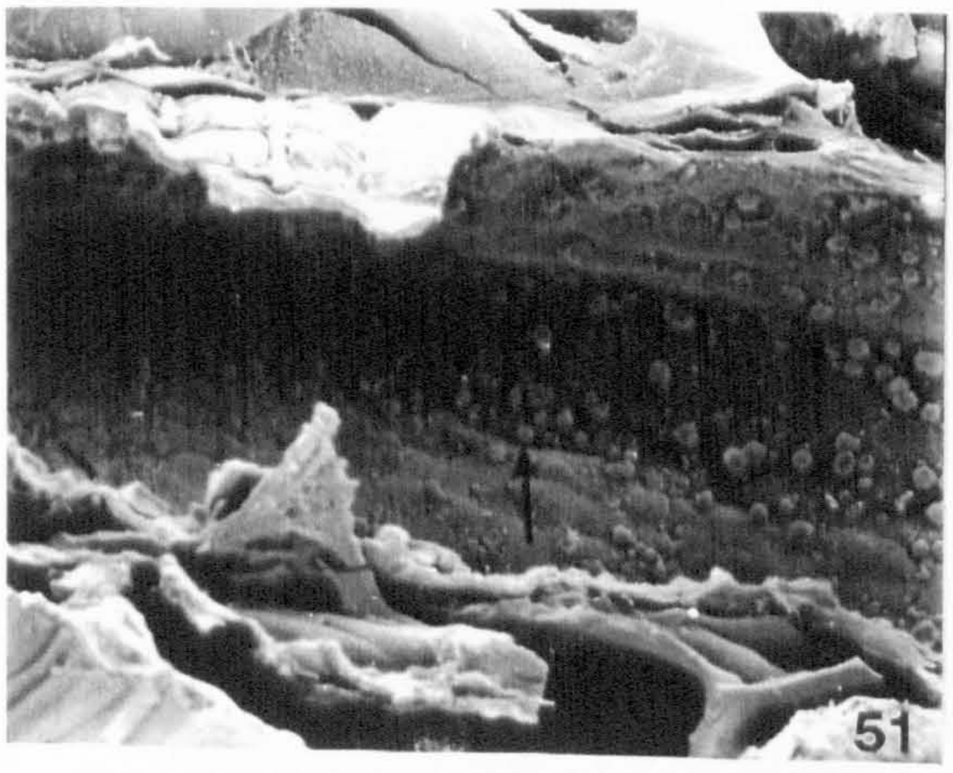
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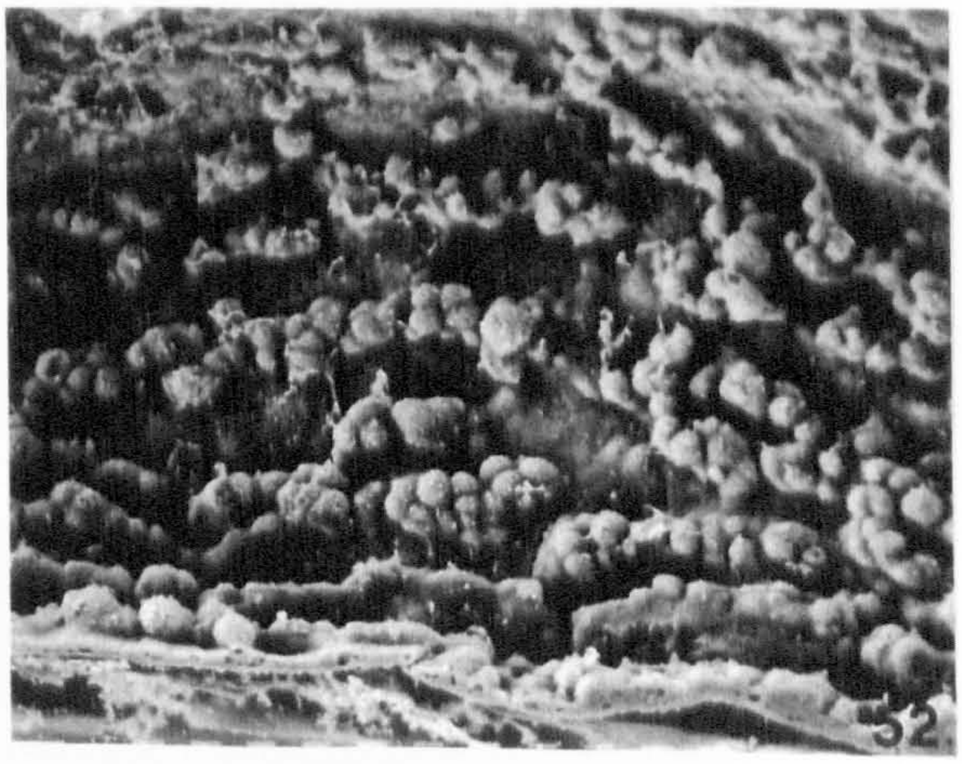
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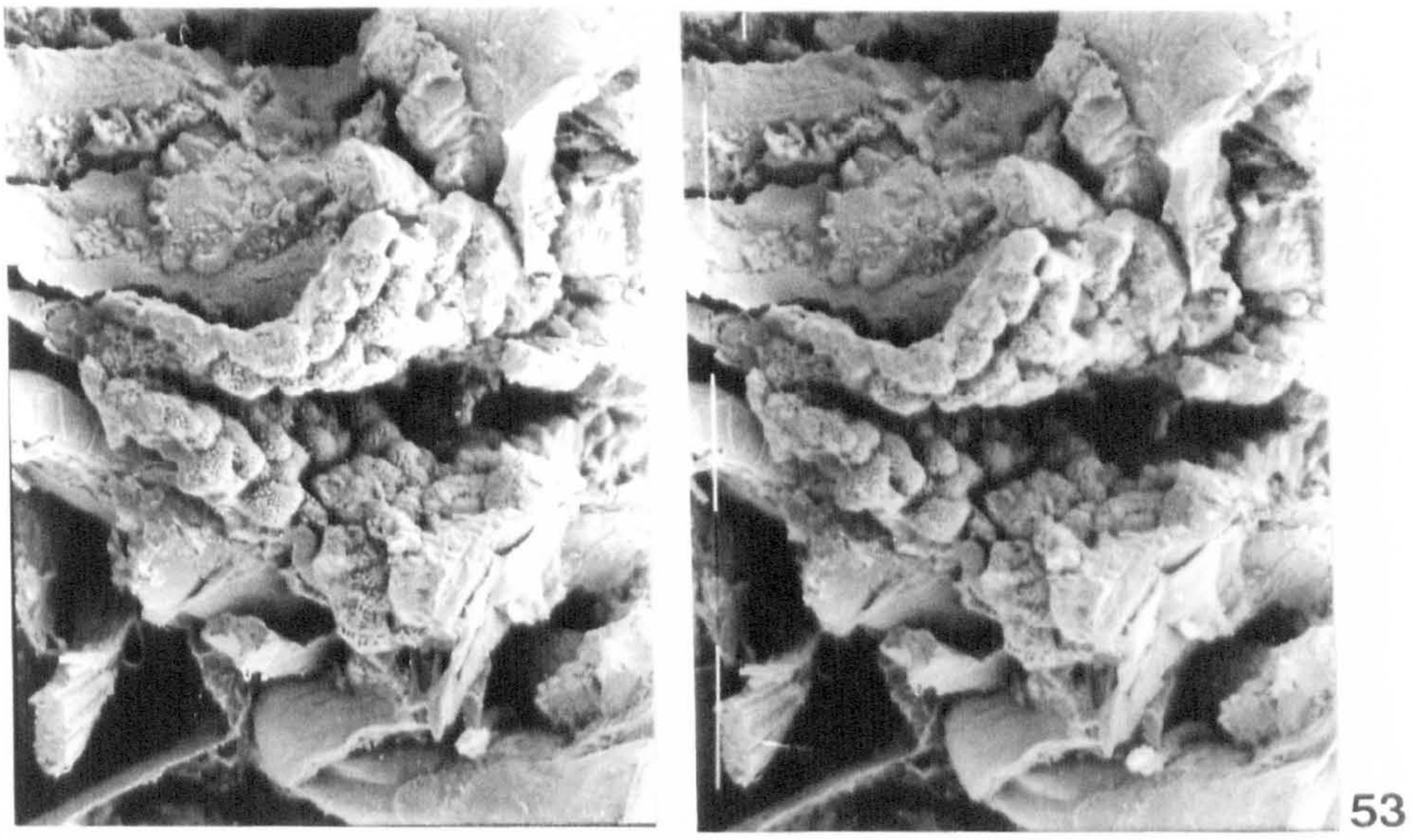
Plate 3.53 Shallow-Crypted gut. Stereo pair of a dry dissection at the level of the narrow junction between anterior and mid midgut. The dorsal part of the louse is to the left, the right is towards the top of the photograph and the anterior away from the viewer. There are almost no areas of Thin gut. The cells, or the ridges, are not tall enough to be considered as Medium Crypted. This gut was classified as Rough on the live louse. SEM 160X.

Plate 3.54 Stereo pair of the anterior midgut open longitudinally with microtome-paraffin carving technique. Note that each ridge (asterisk) of the epithelium is made up several R-cells. The anterior part of the louse is towards the top of the picture, the ventral to the right. Note a portion of the oesophagus (star) inserting into the floor of the anterior midgut. SEM 640X.

Plate 3.55 Stereo pair of the anterior midgut open transversally by dry dissection. The dorsal part of the louse is towards the top right corner. This is a combination of Shallow-Crypted (floor of the gut, long arrow) and Medium Crypted gut (dorsal and lateral areas). SEM 160X.



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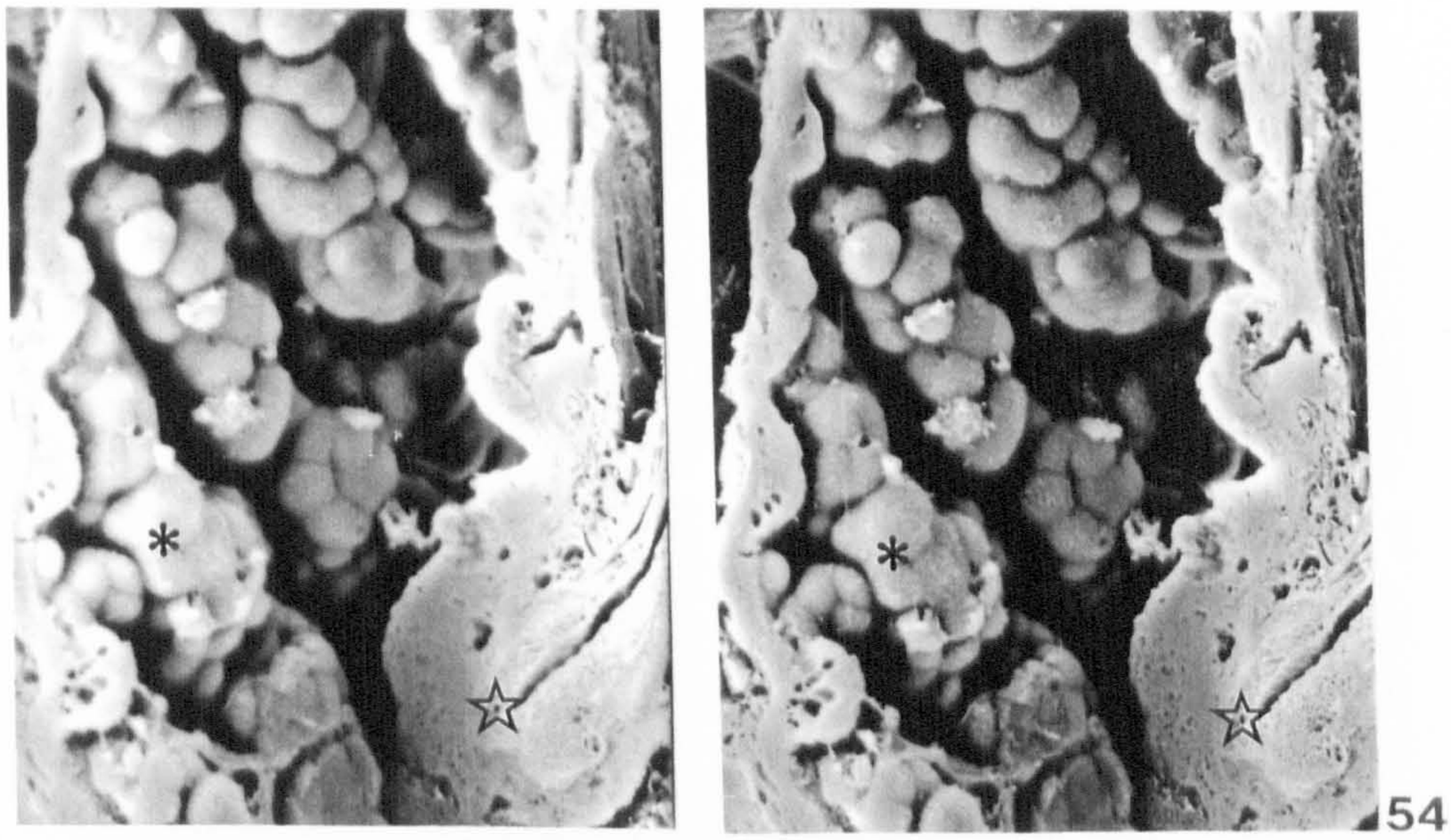

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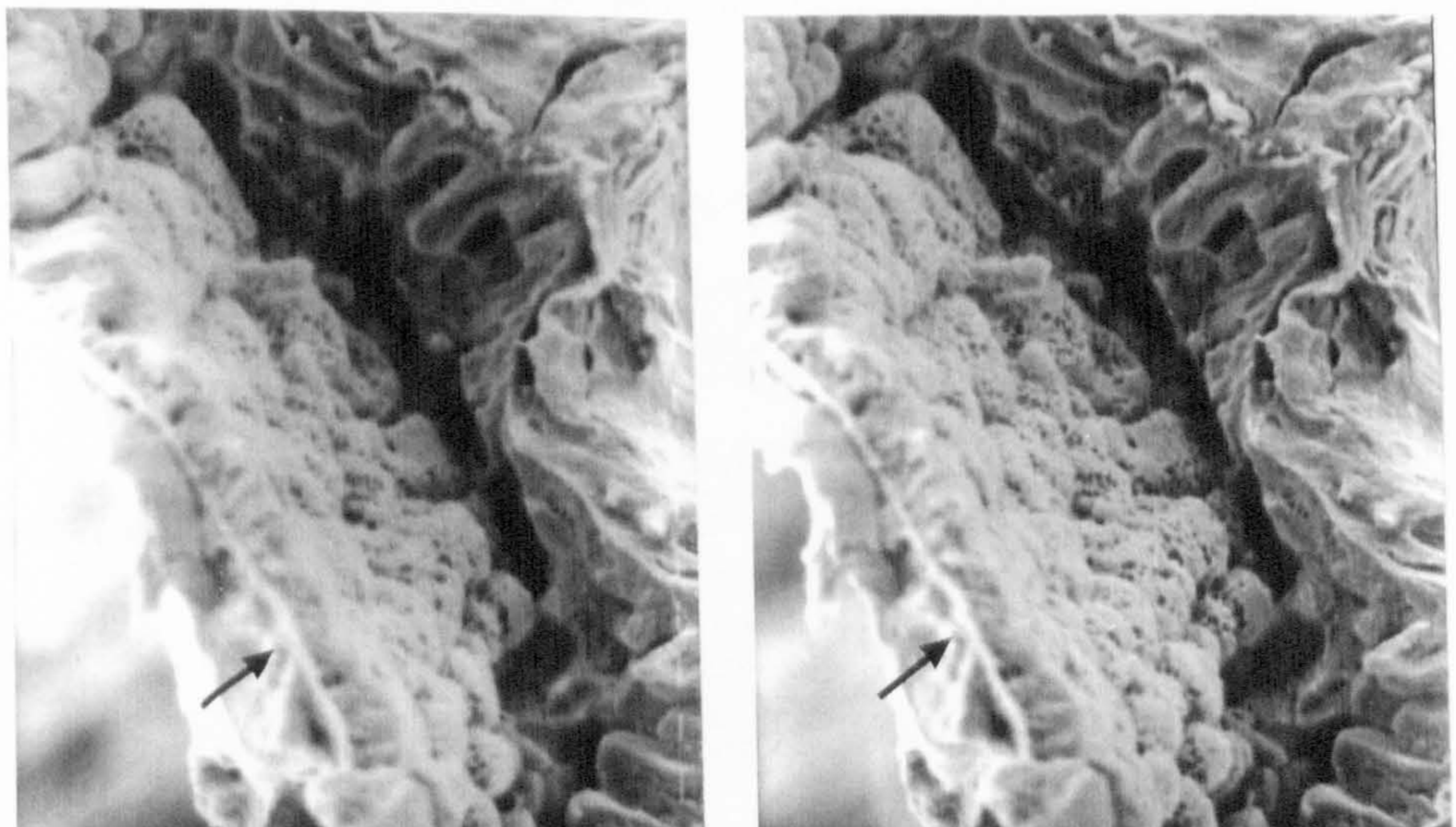

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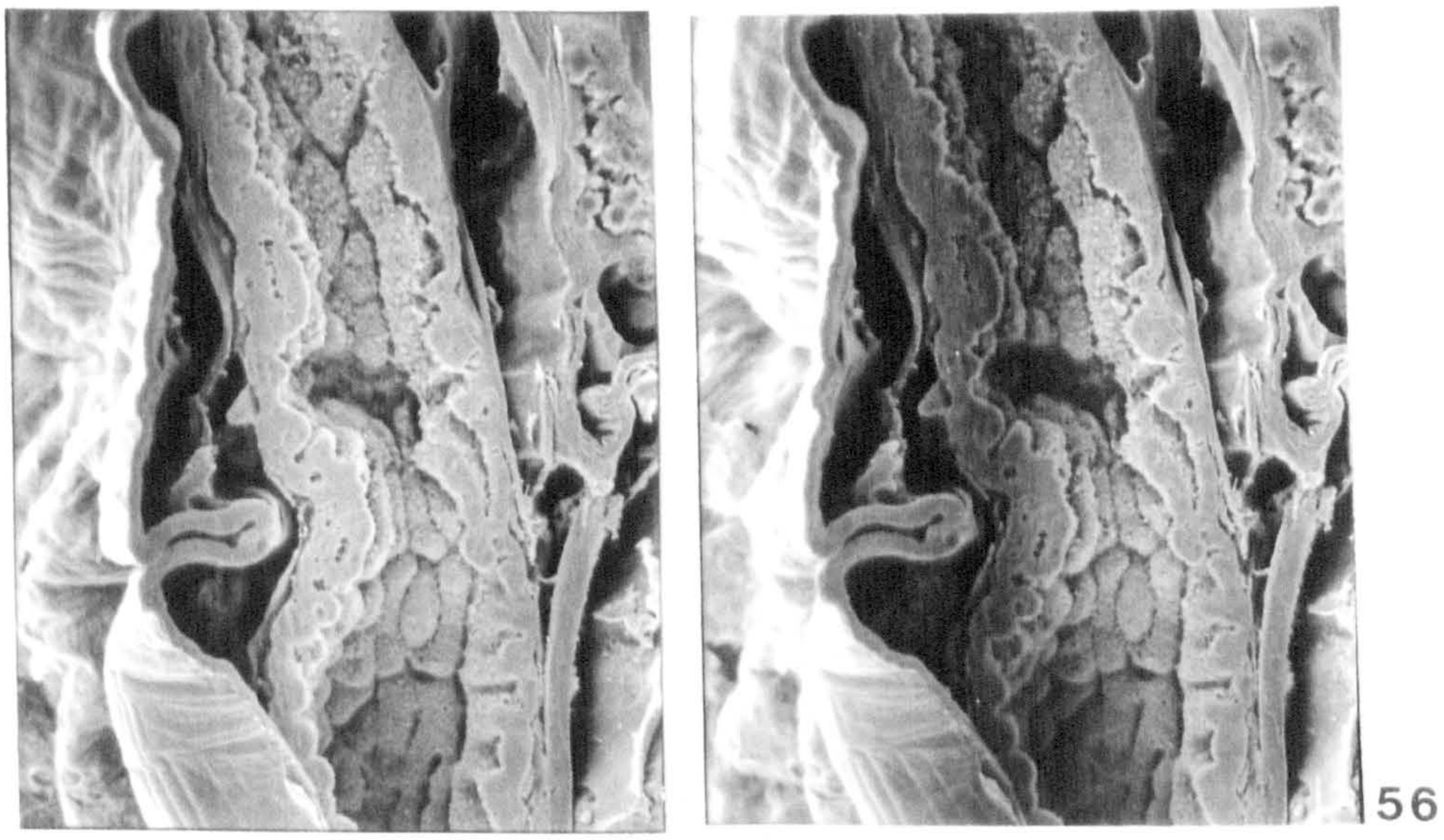
Plate 3.56 Longitudinal section through the transition between mid midgut (towards the top of the picture) and posterior midgut. The ridges are close together, but with the 3-D effect the depth of the crypts can be appreciated. SEM 160X

Plate 3.57 Stereo pair of the mid midgut open longitudinally by paraffin carving. Note the deep crypt formed between the two ridges. The ridge on the left is about 130 μm high. All cells in this long finger-like projection will have contact with the basal membrane through narrow necks passing at the base of the projection. SEM 640X

Plate 3.58 Transverse section of a Deep Crypted gut. L: lumen. LMR. Toluidine blue. Scale bar 10 μm..



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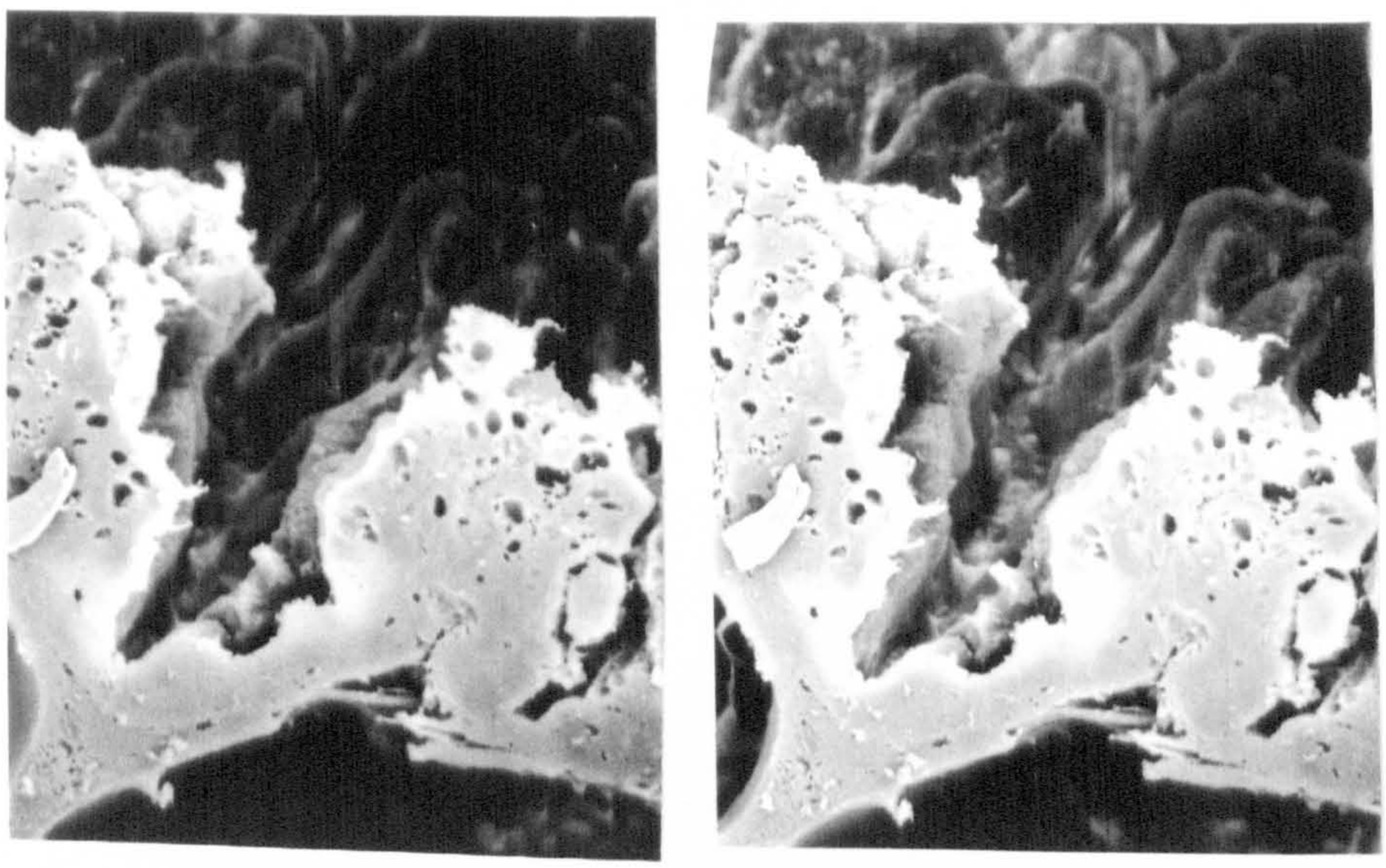

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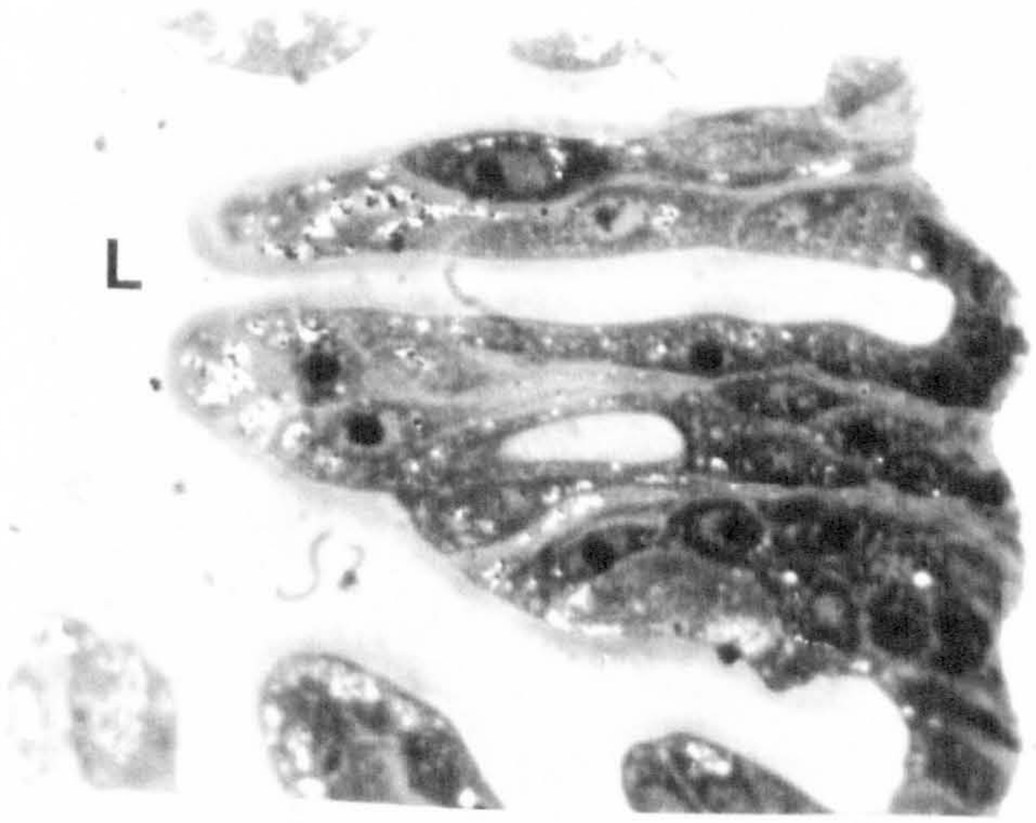

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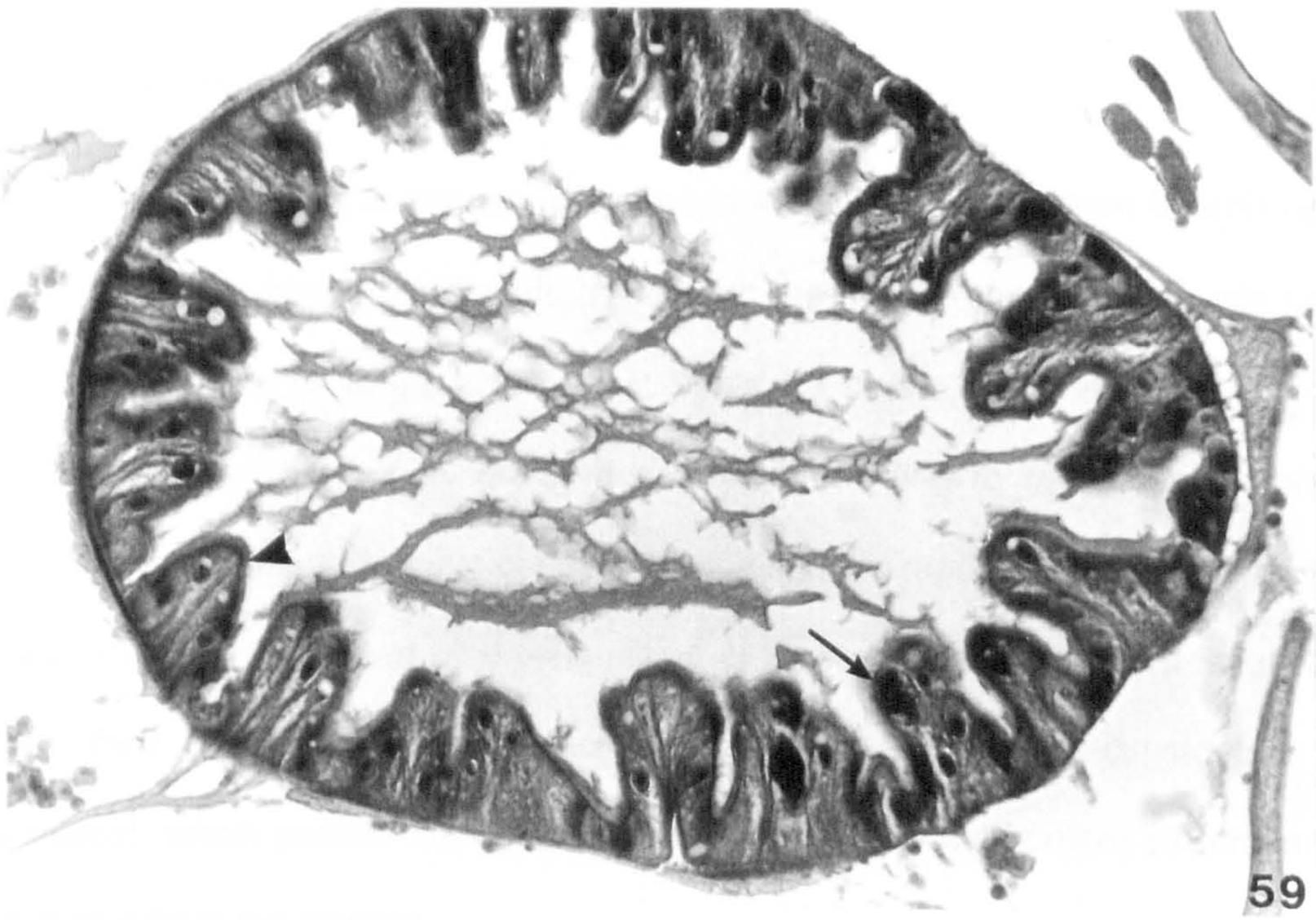
Plate 3.59 Transverse section through the mid midgut showing a Medium Crypted gut. The brush border is very conspicuous with this stain (arrow head) A B-cell can also be appreciated (arrow) LMP. Cason's + alcian green stain. Scale bar 50 μm..

Plate 3.60 Dry dissection of a posterior midgut corresponding to a Medium Crypted gut. On the live louse, this gut was a Rough type. On a transverse section these abundant ridges would appear as pseudovilli, as in the dorsal or lateral part of the anterior midgut depicted in Plate 3.55. The hindgut is at the lower right corner. SEM 80X..

Plate 3.61 Transverse section of a Stacked gut. LMR. Note the appearance of a pseudostratified epithelium. A B4 cell is protruding at the apex of this pseudovillus. Toluidine blue. Scale bar 10 μm..



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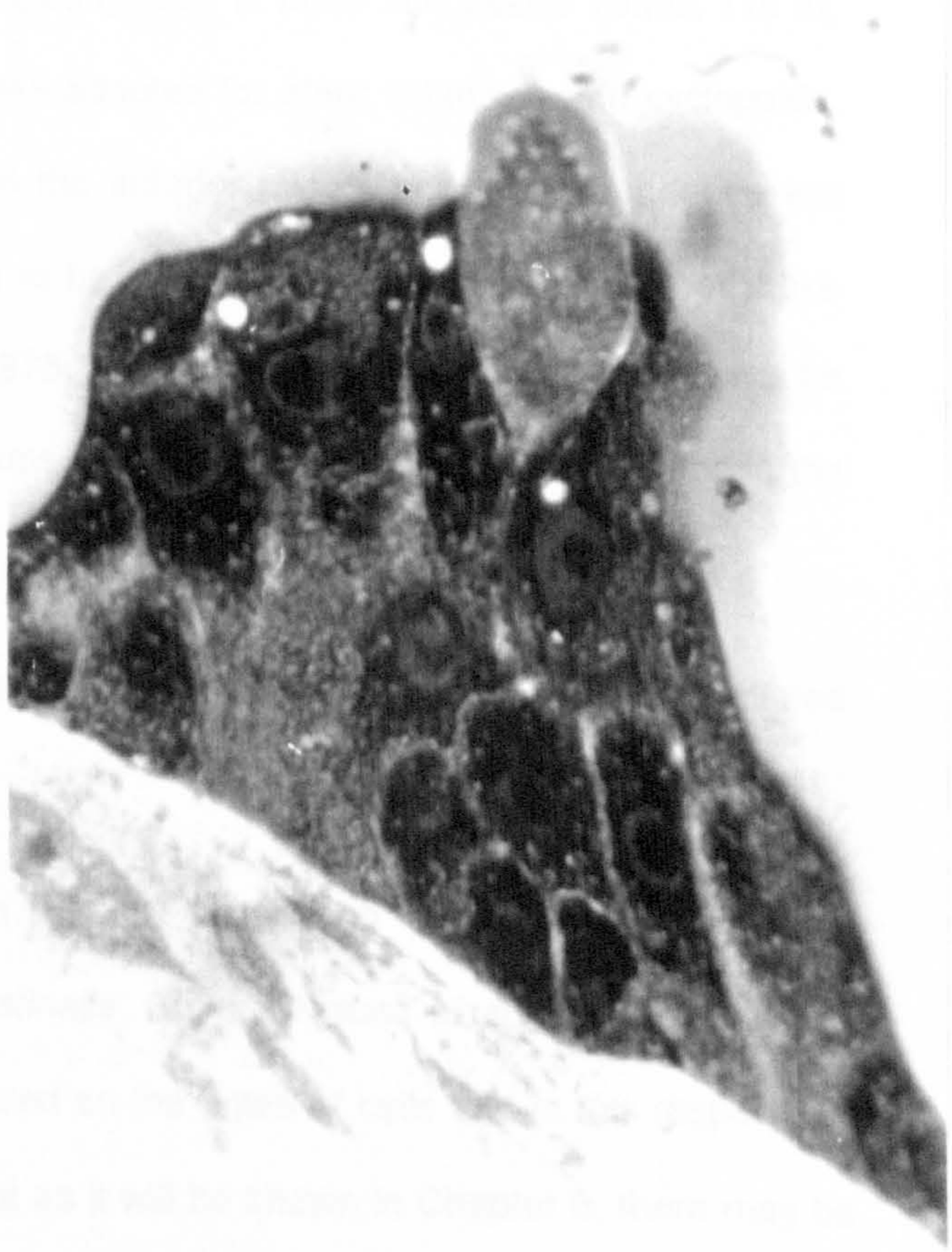

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3.3 DISCUSSION

The *L. salmonis* digestive canal appears to correspond to the typical pattern for arthropods in that it bears a cuticularised foregut and hindgut. In general, the midgut of most copepods can be divided in two regions according to its gross structure. Table 3.3 shows how the midgut of several orders of copepods has been divided by different authors, according to the gross morphology or the function of different sections. In most copepods the midgut is reported to have basically two zones, anterior and posterior, separated by a constriction or a valve. This constriction is located about the level of the third and four pedigerous somites (Boxshall 1992). The constriction leads to the posterior midgut, which passes through the genital and abdominal somites to terminate in a valve that separates it from the hindgut.

Arnaud *et al.* (1978) divided the midgut of *Centropages typicus* in three successive zones, I to III, according to the cell types and this division has been adopted for other calanoids and cyclopoids. The zones I and II are in what can be considered the anterior midgut and their boundaries are defined by a change in cell type, and there seems to be enough evidence to conclude that these two zones have specific functions (Arnaud *et al.* 1978, Arnaud *et al.* 1987). Zone II should not be mistaken for the mid midgut described in other groups (see table), which generally is located in the genital segment, not in the thoracic.

Where the midgut has been divided into two functional sections, they have been also differentiated by a change in the cell type (Boxshall 1992).

Other authors have not found any difference in the cell distribution along the midgut, as in *Paranthessius anemoniae* Claus or *Mytilicola intestinalis* Steuer. Nylund *et al.* (1992) also could not divide the midgut of *L. salmonis* into zones based on the types of cells and in this respect the results of this Chapter agree with their findings. But as it will be shown in Chapter 6, there may be differences in the occurrence of certain types of cells in different parts of the midgut of *L. salmonis*. However the differences are subtle and certainly they cannot be spotted unless a cell type count is done for each of the different regions and in various individuals.

The structure of the oesophagus in this study coincides with that of Nylund *et al.* (1992) except for the description of the junction of the foregut with the midgut. They reported that "the opening between the oesophagus and the midgut is closed by two valves". A similar description for this junction is given for *Phrixocephalus cincinnatus* describing two large palps guarding the junction. A longitudinal section passing exactly through the centre of the papilla has the appearance of comprising 2 components, however, this is not the case. The papilla, at least in the case of *L. salmonis*, is instead a cylindrical continuation of the oesophagus that projects into the floor of the anterior midgut. The same morphology is described in *L. salmonis* larval stages (Bron *et al.* 1993). In other copepods, this junction is not mentioned specifically, but from the drawings, or in some cases plates, it is likely that the same sort of structure might be generally present.

In the presen study it was preferred not to regard the anterior diverticulum as an "anterior caecum" as it is has been named by other authors (*eg.* Boxshall 1985a, Boxshall 1992, Bron *et al.* 1993). Arnaud *et al.* (1980) also preferred to call it a diverticulum in *Centropages typicus*, considering it to be a possible vestigial caecum. Brunet *et al.* (1994) also point out that these anterior extensions of the midgut observed in some calanoid and harpacticoid copepods are not true caeca and should not be confused with them. In other crustaceans like cirripedes or amphipods, true caeca (blind ending tubular extensions) are present, connected to the anterior or the posterior midgut (Schmitz 1992, Walker 1992). The gross morphology of the midgut is associated with the feeding strategies in most copepods (Boxshall 1985a). In gorging and predatory forms, an anterior caecum is well developed or the anterior midgut itself is capable of great distension. Well developed lateral caeca occur also in forms that have occasional meals, and therefore need storage capacity to accommodate as much food as they can in the rare event of a meal (Boxshall 1985b). In true parasites, like sea lice and other siphonostomatoids like *Lernaea*, there are no true caeca. In these animals there is no need for storage of food as this is constantly available (Boxshall 1985a). These lobes, or globular lateral and frontal projections, like those observed here in *L. salmonis*, could well be vestiges of true caeca.

The basal membrane of *L. salmonis* was reported by Nylund *et al.* (1992) to be an amorphous, elastic, thick structure, although no mention was made the porosity which is described here. In the present study the histochemical characteristics of the basal membrane indicate that its structure is proteinaceous, in a matrix of neutral polysaccharides. These results are similar to those reported by Francois & Graf (1988) for the gammarid *Niphargus virei* and for the freshwater shrimp *Caridina denticulata* L. (Miyawaki, Taketomi & Mishima 1985).

Francois & Graf (1988) classified basal membranes into four groups (see their paper for references): 1- layers made of dense granulated beads, without bridges and regular substructures, reported in dipterans and coleopterans; 2- regular grid-like structures, held together by bridges, reported in dipterans, coleopterans, fleas and slugs; 3- interconnected hexagonal plates, like in heteropterans and gammarideans; 4- massive plates of fibres that form networks protruding into the connective tissue, connected by thin microfilaments and described in some decapods.

The basal membrane of *L. salmonis* does not fall into any of these groups as its matrix is made of finely granular homogeneous material, which adds another category to the above classification. A similar membrane to the one reported here is described by Briggs (1977) in the parasitic copepod *Paranthesius anemoniae*. In most of the studies of the ultrastructure of the midgut of copepods, the attention is centred on the enterocytes and there is little detail about the structure of the basal membrane. A exception is the free living copepod *Calanus helgolandicus*, in which the basal membrane of the midgut diverticulum is composed of a number of parallel layers of electron dense material intervening with electron-lucent ones (Ong & Lake 1969), peculiar enough to add even another category to those proposed by Francois & Graf (1988).

Table 3.3 Divisions of the midgut (of adults) in several groups of copepods. "Gross divisions" indicates when the authors have divided the midgut in zones according to the overall morphology of the digestive tract. "Functional divisions" shows when the midgut has been divided in zones according to the cellular distribution and structure , attributing different functions to each zone. When an anterior diverticulum or caecum has been considered as a separate zone by the original author, for practical purposes it was considered for these comparisons as part of the anterior midgut. † indicates when a zonation is not mentioned in the original paper but it can be inferred from figures or plates. A question mark indicates when no zonation is especifically mentioned and it cannot be inferred from the figures, plates or descriptions. F: free living; P: parasitic; C/P: commensal/parasitic

Species	Mode of life	Gross divisions	Names of divisions	Valve or constriction	Functional divisions	Name divisions	Source
CALANOIDA							
Centropages typicus	F	2 †	Anterior Posterior	C †	3	I - III	Arnaud, Brunet, and Mazza, 1978
Calanus finmarchicus *Centropages hamatus* *Pseudocalanus elongatus* *Paracalanus parvus* *Acartia clausi* *Acartia bifilosus* *Acartia longiremus* *Oithona similis*	F	2 †	Anterior Posterior	C †	2	anterior posterior	Dakin, 1908
Calanus finmarchicus	F	2 †	Anterior Posterior	C †	none	none	Ong and Lake, 1969
Calanus helgolandicus Claus	F	2 †	Anterior Posterior	C †	3	Anterior Glandular Posterior	Hallberg *et al.*, 1980
Calanus finmarchicus	F	2 †	Anterior Posterior	C †	3	Anterior Glandular Posterior	Hallberg and Hirche, 1980
Epilabidocera amphitrites (McMurrich, 1916)	F	3 &	Anterior Mid Posterior	?	3	Anterior Mid Posterior	Park, 1966
Eudiaptomus gracilis	F	2	not named	C	3	I - III	Musko, 1983; Musko, 1988
Euaugaptilus placitus Sars	F	4	Anterior chamber Mid chamber Posterior chamber 4th region	C	3	I - III	Boxshall, 1985; Boxshall, 1992
Lophothrix frontalis Giesbrecht	F	?	-	-	3	I - III	Nishida, Oh, and Nemoto, 1991
Scottocalanus securifrons (T. Scott)	F	?	-	-	3	I - III	Nishida *et al.*, 1991
CYCLOPOIDA							
Macrocyclops albidus	F	2 †	Anterior Posterior	C †	3	Im1, Im2, Im3	Defaye, Such, and Dussart, 1985
Cyclops vicinus	F	2	not named	C †	3	I - III	Musko, 1983
HARPACTICOIDA							
Tigriopus californicus (Baker)	F	2	Anterior Posterior	C	2	Anterior Posterior	Sullivan and Bisalputra, 1980
Tigriopus japonicus	F	2	Anterior Posterior	C †	2	Anterior or "glandular" Posterior	Yoshikoshi, 1975

Species	Mode of life	Gross divisions	Names of divisions	Valve or constriction	Functional divisions	Name divisions	Source
Diarthrodes cystœcus	F	2	Anterior Posterior	V	-	-	Fahrenbach, 1962
Paramphiascella fulvofasciata	F	none	-	-	-	-	Dahms, 1993
Avdeevia antartica Bresciani & Lutzen, 1994	F	2	Anterior Posterior	C	2	Anterior Posterior	Bresciani and Lutzen, 1994
POECILOSTOMATOIDA							
Paranthessius anemoniae Claus	P	2	Anterior Posterior ("hindgut")	-	2	Anterior Posterior	Briggs, 1977
Ergasilus sieboldi	P	2-3?	?	?	?	?	Einzporn, 1965
Sapphirina angusta	C/P	2	Stomach Intestine	-	2	Stomach Intestine	Marino and Onesto, 1970
Mytilicola intestinalis Steuer	P	?	-	?	none	-	Gresty 1992
SIPHONOSTOMATOIDA							
Phrixocephalus cincinnatus (Wilson)	P	2	Anterior (stomach) Distal, Posterior	?	none	-	Perkins 1994
Pseudocycnus armatus	P	2	Stomach (=anterior) Intestine (=posterior)	C	2	Stomach Intestine	John and Nair, 1975
Pseudocharopinus dentatus	P	3	Anterior stomach Posterior stomach intestine	none	2	stomach intestine	Rigby and Tunnell, 1971
Allanthogynus delamarei	P	3	Anterior Mid Posterior	C †	3	Anterior Mid Posterior	Changeux, 1960
L. salmonis	P	2	Anterior posterior	V	3	Anterior Mid Posterior	This study
L. salmonis	P	none	-	-	none	-	Nylund, Økland, and Bjørknes, 1992
L. pectoralis	P	2	stomach intestine (by Scott)	C	-	-	Scott, 1901; Boxshall, 1985
Caligus minimus Otto	P	2	Anterior Posterior	C	2	Anterior Posterior	Poquet, 1980
Lernaeocera aceratus Ho & Honma	P	3	Anterior Mid Posterior	-	3	Anterior Mid Posterior	Honma and Ho, 1988
Lernaeenicus sayori Yamaguti	P	3	Anterior Mid (vacuolar) Posterior	-	3	Anterior Mid Posterior	Honma *et al.*, 1988
Lernaeocera branchialis	P	2	stomach intestine	none	2	stomach intestine	Capart, 1948
MISOPHRIOIDA							
Benthomisophria palliata Sars	F	2	?	C	?	?	{Boxshall 1982 #476}
MORMONILLOIDA							
Mormonilla phasma	F	3	Anterior chamber Middle chamber Posterior region	C	2?	Anterior Posterior	{Boxshall 1985 #475}

Perkins (1994) also described an unusual basal membrane in the pennellid copepod *Phrixocephalus cincinnatus*, which consists of a meshwork formed by smooth tubules, mitochondria, striated muscle cells oriented in different planes and collagen fibres. The structure of this basal membrane seems peculiar enough to add even another category to the classification of Francois & Graf (1988). Raymont *et al.* (1974) strangely do not mention the presence of a basal membrane *per se* in their description of the alimentary canal of *Calanus finmarchicus.* They do mention two layers of smooth muscle just beneath the gut epithelial cells which apparently exists throughout the length of the midgut. The inner layer, the one next to the epithelium, is said to be formed of 12 to 15 layers of, citing the authors, "...contractile tubulous fibrillae, which are arranged so that they form an undulating band round the alimentary canal...". This description and the photographs presented resemble closely those of the basal membrane of *C. helgolandicus* (Ong & Lake 1969, see above) and the basal membrane described in the midgut of the amphipod *Corophium volutator* (Icely & Nott 1984). It seems possible that the authors probably have mistaken the true basal membrane as a smooth muscle layer, as it was later clarified by Hallberg & Hirche (1980), who describe it as having lamellae arranged in undulating bands with more electron dense portions at intervals, and thus belonging to the first category of the classification (see above). An epithelium without basal membrane seems rather unusual and has never been reported in other arthropods. Moreover, continuous muscle sheaths apparently do not occur in arthropods (Cioffi 1984).

So it seems that at least two types of basal membrane exist in copepods, an amorphous, finely granular one, and a more complex one with parallel lamellae. More attention to this layer in future studies of other copepods might shed more light on the distribution of different types of basal membranes in different groups. Such differences in basal membrane structure may also reflect different ways of dealing with the passage of nutrients from the midgut to the haemolymph. In an amorphous basal membrane like the one described here there is no barrier between the midgut and the haemolymph for the passage of substances between them as they are in constant contact owing to the porosity of the basal membrane. In the case of more complex basal membranes constituted of two or three layers and with limited or no porosity, as in *N. virei* (Francois & Graf 1988), the crab *Menippe mercenaria* Say and the lobster *Homarus americanus* (Factor 1981) or

the shrimp *Caridina denticulata* (Miyawaki *et al.* 1985), there must be another mechanism for the passage of substances to the haemolymph.

Koefoed (1987) and Francois & Graf (1988) suggest that the basal membranes in the organisms they studied may be able to protect the midgut against variations in osmolarity of the haemolymph. While this may be true for complex basal membranes like those mentioned above, it is difficult to imagine how this can hold true for *L. salmonis*, in which the haemolymph is in intimate contact with the basal cell membrane of the enterocytes by means of the pores of the basal membrane.

Basal membranes with pores have been recorded also in insects (Cioffi 1984).

The observation made in this study that the longitudinal fibres of the myoepithelial network surrounding the midgut are actually branches of the circular muscles has been observed before by Loizzi (1971) in the digestive gland of the crayfish, which has a similar network of muscle fibres. He mentions that these longitudinal muscle fibres share the same sarcolemma of the circular ring. This muscle network of the midgut and digestive gland has been described in a number arthropods (Cioffi 1984). This latter author suggests that a continuous muscle sheath would constitute a permeability barrier in organisms with limited or no capillary system, as in insects and crustaceans, and the need for muscular movement in the midgut to move the gut contents is therefore accomplished by this network of muscles firmly connected to the basal membrane of the epithelium, while at the same time allowing no barrier between the haemolymph and the epithelium. She examined the muscle layers surrounding the midgut of several lepidopteran larvae and found that "... each of the muscle blocks is surrounded by a sheath of basement membrane from which the thin filaments extend to contract the basament membrane surrounding the adjacent muscles and the basement membrane underlying the epithelium". Schrehardt (1987) reported a similar relationship of the circular muscles and the basement membrane in the gastric caeca and the midgut of *Artemia.* In the present study it was also observed that what surrounds the muscle myofibrils of *L. salmonis* is connective tissue, morphologically similar to the basal membrane. But, immediately below this sheath, there is a layer of connective tissue of similar composition to the mesenteries. The ramifications of this mesenteric tissue is what actually interlocks with the pores of the basal membrane. This interlocking mechanism apparently has not been reported before.

Loizzi (1971) states that muscle fibres, both circular and the laterally projecting, being part of the same cell, can then contract together preventing the "ballooning" that would occur if the circular muscle contracted alone. The mode of communication between successive circular fibres could then be partially explained. If the sarcolema of successive muscle rings is shared by means of the lateral projections, the rings can act as a continuous muscle, as in vertebrates (Cioffi 1984), passing the contracting waves of peristalsis from one ring to another without the need for individual nervous terminals for each ring. Very little is known about control of several midgut functions, such as cellular proliferation, absorption and myofibril contraction, and Dall & Moriarty (1983) mention that it is possible that one or more of these functions could be under endocrine control.

Similar suspension of the gut by connective tissue as the one found in the present study has been reported by Raymont *et al.* (1974) for *Calanus finmarchicus* although no details of structure or mode of attachment to the gut wall are given.

Koulish (1971, 1976) and Rainbow & Walker (1977) have ascribed some properties to a special layer, which has been variously called the "stratum perintestinale", "special parenchymal cells" , "perintestinal layer" or "morphologically polarised cells" in some barnacles or "perintestinal cells" in the cephalocarid *Hutchinsoniella macracantha* Sanders (Elofsson *et al.* 1992). In all cases the cells of this layer send "processes" through the basal lamina to make contact with the midgut epithelial cells and apparently they are involved in movement of ions and small molecules and are probably also neural in nature. Walker (1992) further states that this *stratum perintestinale* of the barnacles is ideally placed to carry out an hepatic role, controlling the release of absorbed materials to the haemolymph. Elofsson *et al.* (1992) suggest that this tissue deals with the translocation of metabolites from the epithelial cells. In *L. salmonis,* the morphology of the mesenteric tissue and its attachments suggest that its function may be similar to the processes of this special tissue of the barnacle midgut, as much as that the mesenteric projections passing through the basal membrane also are in close contact to the enterocytes basal cell membrane and have similar microstructure. In the louse, though, the processes and its projections have fine-fibre bundles inside, instead of the microtubule bundles described for barnacles. It is therefore possible that the mesenteric tissue of *L. salmonis* could also have a transport, storage, sequestering, excretory and/or neural role, apart

from mechanical support. The retention of neutral red in these specific tissue may point to an excretory or sequestering function. Defaye *et al.* (1985) report a mesentery completely encasing the alimentary canal of the freshwater copepod *Macrocyclops albidus*. They also attribute a transport role to this tissue, where the products of digestion pass through the interior of the animal, although the characteristic contact of the projections of the mesenteric cells with the basal cell membrane of the enterocytes described for barnacles and the cephalocarid (see above) or in the present study is not mentioned.

Further detailed studies of the mesenteric tissue of the lice are necessary to confirm its specific function.

In copepods, the circulatory system is reduced compared to other crustaceans, and it is only in the calanoids and a few misophrioids that a heart exists (Boxshall 1992). The basic circulation pattern for copepods described by Boxshall (*op cit.*) seems to apply also for *L. salmonis*. However such a pattern of lacunae through which the haemolymph flows, formed by the mesenteric tissue, apparently has not been described before for *L. salmonis* nor has the valve observed in the cephalothorax. It could be considered a primitive condition or a remnant of what was a more complex system. It is possible that other valves exist in the cephalothorax to control and direct the flow of the haemolymph, but more detailed observations are needed to confirm this and to describe completely the set of sinuses and cavities that conform the circulatory system of the sea lice.

Finally, the origin of the basal membrane is uncertain. It is generally considered as a product of the epithelial cells (Francois & Graf 1988) but, on the other hand, Miyawaki *et al.* (1985) consider that the basal membrane of decapods is formed by a part of the cytoplasm of the underlying connective tissue cells. As we have seen here, in *L. salmonis* the sheath that surrounds the mesenteries seems to be continuous with the basal membrane. It is possible then that, following the proposition of Miyawaki *et al.* (1985), the basal membrane is indeed a product of the connective tissue cells, in this case the mesenteric cells. It seems less likely that the enterocytes, apart from being involved in the demanding digestive process, would embark *also* in the huge task of producing the material that ensheaths the mesenteries of the rest of the body.

Still, further studies, both biochemical and physiological are necessary to establish in detail the functions of this apparently very versatile mesenteric tissue.

The midgut epithelium cells of calanoid copepods have been grouped into four cell types, which correspond to the cell categories established long ago by Hirsh & Jacobs (1928) (see Gibson & Barker 1979) for decapods. Arnaud *et al.* (1978) also classified the cell types of the midgut of the copepod *Centropages typicus* according to this nomenclature. This E, R, F and B-cell classification has been adopted by other authors in other groups of copepods (Arnaud *et al.* 1980, Hallberg & Hirche 1980, Boxshall 1992, Gresty 1992). Nevertheless, the ultrastructural characteristics used by Arnaud *et al.* (1978), mainly for those of R- and F-cells, differ in some respect than the original descriptions for decapods and they did not match properly with the cell types found in this study. Therefore, the assignment of the cell types found in the gut of *L. salmonis* to the types E-, R-, F- and B-cells was based on the characteristics for decapod enterocytes outlined by Gibson & Barker (1979).

The R-cells were the most abundant in the gut epithelium in this study. Their characteristics, such as presence of long microvilli and numerous mitochondria located in the apical part are indicative of their function as absorbing cells. These absorbing cells have been reported in copepods under different names apart from R-cells (see Table 3.3) and they are always the most abundant type.

Changeux (1960) found ALP activity in the enterocytes of most of the midgut of *Allantogynus delamarei* but, in contrast with the present study in which the ALP activity was restricted to the brush border of R or F cells, the activity he reported was in the cytoplasm of the cells along with secretion granules, but never in the interior of these granules, or in the brush border. ALP is a membrane bound enzyme which, in absorptive cells, is activated when it reaches the apical membrane (Alberts, Bray, Lewis, Raff, Roberts & Watson 1994), and Changeux's results could have been the consequence of the diffusion of the reaction product into the cytoplasm.

Table 3.3 Names of the different types of midgut epithelial cells of copepods after several authors..

Author	Species or group	Mode of life	Function of the cell type: Replacement of cells	Absorption	Secretion of digestive enzymes	Intracellular digestion
Gibson & Barker 1979	Decapods		E	R	F	B
Park 1966	*Epilabidocera amphitrites*	F		non-vacuolated	-	Vacuolated
Raymont et al. 1974	*Calanus finmarchicus*	F	-	Non vacuolar	-	Vacuolar
Arnaud et al. 1978	*Centropages typicus*	F	E	R	F	B
Hallberg & Hirche 1980	*Calanus finmarchicus* *Calanus helgolandicus*	F	-	R	F	B
Nott et al. 1985	*Calanus helgolandicus*	F	-	R	F	B
Nishida et al. 1991	*Lophothrix frontalis* *Scottocalanus securifrons*	F	-	R	F	B
Defaye et al. 1985	*Macrocyclops albidus*	F	-	Non vacuolar	With small vacuoles	Vacuolar cells
Musko 1986, Musko 1988	*Cyclops vicinus*	F	-	Cylindrical	Pyramidal	Vacuolar
Yoshikoshi 1975	*Tigriopus japonicus*	F	-	Non vacuolar	-	Vacuolar
Sullivan & Bisalputra 1980	*Tigriopus californicus*	F	Type 1	Type 4	Type 2	Type 3
Rigby & Tunnell 1971	*Pseudocharopinus dentatus*	P	-	Columnar cell	-	Gland cell
Nylund et al. 1992	*L. salmonis*	P	-	Type II	Type I	Type III
Bron, J.E. 1993, Bron et al. 1993	*L. salmonis* (larvae)	P	Stem	Microvillar or R/F	Microvillar or R/F	Vacuolar
This study	*L. salmonis*	P	E	R	F	B
Poquet 1980	*Caligus minimus*	P	-	Type A or C	-	Type B
Durfort 1977	*Mytilicola intestinalis*	P	-	Type B	-	?
Gresty 1992	*Mytilicola intestinalis*	P	-	R	F	B
Honma & Ho 1988	*Lernaeocera aceratus* *Lernaeenicus sayori*	P	-	"Secretory"	-	Vacuolated
Changeux 1960	*Allanthogynus delamarei*	P	-	Adipose cells	-	Secretory cells
Briggs 1977	*Paranthessius anemoniae*	P	-	Columnar	-	Ameboid
Rybakov & Dolmatov 1992	*Cucumaricola curvatus Avdeev*	P	-	Cuboidal cells	-	Secretory cells
Perkins 1994	*Phrixocephalus cincinnatus*	P	-	Non vacuolar	-	Vacuolar

But the concurrent absence of ALP reaction in the brush border is difficult to interpret as this is the place where it is usually located (Van Goor, Gerrits & Hardonk 1989). Barker & Gibson (1977) also found intense phosphatase activity in the brush border of the R-cells of *Homarus gammarus*. Phosphatase enzymes are known to occur at sites of active metabolite transfer across membranes (Saev 1963) and the presence of ALP in the microvillar cells may be concerned with the mechanism of absorption and also probably ion and water transport (see below).

Acid mucopolysaccharides were found to be present also in the brush border of R-cells of *Homarus gammarus* (Barker & Gibson 1977). They suggested that these mucopolysaccharides might have a role in the pinocytotic or phagocytic processes, acting probably as binding agents on the cell membrane. Bron (1993) did not find evidence of acid mucopolysaccharides in the brush border of microvillar cells of larval *L. salmonis* and this contrasts with the present study. It is possible that he encountered the same low or negative staining in plastic sections as happened here or that the copepods were in a stage where these compounds were poorly represented in the brush border.

The number of organelles per cell, including mitochondria, can be regulated precisely according to need and increases of 5 to 10 fold are reported when cells are in an energy demanding period (Posakony, England & Attardi 1977). Active R-cells have many mitochondria in their apical part, reflecting the high energy demands that must be occurring at their peak of absorption activity. The E-cells have few mitochondria, so there must be a stage where mitochondrial division takes place to account for the huge numbers of them in the later stages as R cells. The proposed early R-cell in *L. salmonis* seems to fill this gap. Long mitochondria in a cell may be indicative of mitochondrial division process (Alberts *et al.* 1994). However, this process must be very rapid judging by the apparently low occurrence of this type in the cells. The RER seems to be present in these early R-cells must be later transformed into smooth type.

Mature R-cells are characterised by the accumulation of lipid droplets and glycogen, or they may store only lipids, probably depending of the type of diet of the organism (Brunet *et al.* 1994). In *L. salmonis*, glycogen was never observed in R cells and the storage of lipids seemed to be related

to the diet of the louse, the lipid droplets being more frequent and bigger in lice that had fed on blood. This issue will be further discussed in Chapter 5.

Yoshikoshi (1988b) also observed that one of the functions of the "nonvacuolar cells" (possibly equivalent to the R-cells of the present study) of *Tigriopus japonicus* was the storage of lipids. In starved animals these lipids were consumed and in animals fed with fresh milk these lipid droplets increased in number and size. In this respect, the R-cells in this study are comparable to the cell type II found by Nylund *et al.* (1992), to the R/F cells of Bron *et al.* (1993), also in *L. salmonis.*, adult and larvae respectively, and to the R-cells described for calanoid copepods (Arnaud *et al.* 1978, Arnaud *et al.* 1980, Hallberg & Hirche 1980, Nishida *et al.* 1991)

The extracellular network of the R-cells suggest that R-cells are not only dedicated to the absorption of nutrients but also may have a role in the transport of ions and water. In other groups, like amphipods, the cells in the posterior midgut probably contribute less to the digestive process and the cells of this region, which possess an extensive SER associated with numerous mitochondria, correlate to the mentioned function of transport of ions and water (Icely & Nott 1984). Cells with a similar extracellular network to the R-cells of *L. salmonis* are found in the midgut posterior caeca of amphipods (Graf & Michaut 1980), where the cells in the proximal segment are mainly involved in the transport of ions and water. Brunet *et al.* (1994) suggest that the R-cells in the posterior midgut of calanoid copepods probably engage in the absorption of a water fraction which might contain soluble components involved in osmotic regulation.

R-cells in *L. salmonis* that present this characteristic extracellular network are found not only in the posterior midgut, but throughout the midgut. It is possible that the extracellular network might be more frequent or developed in the mid and posterior midgut, but the observations carried out in this study are not sufficient to substantiate this. Nevertheless, it is possible that the role of the enterocytes of *L. salmonis*, as in other crustaceans, is not only nutritive but also related to osmotic and ionic regulation. Secretion of digestive fluid of a particular osmotic or ionic composition may aid in regulation and composition of haemolymph, apart of course from the process of digestion (Dall & Moriarty 1983). The extent of the extracellular network seemed to be variable from sample to sample and from individual to individual, and these changes may be related to environmental

conditions, particularly salinity, prevailing just before and/or at the moment of fixation, or they could be related to the nutritional state of the louse. The parasitic copepod *Lernaeocera branchialis* has an internal medium 57-82 % of the concentration of the surrounding sea water, and that of its host's blood is somewhat lower 42%. But when the parasite is isolated in sea water it becomes isosmotic to the medium. This happens also with other copepod and bopyrid parasites (Vonk 1960). For a long time the gut has been considered as a site of ion-dependent fluid absorption. Oral and anal drinking has been recorded in a wide range of crustaceans and it has been proven to be related to osmotic and ionic regulation (Dall & Moriarty 1983). With the apparent lack of specialised structures for ionic regulation, like the branchiae or caeca of other lower crustaceans it is possible that the gut of *L. salmonis* may take part in this metabolic process. This has been proposed before for *L. salmonis* by Hahnenkamp & Fyhn (1985). They studied the osmotic response of lice attached to the host and free swimming ones during transition from sea water to fresh water. Free swimming lice survived up to 8 h in fresh water while the attached lice could survive for the whole length of the experiment, which was of 7 days. The authors suggest that for atttached parasites, the body salts lost when the haemolymph is diluted may be replaced through feeding on the mucus and body fluids of the salmon host. Thus, it seems that in *L. salmonis* the gut may have a role in osmotic regulation and ion balance, but more studies are necessary to understand on what extent and how is the gut involved in this mechanism.

But without proper quantification is not possible to substantiate this. Nevertheless, it opens up possibilities for future research.

While F-cells in this study share many characteristics with those described for decapods, it was difficult to relate them to the descriptions of F-cells for calanoid copepods of Arnaud *et al.* (1978) and Arnaud *et al.* (1980), except for the presence of abundant RER and zymogen granules. F-cells of *L. salmonis* do not have numerous phagosomes and short microvilli. The length of microvilli is always comparable to those of their neighbouring R-cells, except when the F-cells reach the F-B stage (see below). Moreover, the F2 stage seen in calanoids, with stocky or cylindrical and even shorter microvilli was not seen either. This F2 stage of calanoids also shows the upper part of the columnar cells projecting into the intestinal lumen with a swelling that contains phagosomes. This

description is very similar to the late stages of R-cells observed in *L. salmonis* , although the microvilli are not short or stocky.

F-cells in this study correspond to the cell type II of *L. salmonis* described by Nylund *et al.* (1992), to which they attribute a secretory function comparable to F-cells of decapods. Other equivalencies to cells described in other species are listed in Table 3.3

In this study, F-cells usually had few zymogen granules in the apical part. Brunet *et al.* (1994) mention that the reason why zymogen granules are rarely mentioned in Crustacea is possibly owing to the shortness of their cellular phase. In *Penaeus semisulcatus* the production of zymogen granules takes place within the first 2 h after feeding. In the specimens studied, F-cells with zymogen granules came usually from lice with signs of having food in the midgut, usually a blood meal, and that had been fixed immediately after removal from the fish. Therefore, it is possible that the peak of digestive enzyme production of the F-cells is also short in *L. salmonis* and hence, easily missed.

As is the case in the majority of studies of copepods, it was not possible to differentiate R-cells from the other types of microvillar cell with the light microscope. Even under TEM, most authors have found cells with intermediate characteristics of R-and F-cells. Arnaud *et al.* (1980) also found that in *Calanus helgolandicus* and *Acartia clausi* the F cells were less typical due to the lack of phagosomes, and that the endoplasmic reticulum had a vesicular and hybrid aspect. In this study, there were cases where mixed characteristics were found. It is thought that they may correspond to late E-cells which are in the process of differentiation either into R or F-cell. If E-cells are a common stage for both, it is likely that at some point one has to encounter mixed characteristics.

Also, due to the mixed characteristics it has been suggested that R and F-cells form a sort of continuum (Arnaud *et al.* 1980, Nylund *et al.* 1992, Bron, J.E. 1993), and Nylund *et al.* (1992) proposed that R-cells are a late phase of F-cells .

In the present study it has been shown that R-cells undergo a clear transformation into what was called the A-cell stage before being extruded at the end of their cycle. It was also illustrated here that there are F-cells that show a phase that could be an intermediate stage of transformation into

B-cells. F-cells then cannot be a stage before R-cells in *L. salmonis* . They cannot be a stage after R-cells either, as the final stages of R cells were demonstrated. The only possibility that remains, assuming that R and F-cells are a continuum, is that F-cells arise from R-cells. But one characteristic of R-cells is the high number of mitochondria and the number of mitochondria in F-cells is clearly diminished. Therefore the existing mitochondria of the presumed R-cell in the process of becoming an F-cell would have to be purged, and the signs of this process would be seen as an increased number of autophagosomes and myelin figures in the resulting F-cells. But these signs of organelle destruction were not seen in F-cells of *L. salmonis*. The signs were conspicuous in R-cells though, indicating the high turnover and metabolic activity of the R-cells at the peak of their absorptive function, and become even more common as the R-cell approached the end of its cycle to gradually become A-cells.

Interestingly, the fate of R-cells has seldom been approached by other authors and its understanding may represent a vital clue to comprehending the main patterns in the digestive cycle of crustaceans. Degenerate R-cells have been observed, for example, by Loizzi (1971) in crayfish, by Arnaud *et al.* (1978) or Nott *et al.* (1985) in calanoids. Yoshikoshi (1975) considered the dark staining columnar cells in *Tigriopus japonicus* to be necrotic. Nylund *et al.* (1992) observed breakdown of I and II cell types in *L. salmonis*, with the consequent discharge of cytoplasmic contents into the lumen. In all cases, as a result of the breakdown, the basal membrane was left exposed.

Thus, the fate of R-cells has been assumed as "discharge", "extrusion" or " degeneration" (Brunet *et al.* 1994), or even as "necrosis". Arnaud *et al.* (1978) mentions that R-cells undergo progressive degeneration and the stages leading to necrotic cells (N-cells) with numerous membrane ruptures can be followed. The picture of N-cells that they describe has indeed all the signs of necrosis but it is also possible to observe these features after fixation with fixatives for which the osmolarity has not been adjusted specifically for the tissue to be fixed, resulting in a lack of proper preservation of the cell architecture (see Chapter 2). Also B-cells have been reported to undergo such degenerative changes (for example Arnaud *et al.* 1978, Arnaud *et al.* 1980, Nott *et al.* 1985, Hallberg & Hirche 1980).

Moreover, epithelial cell sheets by definition are selective permeability barriers to the movement of water and solutes from one body compartment to another one with different chemical composition. The tight junctions of the epithelial tissue keep the cells together and act as a diffusion barrier. If tight junctions are disrupted, undesirable and dangerous leakage of body fluids can occur (Alberts *et al.* 1994). Necrosis of enterocytes, with their consequent extrusion leaving the basal membrane exposed must be considered thus as an artefact or as a pathological condition. In a tissue with a rapid turnover of cells as the epithelial, the numerous gaps and breaches of integrity of the epithelial sheet that would be generated by this harsh degenerative process would cause a tremendous leakage of solutes, nutrients and body fluids that could prove to be fatal. Indeed, one of the main strategies in the search for vaccines against sea lice is precisely to achieve the disruption of the structure of the gut epithelium (Jenkins *et al.* 1993, Andrade-Salas *et al.* 1993), This strategy is based on the success of vaccination of cattle against ticks, in which ticks feeding on vaccinated animals showed extensive damage of the gut and leakage of lumen contents into the haemolymph (Willadsen *et al.* 1988, Wong & Opdebeeck 1989).

For long it has been known that there are other structures indicative of cell death apart from necrosis, but it was until 1972 that the term apoptosis was coined to characterise this second type of cell death (see Searle, Kerr & Bishop 1982). Apoptosis (which means "dropping off"; the second "p" is silent), or "programmed cell death", affects scattered cells. It involves rapid condensation of both the nucleus and the cytoplasm, with abundant protrusion of the cell surface. The chromatin accumulates in the periphery of the nucleus and there is a gradual condensation of the cytoplasm that leads to cellular budding to produce a cluster of membrane-bounded apoptotic bodies in which organelles are well preserved. The fate of apoptotic cells depends on the tissue involved. Generally they are phagocytosed and degraded by resident tissue cells (Searle *et al.* 1982) or they can be exfoliated to the luminal side of the organ (Han, Iwanaga & Fujita 1993). Unlike necrosis, apoptosis is not associated with inflammation and it does not induce scarring. It is a process of active cellular self-destruction, not degeneration (Wyllie, Kerr & Currie 1980)

Necrosis on the other hand, is a completely different process of cell death, called also "accidental cell death" (Wendelaar Bonga & van der Meij 1989). It is characterised by swelling of all cellular

components, rupture of internal and plasma membranes, dissolution of organelles and eventual loss of chromatin. All this is the result of an irreversible disruption of the vital processes that maintain cellular integrity. More importantly, necrosis usually affects groups of contiguous cells and *is always pathological* (Searle *et al.* 1982).

L. salmonis A-cells characteristics conform properly to the definition of an apoptotic cell. Their organelles are well preserved even when the cells have been extruded and they leave no scar in the underlying tissue. Enterocytes exfoliated into the lumen as entire cells containing a nucleus have been reported in mice and rats by Han *et al.* (1993) and their morphology resembles closely the A-cells of *L. salmonis*. Moreover, Pipan & Sterie (1986) observed that the apoptotic bodies in the gastric epithelium of the mouse initially remain tightly bound to viable cells, and this close connection is lost slowly in a step-by-step process which eventually leads either to their engulfment by adjacent cells or extrusion to the gland lumen, and in the latter case, the apoptotic cells seldom deteriorate afterwards. Potten & Allen (1977) noted that apoptotic cells close to the villus tip of mouse small intestine are probably extruded rather than undergoing the typical engulfment by neighbouring cells. In *L. salmonis*, engulfment of A-cells by neighbouring cells was not observed but should not be discarded, as both types of cell disposal are common in digestive epithelia (Wendelaar Bonga & van der Meij 1989, Benedetti, Mancini, Marucci, Paolucci, Jezequel & Orli 1990).

Programmed cell death is a common characteristic of normal animal development, and it probably makes up for an important fraction of the fate of the cells produced in most animals (Ellis, Yuan & Horvitz 1991). Genes that regulate this cell death have been identified and the aminoacid sequences of the proteins (called *Ced* [cell death abnormal] proteins) they express are known. More importantly, they seem to be highly conserved in evolution. When the human gene that represses the death programme is transferred to the nematode *Caenorhabditis elegans*, it inhibits normal cell death in the worm (Vaux, Weisman & Kim 1992), confirming that this capacity of programmed cell death is a primary property of animal cells (Alberts *et al.* 1994). Therefore, the identification of apoptosis in the midgut epithelium of *L. salmonis* should not be surprising.

Apoptosis has never been put as a mechanism for enterocyte cell death in crustaceans. This novel view could bring a different understanding of the processes involved in the midgut epithelium tissue homeostasis.

Cells morphologically similar to A-cells have been described in the midgut of *Artemia* (Schrehardt 1987). These cells were called degenerated epithelial cells because they show signs of necrosis. But the picture of a cell at the "beginning of degeneration" shows a cell protruding into the lumen with its microvilli disturbed in a similar fashion as the one observed in the present study. Its nucleus shows signs of chromatin fragmentation and clumping and other organelles are still recognisable. But more advanced stages of these cells lose integrity and only nucleoli, a few mitochondria and lipid drops are still detectable. It is possible that these cells of *Artemia* might also be apoptotic cells.

Cells with a structure similar to the B-cells found here in *L. salmonis* have been described in other copepods and other crustaceans (see Table 3.3). In general they are all characterised by the presence of many vacuoles of different sizes and densities in the cytoplasm.

Unlike the B-cells in calanoid copepods which are restricted and dominant in a distinct zone of the anterior midgut, the B-cells of *L. salmonis* can be found in the whole length of the midgut and with much less frequency than R-cells. These results are comparable to those obtained for the same parasite by Nylund *et al.* (1992) and Bron *et al.* (1993) and for other groups of copepods (Fahrenbach 1962, Rigby & Tunnell 1971, Yoshikoshi 1975, Briggs 1977, Sullivan & Bisalputra 1980).

It is generally agreed that the cytological and cytochemical characteristics of the B-cells in decapods are associated with intracellular digestion (Loizzi & Peterson 1971, Monin & Rangneker 1975, Barker & Gibson 1977, Kumari, Rao & Shyamasundari 1983, Al-Mohanna *et al.* 1985a, Al-Mohanna & Nott 1986, Al-Mohanna & Nott 1987b, Arnaud *et al.* 1991). A similar function has also been demonstrated through ultrastructural studies and cytochemical techniques in the B-cells of several free living (Arnaud *et al.* 1983, Arnaud *et al.* 1984b, Arnaud *et al.* 1984a, Nott *et al.* 1985) and parasitic copepods (Moore *et al.* 1978, Yoshikoshi & Ko 1991b, Yoshikoshi & Ko 1991a) which

have shown that the vacuoles or vacuolar apparatus of B-cells possess lysosomal enzymatic activity. The non-specific esterase activity found in the vacuoles of the B-cells of *L. salmonis* in this study and the intense pinocytotic activity suggest that, also in this species, the function of B-cells could be intracellular digestion.

A characteristic difference between the B-cells of free living and parasitic copepods seems to be that only endocytic vacuoles are conspicuous in the vacuolar cells of free living forms (Yoshikoshi & Ko 1991a). In *Centropages typicus* the clear bodies are predominant and only a small number of dense bodies are recorded. (Arnaud *et al.* 1978).

Many dense primary lysosomes were found in *L. salmonis* in this study and by Nylund *et al.* (1992) and Bron *et al.* (1993) and seemingly they are also a common feature of B-cells of other parasitic copepods, like those studied by Yoshikoshi & Ko (1991a) and, for example, in *Ergasilus sieboldi* (Einzporn 1965a), *Caligus minimus* (Poquet 1980), *Mytilicola intestinalis* (Gresty 1992), *Cucumaricola curvatus* (Rybakov & Dolmatov 1992) and *Phrixocephalus cincinnatus* (Perkins 1994).

These dense vesicles or primary lysosomes stain strongly with PAS as well as for protein. The strong staining for protein is probably due to the high concentration of enzymes and transport proteins in the lysosome. Most soluble and membrane-bound proteins that are made in the endoplasmic reticulum, including those that are to be transported to the Golgi apparatus, lysosomes, plasma membrane or extracellular space are glycoproteins. During protein glycosilation, an oligosaccharide is transferred to an NH_2 group of an aminoacid in the protein, and thus the protein is said to be N-linked (Alberts *et al.* 1994). All lysosomal hydrolases are N-linked and these oligosaccharides are the probable cause of the PAS positive reaction observed in these vesicles. Thus, this PAS positive content of these vesicles is unlikely to be due to concentration of host mucus taken up by the B-cell as it has been suggested by Briggs (1977) and Bron *et al.* (1993) and also because, being primary lysosomes, they have not yet come in contact with the endosomes that carry the material to be digested.

It is difficult to interpret the reason for such difference in the vacuoles of the free-living and parasitic copepods unless it is related somehow with the different type of food they are ingesting.

Primary lysosomes in the B-cells of *L. salmonis* were larger in the middle of the complex and smaller towards the apical part or towards the nuclei. This can suggests that they are being formed in the vicinity of the nuclei by the Golgi apparatus and as several small ones coalesce they get bigger towards the apical part where they meet the endocytic vacuoles.

Because there is no distinct material inside the endocytic vacuoles formed during pinocytosis it seems likely that these cells absorb substances already partially digested in the gut lumen (Arnaud *et al.* 1978)

It is commonly mentioned in the literature that B-cells release their contents to the lumen by holocrine, merocrine or apocrine secretion. Can the release of the products in the interior of a B-cell be called holocrine *secretion*? If the function of B-cells is *intracellular digestion* of partially digested material, then the purpose of their extrusion into the lumen would be to liberate these further digested nutrients, so that they in turn can be absorbed by the cells dealing with absorption, say R-cells. If B-cells were liberating enzymes or other proteins that will aid in the extracellular digestion or another process in the lumen, then it could be called *secretion*. Perkins (1994) considers the dense vesicles of the B-cells of *Phrixocephalus cincinnatus* as secretory vesicles, but at the same time she mentions that the endocytic vesicles fuse with primary lysosomes to become later the characteristic electrolucent secondary lysosomes. If the dense bodies are primary lysosomes then they cannot be secretory vesicles and viceversa, as they are different specializations of the vesicular transport of the cell serving completely different functions (Alberts *et al.* 1994). From the histo and cytochemical tests (already discussed above) it is known that these dense vesicles of B-cells are indeed lysosomes, primary lysosomes. Therefore it seems more likely that the function of the B-cells, with this lysosomal apparatus is intracellular digestion, not secretion, and if they are not secreting, then their extrusion to the lumen cannot be called "holocrine secretion". Vogt (1993) demonstrated by immunohistochemistry with antibodies that B-cells are not involved in the synthesis of a digestive enzyme called astacin, and considers that F-cells are the responsible cells for digestive enzyme synthesis and secretion.

Cells that are specialised for secreting products on demand, concentrate these products in secretory vesicles, or secretory granules. These secretory vesicles in turn gather and "wait", usually near the site where exocytosis is going to take place, until the cell receives the signal to secrete (Burgoyne & Morgan 1993) and then they are liberated by exocytosis (a process that apparently happens in F-cells) or by holocrine, merocrine or apocrine secretion (which has not been recorded to happen in F-cells).

Al-Mohanna & Nott (1986) considered that F-cells transform into intracellular digestive cells which also can absorb nutrients, and considered them as performing the *main* absorbing and digestive functions of the gland. Indeed, in their proposed routes of metabolites within R-cells, there are no routes *out* from the cell, which seems rather unusual for an absorptive cell. Vogt (1993) did not agree with the absorptive function of B-cells because the role of R-cells as being mainly responsible for absorption and metabolization of nutrients has been well established (see Vogt op cit. for references). While this is true, it does not exclude the possibility that B-cells are also able to move material from the lumen to the haemolymph. Endocytosed material from outside the cell is not always delivered to the lysosomes. It is a selective process and, depending on the type of material, the endosome is directed to the lysosomes for degradation or to the basolateral membrane, where its contents are liberated. This latter process is called transcytosis (Alberts *et al.* 1994). Once in the intercellular space, this material can then pass to the haemolymph. But specific studies would have to be done to elucidate what are the pathways that different materials follow within the B-cells.

Excluding digestion and nutrient absorption as functions of the B-cells, Vogt (1993) then concluded that excretion was the role of these cells, excretion in the sense of "clearing of the lumen of the hepatopancreas tubules from waste products of digestion and degradation of the pinocytosed material". In a sense, this function also had been proposed earlier by Hopkin & Nott (1980) because they found central vacuoles or entire B-cells in the faeces and by Al-Mohanna & Nott (1987b) where they mention that after the intracellular digestion and assimilation, the loss of B-cells by "holocrine secretion" involves the disposal of waste products.

As already discussed, B-cells of copepods also undertake intracellular digestion. But at the end of the process the cells are extruded and disintegrate into the lumen of the midgut (Brunet *et al.* 1994). This led Arnaud *et al.* (1978) to conclude that the digested material was made available in this way to the R-cells to be subsequently absorbed. Also, as in decapods, a double function for B-cells has been suggested: intracellular digestion and detoxification (Arnaud *et al.* 1987).

In *L. salmonis* B-cells in the lumen were extremely rare and were totally absent in the faeces. Nylund *et al.* (1992) and Bron *et al.* (1993) also did not find signs of B-cells in the lumen of adult and larval *L. salmonis.* Nylund *et al.* (1992) suggested that the B-cells in *L. salmonis* are expelled complete, although he did not actually observe these late stages of B-cells. This suggests that after their extrusion they probably disintegrate very quickly. In this way, any digested material can be accessible for absorption by the R-cells and the waste products can be incorporated in the faecal pellets. Thus, apparently in *L. salmonis* the B-cells have also the double function of intracellular digestion and excretion. Absorption of nutrients is probably not an important function of the B-cells of *L. salmonis*, as these cells detach from the basal membrane early in their development.

Several stages of development of B-cells were recognised in *L. salmonis* . Similar evolution of B-cells was reported by Arnaud *et al.* (1978) with the difference that in *L. salmonis* there was no formation of a large clear vacuole and that in the final stage of extrusion the B-cell did not suffer fragmentation and disintegration but gradually detached first from the basal membrane, and the cells surrounding them encroach below it until eventually the B-cell pinches off. No spaces with bare basal membrane in between R or F cells are left.

In the present study these late stages were very scarce, and this might suggest that the actual process of extrusion takes place very quickly. In decapods, for example in *Penaeus semisulcatus* (Al-Mohanna & Nott 1986), the evolution of B-cells has also been recorded, and at the last stages the cells are extruded complete. The difference with *L. salmonis* is that this final stage takes place after the intracellular digestion has been completed, judging by the transformation of all primary lysosomes into translucent digestive vacuoles, that later coalesce to form a big clear digestive vacuole that occupies most of the cell volume. The B-cells of *L. salmonis* are being extruded with

most of their digestive bodies or primary lysosomes in the first stages of activity (see Plate 3.36), and the cells look similar to the C and D stages of B-cells of *P. semisulcatus* i.e., in between the final stages of pinocytosis and initial phase of intracellular digestion. Whether the intracellular digestion continues after the B-cell has detached is not known, but it seems unlikely, as isolated B-cells in the lumen were never observed. They were never observed in the faeces either, suggesting that after the extrusion, B-cells probably eventually fragment, liberating the products of the digestion in the lumen so that they can be absorbed by the R-cells as has been proposed here.

Most of the knowledge of digestive processes in crustaceans has come from studies in decapods. Also, most of the reviews on this matter have dealt almost exclusively with the decapod system (see Brunet *et al.* 1994). Recently, these latter authors in their excellent review have put in perspective the digestive cellular processes occurring in all Crustacea.

The issue of cellular function and differentiation in the different groups of crustaceans is still in debate. Here, two models, of decapod and copepods, will be considered to elaborate on what might be occurring in the midgut of *L. salmonis*. (see Figure 3.2) In decapods, the main patterns in the digestive cycle proposed by Al-Mohanna & Nott (1987b) for *Penaeus semisulcatus* seem to be the most widely accepted. In it, a two-cell line concept is assumed, in which E-cells transform into R- and F-cells and the F-cells in turn transform into B-cells (Brunet *et al.* 1994). But recently another model was suggested by Vogt (1993). A three cell-lineage is proposed for *Penaeus monsoon* in which R, F and B cells originate independently from embryonic E-cells.

Most of the work on digestion of copepods has been done in calanoids (Hallberg & Hirche 1980, Arnaud *et al.* 1983, Arnaud *et al.* 1984b, Arnaud *et al.* 1984a, Arnaud *et al.* 1987, Nott *et al.* 1985, Arnaud *et al.* 1991) which Brunet *et al.* (1994) has summarised. In this group no direct link has been found between B cells and F cells. Also, a relationship between R- and F-cells has been proposed due to the common occurrence of intermediate cell types (Arnaud *et al.* 1980). In *L. salmonis* these mixed morphologies also have been reported (Nylund *et al.* 1992, Bron *et al.* 1993)

In the present study there were mixed morphologies between R- and F-cells, but these similarities are thought to be due happen in the early stages of both cells. Once the cells have reached maturity, the differences are quite obvious. A strong polarisation in R-cells, with many mitochondria at the apical part, forming bulges that project into the lumen, which does not happen in F-cells. Moreover, the finding of the terminal stages for both cells, A-cells for R-cells and B-cells for F-cells suggest that R- and F-cells develop independently from E-cells.

The occurrence of intermediate stages between F- and B-cells was very low. This could be due to a) the low occurrence and scattered distribution of B-cells, which make the chances to find intermediate stages very slim and/or b) the high speed at which this transformation can take place. Tinley & Cardell (1970) observed that a dramatic decrease in microvilli number in the small intestine of the salamander, due to fragmentation and retraction, could be appreciated under 20 min after the application of the stimulus (pressure). The F-cells showing transition to pinocytotic B-cells in this study show this fragmentation of microvilli, and if this happens in such a short time in low-occurrence cells, the probability of coming across one must be very low indeed.

In *L. salmonis* there does not seem to be a zonation where B-cells are more common, as in calanoids. This diminishes even more the chances of finding these F/B-cells stages.

The evidence presented here calls for a relation between F- and B-cells and therefore the scheme of cellular differentiation, at least in *L. salmonis*, looks similar to the one proposed for decapods, based mainly in studies on *Penaeus semisulcatus* (see Figure 3.2).

Although Perkins (1994) found two type of cells, vacuolar (similar to B-cells of *L. salmonis*) and non-vacuolar, in the parasitic copepod *Phrixocephalus cincinnatus*, she suggests that they may represent different developmental stages of a single epithelial cell. Immature cells differentiate into nonvacuolar cells which engage in absorption and then begin synthesising enzymes that are

packaged into vesicles. This in turn accumulate in the cell, and along with the digestive vacuoles within the cytoplasm the cell gives rise to a vacuolar cell. B-cells are thus regarded by Perkins as secretory (of digestive enzymes) and digestive (intracellular digestion).

But as Vogt (1993) points out, it is unlikely that B-cells, with the complex lysosomal apparatus engage as well in synthesis of enzymes for secretion.

The enzymes that are directed to the lysosomes are designed to work within the lysosomal environment (Alberts *et al.* 1994). Therefore it is unlikely that they have a role in the extracellular digestion that takes place in the lumen, as some authors have suggested (see for example Nott *et al.* 1985). The digestive enzymes that do this function are secreted through a pathway different from that of the production of lysosomes (Hong & Tang 1993) and the morphology of B-cells, with their profuse endocytic activity in the apical part probably leaves no chance for an exocytic pathway to exist.

The results found in the present study do not agree with Perkins' (Perkins 1994) theory of a one cell-line theory or double-purpose (secretory/digestive) B-cells, at least in *L.* salmonis .

More experiments and observations are needed, mainly with the use of tracers and microanalytical and cytochemical techniques, to corroborate specific function of each cell type and the genealogy and affiliation between the different cell types found in the midgut epithelium of *L. salmonis* in this study.

During the present study, literally hundreds of *L. salmonis* adult females were screened under the dissecting microscope. This allowed for the differentiation between different appearances of the digestive tract which were related to the state of the midgut epithelium. This approach has not been reported in other crustaceans. The characteristics of the sea lice, with a somewhat transparent cuticle on the ventral side and their size made this task possible.

Figure 3.2 Diagram summarising the main functions of the digestive epithelial cells in decapods, calanoid copepods and *L. salmonis*. For decapods two of the most accepted models are depicted at the left.

DECAPODS
Penaeus semisulcatus
(After Al-Mohanna & Nott 1986, 1987; Al-Mohanna et al. 1985)

COPEPODS
Centropages typicus
(After Brunet et al. 1994)

Storage of lipids and glycogen Accumulation of minerals

Uptake of material from the haemolymph.

Storage of lipids (Zone III)

Uptake of material from the haemolymph

Uptake of material from the haemolymph

Intracellular digestion and assimilation

Intracellular digestion and assimilation

Contact digestion and absorption

Exocytosis of enzymes

Exocytosis of enzymes

Extrusion of whole cell

Pinocytosis of nuenttris

DIGESTION IN LUMEN

Pinocytosis of nutrients

Extrusion of whole cell

Exocytosis of enzymes

Exocytosis of enzymes

Intracellular digestion and assimilation

Intracellular digestion and assimilation

Contact digestion and absorption

Uptake of material from the haemolymph

Storage of lipids and glycogen

Uptake of material from the haemolymph.

Storage of lipids

DECAPODS
Penaeus monodon
(After Vogt 1993)

Lepeophtheirus salmonis
(This study)

The almost constant 3:1 proportion between rough and smooth guts is noteworthy. Mature R-cells are more common in "crypted" epithelium. Moreover, these cells are the reason why the gut is crypted. This could mean that rough or crypted guts have an epithelium which is in an active absorptive phase. Although the majority (70%) of lice present this type of gut in "natural" (sea cage) conditions, there is always a small percentage (30%) of lice which have an epithelium which maybe in a sort of "resting" state, preparing probably for another meal. Nott *et al.* (1985) found that the copepod *Calanus helgolandicus* required a non-feeding period to complete regeneration of the midgut epithelium. They further suggested that the supply of B-cells may be a limiting factor in the duration of feeding, because, at a certain point of the feeding cycle, B-cells are numerous and are lost at such a high rate that it seems unlikely that they can be replaced immediately. This non-feeding period required is naturally accomplished during the diel migration of the free living copepod, in which there is little food available. On the other hand, in *Acartia tonsa* Hassett & Blades-Eckelbarger (1995) demonstrated that production of B-cells does not appear to be a limiting factor in the feeding cycle of this copepod, as B-cells seem to have a life cycle greater than the feeding period. As Hassett & Blades-Eckelbarger (1995) point out, these two copepods, *A. tonsa* and *C. helgolandicus*, employ different strategies to deal with variation of food. *C. helgolandicus* posseses lipid reserves that it uses when low levels of food occur. On the other hand Hassett & Blades-Eckelbarger (1995) mention that *A. tonsa* does not possess reserves and therefore is very susceptible to starvation. As its migratory range is limited, it has food always available. Its feeding activity is diminished during the day but it still continues to feed. These basic differences are probably the reason for the different digestive strategies observed.

In other chapters, the effects of starvation in *L. salmonis* (Chapter 5) and the feeding cycle (Chapter 6) will be considered and this issue of the relationship of feeding activity on the morphology of the midgut epithelium and occurrence of cell types will be discussed further.

But what seems clear is that the midgut epithelium of *L. salmonis* has a high degree of plasticity. The shape, the topography of the epithelium seems to be in constant change. A louse with 'rough' gut at one moment could show a smooth gut after several hours. And the rough guts can present a variety of topography themselves, form shallow crypts to very deep crypts. In other chapters we

will see evidence that the R-cells are in constant renewal, judging by the production of membranous pellets full of shed A-cells (See section 4.3.4)

Bron (1993), based on the lack of boundaries or zonation of cell types in *L. salmonis* larvae and the same findings by Nylund *et al.* (1992) in adults, suspected that a high degree of plasticity must be present in the composition of enterocytes, at least in *L. salmonis*, but that this might also be the case in other copepods.

From the observations of crypted guts under the SEM a pattern of crypts can usually be appreciated. Rows of mature R-cells are generally found orientated longitudinally (see for example Plates 3.52 , 3.56 and 3.60) and there should be a physiological explanation for that. However with the available information it is not sufficient to provide an explanation. Maybe the cell renewal follows a longitudinal pattern and from there the cells migrate sideways to replace the cells that are being lost in the pseudovilli. The mesenteries are also attached to the basal membrane forming a continuous longitudinal contact with the enterocytes. Maybe the mesenteries send the appropriate signals that control cell proliferation. Punin (1981) has reported that the basal cell system of the midgut of the aphroditid polichates has a role in the reproduction and differentiation of enterocytes. The signals that the mesenteries could send to the enterocytes could be of neurosecretory nature, judging by the findings of Punin (1986) of endocrine-like cell elements in the midgut of annelids and molluscs. Unfortunately, the work in this field that has been carried out by Punin and co-workers have only been published in Russian and the details of their experiments are therefore not readily available. As discussed before, barnacles also present a particular mesenteric arrangement in intimacy with the enterocytes that suggest a close communication between these two tissues. Certainly, more research in this area can open up a new understanding of the digestive processes and tissue homeostasis in Crustacea.

E-cells also seemed to be distributed throughout the midgut. There are no apparently specialised or localised foci of cell renewal as there are in the digestive gland of decapods. The Stacked type of gut, in which E-cells seem to be more abundant, could be a sign that cell renewal is taking place, judging by the size of the cells, their clumping and their the occurrence of double, triple or

dissociated nucleoli. Dissociated nucleoli are present in cells that either are preparing for mitosis or the mitosis has taken place. Double or triple nucleoli are characteristic of cells that have undergone mitosis and are in the early stages of G1 phase (Alberts *et al.* 1994).

For a better understanding of cell proliferation, it would be necessary to follow a different approach. Cellular renewal was measured in the copepod *Tigriopus japonicus* (Yoshikoshi 1980) using colchicine to arrest the cells in division and count the mitotic figures. By this method the author estimated a turnover time of the enterocytes of 9.6-10.2 days for glandular cells and 13-15 days for absorptive cells. Also, he observed that the epithelial cells arise from undifferentiated cells scattered on the basal membrane of the epithelium. But as the author points out, the problem with the use of colchicine is its high toxicity and its use at sub-lethal concentrations may not be sufficient to arrest mitosis completely, resulting in an overestimation of the renewal rate.

Other methods are available for the histologic identification of proliferating cells. Tritiated thymidine autoradiography has been known since the 1950s but more safe and efficient methods have been developed recently. One of the most widely used requires the pre-administration of the marker bromodeoxyuridine (BrdU) which is incorporated into the DNA of the replicating cells during the S phase of the cell cycle and made visible immunohistochemically by an antibody to this compound. Also recently, an immunohistochemical assay was developed which utilises an endogenous marker, proliferating cell nuclear antigen or PCNA, which is highly conserved and is directly involved in DNA synthesis. Other endogenous markers have been discovered since then. For further information the reader is directed to the most recent reviews for assessing cell proliferation (Hall & Woods 1990, Yu & Filipe 1993, Yeo & Toh 1994, Boulton & Hodgson 1995, Treré 1996). But of particular interest are the works of Moore, Leavitt, Shumate, Alatalo & Stegeman (1994) and Ortego, Hawkins, Walker, Krol & Benson (1995) who have developed assays for the detection of proliferating cells in aquatic animals using BrdU and anti-PCNA antibody respectively. Moore *et al.* (1994) could detect proliferating cells in the midgut and reproductive system of the copepod *Pseudodiaptomus coronatus* Wilson after bath exposures to bromodeoxyuridine. This technique could prove very useful to find more about the cell kinetics in the midgut of *L. salmonis*. With the use of double and triple labelling techniques it could be possible to measure not only the labelling

index but also the duration of the S-phase as well as the total cell cycle time. PCNA could be of particular interest because it does not require administration of any marker, so the problem of handling the parasite is avoided. At the end of this study some attempts were done to evaluate the use of PCNA or BrdU in *L. salmonis* with negative results. This does not mean that these methods are not suitable, but they need to be refined for this particular system to find out the adequate dilutions and incubation times for these immunohistochemical techniques.

It is worth noting that many differences in cell structure between species and groups could be due to the fixation process performed for a particular study. In this investigation, during the search for the more appropriate fixation schedule, a whole range of artefactual morphologies were found related to unsuited fixation. The fixative was not the problem, but the final osmolarity of the fixative. A number of the artefacts observed in this study seem to be common in the literature, and by looking at the particular fixation methods it becomes clear that no or little attention was given to the osmolarity of the fixatives. Einzporn (1965a) did make the observation that considerable divergences found by different authors regarding the cellular structure of the intestine result probably from the specific action of the fixatives. During the preliminary tests of fixatives in the present study, the ultrastructure of the enterocytes using the conventional fixatives mentioned in numerous works was remarkably different compared to properly fixed tissue using a fixative with the correct osmolarity. For example, A-cells were never found, differences between microvillar cells were impossible to detect, sloughing and "degenerating" cells were extremely common as well as figures of "apocrine secretion", "holocrine secretion" (involving lysis of the apical membrane and discharge of the cellular contents into the lumen), vesiculated SER, swollen Golgi, etc. Many of these characteristics have been considered in many studies as part of a cellular process (as it was also done at the beginning of this study before realising they were artefacts). The author feels strongly that many of the differences and discrepancies in the structure of epithelial cells in copepods could be due to fixation artefacts and that the overall organisation and functioning of the digestive epithelium in copepods, and maybe in other crustaceans, might be more homogeneous than it is thought. This problem will be dealt in detail in a separate publication (Andrade-Salas, in prep.) as it deserves special consideration.

4. FEEDING HABITS.

4.1 MATERIALS AND METHODS

4.1.1 Staining the fish skin to monitor the feeding activity of lice.

In order to assess the possible ingestion of host's skin epithelial cells by the louse, two vital dyes were tested to stain the skin of the salmon: Neutral red and methylene blue.

The fish was placed in a small aquarium with aerated seawater (33-34 ‰) and constant temperature (10°C). Enough dye was added to get an approximate final concentration of 0.1%. The fish was left in the dye for 2-4 hours and then transferred to another aquarium with clean seawater. Three or four subsequent changes of seawater were necessary to wash out the excess of dye attached to the fish skin. When no more dye washed out, the fish was ready to be infected with lice.

4.1.2 Observations of movements of lice on fish.

A fish was placed in a small rectangular aerated aquarium so that the fish would find it difficult to turn. The fish was left to acclimatise to the small environment for two to three hours before attempting any infection with lice. During this time, the lid of the aquarium was lifted frequently and light from a halogen pocket torch was directed to the body so that the fish got used to the activity and presence of the observer. Then, an adult female louse that had been starved for 1-2 days was taken from the stock with fine curved forceps and placed manually on the dorsal part of the fish, between the dorsal fin and the head. From then on, the position of the louse on the salmon was recorded on a specially designed form, which had the outline of both sides of the fish printed (Figure 4.1). The starting position was recorded with the number "0" and subsequent change of positions with sequential numbers along with the time elapsed between each movement. The observations were done at regular intervals, every five minutes for the first half hour, and then

every 15 minutes. The duration of the observations was variable, from a few hours to several days. In other cases the fish was infected with several lice at a time and their position recorded in a similar way but using a different log form, with the outline of the fish in dorsal view. In these observations it was not possible to follow the change of position of individual lice, except in some cases where a particular louse was easily recognisable from the others due to its colour or other characteristic.

4.1.3 Evaluation of the food ingested by the lice.

4.1.3.1 Analysis of gut contents.

4.1.3.1.1 Histological sections.

Lice collected at the fish farms and lice from the feeding experiments performed for this Chapter and also for Chapters 5 and 6 were fixed and processed for plastic and paraffin embedding and for SEM. Transverse and longitudinal sections of the embedded material were prepared and stained with selected general purpose stains. All methods were according to the techniques detailed in Chapter 2.

4.1.3.1.2 Gut contents by dissection

Lice were put on neutral red-stained fish as described previously and were allowed to stay on the fish for periods between 1 and 2 h. After this, the lice were collected from the fish and observed under the dissecting microscope to check if they had fed. If signs of food were observed, the louse was put in a small Petri dish, immersed in fixative and then the gut carefully dissected using opthalmological scissors and fine tweezers. The dissected gut was put in a new Petri dish with fresh fixative and opened carefully with the scissors. The food material that leaked out from the gut was collected with a fine-pointed Pasteur pipette and put in a vial for further processing for SEM.

4.1.3.2 Analysis of faecal pellets.

4.1.3.2.1 Collection and processing of faecal pellets.

4.1.3.2.1.1 From sea lice samplings.

Once lice had been transported to the laboratory and before sorting them as detailed in Chapter 2, the seawater from the bags where the lice had been transported was passed through a plankton net (60 μm mesh size), and the retained material concentrated in a small amount of seawater, about 25 ml, in a fix pot. After a gentle agitation, this sample then was distributed evenly in the wells of a 6-welled tissue culture plate and observed under the dissecting microscope. The faecal pellets were fished from the debris with a fine tip Pasteur pipette and placed in fixative for further processing for light, scanning and electron microscopy following the methods detailed in Chapter 2.

4.1.3.2.1.2 From lice feeding experimentally on fish.

Pellets also were collected from lice feeding on experimental fish and processed as above. The details of the procedure to collect the pellets are described in Chapter 6.

4.1.3.2.2 Histochemical staining.

Several histochemical techniques to detect mucopolysaccharides, proteins and lipids were used to analyse the pellet composition. Staining of pellet sections was done according to the histological techniques detailed in Chapter 2. In case of whole pellets, the staining steps were carried out in glass embryo dishes or 6-welled tissue culture plates. The stained pellets were then mounted in Pertex.

4.1.3.2.2.1 Chitin stain.

For the localisation of chitin or chitin precursors, colloidal gold labelled with the lectin wheat germ agglutinin (WGA) was used. WGA binds specifically to N-acetyl-D-glucosamine (NAGA) and has an extremely high affinity to chitin (Becker & Peters 1985) but it also binds to other compounds, such as glycoproteins which contain NAGA (Peters & Latka 1986) . Pre-treatment of the sections

with chitinase allows the specific identification of chitin as the substance binding WGA-gold in untreated sections. The method is as follows (Martin & Kirkham 1989):

WGA-gold (10 nm, Sigma L-1894) was diluted 1:4 with Tris buffered saline (TBS) containing 0.01 mM phosphate buffer, pH 7.0, 0.8% NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. Sections mounted on nickel grids were incubated for 5 min in 0.05% bovine serum albumin (BSA) in TBS followed by 60 min in WGA-gold before being rinsed in deionized water. and stained with uranyl acetate and lead citrate as detailed in Chapter 2.

Control sections were incubated for 5 min in chitinase from *Streptomyces* (Sigma, 6.5 units/ml 0.05 M Tris buffer, pH 8.0) prior to BSA.

4.1.4 Structure of the oral cone.

Material was fixed and embedded in LR White (processed as described in Chapter 2). Under the dissecting microscope, the block with the cephalothorax of the louse was marked for trimming, so that an exact longitudinal face of the mouth cone could be later trimmed first with a metal saw and later using an ultramicrotome. Serial 2 µm thick longitudinal sections of the mouth cone were cut with glass knives in the ultramicrotome and stained with toluidine blue. Drawings were made with the aid of a camera lucida.

For scanning electron microscopy, the mouth cones of several specimens were dissected out from the lice by grasping the oral cone from its base with fine pointed tweezers and pulling it away in a sharp anteriorly-directed movement. In this way, the complete mouth cone and the oesophagus along with its papilla were neatly removed. Once dissected they were fixed and processed as described in Chapter 2 and mounted on aluminium stubs with double-sided sticky tape in different positions. Some mouth cones mounted vertically were cut in half longitudinally with a sharp razor blade to separate labium and labrum.

4.2 RESULTS

4.2.1 Staining the fish skin to monitor the feeding activity of the lice.

Methylene blue did not give satisfactory results in staining the skin of the fish. Even after 4 hours of dye bath, the skin remained almost unstained, just with a hint of blue colour. This weakness of staining of the epithelial cells was not useful for the purpose of the experiment.

Neutral red was more efficient in staining the skin of the salmon. Even though the cornea of the eye was also stained red, the fish did not seem affected by this procedure. They continued to feed well with no apparent signs of stress. The skin remained stained for several days.

With this method it was very easy to evaluate if a louse was eating because under the dissecting microscope one could see small reddish or pink fragments inside the intestine which, under higher power, proved to be fragments of salmon skin.

During the observations detailed in section 4.2.2 it was noted that the gut of the lice feeding on a stained fish also acquired the red colour of the stain. This was not only because the gut contents were red, but the gut epithelial cells also took up the stain. When some of these lice with a stained gut were removed from the fish and held over for observation, the gut could remain stained for several days. By day seven, most of the dye had vanished.

4.2.2 Observations of movements of lice on fish.

Figure 4.1 shows a sample of the logged movements of a louse on a fish. The starting point where the louse was put is indicated by the number "0", on the left flank of the fish. During the first five minutes the louse moved about apparently searching for a place to settle, as the movements were almost continuous, following the line indicated in the figure. By 5 minutes the louse was in the dorsal region (1). During this time the fish was also moving actively, apparently irritated by the presence of the louse. By five minutes the fish had settled down. By 10 minutes the louse had moved to position "2", where it stayed for 12 minutes before going forwards to "3", where it stayed for 11 minutes. The louse was picked up from the fish at this time and observed under the

dissecting microscope. Inside the gut, red skin fragments were seen moving back and forth along the anterior and mid midgut, indicating that the louse had been eating during this time.

The same type of observations were carried out on 10 lice, placed individually on a fish, for periods ranging from 30 min to one hour. The behaviour was similar in six cases. Lice would move one or two centimetres every five or ten minutes, settling down on one place, with very little movements while on one spot. It was indicated that these six lice were in fact feeding, as there were variable amounts of skin fragments in the gut after being removed from the fish. In the remaining four cases the lice did not move at all during 30 minutes. When picked up from the fish and checked, these lice did not show any signs of having ingested any food. One of them was left on the fish for 20 hours. During this time it remained in the same place and there was no evidence that it had ingested any food at all.

Figure 4.2 shows the log of activities of a group of 18 lice on one salmon smolt during 12 days. In this case the fish was held in a bigger tank, therefore, a log sheet with the outline of the dorsal part of the fish was found more appropriate. Only the days where there was a significant change in the pattern are illustrated. This change in pattern usually corresponded to the detachment of some lice from the fish. Detached lice that were not found in the tank were accounted as dead, as the tank had an Eheim filter which would have sucked up a dead louse. In other instances, detached lice were found attached to the sides of the tank. These were picked up and observed under the dissecting microscope. In all cases except one, these detached lice showed no signs that they had been eating, at least recently, and possibly since the start of the experiment, although this fact could not be corroborated.

This particular set of observations was interesting because it was one of the very few cases where lice apparently fed on blood which was apparent due to the red colour of their guts. By the second day eight of the 18 lice had red guts. It is not known exactly the time they started to feed as this was recorded in the first observation of the second day, so they could have started at various times between the last observation on day one, around 7 PM, and 9 AM on the second day.

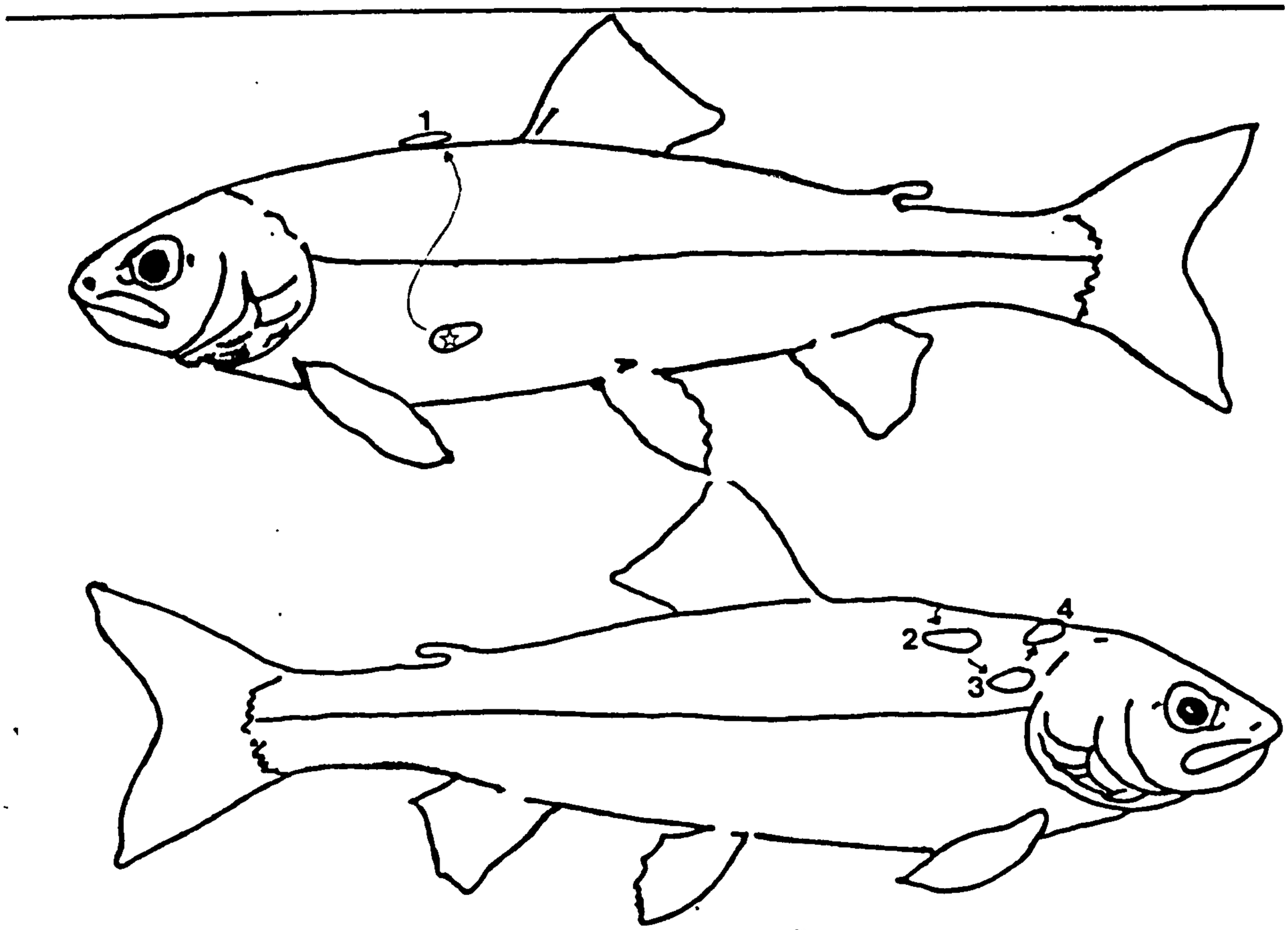


Figure 4.1 Sample of a form where the activity of lice on its host was recorded. See text for details..

One of them was taken from the fish, fixed and processed for further histological analysis.

By day three, four lice had detached from the fish. Probably amongst them were two of the four that were on the head the day before. Only six lice had red guts. It is not known if the two missing lice with red guts were among those that had detached.

By day five, two lice had disappeared. There was little change in the position of the remaining lice. One with a red gut apparently moved forward from behind the dorsal fin to just beside its anterior edge. Six again had red guts. It is not known for sure if these were the same six that had red guts the day before, although it seems likely.

By day seven, two lice had disappeared and now seven had red guts. Apparently one louse from the posterior part of the fish had started blood feeding, as in the anterior part there was still the same number of lice with and without red guts. (four and two respectively).

By day 12, when the observations were finished, only five lice remained, four of them with red guts. All lice were examined under the dissecting microscope. All lice had skin fragments in mid or posterior midgut. All lice were fixed and processed for histology. Red blood cells were not found in any of the histological sections of any louse.

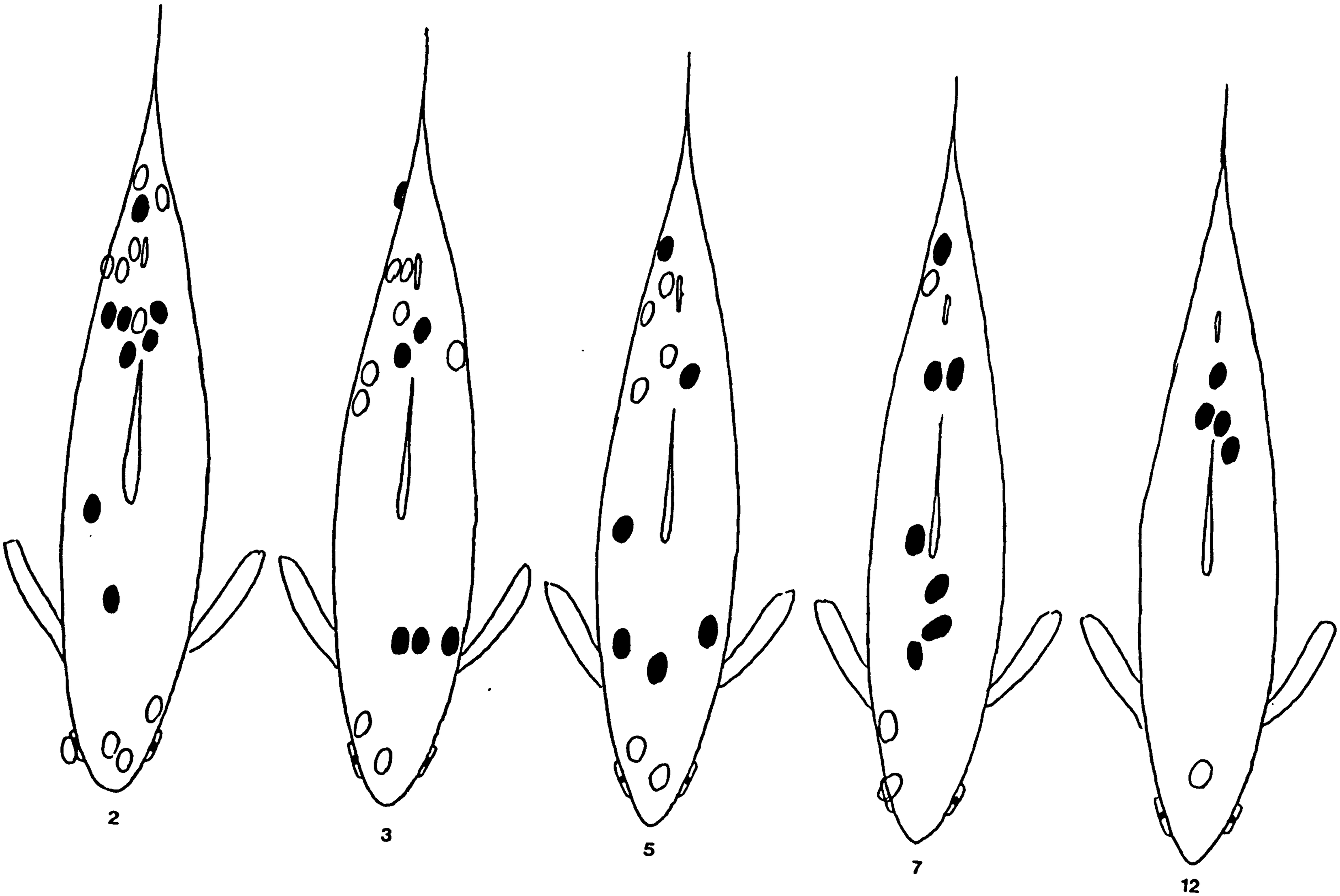
During the pellet production experiments (see Chapter 6), there were invariably scales from the fish in the sediment from the aquarium. On some occasions, one could appreciate under the dissecting microscope that a particular scale had a set of lines oriented in a specific pattern, like "V"s. These scales were processed for SEM. Plate 4.1 shows that these marks, because of their shape and regular pattern, could have been left by a louse. Note for example the three curved lines. Their size correspond to the margin of the cephalothorax of an adult female louse. At higher magnification, a mark left probably by the postantennary process or by the second antenna can be appreciated (Plate 4.2).

Lice spent most of the time on the dorsal and lateral parts of the fish. They never went for the ventral part, or if they did, they did not stay long.

From the observations of the lice on the fish, one can assume that although they move about, their movements are restricted within a certain area. They are not browsing over the entire surface of the fish. Their movements can also be conditioned by the number of lice present on a fish. During observations, just after putting lice on a fish, there is a period of settlement where lice search for a suitable area, and during this movement, when one louse touched another one that had settled, the other one would start moving in response to the contact. Once they had dispersed the movements would decrease as they tended to settle down.

Lice that were not feeding while on the fish usually tended to remain in one place for a longer time compared to those feeding. It is likely that those feeding probably have to move about to find new areas of skin to feed on.

Figure 4.2 Log of activity of lice on a salmon smolt during 12 days. The initial infection was 18 lice. Symbols indicate the position of lice at different times during the 12 days. Only selected days are shown, mainly when significant changes in the position of the lice occurred. Symbols filled in black indicate that that particular louse had a red gut apparently due to blood feeding.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3
5
7
12

4.2.3 Gut contents analysis.

There was no clear evidence that the lice were feeding solely on mucus or that the main food taken was mucus. In histological sections there was again no evidence of mucus in the lumen of the digestive tract nor did all the guts examined contained skin fragments. When there were gut contents, it was usually chunks of skin epithelium three or four layers thick (Plate 4.3). These skin fragments were found in variable states of digestion, but the more common occurrence was skin with few signs of having been digested.

There seems to be a satiation signal, otherwise we would find lice with skin fragments in the gut most of the time, which was not the case. Usually, lice with a pellet in the mid-post midgut did not have loose skin fragments moving about in the lumen. Also, in lice which did have loose skin fragments moving in the lumen, the pellet had not been formed yet or some signs of packing of skin fragments in the posterior midgut had started to form, but they were not yet well compacted as the case of a well formed pellet.

Very seldom there were red blood cells from the host in the sections.

In a very few cases lice were seen feeding on blood in experimental fish. It was a rare and unpredictable event. The findings about lice feeding on blood are described later in this chapter (see section 4.2.3.2). Frequently the gut epithelium of the louse was stained red due to the neutral red dye in the skin of the fish. This was evident only when the lice were observed under the dissecting microscope. The intensity of the staining varied from louse to louse from no staining to a bright red colour. The intensity of this staining did not seem to be correlated with the amount of food that the louse had in the gut, as there were several cases of lice with no staining at all and a gut full of pinkish epithelial cells. The gut contents observed were then of two kinds: epithelial cells from the skin and blood. Both will be dealt with separately.

4.2.3.1 Epithelial cells.

Most of the gut contents of *L. salmonis* observed after dissection of the digestive tract were small loose fragments of the host epithelium. These could be clearly identified because of their red colour due to the neutral red. These fragments usually were quite constant in size. Some of these skin fragments were processed for scanning electron microscopy to obtain more information about their shape and nature.

Plate 4.4 shows 17 fragments of skin recovered from the gut of a louse that had been feeding on the salmon for an hour. These were half of the fragments that this louse had in the entire gut. On closer examination (Plate 4.5) it can be appreciated that the skin fragments are elongated blocks of quite regular dimensions. It is also worthy of note that all the fragments were curved, the concave part being the outer epithelium surface.

Table 4.1 shows the results of the measurements of the individual fragments and an estimation of their area and volume and the total area and volume of the 17 fragments. The dimensions of the skin fragments were estimated from SEM photographs.

The total area of all the chunks was 0.1595 mm^2. This is equivalent to a square of skin of 0.34 by 0.34 mm. On the same line, the volume would be equivalent to a cube of 0.157 mm dimension. These values correspond to half of the total of skin fragments eaten by this particular louse (17). If we consider these estimates as representative for the other half that was not measured, then this louse eat, within an hour, the equivalent of a piece of skin of 0.56 by 0.56 mm square or a cube of skin of 0.198 mm by a side or a long strip of skin of 7.7 mm in length.

We do not know if the louse had satiated its hunger or if it was going to continue eating. This estimate provides only an estimate of the speed at which the louse might ingest a certain volume of food. It also tells us the area of fish skin affected.

Another more accurate estimate could be made using the volume of the faecal pellets. The volume of cells of a faecal pellet could be used to calculate the area of fish epithelium affected because we know now that the louse is taking chunks of skin of a relatively constant thickness (of 24.6 µm on average). Then, if a record of the production of faecal pellets is kept against time and the volume

of them calculated, one could arrive at a close estimate of the feeding rate and the area affected to accomplish it.

Lets take for example an average pellet. For practical purposes we can assume that the shape of the pellet is a cylinder, in this case with a radius of 45 μm and a length of 781 μm. The volume ($\pi \times r^2 \times$ length) of this pellet would be approximately of 4,968,507 μm^3, or 0,005 mm^3. This volume corresponds to 42 strips of skin (average length, width and thickness of each one being 226.5, 41.2 and 24.6 μm respectively, see Table 4.1) which together would make a strip of 9.5 mm long. This in turn can be expressed on surface figures, 0.39 mm^2 (equivalent to a square of 0.62 mm sides). But these numbers do not represent the total amount of skin that the louse eats, as we do not know how much of what entered the gut was digested. But what can be said is that the particular louse that voided these pellets ate *at least* this amount of skin.

With appropriate lighting (two fibre optic lights situated at the both sides of the louse and no transmitted light), one could also observe a certain iridescence within the gut lumen, presumably from the iridiophores of the salmon skin. Another common finding in the gut contents, in histological sections, was melanin granules, presumably from the melanophores of the salmon skin.

Table 4.1 Measurements of the fragments of salmon skin retrieved from the gut of a sea louse.

Skin piece	Length μm	Width μm	Thick μm	Area μm^2	Area mm^2	Volume μm^3	Volume mm^3
1	266.7	42.6	31.1	11361.4	0.01136	353340.2	0.000353
2	366.7	41.3	20.0	15145.2	0.01515	302796.3	0.000302
3	200.0	40.0	28.9	8001.0	0.00800	231057.4	0.000231
4	200.0	40.0	26.7	8001.0	0.00800	213283.8	0.000213
5	300.0	42.6	22.2	12781.6	0.01278	283934.1	0.000283
6	150.0	40.0	28.9	6000.7	0.00600	173293.1	0.000173
7	200.0	41.3	26.7	8261.0	0.00826	220215.5	0.000220
8	200.0	42.6	26.7	8521.1	0.00852	227147.2	0.000227
9	383.4	44.0	20.0	16868.8	0.01687	337255.0	0.000337
10	166.7	40.0	20.0	6667.5	0.00667	133302.4	0.000133
11	166.7	41.3	24.4	6884.2	0.00688	168220.2	0.000168
12	266.7	42.6	24.4	11361.4	0.01136	277624.4	0.000277
13	250.0	40.0	28.9	10001.3	0.01000	288821.8	0.000288
14	166.7	40.0	26.7	6667.5	0.00667	177736.5	0.000177
15	133.4	42.6	20.0	5680.7	0.00568	113573.6	0.000113
16	266.7	40.0	22.2	10668.0	0.01067	236982.0	0.000237
17	166.7	40.0	20.0	6667.5	0.00667	133302.4	0.000133
SUM	3850.5	700.9	417.6	159539.9	0.15954	3871886.0	0.003872
MEAN	226.5	41.2	24.6	9384.7	0.00938	227758.0	0.000228
STD.	71.4	1.3	3.7	3162.1	0.00316	69882.4	0.000070

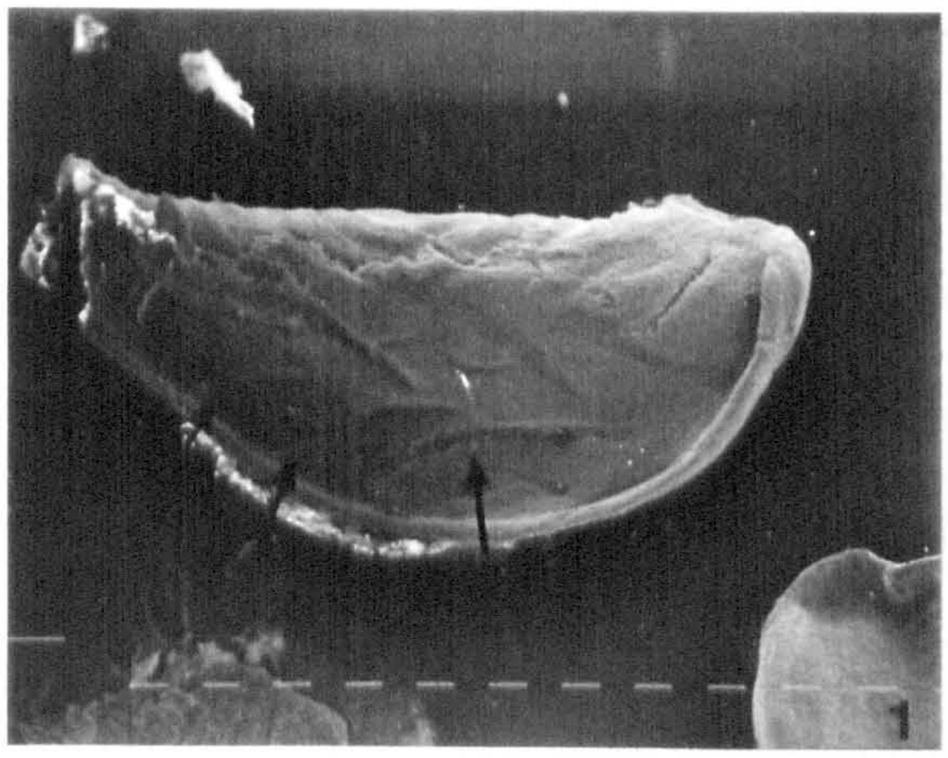
Plate 4.1 Salmon scale where imprints of the louse cephalothorax can be seen. There are three imprints in succession (arrows) apparently made by the antero-lateral margin of the cephalothorax of a louse. SEM 40X.

Plate 4.2 Zoom of an area of the scale depicted in Plate 4.1 . The long curved lines (arrows) could be the result of the pressure of the antero-lateral margins of the cephalothorax. Note a sharper scar (long arrow) caused probably by the second antenna of the louse. SEM 160X..

Plate 4.3 TS through the posterior midgut of a louse with several pieces of salmon skin in the lumen (L). Note that the epidermal cells are almost intact.. LMR. Toluidine blue. Scale bar 20 μm..

Plate 4.4 Fragments of salmon skin epithelium recovered from the gut of a louse. Note the regular shape and size of the chunks. SEM 40X.

Plate 4.5 Top (micrograph on the right) and underside (on the left) view of a salmon skin fragment found in the gut of a sea louse. Note the integrity of the tissue and the cells. SEM 640X.



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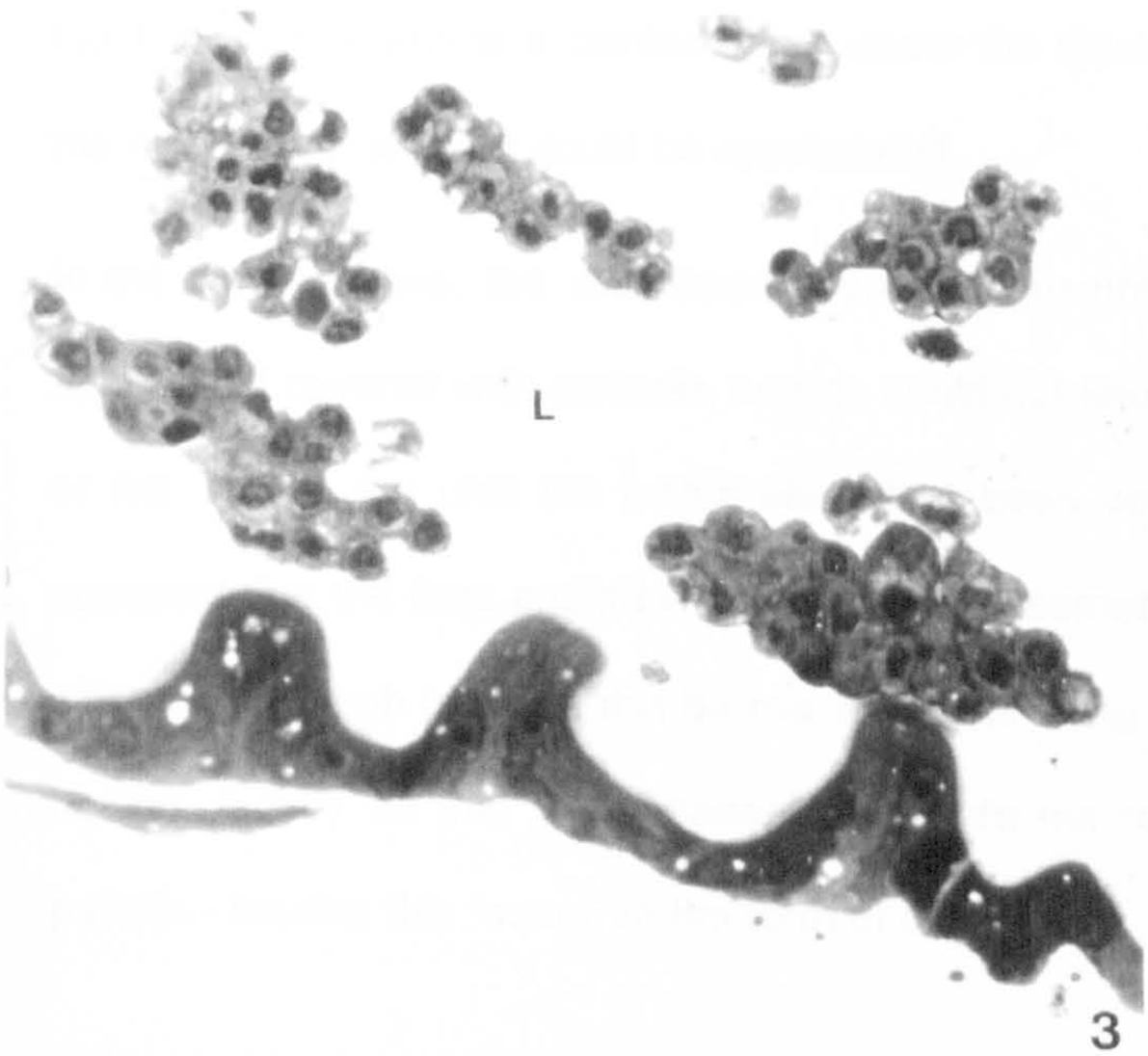

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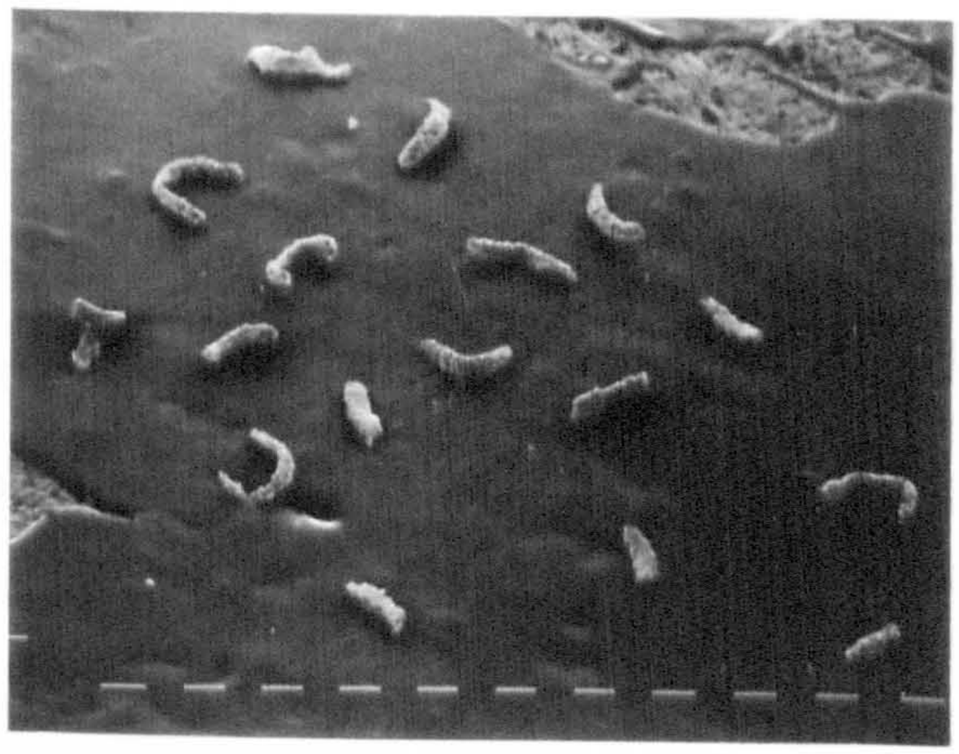

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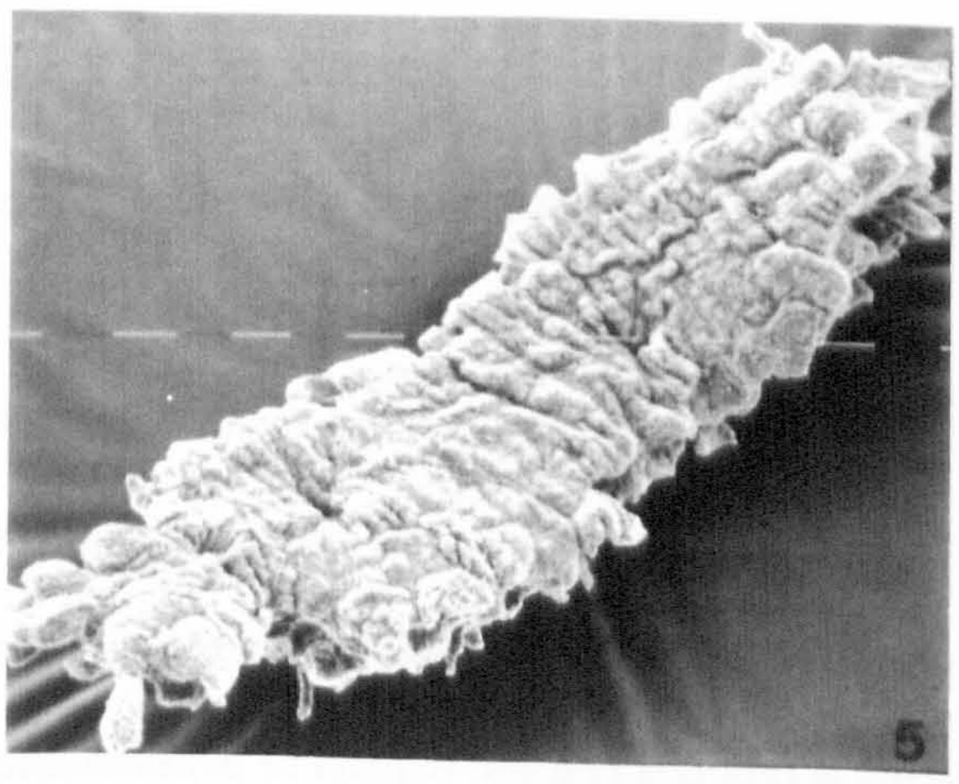

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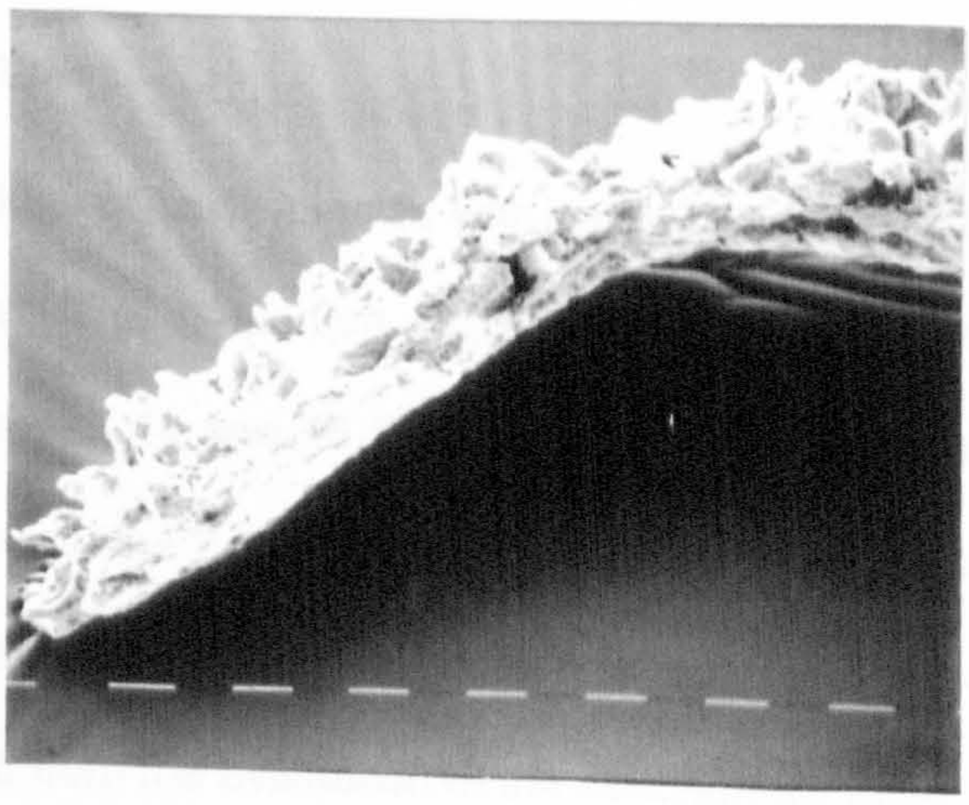


Some fish that were used for this and other experiments were anaesthetised and the skin scanned under the dissecting microscope to see if there were any signs left of the activity of the lice. A common finding in the areas where the lice had been for longer periods were light lines of about 5-10 mm long. At higher power, the lines seemed to be grooves left in the uppermost part of the dermal layer where the melanophores are located - just immediately below the basal membrane of the skin epithelium. Two pieces of this skin with lines were cut from a sacrificed fish and processed for plastic embedding. Transverse sections of the skin were cut and stained with toluidine blue and H&E. No sign of damage was evident in the epidermal or dermal layer. But when the same plastic block was observed at a slanted angle under the dissecting microscope, some grooves carved in the melanophores layer could be appreciated.

In the skin sections, the mentioned grooves were not seen, probably because there are natural spaces not covered with melanin, so one could not know that the gap observed was from a groove or not. It was not until the whole block had been observed at an angle that evidence became apparent that the lines could be areas where presumably the lice had been feeding, lifting strips of skin deep enough to reach this dermal layer and lifting the melanophores with it. After the skin had healed, it may be that the melanophores were not replaced - at least not following the original pattern - leaving this "scars" in the form of clear lines.

4.2.3.2 Blood.

4.2.3.2.1 Percentage of lice with red gut in samples..

Table 4.2 shows a contingency table of the proportions of adult females with and without red guts in different samples at different times of the year. The proportion of lice with red guts was remarkably close between samples, fluctuating between 14 and 16 %. The last sample's results were quite unusual. As will be commented upon in section 4.2.4.1.2 , the physiological condition of the lice in this sample was affected in some way. Therefore, this sample is not considered representative of normal feeding lice and is excluded from the evaluation of the contingency table.

The chi-square analysis of these proportions demonstrate that this 16% is highly significant ($p<0.001$)

Table 4.2 Contingency table of the number of lice with and without red guts in several field collections. † indicates the sample that was not included in the chi-square analysis. See text for details.

Date	Lice without red guts	Lice with red guts	TOTALS
11/1/94	218	38	256
	(85.2)	(14.8)	
18/5/94	336	57	393
	(83.9)	(16.1)	
09/8/95	267	49	316
	(84.5)	(15.5)	
01/11/95	292	56	348
	(83.9)	(16.1)	
21/2/96	321	61	382
	(84.0)	(16.0)	
11/1/96 †	294	2	296
	(99.3)	(0.7)	
TOTALS	1728	263	1991

4.2.3.2.2 Serial sectioning of red-gutted lice.

The absence of red blood cells in sections of red gutted lice poses an intriguing question. To discard the possibility that we were not finding red blood cells because of a small number of them in the gut, paraffin wax serial transverse longitudinal sections of 22 bright or dark red-gutted lice were produced. Care was taken to keep the gut contents of lice by tying their anus (as described in Chapter 2) immediately after being lifted from the fish and before fixation.

In none of these 22 lice was there any evidence of red blood cells OR skin fragments. Analysis of sections from many other red-gutted lice from other samples gave similar results, except for only two cases. In these, red blood cells were clearly seen amongst skin fragments. These red blood cells seemed intact, with no indication of having been digested at all (Plate 4.6).

4.2.4 Faecal pellets.

Another way of assessing what is entering the gut and what processes follow is the analysis of the faecal pellets. This can give much information about the morphological and physiological state of

the digestive system, as well as information about the feeding habits. With this in mind, a closer look at the faecal pellets of the louse was carried out.

4.2.4.1 Types.

It was found that lice produce two types of pellets. A "ribbon" or "tape" flattened, gelatinous pellet and a solid, cylindrical or spindle shaped pellet. The latter could be divided in two sorts, depending on the colour: A "clear" pellet and a "dark" pellet. A description of them follows.

4.2.4.1.1 Clear and dark pellets.

Clear and dark pellets (Plate 4.17) were the most common types voided when the lice were feeding on the fish. Plate 4.7 and Plate 4.8 show the anterior and posterior end of one clear pellet. Their general shape is relatively constant, with a rounded end which is the one that is voided first, and a tapered end on the other side. Although they were not measured systematically, their length seemed to be variable, apparently according to the size of the louse voiding it. The pellets are covered completely by a very thin peritrophic envelope (Plate 4.9). Under SEM, below the membrane the skin fragments of the salmon skin can be seen tightly packed (Plate 4.10). When the peritrophic envelope is broken, the epithelial cells of the salmon are clearly seen. Under the dissecting microscope, they look semi-translucent, hence the name of clear pellet. On a louse that had fed on a salmon which skin was stained with neutral red, the pellet showed a translucent pinkish colour. Under the light microscope, the packed skin cells seem to be surrounded by a clear matrix. This matrix was more evident in areas of the pellet where there were few or no skin cells and, at higher magnification, loose melanin granules can be seen (Plate 4.11). When stained using the PAS-alcian blue pH 2.5 technique, only the mucous cells of the salmon skin showed positive for PAS, and a very slight reaction of the pellet matrix for alcian blue (Plate 4.12).

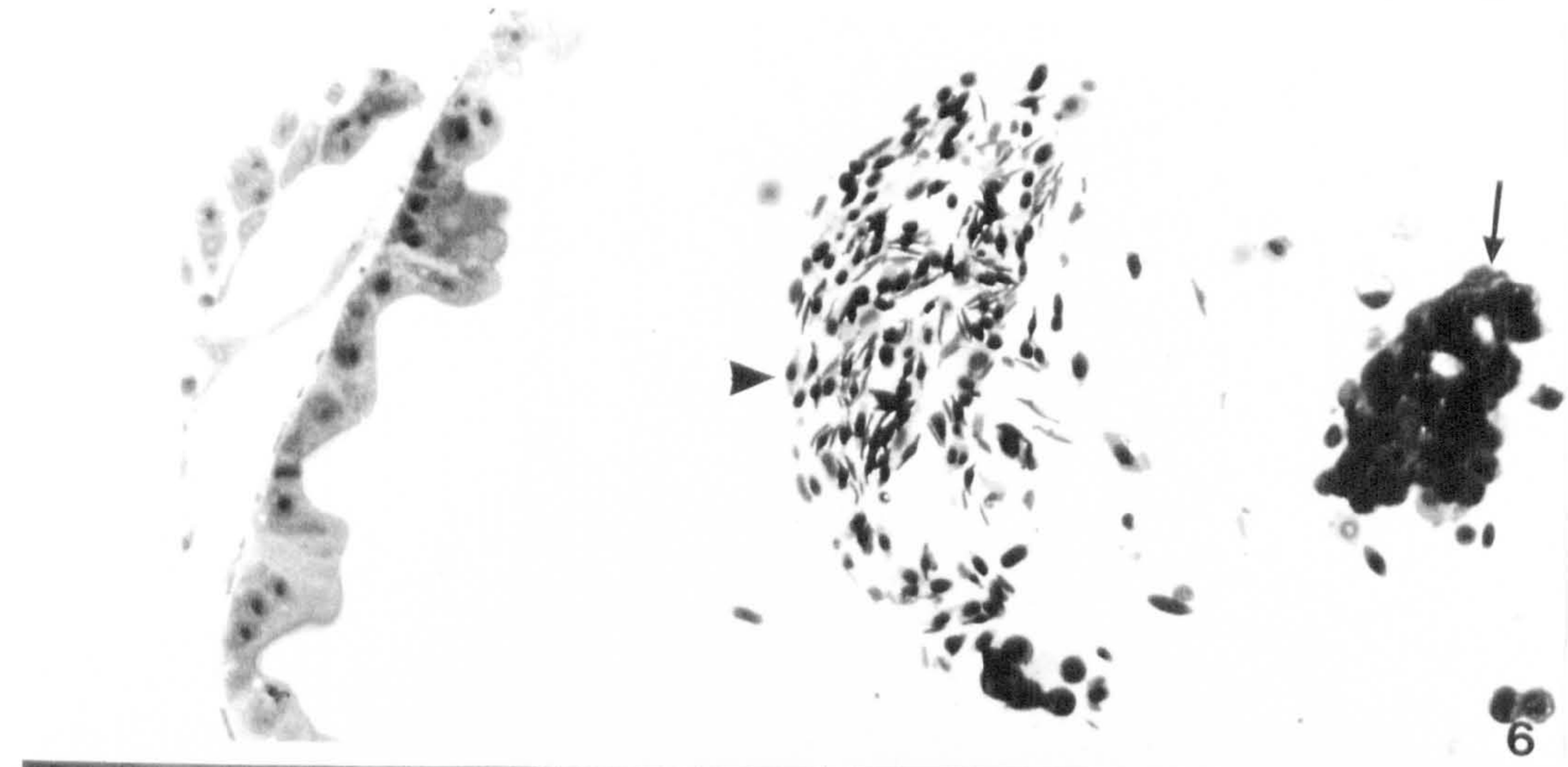
Plate 4.6 Red blood cells (arrow head) and salmon skin fragments (arrow) in the lumen of the posterior midgut of a red gutted louse. LMR. Toluidine blue. Scale bar 50 μm..

Plate 4.7 Posterior part of a clear pellet. Note the thin peritrophic envelope that surrounds the epithelial cells of the salmon skin, which are tightly packed. Note also the flattened end of the pellet (at the top left of the micrograph), sealed by the peritrophic envelope. This part is the last to be voided. SEM 320X.

Plate 4.8 Anterior part of the same pellet shown on Plate 4.7 . Note the rounded end, which is the first to appear when the louse is defaecating. Some individual blocks of fish skin can be recognised below the peritrophic envelope (arrows). SEM 160X.

Plate 4.9 Clear pellet in which the peritrophic envelope has been partially removed to show the epithelial cells of the salmon inside it. Note the extremely thin peritrophic envelope (arrow). SEM 2,500X.

Plate 4.10 Stereo SEM micrograph of a faecal pellet split to show a strip of salmon skin that was packed inside the peritrophic envelope. 640X.



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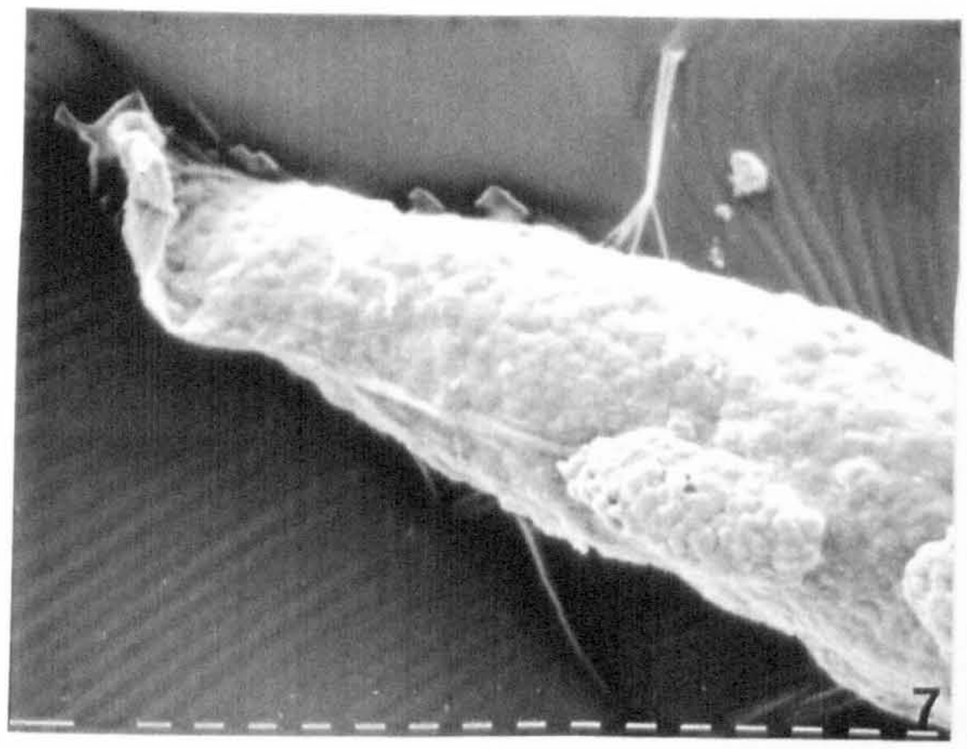

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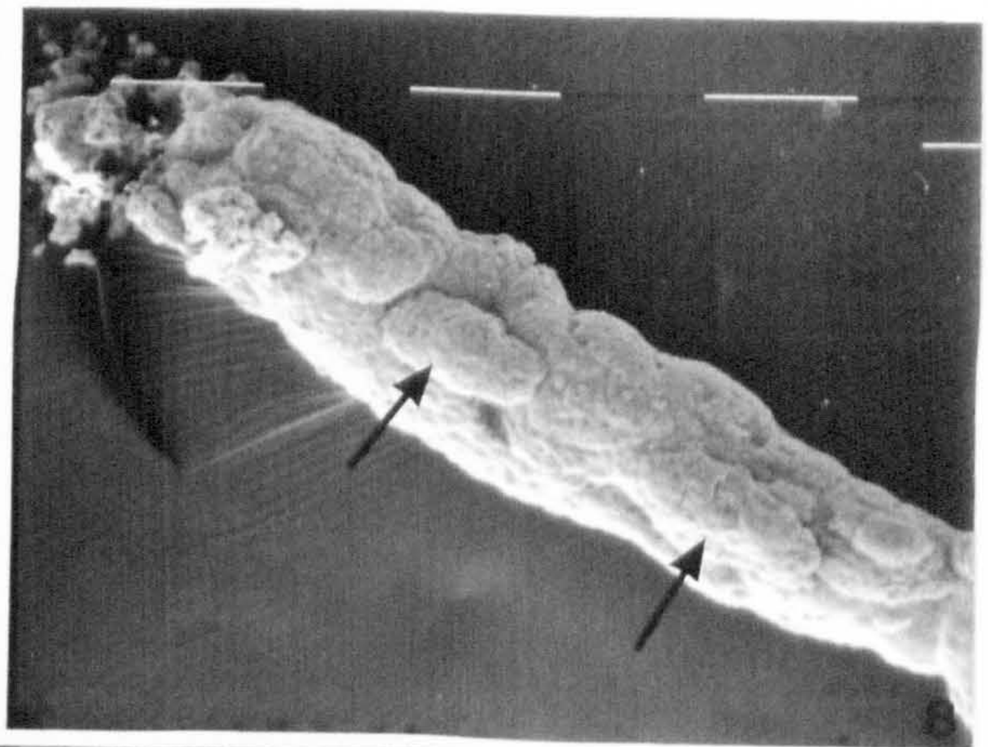

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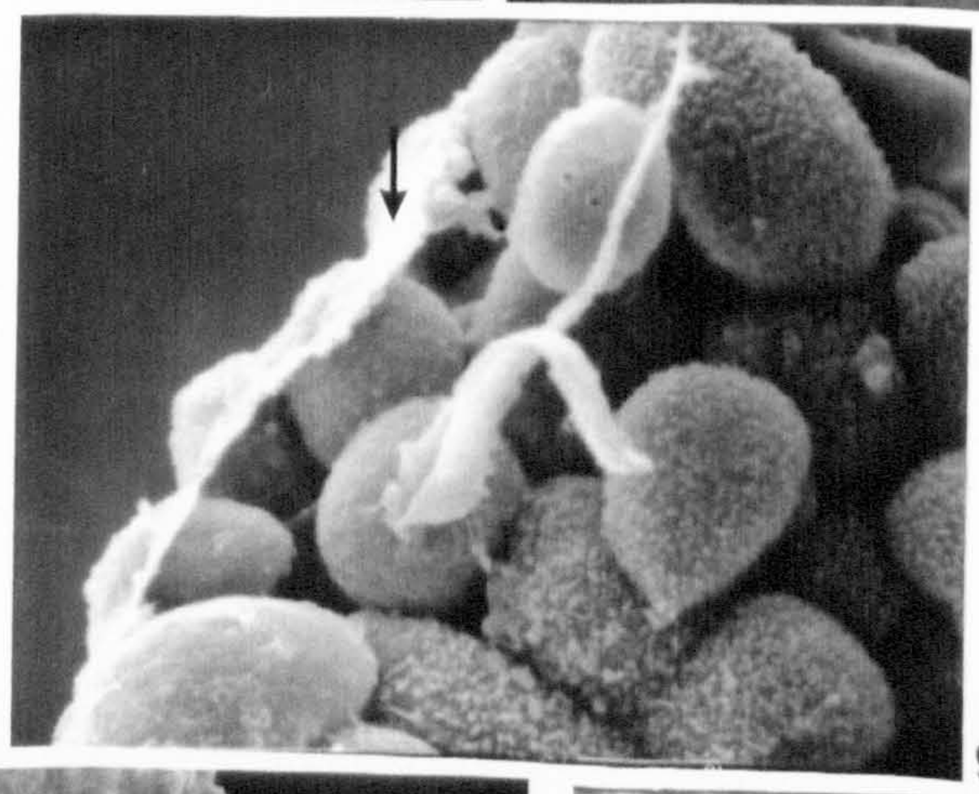

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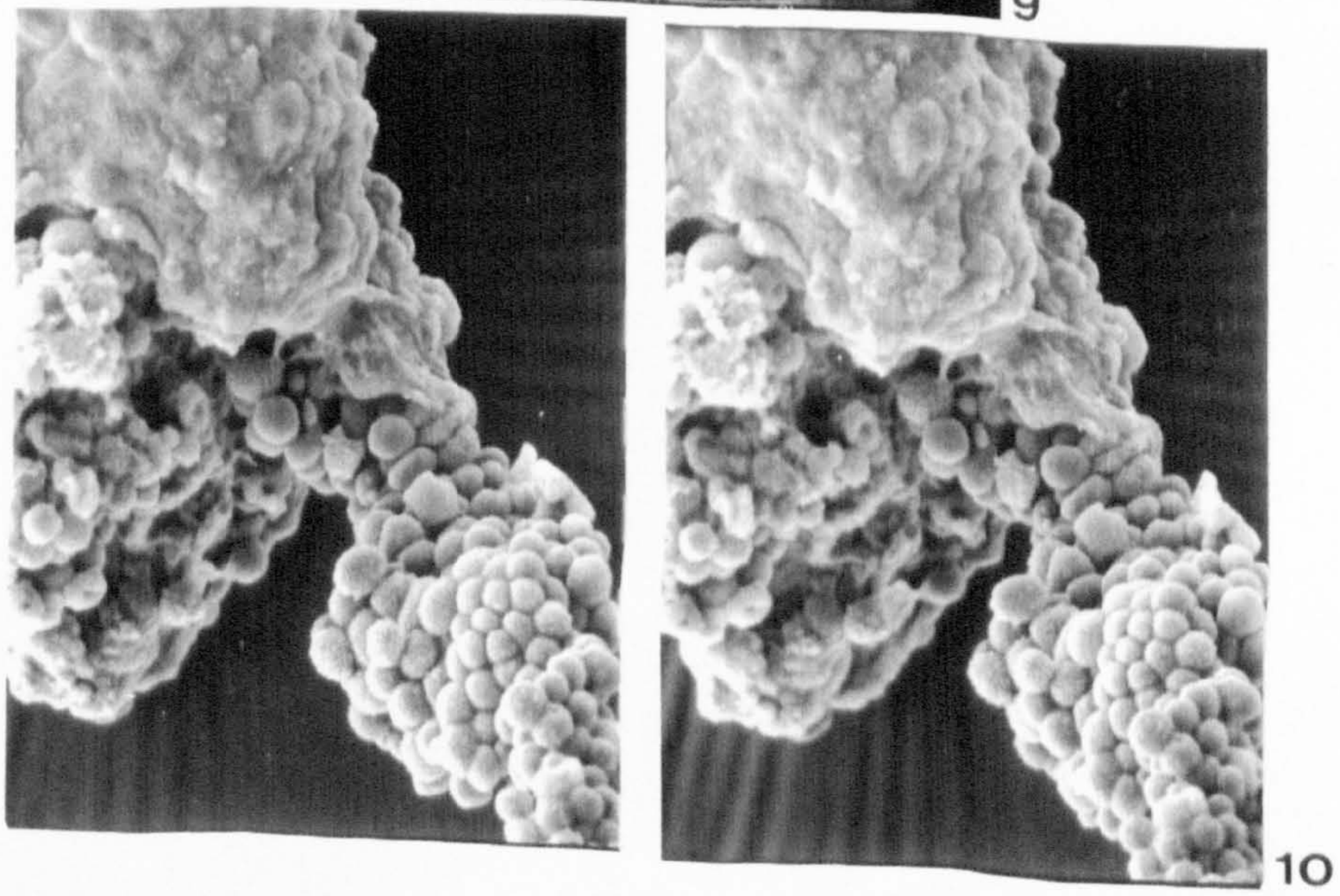

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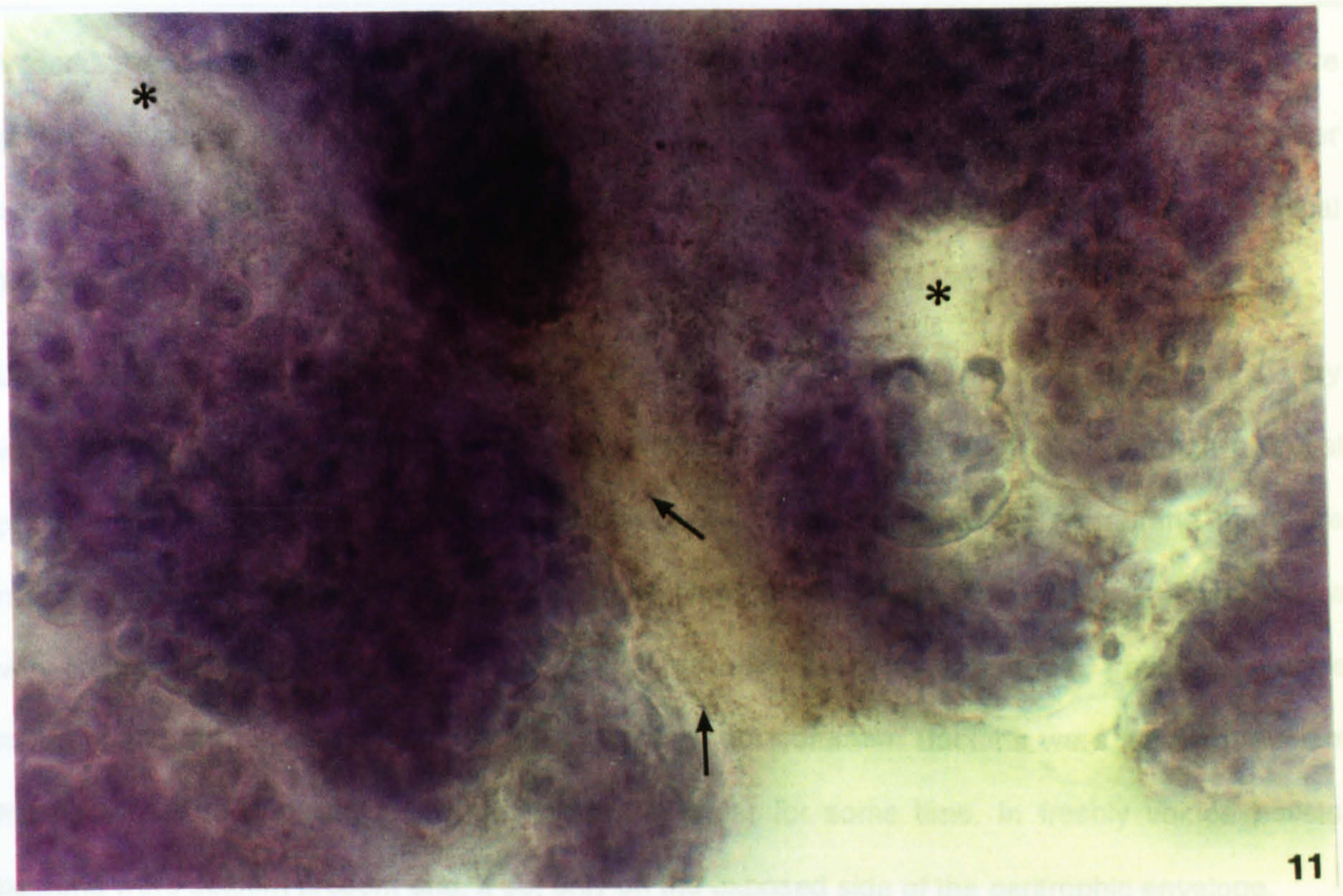
Plate 4.11 Clear pellet stained with haematoxylin. Note the nuclei of the epithelial cells of the salmon and the matrix of the pellet (asterisks) in between fragments of skin. The small granules in the matrix (arrows) are melanin granules. Scale bar 20 μm.

Plate 4.12 Clear pellet stained with PAS-alcian blue. There is a very faint reaction for alcian blue in the matrix of the pellet. The goblet cells of the salmon skin react strongly for PAS. Scale bar 40 mm.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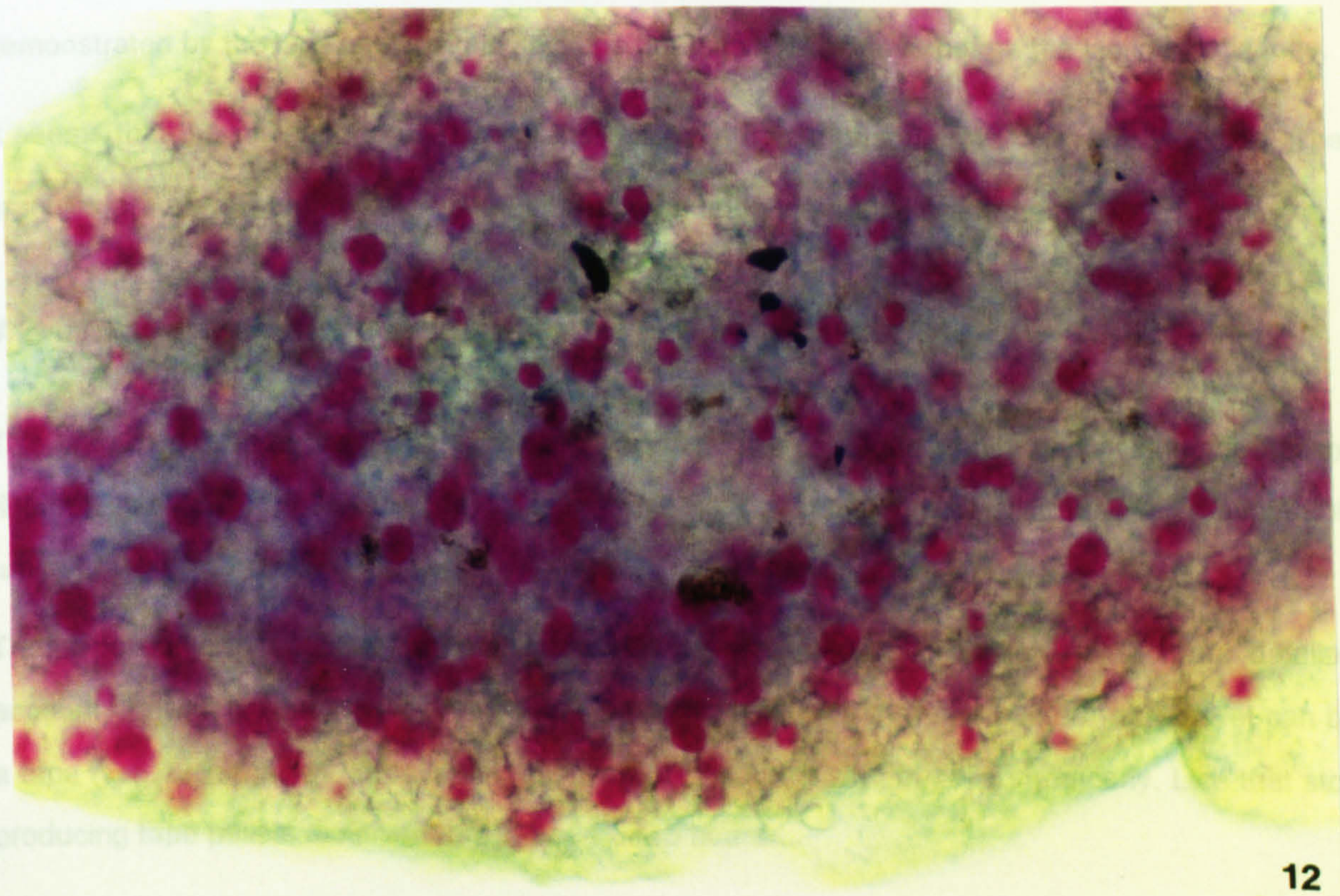
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A "dark" pellet is similar to a clear one except that it has dark inclusions. These dark pellets are quite conspicuous and can even be seen inside a louse with the naked eye. The dark matter comes from the accumulation of melanin granules, tightly packed and always within the faecal pellet matrix.

Under TEM, the nature of the peritrophic envelope can be appreciated. Its thickness is very variable: from 1.5 μm to up to 14 μm. Plate 4.13 shows a transverse section through a faecal pellet. Sometimes several layers can be appreciated. In the matrix of the membrane there are melanin granules, membrane debris and collagen fibres (Plate 4.14), although the latter were not a very common finding. Other types of very fine fibres were also found (Plate 4.15) but their precise nature could not be determined. These again were not common. Bacteria were also a prevalent finding in pellets that had been standing in the water for some time. In freshly voided pellets, bacteria were seldom present and, if so, only on the exposed side of the peritrophic envelope.

Incubation of pellet ultrathin sections with WGA gold-labelled lectin resulted in a strong binding of the lectin to the peritrophic envelope (Plate 4.16), indicating the presence of chitin, as demonstrated by the lack of binding in sections treated with chitinase before the lectin incubations.

It seems that the melanophores release their melanin contents soon after they are ingested. The granules are always found either loose or trapped among the faecal pellet matrix.

4.2.4.1.2 Tape pellets.

This type of pellet, as the name indicates, take the form of a long fragile and gelatinous tape or ribbon (Plate 4.18) 25-35 μm wide. Its length was very variable. The smaller ones were about 600 μm but they could reach 5 mm in some cases.

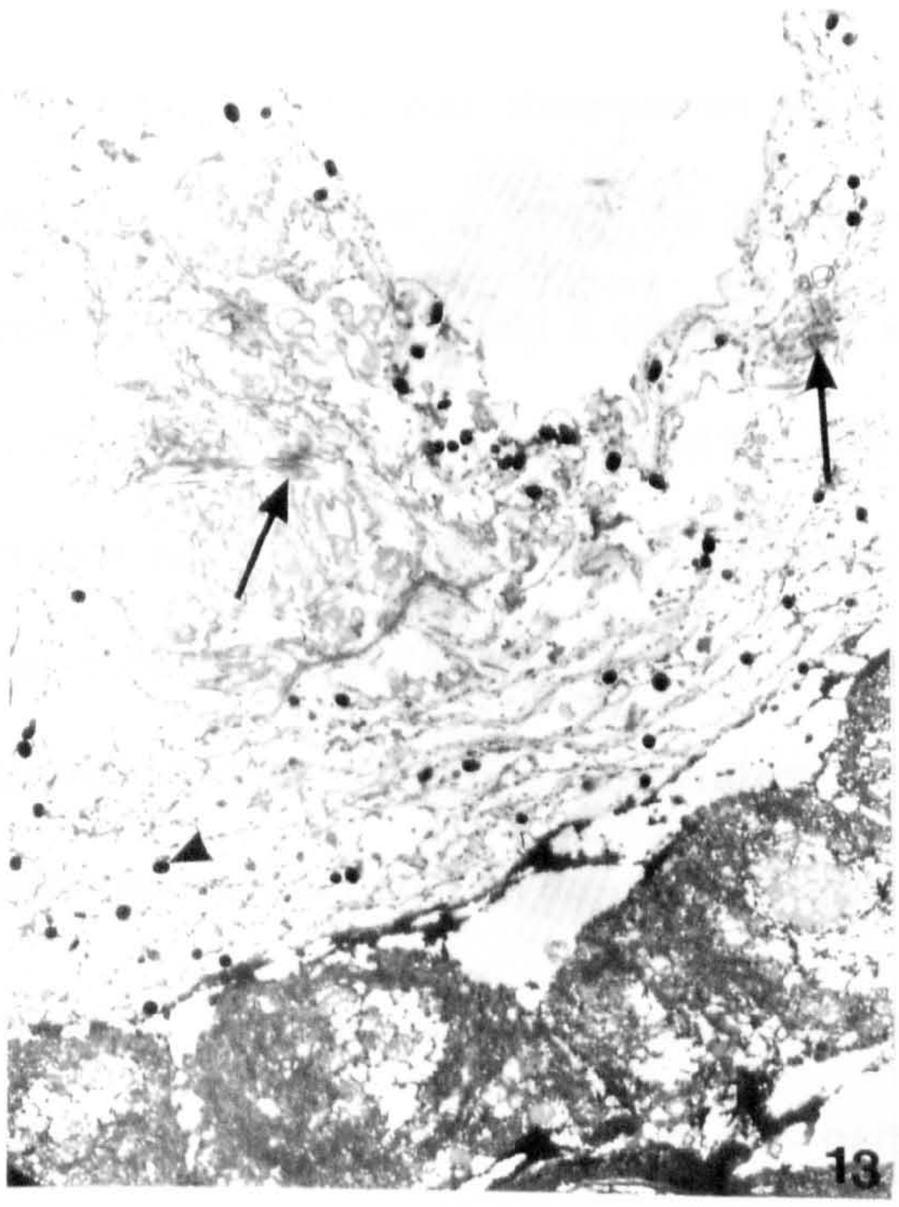
These pellets are produced when the louse is not eating (see Chapter 6 for a more detailed account of pellet production), although sometimes the posterior part of a clear or dark pellet can be a tape type. Lice that are kept in starvation will produce this kind of pellets regularly. Lice that stop producing tape pellets die invariably after 24 or 48 hours.

Plate 4.13 Peritrophic envelope of a louse clear faecal pellet. The remains of salmon skin cells lie below it. Collagen fibres can be seen (arrows, see magnification of them in the next plate), and melanin granules (arrow head). TEM 2800X.

Plate 4.14 Collagen fibres found in the peritrophic envelope matrix of some faecal pellets. These collagen fibres are likely to be of host origin rather than normal constituents of the peritrophic envelope, as the majority of peritrophic membranes did not show them. TEM 43,000X

Plate 4.15 Fine fibres found in the matrix of the peritrophic envelope of a clear faecal pellet. The nature and origin of these fibres could not be determined. TEM 75,000X.

Plate 4.16 Binding of gold-labelled WGA lectin to the peritrophic envelope of a faecal pellet. The gold particles can be seen within the peritrophic envelope (pe), but not within the contents of the pellet. TEM 22,000X.



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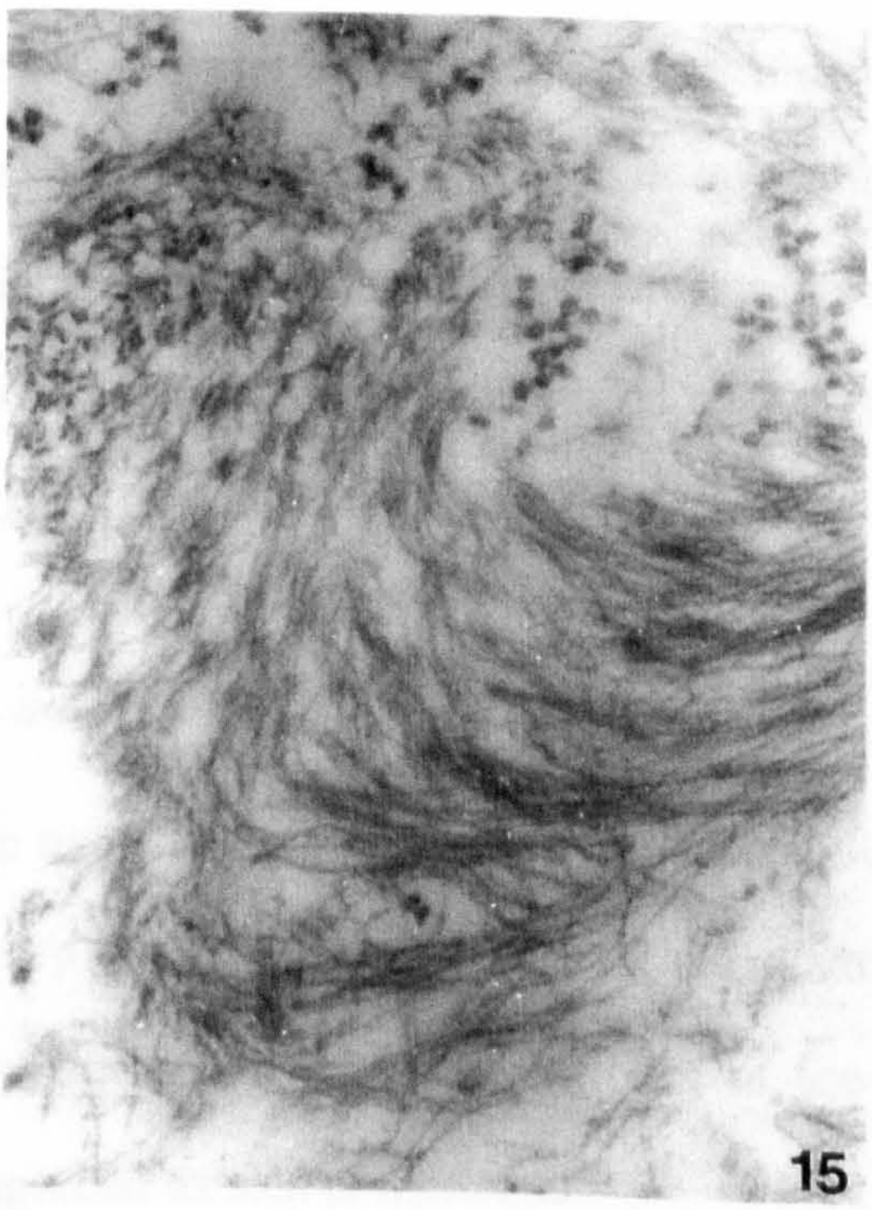

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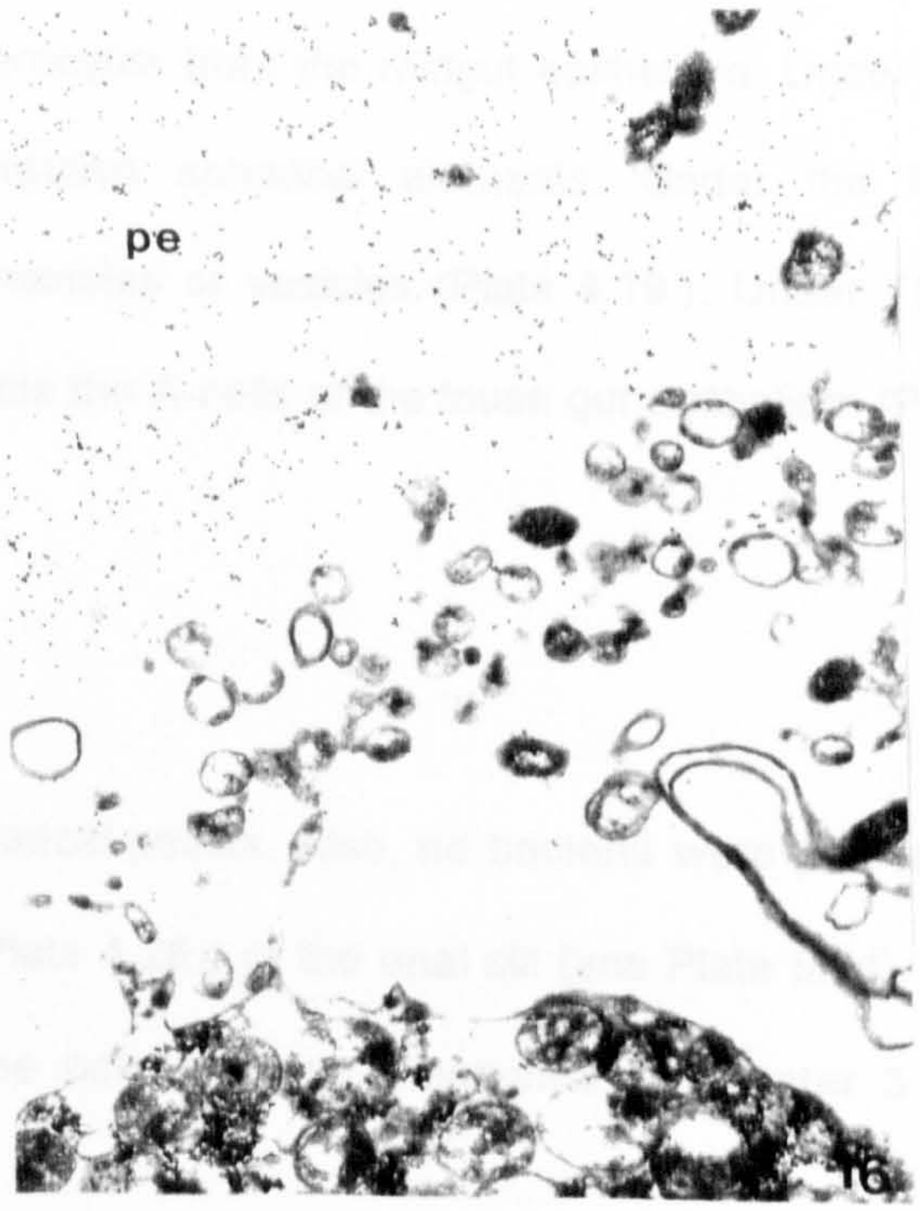

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One unusual event was observed on the 11/Jan/96 during a collection of lice. After arriving at the laboratory, the water in which the lice were transported was passed through a plankton net to collect faecal pellets. Only 8 tape pellets were found. No clear or dark ones, even though the number of lice collected surpassed the 300 individuals. Usually in a sample of this kind one could collect hundreds of tape pellets, and many clear and dark ones. The temperature of the water at the collection site was 4°C and the salinity was 20‰. After being transferred to full seawater strength (33‰) and to the temperature of the cold room (10°C), lice began to void tape pellets again after a few hours. In this sample, also only 2 lice with pinkish guts were found.

In other "normal" samples around the same season there were similarly days with low temperatures approaching that of this particular sample, but the salinity was not as low, lying between 25 and 30‰. On the other hand, there were samples at other times of the year where the salinity was low but the temperatures were higher. In none of these cases was there such an absence of production of tape pellets and there were always lice with red guts.

This tape pellet is made almost exclusively of enterocytes from the midgut epithelium. Under the dissecting microscope one can see many refractive spherical elements. Under the light microscope, these refractive elements resemble vacuoles or vesicles (Plate 4.19). Under TEM, these vesicles appear to belong to cells that resemble the A-cells of the louse gut epithelium (Plate 4.20).

4.2.4.2 Bacteria

No bacteria were present on any recently voided faecal pellets. Also, no bacteria were present in any part of the gut of lice, except the mouth (see Plate 4.26) or the anal slit (see Plate 5.24). The only exception to this is the case of lice with the pale gut type mentioned in Chapter 3 and regarded as sick or moribund.

Plate 4.17 Clear and dark pellets as seen through the dissecting microscope. The epithelial cells of the salmon skin can be appreciated tightly packed inside the pellet. Scale bar 1 mm.

Plate 4.18 A tape pellet being voided by an adult female as seen through the dissecting microscope. The length of this pellet at the end of the defaecation process was about the same length as the length of the louse. Scale bar 1 m.

Plate 4.19 Tape pellet stained with PAS-alcian blue. Some vacuoles are PAS positive but the majority do not react. The matrix of the pellets reacts perceivably to alcian blue and which probably is due to the brush border of the enterocytes packed in the pellet. Scale bar 30 mm.

Plate 4.20 Cell embedded in the matrix of a tape pellet. Because of the condensed cytoplasm and the large vacuole it is possible that this is an A-cell sloughed from the louse gut epithelium. TEM 4,300X..

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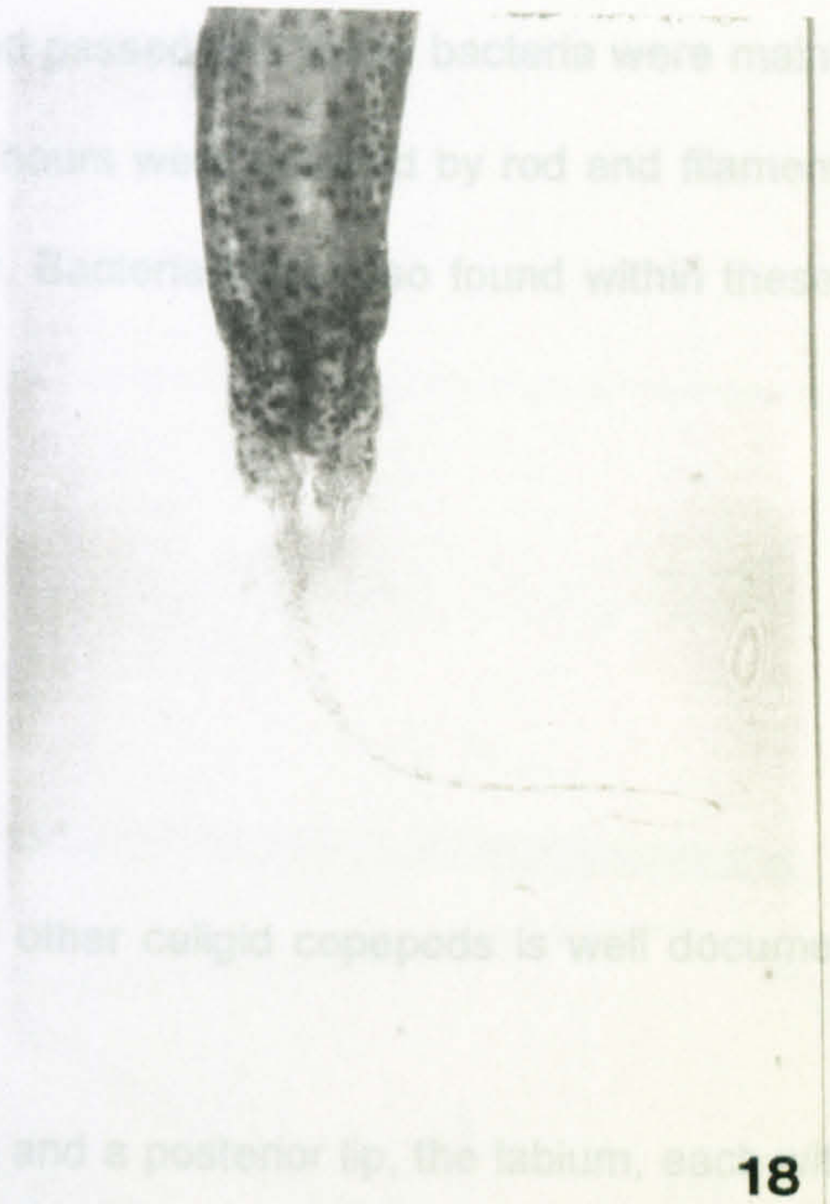

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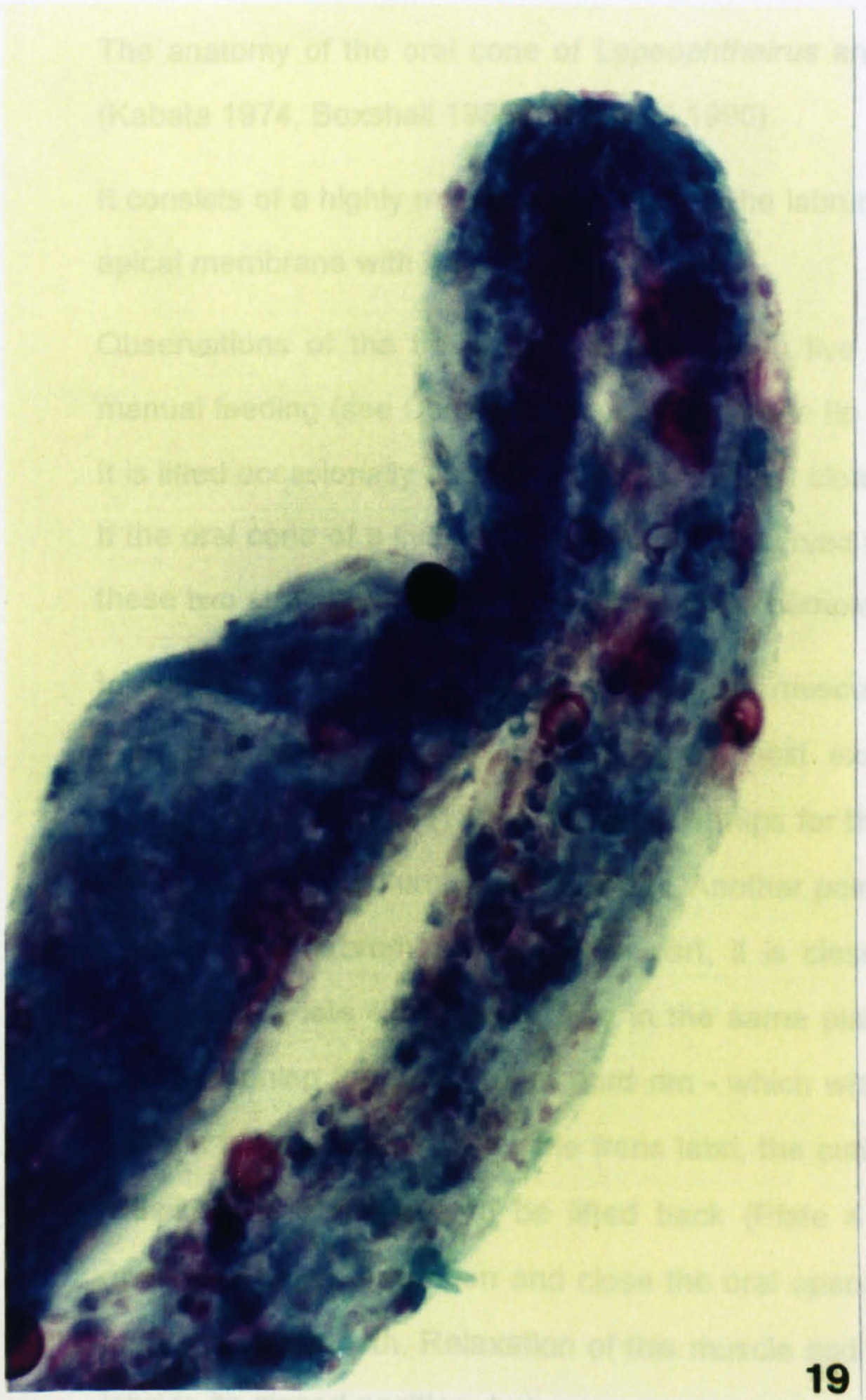

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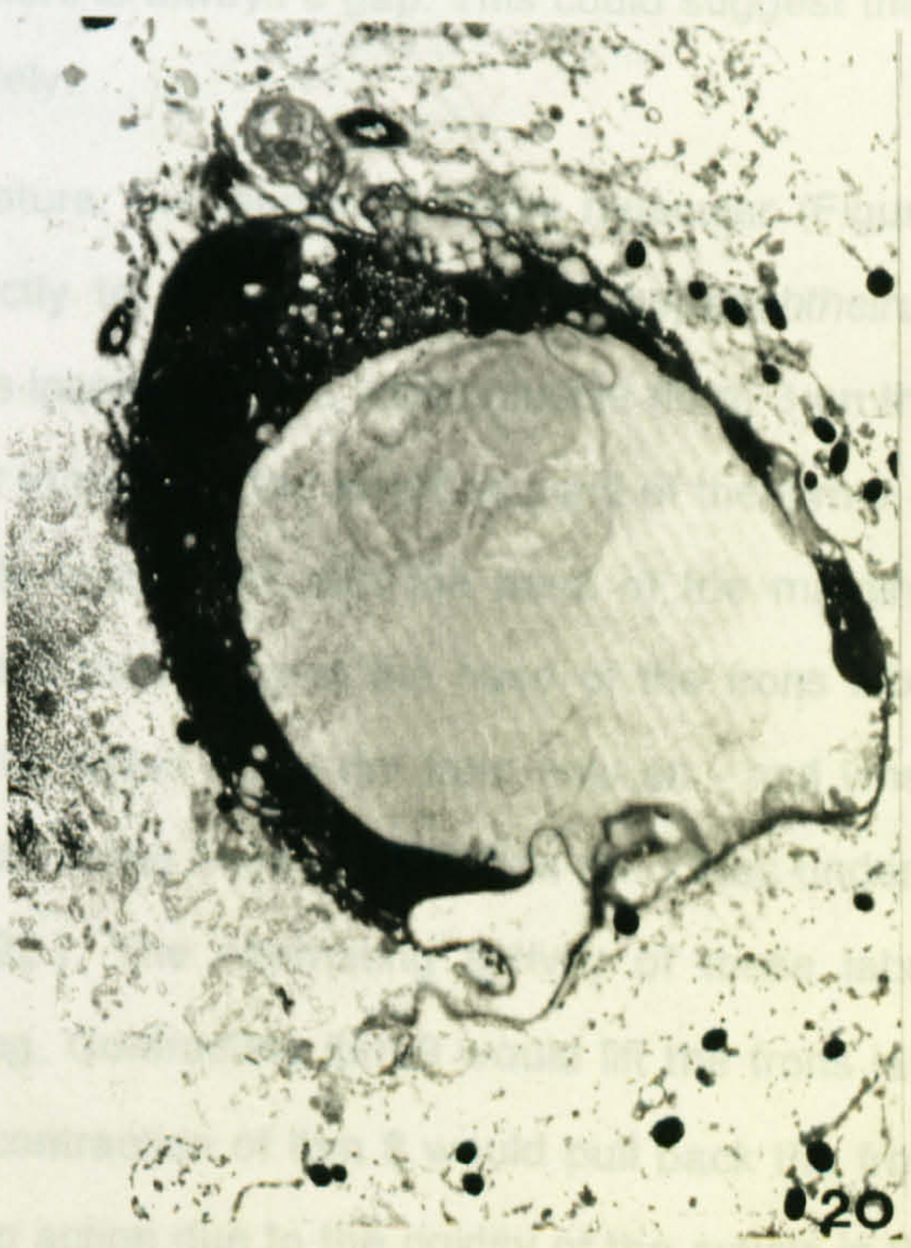

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This absence of bacteria in pellets was maintained for several hours. Pellets left in seawater were colonised by bacteria only after more than 18 hours had passed and these bacteria were mainly at the surface of the pellet. Pellets left for more than 24 hours were covered by rod and filamentous bacteria, which probably originated from the seawater. Bacteria were also found within these old pellets.

4.2.5 Food uptake

4.2.5.1 Structure of the oral cone.

The anatomy of the oral cone of *Lepeophtheirus* and other caligid copepods is well documented (Kabata 1974, Boxshall 1985a, Boxshall 1990).

It consists of a highly muscular anterior lip, the labrum and a posterior lip, the labium, each with an apical membrane with a short row of setae

Observations of the tip of the mouth cone in live lice under the dissecting microscope during manual feeding (see Chapter 5) revealed that the tip of the labrum, the frons labri, is highly mobile. It is lifted occasionally and closed again. It is not clear if these two structures can close completely. If the oral cone of a live louse is lifted and observed there is always a gap. This could suggest that these two structures may not be able to close completely.

In contrast to the labium which possess no musculature, the labrum is highly muscular (Figure 4.3). The groups of muscles conform almost exactly to those described for *Lepeophtheirus pectoralis* by Boxshall (1985a), except perhaps for the insertion of the labral muscle (lbm) 3 on the inner part of the labrum, which is wider. Another point to make is the insertion point of the lbm 9 at the tip of the labrum. At its medial part, it is closely associated with the base of the marginal membrane (Plate 4.21). Note also in the same plate a thickening at the base of the frons labri. This thickening forms a kind of hard rim - which will be called labral rim from now on - and when the lbm 9 contracts and pulls the frons labri, the cuticle at the base of the labral rim slides under it allowing the frons labri to be lifted back (Plate 4.22). The alternating activity of these labral muscles 8-9 serve to open and close the oral opening. Contracting lbm 9 would lift the frons labri and open the mouth. Relaxation of this muscle and contraction of lbm 8 would pull back the frons labri to its closed position, helped probably by a spring action due to the rigidity of the cuticle in this area.

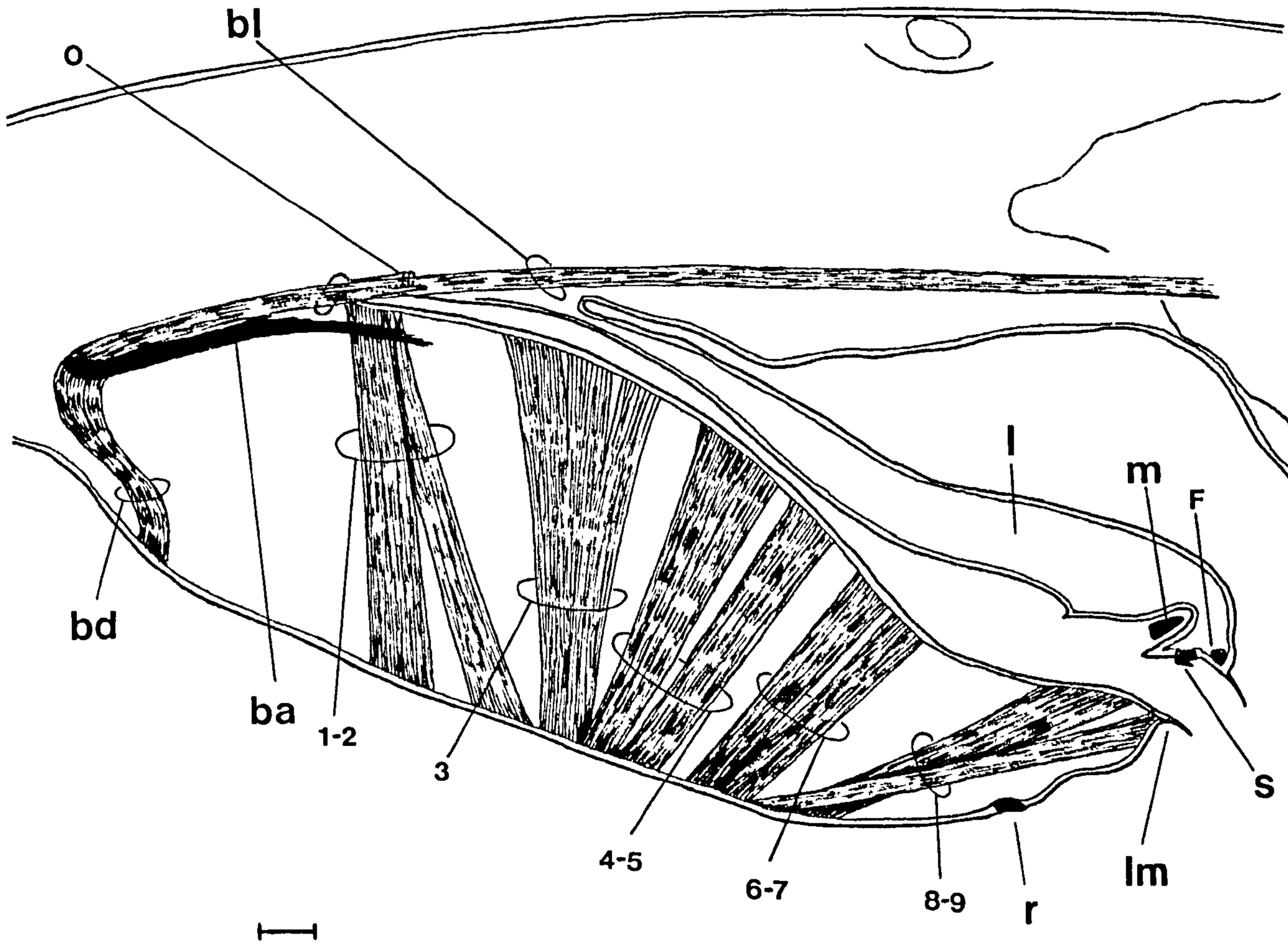


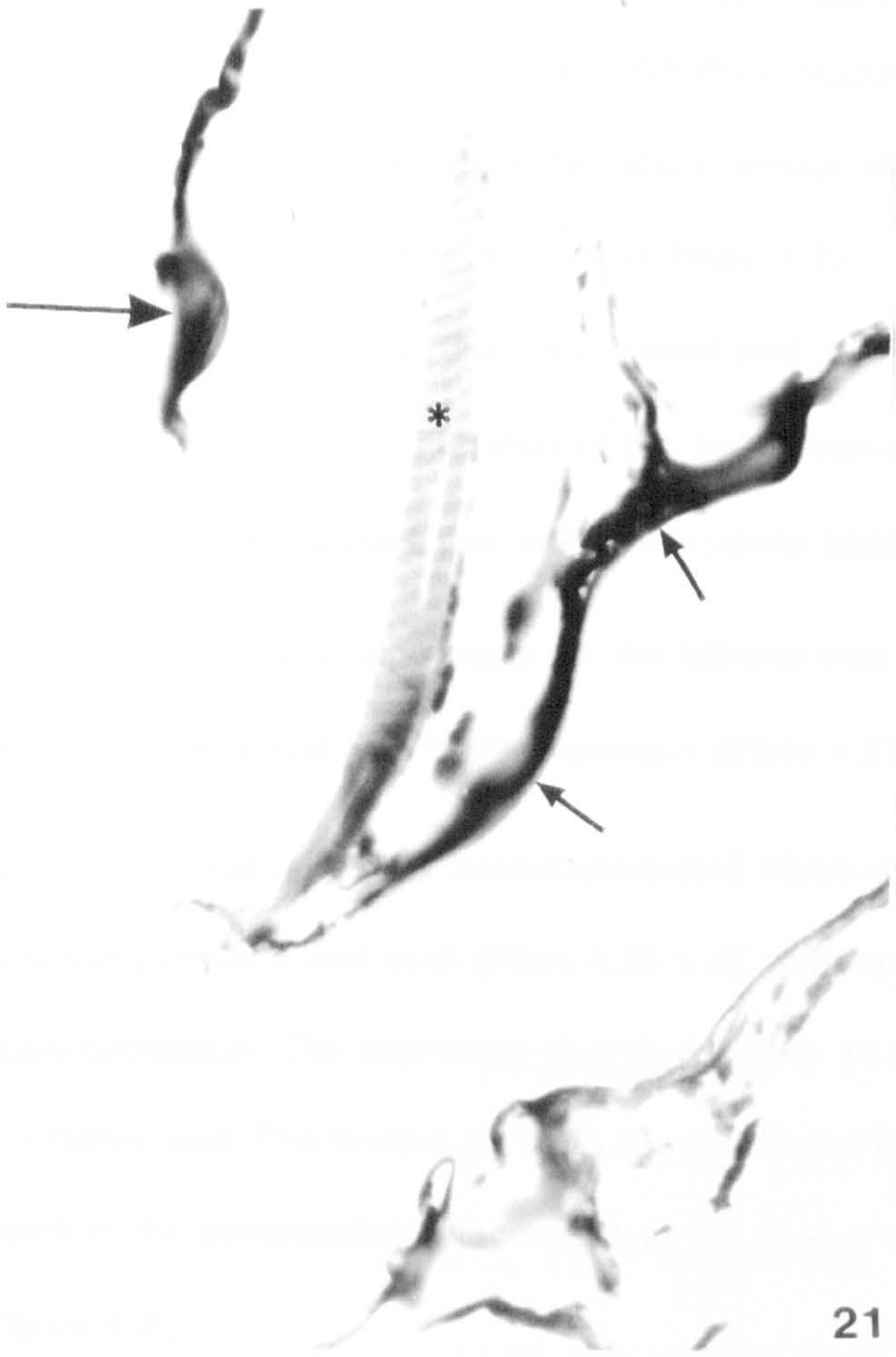
Figure 4.3 Diagrammatic longitudinal section through the oral cone of *L. salmonis*. **ba**: buccal apodeme; **bl**: buccal levator muscle; **bd**: buccal depressor muscle; **f**: labial fold; **l**: labium; **lm**: labral marginal membrane; **m**: mandible; **o**: oesophagus; **r**: labral rim; **s**; strigil. Numbers 1 to 9: labral muscles. Scale bar 50 µm..

Plate 4.21 Longitudinal section of the oral cone of *L. salmonis* showing the insertion point of the lbm 9 - the distal labral muscle (asterisk)- at the base of the marginal membrane of the frons labri. Note the thicker cuticle in the lining of the buccal cavity (arrows) and the labral rim - the sclerotised thickening behind the frons labri (long arrow). This thickening forms a sort of rim at the base of the frons labri (see next Plate where the same structure is marked with a long arrow as well). LMR-Toluidine blue. Scale bar 20 μm..

Plate 4.22 Oral aperture of *L. salmonis* illustrating the frons labri which by action of the labral muscles has been pulled open and back. Note the rim or thickening behind the frons labri (arrow) and the two mandibles with denticles. SEM 320X.

Plate 4.23 Luminal view of the labium. The labrum has been removed. Note the concavity in the medial part of the labium floor. The cuticular lining here is thinner than in the rest of the labium. Note the strigil (star), the labial fold (arrow) and the marginal membrane (asterisk) with setae. The left mandible (M) is also evident here resting in its respective groove in the labium. SEM 320X.

Plate 4.24 Longitudinal section of the oral cone of *L. salmonis* showing the labial cuticular sclerotised plate (arrows) continuous from the base of the oesophagus (arrow head) through the strigil (small arrow). LMR-Toluidine blue. Scale bar 100 μm..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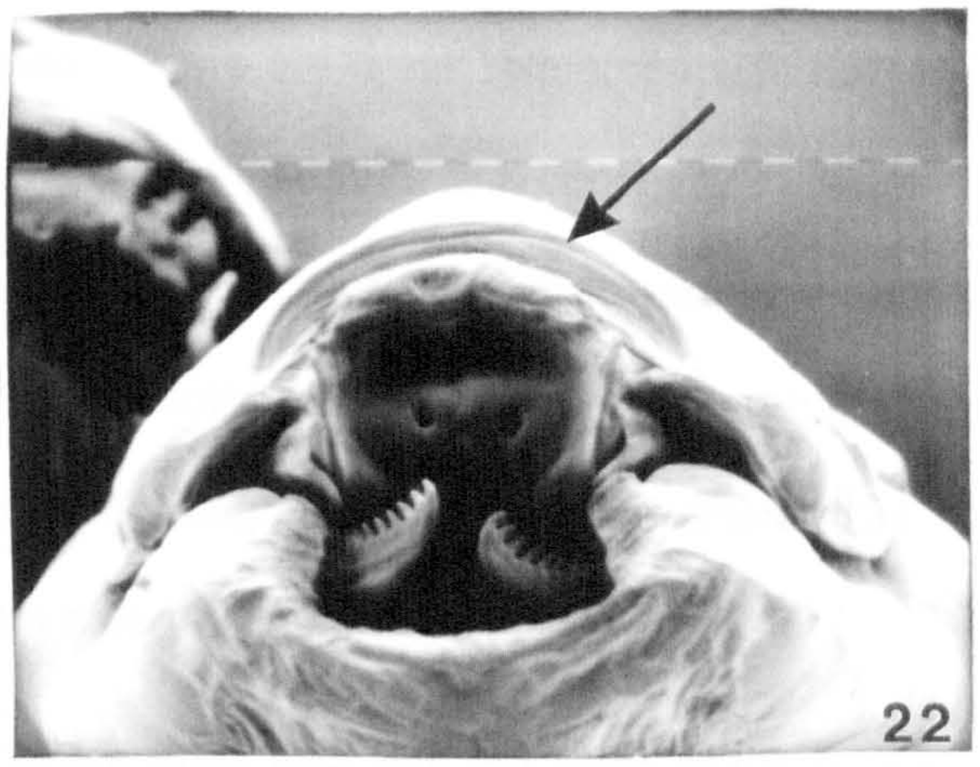
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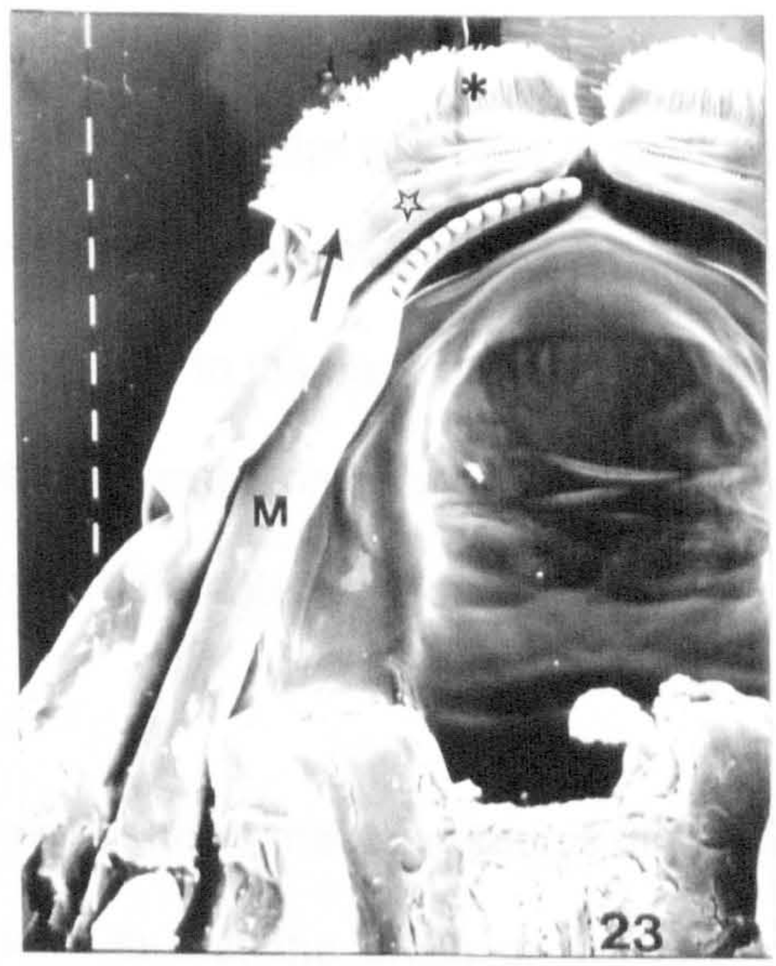
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The lining of the buccal cavity is not uniform in thickness. There are areas reinforced with thicker cuticle along the whole length of the labium except in the medial part, where a concavity of thinner cuticle is formed (see Plate 4.21 and Plate 4.23). This thick sclerotised cuticle probably adds rigidity to the labium. Away from the medial part, this thicker cuticle runs along all the length of the labium, from its origin at the start of the oesophagus to the strigil on the other side (Plate 4.24) forming a continuous plate that makes the whole labium a very rigid structure.

The other thickening of cuticle is on the luminal side of the frons labri and the lining of the buccal cavity near the junction to the oesophagus (Plate 4.21)

As in *L. pectoralis* a pair of buccal apodemes serve as a lever by which the oral cone is swung to its feeding position and back (Plate 4.25). At each apodeme attach a pair of levator muscles and a single depressor. The depressor muscle attaches on the other side to the base of the oral cone, on its anterior wall. The levator muscles run beside the oesophagus, pass through the nerve ring and attach to the postmaxillulary apodeme at the level of the start of the anterior midgut caecum (Figure 4.3).

In concordance with Boxshall (1990) for *L. pectoralis*, this work shows that *L. salmonis* differs from the study of Kabata (1974) of *Caligus elongatus* Nordmann in that the origin of the oral cone levator muscles is in the postmaxillulary apodemes and not on the ventral wall of the cephalothorax.

Plate 4.23 shows the distal part of the labium in which the labrum has been removed. The mandible is curved distally and it has 12 denticles along the concave margin. It rests confined in a depression of the labium, or labial guide, which in turn is formed on its distal part by the proximal wall of the strigil. The strigil is a hard cuticular bar which bears in its distal part a row of small sharp teeth denticles, between 60-70 on each half of the bar. Next to the strigil is a ridge of hard cuticle, the labial fold, which in turn is fringed with the marginal membrane and its flexible setae. The marginal membrane is interrupted in the median part by a notch.

The distal part of the labium is formed by the frons labri (Plate 4.26). Just behind it there is a rectangular pad of cuticle that could be sensorial, as most of this area, including the frons labri, seem to be innervated by nervous tissue below the cuticle (Plate 4.28). On its medial part, there are three depressions, two big and one small (Plate 4.27). No ducts from glands of any sort were found associated with any of these depressions, so they are presumed to serve a sensorial function. The tip of the frons labri has a marginal membrane with short setae on its external side, but on the internal side the setae seem to be highly worn (see Plate 4.27). A median notch separates the marginal membrane into two halves. In the middle of the notch there is another depression that again could have a sensorial function.

The sharp buccal stylet points to the median part of the labrum. It seems to be a stop for the closing movement of the frons labri, and its outer part seems to serve as a guide for the labium when the frons labri is closing. When the frons labri is closed, the buccal stylet rests in a small groove on the labrum, just on the face of the strigil (Plate 4.28).

4.2.5.2 Feeding mechanism for epithelial cells

Feeding in caligid copepods has been described by Kabata (1974, 1979) and, although the basic mode of feeding is thought to remain basically the same as Kabata's description, further speculation is necessary in the light of the observations and findings in this study. The following mechanism is proposed.

While the louse is finding a place to settle the mouth cone is held in a posteriorly-directed position. Although it was not directly observed, it is possible that from time to time the oral cone is erected towards the substrate and the frons labri pulled backwards by the contraction of the labral muscle 9 and put against the substrate to sample the surface in the search for an appropriate spot to feed. The main sniffing could be carried out by means of chemoreceptors situated at or below the notch in the marginal membrane, which is the closest to the substrate.

Plate 4.25 Longitudinal section of the oral cone of *L. salmonis* showing the buccal apodeme (asterisk) and its levator (arrow) and depressor (arrow head) muscles. Note the depressor muscle inserted to the frontal side of the base of oral cone. LMR.-Toluidine blue. Scale bar 100 μm..

Plate 4.26 Stereo scanning electron micrograph of the distal part of the labium. The labrum has been removed to show the buccal cavity side of the labium. Note the frons labri (asterisk) folded down and backwards, the rectangular pad (open star) behind the horse-shoe shaped floor of the frons labri and the two sensory papillae (arrow head pointing to the right one). Arrows point to small clusters of rod shaped bacteria. SEM 320X.

Plate 4.27 Zoom of the luminal side of the frons labri to show the three orifices that open in its medial part. Note the buccal stylet (asterisk) and the marginal fringe (star) with greatly frayed setae (arrow) and its medial notch (arrow head). SEM 640X.

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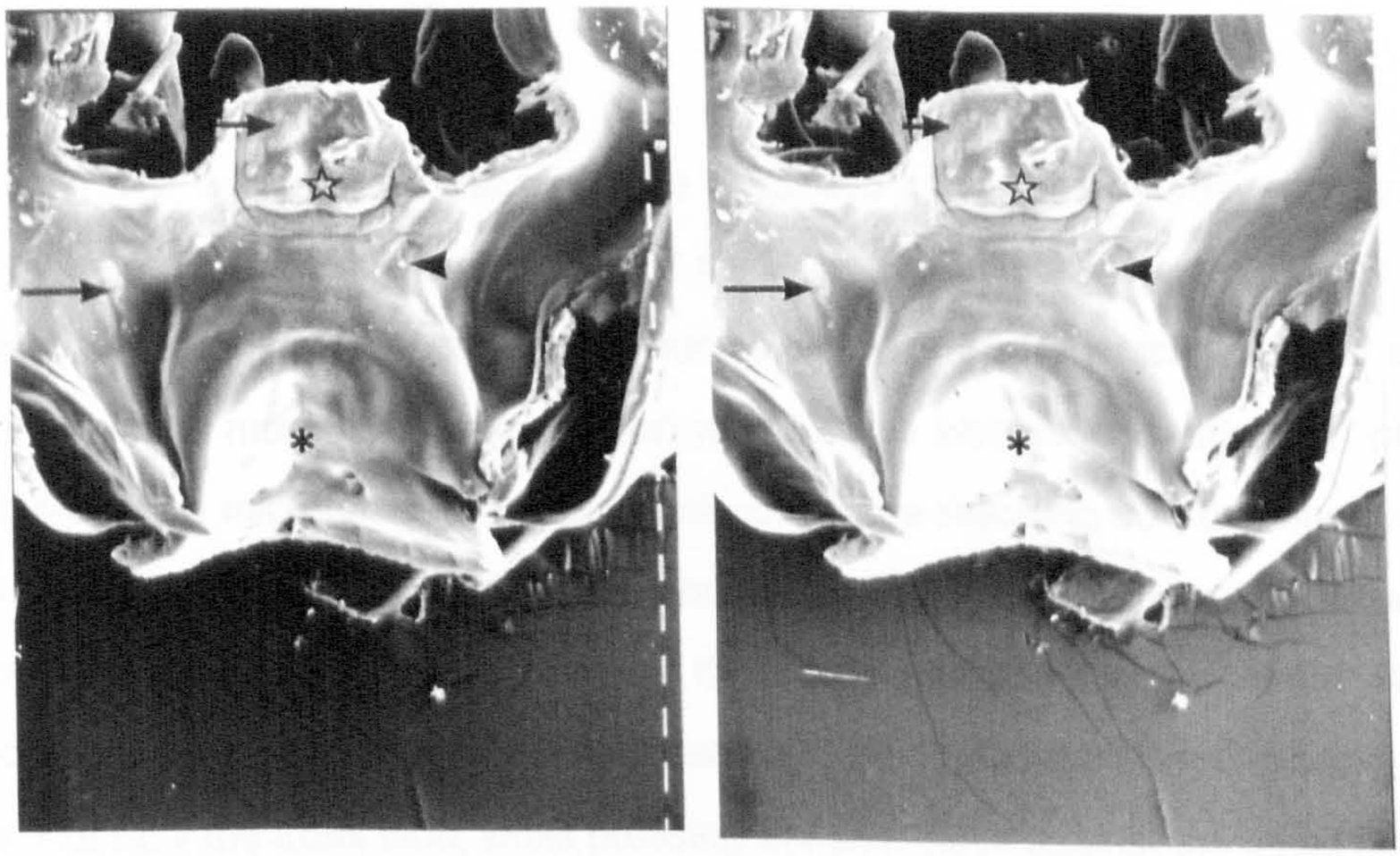

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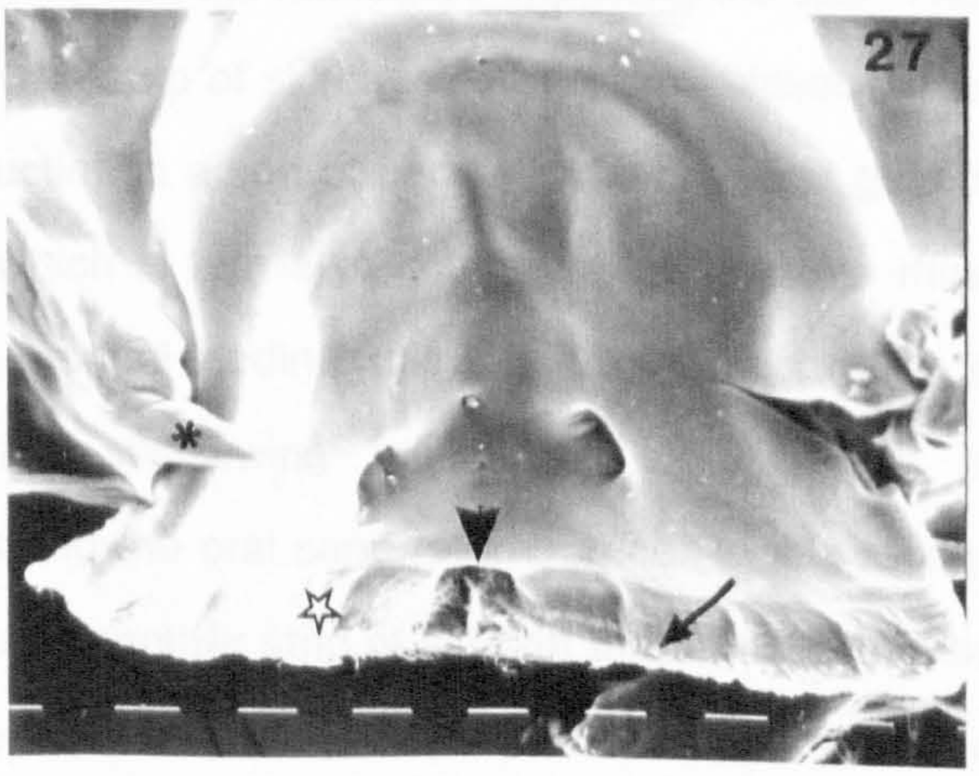

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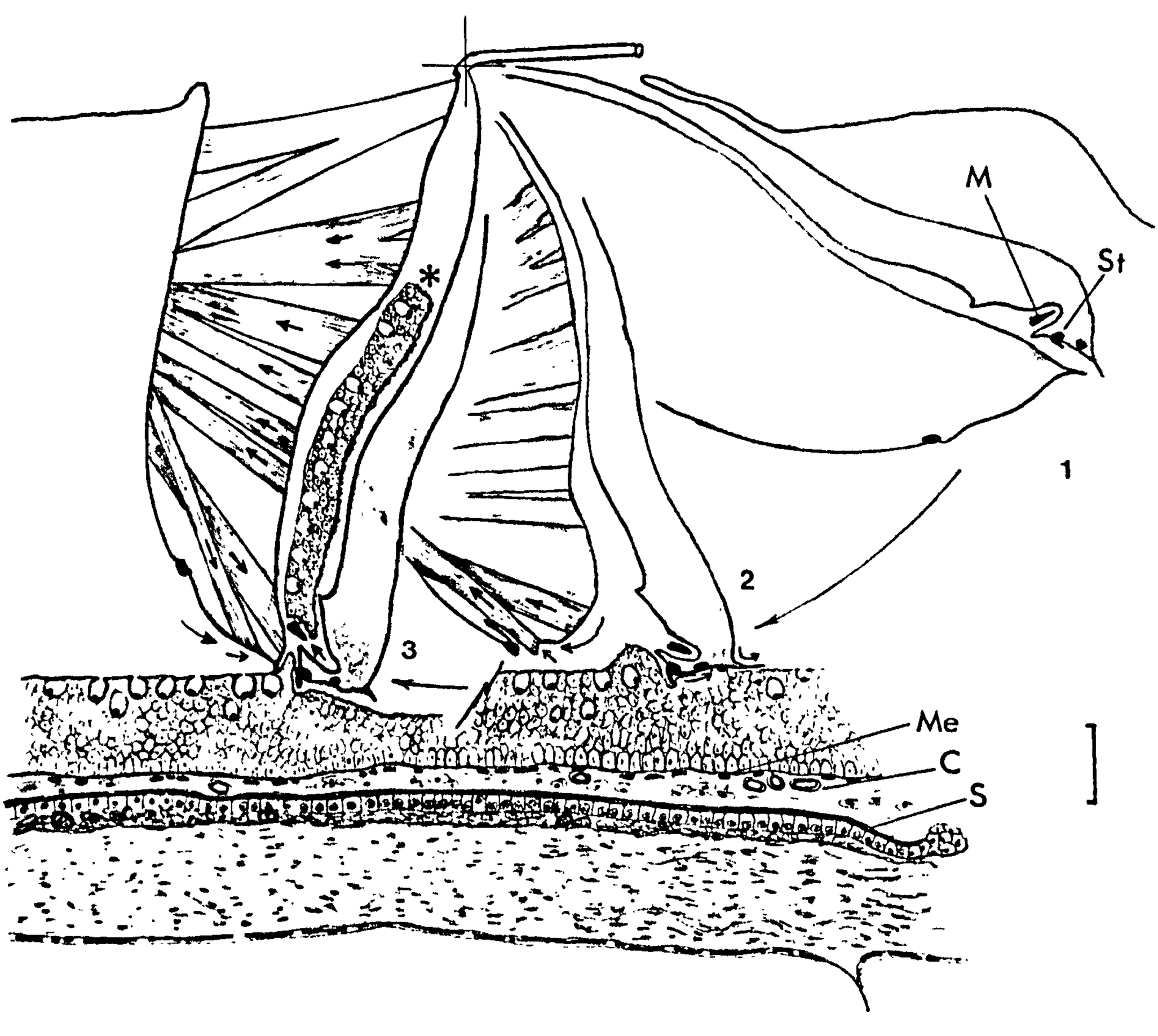
Figure 4.4 illustrates diagramatically the proposed sequence of events during the ingestion of a piece of host' skin. At rest, the oral cone is held at an angle of about 25°. Once the louse has found an appropriate place and is ready to feed the oral cone is erected by the contraction of the buccal levator muscles which in turn pull the buccal apodemes. The oral cone raises and at about 65-70° comes in contact with the substrate, spreading the marginal membrane and the labial fold backwards and exposing the strigil teeth. Before this, the levators of the frons labri contract, lifting the frons labri to give way for the strigil to come in contact with the substrate, otherwise the labrum would stand in the way of the labium. The tip of the strigil is then inserted into the skin. If the movement of the oral cone continued forwards without further adjustment of the position of the ventral cephalothoracic wall, the labium would have to penetrate deeply into the skin, which mechanically may be impossible.

With these continuous adjustments, the oral cone continues its travel forwards, scooping a strip of skin on its way, which is taken inside the buccal cavity by the combined action of the mandibles, the frons labri and possibly by a slight drop in buccal cavity pressure which could be achieved by contraction of the labral muscles. The frons labri would serve also as an "antirolling" plate, just as the antirolling plate of a microtome for frozen sections. Before reaching 90°, the labral muscles 6-7 start to contract slightly to accommodate the ribbon of skin that by this time would have reached this area, followed later by the contraction of the labral muscles 5-6. At about 100-110° the maximum forward movement of the oral cone is reached, the frons labri closes, grasping the end of the skin strip. Possibly the frons labri, while pressing the skin block against the strigil could start the cut as in a sellotape dispenser with the mandibles exploiting the tear. The mandibles then could act as a guillotine to cut away the strip of skin against the semi closed frons labri. The labral muscles then contract producing suction in a sort of peristaltic fashion and gulp the piece of skin, forcing it through the oesophagus which in turn also contracts and relax its muscles to swallow the food. From observations during manual feeding, this swallowing mechanism of the oesophagus can be done separately from the swallowing mechanism of the oral cone. Then, the ventral cephalothoracic wall rises and the oral cone returns either to its resting position or straight to the starting position for scooping another strip of skin on the same place, but now deeper in the skin or in a different place after the louse advances a short distance, as the marks found on the skin of the salmon suggest (Plate 4.2).

Figure 4.4 Diagrammatic representation of the possible mode of feeding of *L. salmonis* on its host epidermis. Drawings of the oral cone and the skin were done separately, at the same scale, with the aid of a drawing tube. The junction between the oral cavity and the oesophagus (cross hairs) was taken as the pivoting point for the swinging movement of the oral cone. The angles mentioned in the text are in reference to the axis formed by aligning the junction with the strigil and the ventral cephalothoracic wall, which in turn is considered parallel to the substrate. See text for the description of the mode of feeding. Refer also to Figure 4.3 for the identification of some structures mentioned in here and in the text and not indicated in this diagram. 1: Resting position of the oral cone; 2: position of the oral cone at the beginning of the "scooping movement", when the strigil is inserted into the host' skin. For this to happen, the frons labri had to be lifted beforehand and this is done by the contraction of the distal labral muscles (lm8 and lm9); 3: position of the oral cone at the end of the scooping movement when the strip of host' epithelium (asterisk) lifted by the strigil and ingested by the louse is cut by the action of the mandibles and starts to be gulped helped by successive contraction of the labral muscles. C: blood capillary; M: mandible; Me: melanin; St: strigil. Scale bar 100 μm. .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The rigidity of the labium due to the sclerotised plates seems to be an important feature, as strong pressures will be exerted by it while scooping strips of host skin. This may account for the lack of muscles in this structure.

Plate 4.29 shows a scar found on the surface of another scale. Its dimensions are 280 μm long and 60 μm width at one end and 28 μm at the other. This dimensions resemble the average dimensions of the skin strips found in the gut of feeding lice. Plate 4.30 shows a strip of skin at the same magnification of that of this scar. Note as well that one end of the skin strip is wider than the other, characteristic that the scar also shares.

The wider end of the scar could be the place where the labium's strigil first cuts into the skin. As it starts to dig, lifting the skin like a gouge would lift a strip of wood. This gouging on the skin by the louse mouth is validated by the shape of the skin fragments, which are curved, just as the rolls of wood are formed during carving. This bending is not the result of artefact during fixation or dehydration, because that was the original shape of the fragments when they were retrieved from the gut of the lice. Also note that in Plate 4.5 the outer surface of the skin fragment has creases in the middle part, which probably are stress lines formed when the skin bent while it was being carved out.

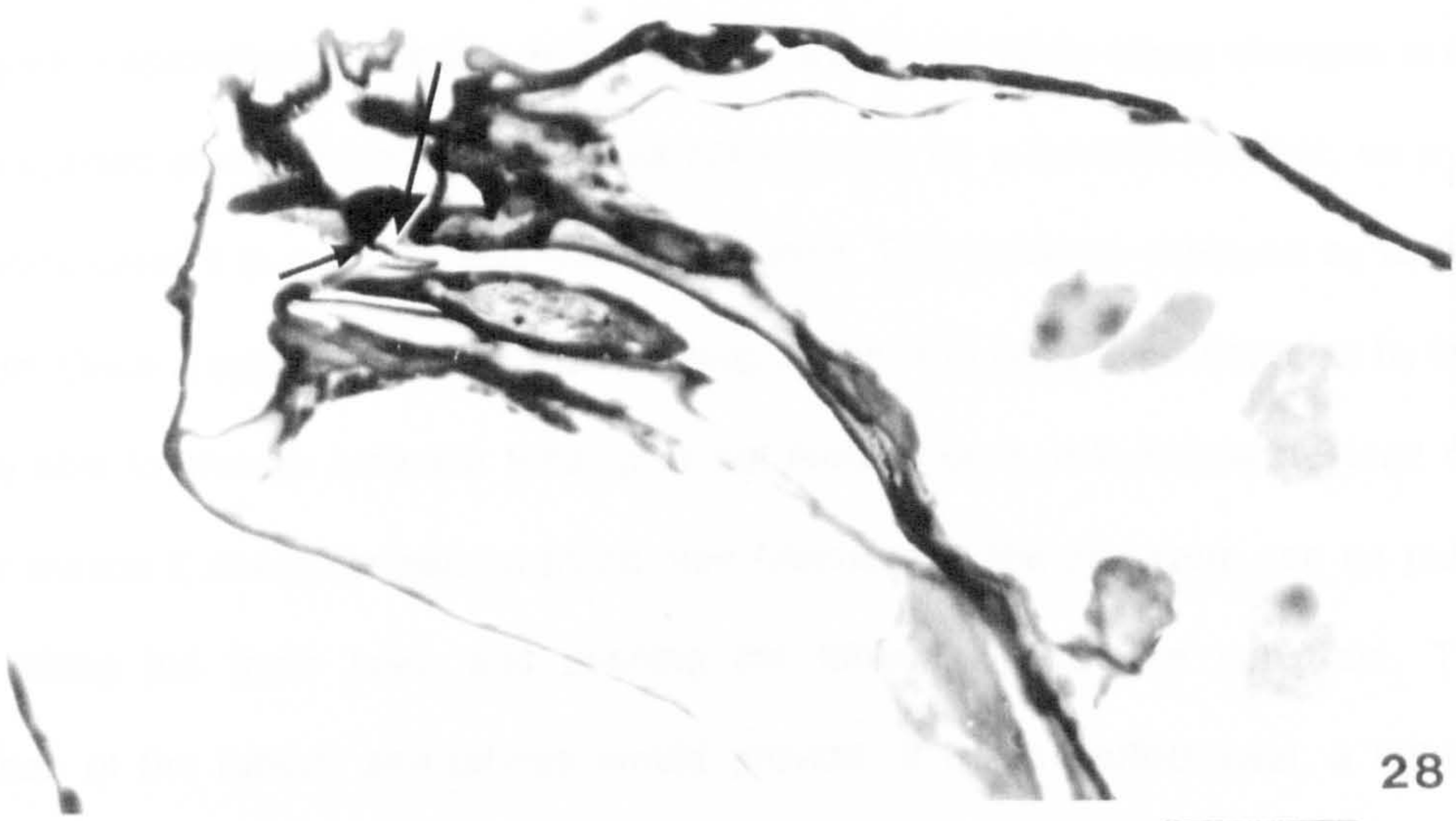
The function of the buccal stylet is not very clear, but it could serve as "guide" for the strip of skin or a guide for the closure of the labium and labrum.

The long lines found in the skin and the imprints on the scale (Plate 4.2) could be the result of a louse "ploughing" along the skin, taking one strip of skin after another in a continuous line. That could also explain the individual movements of a louse detailed in section 4.2.2 .

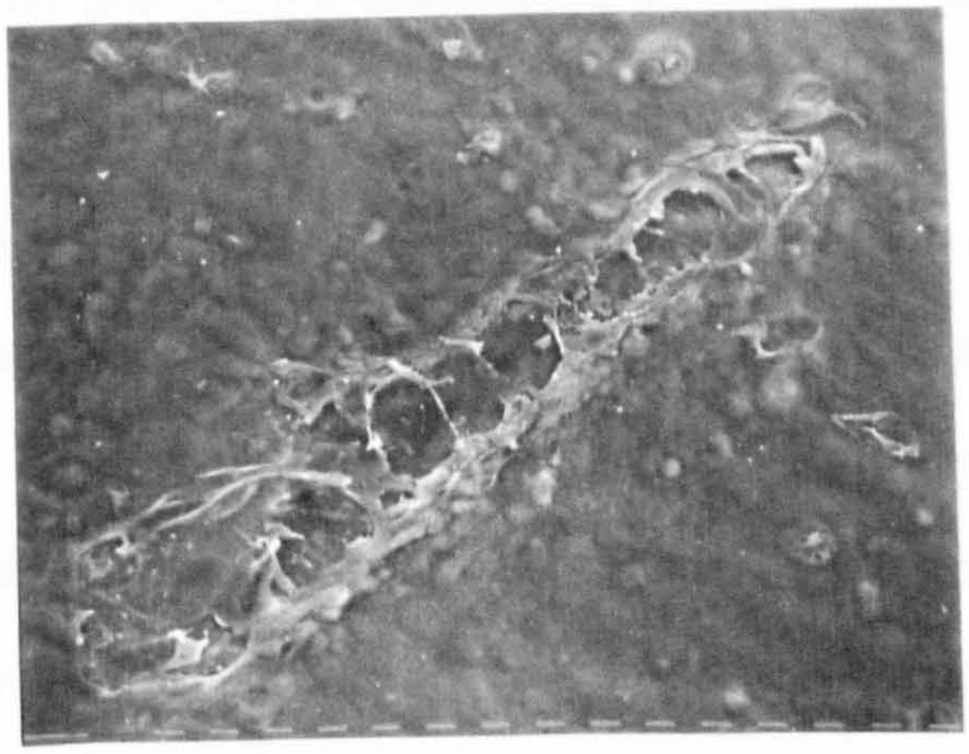
Plate 4.28 Sagital section of the mouth cone showing the notch (short arrow) that exists laterally in the luminal side of the strigil. This notch houses the buccal stylet (long arrow) when the louse closes the frons labri. LMR-Toluidine blue. Scale bar 50 μm..

Plate 4.29 Scar on the skin surface of a salmon scale. The size and length of this scar corresponds to the dimensions of the skin fragments found in the louse gut (see Plate 4.30 and Plate 4.5). SEM 320X.

Plate 4.30 Strip of skin from the gut of a louse. Compare dimensions with the scar depicted in Plate 4.29 . SEM 320X



28



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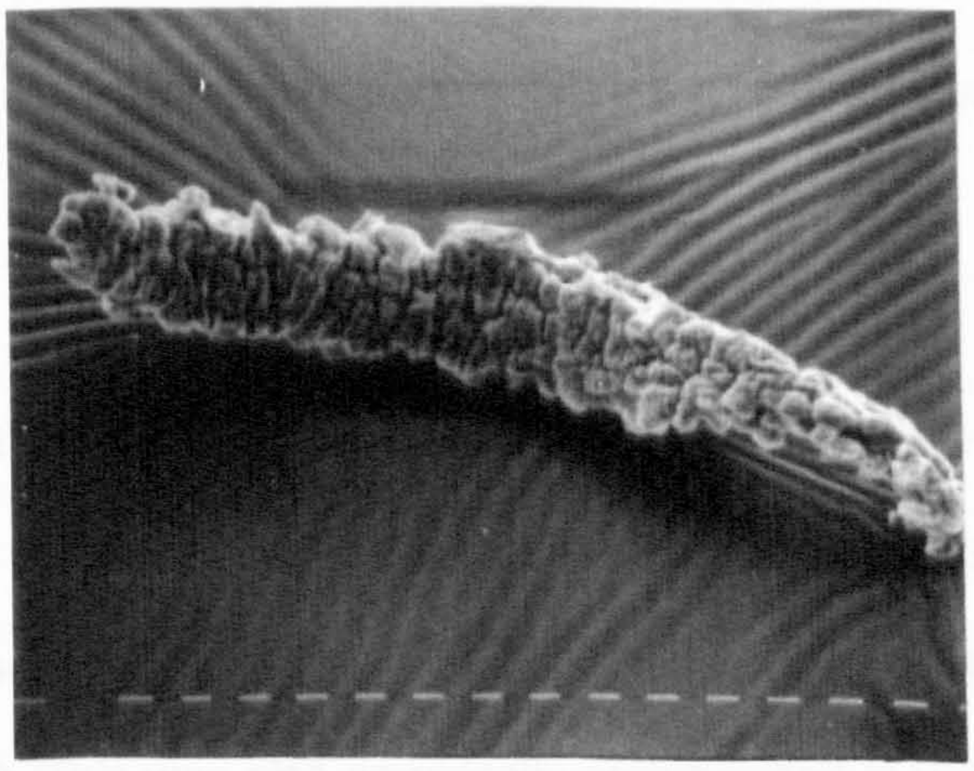

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4.2.5.3 For blood

Switching from epithelium feeding to blood feeding would not imply many changes in the mode of feeding proposed above. Blood feeding does not seem to be a random incident, so a louse would have to know when it is available and how to look for it. This could be achieved by taste sensors at the labrum. Once a capillary is hit by the scooping action, it is likely to be detected by the louse and it may be able to choose between feeding or not feeding on it. If it decides to feed on blood (for whatever reason it needs to feed on blood, see Discussion) the oral cone can be put against the capillary lifting the frons labri, and pushing the labium against the substrate. The marginal membranes of the labium and labrum would provide, if not a perfect seal, a tight grip on the substrate, helped by a drop of pressure in the buccal capsule. It then may allow the blood to flow freely into the buccal cavity and into the oesophagus or, with slight pumping contractions of the labral muscles.

This view is supported by observations of the louse behaviour during manual feeding that will be described later in Chapter 5, when a louse would suck from the tip of a pipette inserted just at the tip of the oral cone. But, during this type of feeding (from the tip of the mouth), a full gut of blood was never accomplished, no matter how long the manual feeding lasted. The louse would gulp small amounts of blood one by one and the hindgut would never appear to be full. When the manual feeding was done by placing the tip of the pipette at the origin of the oesophagus and applying pressure, the louse would let the blood pass freely and the gut was filled in seconds, giving the same picture as the red-gutted lice that had fed on blood naturally.

4.3 DISCUSSION

It has been reported that lice have a preference for certain parts of the body. Atlantic salmon from cages had the highest density of adult *L. salmonis* on the head, operculum, dorsal part, but relatively more on the head and operculum of small salmon and post anal area in large salmon (Jaworsky & Holm 1992). This was also empirically corroborated in the field during the collections, where most of the time the greatest aggregation of lice, particularly adult females was behind the dorsal, adipose and anal fins. The preference for these areas was not evident in the experimental

fish in the present study, probably due to the size of the fish or its activity in the aquarium tank, which evidently is different to the activity of a larger fish inside a sea cage. Lice behind the anal fin were never recorded, for example. Jónsdóttir *et al.* (1992) suggest that lice would prefer areas where they are less likely to be displaced by the water flow. But in experimental conditions and with small fish as in the present study, these constraints probably do not occur, resulting in a more even distribution of the lice on the fish. When a louse on an experimental fish confined to a small tank produces egg strings, these are usually curled, not straight as those found always in lice from cultured fish (personal observations). The current generated by the swimming fish in a cage seems to shape the egg strings as they are extruded. It is possible then that the distribution of lice on cultured fish is the result of lice trying to avoid areas with greater drag.

It has also been mentioned that *L. salmonis* would prefer areas where the skin is thinner, as it is thought that a louse would feed more easily in these areas (see Jónsdóttir *et al.* 1992). From the observations in this study, this seems not to be the case. It was observed that lice are feeding on the epithelial layer of the salmon skin. There is apparently no need for deeper penetration that would support the mentioned hypothesis of easier feeding on thinner skin. In the case where a louse requires a blood meal there would be apparently no difficulties. Judging from the size of the skin blocks that the louse is feeding on, blood capillaries are within easy reach of the mouth cone of the louse, just below the epithelial layer, above the scales.

A common observation was that, although the louse was firmly attached on the fish, this did not mean that it was feeding. There could be several reasons for this. Non-feeding lice could be senile. During this study it as observed that a number of females die within one or two days after their collection from the field, while others can survive for longer periods in starvation. It is possible that these lice that die sooner have reached the end of their life span. Further observations on survival and occurrence of feeding in different age groups could shed more light on this possibility.

Other causes of lack of feeding could be that the starvation period has affected their physiology enough to have reached a point of no return where they are no longer able to recuperate. The stress of handling could be another factor. In general, it was observed that a non-feeding louse

would stay in the same place on the fish for long periods of time. This issue of the high occurrence of non-feeding lice will be discussed further in Chapter 6.

The observations of the movement of lice on the fish revealed that they tend to remain within a specific area, but not the same spot. In natural or culture conditions their coverage probably is more restricted, as they may try to keep sheltered from the currents generated by the swimming fish. With more lice crammed and feeding in a narrow area, the skin of the fish is not allowed to heal properly and would be more likely to develop the localised wounds commonly observed on the fish.

There seem to be in the literature few attempts to observe the actual behaviour of *Lepeophtheirus* or other caligids or branchiurans (which have a similar mode of life) on their hosts. Kabata & Hewitt (1971), studying the locomotory mechanisms of caligids described "settling" movements of *Lepeophtheirus* and *Caligus*. These movements resemble the settling movements observed in this study, although their observations, in the case of *Lepeophtheirus*, were done in isolated individuals off their hosts. Histopathological studies related to *L. salmonis* are relatively more ample (see Boxshall 1977, Jones *et al.* 1990, Jónsdóttir *et al.* 1992, Tully 1992, Grimnes & Jakobsen 1996 for histopathological studies of the effect of adult and juvenile stages of *L. salmonis*, Bron, Sommerville, Jones & Rae 1991and Johnson & Albright 1992 for the effect of the larval stages). Very few histopathological studies exist on of the effect of *Caligus* on its hosts. Mackinnon (1993) and Bennett & Bennett (1994) studied the effect of the larval stages of *Caligus elongatus* and *Dissonus manteri* Kabata respectively and Roubal (1994), the effect of various stages of *Caligus epidemicus* Hewitt. For branchiurans Avenant-Oldewage (1994) is the only recent account of the pathological effect of these parasites on their host.

But only in two of the studies mentioned above, the actual damage caused to the surface of the skin of the host has been studied by SEM and associated into their mode of life. In the first one, Jónsdóttir *et al.* (1992) found that *L. salmonis* leaves an imprint on the skin of the fish due to the pressure made during attachment. They noted that this imprint was more pronounced anteriorly, due probably to a stronger application of the anterior cephalothorax to the skin. The imprints found on the detached scales in this study apparently were made by the anterolateral part of the

cephalothorax and therefore seems to agree with their observations. It also points to the mode of moving about on the surface. The settlement movements observed in this study, with short advances of the parasite, seem to be the reason for these successive imprints of the cephalothorax. They could also be the result of the pressure exerted whilst pulling itself onto the host' surface to facilitate the scooping into the skin with the oral cone.

The latter authors also observed the actual damage caused by the mouth tube, which apparently left a groove on the skin. In the scar left by the mouth cone of *L. salmonis* in this study, a groove was not evident because, apparently, some healing had taken place in the wound. It has been observed that within 1 h, epidermal cells of the salmon skin begin a quick migration to cover any wound inflicted (Bullock & Roberts 1992). In such a small wound like the one inflicted by a louse, this healing of the surface probably takes even less time, therefore, there are few chances to observe a fresh wound.

In the study of Avenant-Oldewage (1994) she found similar damage to the skin of the catfish *Clarias gariepinus* (Burchell) inflicted by the branchiuran *Dolops ranarum*. Puffed skin as a result of the suction action of the cephalothorax similar to that found by Jónsdóttir *et al.* (1992) and the imprint of the claws of the prehensile appendages and the edges of the cephalothorax on the skin, as found by the latter author and in the present study, were also characteristic features. Penetration of the claws of the second antennae and the maxillipeds usually happens in the epidermis only, but in *L. pectoralis,* in some cases, it was observed to enter into the dermis of the flounder *Platichthys flesus* (L.), and their movement could cause severe mechanical erosion of the epidermis (Boxshall 1977).

Avenant-Oldewage (1994) suggests that, in natural infestations, the hosts usually survive the presence of *Dolops ranarum* because the number of parasites per unit area on the host is normally low. But, as the number of parasites increases in an infection of these types of ectoparasites, the chances for the skin to heal become diminished. The gradual loss of the mucus-producing layer, which is thought to have antibacterial properties, gives way to secondary infections, haemorrhages and loss of scales which expose the dermal layer and subject the fish to osmotic stress (Jónsdóttir *et al.* 1992).

Several attempts have been made to describe the mode of feeding of parasitic copepods but, due to the obvious difficulties of direct observation, the ideas on how they feed come mainly from interpretations of the morphology of the parts presumed to be involved in the food uptake (Kabata 1981) .

In this study, this statement also holds true. Although the interpretation of the mode of feeding of caligids proposed by Kabata (1974) remains basically the same, novel details are added in this study.

The labrum, or more exactly, the frons labri had not been thought to play a significant part in feeding. The possible sensorial role of its tip, presumably as a chemoreceptor, and its likely function in securing in place the pieces of skin that are scooped by the strigil before being cut by the mandibles and swallowed, provide reasons to consider the labium as a very active structure. Bron (1993) described a "distal labral organ" at the tip of the labrum in *L. salmonis*. Although the function of this organ is uncertain, he suggested that it may be nervous in origin, supporting the proposition put in the present study.

It is difficult to imagine how the "sawing motion" of the two halves of the strigil proposed by Kabata (1974) can occur. The strigil is a fixed structure, continuous with the sclerotised cuticle of the labrum, incapable of side movements. Because it could be considered as "pedunculated", the only movement possible when pressure is applied to this structure would be up-down, but not sideways. The nature and uniformity of the tissue strips ripped from the skin of the fish has never been documented in other caligids. This suggests that the oral cone is a very precise structure that not only "dislodges" tissue fragments, but does it in a very particular and constant fashion, and with surgeon-like accuracy. The presence of bits of fish-skin with melanophores in the gut of *L. salmonis* had been documented before by White (1942) but no mention was made in respect to their size or shape.

It has been mentioned that the diameter of the nerve ring limits the size of particles that can pass through the oesophagus and that in parasitic siphonostomatoid copepods, this limits the ingestion to fluids only (Boxshall 1992). Boxshall (1985a) also mentions that the fragments of tissue detached by the strigil of *Lepeophtheirus* must be further fragmented before they can pass through

the oesophagus and the ridged inner walls of the buccal cavity may act as a gizzard accomplishing this mechanically, or the secretions released into the buccal cavity by the labral glands may have a pre-ingestion digestive function (Boxshall 1990). Also Boxshall (1977) suggested that *L. pectoralis* feeds on macerated tissue because no intact host cells were found in the intestine of this parasite.

This does not seem to be the case in *L. salmonis*, where the main form of food is in the form of whole chunks of skin lifted from its host. Therefore, the diameter of the nerve ring seems not to limit the size of particles that can be ingested by *L. salmonis*. It is possible that the same feeding strategy is followed by the other caligid copepods, in which the morphology of the mouth parts is similar. Also in *L. salmonis*, Bron (1993) found similar results as in this study, in the sense that a copepodite could take large pieces of tissue from the host. Nevertheless, they were only found filling the entire buccal cavity of the copepodites, but not in the midgut. He suggested that these pieces of tissue might be subjected to preliminary digestion by labral or other glandular excretions because, what he found in the foregut or anterior midgut, were host cell fragments and amorphous material instead of large boluses of tissue. The integrity of the host tissue found in the midgut and in the pellets of the lice adult females in this study does not support this assumption that there may be an enzymatic pre-treatment of the food at the level of the buccal cavity. At the moment of fixation, lice usually void part of their gut contents, and it is likely that the absence of big pieces of tissue in copepodites could have been due to this fact. In this study the anus was ligated to ensure no loss of gut contents.

Kabata (1979) mentions that a slight drop in intrabuccal pressure is necessary to maintain firm contact between the mouth and the skin. This does not seem to be necessary in the case of *L. salmonis*, but this drop in pressure could still work to suck the block of salmon skin while it is being scooped and accommodate it in the buccal cavity.

The "trails" observed in the skin of the salmon in the present study are probably the result of the lice feeding activity. The feeding mechanism proposed in this study can explain these trails. It seems that a feeding louse takes several skin blocks as it advances. As it was shown, an average pellet can contain approximately the equivalent of 55 of these skin chunks which in turn would make a strip of skin with similar dimensions to the trails observed. It is thought that each of these

trails may represent a louse "meal". In field conditions, where the lice are thought to have more restricted movements, or at least restricted to a small area on the fish, as lice shelter from strong water currents generated by the swimming fish, feeding lice probably feed over and over in the same area and the healing process of the skin of the fish cannot keep up with the loss and erosion, eventually leading to the haemorrhagic lesions commonly seen. Moreover, it is clear that the lice are reaching the dermal layer during their feeding, as the presence of melanin granules and iridescent material in their gut suggests. The dermal layer takes longer to heal. In normal salmon, the dermis still showed high fibroblast activity even 28 days after an experimental wound was made on the skin (Bullock & Roberts 1992). This might explain the permanence of the trails in the skin. It is possible that the melanophores lost due to the parasite feeding had not been replaced, even though the epidermal layer had been healed completely.

It has been suggested that mucus is the main food component of *L. salmonis* (Scott 1901, Egidius 1985) but this is not supported by the findings in this study where the main food item seems to be epithelial cells. Little digestion of these epithelial cells seems to occur during their residence in the gut of the louse. The possible reasons for this will be discussed in Chapter 7 at the light of results from other chapters. These latter authors also mention that *L. salmonis* occasionally feeds on blood, which seems to be a common observation (White 1942, Brandal *et al.* 1976, Nylund *et al.* 1992)

The question that arises here about the 16% of the adult female lice feeding on blood is challenging. Why such a constant proportion?

Why we don't see a higher proportion of females feeding on blood? or more variability? Is it that only in certain circumstances (possibly physiological state) is when lice feed on blood?

In the case that *L. salmonis* females fed on blood by chance, i.e. when a capillary in the skin is hit while feeding, one would expect a more variable proportion of females with blood in their guts, therefore, other factors must be in play. In the next Chapter it will be shown that blood feeding may be associated with the presence of lipid reserves in the enterocytes of the lice.

In this study it was observed that only on very rare occasions were red blood cells found in the gut. This suggests that the red blood cells are haemolysed quite quickly. This has been observed in bloodsucking insects where two distinct patterns of blood digestion are recognised. The first one, observed in lice and fleas, is remarkably similar to the one observed in *L. salmonis*. It is characterised by a rapid haemolysis and liquefaction of the blood meal within 6 h after feeding, absence of formation of peritrophic membranes and the blood remained liquid throughout the 48 h observation period. In the second pattern, observed for bed bugs, sand flies and mosquitoes, there was a lag time of 6-18 h before substantial breakdown of erythrocytes began, peritrophic membranes were present surrounding the blood meal and the blood meals were clotted. So, the authors suggest that these two feeding patterns reflect two different feeding strategies. They mention that lice and fleas maintain a close association with the host and take relatively small but frequent blood meals. And because the availability of blood is unlimited, red blood cells are degraded quickly, most likely to assist on a rapid turnover of nutrients necessary for a constant output of eggs. Therefore, it could be possible that, in the case of *L. salmonis*, blood feeding, which seems to provide ample amount of lipids and for sure many other nutritious materials, is associated with vitellogenesis and the production of eggs. To explore this possibility, more refined and accurate samples that also take into account the developmental stage of the lice and the state of maturation of the genital system are required to further substantiate this matter. This aspect of blood feeding will be further explored in the next chapter.

Numerous marine and estuarine crustaceans egest faecal material which is bound together in the form of pellets by a peritrophic envelope. The faecal pellets are considered as an important food source in aquatic ecosystems (see a comprehensive review given by Turner & Ferrante 1979). They have also been used in taxonomic investigations (Kraeuter & Haven 1970). But despite their importance and the wealth of studies about pellet production in the zooplankton and their contribution to the energy flow in the marine ecosystem, the study of their morphology is reduced to a few accounts where morphological observations are made incidentally. Martens (1978), Hiromi, Hiyama & Kadota (1988) and Uye & Kaname (1994) are among the few works that have studied the morphological specificity of the copepod faecal pellets and Lautenschlager, Kaushik & Robinson (1978) seems to be the only instance where the structure of faecal pellets has been

studied under SEM. The present study, therefore seems to be the first account of the detailed morphology and composition of faecal pellets in parasitic crustaceans.

Basically two types of faecal pellets were produced by *L. salmonis*. The so-called clear and dark pellets of the cylindrical type that is continuously voided by the lice when they are on their host and the tape type. The contents clearly demonstrate that the main food item is the host's epithelial cells. The presence of melanin granules in the dark pellets is indicative that lice are reaching the dermal layer during their feeding. But the absence of melanin granules in the faecal pellets does not always mean that they are feeding superficially, but could depend on the state of pigmentation of the skin of the host. On a silvery skin the melanin in the melanophores of the skin would be concentrated in the centre of the cell (Alberts *et al.* 1994) and therefore less available. When a louse feeds on a fish with silvery skin, the chances to ingest melanin probably are less compared to those of a louse feeding on a dark skin. In the latter, the melanin is dispersed throughout the cytoplasm of the melanophore and therefore more readily available.

Peters (1992) mentions that in most crustaceans the faecal pellets are simple rod-like forms which may be tapered at the ends. This statement applies also for the faecal pellets of *L. salmonis*. The tapered ends were not symmetrical, and an anterior rounded end could be distinguished from the posterior tapered end. Both ends were sealed by the peritrophic envelope. This contrasts with the faecal pellets voided by *Gammarus lacustris* Sars where nearly all had an open-ended envelope (Lautenschlager *et al.* 1978).

Peritrophic membranes seem to be an ancestral and constant feature of the Crustacea (Peters 1992). The chitin-containing microfibrils that make up the peritrophic membranes can be arranged either in a random or felt-like structure or an hexagonal honeycomb texture. In *L. salmonis*, the peritrophic membranes surrounding the faecal pellets are of the first kind, suggesting that the peritrophic envelope is formed at the tips of the microvilli of the enterocytes. An hexagonal texture is characteristic of species in which the polymerisation of the microfibrils occurs in the interstices of the microvilli (Peters 1992). Peritrophic membranes have been recorded before in a number of copepods (Yoshikoshi & Ko 1988), and the presence of chitin was confirmed by the

chitosan test. Other aspects of peritrophic membranes in *L. salmonis* will be discussed in Chapter 5 and 6.

The shape of the faecal pellets of *L. salmonis* was relatively constant, although they were not measured systematically, their length seem to vary according to the size of the louse voiding it. The correlation of pellet dimensions with the animal body size has been recognised in several instances. Hiromi *et al.* (1988) studying the faecal pellets of several planktonic copepods found that all were cylindrical in shape but their size (length to width ratio) was strongly associated with the width of the copepod anal segment, and therefore, quite specific. Martens (1978) also had found this, particularity of faecal pellets in planktonic organisms and suggested that the analysis of the distribution of the size of faecal pellets in a natural population could lead to identification at the species level. Similar findings were made recently by Uye & Kaname (1994), where the pellet volume was highly correlated with animal body size for copepods (10 species combined).

Bron *et al.* (1993) reported faecal pellets produced by the larvae of *L. salmonis* and described them as possessing a rigid peritrophic envelope. Although no account was made concerning the composition of these faecal pellets, judging from the photograph (their Plate IIId) they are very similar to those observed in this study in the sense that they seem to be packed with epithelial cells from the host' skin. They did not have the chance to observe the faecal pellets *in situ*, as, as they mention, the specimens appear to void the gut contents when stressed or at the moment of fixation. Probably this is also the reason why Nylund *et al.* (1992) did not report faecal pellets or peritrophic membranes in the gut of *L. salmonis* in their study. In the present work, it was observed that when a weak fixative was used, the stressed lice took longer to die and in the process they voided the gut contents. Karnovsky-type fixatives, as the one used by Nylund *et al.* (1992) had this effect. If a stronger fixative is used, for example 4% gutaraldehyde, the louse dies very quickly and the gut contents generally stay in place, particularly if the contents are solid faecal pellets. Another way to secure the gut contents in place is to tie the anus as was done in the present study, although this would be impractical in the smallest larval forms.

Gelatinous ribbon or tape pellets as those described in this study are seldom mentioned in the literature. Lautenschlager *et al.* (1978) reported pellets with a watery gel-like consistency which

were produced by *Gammarus lacustris* that had been previously starved for 24 h. These pellets seemed to consist of peritrophic membranes only, with little food material from previous feedings. They also report this type of envelope extending over one end of some faecal pellets like a collapsed transparent sheath. A similar trailing membrane is depicted, although not mentioned, for the faecal pellets of larval *L. salmonis* (Bron *et al.* 1993) and it was a common feature in most of the pellets observed in the present study.

Tape pellets seem to be constituted of sloughed enterocytes, mainly A-cells, from the louse midgut epithelium surrounded by peritrophic membranes. What is particular to these tape pellets is that they are observed in lice that are not eating or lice that are put on starvation. This indicates that although there is no digestion, the cell loss -and probably the cell renewal- in the midgut epithelium continues. R-cells are thought to be involved in detoxification by accumulating undesirable materials, for example metals, in their cytoplasm (Hopkin & Nott 1979, Al-Mohanna & Nott 1987b). It is possible then that tape pellets are the result of the elimination of waste products which are routed through the midgut epithelium.

When lice are not producing tape pellets, it is usually a sign that they are under some kind of physiological stress. The finding of only 2 lice (of several hundreds) with pinkish guts and just a few pellets in the sediments of that sample of the 11/Jan/96 (see section 4.2.4.1.2) was very unusual, as commonly one can find 16-17% of red-gutted lice (see section 4.2.3.2.1) and hundreds of tape pellets in a "normal" sample. The pinkish guts mean that probably the last blood meal of these 2 lice was 10 to 12 hours previously (see section 5.3.1.1) and this could be the case for the rest of the lice in the sample. It is possible that the combination of low temperature and low salinity affected the lice to an extent that they stopped feeding, and even stopped the "excretory" activity of the midgut, if we consider the presence of A-cells in the tape pellets as an indication of waste disposal. Another indication that lice which do not produce tape pellets may be physiologically compromised is that these lice die within 2 days of stopping voiding pellets. Peritrophic membranes have been related to excretion in other groups of arthropods. DDT-resistant strains of the mosquito larvae *Aedes aegypti* L excreted nine times as much peritrophic membranes and six times as much DDT as a susceptible strain. The membrane protruding from the anus reached a length of 3

mm, about half as long as the mature larvae, and it was not produced in the absence of DDT (Peters 1992). The size of the tape pellets voided by *L. salmonis* in the present study could reach in some cases almost the whole length of the louse. In Onychophora, peritrophic membranes are used also to wrap crystals of uric acid as excretion products (Peters 1992).

More studies are necessary to establish what are the exact causes of cessation of the production of peritrophic membranes but this has also been observed in free living marine copepods in certain cases. Over-wintering is associated with drastic physiological changes, such as reduced metabolism, discontinuation of feeding, lack of faecal pellet production, empty stomachs, decreased digestive enzyme activities and reduced midgut epithelium (see Hallberg & Hirche 1980 for citations). Some of these physiological conditions have been thought to indicate some kind of dormancy. Pagano & Gaudy (1986) found that differences of 10 ppm in salinity over or under the natural salinity level led to a decrease in faecal production of the brackish copepod *Eurytemora velox* Liljeborg, suggesting unachieved acclimatisation to salinity variation due to a too short acclimation period before their experiments. It is possible then that certain environmental conditions such as a combination of low temperature and/or low salinity might trigger a similar physiological response in *L. salmonis* that affects directly its feeding behaviour and/or puts them in a sort of dormancy or reduced physiological state. Further experiments which specifically address this issue would be necessary to clarify this.

There are no comprehensive summaries of the presence of intestinal microflora in marine invertebrates and simultaneous observations of guts and faecal pellets are very scarce. Nagasawa (1992) established from his own observations and from the findings of other authors a direct correlation between presence or absence of bacteria in the gut and the presence or absence of bacteria in the pellets and the rate of its subsequent colonisation once they are voided.

Guts with bacteria have been observed in the amphipods *Caprella kroyeri* (Pallas)(see Nagasawa & Nemoto 1988) and *Hirondellea* sp. and in the decapod *Homarus vulgaris* L. (see Boyle & Mitchell 1978) the copepods *Calanus pacificus, C. plumchrus* Murakawa, *Eucalanus bungii* Giesbrecht and *Acartia omorii* Dana(see Nagasawa & Nemoto 1988, Nagasawa 1992). In these copepods and the amphipod *C. kroyeri,* ready colonisation of the faecal pellets by bacteria was observed within 2

hours after the pellets had been released. Other copepods possessing gut microflora, but unconfirmed presence of bacteria in their faecal pellets, are *Acartia tonsa, Pontellopsis regalis* Harlan, *Labidocera aestiva* Wheeler, *Centropages furcatus* L. and *Pleuromamma* sp. Giesbrecht (Sochard, Wilson, Austin & Colwell 1979). Nagasawa (1992) postulates that in the faecal pellets of these copepods, probably a rapid bacterial colonisation takes place since they possess gut microflora.

In contrast, digestive tracts without bacteria have been reported in the marine wood-boring isopods *Limnoria tripunctata* Menzies and *L. lignorum* L. the wood-inhabiting amphipod *Chelura terebrans* Philip and the terrestrial isopod *Oniscus asellus* (L.) (Boyle & Mitchell 1978), in the copepod *Pseudocalanus minutus* Krøyer, and the amphipod *Themisto japonica* Bovallius (Nagasawa & Nemoto 1988, Nagasawa 1992) . In all these cases, the faecal pellets are not readily colonised by bacteria for up to 24 hours.

Freshly voided faecal pellets of a number of other copepods (see Nagasawa & Nemoto 1988 and Nagasawa 1992) and in the gammarid *Gammarus lacustris* (Lautenschlager *et al.* 1978) are reported to be devoid of bacteria and are colonised by ambient bacteria only after several hours or days, and according to Nagasawa (1992), it is likely that the guts of these crustaceans do not have microflora.

The findings in this study contribute to corroborate Nagasawa's hypothesis. No bacteria were found in the gut or in the freshly ejected faecal pellets of *L. salmonis*, and the faecal pellets were colonised by bacteria only after about 18 hours of being voided, suggesting that the same inhibitory mechanism also takes place in the faeces of this parasitic copepod. Nylund *et al.* (1992) reported the presence of bacteria in the gut of some specimens of *L. salmonis*, inserted parallel to the microvilli and also inside the enterocytes, but concluded that these bacteria were not common to the gut because the majority of the individuals observed did not have it. These specimens with bacteria in their guts may be the same sort of individuals that presented pale guts in this study and believed to be morbid.

The mechanisms involved or causes of this event are still unknown and more simultaneous observations of gut and faecal pellets are necessary for a better comprehension of this issue (Nagasawa 1992). The present study is the first simultaneous observation of guts and faecal pellets of a parasitic crustacean.

5. PHYSIOLOGY.

5.1 MATERIALS AND METHODS.

5.1.1 Function of the digestive system.

5.1.1.1 Observations on red gutted lice.

Lice with conspicuous red guts were collected from cultured salmon between 7-8 AM in fish farms off the west coast of Scotland. Lice were transported to the lab as detailed in Chapter 2. On arrival at the laboratory lice, still with conspicuous red guts, were selected and accommodated in a device to observe them individually without disturbing them. The device consisted of two rectangular glass plates 15x4x0.5 cm and a piece of garden plastic mesh of ½ inch mesh size. The thickness of the mesh was 3 mm. The plastic mesh was cut to the same dimensions as the glass plates such that the final piece of mesh consisted of 2 rows of 9 squares of ½ inch each. To load the lice in the device, the plastic mesh was put on the first glass plate, then, one louse was put in each square. Once all the squares had a louse in them, the second glass plate was put on top of the mesh and the two glasses were kept together by means of two rubber bands. Each square was numbered on the glass and the whole device was immersed in a bowl with aerated sea water. The system was kept in a cold room at 10 °C during the length of the observations. This device accommodated 18 lice. Observations began at 1 PM. Every two hours the bowl was observed under the dissecting microscope and the state of the gut of each lice recorded (gut movements, colour, presence of faecal pellets, general activity).

5.1.1.2 Video recording.

During the observations of lice under the dissecting microscope, there were certain details of the activity of the digestive system that were difficult to determine due to the resolution of the microscope and the speed at which they happened. To overcome this difficulty, a compound microscope equipped with a video recording equipment was put to use.

The same chamber used to hold the lice for manual feeding (see section 5.1.3) was utilised for these observations. Because its dimensions were the same as a glass slide, it fitted well into the microscope stage. Red-gutted lice from collections in the fish farms or from manually fed lice were loaded in the chamber as described in section 5.2.4. Observations were carried out under 1X, 4X and 10X objectives and recorded on a videotape for further analysis. As the equipment was in a laboratory kept at ambient temperature (18-20 °C), the water in the chamber was continually renewed with seawater kept in ice to maintain a low temperature for the lice. In this way a louse could be maintained in apparently good condition during the observations, which lasted between 30 to 60 min.

5.1.2 Structure of the midgut under different physiological conditions.

From the observations in this study, lice were seen to feed in normal conditions either on blood or skin epithelium, or both. The aim here was to assess if there were any differences in the structure of the midgut under these two basic feeding conditions. A third condition was added, starved lice, as a form of induced pathology to appraise any changes in the midgut structure under "abnormal" conditions.

The chitin stain detailed in Chapter 4 was applied to selected ultrathin sections to evaluate the presence of chitin in the peritrophic membranes.

5.1.2.1 Red gutted lice.

Lice with conspicuous red guts were collected from cultured salmon, the anus tied with a hair, fixed immediately in MIF fixative or modified Karnovsky and processed for LR White embedding, sectioned and stained as detailed in Chapter 2.

5.1.2.2 Lice feeding on fish epithelium.

A number of lice used in the observations detailed in section 4.4.2. that had fed on salmon skin were processed for LR White embedding, sectioned and stained as detailed in Chapter 2.

5.1.2.3 Starved lice.

Lice collected from cultured salmon were put in an aquarium in a constant temperature room at 10°C. Every two days a sample of 4 lice was taken and processed for LR White embedding, sectioned and stained as detailed in Chapter 2.

5.1.3 Manual feeding.

A method was designed to allow the artificial feeding of lice with different substances. The system consisted of a dissecting microscope, a micromanipulator and a specially designed chamber to hold the louse immobile during the procedure.

The chamber was made with glass slides and coverslips. Figure 5.1 illustrates and describes how the chamber was assembled. The chamber has a square well in the middle where the louse was placed on its dorsal surface and immobilised by a lid. A slit of about 1 mm wide was left in the middle of this lid. This slit allowed an easy access to the oral cone while restricting the movements of the louse at the same time.

Micropipettes were made in a capillary glass tube stretcher. The capillary glass had a length of 45 mm with 0.25 and 0.1 mm of external and internal diameter respectively. The micropipette end was cut to give a tip with an external diameter of approximately of 45-50 μm

The micropipette was connected by Teflon tubing to a 800 μl microsyringe equipped with a knob to control of the quantity to be dispensed. The minimum graduation was 0.1 μl.

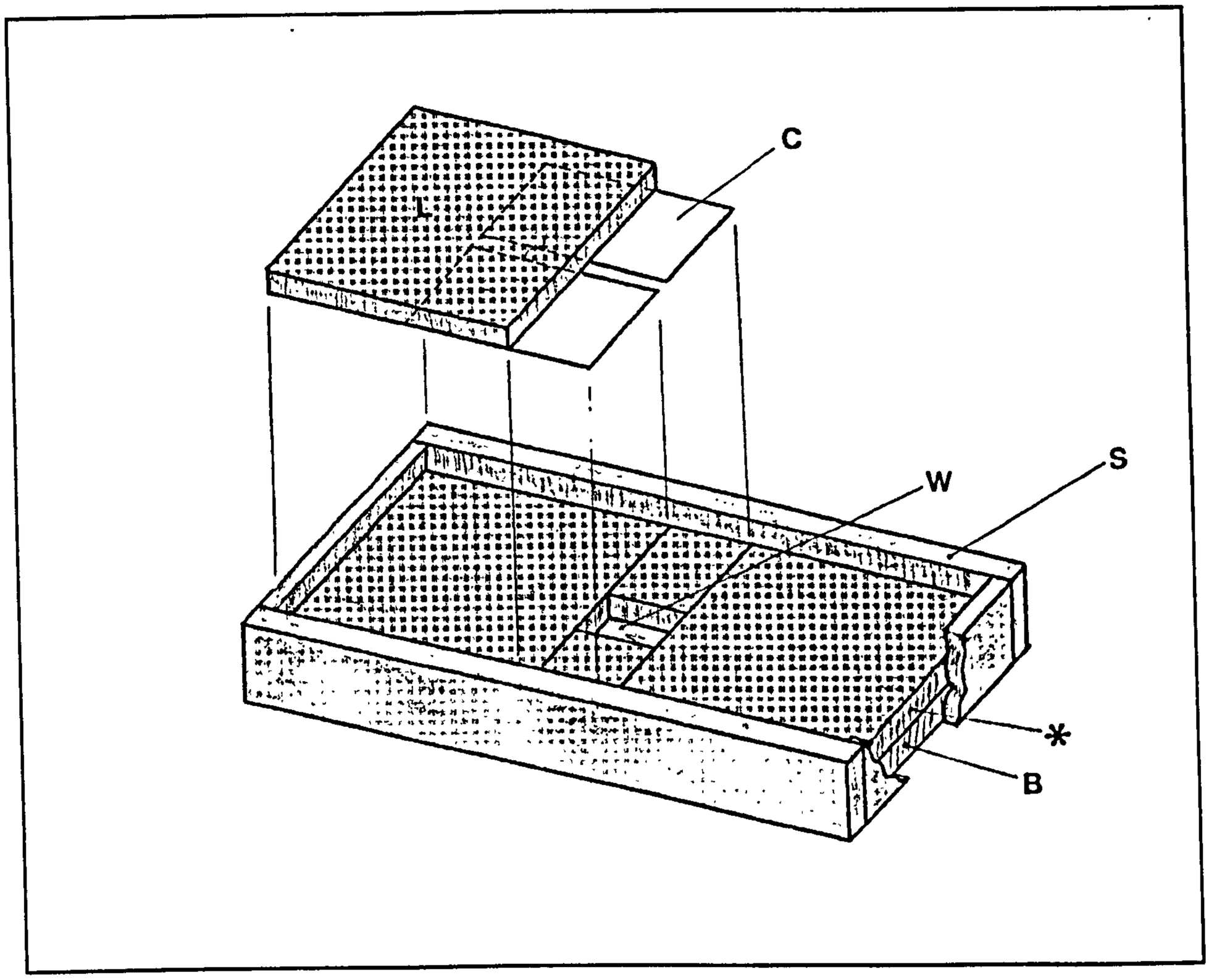

Figure 5.1 Illustration of the glass chamber used to hold the lice during manual feeding procedure. The chamber was made with glass slides and coverslips. A glass slide served as a base (B). Another glass slide (asterisk) was cut in four pieces and glued to the base leaving a space in the centre which formed a well (W). Four glass slide strips (S) were cut and glued to the sides of the chamber. This chamber could hold about 10 ml of seawater. The lid (L) consisted of a square of glass slide to which two pieces of coverslips were glued underneath it as shownand leaving a slit of about 1 mm in the centre. A louse was put on its dosum inside the well and covered with the lid. While the coverslips held the louse immobile the slit allowed easy access to the mouth cone. .

The manual feeding process was carried out in the following manner:

The micro syringe and the tubing of the micromanipulator system were filled with liquid paraffin taking care that no air bubbles were left. The micropipette was then filled from the wide end with the liquid to be injected using a 1 ml syringe fitted with a needle. A meniscus was left at the end of both the micropipette and the tubing filled with liquid paraffin, so when these two were joined there were no air bubbles trapped in between.

A louse was then loaded into the chamber, a drop of seawater added in the well containing the louse and the lid put over it. The chamber was then placed in a plastic Petri dish and the Petri dish and the chamber filled with fresh, cold seawater (at the holding temperature of the cold room,

10°C). This was necessary to avoid a rapid rise in the temperature of the water due to the heat generated by the lamp of the dissecting microscope. Working now under the dissecting microscope. the Petri dish was manipulated with one hand while the micromanipulator was operated with the other. The micropipette was brought towards the oral cone of the louse at an angle of about 35°. Coarse movements to direct the tip of the pipette into the oral aperture of the louse were done with the hand holding the Petri dish while micromanipulating with the other to insert the tip of the micropipette into the mouth. To do this, it was necessary first to lift the frons labri with the tip of the pipette and then slide it into the mouth.

Two ways of feeding the louse were tried. One was placing the tip of the micropipette just in the oral aperture and letting the louse suck the liquid by itself, which was delivered by capillarity from the micropipette. The other method was carried out by introducing the micropipette all the way up to the back of the buccal cavity, pressing the tip against the opening of the oesophagus and forcing the liquid into the oesophagus.

Although the louse could be fed well with the first method, it was very slow. The louse would gulp the liquid gradually but it could take more than 20 minutes to half-fill the gut. The second method was adopted instead. In this way, the gut could be filled in seconds with the sample liquid with no perceptible negative effect on the louse.

During the observations detailed in section 4.4.1, it was noted that the proportion of lice that fed once they were on the fish was very variable. To increase the probability that the lice chosen for manual feeding were lice "eager" to eat, one day before the manual feeding, several lice were put onto a neutral-red stained salmon smolt for 5 hours, picked up from the fish and checked. Those with evidence of food in their guts were selected for manual feeding for the next day.

5.1.3.1 Salmon blood.

Blood was taken with an heparinised syringe from the caudal vein of naive smolts, where possible, or from cultured marine salmon when naive smolts were not available. The blood was kept in ice or in the fridge and used within 24 hours. Lice were fed this blood manually as detailed in the previous section.

5.1.3.2 Milk.

This was an attempt to evaluate morphological changes in the midgut of the lice after a meal rich in lipids. Fresh cream was tried on a few lice but it coagulated in contact with water and it could not be readily swallowed by the louse. In one instance some milk coagulum remained stuck for several hours in the middle of the oesophagus of one louse. Diluting milk with distilled water did not work either.

Evaporated non-skimmed milk was tried instead with success. The composition of the milk according to the label of the product was 0.2% NaCl, 5% protein, 22% solids and 9% fat, of which 5.8% was saturated and 0.2% unsaturated. Ten µl of 1% neutral red was added to 200 µl of evaporated milk and this mixture was fed manually to the lice as detailed in section 5.1.3. Neutral red was added to the milk because it helped to visualise better the solution while it was being injected into the lice.

Seven lice were used. These lice were chosen from a batch of lice that was known to have fed on an experimental salmon smolt and they were then starved for 3 days. After the manual feeding, lice were put into individual containers of fresh sea water which was constantly aerated and later fixed with MIF fixative (see Chapter 2) after different times: two lice after 50 minutes, two after 1:30 h and then one louse after 3:30, 4:30 and 5:30 h. After fixation, 4 lice (0:50, 1:30, 3:30 and 5:30 h after feeding) were processed for LR white embedding as detailed in Chapter 2. The other 3 lice (0:50, 1:30 and 4:30 h after feeding) were processed first for lipid staining using the p-phenylenediamine method (Boshier *et al.* 1984). It consists of soaking the tissue in 1% ρ-phenylenediamine (Sigma P6001) in 70% ethanol or acetone after the osmication step in the dehydration rinse and a 70% ethanol or acetone rinse before dehydration and embedding are continued. In this case, ethanol was used as the LR White embedding is incompatible with acetone.

5.2 RESULTS.

5.2.1 Function of the digestive system.

5.2.1.1 Macroscopical changes and activity of the digestive tract of lice which had fed manually on salmon blood.

The appearance of the gut of the 18 lice observed was variable in terms of the colour that the gut contents gave to the digestive tract of the lice. The colours observed at the start of the observations were red, dark red, brown red and pale pink.

Table 5.1 State of the gut at the start of the observations and time elapsed for the gut to be clear of red material.

Colour	No. of lice	Time (hr) elapsed to reach a clear gut	Average (SD)
Red	10	8,10,10,12, 14 16,18,24, 24,26	16.2 (6.5)
Brown red	2	6,6	6
Pale pink	6	3,4,6,8,8,10	6.5 (2.6)

During the time the observations lasted the colour changes seem to occur in this order: Red ⇨ pale red or pink ⇨ yellowish-brown ⇨ greenish ⇨ clear.

The state of the gut at the start of the experiment, the number of lice and the time it took to each lice to show a greenish or clear gut is shown in Table 5.1 .

Adding the time between the collection and start of the experiment (about 5 hr) it is shown that the time needed for the gut to lose the red colour is in the range of 13 to 31 hours.

Between the lice with red guts, there were some variations in the intensity of the red: dark red, bright red or pink. The only one with dark red took 14 hours to lose the colour. The only pink one took 18 hours. These two were included with the "reds" in the calculations for the Table 5.1 .

The activity of the gut was variable, with periods of no or slight contractions of the circular muscles and very active periods when peristaltic movements could be seen along the whole length of the midgut or in specific areas of the anterior, mid or posterior midgut.

To help describe the movements of the material inside the digestive tract, areas of the midgut will be referred as letters. Refer to Figure 5.2 for the localisation of these areas.

No specific pattern of peristaltic movements was observed, although a common one was periods of a continuous peristalsis from the posterior midgut (G) to the mid midgut (B) and back peristalsis from the anterior caecum and anterior lateral lobes (E) to the junction of the anterior-mid midgut (D).

In some instances the contractions began at the posterior midgut, pushed the fluid towards the valve at D which remained closed, then it opened quickly and the fluid filled the anterior part of the anterior midgut (E). The muscles in the latter then contracted and the material was pushed again towards the valve. As this happened and because the valve remained closed, the lateral lobes (C) at this part of the midgut expanded, receiving the fluid. The valve then opened again to let the fluid pass through the mid midgut.

In other cases, the valve remained closed and the fluids moved back to the anterior part when its muscles relaxed again.

As the colour of the midgut reached the pale pink stage, the activity of the midgut decreased. A greenish or clear gut would hardly have peristaltic or other gut movements.

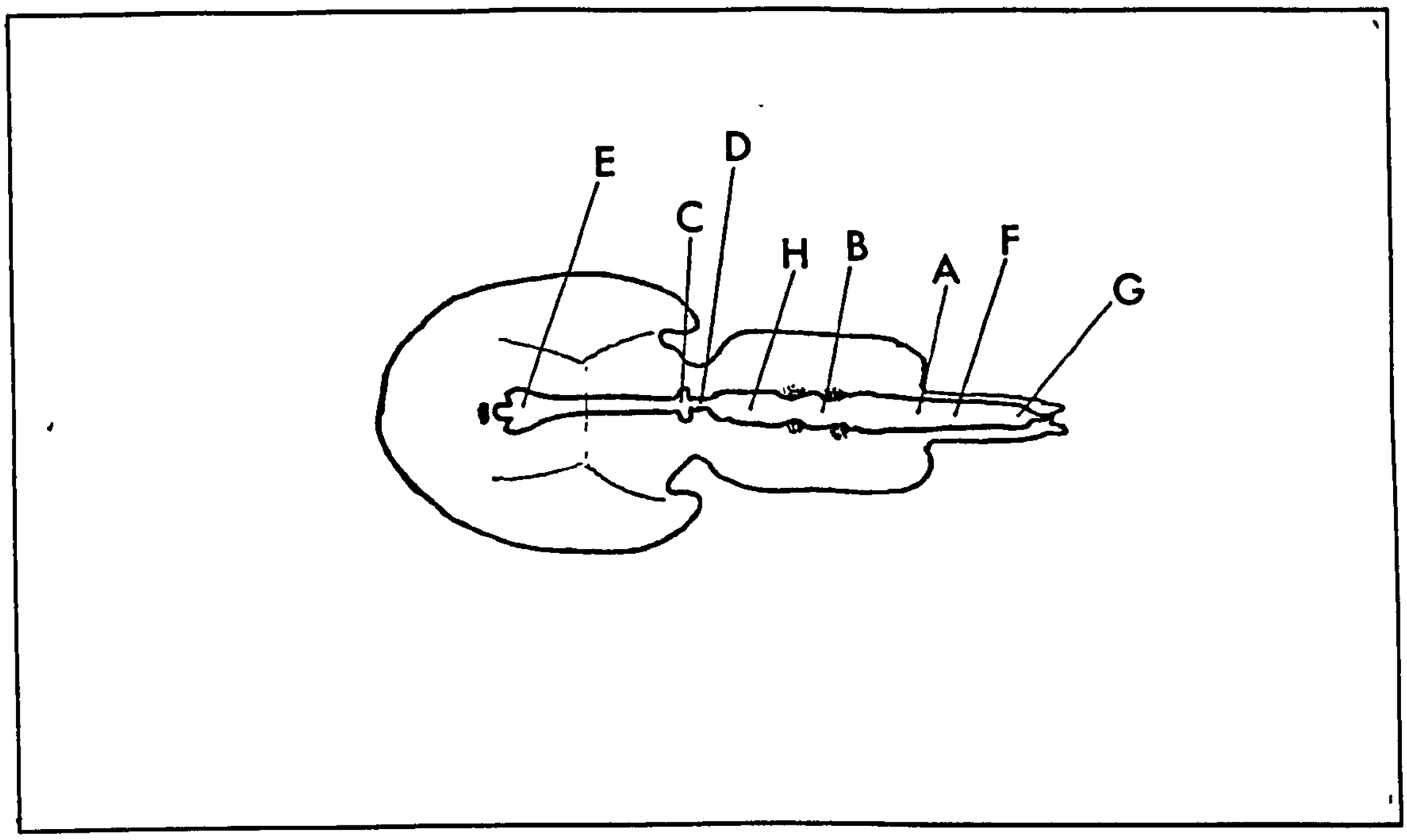

Figure 5.2 Regions of *L. salmonis* midgut. See text for details. A: boundary between mid and posterior midgut; B: mid midgut; C: posterior part of anterior midgut, including lateral lobes; D: Valve; E: Anterior caecum and ventrolateral lobes; F: posterior midgut; G: posterior part of posterior midgut; H: anterior part of mid midgut..

Seven lice had food other than blood in their guts at the start of the experiment. Epithelial cells from their hosts formed dark pellets that were voided within 6 hours of the observations, mostly between the first two or four hours. They were usually at the posterior midgut although they could also be seen in the mid midgut. During the peristalsis the pellet did not move. Only when the pellet was about to be expelled did the peristalsis, in this case in antero-posterior direction, move the pellet.

Tape pellets were also produced during the observations. In two cases where the process was actually seen, two lice produced this kind of pellet which was about half their length. The continuous voiding of these pellets lasted at least 2 h but always within 4 h. The production of the tape-pellet was by means of alternate contractions of the muscles of the hind gut which shape it and "pull" it towards the exterior (see section 5.2.4 for more detail about the defecation process).

5.2.1.2 Observations during manual feeding.

As mentioned in the methods section, two ways of manual feeding of the lice were used.

The first one, letting the louse "suck" the fluid from the tip of the micropipette was very time consuming. The louse contracted the labral muscles rhythmically and, in this case, the blood filled briefly the buccal cavity but the blood was not gulped. Instead, it leaked out of the mouth after the labral muscles had relaxed. It was only occasionally that a mouthful of blood was gulped and passed to the midgut. Although this happened very quickly, the dilation of the oesophagus could be appreciated as the blood passed through it before being released into the anterior midgut. It is worthy of note that there were some cases where a louse would not take any blood at all.

Several lice were observed, and each one behaved differently from the others. While one could suck blood avidly from the micropipette and fill its gut in about 10 minutes, another would take 30 or 40 minutes to do so. Nevertheless, a general pattern of the early activity of the midgut after a blood meal could be established and is described below.

Immediately after the blood was sucked it was quickly passed to B. It is not clear how the blood is stopped from passing beyond A as there is no contraction or valve there. For a variable amount of time (between 10 minutes and 1 hour) the blood was moved from B to C and E and back, but without reaching F or G. Blood that is in B is rather "sucked" by a simultaneous distension of C and opening of the valve in D, as there are no contractions of B while this happens. Occasionally B contracted, but in a peristaltic fashion by contraction of the circular muscles. However, the blood there did not move during these contractions. As blood passed to C, it "pushed" the rest to E, which expanded, accumulated there and then started to move in the E-C-D in reverse D-C-E directions several times, keeping D closed. When D opened again, some blood passed to B and the cycle continued.

After 10-20 minutes, blood started to reach F and G, but in small quantities, and remained statically there. Occasionally, peristaltic contractions occurred in this area, but they were mild and did not seem to displace the blood in it towards B.

The valve in D clearly prevents blood from flowing in either direction. It is only when D opens that the blood flows, establishing the importance of this valve in conditioning the movements of food throughout the gut.

After the initial observations during manual feeding, a fed louse was put back in an individual aerated container so that its behaviour following feeding and gut activity could be monitored, including the possible evacuation of a faecal pellet. Every 5 or 10 minutes for the first two hours, and from then on every two hours, the container with the louse was put under the dissecting microscope and the observation of the louse recorded.

The behaviour was variable. In general, a louse would sink to the bottom and remain lethargic for 5 to 10 minutes. Then it reacted and swam to attach to the sides of the pot.

From then on, the movements of the gut would be more or less the same as those described previously, except that the food in the posterior midgut began to be moved into the rest of the gut. After this, the gut contents in all the length of the midgut are mixed by peristaltic movements. The basic pattern observed was as follows.

Reverse peristaltic waves started at the posterior end of the posterior midgut from G to A. From A, the same material was passed quickly to H. This area expanded to hold the material, then contracted, the valve in D opens and the material passes to C, which expands when receiving it, then contracts again and the material was passed to E, which expanded to receive it. It then contracts and the pattern is repeated in the opposite direction. While this is happening, other waves of peristalsis start at G making these basic patterns overlap.

Some lice would keep the red colour of the gut for quite a long time, some 6 to 12 hours, passing through the pink gut stage before becoming clear. During this time, tape pellets were voided by some lice, but with no sign of undigested red blood cells in them. A few lice did not void any type of pellet but cleared the gut in a very short time, 1-2 hours, with bursts of blood that were seen to be coming out from the anus.

5.2.2 *Structure of the midgut under different physiological conditions.*

5.2.2.1 Red-gutted lice.

The lice that are referred as red-gutted came from lice naturally feeding on salmon blood in sea cages.

Under the light microscope, the histological picture of the gut of these lice was very characteristic. The first thing that captured the attention was the high quantity of lipid droplets in the apical part of most of the enterocytes (Plate 5.1). Each R-cell may contain up to 8 large lipid droplets, which sometimes distorted the shape of the nucleus or displaced it to one side of the cell (Plate 5.2). The gut type was usually Medium-Crypted (MC) and the lumen was completely filled with a dense homogeneous material which had many lipid droplets evenly distributed within it. This material stained well for protein, and PAS and was negative for acid mucopolisaccharides. Histochemical tests for haemoglobin or for iron were always negative.

With the light microscope, the microvilli of the enterocytes could hardly be discerned from the homogeneous material in the lumen. Under TEM the microvilli started to be conspicuous by 5000-6000X (Plate 5.3). One common characteristic of the microvilli of red-gutted lice is its profuse vesiculation Plate 5.4 .

Lice with pinkish guts did not show the characteristic homogeneous gut contents of the red-gutted ones. The lumen seemed empty with only occasional small areas of homogeneous material. But the microvilli still showed signs of microvesiculation and some homogeneous material could still be seen associated with the microvilli (Plate 5.5 and 5.6). Lipid droplets in the R-cells were less abundant compared to those of the red-gutted lice.

Although not quantified, the B-cells seemed to be more abundant in blood-feeding lice than in lice feeding on fish epithelium. The type of gut was generally Medium-Crypted but sometimes Deep-Crypted. Lipid droplets were usually fewer and smaller in the anterior midgut compared to the mid or posterior midgut.

Peritrophic membranes were never present in lice that had fed on blood.

5.2.2.2 Lice feeding on fish epithelium

The gut epithelium of lice feeding on fish epithelial cells was usually Shallow- or Medium Crypted type and the Deep-Crypted type was not common. Lipid droplets in the cytoplasm of R-cells, when present, were small and never as abundant as in a blood-feeding louse. The microvilli of the microvillar cells were conspicuous and contrasted well with the lumen, which was usually clear. Pieces of salmon skin epithelium could sometimes be seen loose in the lumen (as in Plate 4.3 in the previous Chapter), or packed as a faecal pellet.

5.2.2.3 Starved lice.

One of the main characteristics of the gut of starved lice was the reduction in thickness of the epithelium as seen histologically. But this was fully evident after 6 or 7 days of starvation. The appearance of the gut during the first 5 days of starvation was very variable. Deep-Crypted type guts were never seen, but Shallow-Crypted and to a lesser extent, Medium-Crypted guts were common. There were not enough morphological differences in these latter cases to differentiate them from a "normal" gut of a lice feeding on fish epithelium.

From day 6, the most common gut type started to be the Thin type, mixed with the Shallow-Crypted and, by day 10, most guts were of the Thin type with occasional foci of thicker cells and with evident reduction of organelles and rough endoplasmic reticulum (Plate 5.7). Measurement of the height of the cells at 5 random points along each one of these four guts yielded an average of 7.17± 2.1 μm (n=20), whereas in a "normal" Shallow-Crypted gut (of feeding lice) the height of the cells *at the thinnest areas* was 12.1±1.7 μm (n=15).

By day 17 the predominant gut type was Very Thin with an average cell height of 3.55±1.9 (n=10), although there were some areas where only the apical and basal membrane of a flattened cell separated the lumen and the basal membrane. When ridges of the gut epithelium were evident, these were made of thin cells (Plate 5.8). The nucleus of a thin cell filled most of the scarce cytoplasm, which appeared clear and with almost no reaction to RNA. Some cells show detachment of the basal plasma membrane from the basal membrane.

By day 20, the last day of the observations, the gut epithelium was extremely thin. The thickest areas corresponded to the bulges of the nucleus of the remaining cells, which apparently had diminished greatly in numbers and therefore had flattened and spread to retain the lining of the gut (Plate 5.9). For example, 5 of these flattened cells covered about 113 μm whereas the same length was covered by 10-15 cells in a normal gut.

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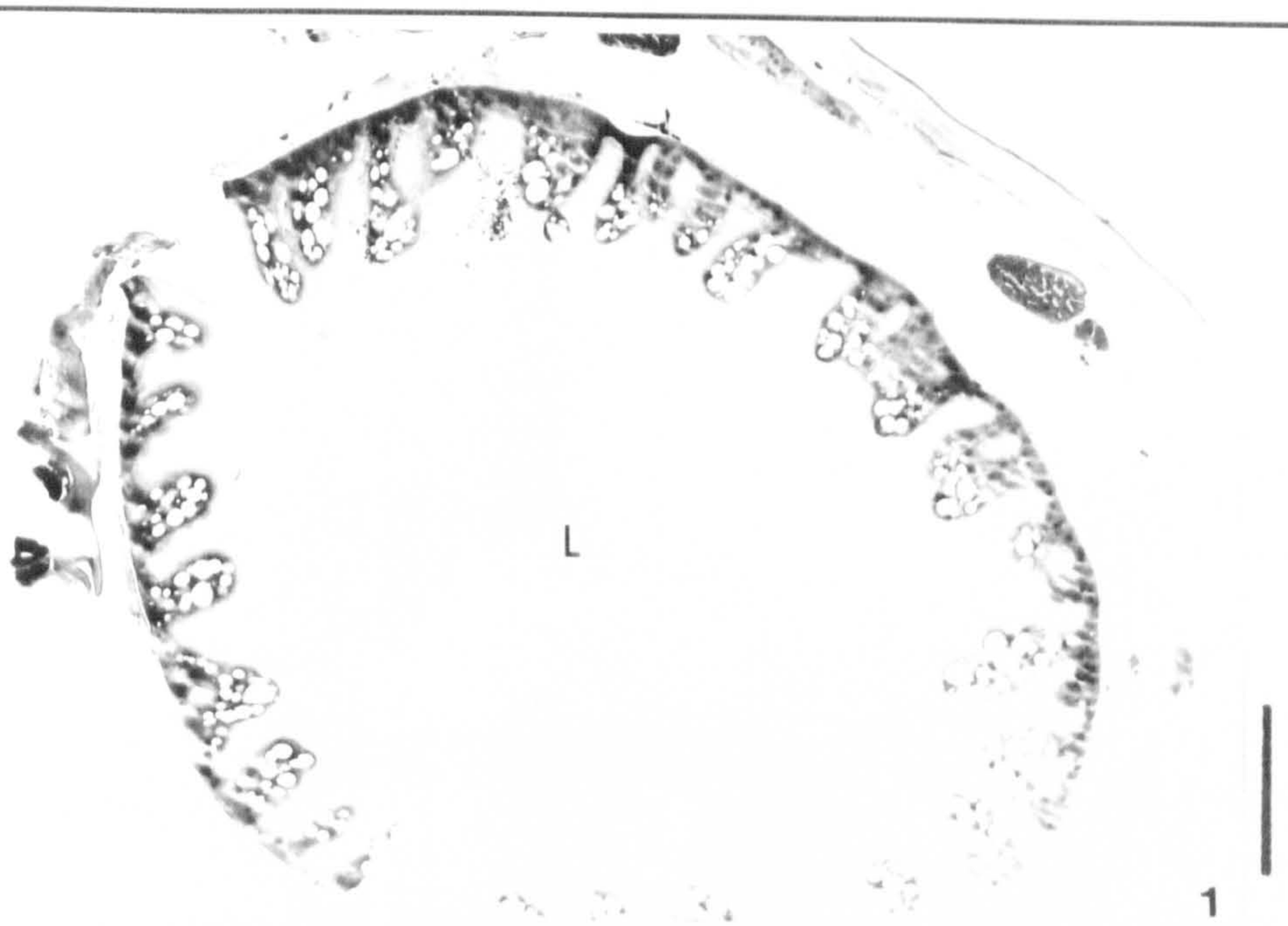
Plate 5.1 TS through the mid midgut of a red-gutted louse. Note the Medium-Crypted gut type, the presence of lipid droplets in the apical part of most of the cells and the homogeneous material in the lumen (L). LMPW. Cason's stain + alcian green. Scale bar 50 μm.

Plate 5.2 Group of enterocytes from a red-gutted louse. Note that the microvilli are barely discernible from the homogeneous lipid droplet-rich material in the lumen (L). A B-cell (stage 3-4) with dense primary lysosomes (arrow) and some E-cells (arrow heads) can be seen. Scale bar 20 μm.

Plate 5.3 Apical part of an R-cell of a red-gutted lice. Note the homogeneous material in the lumen and its lipid droplets. The microvilli start to be evident at this magnification. TEM 5,900X.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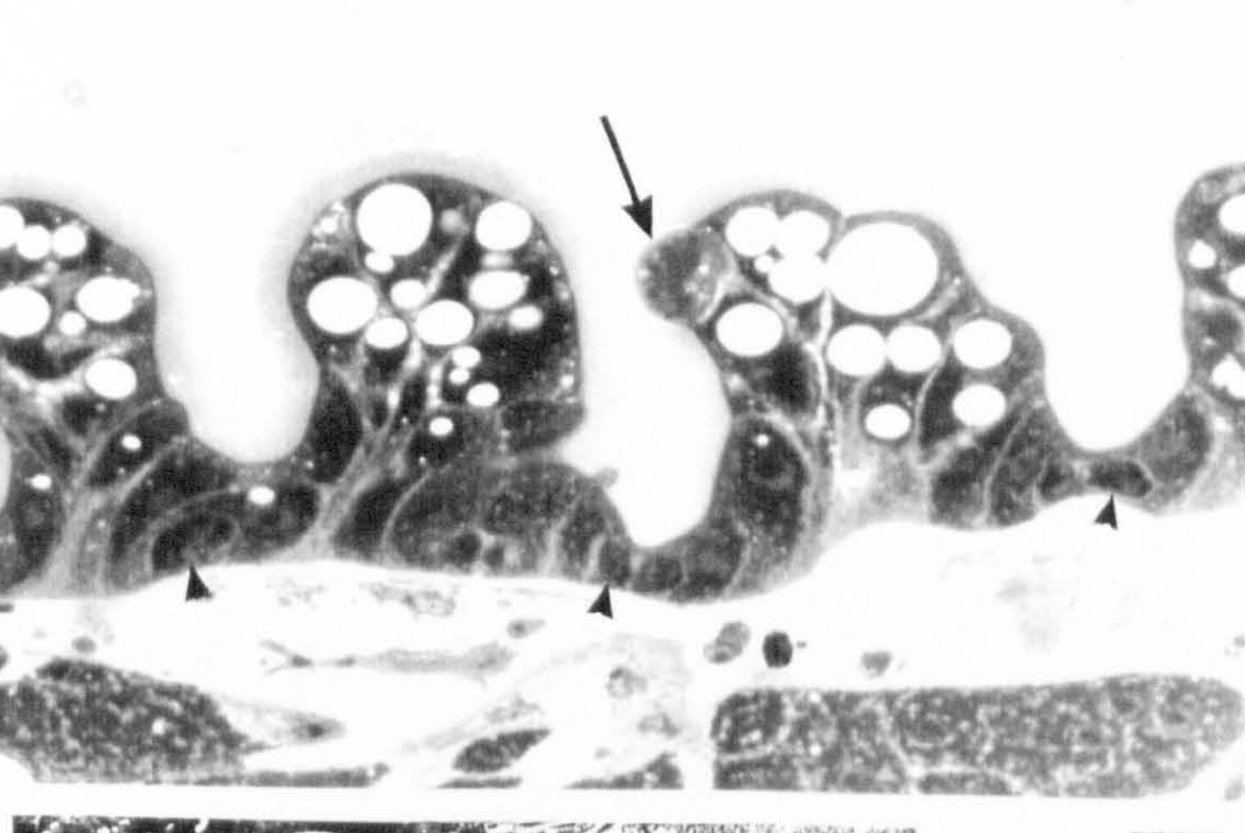
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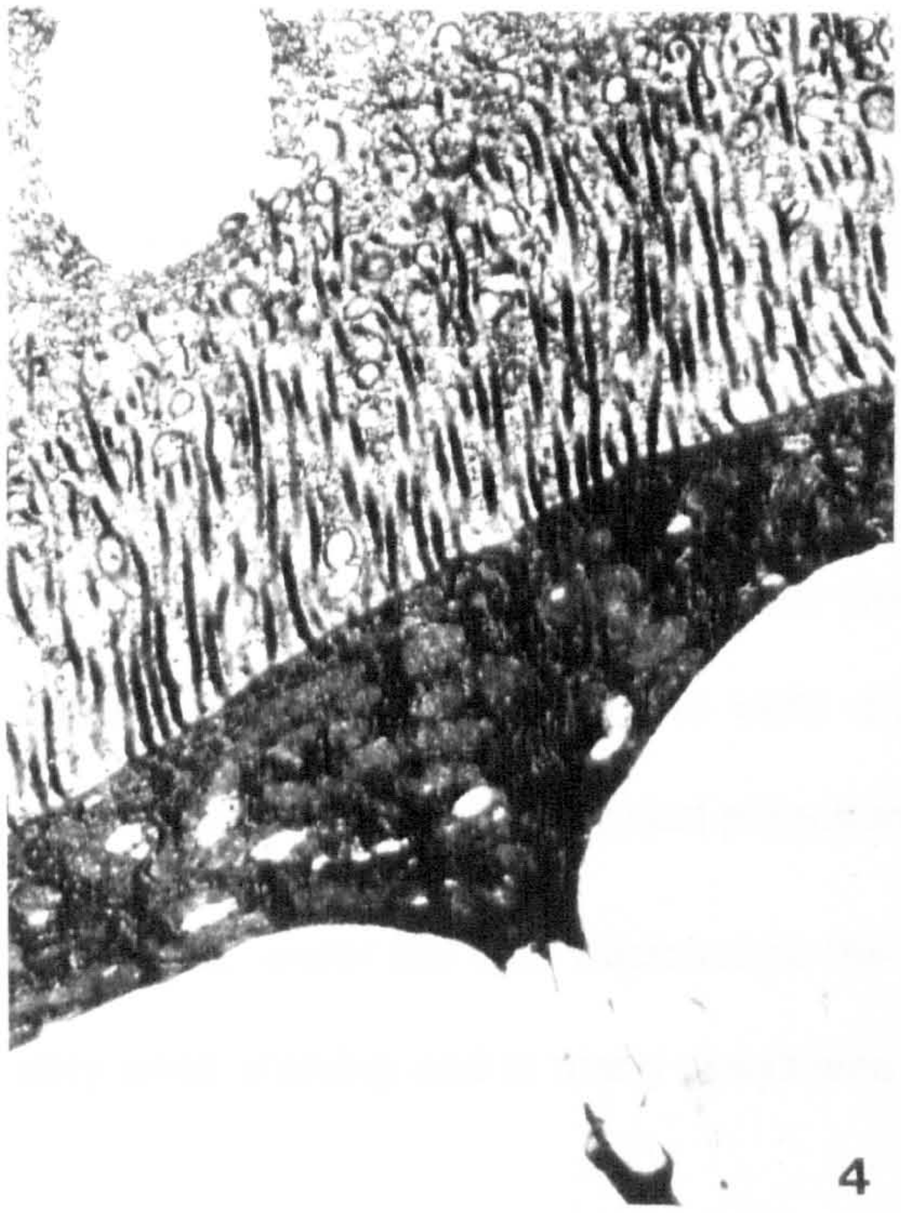
Plate 5.4 Higher magnification of the apical part of the cell depicted in Plate 5.3 . Note the vesiculation of the microvilli and the mitochondria aggregated in the apical zone. TEM 13,000X.

Plate 5.5 Apical part of two R-cells of a pink-gutted louse. There is some homogeneous material in close association with the microvilli (long arrow). The lumen seems otherwise empty. Many autophagic vacuoles (arrow) and mitochondria (arrowhead) can be seen. TEM 4,300X.

Plate 5.6 Higher magnification of the apical part of the cell depicted in Plate 5.5 to show the microvesiculation of the microvilli that still can also be seen in this type of pink-gutted lice. TEM 22,000X.



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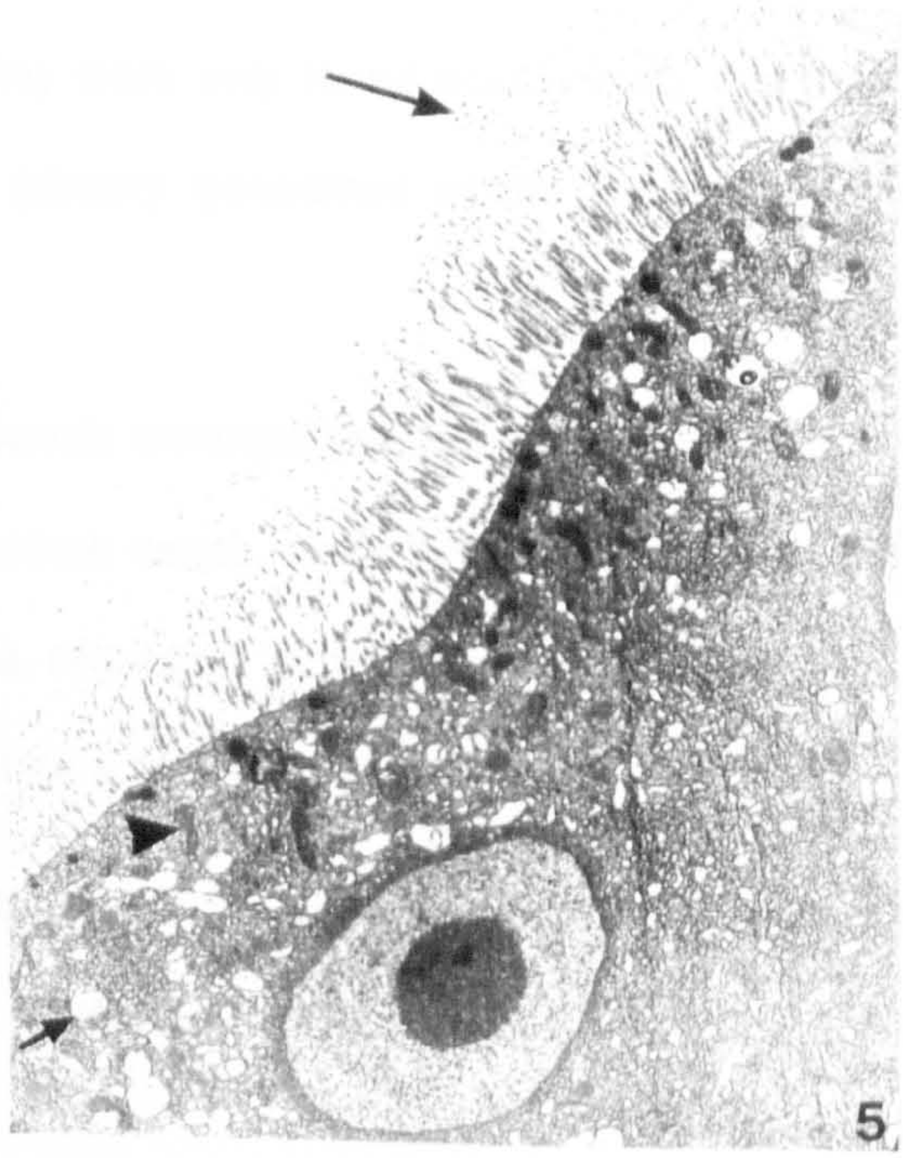

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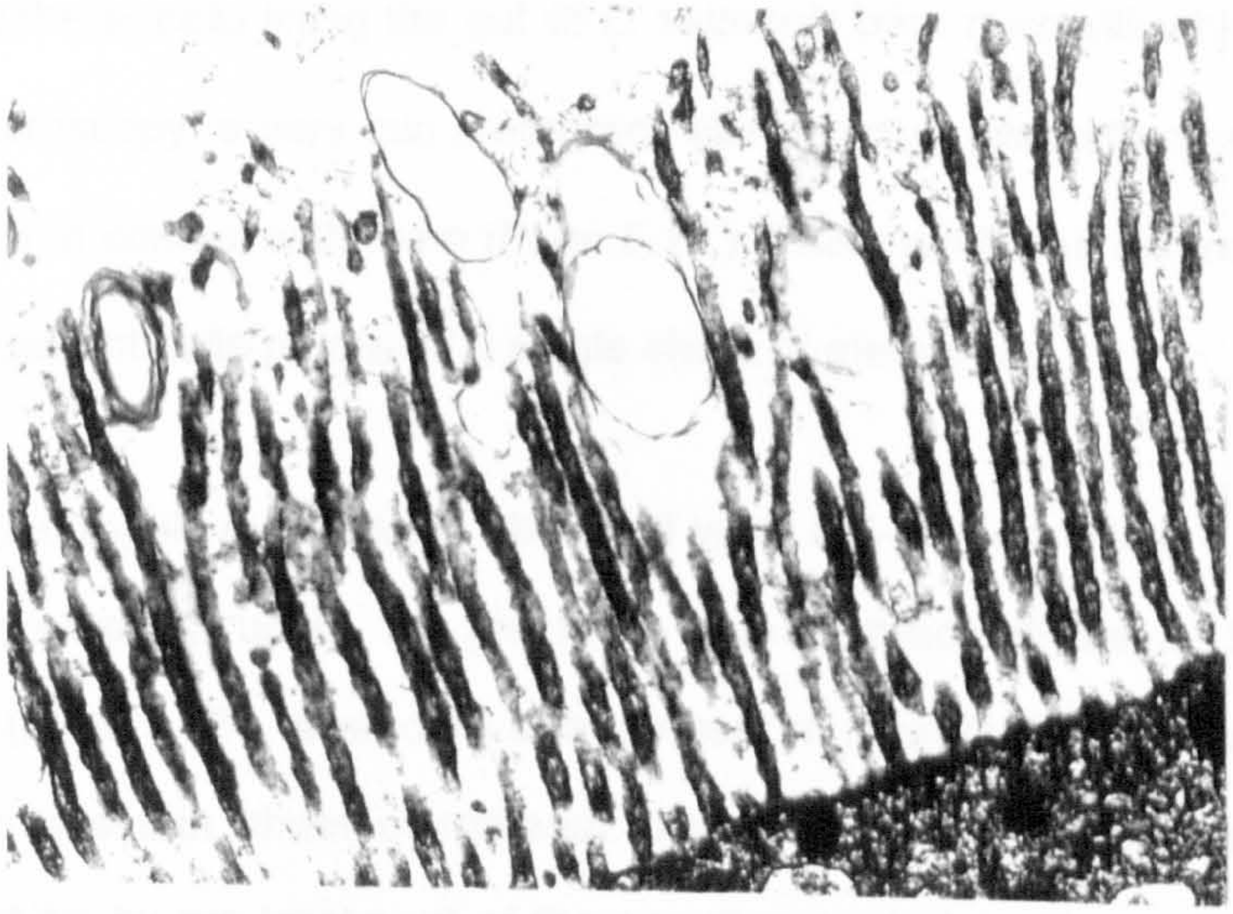

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B cells were scarce in starved lice. By 10 days they were only found occasionally, but they were small and did not show the characteristic large primary lysosomes of the B-cells of a normal epithelium (Plate 5.10).

Under TEM, a common finding from 10 days onwards amongst the flattened epithelium was the presence of large autophagic vacuoles which, in some cases, were larger than the nucleus itself (Plate 5.11). Also, most of the cells showed a reduction in the number of organelles. The occurrence of large hypertrophied mitochondria (Plate 5.12) was also common.

In general, under the light microscope the brush border of the enterocytes of starved lice showed very weak staining and in many cases was almost indistinguishable, usually from day 10 onwards.

5.2.3 Peritrophic membranes.

Most of the epithelial cells lining the gut of *L. salmonis* bear microvilli which extend into the lumen. Under light microscopy, a very thin membrane-like structure was often seen just above the tips of the microvilli. or in contact with them (Plate 5.13). This membrane extended over several cells. As many as 25 adjacent cells produced a single sheet of membrane

The thinnest peritrophic membranes observed were 0.2-0.3 μm (Plate 5.14 and 5.17), but thicker peritrophic membranes, up to 1.5 μm thick were common (Plate 5.15 and 5.18). At higher magnification (Plate 5.16), a random arrangement of microfibrils could be appreciated. WGA-gold was bound to this thin sheet of material. This component of the peritrophic membranes was identified as chitin by pre-treatment of the negative control sections with chitinase, which blocked the lectin binding sites. In these latter sections, no binding of WGA-gold was observed.
Very little binding of WGA-gold was observed between the microvilli and no binding was observed inside the cell itself.

Plate 5.7 TS through the mid midgut of a louse starved for 10 days. Most of the gut is of a Thin type with occasional protuberances. An A-cell is pinching off from the epithelium (arrow). Note that the brush border cannot be distinguished. Scale bar 50 μm.

Plate 5.8 TS through the posterior midgut of a louse starved for 17 days. The epithelium is Very Thin type. Note that although there are ridges, these are made of very flattened cells. The brush border is not evident. Scale bar 50 μm.

Plate 5.9 Flattened epithelial cells of a louse starved for 20 days. The cytoplasm can barely be distinguished and only the nuclei (arrow head) are conspicuous. In some areas (arrow) practically only the cell membranes are separating the lumen from the basal membrane. Scale bar 20 μm.


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Thick peritrophic membranes are probably the result of several sheaths of thin peritrophic membranes packed together. Plate 5.19 shows a section through the posterior midgut in the process of packaging a faecal pellet. The successive deposition of peritrophic membrane sheaths seems to wrap and package the gut contents. The peritrophic membranes produced at the tips of the microvilli do not remain as layers that can be individually distinguished in the final pellet. They seem to coalesce and form, together with the debris of digested material, the matrix and the envelope of the faecal pellet.

Although sometimes small sheaths of thin peritrophic membrane were seen in the anterior midgut, the production of peritrophic membranes seems to be taken place mainly in the mid and posterior midgut, which is where the pellet is formed. A pellet was never seen in the anterior midgut.

5.2.4 Defecation process.

The posterior midgut leads to a cuticularised hindgut. They are separated by a series of strong circular muscles at the end of the posterior midgut, which serve as a sphincter. The hindgut is a laterally compressed chamber made of two lateral, cuticularised convex plates. Plate 5.20 shows the luminal side of one plate. The first two thirds look smooth and have small longitudinal ridges, but at higher magnification, very small cuticular spines of about 0.6 μm in length that point posteriorly become evident (Plate 5.21). Under TEM, these spines stand out as clear sclerotised structures within the cuticle (Plate 5.22). In the last third of the hindgut, the cuticle becomes rough, with abundant small ridges that lead to the anal slit (Plate 5.23). The anal slit walls are quite rough, have many folds and a very distinct band at the edge with small spines pointing posteriorly and of similar size as those found lining the lumen of the hindgut.

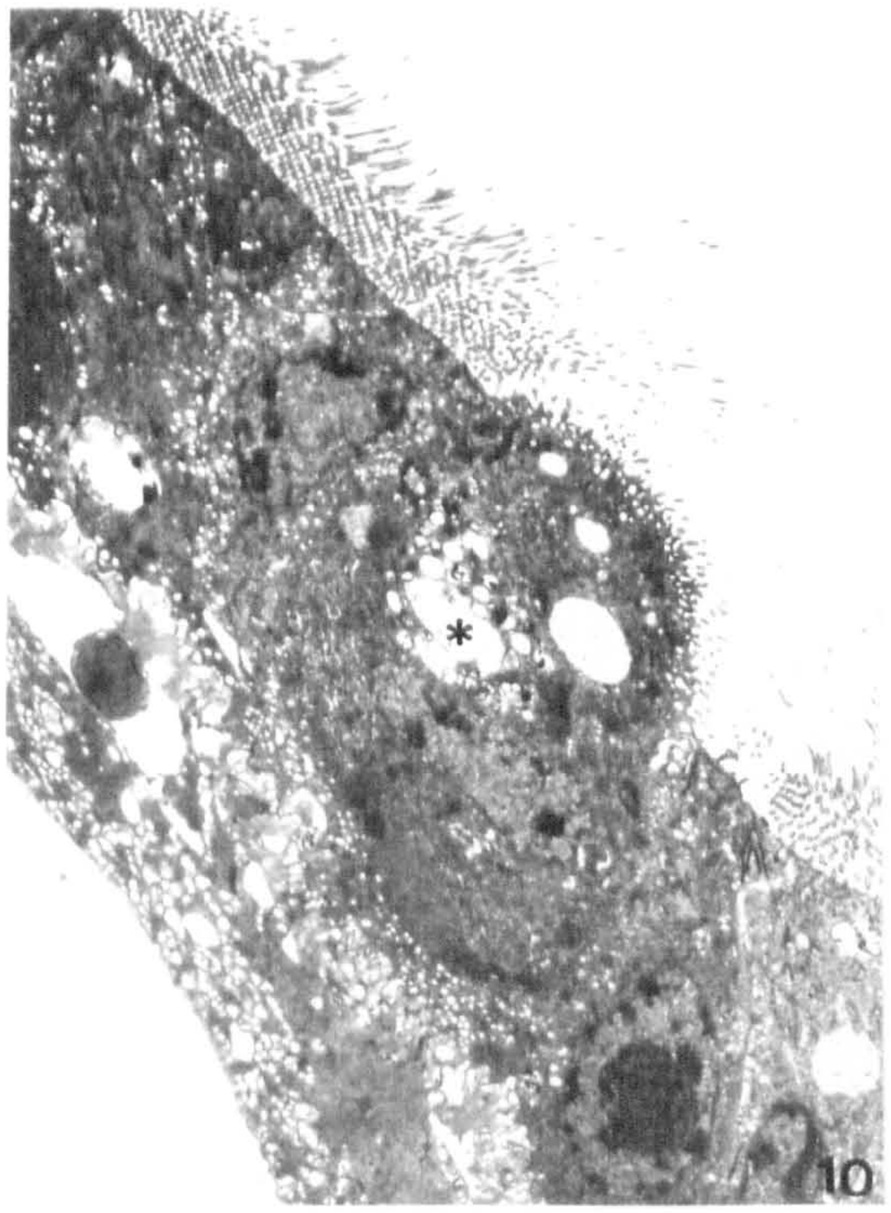
The musculature associated with the hindgut of *L. salmonis* conforms exactly to the description of the same area of *L.. pectoralis* by Boxshall (1990). Seven muscle groups on each side can be recognised (Figure 5.3). These muscles serve to dilate the lumen of the hindgut to accommodate the faecal pellets. These muscles also serve to assist in the voiding of pellets.

Plate 5.10 B-cell of a louse starved for 10 days. Primary lysosomes are scarce and small and what predominates are the electron lucent secondary lysosomes (asterisk). Note the thin epithelium (about 6-7 μm in height) TEM 4,300X

Plate 5.11 Epithelium of a louse starved for 10 days. A huge autophagic vacuole fills most of the cell displacing the nucleus to one side. Note also the hypertrophied mitochondria at the sides of the vacuole. TEM 13,00X.

Plate 5.12 Epithelium of a louse starved for 10 days. Note the hypertrophied mitochondria (arrows) and the autophagic vacuole (asterisk). TEM 7,500X.

Plate 5.13 Transverse semithin section of the midgut. A very thin layer of peritrophic membranes can be seen near the epithelium (arrow head). A low contrast and long exposure had to be used while printing the micrograph to be able to show the thin peritrophic membrane. An A-cell is pinching off from the underlying epithelium (arrow). LMR, toluidine blue, scale bar 10 μm.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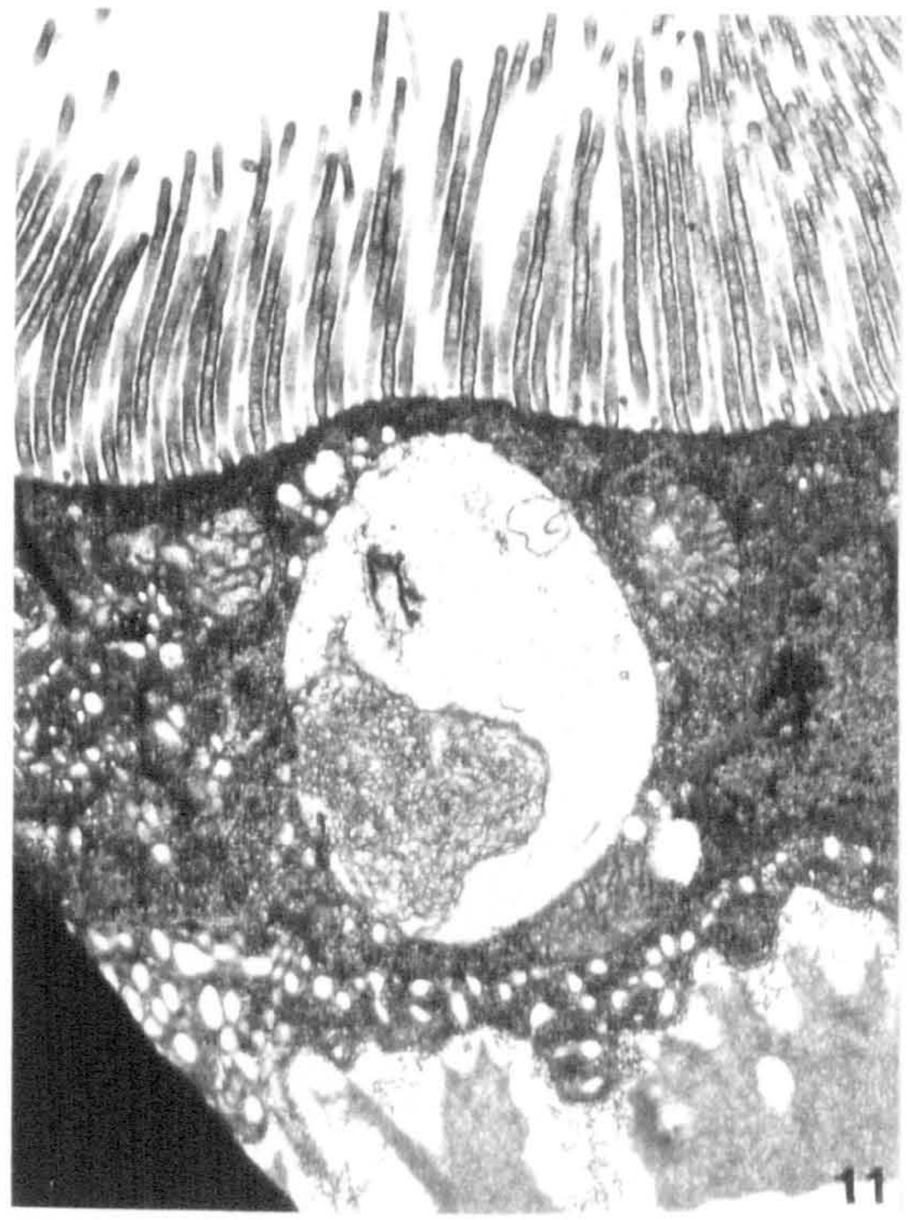
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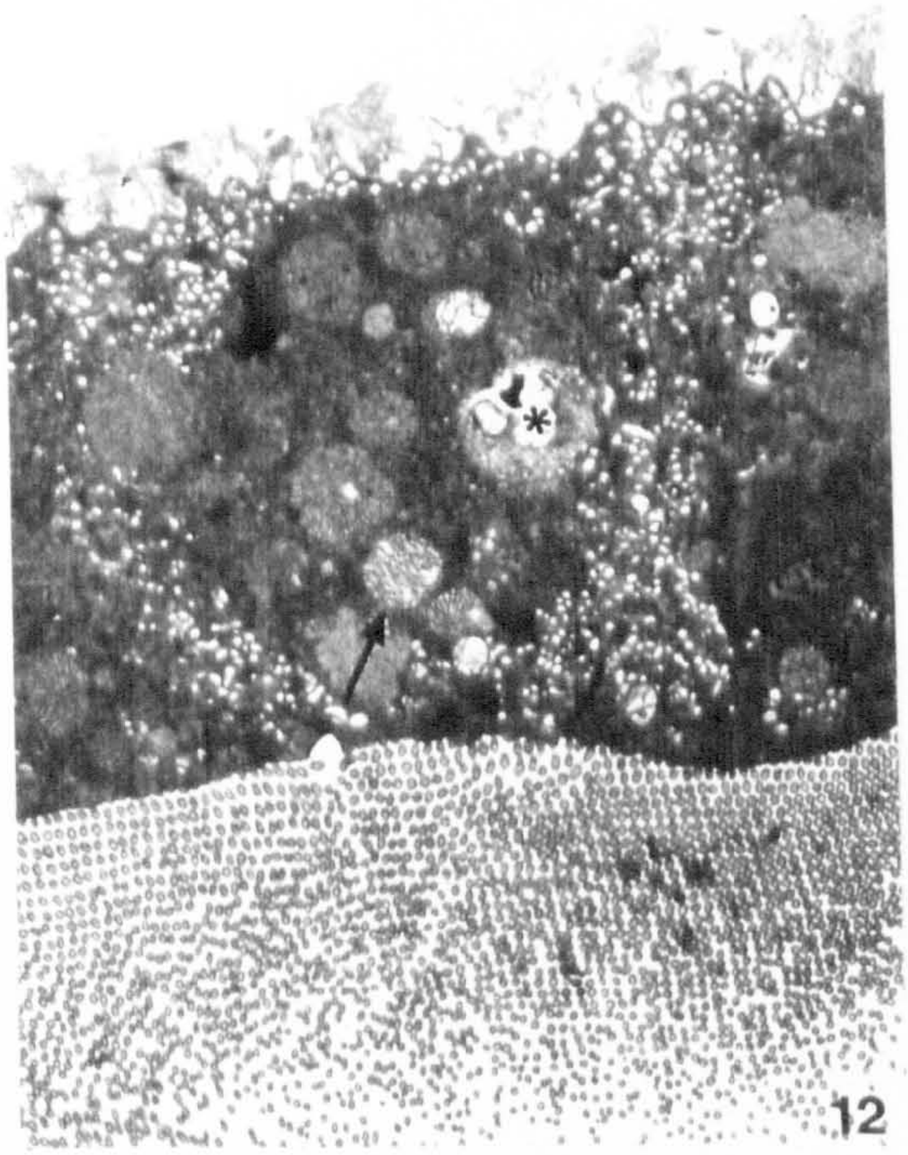
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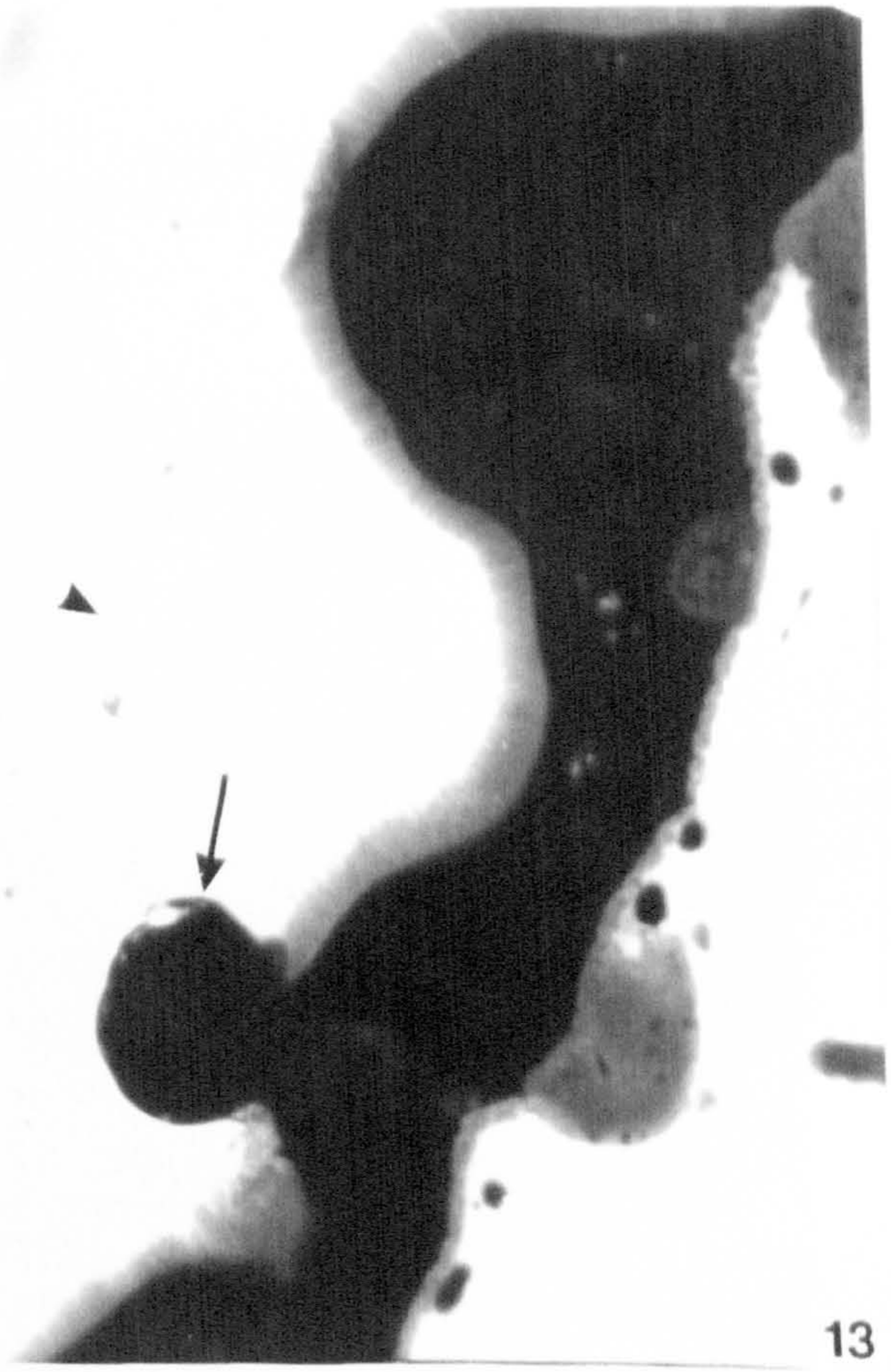
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Plate 5.14 Thin peritrophic membranes associated with the epithelium (arrow). TEM 5,900X.

Plate 5.15 Thick peritrophic membranes (arrow) in close association with the microvilli of an R-cell. TEM 5,900X..

Plate 5.16 High magnification of thin peritrophic membranes (asterisk) in close association with the microvilli (arrow). The peritrophic membranes were stained with WGA-gold to test the presence of chitin Note the gold label within the peritrophic membranes (arrow head). TEM 43,000X..

Plate 5.17 Surface of the posterior midgut epithelium. Note a thin sheet of peritrophic membranes on top of the microvilli of several enterocytes. Note the A-cells on the way to be extruded, still under the peritrophic membranes. Another A-cell in the foreground is partially covered with peritrophic membranes SEM 1250X.

Plate 5.18 Longitudinal section through the posterior midgut. Thick peritrophic membranes can be seen on top of the microvilli of several cells (arrow). A sectioned A-cell is about to pinch off (arrow), and the bulges of other A-cells below the peritrophic membranes can also be appreciated (big arrow) SEM 2500X.

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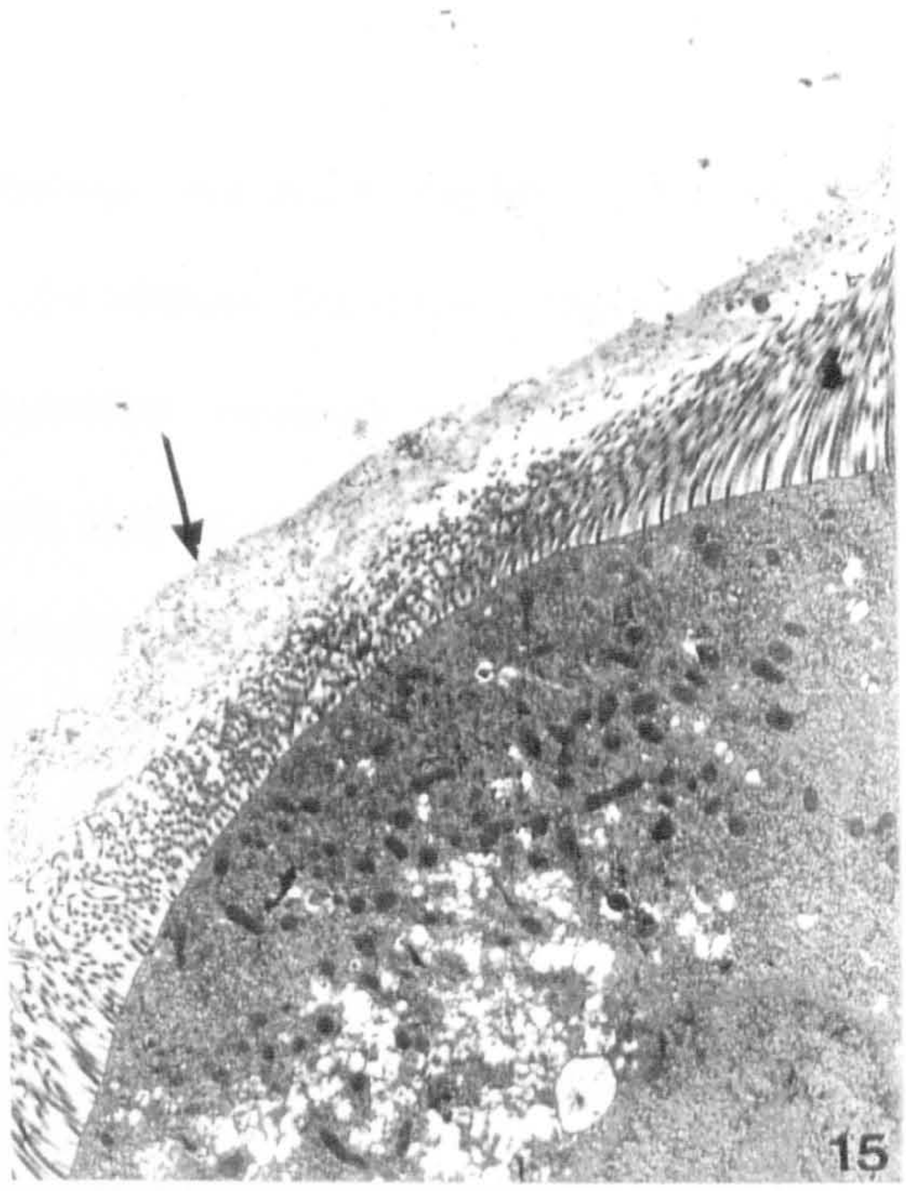

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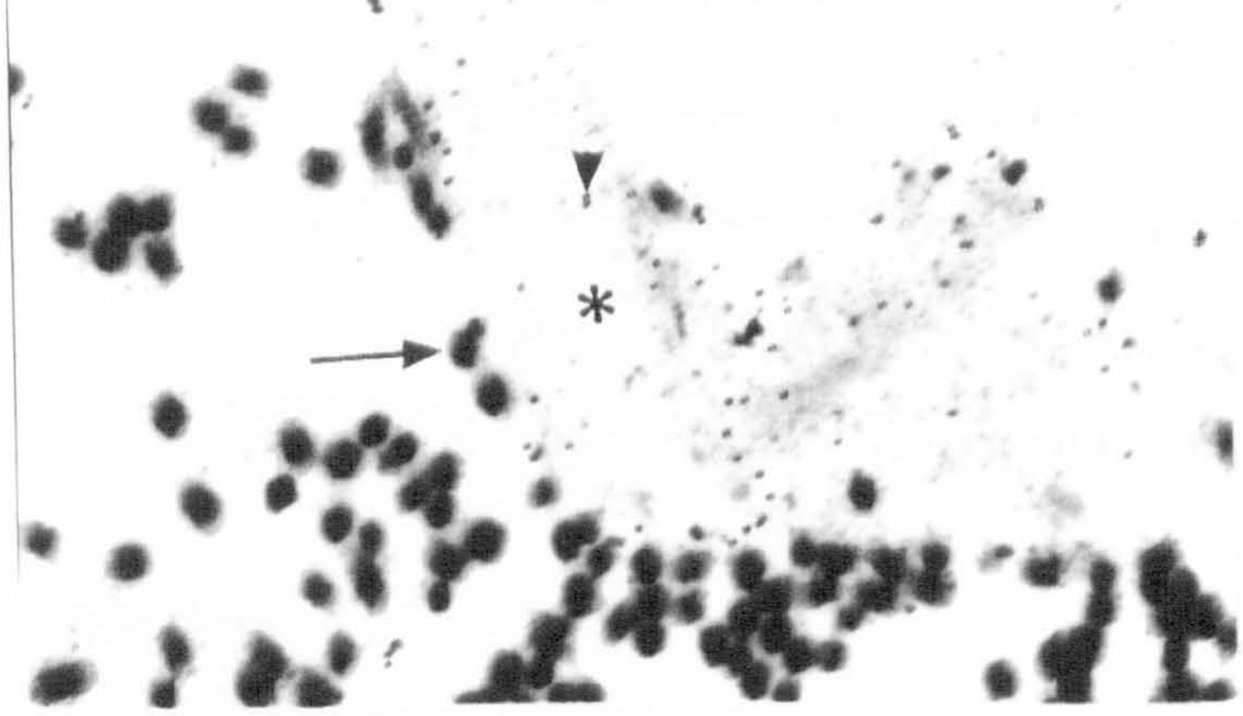

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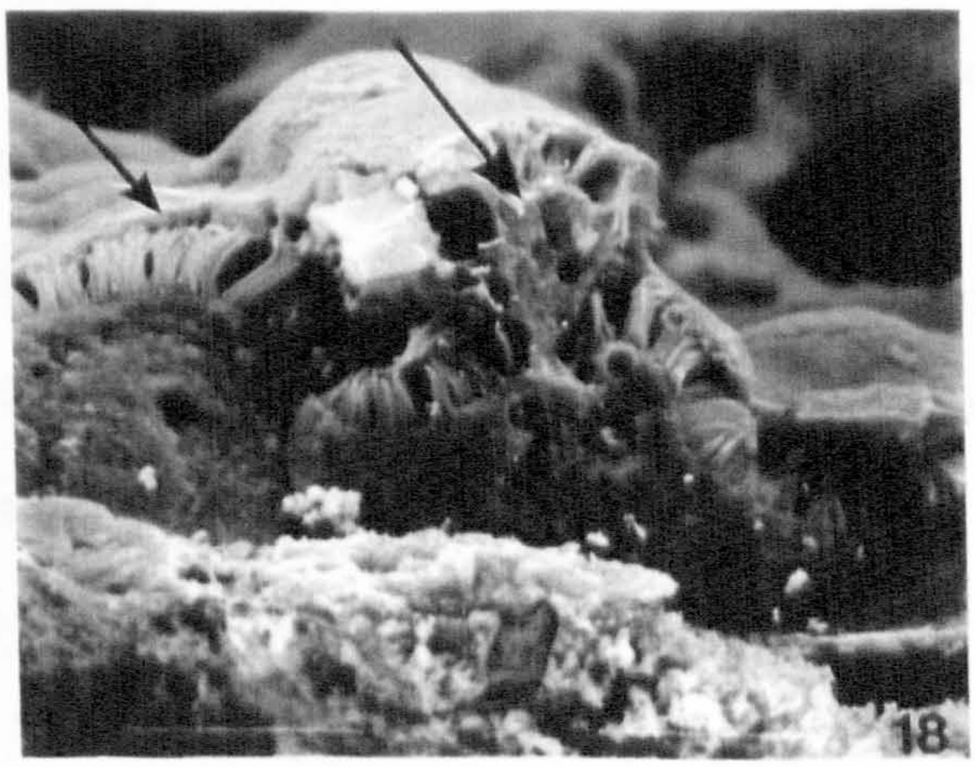

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Clear and dark pellets, due to their size and volume, are easily expelled by peristalstic waves coming from the midgut, that push the pellet into the hindgut. But once in the hindgut they have to be expelled by a different mechanism than peristalsis, because the hindgut does not posses circular muscles to produce the sort of peristalsis that would push the pellet out. This is more important in the case of tape pellets, which are very thin and flexible. Here is where the small anal spines come into play. When part of the pellet has been pushed into the hindgut by action of the posterior midgut peristalsis, the cuticular spines "engage" in the peritrophic envelope of the pellet securing it in place. Subsequent alternate contraction of the muscles of the hindgut make the pellet advance posteriorly. When the tip has arrived near the anal slit, other muscles come into play together with the spines of the anal slit. The muscles 4 and 7 of one side bring the anal edge that has the spinules towards the lumen of the hindgut. Then a relaxation of muscle 7 makes the anal edge with the spinules compress the pellet against the plate on the other side. A relaxation of muscle 4 brings that half of the anal slit to the original position, sliding along the opposing cuticular plate and pulling the pellet, which has been grasped by the spinules, a small distance. Alternating movements of each side like the one described keep pulling the tape pellet until it is completely voided. The tape pellet is shaped as a ribbon during this passage through the hindgut.

5.2.5 Manual feeding.

As mentioned before, the results of force feeding were very variable. In some lice the blood could be introduced into the midgut relatively easily. In other cases, finding the correct position of the tip of the micropipette against the mouth of the oesophagus needed a lot of adjustment, with the consequent stress for the louse. These lice usually stayed lethargic for a longer time land emptied their guts via the anus once put back in the recovery vessels. Also, some of them produced tape pellets within 2-6 hours of recovery. In others, there was little or no peristaltic movement of the gut, which remained static most of the time, with only occasional contractions.

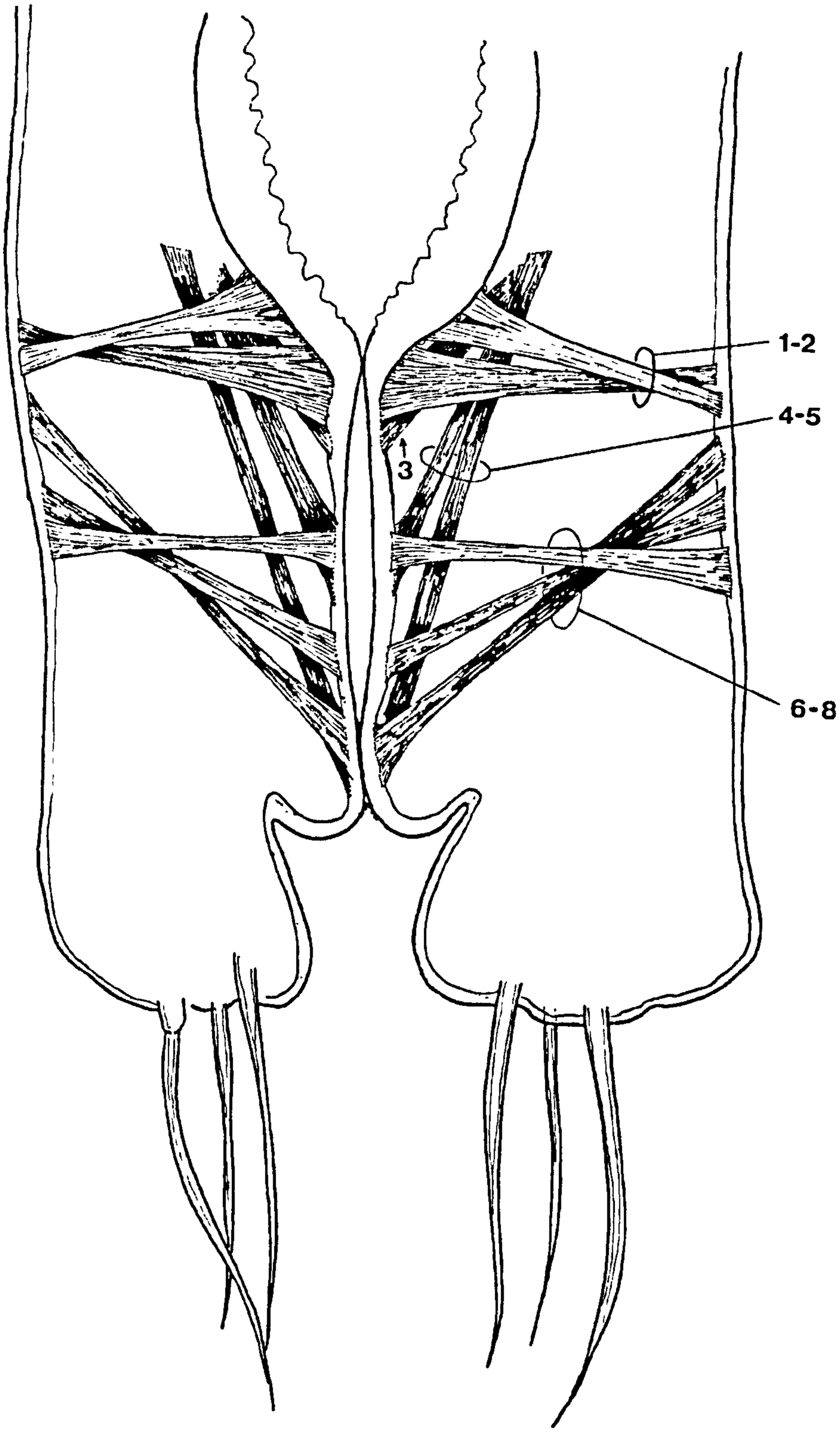
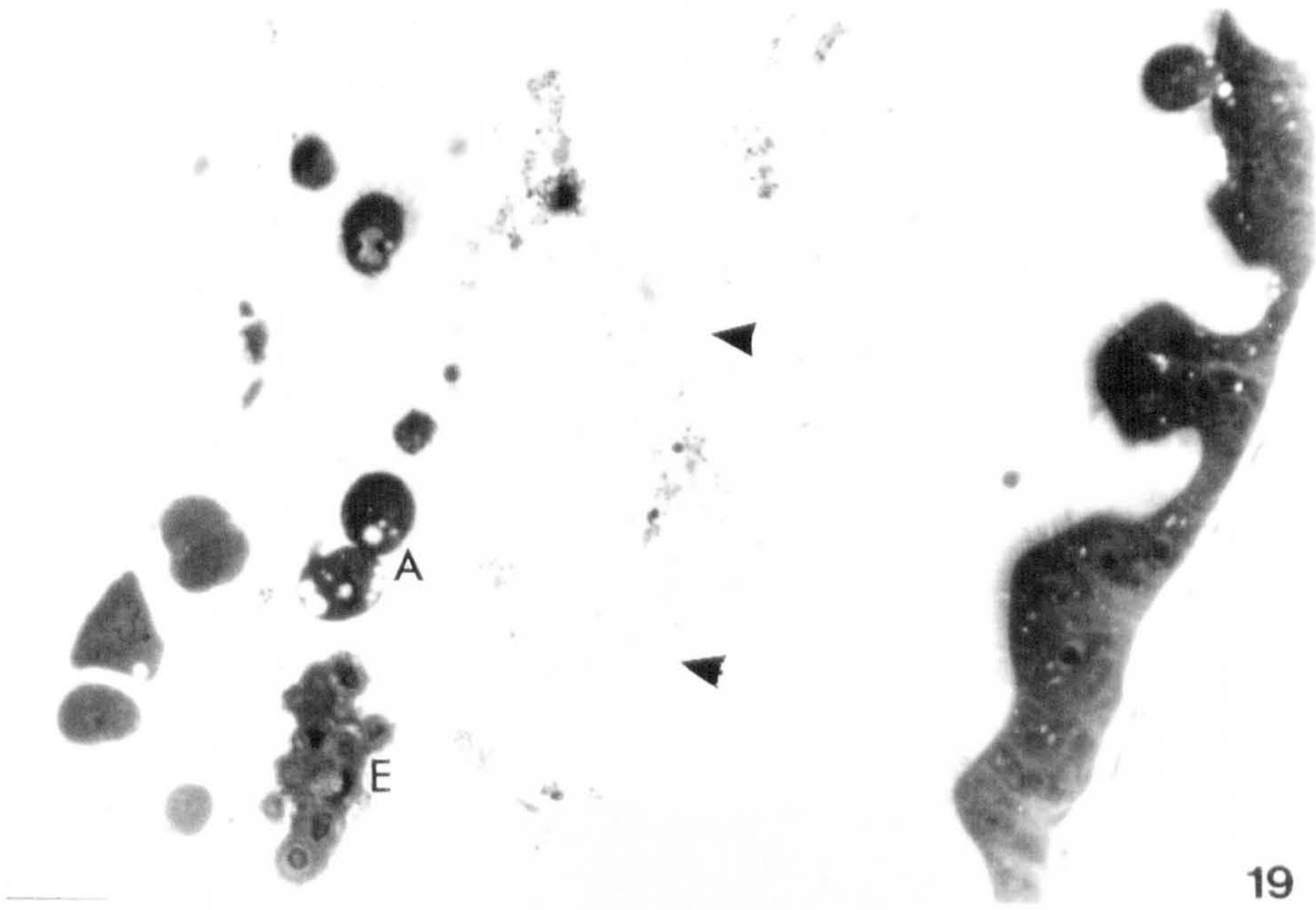


Figure 5.3 Ventral view of the posterior end of *L. salmonis* showing the musculature associated with the hindgut..

Plate 5.19 Pellet in the process of being packed with peritrophic membranes. Note the sheets of peritrophic membrane (arrow heads) which in turn will form part of the pellet matrix. A-cells (A) and salmon epithelial cells (E) have already been entrapped by the peritrophic membranes. Scale bar 20 μm..

Plate 5.20 Lateral view of the posterior end of *L. salmonis* showing the hindgut. The left half of the hindgut has been dissected away to reveal the structure of the inner wall of the right hindgut cuticular "plate" (asterisk). SEM 160X..

Plate 5.21 High magnification of the luminal surface of the hindgut showing the small cuticular spinules that cover it. SEM 1250X



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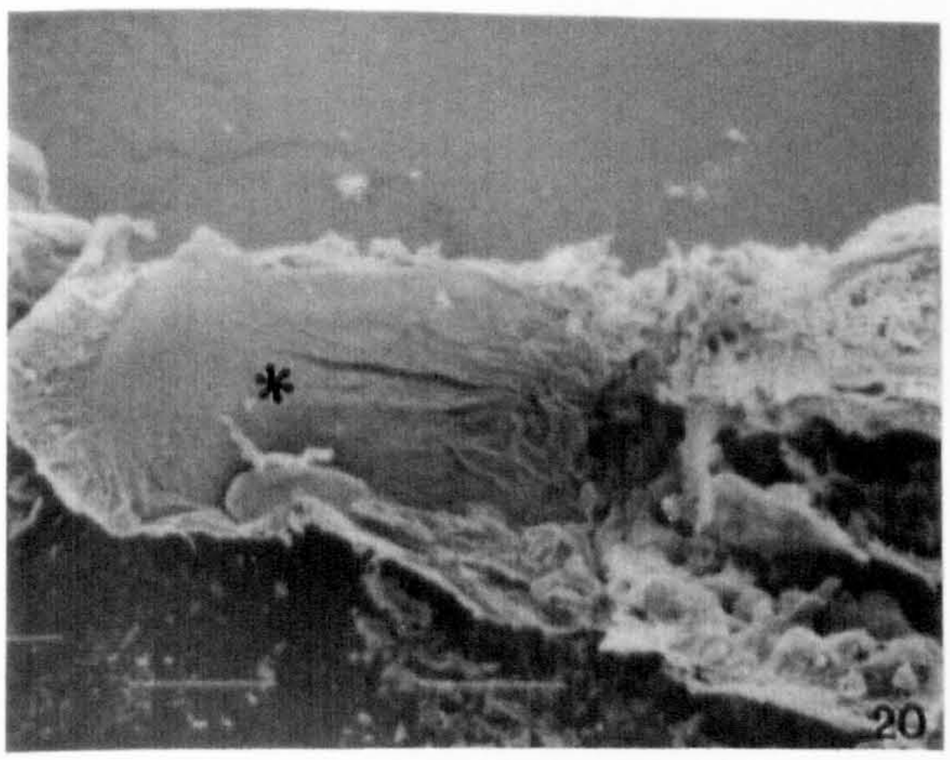

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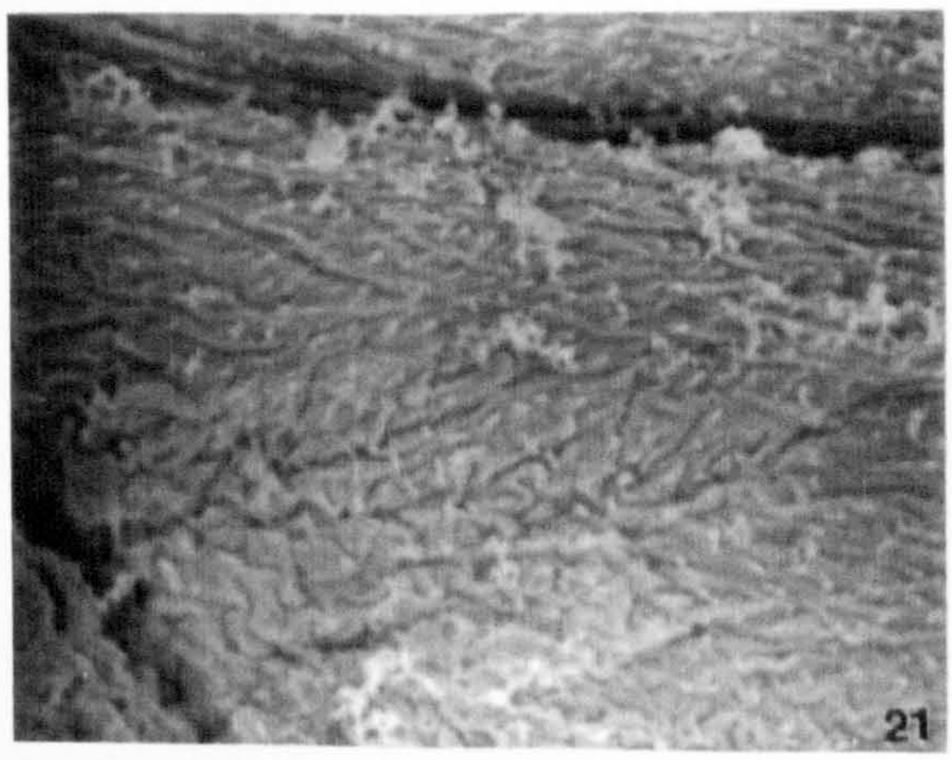

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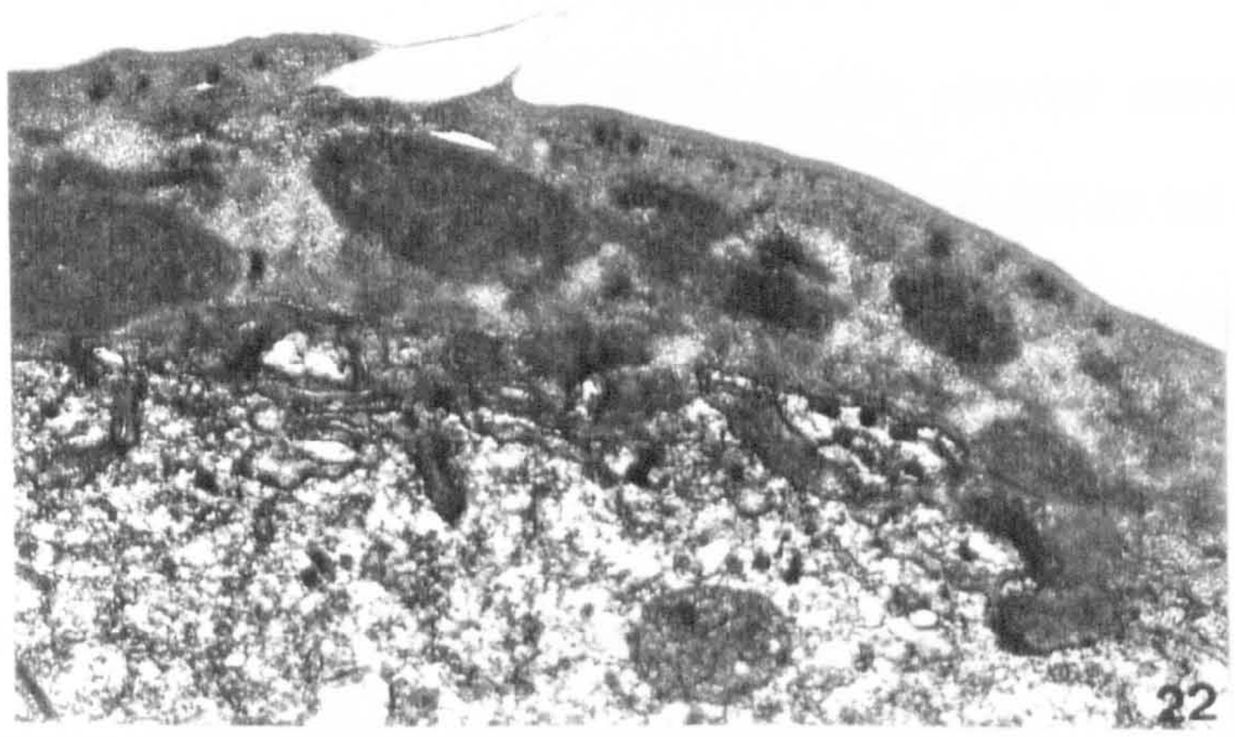
Plate 5.22 TEM of a cuticular spine from the hindgut cuticular lining. TEM 22,000X..

Plate 5.23 Ventral view of the posterior part of *L. salmonis* showing the anal slit. Note the rugosity of the walls of the slit. SEM 320X..

Plate 5.24 Zoom into the edge of the anal slit showing the small spinules. Note a small colony of rod-shaped bacteria (arrow). SEM 2,500X..



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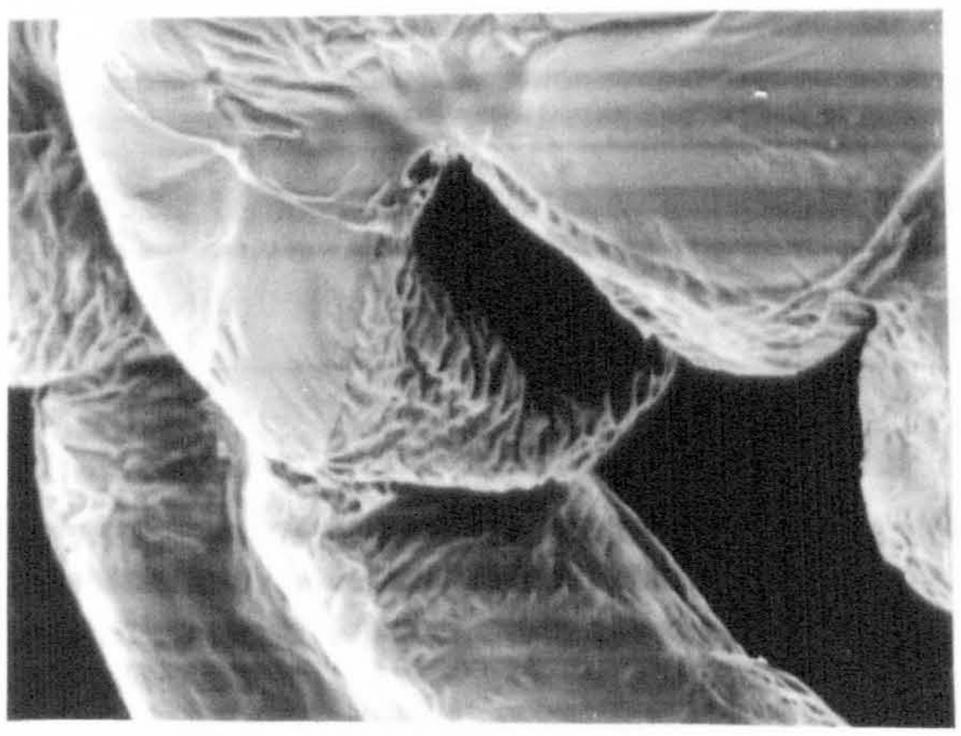

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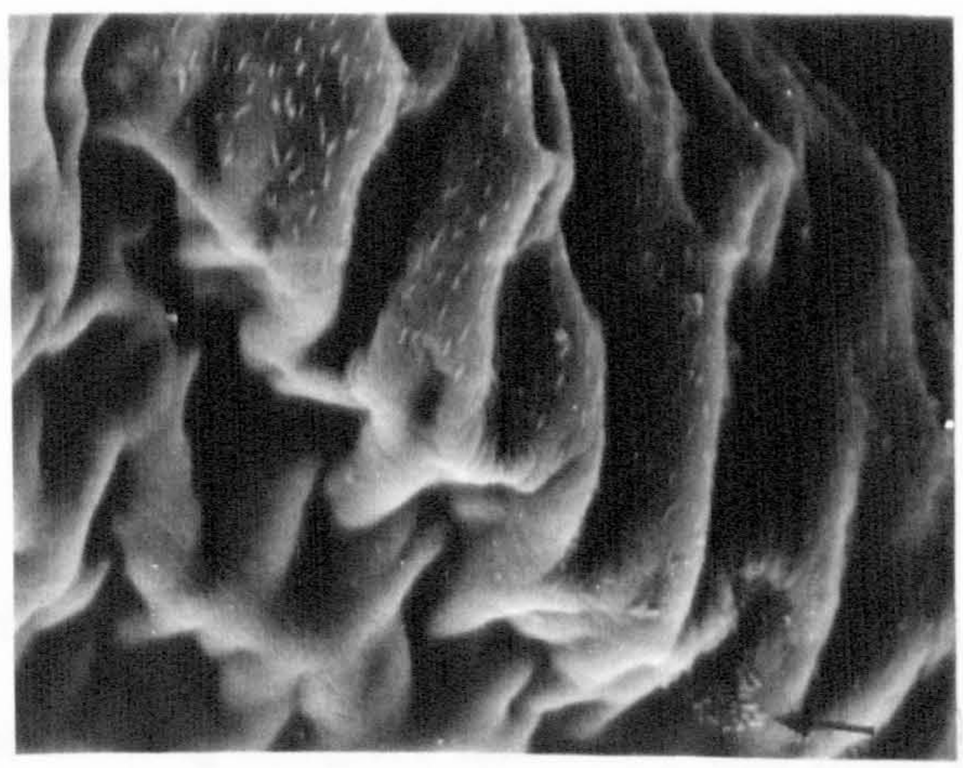

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The lice chosen for further processing were those in which the feeding had been rapid and did not show much signs of stress. These lice usually recovered quickly, in 1-5 minutes, and usually retained the blood meal in their guts which in turn showed active peristaltic contractions. Unfortunately, the proportion of problematic lice, which could not be fed well or their recovery was not satisfactory was very high. Only a small amount of lice were useful for further processing.

5.2.5.1 Salmon blood.

Of about 40 lice manually fed with blood, only 10 were useful for further analysis. Table 5.2 shows a summary of the results.

As is evident from these results, the key characteristics of a gut from a louse that had fed naturally on blood could not be replicated by manual feeding. Homogeneous gut contents with many lipid droplets and absence of any sign of red blood cells and enterocytes with lipid droplets in their cytoplasm were never seen in these experimental lice. Even after 7 h the red blood cells could be found intact in the midgut. The midgut of this louse and the louse that was left for 19 hours before fixation never showed the gradual changes of colour described for red-gutted lice in section 5.2.1.1.

Only one louse voided a pellet during these observations. It was from a louse that was fixed 5h after manual feeding. The louse (along with another 2 lice) could not be analysed for histology because the LR White resin did not polymerise properly in that batch. But the pellet did. It had no peritrophic envelope and the red blood cells were intact, with no sign of any alteration. The picture was exactly the same as the pellet inside the posterior midgut shown in Plate 5.26 .

5.2.5.2 Milk.

Observations of the lice before fixation. The behaviour of lice after being manually fed with milk was similar to that following being fed with blood. Lice would remain lethargic for a few minutes after being put into their individual vessels, probably due to the stress of the handling. Then they became active, adhering to the sides of the vessel. The two lice fixed after 50 minutes had some opaque, loose clumps of curdled milk in the posterior midgut, not yet compacted to what could be

considered a pellet. The anterior and mid midgut enterocytes had acquired a pinkish colour due to the neutral red used as a marker for the milk.

Table 5.2 . Summary of the histological observations on 10 lice that were manually fed with salmon blood. AM: Anterior midgut; MC: Medium-Crypted gut type; MM: Mid midgut; PM: Posterior midgut; RBC: red blood cells; SC: Shallow-Crypted gut type; TH: Thin gut type; VTH: Very Thin type

Time after blood Feeding (h)	Observations
0:30	Almost no gut contents except for a few RBC. AM TH type. MM and PM SC-MC type
0:35	All three portions of the midgut MC type and full of intact RBC, no signs of being digested, no haemolysis.
0:40	All three portions of the midgut full of intact RBC. Many A-cells among the red blood cells in PM . PM TH type. (see Plate 5.25) AM and MM MC type.
0:50.	No gut contents. AM SC type. MM and PM MC type
1:30	Some intact red blood cells in AM and MM. In PM a pellet of almost intact RBC is being formed; these RBC have little signs of degradation in the cytoplasm, but are not lysed and nucleus and cytoplasm is still recognisable in most. No sign of peritrophic envelope. Some homogeneous contents outside the pellet similar to those found in "natural" red-gutted lice (see Plate 5.26). AM SC type. MM and PM MC type.
2:00	A few intact RBC scattered in AM and MM. No sign of lysis or digestion. A-cells among the groups of red blood cells. In PM, RBC loosely grouped as a early pellet with no sign of peritrophic envelope. Some RBC scattered outside this group. AM And MM MC type. PM mostly TH type, some shallow crypts.
3:00	Similar to above. Large pack of RBC in PM, looking like a pellet but no sign of a peritrophic envelope. AM SC type; MM TH type; PM VTH type.
3:00	No sign of gut contents. All three midgut regions TH-SC type
7:00	All three midgut regions highly contracted. AM and MM packed with intact RBC; PM with no contents, but several A-cells being produced.
19:00	No gut contents. AM TH type; MM and PM TH-SC type.

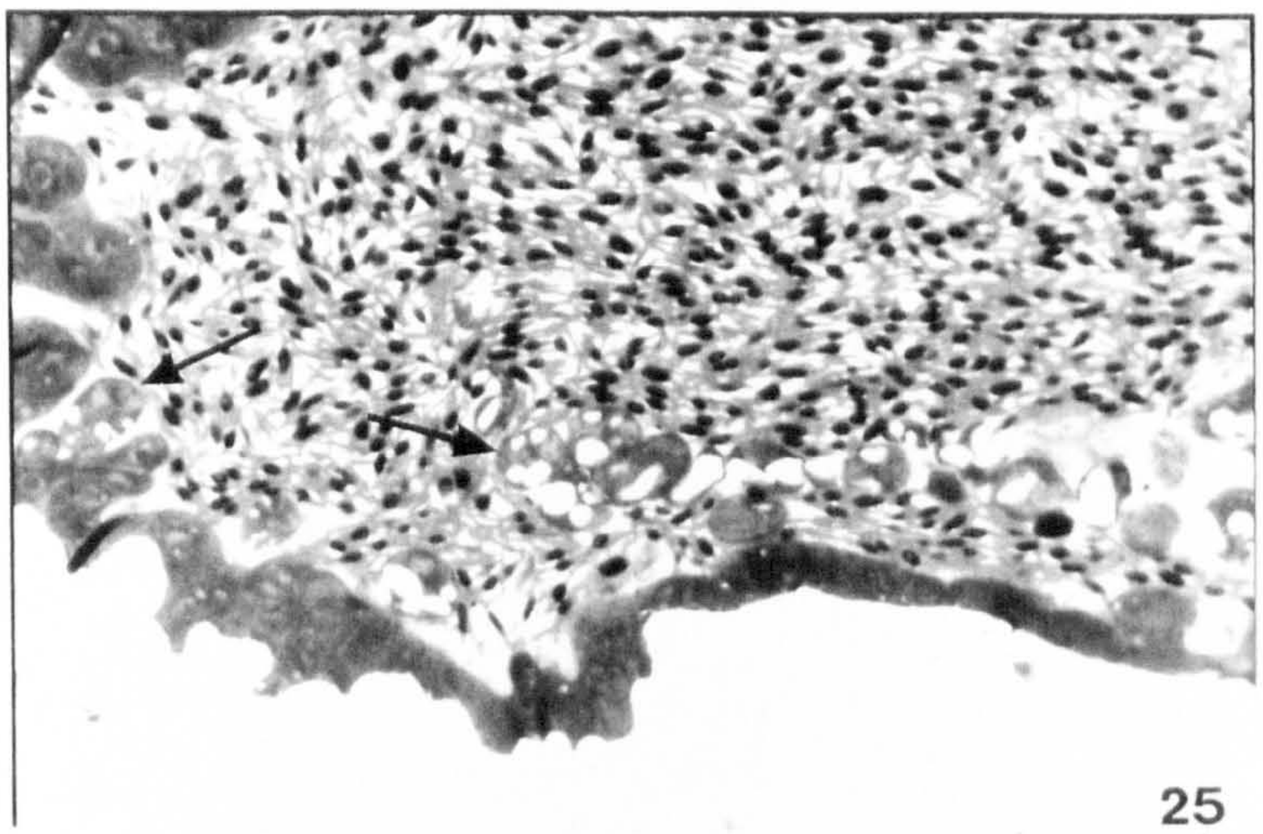
One louse fixed after 1:30 h had a well formed, pale looking clump of material in the posterior midgut. The rest of the gut seemed empty and the enterocytes did not stain with neutral red. The other louse had a similar pale clump in the posterior part of the mid midgut and only the anterior midgut had a pinkish colour.

Lice fixed after 3:30 and 4:30 h both had a pale clump in the posterior midgut. One was without pink stain and, in the other one, the anterior and mid midgut were pinkish.

The last louse, fixed after 5:30 h, did not have any pellets or clumped material in the gut . Also the gut was not stained pink by the neutral red.

Plate 5.25 TS through the posterior midgut of a louse 40 min after manual feeding with salmon blood. Red blood cells do not show any sign of decay or alteration. Note some A-cells from the louse (arrows) among the red blood cells and another one about to pinch off from the epithelium (arrow) and the Thin type gut epithelium. LMR. Toluidine blue. Scale bar 20 μm..

Plate 5.26 TS through the posterior midgut of a louse 1:30 h after manual feeding with salmon blood. A pellet of red blood cells is forming . Note the absence of peritrophic envelope and the integrity of the salmon haemocytes. The gut type is Medium-Crypted. LMR. Toluidine blue. Scale bar 50 μm..



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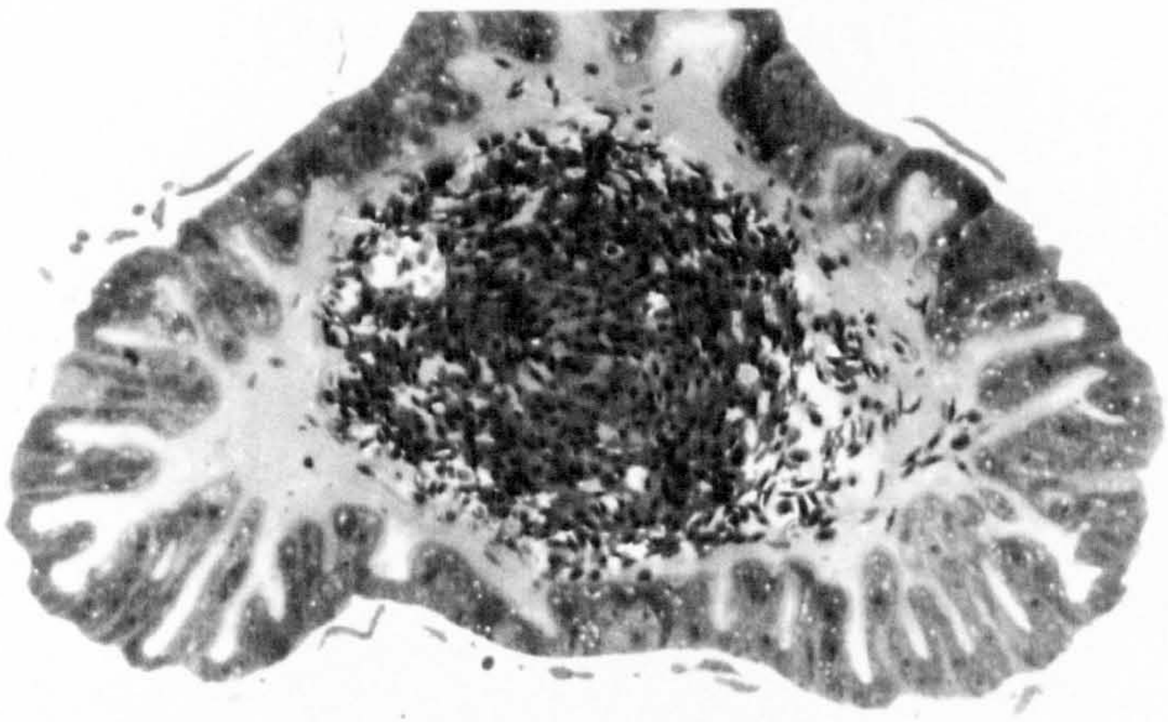

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Light microscopy. Unfortunately, the embedding of the lice processed for lipid staining was not satisfactory. The LR White did not penetrate properly to the tissues and it was impossible to get any section from them.

There was no problem with the remaining blocks of the four lice that were not processed for lipid staining, even though they were processed in the same batch.

Under the light microscope, the morphology of the enterocytes in all these lice was very similar, between them and between the anterior, mid and posterior midgut of each individual. In all cases the gut type was basically Very Thin, with short microvilli, occasionally with small areas with bulges that did not reach the characteristic of a Shallow-Crypted gut and is referred in Table 5.3 as VThin-Shallow-Crypted (VTh-SC) type. B-cells, when present, did not have dense granules, which is a common finding in VeryThin gut type.

Table 5.3 Summary of some morphological features of the midguts of 4 lice after manual milk feeding . Th:Thin, VTh: Very thin, SC: Shallow-Crypted. + - ++++. Relative number of cells.

Time After Feeding (h)	Gut section	Gut type	B-cells	A-cells
0:50	Ant	VTh	-	-
	Mid	VTh	+	+
	Post	VTh	-	-
1:30	Ant	VTh	-	-
	Mid	VTh-SC	-	+
	Post	VTh	-	++
3:30	Ant	VTh-SC	+	-
	Mid	VTh	+	++++
	Post	VTh	+	++++
5:30	Ant	VTh-SC	-	-
	Mid	VTh-SC	-	-
	Post	VTh	-	-

5.3 DISCUSSION.

The contents of the gut in the Crustacea are propelled by peristalsis (Vonk 1960). In *L. salmonis*. peristalsis took place along the whole length of the midgut and reverse peristaltic movements were also very common. The thorough mixing of the gut contents was witnessed also by Scott (1901). This may explain why the morphology of the epithelium seems rather uniform in different areas as

reported by Nylund *et al.* (1992), and therefore the secretion of enzymes and absorption of nutrients could easily take place along its whole length .

Despite their importance, digestive processes have seldom been studied in parasitic copepods. Observations on the function of the intestine and the successive stages of blood digestion of haematophagous crustaceans are restricted to rather old accounts. Capart (1948) made a detailed description of the changes that the gut contents of *Lernaeocera branchialis* undertake during the digestion of a blood meal. In his description the colour of the gut contents at the beginning is dark red and, after the destruction of the blood corpuscles, they become a flocculate brownish paste. By the end of the digestion, the contents are pale brown and still flocculate, finally becoming yellowish brown and clear. The process lasts 5-8 h and it can be followed by these changes in colour.

These changes are very similar to those described for *L. salmonis* in this study, with the major difference that in the case of *L. salmonis* the process took longer. The common absence of red blood cells in the gut contents has been documented before for lernaeids (Van Der Berghe 1933) but Capart (1948) was of the opinion that this absence depends on when the parasite is fixed. He had in his samples parasites fixed just after a blood meal, and he found the digestive tract full of blood corpuscles from the fish. As stated in the previous Chapter, in the present study, even though great care was taken to preserve the gut contents in place by tying the anus of the lice, red blood cells were very seldom found and in extremely low numbers. This indicates that the lysis of the host's blood must take place very quickly indeed. The absence of positive staining for haemoglobin also indicates that the blood is transformed fast to a form that is not detectable by the histochemical test employed here but that still preserves its spectrum absorption characteristics, as Brandal *et al.* (1976) demonstrated host's haemoglobin in the gut contents of red-gutted lice. The same histochemical test has been used in ticks with positive results (Agyei *et al.* 1992) but, in this group or arthropods, the haemoglobin remains intact in the lumen and for some time inside the digestive cells because all the digestion takes place intracellularly (Grandjean 1984). It is not known what happens to the haemoglobin after *L. salmonis* has digested it. The absence of a positive reaction for iron is puzzling. In other blood sucking arthropods in which the haemoglobin digestion is known, such as bed bugs, mosquitoes and fleas, the haemoglobin is broken down in

the gut lumen and transformed to haematin which is then disposed of in the faeces (Wigglesworth 1943). In this way, iron is never absorbed or catabolised, and there is no problem of accumulation of this otherwise toxic element. In ticks, because they digest the haemoglobin intracellularly, the problem of accumulation of toxic iron is solved by concentrating it into indigestible haematin in residual bodies which accumulate in the cytoplasm. When the cells are full of residual bodies, the cells are discarded from the epithelium and the haematin is removed by defaecation (Coons, Rosell-Davis & Tarnowski 1986, Tarnowski & Coons 1989). Haematin is difficult to miss because it forms characteristic brown birefringent granules which can easily be detected by the histochemical technique employed here. If this pigment was never found in blood feeding *L. salmonis* it could mean that its production and release from the gut is quick and it was missed or that another mechanism for the digestion of haemoglobin is taking place. The haematophagous copepod *Cardiodectes medusaseus* is unique in its mode of dealing with blood. The haemoglobin is completely catabolised and releases free iron into its own cytoplasm. It then synthesises ferritin, an iron detoxification and storage protein which is accumulated in crystals inside the cytoplasm of the attachment organ, which is a syncytium (Perkins 1985). The ultrastructure of these crystals is very characteristic and definitely they were not observed in the present study. Apparently there are no other studies of the fate of haemoglobin in haematophagous crustaceans. More detailed studies would be necessary to elucidate what is happening in *L. salmonis* to the ingested haemoglobin.

Other studies on blood-sucking crustaceans comprise morphological and histological descriptions of the alimentary canal but rarely address the issue of what happens to and/or what is the nature of the gut contents (e.g. Bower-Shore 1940, Ho & Honma 1983, Perkins 1985, Juilfs & Wägele 1987, Honma & Ho 1988, Perkins 1994).

The digestive processes of haematophagous insects and ticks are better studied. Ticks have the peculiarity that the digestion occurs intracellularly by heterophagy and is very slow (Grandjean 1983, Grandjean 1984, Coons *et al.* 1986, Koh, Shiraishi & Uchida 1989, Tarnowski & Coons 1989) and it is not readily comparable to the present study. On the other hand in most haematophagous insects, blood meal digestion occurs extracellularly (Gooding 1975) and two patterns can be recognised (Vaughan & Azad 1993). In one pattern, observed for bed bugs

(Hemiptera: Reduviidae), sand flies (Diptera: Psychodidae) and mosquitoes (Diptera: Culicidae), the blood starts to be broken down around 12 h after being ingested, the blood meals remained clotted and peritrophic membranes were produced. In the other pattern, observed in lice (Anooplura) and fleas (Siphonaptera), the haemolysis and liquefaction of the blood meal occurred within 4 h after feeding, the blood remained liquid throughout the 48 h observation period and no peritrophic membranes were produced. This latter pattern is remarkably similar to the one observed in this study. Vaughan & Azad (1993) add that these patterns reflect the feeding strategies employed by these two groups of insects. On one hand, lice and fleas maintain a intimate association with their host, taking small and frequent blood meals which are degraded quickly. There is no need to store and wait long to slowly digest the meal because the food is practically unlimited. On the other hand, bugs and flies take large infrequent blood meals and use multiple hosts and digestive efficiency may take precedence over rapid cycling, digesting small portions of the meal at any one time.

These two groups of insects also show different reproductive strategies (Vaughan & Azad 1993). Lice and fleas display a constant output of small batches of eggs while bed bugs and sand flies produce few and bigger batches of eggs. A similarly contrasting pattern is seen in two families of ticks: the argasid pattern lacks the ability to produce large amounts of vitellogenin. They live in close proximity to the burrow or nest of their hosts and require small batches of eggs per feeding to ensure successful parasitism. On the other hand, ixodids live on multiple hosts and have little contact with the nest or home of the host and therefore need to produce thousands of eggs to guarantee successful parasitism. To achieve this, they ingest large quantities of blood to produce large amounts of vitellogenin within a relatively short time (Coons *et al.* 1986).

Although figures for the number of broods produced by a single *L. salmonis* female have not been published, Ritchie *et al.* (1993) estimate that a likely figure is at least 6. The maximum number of clutches recorded in *L. thompsoni* was 10 with intervals of 6-8 days between them. Also, no published information exists on the life span of *L. salmonis* adult females on the host. Adult females of *L. thompsoni* and *L. europaensis* are reported to live up to 135 days, from egg hatching to death or 91 days from the first egg laying until they die (De Meeüs *et al.* 1993a). Preliminary

results from current research at our premises shows that the life span of gravid *L. salmonis* adult females on their host is about 20 days (Shinn pers. com.) and the interval between clutches is of 4-5 days (McAndrew pers. com). From this data the rate of production of eggs would be *at least* two broods every week. These strategies showed by *L. salmonis*, reproductive and feeding ones, resemble the strategies used by lice and fleas and by argasid ticks. The blood digestion pattern observed here in *L. salmonis* also resembles closely the pattern demonstrated in lice and fleas.

Blood feeding in haematophagous insects and in ticks is closely associated with reproduction, therefore it may be valid to assume that gonotrophism could also be happening in the sea lice. There is some evidence that suggest that another species of *Lepeophtheirus* might have similar feeding strategy linked with reproduction. *L. thompsoni* lives on the body surface of the turbot (*Psetta maxima* L.). After mating, the females move into the gill chamber and then to the gill filaments, where they become haematophagous . The females of *L. europaensis,* a closely related species parasite of brill (*Scophthalmus rhombus* L.) and flounder (*Platichthys flesus* L.), also move to the gill chamber after mating, but they remain there feeding on mucus like the other stages (Zeddam *et al.* 1988). Comparing the life history of these two species De Meeüs *et al.* (1993a) showed that *L. thompsoni* females are more fertile than *L. europaensis* females and suggested a possible correlation with the haematophagous diet.

In ticks and haematophagous insects the blood meal is used for lipogenesis and to initiate vitellogenesis (Mitchell & Briegel 1989, Coons *et al.* 1986). From the observations in the present study, it seems that *L. salmonis* may be using the blood meal for lipogenesis, as the common occurrence of lipids inside the R-cells of blood-feeding females testifies. What happens afterwards with these lipids is not known, but it is possible that these may be used for vitelogenesis. It is not known if vitellogenesis in *L. salmonis* can occur without a blood meal. Further experiments would need to be carried out to assess if blood feeding is required for successful reproduction in *L. salmonis*.

The characteristic vesiculation of the microvilli observed in this study can also be associated with the digestion of a rich meal like blood. These microvesicles have been observed in the apical surface of the polarised enterocytes of the small intestine of vertebrates, and were increased after

feeding (Black, Yoneyama & Moog 1980, Berteloot, Chabot & Hugon 1981a) and particularly after fat feeding (Deschryver-Kescskemeti, Elikaim, Carroll, Stenson, Moxley & Alpers 1989). Hobbs (1980) showed that the formation of these vesicles occur by budding from the microvillous membrane and proposed that these microvesicles may serve to increase the area available for enzymatic digestion. Other studies have revealed that brush border enzymes are released from the enterocyte by microvesiculation and that 90% of the enzymatic activity could be membrane bound (see Özgünes 1996). These microvesicules were absent in lice feeding on host's epithelium indicating the possible association between the "richness" of the meal and the production of microvesicles. The absence of peritrophic membranes seems justifiable then when *L. salmonis* is feeding on blood, because probably it may interfere with this "contact digestion". The microvesiculation of the microvillous membrane results in shedding of membrane fragments along with the attached digestive hydrolases (Berteloot, Chabot & Hugon 1981b) and this may be advantageous for nutrients that require "membrane" digestion (Jacobs 1983), which are probably abundant in a blood meal. Alkaline phosphatase is a membrane bound enzyme which is present in these microvesiculations of the microvilli and other membranous structures called surfactant-like particles, which together seem to play a major role in fat absorption in the enterocyte of the rat (Alpers, Mahmood, Engle, Yamagishi & DeSchryver-Kecskemeti 1994) and possibly many other animals (Özgünes 1996).

Another important feature found in the present study is that the pattern of blood digestion observed in *L. salmonis* females in "natural" conditions could not be reproduced by manual or force feeding.

Proteins are probably the most abundant nutrients of the blood meal and, at least in haematophagous insects and ticks, the major digestive enzymes are proteases (Lehane 1994). There is no information on this aspect for haematophagous crustaceans but, if they are to digest such a protein-rich meal, proteases should also be among the primary enzymes. It is a general characteristic in most animals that the digestive enzyme activity increases after feeding, reaches a maximum and declines as the meal is digested (Gooding 1975). The failure of *L. salmonis* to digest manually-fed blood could mean that whatever triggers the mechanism for blood digestion was lacking in these lice. In insects, the mechanisms of control of levels of digestive enzymes are well

studied. There seem to be two possible mechanisms of control, a hormonal or paracrine and a secretagoge, also called prandial (Gooding 1975, Lehane, Blakemore, Williams & Moffatt 1995). In the hormonal mechanism, some factor in the blood meal is detected and a message is sent via the haemolymph to an endocrine gland, which releases a hormone into the haemolymph which in turn stimulates the digestive cells in the gut to synthesise and release the enzymes. In the prandial mechanism a chemical in the blood stimulates the secretory cells directly causing them to liberate the appropriate enzyme. Distinguishing between the two mechanisms is not an easy task as these two mechanisms could be interrelated (Lehane *et al.* 1995). These authors consider that hormones probably play a role in modulating, but not controlling, the levels of digestive enzymes and that feeding can affect the control of these enzymes. However, the presence of a meal may not be enough to trigger a digestive response. *L. salmonis* in the present study was unable to digest a blood meal after being force fed, so other factors may be involved. In mosquitoes physiological factors such as age, nutritional state, presence of eggs, mating condition, circadian rhythmicity and the number of gonotrophic cycles completed can modulate the host's seeking and feeding behaviour and a mosquito may or may not engage in host seeking or feeding even though the stimulus that normally triggers this behaviour is always present (Klowden 1996). Diapausing culicid females eject most of the blood ingested when they were induced to feed. This and the reduced avidity for blood indicate that they are not physiologically programmed for taking and retaining blood, and it is only when diapause is terminated that they begin to take blood again and develop eggs (Mitchell & Briegel 1989).

The experiments forcing lice to ingest milk were also unsuccessful and the fact that all guts were of the thin type could mean that there was not any reaction from the lice to the food introduced into the gut. Although pellets of milk were seen under the dissecting microscope, they could not be confirmed in sections, nor were peritrophic membranes found associated with them. It may be that the milk just had the appearance of a pellet. The salt water could have made it thicker and contributed to this artefact.

It is possible that *L. salmonis* needs also some external stimuli to be able to trigger a digestive response, such as a mechanical contact with the host or some factor or factors in the skin. Also it

is possible that the stress induced by handling or by being away from its host may have a detrimental effect in the normal physiology of the louse

But the fact that lice are not always feeding on blood might suggest that there may be an internal factor or factors that trigger blood feeding behaviour. The state of the reproductive system may be a strong candidate and then, as in haematophagous insects, a gonotrophic mechanism may be involved.

It would be interesting to see if blood feeding occurrence changes significantly between summer and winter generations, as it has been demonstrated that *L. salmonis* females in winter produce significantly longer egg strings with greater number of smaller eggs than females from summer generations (Ritchie *et al.* 1993). These authors suspect that the levels of reproductive investment change seasonally, therefore, if the meal type is related to reproduction, it is possible that one could observe as well a seasonal change in the occurrence of blood feeding strategy showed by *L. salmonis*.

The results on the starvation of *L. salmonis* in the present study show a pronounced change in the morphology of the gut epithelium during the 20 days of starvation. The first noticeable feature was the reduction and eventual loss of lipid droplets, followed by a gradual reduction of cell volume and number of organelles, until the only distinguishable component of the cell was the nucleus.

Only three studies on the effect of starvation on the morphology of the gut epithelium in copepods were found in the literature. One study corresponds to a parasitic form in which the aspect of the effect of starvation was examined very briefly: Briggs (1977) observed in *Paranthessius anemoniae*, which was starved for "a few days", that the number and size of lipid droplets in the columnar cells was diminished and the dense material in the central vacuole of the B-cells (amoeboid cells originally) was replaced by material of lower electron density. Also it was observed that the depth of the midgut epithelium was reduced almost by half, leaving an epithelial layer of less than 3 μm thick. The second study (Hallberg & Hirche 1980) was not strictly a starvation experiment. The authors investigated the digestive enzyme content and fine structure in various stages and generations of the free living copepods *Calanus finmarchicus* and *C. helgolandicus*, and one of these generations was an over-wintering one, which is characterised by a reduction of

metabolism, decreased activity of digestive enzymes and discontinuation of feeding. In these overwintering generations, the main morphological changes observed were reduction in number of B-cells and reduction in the volume of the gut epithelium. The third study was on *L. salmonis.* Turnbull (1991) starved adult females for up to 30 days. By day 14 he observed signs of cellular degeneration, loss of brush border, vacuolation and reduction in the folding of the epithelium. The gut epithelium had flattened by day 21 and by day 30 the flattening was extensive.

A reduction in the depth of the gut epithelium during starvation is commonly observed in starvation studies in other Crustacea such as Decapoda (Storch *et al.* 1982, Storch & Anger 1983, Papathanassiou & King 1984, Anger *et al.* 1985), Isopoda (Storch & Lehnert-Moritz 1980, Štrus *et al.* 1985, Štrus 1987), Amphipoda (Storch & Burkhardt 1984) and Cladocera (Schultz & Kennedy 1976, Elendt & Storch 1990). It is known that the intestine of animals in general has a very active metabolism and a high rate of cell loss and renewal compared to other tissues. Energetically, it is an expensive tissue to maintain and animals that are put under energetic strain may not be able to keep it in top condition (Cossins & Roberts 1996). The result is a noticeable reduction in cell number and volume.

Probably the most detailed study of the morphological changes of the intestinal epithelium during starvation in non-decapod crustaceans is the one of Elendt & Storch (1990). Following the ultrastructure of the daily changes they found that by day 4 of starvation in *Daphnia magna* the cell size was reduced to half and, by the eight day, the maximum time *D. magna* could survive, the epithelium had reduced to about 6 μm, one-fifth of the original height. The microvilli showed also gradual reduction in height from 8-10 μm at the beginning of the experiment to 1-2 μm at the end.

The dramatic reduction in cell height observed by the 20th day in the present study has not been recorded before in crustaceans. This is probably because in the few works on this subject the observation period has not been long enough or, as in the above case of *D. magna,* because the organism is too susceptible to starvation and dies before further reduction of the thickness of the gut epithelium takes place. An exception is the case of isopods, which can endure long periods of starvation. Observations have lasted for up to 15 weeks, and although substantial reduction of the

height of the enterocytes was observed (Storch & Lehnert-Moritz 1980), apparently it did not reach the levels observed in the present study.

Some *L. salmonis* females seem to have an unusual capacity to survive very long periods of starvation. There are no published studies on the survival of *L. salmonis* off its host, but current research on this matter shows that by day 6-7 half of the lice die and from then on the drop in lice numbers is sharp such that by day 10 only about 20-30% of the original numbers remain (Tucker, pers. com.). This suggests that *L. salmonis* survival under starvation could be similar to the observed in some free living copepods. It would be interesting to investigate if the large reduction in cell volume and number observed in the present study also happens in those free living copepods that survive for relatively long periods (2-3 weeks) in food deprivation (e.g. Cox & McLaren 1981, Runge 1984, Gill & Harris 1987, Hassett & Landry 1990).

The loss of contact of the basal plasma membrane with the basal membrane observed in the present study has also been recorded in individuals in advanced stages of starvation in *D. magna*. Animals with this features soon die (Elendt & Storch 1990).

Other common reported changes in the enterocytes associated with starvation which also were observed in this study include: reduction in size and number of lipid droplets after the first day and almost complete depletion of them within 2 and 4 days of starvation; higher occurrence of autophagosomes; swelling of mitochondria; reduction of amount of endoplasmic reticulum and number of Golgi and mitochondria and loss of contact of the basal plasma membrane with the basal lamina (Storch & Lehnert-Moritz 1980, Storch & Anger 1983, Papathanassiou & King 1984, Elendt & Storch 1990).

The reduction in number of B-cells and slight change in its characteristic morphology has also been reported in the prawn *Palaemon serratus* (Pennant) (Papathanassiou & King 1984). In the copepod *Paranthessius anemoniae* the characteristic dense material in the central vacuole (primary lysosomes?) of the B-cells (amoeboid cells) becomes more electron lucent. This reduction in primary lysosomes was also a common feature in the present study.

In overwintering generations of planktonic copepods there is a marked reduction in the production of digestive enzymes (Hallberg & Hirche 1980). Also, in *Palaemon serratus*, 56 h of starvation were

enough to affect negatively the absorption and protein synthesis mechanisms, judging by the effect on the R- and F-cells (Papathanassiou & King 1984). A similar effect seems to be taking place in *L. salmonis* considering the substantial reduction in rough endoplasmic reticulum and mitochondria. The faintness in the staining of the brush border in the first stages of starvation up to the almost complete lack of staining in last stages could be due to the reduction and eventual loss of synthesis of membrane bound enzymes.

Membranous material surrounding the food residues in arthropods have been reported since the 18th century (see Peters 1992). This latter author suggests replacing the widely used singular term "membrane" by the plural "membranes". "Membrane", he says, could be misleading in the sense that it has nothing to do with plasma membrane and also because the "membrane" is in fact a group of several membranous materials. This nomenclature is also adopted in the present study. Also, this group of membranes wrap around the food mass forming a peritrophic "envelope", which is the other term that Peters suggests to use.

The variable thickness of the peritrophic membranes observed in the present study is probably a function of the number of individual membranes that are deposited one over another one, although the net result is an envelope where there is no distinction of individual membranes. The local peristaltic movements observed in the posterior midgut of *L. salmonis* probably aid in packing the peritrophic membranes together against the food residues.

In Crustacea, the chitin-containing microfibrils are found arranged either in a random or an hexagonal texture. The peritrophic membranes of *L. salmonis* in the present study belong to the random type of microfibrils. This characteristic has also been observed in a number of copepods, including several commensal and parasitic forms (Yoshikoshi & Ko 1988). The formation of the peritrophic membranes with hexagonal or orthogonal texture is probably because the precursors of the peritrophic membranes are secreted at the base of the microvilli and polymerise while still in between them. The microvilli act as a template, so when the peritrophic membrane is finally shed it bears the hexagonal pattern of the arrangement of the microvilli (Martin & Kirkham 1989, Ryerse, Purcell & Sammons 1994). In species where the peritrophic membranes have a random texture,

the final assemblage takes place at the tips of the microvilli and therefore no pattern is formed (Becker & Peters 1985, Martin & Kirkham 1989).

In the present study, the peritrophic membranes were recorded in all three parts of the midgut, but probably the occurrence is substantially greater in the mid and posterior midgut. This issue is studied in deeper detail in Chapter 6. But it is worthy of mention here the report that in the copepod *Calanus helgolandicus* the peritrophic membranes are formed in the posterior midgut with the contribution of necrotic epithelial cells (Nott *et al.* 1985). As was discussed in the previous chapter, necrosis is always pathological. Moreover, leaving the basal membrane naked due to the sloughing of "necrotic" cells as it is reported by these authors would be a dangerous procedure, leaving in contact two completely different compartments that should be separated, the lumen of the gut and the haemolymph. There is little doubt that the faecal pellets are composed of the digestion residues and also extruded epithelial cells. What is being questioned here is the allegedly necrotic state of the epithelium, which is definitely not normal in a healthy animal. It is considered here that the observations of those authors with respect to the contribution of the epithelial cells to the formation of the peritrophic membranes are likely to be the result of fixation artefacts (refer to Chapter 2 for more details about fixation problems and Chapter 7 for further discussion).

Several physiological functions have been proposed for the peritrophic membranes (see Peters 1992 for a thorough review on peritrophic membranes in invertebrates).

They may act as protection against mechanical damage of the midgut epithelium by coarse particles in the lumen (Sudha & Muthu 1988). Yoshikoshi & Ko (1988) found that all of the free living copepods studied produced peritrophic membranes in the whole length of the gut, and in the anterior midgut the thickness of it was greater then in the posterior midgut. Moreover, in most of the commensal and all the parasitic copepods thin peritrophic membranes were only present in the posterior midgut. The content of the midgut in free living copepods is usually composed of hard and/or sharp fragments, in opposition to commensal or parasitic forms, in which the gut contents are soft material coming from the tissues of the host. The function of the peritrophic membranes as protection against abrasion seems to fit in the case of copepods, including *L. salmonis*.

However, if the latter was the only function of peritrophic membrane then one would expect to be absent in fluid feeders, which is not the case. Other functions have been sought. They could have protection properties against foreign harmful molecules, with receptors (e.g. lectins) to identify such materials. As some digestive enzymes have been localised in peritrophic membranes it is thought that they could serve as a place to immobilise enzymes during digestion and preventing them being lost too quickly, or at least at a lower rate than those in the lumen (Peters & Kalnins 1985).

Peritrophic membranes may also provide compartments for different digestive processes. Blackburn, Wallbanks, Molyneux, Lavin & Winstanley (1988) suggested that the peritrophic membranes of the blood feeder sand fly *Phlebotomus papatasi* Luc serve for the confinement of large proteins, particularly serum trypsin inhibitors, to the endoperitrophic space while allowing sandfly proteases access to the blood meal periphery. Terra, Ferreira & Bianchi (1979) demonstrated that digestion in insect larvae takes place in spatially organised steps where the endo and ectoperitrophic spaces play an important role and this specialisation may be an adaptation to conserve digestive enzymes.

Terra & Ferreira (1981) proposed that if the latter function is correct one should find peritrophic membranes only in organisms with midgut luminal digestion. Most insects, including those feeding on blood and crustaceans have luminal digestion and the peritrophic membranes are present in most of them. And in those groups of insects in which the digestion is extra intestinal, the peritrophic membranes are absent. Although this theory seems to be plausible, there are exceptions to this rule, and *L. salmonis* seems one of them. Although *L. salmonis* produces peritrophic membranes, these seem to be absent when it switches to blood feeding. As it has been discussed before in this chapter, there are other blood sucking insects that share the similar pattern of blood digestion of *L. salmonis*, and also in them there is no production of peritrophic membranes. Therefore, the compartmentalisation of the midgut by peritrophic membranes may not be an important issue in these organisms. Moreover, Ramasamy, Raschid, Srikrishnaraj & Ramasamy (1996) found that in *Anopheles tessellatus* Theobald the formation of the peritrophic membranes was inhibited by anti-midgut antibodies, and were not essential for the survival of the mosquito.

Blackburn *et al.* (1988) also mention that another of the membranes' main functions is probably the prevention of clogging of the microvillous brush border by the blood meal. This seems not to apply in the case of *L. salmonis*, and as has been discussed above, this close contact between the blood meal and the microvilli may have an important significance for membrane contact digestion.

Another possible function of the peritrophic membranes associated with the excretion of unwanted material via the tape pellets has already been discussed in the previous chapter.

As Peters (1992) points out, peritrophic membranes may have other roles in digestion which as yet have not been identified and which require further investigation.

The hindgut of microcrustaceans such as copepods is regarded as a simple cuticle-lined tube (Dall & Moriarty 1983). In the present study, the structure of the midgut is not as simple as it seems when looked at closely. On the one hand, it is not a simple tube, as it is formed of two lateral cuticular plates that allow a variety of pseudoperistaltic movements comparable to the true peristalsis that would be performed by a set of circular muscles. On the other hand, the back-pointing spinules of the cuticular lining have never been recorded in copepods. And the reasons are clear. The spinules are very small, they cannot be detected under light microscopy. Under TEM a sagittal section through the right place would be necessary to spot them. SEM would be certainly the easiest way to confirm their presence, but morphological SEM studies usually do not look inside the lumen of the midgut or hindgut. Also, a thorough scan of the whole surface would be necessary because the distribution of the spinules is not uniform with some areas devoid of them. It would be a painstaking process to check every morphological description of the alimentary canal of all crustaceans to confirm if these structures have been recorded in other organisms. Looking into the reviews of crustacean groups could be a short-cut in this process. For the non-decapod crustaceans, no report on the microstructure of the hindgut lining was found in these reviews (see Harrison & Humes 1992). For decapods, it was fortunate to find the SEM study of Harris (1993b) who looked at the topography of the hindgut of 11 crabs and 5 thalassinid prawns while looking for the occurrence of bacteria. The two prawns of the genus *Upogebia* possess a convoluted smooth cuticular lining, with no spines or brush-like processes. The other 3 *Callianasa* prawns and all the crabs showed a cuticular lining with some regions with regularly spaced brush like projections of

the cuticle. Unfortunately, the author wrongly assumed that these cuticular projections were part of the epithelial brush border of the gut epithelium and even suggested that "the brushes on the lining... may aid in shedding the peritrophic membrane", a function clearly belonging to the microvilli of the midgut. Therefore, there are no relevant comments in his work regarding the possible function of these structures.

The description of the morphology of the hindguts is limited in this study of Harris (*op. cit.)*, as the main objective of his work was to look at the occurrence of bacteria. Nevertheless, the photographs are good and self explanatory and show an evident morphological diversity of these cuticular processes which possess a distinct pattern shared by the species of the same genus, but are different enough to be singled out specifically.

As observed in *L. salmonis* in the present study, there were zones with a smooth or convoluted cuticle, devoid of spines and areas with regularly spaced spines. The organisation of the spines in the organisms studied by Harris (*op. Cit.*) is variable. They can project individually as in *Sesarma catenata* Ortmann and *Uca* spp, or they can be grouped in regularly spaced tufts of variable number of spines, from 2 to 3 in *Panoepus hebstii* Milne-Edwards or *Callianasa australiensis* (Say) to 7-12 as in *Sesarma cinereum* Bosc or *Mictyris longicarpus* Latreille. The orientation of the spines is not mentioned but it is assumed that the spines point posteriorly as they do in the hindgut of *L. salmonis*. Hopkin & Nott (1980) also described the morphology of the cuticular lining of the hindgut of *Carcinus maenas* and reported similar spines. They are arranged in tufts of several spines and the groups are separated regularly. The arrangement looks similar to the one of *Mictyris longicarpus* depicted in Harris (1993b) In other decapods, longitudinal rectal pads covered with spinules have been reported (Pillai 1960). In penaeids, six smooth-surfaced pads of spongy tissue fill the lumen of the hindgut and taper posteriorly to longitudinal ridges of muscle . These longitudinal pads grasp the faecal pellet in its peritrophic envelope and expel it rhythmically (Dall & Moriarty 1983). This latter author suggests that the longitudinal ridges in other decapods have probably a similar function. Pugh (1962), Hopkin & Nott (1979) and Lovett & Felder (1989) have also reported spines in the hindgut of decapods. The function of these spines is very likely to aid in the defecation process, probably in a similar way as it happens in *L. salmonis*, preventing the

anterior movement of the faecal pellet during the muscular contractions that move it through the hindgut (Hopkin & Nott 1980). The hindgut spines of *L. salmonis* are very small compared to those of decapods. While in *L. salmonis* they measure about 0.6 μm in length, in the examples noted above the length of the spines range from 2 μm in *Myctiris longicarpus* to 13 μm in *Uca minax* LeConte.

The arrangement of the musculature of the hindgut in *L. salmonis* was almost identical to the one described by Boxshall (1990) for *L. pectoralis*. He states that these muscles dilate the lumen of the hindgut for elimination of faecal pellets. They also seem to have not only the function of dilating the lumen, but to actively aid in the pulling of the faecal pellets out of the hindgut. The activity of the hindgut when there were no pellets to expel is probably associated with the pumping of water into the gut. Anal drinking has been observed in many crustaceans and it could be associated with respiration or osmotic regulation (Dall & Moriarty 1983)

It would be interesting to investigate if the pattern of hindgut cuticular spines could be used as a fingerprint that could aid in the taxonomic identification to species level in crustaceans.

6. THE DIGESTIVE SEQUENCE.

In the previous chapters some aspects of the morphology of the digestive tract of *L. salmonis* have been studied. Cell types, suggestions of their possible role, the nature of its food and how the louse might be taking it have been established. Some morphological differences of the gut epithelium depending on the food ingested have also been pointed out. The objective of the present Chapter was to try to follow the morphological changes of the midgut epithelium during a digestive cycle.

But the duration of a digestive cycle, if it existed, was not known. It was thought that the investigation of the production of faecal pellets of *L. salmonis* might gave some idea of the sort of time scale that one could encounter for a digestive period.

Studies of faecal pellets can provide valuable information about the feeding behaviour of invertebrates that produce them. There is a wealth of studies regarding faecal pellets, in particular for copepods, according to Butler & Dam (1994). The production of faecal pellets has been used commonly as a rough measure of the ingestion rate (Ayukai & Nishizawa 1986) and as an indirect measure of the feeding period, where the feeding period is the time between pellet releases (Paffenhöfer, Bundy, Lewis & Metz 1995). With this in mind, a series of observations on the production of faecal pellets by individual lice feeding on their host were carried out to try to determine the feeding period that sea lice show or whether feeding is continuous. This information was necessary to establish the sampling strategy to follow the changes in morphology of the midgut during a digestive period.

6.1 MATERIALS AND METHODS

6.1.1 Monitoring of pellet production.

Small rectangular aquaria of 5 l capacity were used, fitted with an airstone and a lid. The size was only slightly longer than the salmon smolt in order to restrict its movements. Three aquaria were used, one fish in each. Fish were left to acclimatise to the frequent handling and restricted space

for 2-3 hours. During this time, the observer would check the fishes, move the aquaria and lift the lids regularly. Three lice were chosen from a batch that had been under starvation for five days. At the start of the observations, time zero, one louse was carefully handled with fine tweezers and put on the dorsum of each fish.

To assess pellet production, a spare aquarium was filled with seawater and put beside the aquarium to be checked. The fish, which by now was used to the presence of the observer, was carefully passed to the spare aquarium, using both hands, in a quick movement, putting the lid and the airstone back. The water of the aquarium was then passed through a plankton net which was tightly fitted to a PVC cylinder. With the aid of a jet bottle filled with seawater, the solids retained in the net were carefully concentrated in approximately 30-40 ml in a labelled 50 ml beaker. The same procedure was repeated for the remaining fishes.

The contents of the beaker were then distributed over the wells of a six-well tissue culture plate and the presence of faecal pellets determined under the dissecting microscope. A record was kept of the time of the observations, the type of pellets found recorded as a quick drawing of its shape and certain characteristics such as colour or dark spots.

Collection of pellets were carried out approximately every 3-4 hours. The light regime was 12 h light- 12 h darkness. Observations were concluded after 91:30 h. At this time, the lice were removed from the fish, observed under the dissecting microscope to record their overall aspect and presence of food in the gut. After this they were fixed and processed for LR White embedding, following the procedures detailed in Chapter 2.

Another similar set of observations was made with lice that had been on a fish for about 30 hours. In one case ("louse #4" in results), a fish had been infected with several lice in order to select feeding lice for other experiments. Whilst checking this fish it was noted that one of these lice was voiding a pellet at that precise moment. This moment was considered as time zero, the rest of the lice were taken off the fish, leaving this one in order to record pellet production following the same procedures described above, except that the observations were made every hour during 48 hours. The second case involved a fish that had been infected with several lice for a feeding trial. Lice

had been on this fish for about 24 h by the end of this trial. Those lice left on the fish were taken off, except one ("louse #5" in results). The pellet production of this louse was followed for 25 hours, taken samples as described above every hour during the first 9 hours, and then every 2-3 hours.

These last two set of observations were carried out in a constant light regime.

6.1.2 Cell cycle experiment.

6.1.2.1 Selection of lice for the experiments.

Although the observations of pellet production of section 6.1.1 gave an idea of how long the digestive cycle could be, it did not give enough clues as to the time lice would take to feed once they were put on the fish. Also, it was observed that not all lice that were put on the fish fed. To increase the probabilities that the lice that were put on a fish for experimental purposes were "feeding" lice, batches of variable amounts of lice were put on a neutral red stained salmon and after one or two hours they were taken off the fish and observed under the dissecting microscope. Those that showed food in the midgut were then put apart to be starved and used for further experiments. But this did not improve the amount of lice feeding, and the idea was abandoned.

Therefore, adult females collected in the usual way (Chapter 2) and starved for 24-36 h were used for the experiments.

These selected lice were put in 500 ml beakers filled with seawater with aeration. Water was changed daily.

6.1.2.2 Setting of sample intervals and sample size.

From the results of pellet production study, it seemed likely that most of the changes that could be observed during a digestive cycle, if there was one, were going to occur within the first 2-3 hours after feeding, therefore, the sampling intervals were set to be every 15 minutes during the first 2 h, then every 30 min for two hours and every one hour there on until 8 hours were completed.

It was decided to take 3 lice from each sampling period which would total 48 lice for the 16 samples proposed. In view of the early observation that not all lice were going to feed on the fish,

this quantity had to be doubled. Therefore, 96 lice had to be put on a fish at one time. As this was impracticable it was decided to divide the lice in two batches of 48 The first batch for the first two hours and a second batch for the rest.

6.1.2.3 Experimental set up.

The procedure for this experiment was the same as for the one described in section 6.1.1 with the difference that this time several lice had to be put on the fish in the minimum time possible.

Two methods were tried. The first one involved anaesthetising the fish with Benzocaine. Once the fish was anaesthetised, it was washed several times passing it in 3 different changes of seawater in order to wash out any excess of Benzocaine on the skin. It was then put on one side in an oval bowl filled with seawater, so that the water almost covered the flank of the fish, but leaving a little area of the skin uncovered. Previous to this, the number of lice required were put on a piece of wetted acetate sheet and kept slightly wet until the fish was ready to receive the lice. Then, one edge of the acetate was put against one side of the salmon, the one not covered with water and with a brush, all the lice were gently transferred to the fish. As soon as the lice touched the fish, they dispersed on the skin leaving room for the lice that were being pushed behind. In this way, it was possible to put a good amount of lice on the fish in less than a minute. Nevertheless, the method was found to be inappropriate because about one third of the lice detached from the fish in the first 5 minutes after the fish was put in the aquarium. It was thought that probably the lice were affected by the Benzocaine despite the efforts to remove it from the fish.

It was decided then to put each batch of lice one by one. With practice, this method proved to be quite quick. Fifty lice could be put on a fish in less than four minutes. For this purpose, the lice to be used were put first on a thick glass plate14x7 cm. The glass plate with the lice was held in one hand while working with the other one with a pair of long curved pointed tweezers. One by one, each louse was pushed to the edge of the glass plate until the thorax stuck out from the edge. Then, it was taken carefully with the tweezers by the posterior part of the thorax and quickly put on the fish, letting the louse make slight contact with the skin before letting it go. The first 20 or so lice were put on the anterior dorsal part and sides of the fish and the rest on the sides and dorsum

behind the dorsal fin. In this way one could be fairly sure that the first samples of lice would be from the lice that were put on the fish first.

6.1.2.4 Sampling Procedure.

Lice were taken from the fish with tweezers. It was easy to pick up the lice from the fish without disturbing it much by sliding one side of the curved tweezers below the thorax of louse to be sampled and closing them on one side of the thorax to remove it from the fish and put in a Petri dish. Then, the lice were taken to the dissecting microscope to check if they showed any signs of having fed. Those lice with clear signs of feeding activity like food material in the midgut and/or midgut stained red from the neutral red of the salmon skin. It was easy to discern the unwanted lice, from the feeding ones. The non-feeding ones showed no sign of activity in the midgut, no peristalsis or contractions, no sign of any food material inside the midgut and no sign of neutral red stain in the midgut. Three lice were fixed first immediately before starting the experiment and were labelled as 0:00 h. These would be the starting reference. "Feeding" lice of each subsequent sample were dissected and fixed according to the procedures detailed in Chapter 2, including the tying of a knot around the hindgut with a piece of hair prior to fixation. This operation, apart from holding the gut contents inside the louse during fixation and processing, allowed to identify each individual louse by associating it with the type of knot. Seven "types" of knot were easily produced by cutting the ends at different lengths, very short, medium and long ends. This permitted a considerable amount of time saved in processing, because three or four lice from the same sample could be put in one vial for fixation, buffer wash, dehydration, etc.

All lice were divided in three portions and embedded in LR White as detailed in Chapter 2, section 2.2.4. Two 2 μm thick sections from each louse were cut, mounted on glass slides and stained with Toluidine blue. Special care was observed during embedding and sectioning so that all lice were cut at about the same level in each gut portion. The frequency of the following characteristics was recorded for each section and each portion of the gut of each louse: Cell types (A- and B-cells); gut types; gut contents (absent, salmon epithelium, blood, melanin) and presence or absence of

peritrophic membranes. The raw data were fed to a *ad hoc* designed database to be able to correlate the large amount of data that was generated.

When mixtures of gut types or gut contents were found, they were recorded in descending order of predominance.

6.1.3 Statistical methods.

The Chi square test was used to test the hypothesis that the frequencies of occurrence in the various categories (usually present or absent) of one variable (gut type, gut section, gut contents, cell type, peritrophic membranes) were independent of the frequencies of the second variable. Data were tabulated in contingency tables for this purpose. In the cases of 2X2 contingency tables, they were assessed using the Yate's correction for continuity. Three-dimensional contingency tables were used to test more than one hypothesis regarding the independence of more than two variables, first testing for mutual independence and if Ho was rejected, the analysis was continued for partial independence and the contingency tables subdivided. Significance level (α) was 0.05. All statistical methods were from Zar (1996).

6.2 RESULTS.

6.2.1 Pellet production.

Figure 6.1 shows the faecal pellet production over time of five lice, each feeding on a single fish.

At the beginning there was a problem with louse #2, which detached during the second sampling , was put again on the fish and detached again in the third sampling interval. A different louse was then chosen and put on the fish.

As the sampling intervals for lice #1, #2 and #3 were between 3 and 4 hours, the release of pellets could have happened anytime between this sampling interval. This implies that the highlighted periods when no pellets were released are the *minimum* times when pellets were not produced, the maximum being up to 6-8 hours *more* than the time stated (adding 3 to 4 hours to either side), as we do not know exactly when a pellet was voided during the time between samples.

A common characteristic in the first three lice was the production of tape pellets during the first hours of the observations. Once a louse started feeding, the production of tape pellets ceased and did not appear again, except in association with a clear pellet (indicated in Figure 6.1 as clear/tape: "CL/TP"). This production of tape pellets had been observed before in lice put under starvation (Chapter 4).

The time elapsed between the start of the experiment and the first appearance of a pellet, indicative that the louse had fed, was variable: 9-12 hours in louse #1 and 39-43 hours in louse #2. Louse #3 did not produce a clear or dark pellet during 31 hours. Unfortunately, an accident occurred, when the fish jumped out of the aquarium and died and no further observation could be made on louse #3.

The number of pellets observed at each sampling could be misleading due to the fragility of the pellets, particularly the longer ones, which sometimes broke into several parts. Careful observation of the pellets was then necessary to establish the actual number of pellets produced. It was found that the pieces could be put together again as the pieces of a puzzle. In one occasion, up to 4 fragments belonging to one long dark pellet were found.

The longest interval between releases of faecal pellets was 14 hours. The shortest was 3 hours in lice #1 and #2 and 1 hour in lice #4. Table 6.1 summarises the events depicted in Figure 6.1

The faecal pellet release was very similar in lice #1 and #2. Of nine occasions where louse #1 released a clear or dark pellet, louse #2 voided a similar one in five, a tape pellet in two occasions (intervals 9-12 h and 24-26 h) and nothing in two (intervals 21-23 and 81-84 h). At the end of the observation, both lice had a well formed dark pellet in the mid-posterior midgut and since both had not released a pellet in the previous seven hours, it is likely that they were very near the time of defecating. From the time of the release of the first clear or dark pellet until the end of the experiment, the pellet production of lice #1 was 0.13 pellets/h (12 pellets in 91 h) and that of lice #2 was 0.10 (6 pellets in 61 h).

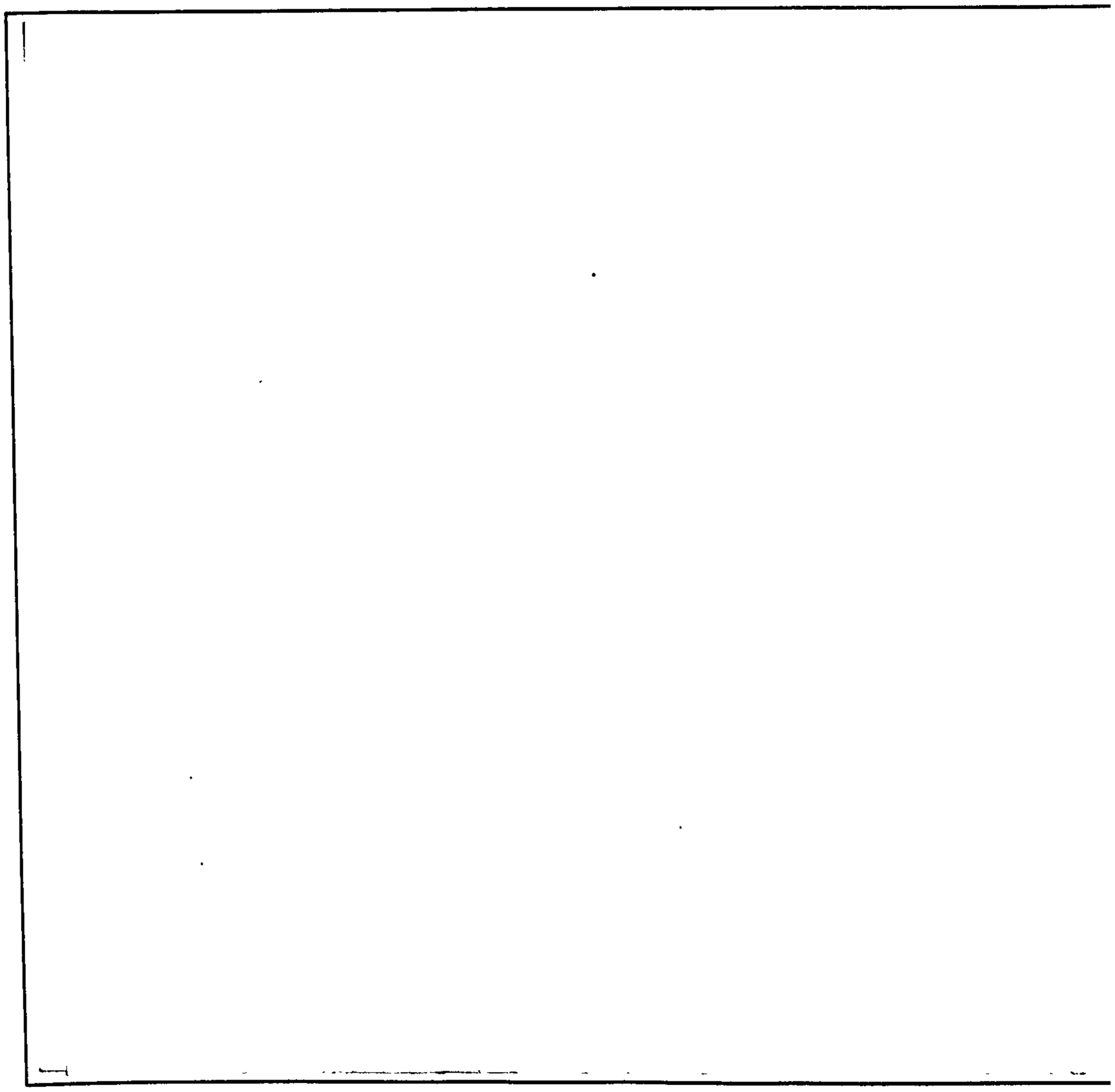
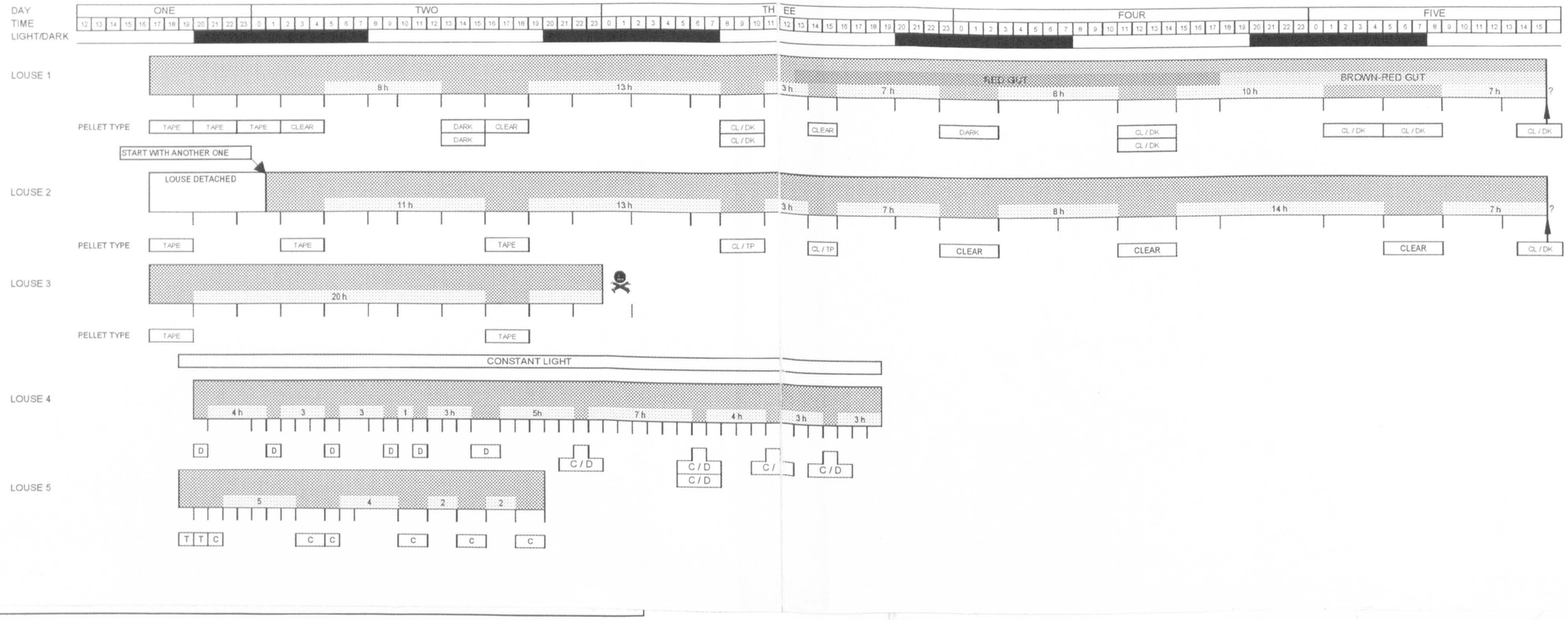


Figure 6.1 Graphic representation of the faecal pellet release by five lice, each feeding on a salmon smolt, over time. The tick marks below each bar indicate when a sample was taken. The clear bars within the main bars indicate the hours elapsed with no pellet release. C/D: mixture of clear and dark pellet; D: dark pellet; T or TP: tape pellet.

Table 6.1 Intervals between releases of faecal pellets by individual adult females. Lice No. 1 and 2 were under a regime of light and dark 12-12 and lice 4 and 5 under constant illumination. Pellets/h is the number of pellets released by the louse divided by the time elapsed from the release of the first clear or dark pellet until the end of the observations. CV: coefficient of variation; SD: standard deviation.

Louse No.	No. Intervals	Mean (h)	SD (h)	CV (%)	Pellets/h
1	7	8:00	3:03	38.1	0.13
2	7	9:00	3:52	43.0	0.10
4	10	3:36	1:34	43.8	0.23
5	4	3:15	1:30	46.2	0.24



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Louse No.	No. Intervals	Mean (h)	SD (h)	CV (%)	Pellets/h
1	7	8:00	3:03	38.1	0.13
2	7	9:00	3:52	43.0	0.10
4	10	3:36	1:34	43.8	0.23
5	4	3:15	1:30	46.2	0.24

But louse #4 behaved differently. Intervals between pellet release were generally of 3 hours (5 cases), but 1 h (1 case) and 4 hours (2 cases) or 5 or 7 (one case respectively) were also recorded. It is worth noting that this louse had been established on the fish for about 30 hours before the start of the observations. But the time on the fish is probably not the reason for these shorter intervals between pellet releases because in lice #1 and #2, after a similar period on the fish, the interval between pellet production did not reduce that much, except for the 43-45 h interval.

Louse #5 also had shorter intervals between pellet release, 5, 4 and 2 h,

In the first 24 h period in lice #4 and #5, each one produced 6 pellets, while in a equivalent period of time once they had established, lice #1 and #2 produced half this amount (for example between 8 AM of day 3 and 8 AM of day 4.). Pellet release rate of lice #4 and #5 was 0.23 and 0.24 pellets/h (11 pellets in 47 h and 6 pellets in 25 h respectively). The coefficient of variation was very similar in the four lice.

Most of the lice observed during this study that had a pellet in their mid and posterior midgut did not have loose food in the rest of the gut, and vice versa, those with loose skin fragments in the midgut did not have a pellet formed or, sometimes one could observe accumulation of food in the mid and posterior midgut but still not completely compacted in a formed pellet. In several cases lice voided a clear or dark pellet after they were taken from the fish during various samplings for this experiment and for other observations in this study. Whether the voiding of the pellet was induced by handling stress or not is unknown but, the fact is that there was no sign in the midgut that the louse was having, or had had a new meal.

Loose food material was found only in lice that had been under starvation and were put on a fish and retrieved within one hour. In all these cases (*i.e.* isolated observations during trials, observations described in chapters 4, 5 and this one), a pellet had not yet been formed, and the loose skin fragments of the fish were being moved back and forth due to peristalsis. In the above mentioned trials, the shortest time of first feeding after a louse was put on a fish was 30 minutes (see Table 6.2). The longest was 1:30 h in one case and the mode was 60 min. It should be taken

into account that all these observations were made under conditions of constant illumination. So, taking into account that the time between samples was 15 minutes, this means that between 30 and 45 minutes or 45 and 60 minutes after being put on a fish, a louse begins to feed. From these observations, is seemed unlikely that a louse would start to feed immediately after being put on a fish.

The most frequent time for the first sign of pellet in the posterior midgut was 3:00 h (Table 6.2), ranging from 2 to 3:30 h. This range of time matches with the average interval between pellet releases in the case of louse #4 and #5. This could mean that a feeding period is probably completed in the range of 3-5 hours, under constant light or probably longer, twice the time, in a light/dark regime. The critical time then to observe possible physiological and morphological changes during the first stages of the digestion of a meal seemed to be within this range. The sampling periods and duration of the "digestive period" experiment were set up with this in mind.

It has to be stated that the time of first feeding and first appearance of a pellet in the midgut indicated in Table 6.2 comes from lice that actually fed. During the many trials and observations in this study it was noticed that a large proportion of lice did not feed at all when put on the fish. Table 6.3 and the accompanying histogram of the frequencies of these proportions (Figure 6.2) illustrate this. A record was kept of the lice put on a fish and the number of lice that did not feed or that repeatedly detached from the host (and which were counted as "not feeding"). There were extreme cases on both sides of the spectrum. For example, there were 3 batches of lice in which a great majority (80-83%) of lice fed within 4 h, but also there were 3 batches in which none or just one of the lice put on the fish (<10%) started to feed within one or two hours of being on their host. Interestingly, these lowest percentages corresponded to lice with the shortest starvation period (8 h) In those batches in which lice were allowed to stay longer on the fish (11 and 15 hours) the resulting proportion of lice feeding remained low (53.5 and 33.3 % respectively). The most common values fell into the 50s% range. This posed a considerable problem for any feeding experiments because twice the lice needed for a particular experiment or observations had to be put on the fish in order to get the required amount of data. It was thought that if one selected "feeding" lice for

subsequent experiments this percentage was going to increase, but the result was the same. In Table 6.3 is shown that the proportion of these "feeding" lice (marked with ⇧) also remained low.

Table 6.2 Minimum times recorded before indication that a louse had fed and appearance of faecal pellet in the mid and/or posterior midgut of lice. Times were converted to decimal notation for the calculation of average, standard deviation (SD) and coefficient of variation (CV). The observations were done under continuous illumination.

Observation	Time before Indication of food	Time before indication of faecal pellet
1	0:30	3:00
2	0:30	3:15
3	0:45	3:30
4	0:45	3:00
5	1:00	3:30
6	1:00	3:00
7	1:00	2:00
8	1:00	2:45
9	1:15	2:30
10	1:15	3:00
11	1:30	3:00
Mean	0:56	2:57
SD	0:17	0:25
CV	30.1%	14.5%

The unpredictability of lice feeding behaviour was illustrated by louse #1. Sometime after 46 or 47 hours this louse apparently began feeding on blood because the gut was completely red. By the next sample, the red colour in the gut was even more intense. The red colour was maintained until the end of the experiment with possibly a slight change by 74 h where it looked brownish red. As the louse was on the fish, the exact colour and condition was difficult to evaluate, but it was definitely red. However, contrary to expectations, the next pellets produced by this louse after the red gut condition started, were similar to a "normal" dark pellet, with undigested salmon epithelial cells tightly packed.

Either the pellet with remnants of red blood cells was missed or lost or the red blood cells were completely lysed and the unrecognisable remnants mixed with the epithelial cells and pellet matrix. The first option is unlikely. The louse must have fed on blood more times during the remaining time

because the gut continued to be red until the end of the experiment. If the louse had fed only once, the red colour would have vanished by 24 h (see Chapter 5, section 5.2.2). Pellets with vestiges of red blood cells were not found in the rest of the pellets produced by this louse.

6.2.2 Morphological changes during the digestive period.

Despite the preliminary experiments to determine the sampling schedule, the sampling programme was difficult to maintain because of the low proportion of feeding lice and the unpredictable nature of their feeding behaviour. As a result only one or two lice were suitable for each sampling period or selecting suitable specimens resulted in delays in lice being taken lice taken in-between the proposed periods. This was more critical during the first two hours, where the sampling periods were planned every 15 minutes.

At the end of the 8 hours, some lice remained on the fish, therefore more samples were taken between 11 and 11:30 h (4 lice) and at 13 h. and 22 h (2 lice respectively). By these times, even after 22 h there still remained lice that had not fed at all. It was therefore difficult to associate any lice taken at a certain hour to the real "time after feeding". The only period where it was possible to be relatively certain about this was those feeding within one hour. Even then it was not known exactly how long ago the lice started feeding as it could have been 15 or 30 minutes before. After the first hour, this uncertainty increased considerably, for example, for a louse taken after 7 hours with a pellet in the posterior midgut; it was not possible to ascertain if this pellet came from the first meal taken 3 or 4 hours before, or from a second meal, the first one having occurred at the beginning of the experiment. Any attempt to associate any changes with time would appear to be unreliable. Nevertheless an attempt was made, and none of the parameters (cell types, gut types, gut contents, peritrophic membranes) showed any relation with time.

The attempts to put a time scale or determine the temporal sequence of cellular events therefore were not successful. However, some interesting observations could be made.

Table 6.3 Number and percentages of feeding and non-feeding lice after being put on a salmon smolt. Time left on fish indicate the time that lice were left on the host before being removed to assess if they had fed on the host. ✞: "feeding" lice that were starved 24 hours before being put back on the host again.

No. LICE PUT ON FISH	No. LICE FEEDING	%	NOT FEEDING	%	TIME LEFT ON FISH
12	10	83.3	2	16.7	4.00
34	28	82.4	6	17.6	4.00
15	12	80.0	3	20.0	2.00
22	15	68.2	7	31.8	1.00
30	20	66.7	10	33.3	3.00
8	5	62.5	3	37.5	1.00
17	10	58.8	7	41.2	2.00
40	22	55.0	18	45.0	4.00
44	24	54.6	20	45.4	3.00
11	6	54.6	5	45.5	1.00
43	23	53.5	20	46.5	15.00
16	8	50.0	8	50.0	2.00
63	31	49.2	32	50.8	2.00
19✞	9	47.4	10	52.6	1.00
60	24	40.0	36	60.0	2.00
33✞	12	36.4	21	63.6	2.00
57	19	33.3	38	66.7	11.00
13	1	7.7	12	92.3	2.00
50	1	2.0	49	98.0	1.00
50	0	0.0	50	100.0	1.00

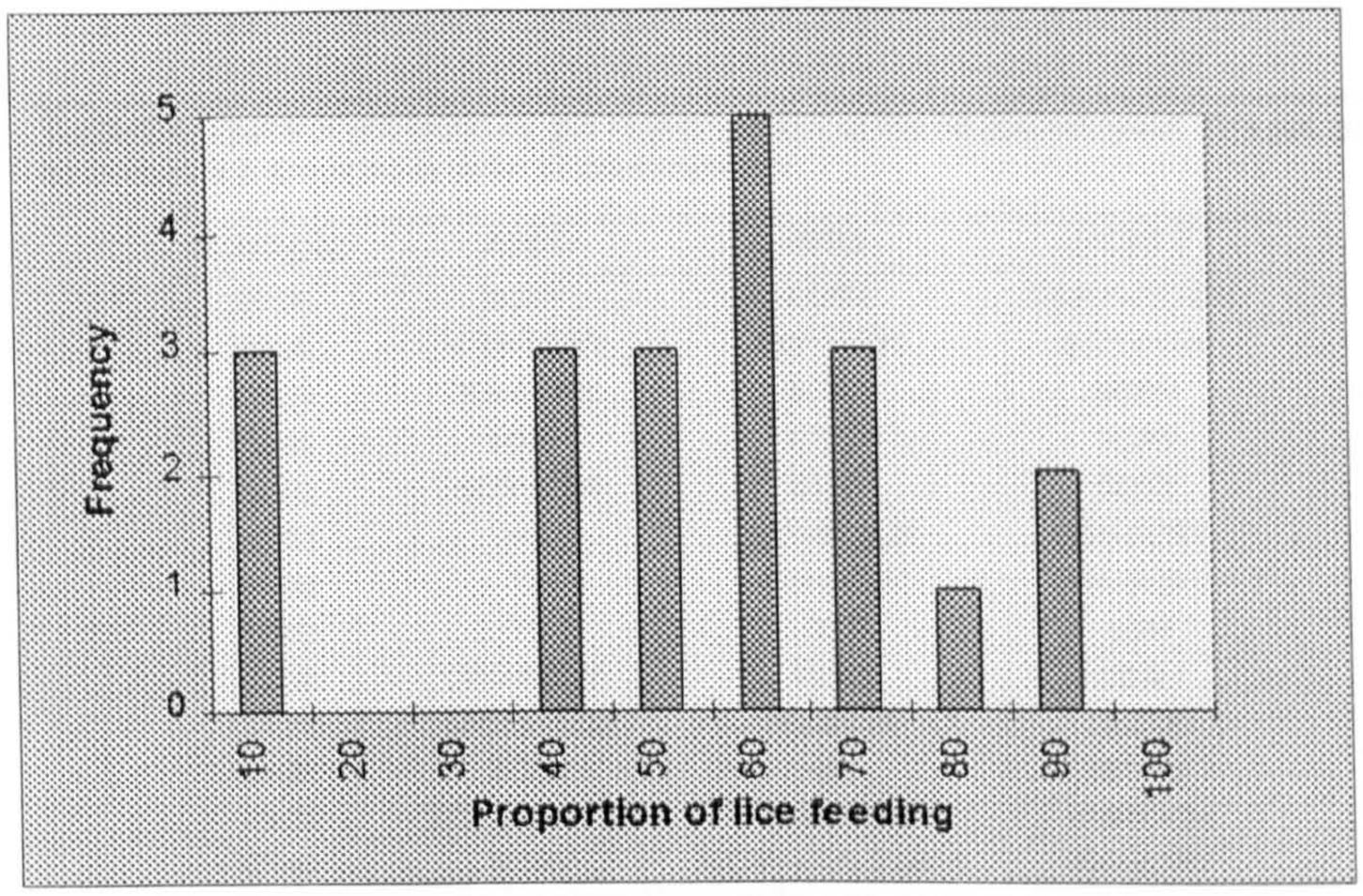

Figure 6.2 Histogram showing the frequency of the proportion of lice that fed on their host after being put on it for at least one hour.

6.2.2.1 Gut types

Table 6.4 shows the types of gut found in each portion of the midgut for each louse sampled.

As mentioned in the methods section, when two or more types of gut were present in the section they were recorded in order of predominance. For example a SCRY+TH+STACK gut means that most of it was ShallowCRYpted, but some areas were THin and other areas had STACKed epithelium.

The most common type of gut was SCRY, either as the predominant type (Table 6.5) or in general occurrence disregarding its predominance (Table 6.6), followed by THin type.

The occurrence of MC and DC guts was very low, 9% and 0.6%, just one case, respectively.

VeryThin guts were not common either, only 13%. Looking at Table 6.4 there are 6 lice that have VTH guts (or TH together with VTH) the three sections of the midgut as first option. These lice make up for almost half the occurrence of VTH guts. These 6 had fed on the host' skin (all of them showed signs of a meal, see Table 6.11), and in three of them, the sign of the meal were in all the 3 gut sections.

Table 6.4 Types of gut found by gut region in each of the 48 lice sampled. The gut types in each case are arranged in order of predominance.

Sample	ANT	MID	POST
00:00	SCRY	SCRY	SCRY+STACK
00:00	VTH+SCRY	SCRY+TH	SCRY+TH
00:30	TH+SCRY	SCRY	SCRY
00:45	SCRY	SCRY	SCRY
01:00	SCRY+TH	SCRY	SCRY
01:00	MC	MC+STACK	TH+SCRY
01:00	TH	TH+SCRY	TH+SCRY
01:00	TH	TH	VTH
01:00	TH+SCRY	SCRY	SCRY
01:25	MC+STACK	TH+SCRY	SCRY
01:35	SCRY	MC	SCRY
01:35	SCRY+MC+TH	SCRY+MC	SCRY+TH
01:40	SCRY	SCRY+TH+STACK	TH+SCRY
01:40	SCRY+STACK	SCRY+STACK	TH+STACK+SCRY
01:40	VTH	TH+STACK+SCRY	TH+VTH
01:45	TH	TH	TH
01:45	TH+SCRY+STACK	SCRY+MC	SCRY
01:45	VTH	VTH+VTH	VTH
01:45	VTH+TH	VTH+TH	VTH+SCRY
02:30	VTH	TH	VTH
03:00	SCRY	SCRY	TH+SCRY
03:10	MC	MC	VTH
03:15	SCRY	SCRY+MC	SCRY
04:00	MC	TH+SCRY	TH+SCRY
04:00	TH+SCRY	MC	TH+SCRY+STACK
04:00	VTH	VTH	VTH
04:35	TH+SCRY	SCRY+TH	SCRY+TH
04:40	TH+SCRY+STACK	TH+STACK	TH+SCRY+STACK
04:45	MC	DC+MC	SCRY
05:20	SCRY	SCRY	SCRY
05:30	SCRY	SCRY+TH	SCRY+TH+STACK
05:35	SCRY	SCRY	SCRY
05:45	TH+SCRY	SCRY+TH	SCRY
05:50	SCRY+DCRY	SCRY	MC
06:15	SCRY	SCRY	SCRY
06:15	TH+SCRY	SCRY	MC+SCRY
06:40	SCRY	SCRY	SCRY
07:50	TH+VTH	TH+VTH	VTH
08:00	SCRY+STACK	SCRY	MC+STACK+DC
08:00	SCRY+STACK	SCRY	SCRY+STACK
11:00	SCRY	SCRY	SCRY+TH
11:00	SCRY+STACK	SCRY+MC	SCRY+MC
11:30	SCRY	MC+SCRY	SCRY+MC
11:30	SCRY+TH+STACK	SCRY	SCRY
13:00	TH+VTH	SCRY+TH+STACK	SCRY+TH
13:00	VTH	VTH	VTH+TH
22:30	SCRY	SCRY	SCRY+STACK
22:30	TH	SCRY	SCRY+DC

Table 6.5 Occurrence of predominating gut types by gut portion. Percentages in parenthesis

	SCRY	MC	DC	TH	VTH
ANT	22	5	0	14	7
MID	29	5	1	9	4
POST	27	3	0	10	8
TOTALS	78 (54)	13 (9)	1 (0.6)	33 (23)	19 (13)

Table 6.6 General occurrence of gut types by gut portion.

	SCRY	*TH*	*VTH*	*MC
ANT	31	24	10	6
MID	34	19	5	10
POST	37	23	9	5
TOTALS	102	66	24	21

Table 6.7 Gut types in the different gut portions grouped in two "families". CRYpteds and THINs.

	CRYs	THINs
ANT	27	21
MID	35	13
POST	.30	18
TOTAL	92	52

Table 6.8 Miniaturised version of Table 6.4 with cells highlighted when TH or VTH gut type were predominant in the corresponding gut portion.

Sample	ANT	MID	POST
00:00	SCRY	SCRY	SCRY+STACK
00:00	VTH+SCRY	SCRY+TH	SCRY+TH
00:30	TH+SCRY	SCRY	SCRY
00:45	SCRY	SCRY	SCRY
01:00	SCRY+TH	SCRY	SCRY
01:00	MC	MC+STACK	TH+SCRY
01:00	TH	TH+SCRY	TH+SCRY
01:00	TH	TH	VTH
01:00	TH+SCRY	SCRY	SCRY
01:25	MC+STACK	TH+SCRY	SCRY
01:35	SCRY	MC	SCRY
01:35	SCRY+MC+TH	SCRY+MC	SCRY+TH
01:40	SCRY	SCRY+TH+STACK	TH+SCRY
01:40	SCRY+STACK	SCRY+STACK	TH+STACK+SCRY
01:40	VTH	TH+STACK+SCRY	TH+VTH
01:45	TH	TH	TH
01:45	TH+SCRY+STACK	SCRY+MC	SCRY
01:45	VTH	VTH+VTH	VTH
01:45	VTH+TH	VTH+TH	VTH+SCRY
02:30	VTH	TH	VTH
03:00	SCRY	SCRY	TH+SCRY
03:10	MC	MC	VTH
03:15	SCRY	SCRY+MC	SCRY
04:00	MC	TH+SCRY	TH+SCRY
04:00	TH+SCRY	MC	TH+SCRY+STACK
04:00	VTH	VTH	VTH
04:35	TH+SCRY	SCRY+TH	SCRY+TH
04:40	TH+SCRY+STACK	TH+STACK	TH+SCRY+STACK
04:45	MC	DC+MC	SCRY
05:20	SCRY	SCRY	SCRY
05:30	SCRY	SCRY+TH	SCRY+TH+STACK
05:35	SCRY	SCRY	SCRY
05:45	TH+SCRY	SCRY+TH	SCRY
05:50	SCRY+DCRY	SCRY	MC
06:15	SCRY	SCRY	SCRY
06:15	TH+SCRY	SCRY	MC+SCRY
06:40	SCRY	SCRY	SCRY
07:50	TH+VTH	TH+VTH	VTH
08:00	SCRY+STACK	SCRY	MC+STACK+DC
08:00	SCRY+STACK	SCRY	SCRY+STACK
11:00	SCRY	SCRY	SCRY+TH
11:00	SCRY+STACK	SCRY+MC	SCRY+MC
11:30	SCRY	MC+SCRY	SCRY+MC
11:30	SCRY+TH+STACK	SCRY	SCRY
13:00	TH+VTH	SCRY+TH+STACK	SCRY+TH
13:00	VTH	VTH	VTH+TH
22:30	SCRY	SCRY	SCRY+STACK
22:30	TH	SCRY	SCRY+DC

Table 6.9 Miniaturised version of Table 6.4 with cells highlighted when SCRY or MC or DC gut type were predominant in the corresponding gut portion.

Sample	ANT	MID	POST
00:00	SCRY	SCRY	SCRY+STACK
00:00	VTH+SCRY	SCRY+TH	SCRY+TH
00:30	TH+SCRY	SCRY	SCRY
00:45	SCRY	SCRY	SCRY
01:00	SCRY+TH	SCRY	SCRY
01:00	MC	MC+STACK	TH+SCRY
01:00	TH	TH+SCRY	TH+SCRY
01:00	TH	TH	VTH
01:00	TH+SCRY	SCRY	SCRY
01:25	MC+STACK	TH+SCRY	SCRY
01:35	SCRY	MC	SCRY
01:35	SCRY+MC+TH	SCRY+MC	SCRY+TH
01:40	SCRY	SCRY+TH+STACK	TH+SCRY
01:40	SCRY+STACK	SCRY+STACK	TH+STACK+SCRY
01:40	VTH	TH+STACK+SCRY	TH+VTH
01:45	TH	TH	TH
01:45	TH+SCRY+STACK	SCRY+MC	SCRY
01:45	VTH	VTH+VTH	VTH
01:45	VTH+TH	VTH+TH	VTH+SCRY
02:30	VTH	TH	VTH
03:00	SCRY	SCRY	TH+SCRY
03:10	MC	MC	VTH
03:15	SCRY	SCRY+MC	SCRY
04:00	MC	TH+SCRY	TH+SCRY
04:00	TH+SCRY	MC	TH+SCRY+STACK
04:00	VTH	VTH	VTH
04:35	TH+SCRY	SCRY+TH	SCRY+TH
04:40	TH+SCRY+STACK	TH+STACK	TH+SCRY+STACK
04:45	MC	DC+MC	SCRY
05:20	SCRY	SCRY	SCRY
05:30	SCRY	SCRY+TH	SCRY+TH+STACK
05:35	SCRY	SCRY	SCRY
05:45	TH+SCRY	SCRY+TH	SCRY
05:50	SCRY+DCRY	SCRY	MC
06:15	SCRY	SCRY	SCRY
06:15	TH+SCRY	SCRY	MC+SCRY
06:40	SCRY	SCRY	SCRY
07:50	TH+VTH	TH+VTH	VTH
08:00	SCRY+STACK	SCRY	MC+STACK+DC
08:00	SCRY+STACK	SCRY	SCRY+STACK
11:00	SCRY	SCRY	SCRY+TH
11:00	SCRY+STACK	SCRY+MC	SCRY+MC
11:30	SCRY	MC+SCRY	SCRY+MC
11:30	SCRY+TH+STACK	SCRY	SCRY
13:00	TH+VTH	SCRY+TH+STACK	SCRY+TH
13:00	VTH	VTH	VTH+TH
22:30	SCRY	SCRY	SCRY+STACK
22:30	TH	SCRY	SCRY+DC

STACKed epithelium was never recorded as first option. This is because this type of epithelium was usually restricted to small areas spanning 10 or 15 cells, and was also generally associated with SCRY gut.

There was no dependence of the type of gut and the gut region ($p=0.587$ and $p=0.628$ respectively), therefore, the number of gut types in each region were pooled (see totals in Table 6.6 and Table 6.5 . This gave significant differences between the proportions of gut types in both cases, as predominant or as general occurrence ($p << 0.001$).

As mentioned earlier, caution is necessary when trying to associate any morphological changes with time. Nevertheless, after grouping gut types into two "families" of similar morphology, like THin, and VeryTHin in one (which will be called "THINs") and Shallow-CRYpted, Medium-Crypted and Deep-Crypted in the other ("Crypteds"), some tendencies were evident.

Table 6.7 shows the occurrence of gut types by gut region after grouping them in these two "families". Again, their number was independent of the gut region (p= 0.229), but from the totals, CRYpted guts remained as the most common type (p<< 0.001)

Table 6.8 and Table 6.9 are miniaturised versions of Table 6.4 made to visualise better the distribution of gut types in time. The gut portion in which the type of gut belonging to the same "family" type as the predominant type is highlighted. THINs are highlighted in Table 6.8 and CRYpteds in Table 6.9 . . .

Table 6.10 Occurrence of gut type families at selected periods of time during the experiment. Percentages are in parenthesis.

	CRYpteds	THINs
Before 4:40 h	42 (50.0)	42 (50.0)
From 4:45 h onwards	50 (83.4)	10 (16.6)
All the time period	92 (63.9)	52 (37.1)

Considering the type of gut in each portion of the midgut as a separate one, from the start to the end of the experiment, it can be seen that "Crypted" types dominated the picture, at a 63.9% (p= 0.0011). But from 4:45 h until the end of the experiment, the occurrence of this type rose significantly to 83.4% (p<< 0.001). Before that time, from 0:00 h to 4:40 the ratio was exactly 1:1 (p<< 0.001). Table 6.10 summarises this point.

6.2.2.2 Gut contents.

The different gut contents recorded for each section of the gut of every lice are displayed in Table 6.11 . The gut contents found were salmon skin epithelium chunks, red blood cells, A-cells and other enterocytes sloughed from the louse gut epithelium, flocculate material and debris and melanin granules from the salmon dermis.

Table 6.12 shows graphically the occurrence of gut contents indicative of a meal in the sections of the midgut. From this table it can be appreciated that the firsts meals were taken after one hour, and from 1:35 h almost all lice have indication of a meal in their guts. This was expected because only lice with evidence of feeding activity were chosen at every sample, as explained in the methods.

Table 6.11 Occurrence of gut contents by section of gut.; A: A-cells; FLOC: flocculate material. LEN: Louse enterocytes; MEL: melanin granules; MV: microvilli; RBC: red blood cells; SEP: salmon epithelium;

TIME	ANT	MID	POST
00:00	A	NO	A
00:00	A	NO	NO
00:30	FLOC	A+FLOC	NO
00:45	NO	NO	NO
01:00	SEP+A	SEP+A	SEP
01:00	NO	SEP	SEP
01:00	FLOC	FLOC	SEP
01:00	NO	NO	NO
01:00	NO	NO	NO
01:25	NO	NO	NO
01:35	NO	NO	NO
01:35	SEP+RBC	RBC	SEP+RBC
01:40	NO	RBC	SEP+RBC
01:40	NO	SEP+MEL	NO
01:40	NO	NO	SEP+MEL
01:45	NO	NO	SEP
01:45	NO	SEP+MEL+DEBRI	SEP+MEL+DEBRI
01:45	NO	RBC (3)	SEP+MEL+RBC
01:45	NO	SEP	NO
02:30	LEN	SEP	SEP
03:00	NO	A	A
03:10	DEBRI	SEP+MEL	NO
03:15	LEN	MEL+FLOC+DEBRI	SEP+MEL
04:00	NO	NO	SEP
04:00	SEP+MEL+DEBRI	NO	SEP+MEL
04:00	SEP+MEL	SEP+MEL+A	SEP
04:35	NO	NO	SEP
04:40	SEP	MEL+DEBRI	SEP
04:45	MV+DEBRI	NO	MV
05:20	NO	NO	SEP
05:30	RBC	SEP+MEL	SEP+MEL+A+RBC
05:35	RBC+MEL	SEP+MEL	SEP+MEL+A+FLOC
05:45	MV	MV	SEP+MEL
05:50	A	SEP+MEL+A	SEP+MEL+A
06:15	NO	NO	SEP
06:15	NO	A	A
06:40	NO	NO	SEP+FLOC+MEL
07:50	SEP	SEP+DEBRI	SEP+MEL+DEBRI
08:00	NO	SEP+MEL+A	SEP+A
08:00	NO	NO	SEP+MEL
11:00	FLOC+LEN+A	A	SEP+MEL+FLOC
11:00	LEN	FLOC+LEN	FLOC+LEN
11:30	NO	NO	SEP+MEL+A+FLOC
11:30	NO	MEL	A+FLOC
13:00	RBC	SEP+MEL+A+RBC	SEP+MEL+RBC
13:00	SEP+MEL	SEP+MEL+A+RBC	SEP+MEL+A+RBC
22:30	SEP+FLOC+MEL+A	SEP+MEL+FLOC	SEP+MEL+FLOC
22:30	NO	SEP+A	SEP

Table 6.12 Miniature version of Table 6.11 in which gut contents indicative of a meal (SEP, MEL, RBC) have been highlighted.

TIME	ANT	MID	POST
00:00	A	NO	A
00:00	A	NO	NO
00:30	FLOC	A+BAC+FLOC	NO
00:45	NO	NO	NO
01:00	SEP+A	SEP+A	SEP
01:00	NO	SEP	SEP
01:00	FLOC	FLOC	SEP
01:00	NO	NO	NO
01:00	NO	NO	NO
01:25	NO	NO	NO
01:35	NO	NO	NO
01:35	SEP+RBC	RBC	SEP+RBC
01:40	NO	RBC	SEP+RBC
01:40	NO	SEP+MEL	NO
01:40	NO	NO	SEP+MEL
01:45	NO	NO	SEP
01:45	NO	SEP+MEL+DEBRI	SEP+MEL+DEBRI
01:45	NO	RBC (3)	SEP+MEL+RBC
01:45	NO	SEP	NO
02:30	LEN	SEP	SEP
03:00	NO	A	A
03:10	DEBRI	SEP+MEL	NO
03:15	LEN	MEL+FLOC+DEBRI	SEP+MEL
04:00	NO	NO	SEP
04:00	SEP+MEL+DEBRI	NO	SEP+MEL
04:00	SEP+MEL	SEP+MEL+A	SEP
04:35	NO	NO	SEP
04:40	SEP	MEL+DEBRI	SEP
04:45	MV+DEBRI	NO	MV
05:20	NO	NO	SEP
05:30	RBC	SEP+MEL	SEP+MEL+A+RBC
05:35	RBC+MEL	SEP+MEL	SEP+MEL+A+FLOC
05:45	MV	MV	SEP+MEL
05:50	A	SEP+MEL+A	SEP+MEL+A
06:15	NO	NO	SEP
06:15	NO	A	A
06:40	NO	NO	SEP+FLOC+MEL
07:50	SEP	SEP+DEBRI	SEP+MEL+DEBRI
08:00	NO	SEP+MEL+A	SEP+A
08:00	NO	NO	SEP+MEL
11:00	FLOC+LEN+A	A	SEP+MEL+FLOC
11:00	LEN	FLOC+LEN	FLOC+LEN
11:30	NO	NO	SEP+MEL+A+FLOC
11:30	NO	MEL	A+FLOC
13:00	RBC	SEP+MEL+A+RBC	SEP+MEL+RBC
13:00	SEP+MEL	SEP+MEL+A+RBC	SEP+MEL+A+RBC
22:30	SEP+FLOC+MEL+A	SEP+MEL+FLOC	SEP+MEL+FLOC
22:30	NO	SEP+A	SEP

It is worth noting that an "empty" gut does not necessarily mean that that gut portion as a whole was empty, because the section plane might have cut through a particular place where there were no gut contents, whereas in other parts of the same gut section, there might have been gut contents. With this in mind, it still may give an approximation of the frequency of gut contents in the different sections of the gut.

Looking at the table 6.12, it is evident that gut contents were more common in the mid and posterior sections of the midgut. Table 6.13 summarises the occurrence of selected gut contents by gut section and also by gut type (CRYpteds or THINs). First, looking at the totals of gut contents we find significant differences between the gut sections in the CRYpteds guts ($p << 0.001$). Mid and posterior midgut show gut contents more frequently than the anterior midgut. This could be the result of the way a louse deals with the food after entering the gut. During the observations of gut activity during manual feeding (Chapter 5) it was noted that the gulped food usually passes quickly to the mid and posterior midgut, where it stays most of the time. That can explain the significantly low occurrence ($p<<0.001$) of gut contents in the anterior midgut (or equivalently, the high occurrence of empty anterior midguts). Interestingly, these differences were significant for the CRYpted guts but not for the THINs. Probably "empty" is not the right word, as an "empty" gut might be lacking solid matter but could well harbour a rich liquid material product of digestion.

Among CRYpted guts there were significant differences between gut sections ($p<<0.001$) and again showing that gut contents were more common in mid and posterior midgut. These differences were not observed in THin guts.

Seven lice had red blood cells in their guts. Of those, only one louse (5:30 h) was recorded as having fed on blood at the time of sampling and fixation because the gut was red from the ingested blood. But despite this, the quantity of red blood cells was relatively low, less than 10 in a transverse section of the gut and in conjunction with salmon epithelium or melanin granules. Also, the dense flocculate material in the lumen, characteristic of red-gutted lice collected in the fish farms (Chapter 4) was absent as well. At the time of fixation, this louse was recorded as also having a full gut, with lots of melanin along with a loose pellet in the mid and posterior midgut. The other 6 lice were reported to have food in their guts but blood was not conspicuous. These could mean that the small quantity of blood was ingested at the time of the louse was feeding on epithelium. The melanin present indicates that probably the louse was feeding deep in the epithelium and, as a result, the capillaries in the dermis were also accessible.

When compared individually by each gut content type against gut portion, CRYpted guts showed significant differences between the anterior portion and the mid and posterior ones for salmon

epithelium (p=0.0019) and melanin (p=0.016) but none for red blood cells (p=0.882). There were no differences in THINs guts.

Table 6.13 Occurrence of gut contents in CRYpteds and THINs type of gut by gut section. The totals in the last 3 rows of the table are totals of guts WITH gut contents. The corresponding percentage is shown in parenthesis. Note that the percentages are not additive as two or more types of gut contents can be recorded in the same gut section.

	CRYpteds			THINs			
No. lice:	27	35	30	21	13	18	
Gut Contents	ANT	MID	POST	ANT	MID	POST	Totals
EMPTY	19 (70.3)	13 (37.1)	5 (16.6)	9 (42.8)	4 (30.7)	5 (27.7)	55
SEP	2 (7.4)	11 (31.4)	18 (60.0)	6 (28.6)	6 (46.2)	12 (66.6)	55
RBC	2 (7.4)	3 (8.5)	3 (8.6)	1 (46.2)	2 (15.4)	3 (16.6)	14
MEL	1 (3.7)	10 (28.6)	11 (28.6)	4 (66.6)	4 (30.8)	6 (33.3)	36
TOTALS	5	24	32	11	12	21	105
Gut Type Totals		61			44		
Gut region Totals (Both gut types)		Anterior: 16	Mid: 36	Posterior: 53			

The figures for the gut contents totals shown in Table 6.13 might be inflated by the fact that more than one type of gut content might be recorded in a gut section. Table 6.14 shows the occurrence of any of the three gut contents indicative of a meal (SEP, MEL or RBC) by gut type and gut section.

Table 6.14 Occurrence of any of the three gut contents indicative of a meal (SEP, MEL or RBC) by gut type. Each table was analysed as a 2X3 contingency table using the chi-square statistic. Significant differences were found for CRYpteds gut (P=0.0004) but not for THINs (P=0.084).

	CRYpteds			THINs		
Gut portion	ANT	MID	POST	ANT	MID	POST
No. of lice	27	35	30	21	13	18
No. Lice with Meal (SEP, MEL or RBC)	4	15	20	7	8	12
No Meal	23	20	10	14	5	6
% with meal	14.8	40.0	70.0	33.3	61.5	66.6

Significant differences between gut regions were observed in the CRYpteds guts, but no differences in the THINs guts.

6.2.2.3 Cell Types.

B- and A-cells

The number of A-cells and B-cells in an histological transverse section of the midgut was recorded for each gut section of each lice.

Table 6.15 shows the occurrence of B cells and A-cells recorded on each gut section for the 48 lice during the experiment.

Table 6.15 Occurrence of B cells and A-cells recorded on each gut section for the 48 lice during the experiment

	B-CELLS			A-CELLS		
Time	ANT	MID	POST	ANT	MID	POST
00:00	NO	1	NO	1	NO	13
00:00	NO	1	3	7	11	18
00:30	NO	NO	NO	1	5	4
00:45	NO	NO	2	1	NO	3
01:00	NO	4	2	NO	2	8
01:00	NO	2	NO	4	4	2
01:00	NO	NO	NO	2	2	NO
01:00	NO	1	1	7	11	15
01:00	2	1	3	4	4	NO
01:25	NO	2	1	NO	5	4
01:35	NO	3	NO	NO	3	2
01:35	NO	NO	NO	2	4	1
01:40	NO	NO	NO	NO	NO	NO
01:40	NO	NO	NO	2	1	7
01:40	NO	NO	NO	1	4	13
01:45	NO	NO	NO	NO	1	NO
01:45	NO	NO	NO	NO	NO	3
01:45	NO	NO	NO	2	NO	NO
01:45	NO	1	NO	1	2	3
02:30	NO	2	NO	NO	4	NO
03:00	NO	NO	NO	10	15	2
03:10	1	3	NO	2	1	NO
03:15	NO	NO	NO	2	6	1
04:00	NO	6	5	NO	7	5
04:00	1	NO	NO	2	3	5
04:00	NO	NO	NO	1	1	NO
04:35	NO	NO	4	NO	2	3
04:40	NO	NO	NO	NO	2	4
04:45	NO	2	1	1	2	2
05:20	NO	6	2	1	2	3
05:30	NO	NO	NO	2	8	5
05:35	NO	2	2	1	2	7
05:45	NO	NO	NO	2	2	1
05:50	1	3	3	3	3	3
06:15	NO	3	1	2	NO	5
06:15	NO	NO	NO	NO	NO	8
06:40	NO	4	1	1	2	6
07:50	NO	NO	NO	NO	NO	NO
08:00	NO	2	NO	1	1	2
08:00	NO	2	1	2	3	5
11:00	NO	1	NO	NO	2	NO
11:00	NO	2	1	NO	4	NO
11:30	NO	NO	NO	3	1	5
11:30	1	4	5	2	2	3
13:00	1	1	2	1	1	4
13:00	NO	NO	NO	NO	NO	NO
22:30	NO	3	3	NO	NO	3
22:30	NO	3	1	1	4	6

There was no apparent relationship of presence or absence of any of these types of cells with time.

Table 6.16 summarises the occurrence of B- and A-cells in the three sections of the gut and shows the number and percentage of lice with that particular cell type in each section of gut. The proportion of lice with cells in each gut section was significantly different ($p<<0.001$) for B-cells but not for A-cells ($p=0.501$). B-cells were more common in the mid and posterior midgut and were present in about half the lice. The number of cells recorded for each gut section was significantly different for both types of cells ($p<<0.001$ for each case). The number of B-cells was higher in the mid midgut (although its significance laid just in the limit, $p=0.055$), followed by the posterior midgut. On the other hand, the number of A-cells was significantly higher in the posterior midgut, followed by the mid midgut ($p=0.014$), demonstrating that, although A-cells occur in the three sections of the gut, their number is significantly different, being more abundant in the posterior, followed by the mid midgut. .

Table 6.16 Occurrence of B- and A-cells in the three sections of the gut showing the number and percentage of lice with that particular cell type and the number of cells in each section of gut

Cell type:		B-cell			A-cell		
Gut section:		ANT	MID	POST	ANT	MID	POST
No. lice without cells		42	22	28	15	10	12
No. lice with cells		6	26	20	33	38	36
% of lice with cells		12.5	54.2	41.7	68.8	79.2	75.0
Lice with	1 cells	5	7	8	13	7	2
	2	1	8	5	12	12	5
	3	-	6	4	2	4	8
	4	-	3	1	2	6	4
	5	-		2	-	2	6
	6	-	2	-	-	1	2
	7 or more	-	-	-	3	5	8
Total No. of cells		7	65	44	75	139	184

The maximum number of B-cells and A-cells recorded in a transverse section was 6 (two cases) and 18 (1 case) respectively.

6.2.2.3.1 A-cells VS B-cells

The presence B cells and A-cells seemed to be interrelated. Table 6.17 presents the chi-square analysis of the mutual independence and partial independence between the presence of A-cells and B-cells in the three sections of the gut. The results indicate that there is a significant dependence of these three variables and also each one of them was dependent on the other two.

Figure 6.3 shows graphically the nature of these interactions. There is a high proportion of cases where A-cells exist when B cells are present OR absent. On the other hand, in more than half of the cases with B cells present in the mid and posterior midgut (61 and 50% respectively) A-cells are present, but when the A-cells are absent, the cases with B cells are even lower (30 and 17% respectively for mid and posterior midgut). It seems then that A-cells can be present whether B cells are present or not but the presence of B-cells seems to be dependent to a certain extent on the presence or absence of A-cells.

Table 6.17 Occurrence of B-cells and A-cells by gut section. Table a) was first analysed as a 2X6X2 contingency table using the chi-square statistic to test for mutual independence between the three variables: frequency of B-cells, A-cells and gut section. As Ho was rejected by this method (meaning that there were interactions between these variables), further tests for partial independence were done to conclude between which variables dependencies and independencies exist. Same table a) and tables b) and c) were analysed then as a 2X6, 3X4 and 2X6 contingency tables respectively. Ho for a): "Presence of B cells are independent of gut section and presence of A-cells". Ho for b): "Gut section is independent of presence of B cells and A-cells". Ho for c): "Presence of A-cells are independent of presence of B-cells and gut section". The three null hypothesis were rejected. Table a) was then subdivided to verify if these dependencies observed were true for each section of the gut, testing the null hypothesis for independence as 2X2 contingency tables (not shown). Ho were not rejected for anterior and mid midgut (P>0.5) but it was rejected for the posterior midgut (0.048). The probabilities resulting from the tests are indicated at the bottom right of each table. A: A-cells.

a)

	A YES			A NO		
	ANT	MID	POST	ANT	MID	POST
B YES	6	23	18	0	3	2
B NO	27	15	18	15	7	10
2X6X2:	P=0.0116	Reject Ho		2X6:	P=3.6E-05	Reject Ho

b)

	A YES		A NO	
	B YES	B NO	B YES	B NO
ANT	6	27	0	15
MID	23	15	3	7
POST	18	18	2	10
			P=0.0034	Reject Ho

c)

	B YES			B NO		
	ANT	MID	POST	ANT	MID	POST
A Y	6	23	18	27	15	18
A N	0	3	2	15	7	10
					P=0.0424	Reject Ho

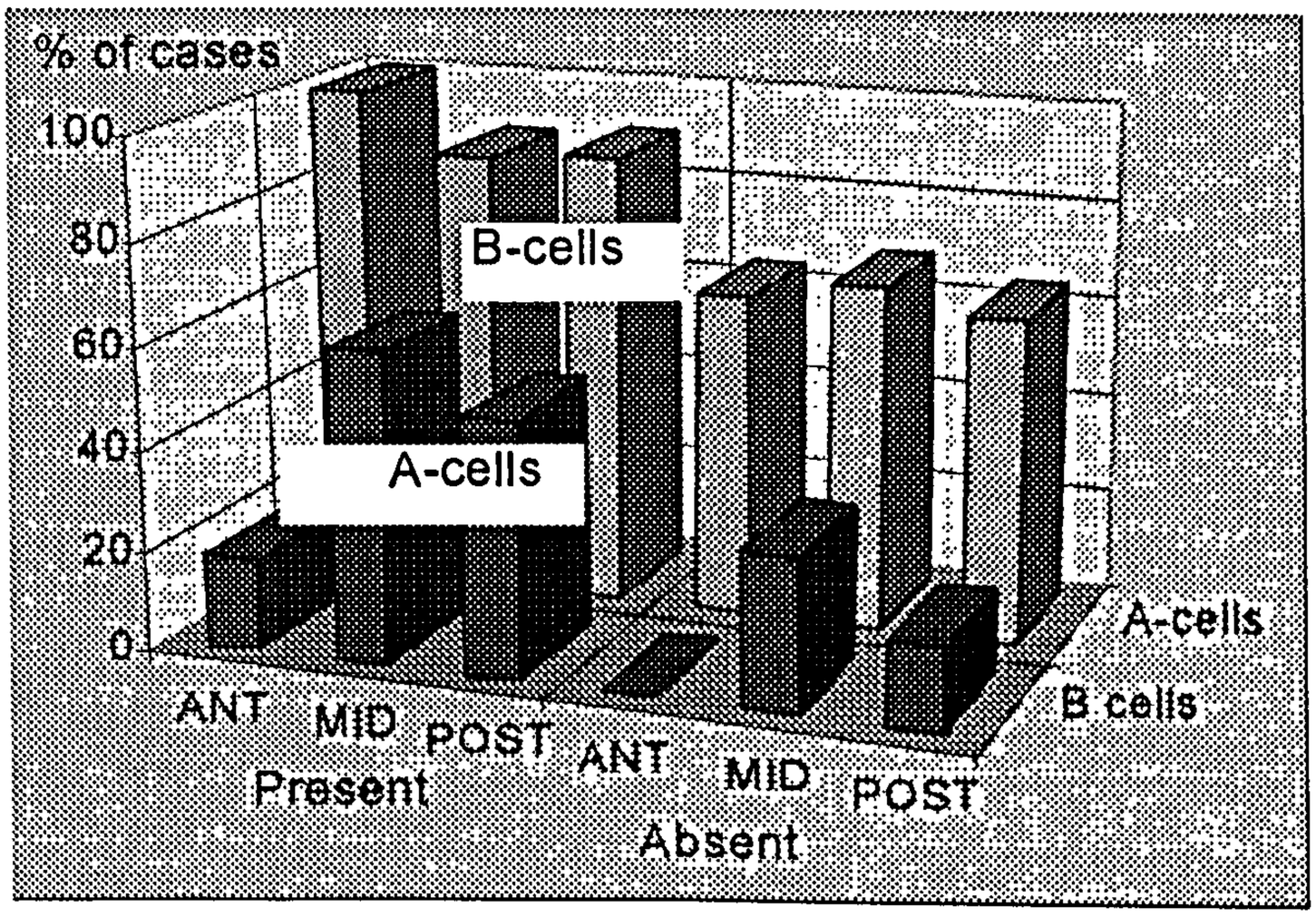

Figure 6.3 Graph presenting the interactions between the presence (or absence) of A-cells with respect to the presence of B-cells in the three sections of the midgut. Bars indicate the percentage of cases where the presence or absence of one type of cell (x axis) was associated with the presence of the other (z axis). Percentages were calculated from the first rows of Table 6.17 a) and c) respectively.

6.2.2.3.2 A-and B-cells VS meal.

In an attempt to further clarify this relationships between B-cells and A-cells, another set of analysis was carried out to question whether the presence of B-cells or A-cells was associated with the presence of food in the midgut. Table 6.18 presents the frequencies found. Table 6.18 a) shows, after the three-way mutual independence test, that presence of a meal, presence of A-cells and gut sections were independent of each other (P=0.135) and thus the analysis could not go further.

But for B-cells Ho was rejected (see Table "b") and further analysis was carried out to see where the interactions were taking place. The results showed that indeed, the three variables were significantly dependent (Tables "c" and "d"). However, earlier in this section, it was shown that presence of a meal and B-cells occurrences were dependent on the gut section, thus, further

division of Table "b" was made to test if this dependence continued after pooling the occurrences of the gut sections. Table "e" shows that this was not the case (P=0.189). Further testing for possible interactions of B-cells and meal in each gut portion (tables not shown) could not demonstrate the presence of any dependence between these variables, confirming that their dependence for the gut section was masking a false dependence between them.

6.2.2.3.3 A-and B-cells VS gut type .

It was shown earlier in this section that the occurrence of gut type "families", (CRYpteds and THINs) were independent of gut section and the same was shown for the occurrence of A-cells. Therefore there was no point in making a 3-way comparison of these three variables (data in Table 6.19 a). Instead, the occurrences of A-cells in the different gut sections were pooled to assess their independence with the gut types as a 2X2 contingency table (Table 6.19 "b"). Ho was rejected (P=0.004) confirming their dependence. The occurrence of A-cells was higher in the CRYpteds guts (83%) than in the THINs (60%).

On the other hand a three-way comparison was done for B-cells, gut type and gut section (Table 6.20 a). The rejection of Ho (P=0.0128) lead to further comparisons for partial independence, which showed dependence between the variables except for the last set of interactions (Table 6.20 c) in which Ho (Gut type occurrences are independent of gut portions and B-cells) was not rejected (although P=0.052 value was just in the rejection level). Since the previous three-way analysis had lead to this assumption that there was a dependence between the other two, occurrences of gut types were pooled (Table 6.20 d).The test confirmed the dependency of these two parameters, (thus supporting the results of the previous analysis in this section concluding that B-cells were more common in the mid and posterior midgut). Table 6.20 e was made pooling the occurrences of the gut portions confirming that in fact B-cells occur more frequently (40%) in CRYpteds guts than in THINs (12%) (P=0.023)

Table 6.18 Occurrence of B-cells and A-cells by gut section in relation with the presence or absence of gut contents indicative of a meal (salmon skin epithelium or melanin granules or red blood cells). Statistical analysis was performed in the same way as in Table 6.16 . Figures in parenthesis in tables b) and d) are the corresponding percentages.

a)

	A Y			A NO		
	33	38	36	15	10	12
	ANT	MID	POST	ANT	MID	POST
MEAL Y	7	14	25	4	6	7
MEAL N	26	24	11	11	4	5
2X6X2	P=0.135	Do not	reject Ho			

b)

	B YES			B NO		
n	6	26	20	42	22	28
	ANT	MID	POST	ANT	MID	POST
MEAL Y	2 (33)	11 (42)	14 (70)	9 (21)	9 (41)	18 (64)
MEAL N	4	15	6	33	13	10
2X6X2	P=4.4E-04	Reject Ho		2X6	P=0.0017	Reject Ho

c)

	B YES		B NO		
	MEAL Y	MEAL N	MEAL Y	MEAL N	
ANT	2	4	9	33	
MID	11	15	9	13	
POST	14	6	18	10	
				P=1.05E-06	Reject Ho

d)

	MEAL Y			MEAL N		
n	11	20	32	37	28	16
	ANT	MID	POST	ANT	MID	POST
B YES	2 (18)	11 (55)	14 (44)	4 (11)	15 (54)	6 (38)
B NO	9	9	18	33	13	10
					P=0.0016	Reject Ho

e)

	B YES	B NO		
MEAL Y	27	36		
MEAL N	25	56	P=0.189	Do not Reject Ho

Table 6.19 Occurrence of cases with A-cells in relation to the gut type and gut section. Table b) is after pooling the occurrences in the three gut sections. Percentages in parenthesis.

a)	CRYs			THINs		
N	27	35	30	21	13	18
	ANT	MID	POST	ANT	MID	POST
BL YES	20 (74)	29 (82)	27 (90)	13 (62)	9 (69)	9 (50)
BL NO	7	6	3	8	4	9

b)	CRYs	THINs		
BL YES	76 (83)	31 (60)		
BL NO	16 (17)	21 (40)		
		P=0.004	Reject	Ho

Figure 6.4 illustrates these interactions. In 85% of the cases where B-cells are present in the posterior midgut the gut is CRYpted type. There are CRYpted type guts without B-cells, but their occurrence is less than half (46%). The same is true for the mid midgut, 77% cases of B-cells present with CRYpted guts, although the cases of CRYpted guts without B cells are higher compared to the posterior midgut (68%). This means that whenever there are B-cells present, the chances that the gut type is CRYpted are high. On the other hand when the gut type is CRYpted, in 57% of the cases there are B-cells and when the gut type is THin (CRYpted absent) the cases with B-cells drop slightly for the mid-midgut (46%) but they drop to a mere 17% for the posterior midgut.

6.2.2.4 Peritrophic membranes

Table 6.21 shows the general occurrence of peritrophic membranes recorded on each section of the gut.

The occurrence of a peritrophic membranes was dependent on the gut section (see Table 6.22). It was more common in the mid and posterior midgut ($p<<0.001$).

6.2.2.4.1 Peritrophic membranes VS Meal.

Further tests were done in order to know if the presence (or absence) of peritrophic membranes in a midgut section was somewhat conditional on the presence of food in that section. Table 6.23 shows the set-up for the chi-square analysis performed on the occurrence of peritrophic membranes in relation to a meal (presence of salmon epithelium, melanin or red blood cells) and midgut section and Figure 6.5 shows graphically the interactions. All three variables were found dependent on each other with high significance. As stated before, the occurrence of peritrophic membranes was higher in the mid and posterior midgut and in more than 70% of the cases this was associated with the presence of food and vice versa. Its absence was also associated with absence of food, although to a lesser extent for the posterior midgut (56%). In the posterior midgut, in almost half of the cases (44%) the peritrophic membranes were absent when food was present. This was far less common in the mid (29%) and anterior (7%) midgut. In fact, when Table a) was subdivided testing the dependence of peritrophic membranes and meal for each gut section, it was found to be the case (dependence of the two variables) for the mid and posterior midgut ($P=0.007$ and 0.005 respectively), but not for the anterior section ($P=0.8$).

Table 6.20 Occurrence of cases with B-cells in relation to the gut type and gut section and analysis for mutual and partial independence between these three parameters. Table d) is after pooling the occurrences in the gut types. Table e) is after pooling the occurrences in the three gut sections. Percentages in parenthesis. See text for details.

a)		CRYs			THINs	
N	27	35	30	21	13	18
	ANT	MID	POST	ANT	MID	POST
B YES	3 (11)	20 (57)	17 (57)	3 (14)	6 (46)	3 (17)
B NO	24	15	13	18	7	15
2X6X2:	0.0128	Reject Ho			4.8E-05	Reject Ho

b)	B YES		B NO		
	CRYs	THINs	CRYs	THINs	
ANT	3	24	3	18	
MID	20	15	6	7	
POST	17	13	3	15	
				9.0E-04	Reject Ho

c)		B YES			B NO	
N	6	26	20	42	22	28
	ANT	MID	POST	ANT	MID	POST
CRYs	3 (50)	20 (77)	17 (85)	24 (57)	15 (68)	13 (46)
THINS	3	6	3	18	7	15
					0.052	Do not reject

d)	ANT	MID	POST
B YES	6	26	20
B NO	42	22	28
		7.4E-05	Reject Ho

e)	CRYs	THINs
	40	12
	52	40
	0.023	Reject Ho

6.2.2.4.2 Peritrophic membranes VS A-cells

The peritrophic membranes were found to be independent of the presence of A-cells (P=0.998). Table 6.24 show the occurrences of both variables in the three portions of the gut. No further statistical analysis for partial independence was then necessary. The independence of A-cells and gut region had been demonstrated above in this section.

Table 6.21 Occurrence of peritrophic membranes on each section of gut of the 48 lice sampled at different times after being put on a salmon.

Time	ANT	MID	POST
00:00	NO	NO	NO
00:00	NO	NO	NO
00:30	NO	YES	NO
00:45	NO	NO	NO
01:00	NO	YES	NO
01:00	NO	NO	YES
01:00	NO	NO	NO
01:00	NO	YES	NO
01:25	NO	NO	NO
01:35	NO	NO	YES
01:35	NO	NO	NO
01:40	NO	YES	YES
01:40	YES	YES	YES
01:40	YES	YES	YES
01:45	NO	NO	NO
01:45	NO	YES	YES
01:45	NO	NO	NO
01:45	NO	NO	NO
02:30	NO	NO	NO
03:00	NO	NO	NO
03:10	NO	YES	NO
03:15	NO	NO	YES
04:00	NO	NO	NO
04:00	NO	NO	YES
04:00	NO	NO	YES
04:35	NO	NO	NO
04:40	YES	YES	YES
04:45	NO	NO	NO
05:20	NO	NO	YES
05:30	YES	NO	YES
05:35	NO	YES	YES
05:45	NO	NO	YES
05:50	NO	YES	YES
06:15	NO	NO	NO
06:15	NO	NO	NO
06:40	NO	NO	YES
07:50	YES	NO	YES
08:00	NO	YES	YES
08:00	NO	NO	YES
11:00	NO	NO	YES
11:00	NO	NO	NO
11:30	NO	NO	NO
11:30	NO	NO	YES
13:00	NO	NO	NO
13:00	NO	YES	YES
22:30	NO	NO	NO
22:30	NO	NO	NO

Table 6.22 Occurrence of peritrophic membranes in the three portions of the midgut. A test for independence (2X3 contingency table) was applied and Ho ("Presence of peritrophic membranes is independent of gut section") was rejected (P<<0.001)

	GUT SECTION		
	ANT	MID	POST
PRESENT	5	13	23
ABSENT	43	35	25

Table 6.23 Occurrence of peritrophic membranes in relation to presence of a "meal" (presence of salmon epithelium, melanin or red blood cells) in the three sections of the midgut of lice. Table a) shows the number of cases with presence or absence of peritrophic membranes which had or had not signs of a meal in that portion of the gut.. It was first assessed as a three-way (2X6X2) contingency table for mutual independence between the three variables (Ho). As the conclusion of the chi-square test for this table was that the three variables were dependent of each other (p<<0.001), the data were further explored for partial independence of one variable against the other two as two-way contingency tables to conclude between which variables dependencies and independencies existed.. In the three cases, Ho was rejected at p<<0.001, and the overall conclusion was that all three variables were dependent on each other. See text for detail.

a)		PM Y			PM N	
N	5	13	23	43	35	25
	ANT	MID	POST	ANT	MID	POST
MEAL Y	2 (40)	10 (77)	20 (87)	3 (7)	10 (28)	11 (44)
MEAL N	3	3	3	40	25	14
2X6X2	1E-11	Reject Ho		2X6: P=	1.1E-09	Reject Ho

b)	PM Y		PM N		
	MEAL Y	MEAL N	MEAL Y	MEAL N	
ANT	2	3	3	40	
MID	10	3	10	25	
POST	20	3	11	14	
				1.1E-05	Reject Ho

c)		MEAL Y			MEAL N	
N	5	20	31	43	28	17
	ANT	MID	POST	ANT	MID	POST
PM Y	2 (40)	10 (50)	20 (65)	3 (7)	3 (11)	3 (18)
PM N	3	10	11	40	25	14
				2X6: P=	1.7E-07	Reject Ho

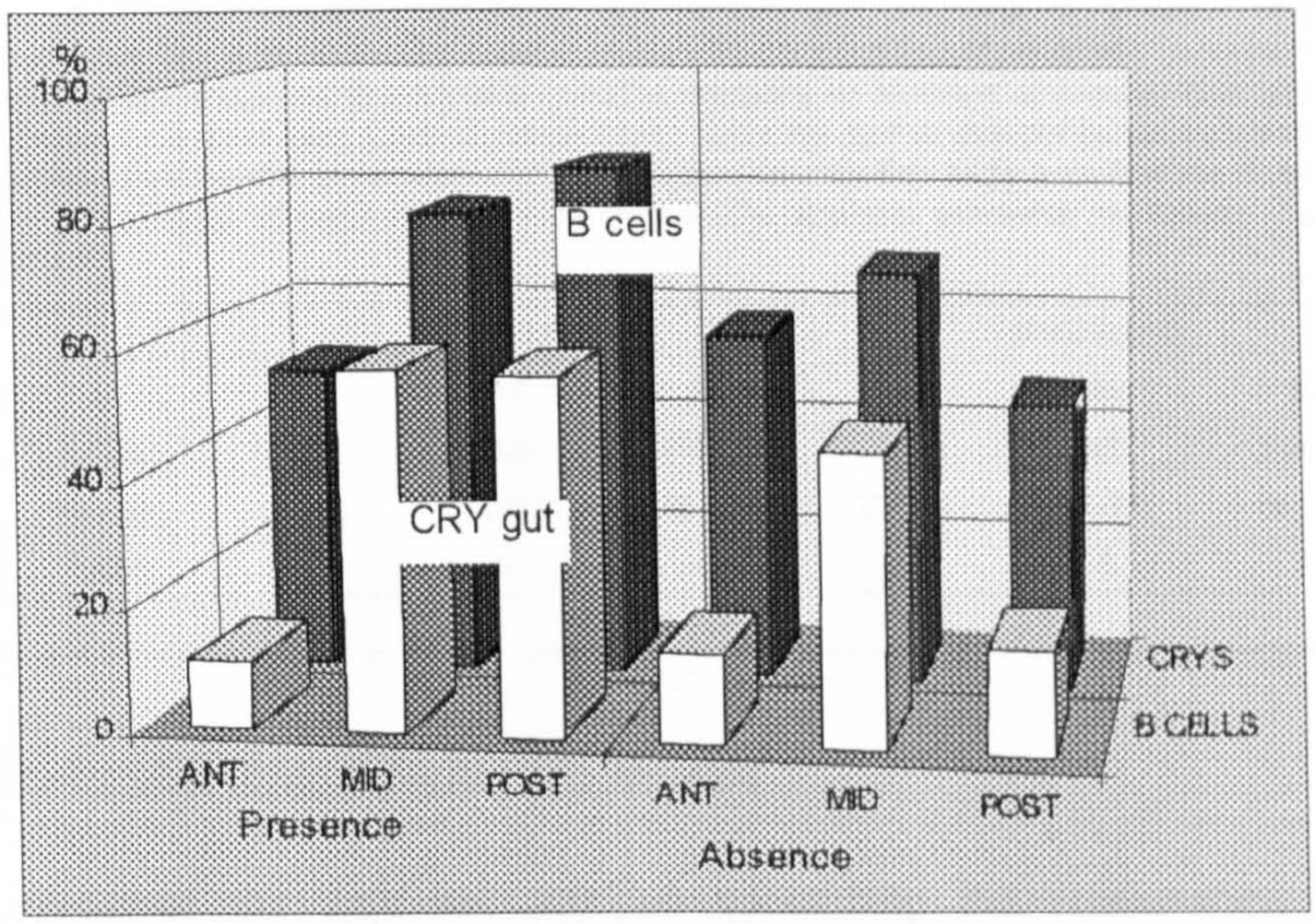

Figure 6.4 Graph presenting the interactions between the presence (or absence) B-cells and CRYpted guts with respect to the concurrent presence of CRYpted guts or B-cells in the three sections of the midgut. Bars indicate the percentage of cases where the presence or absence of either CRYpted gut or B-cells (x axis) was associated with the presence of the other (z axis). Percentages shown in Table 6.20 a) and c) were used.

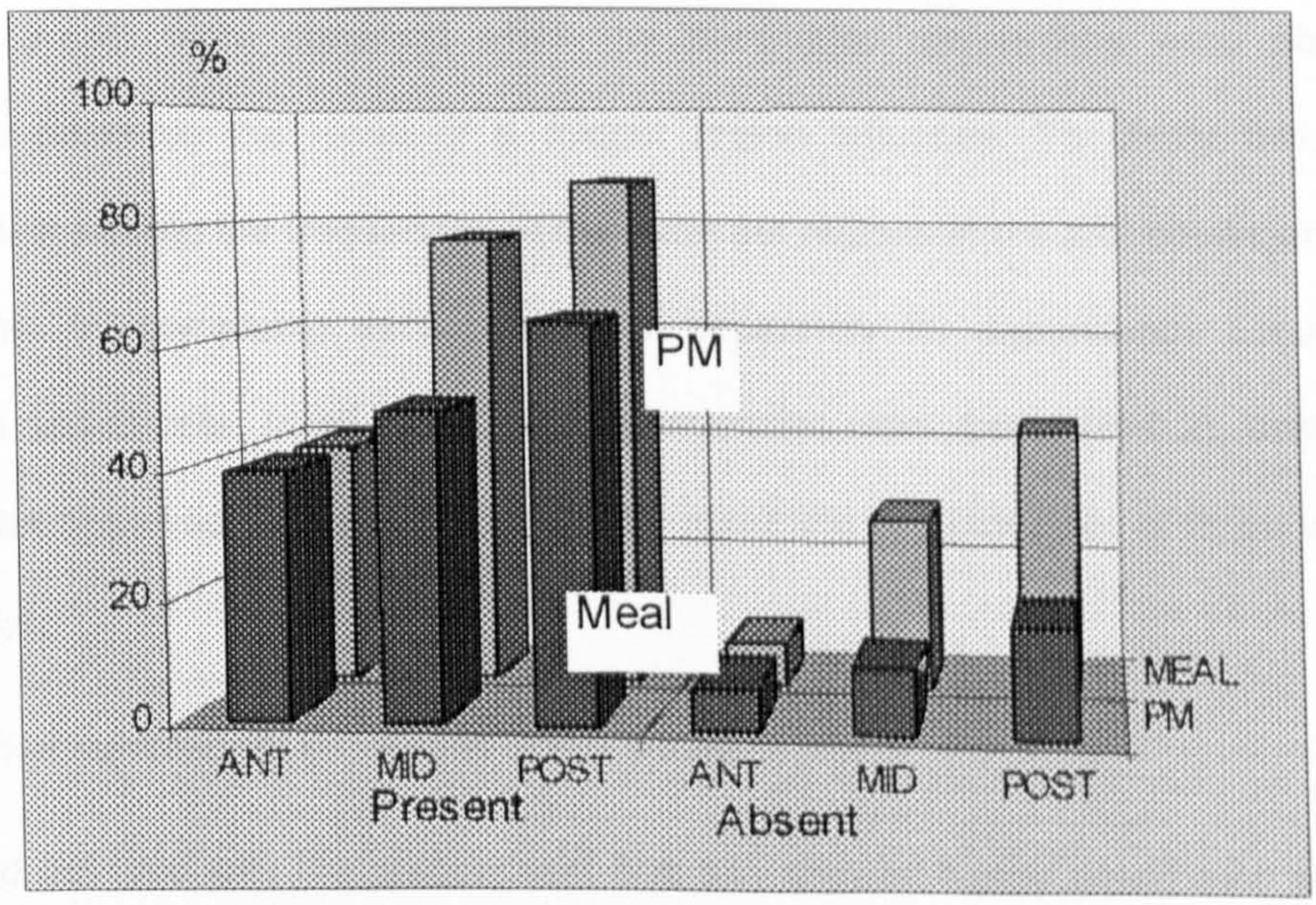

Figure 6.5 Occurrence of peritrophic membranes in relation to presence of a "meal" (presence of salmon epithelium, melanin or red blood cells) in the three sections of the midgut..

Table 6.24 Occurrence of A-cells by gut section and presence or absence of peritrophic membranes. The table shows the number of lice with the condition of peritrophic membranes (n), the number of lice which had that condition and the presence or absence of A-cells. This table was first assessed as a three-way (2X6X2) contingency table for mutual independence between the three variables (Ho). As the conclusion of the chi-square test for this table was that the three variables were independent of each other (p=0.998) then the analysis proceeds no further. See text for comments.

	PM PRESENT			PM ABSENT		
n	5	13	23	43	35	25
A-Cells	ANT	MID	POST	ANT	MID	POST
Present	4 (80)	11 (85)	18 (78)	29 (67)	27 (77)	18 (72)
Absent	1	2	5	14	8	7

6.2.2.4.3 Peritrophic membranes VS B-cells.

Table 6.25 presents the data and analysis concerning the relationships between B-cells, peritrophic membranes and gut regions. The three variables were found to be dependent of each other. Earlier in this section the dependence of B-cells or peritrophic membranes on the gut section was shown. Although peritrophic membranes and B-cells were dependent of each other (Table "a" and "c"), this dependence did not occur often, except perhaps for the mid midgut in which 57 % of cases with B-cells were observed when the peritrophic membranes were absent. But that dependence could be the result of a midgut region influence. To clarify this, table a) was subdivided first pooling the frequencies of the three gut regions into a 2X2 contingency table (Table "d") and also by comparing each gut region separately for dependence between peritrophic membranes and B-cells. In all cases, no dependencies were found (P>0.2), confirming that the dependencies observed before were brought by about the dependencies of those two variables on the gut region.

Next is a short summary of the results of this section:

- CRYpted gut type was the predominant one and showed higher occurrence after 4:45 h after feeding.
- Gut contents were more frequent in the mid and posterior midgut. Salmon epithelium and melanin were the most frequent food items and were associated with CRYpted guts.
- The first feeding was evident after 1 h.

- B-cells occurrence was higher in mid midgut followed by posterior midgut. Their occurrence seemed to be related to the presence of A-cells, it was independent of the presence of a meal and they were more frequent in CRYpted guts.
- A-cells occurrence was higher in posterior midgut followed by mid midgut. They seem to occur independently of the presence or absence of B-cells or a meal and were more frequent in CRYpted guts.
- Peritrophic membranes were more common in mid and posterior midgut, were associated with the presence of a meal and were independent of the presence or absence of A- or B-cells.

Table 6.25 Occurrence of B-cells by gut section and presence or absence of peritrophic membranes. The tables a) and c) show the number (n) of lice with the condition of peritrophic membranes, the number of lice which had that condition along with the presence or absence of B-cells. Figures in parenthesis are the corresponding percentages, for comparison only. In Table d) the frequencies of the three gut sections were pooled to test for independence between peritrophic membranes and B-cells, which was confirmed (P=0.906)

a)		PM Y			PM N	
n	5	13	23	43	35	25
	ANT	MID	POST	ANT	MID	POST
B YES	0 (0)	6 (46)	8 (35)	6 (14)	20 (57)	12 (48)
B NO	5	7	15	37	15	13
2X6X2	P=0.0016	Reject Ho		2X6	P=0.0008	Reject Ho

b)		PM Y		PM N		
		MEAL Y	MEAL N	MEAL Y	MEAL N	
	ANT	0	5	6	37	
B-cells	MID	6	7	20	15	
	POST	8	15	12	13	
					1.22E-06	Reject Ho

c)		B YES		B NO		
n	6	26	20	42	22	28
	ANT	MID	POST	ANT	MID	POST
PM Y	0 (0)	6 (23)	8 (40)	5 (12)	7 (32)	15 (54)
PM N	6	20	12	37	15	13
					P=0.0023	Reject Ho

d)	PM YES	PM NO		
B cells YES	14(34)	38(37)	P= 0.906	Do not reject Ho
B-cells NO	27 (66)	65 (63)		

6.3 DISCUSSION

Most of the studies on defecation rates have been done almost exclusively in free living copepods, and there is a wealth of studies about this subject (see Butler & Dam 1994). Regarding parasitic crustaceans, Yoshikoshi & Ko (1988) report briefly the faecal pellet production of *Lernaea cyprinacea*. This seems to be the second known attempt to measure the defecation rate of a crustacean parasite.

Once sea louse is taken from its host, it is put into a series of "unnatural" conditions which will add unknown variability to any experiment. Starvation and being off the host are likely to be unknown issues to a sea louse because, being a parasite, the supply of food is unlimited and there is no need to be off the host. So, the high proportion of non-feeding lice and the variable delay in taking the first meal amongst the "feeding" lice may well be possible after-effects due to handling and/or starvation. Feeding behaviour has been known to be affected by handling in copepods (Paffenhöfer 1994). Marshall & Orr (1955) also observed a similarly great variation between individual female *Calanus* even under the same conditions, with some *Calanus* eating freely and others not at all, even though they were apparently as healthy and active as the rest. The variability in males was even higher. They could not explain this behaviour but they mention factors like age, or state of maturity and time of year might account for this. These authors also confirmed that a *Calanus* can live even in a rich food culture without feeding. In this study only gravid females were used, but their actual age and life history was unknown and these may account for some of the variation observed. Intensity of light might be another factor which affects feeding. For *Calanus*, even diffuse light reduces the food uptake, affects respiration and can be lethal. The experiments in this study were done in a very well lit cold room, and the negative influence of this bright light cannot be excluded.

Lice #1-#3 were probably of the "non feeding" category. The release of tape pellets during the time before the release of clear or dark pellets was an indicator that the lice were not feeding, as these type of pellets had been observed before in lice put on starvation. This is contrary to what has been observed in free living crustaceans, particularly copepods, in which an enhanced feeding rate, or hunger response, is found when exposed to food after short periods of starvation (1.75 to

30 h) (Hassett & Landry 1988, Mackas & Burns 1986). Between 6 and 12 h of starvation were required to develop such enhanced ingestion capacity, which lasted 1 to 2 h. In lice #1 and #3, once the first clear or dark pellet appeared, the tape pellets did not show again. Interestingly, this also shows that "non feeding" lice, given the appropriate time, can start to feed again. The time threshold, or the "point of no return" for the lice is not known. Storch & Anger (1983) and Storch & Burkhardt (1984) found for crab larvae and amphipods respectively, that starvation resulted in morphological changes that became irreversible beyond 8 to 12 days of starvation. During preliminary trials in this study it was observed that lice with up to 10-12 days starvation still had the capacity to feed again but further experiments are necessary to evaluate this properly. This seemingly necessary long time in contact with the host to acquire the capacity to feed again should be taken into account for such experiments.

Before these experiments took place, non feeding lice were regarded as possibly senescent individuals that had lost its capacity to feed. As adult gravid females were always used in this study, this still remains as a possible explanation, but not for all of them as it was thought.

This long time necessary to feed again after starvation may explain the high percentages of non feeding lice observed, usually of the order of 50 %. But it does not explain why the other 50% did feed. Environmental aspects such as temperature and salinity are suspected to have an effect on the lice feeding behaviour. In Chapter 4, an unusual absence of faecal pellets was recorded in a batch of lice coming from a site with low temperature and salinity, meaning that all lice collected had not fed for at least 8-12 h and that their physiology was affected somehow so as not to produce even tape pellets. Feeding experiments with a detailed look at the history of these environmental variables in each farm where the batches of lice come from would be necessary to further clarify this issue. Also, the feeding behaviour of lice under a set of different controlled conditions, varying salinity, temperature, light regime are also needed to understand more about their physiology and how it affects their feeding behaviour and why some lice are apparently not affected.

"Feeding lice" nevertheless took some time to take a first meal, usually in the order of 1 h. The reason of the delay it is not known, but handling, recognition of the host, finding a suitable place to

feed, acclimatisation after being away from a host could be factors that may account for this delay. There seem to be no studies on parasitic crustaceans that address feeding behaviour experimentally. There are many studies on parasitic insects and mites, but these organisms are used to starvation and, as part of their normal behaviour, would take a meal soon after they are put on the host or in contact with an artificial source of food (see Alburquerque, Neves & Werkhäuser 1992, Abbasy, Stein & Osman 1994, Opdebeeck 1994). Experimental infections with *L. salmonis* are reported, but their aim was to evaluate the damage they caused on the fish skin (Jones *et al.* 1990, Egidius 1985, Jónsdóttir 1992). The difficulty of direct observation of the feeding of these parasites poses a great challenge to the understanding of their feeding behaviour (Kabata 1981)

The two fold increase in pellet release rates of the two lice under constant illumination, compared to the other lice under 12-12 light dark regime suggests that part of the feeding behaviour might be regulated by light, or indirectly by the lice responding to physiological changes in the host induced by the photo period. This two fold factor was obvious also in the shorter intervals between releases of pellets, suggesting that also the ingestion rate was accelerated. Paffenhöfer *et al.* (1995) propound that intermittent, temporally enhanced or reduced feeding in free living copepods could be the result of modification of feeding rates over periods of several hours. This modification could be in response to due to diel changes.

Feeding activity has been reported to be timed to lighting conditions in a number of copepods (see Dagg & Grill 1980, Stearns 1986 and Hassett & Blades-Eckelbarger 1995), but apparently this has never been addressed for parasitic crustaceans. But being an obligatory parasite, with continuous and literally unlimited availability of food, there seems to be little advantage for the louse to adapt its feeding, or increase it, according to the light conditions suggesting that the physiology or behaviour of the fish host may contribute to influence on the feeding habits of *L. salmonis*.

More observations involving more individuals, different regimes of light and dark and replications are necessary to clarify this hypothesis that sea lice feeding activity is influenced somehow by the photoperiod. The observations in this study, although they point to this possibility, are probably not enough to ascertain this matter as the number of lice used was limited.

There seemed to be no influence of light and dark periods on the time of release of pellets in lice #1 and #2. Pellets were released either during light or dark periods. Due to the long intervals without pellet production, probably longer experiments with more lice are needed to establish with certainty if such a relationship exists, but high variability in the release of faecal pellets is known in free living copepods. Paffenhöfer (1994) found higher variability in the release intervals of faecal pellets of adult females of *Eucalanus pileatus* Giesbrecht (CV 23-27%) than in the early copepodites (CV 5-10%) and suggested that this individual variation could be due to the copepodites having little variability in their individual life histories (laboratory), compared to adult females who most likely encountered a wide range of biological, chemical and physical variables during their previous life in the ocean. Other factors he considers are handling effects during collection and experimentation. The high variability observed in this study (CV between 38 and 46%) could have been the result of a number of factors like life history of the lice, as Paffenhöfer suggests and also by handling and host behaviour. Occasionally, the fish, which had restricted movements, would try to get rid of the louse by rubbing its sides on the bottom of the tank, probably in response to the lice feeding activity. These vigorous movements of the fish might alter the ingestion rate. Higher or lower release intervals could also be related to smaller or larger pellet size, respectively (Paffenhöfer *et al.* 1995), and this related either to shorter or longer feeding periods. Unfortunately, the size of pellets was not recorded in this study but would be important in future experiments to be able to asses this suggestion for sea lice. Paffenhöfer *et al.* (1995) observed that certain females of *Paracalanus aculeatus* Claus had a specific longer or shorter release period which they could not relate to lower or higher feeding rates and concluded that each animal appears to possess its specific release rate.

Although it is almost impossible to measure directly the feeding rate of sea lice, the variability in their feeding periods suggests also some individual specificity, which also can change depending on the lighting conditions. But the actual release rate was very close between lice under the same lighting conditions. Donaghay (1988) found that variability of ingestion will not immediately manifest itself in similar variability of pellet release rates and that the latter somehow integrate variability of ingestion frequency. This appears to hold for lice in this study. Nevertheless, more observations involving more lice and a careful measurement of the volume of the pellets produced would be

necessary to establish first, if there is a relationship between defecation rate and ingestion rate as observed in free living copepods (Butler & Dam 1994, Dagg & Walser 1986, Tsuda & Nemoto 1990), and second, what are the causes of the differences in defecation rate. But this poses a technical difficulty that will not be easy to overcome: How can one measure the ingestion rate in the case of sea lice? In free living copepods this is estimated from the difference in food concentration between control and experimental vessel by gut fluorescence quantification (Ayukai & Nishizawa 1986) or, more recently, by continuous observations on the activities of the appendages (see Paffenhöfer 1994). From these studies, we may know how much a louse is defecating, but we do not know how much was ingested in the first instance.

Although the pellets were not measured, during observations under the dissecting microscope, when a pellet was evident in the gut, it usually occupied all the posterior midgut and half or 3/4 of the mid midgut. Dagg & Walser (1986) found that food concentrations sufficient to allow a copepod to fill its gut resulted in the production of faecal pellets of maximum size. As we can consider food concentration to be at maximum in the case of sea lice one could expect under normal conditions that a louse will produce pellets of maximum size, the maximum size being the length of mid and posterior midgut. This length can be very variable depending of the age and stage of maturity of the adult females, so any experiment in the future would have to take this into account and normalise the pellet size or volume with the individual size of the louse to be able to make useful comparisons.

It was pointed out in Chapter 4 that the faecal pellets normally are constituted of almost intact skin epithelial cells from the host and this was again observed during these experiments. We do not know how much food the louse is ingesting, and therefore, its digestive efficiency would be a difficult parameter to measure. But the poor digestion evident in the faecal pellets of the louse points to a probable low digestive efficiency. This has also been observed in free living copepods, leading observers to suppose that digestion went only to a limited extent. Absorption and production of cell tissue may be an energetically costly process relative to the acquisition and digestion of food (e.g. Kiørboe, Möhlenberg & Hamburger 1985). In their study of optimal gut design Penry & Jumars (1987) found that with an unlimited food supply (as is the case with sea

lice), less efficient assimilation may yield greater net benefit and that the digestive process is a function of gut volume and structure, substrate concentration and residence time in the gut, and extent of mixing, as well as enzyme kinetics. With all these factors at play, the relationship between digestive efficiency and concentration of food is likely to overlie a number of other relationships.

The mean time before the first sign of a well formed pellet in the midgut of the louse comes close to the mean interval of pellet release for lice under continuous illumination, around 3 h. This could mean that a feeding period is probably completed in the range of 3-5 hours, under constant light or probably longer, twice the time, in a light/dark regime.

This interval between pellet release is much greater than the rates observed in free living copepods, which range in the order of several minutes, usually 5 or 6 (Marshall & Orr 1955) and maximum 11, for adults (see Paffenhöfer 1994 and Paffenhöfer *et al.* 1995). In *Lernaea cyprinacea,* the only crustacean parasite for which report of faecal pellet production was found (Yoshikoshi & Ko 1988), the defecation took place at regular intervals of 32±3.8 min and in other cases (which are not clear in their brief account) 19±3.1 min, which are closer to the values observed in this experiment, compared to free living copepods as mentioned above or to other "higher" crustaceans (see next).

In the amphipod *Corophium volutator* (Icely & Nott 1985) the production of faecal pellets is quite high (96 pellets/h on average) suggesting that the intervals between pellet release were very small. This amphipod is a deposit feeder, and, as the authors suggest, these high rates reflect the nutritional value of the food. In the crab *Carcinus maenas* the single faecal column is voided in three sections during a single digestive cycle (which can last 48 hours or more) each section corresponding to a phase in the digestive cycle (Hopkin & Nott 1980). In the shrimp *Penaeus semisulcatus* the picture is analogous to the crab, where the cycle takes at least 24 h and the faeces are voided in a similar fashion (Al-Mohanna & Nott 1987b). It is difficult to make comparisons with these organisms because their digestive system is more refined. The food particles are processed by a complex array of muscles, filters and channels in the stomach. The

organic nutrients are then directed to either the caeca or digestive gland diverticulae and the residual particles passed along the midgut as faeces.

This average of 3 h between production of faecal pellets had indicated how long the duration of a feeding period could be. The sampling periods of the second experiment in this Chapter (see section 6.2.2) were therefore made short during the first 2 h in order to try to detect the possible morphological changes of the midgut epithelium that could take place during this time, if there were any. Nott *et al.* (1985) found in *Calanus helgolandicus* that within 1 h after the onset of feeding, B-cells had begun bursting into the lumen and after 2 or 3 h there was an extensive breakdown of the B-cells and by 7 h these cells were depleted.

It is apparent from the present study that a louse can feed on blood AND skin at the same time, or put in other words, that both types of meal can coexist at the same time. The blood meal apparently does not need to be completely processed and evacuated before a skin meal is taken, and vice versa. It is still puzzling that "post blood-feeding" pellets were not recovered when louse #1 was apparently feeding on blood. A broader discussion on this issue of blood feeding by the louse was addressed earlier in Chapters 4 and 5 but it should be noted that in none of the lice in the observations in this chapter there was evidence of digestion of a blood meal. The red blood cells found in the gut were intact and usually associated with the presence of salmon epithelium. This might suggest that the ingestion of the red blood cells occurred while the louse was feeding on salmon epithelium. In Chapter 7 the significance of this observation will be further discussed.

This method for monitoring of pellet production for parasitic crustaceans could be used probably to evaluate the density and even the age composition of population parasitic copepods under laboratory conditions, particularly of those parasites that are difficult or impossible to count on a live host due to their site of attachment, for example the ergasilid copepods, or in cases where handling of the host is either inadvisable or difficult or would be better avoided for a particular experiment. Hiromi *et al.* (1988) demonstrated that copepod faecal pellets can be morphologically distinguished from those of other invertebrates, and even identified to some extent at species level if pellet width, L/W ratio and the species composition of the copepod assemblage are taken into account. Experiments could be devised to try to find what is the relationship between number of

pellets during a given time and population size of a parasite and, if there is, one could devise with a simple model that could predict parasite number at a given time. Pellet dimensions can be associated to the parasite size and therefore the population age composition could also be predicted .

As stated before, one of the main problems encountered in these observations was the enormous uncertainty regarding the time lice fed in the first instance. The possible causes of this have been discussed but, on the practical side, it leaves little room for speculation about the possible changes of the gut morphology that could have occurred during the experiment. It is not possible to make any assumptions about any relationship regarding time and, until a method is devised to be more certain about the time a louse takes food for the first time, any attempt to address sequential morphological changes in the lice gut epithelium will face the same limitation.

With this in mind, the morphological data collected will be considered whole as a sample. Unfortunately a sample with undesirable stressful parameters added, i.e. starvation and handling.

The low occurrence of MD and DC guts was rather unexpected because they seem to be more common in non-experimental lice fixed immediately after sampling in the sea cages (personal observations). It is probable that lice need more time to acclimatise back to a normal physiology. Mackas & Burns (1986) found that *Calanus pacificus* shifted between an inactive and active feeding phase. Inactive individuals resumed feeding in 1 to 3 h Hassett & Landry (1988) observed that this recovery time was longer, 6 to 12 h.

VTH gut types probably represent an artefact introduced due to starving the lice for the experiments. A reduction of the height of the gut epithelial cells has been associated with starvation in other crustaceans (see Elendt & Storch 1990). It is possible that this type of gut does not exist normally, or that its occurrence is low and probably restricted to senescent or sick individuals that have stopped feeding. During the participation in a project to develop a vaccine against sea lice, a considerable quantity of adult females were used regularly for immunohistochemistry screening (Andrade-Salas *et al.* 1993), and VTH type guts were hardly present (personal observations).

A way to corroborate this would be to take a similar sample of lice from a fish farm and compare the occurrences of MD, DC and VTH gut types with the results of the present or similar experiments.

The six lice in which the gut type was VTH in the three gut sections certainly had fed, as there were signs of meal, and in three of them this evidence of meal was in the three sections of the gut. This could indicate that these lice had started to feed a short time before, probably less than an hour. It is possible that for those lice from samples 4:00 7:50 and 13:00 this was a second or even a third meal. If this is so, then this means that a louse that started to feed again after being starved could take several feeding periods to return to normal physiology. Or that a point of no return has been reached and the gut epithelium is not capable of returning to normal condition despite the assumption of feeding behaviour.

TH guts could possibly belong to lice that had just started to feed, but where the gut epithelium had not reacted to the food stimuli, either because feeding had just started to happen or because the "point of no return" for that particular louse had been reached and it was physiologically unable to undertake further digestive activity or because it belonged to the "non feeding" category discussed in the previous section and did not have enough time to "acclimatise" before starting to feed again, as lice #1 and #2 did in the faecal pellet production experiments. These possible reasons may account for the lack of significant differences in the occurrence of a meal in the 3 sections of the gut, which means that the gut contents were distributed more evenly in this type of gut compared to SCRY type, in which a meal occurred preferentially in the mid and posterior midgut. This higher occurrence of food in the mid and posterior midgut confirms the observations made during manual feeding (Chapter 5) that, after a short period of mixing of the food between the anterior and mid midgut by peristalsis, the food is gradually passed to the posterior midgut where it accumulates and a pellet starts to form. This presence of food in the mid and posterior midgut was evident as soon as 1 h .

Although lice were observed to feed even after 7 or 10 days starvation, individual differences make it impossible to generalise that lice would resume normal digestion after being under starvation. Once a louse is taken off the fish, even for a short time, this represents a completely abnormal

situation for the parasite and it is difficult to know how this can affect its physiology, not only the digestive one, but in general, and the repercussions that this would carry in any experiments.

SCRY guts were the dominant type and the fact that they became even more common after 5 h and that there was a significant relationship of this gut type with presence of a meal suggests that this could be the "normal" state of the gut, or a state that is ready to deal with the presence of nutrients. A radial increase in the absorptive surface area increases the uptake of digestive products (Penry & Jumars 1987). An increase of occurrence of MC or DC guts in time was not observed, but probably, the length of time of this experiment was not enough to observe it, or the lice were still in "recovery time", or probably it does not happen, as in the copepod *Acartia tonsa* (Hassett & Blades-Eckelbarger 1995) (see below). It is difficult to make comparisons in this respect, mainly because the studies of morphological changes of the midgut epithelium in crustaceans during a digestive cycle, usually describe morphological changes and/or occurrence of the type of cells (Barker & Gibson 1977, Barker & Gibson 1978, Hopkin & Nott 1980, Al-Mohanna *et al.* 1985a, Nott *et al.* 1985, Al-Mohanna & Nott 1986, Al-Mohanna & Nott 1987b, Al-Mohanna & Nott 1989, Yoshikoshi & Ko 1991a, Icely & Nott 1992, Johnston *et al.* 1993, Vogt 1993), but not the morphological changes of the gut epithelium as a whole. An exception is the work of Hassett & Blades-Eckelbarger (1995), in which the authors measured the changes in the gut cell and vacuole cross-sectional area of *Acartia tonsa*. They report that gut cell volumes changed a little over time, but these changes were not significant. Also, no significant correlation was found with gut fluorescence (as a measure of ingestion or gut fullness).

Increase of gut cell volume during the course of digestion of a blood meal has been observed in mosquitoes. After digestion, the volume then decreases to pre-blood meal levels, but these changes occur over a period of days (Hecker & Rudin 1981). The same has been observed in vertebrates in which feeding after fasting initiates a period of rapid reconstruction, and the weight of the small intestine doubles within a few hours due to an increase in mucosal thickness (Cossins & Roberts 1996). It is likely that this increase in epithelium thickness happens as well in lice, but a different approach would have to be devised to ascertain this.

In *L. salmonis* Nylund *et al.* (1992) make no distinction of midgut zones and report B-cells along all the midgut. They mention that one of the reasons for this lack of differentiation of the midgut in different zones is related to the active peristaltic motion of the gut contents anteriorly and posteriorly, which can mix them thoroughly and the secretion of digestive enzymes can thus occur along the whole length of the midgut.

In this study , although B-cells can be found along the 3 sections of the midgut, their occurrence is higher in the mid midgut, followed by the posterior and it only occasionally that they are found in the anterior midgut. But even though B-cells occur preferentially in these zones, they are not so abundant as to make these gut regions morphologically different, as in the case of calanoid copepods, in which the zone II is morphologically distinct due to the high numbers of B-cells, which in fact are restricted to this zone of the midgut (Arnaud *et al.* 1980, Musko 1983, Defaye *et al.* 1985).

However, even though the gut epithelium of *L. salmonis* seems to be morphologically undifferentiated along the midgut length at any one time, there seem to be reasons to consider 3 functional regions based on the occurrence of cells and the observations of its function *in vivo* (Chapter 5).

It seems that the calanoids is the only group of copepods in which there is such a regionalisation of the B-cell types. In the cyclopoids, although the gut seems to be organised as in calanoids, with 3 zones, B-cells are found in the anterior midgut as well (Musko 1983, Musko 1986).

In harpacticoids B-cells are not reported at all (Fahrenbach 1962, Yoshikoshi 1975, Sullivan & Bisalputra 1980).

In *Paranthessius anemoniae* (Briggs 1977), although the author does not mention any tendency for these cells to occupy a specific zone, it seems that B-cells (amoeboid cells of Briggs) occur only in the equivalent to the anterior and mid midgut, because he reports them to occur in the midgut but not in the hindgut, but from his description, the "hindgut" seems to be in fact the posterior midgut. In another poecilostomatoid, *Mytilicola intestinalis* (Gresty 1992), no regionalisation of B-cells was reported.

Continuing with parasitic copepods, in the caligid parasitic copepod *Caligus minimus*, B-cells are reported to be in equal proportions in the anterior and mid midgut, and absent in the posterior midgut.

Scott (1901) did not find any regional differentiation of the enterocytes in *Lepeophtheirus pectoralis*, and the same holds for *Pseudocharopinus dentatus* (see Rigby & Tunnell 1971) and *Lernaeocera branchialis* (see Scott 1901 and Capart 1948).

From this experience it is recommended that when studying the midgut of copepods, particularly those that do not show regional differentiation, attention is paid to the actual occurrence of cell types along the gut, and thus to be able to ascertain if that lack of differentiation is also functional.

A-cells were also found to occur in all the three sections, with higher occurrence in the posterior part, followed by the mid and anterior. It is difficult to compare this type of cell because they are not reported as such in other crustaceans. A discussion of its possible homologies has been made in Chapter 3. The positive association of its occurrence with the presence of a meal could mean that their function, possibly excretory or part of the epithelium homeostatic mechanism (see Chapter 3), is enhanced during the process of digestion, but not restricted to it, as they are found also when a meal is absent and also in starved individuals (See Chapter 5). This is further supported by the fact that when A-cells are less common or absent, the occurrence of B-cells is low as well, indicating maybe that digestion either has not started in full or it has already occurred. It has been shown that the occurrence of B-cells depends on the feeding cycle in *Calanus helgolandicus* (Nott *et. al.*, 1985), during which B-cells develop, mature and are lost at the end of the cycle, around 7 h after feeding. In contrast, Hassett & Blades-Eckelbarger (1995) did not observe this type of changes in number of B-cells in *Acartia tonsa*, but detected an increase in B-cells vacuole area during feeding.

Due to the problems mentioned with the variability of feeding in the present work it is not possible to ascertain if there is a cycle of digestion *per se*, (as in *Calanus helgolandicus*) or if the cell types persist through several feeding cycles and adjust their occurrence on the same time scale as the feeding cycle is occurring, as in *Acartia tonsa*.

By the same token, the lack of dependence between the occurrence of B-cells and the presence of a meal could be the result of a mixture of different stages of digestion, *i.e.* pre- or post-digestion stages or digestion in process, again due to this feeding variability.

The high occurrence of B-, and A-cells in SCRY type guts seems a logical association. As was discussed in Chapter 3, A-cells are probably a late stage of the R-cells in which they are discarded by apoptosis, and that this process may be related to a type of excretion of waste products as well. Some of the bulges characteristic of the SCRY type gut will eventually grow and pinch off as A-cells. This is a lot less common in THin guts. This supports the idea that the CRYpted gut type is more active and related to the actual digestive process. B-cells are usually found at the sides of the crypts.

Peritrophic membranes arise in Crustacea after food uptake (Brunet *et al.* 1994), so it was not strange to find them closely associated with the presence of a meal in this study. In all marine and freshwater calanoids studied, its presence is confirmed in the posterior midgut (Gauld 1957, Brunet *et al.* 1994, Yoshikoshi & Ko 1988) . The faecal pellet in a louse usually occupies the mid and posterior midgut, hence the higher occurrence of peritrophic membranes in these gut sections in this study. Brunet *et al.* (1994) mention that one or several thinner membranes are also present in the anterior midgut, which was true also for *L. salmonis* in the few cases where peritrophic membranes were found in this section. There seems to be little reason for a higher occurrence of peritrophic membranes in the anterior midgut of *L. salmonis* when most of the food ingested is passed rather quickly to the mid and posterior midgut. In his study of peritrophic membranes of 25 species of copepods, Yoshikoshi & Ko (1988) showed that in all free living and in the commensal notodelphyoid copepods the peritrophic membranes are present in the whole region of the midgut, but in the parasitic forms (11 studied) the peritrophic membranes occur only in the posterior region of the midgut. Also, the peritrophic membranes of the free-living copepods were thicker than the commensal and parasitic forms. The possible role of the peritrophic membranes as protection to the midgut from abrasion that has been suggested before (see Peters 1992 and Sudha & Muthu 1988) seems to be substantiated with these results, as stated by Yoshikoshi & Ko (1988). The absence or low occurrence of peritrophic membranes in the anterior midgut of the parasitic forms

and in *L. salmonis* seems to be then related to the texture of the food particles, which being tissue from their host, present little danger of abrasion to the parasite midgut. The peritrophic membranes are still necessary in the parasitic forms though, as their presence in the posterior midgut ascertains, and the function then could be to serve also as an adaptation to conserve secreted enzymes (by separating two extracellular sites of digestion, endo and ecto-peritrophic) and to selective ultrafiltrate enzymes and end products of digestion (Terra & Ferreira 1981).

The occurrence of A-cells was found to be independent of the peritrophic membranes. They were also independent of a meal. so they seem to exist whether there is digestion or not or they may, as it was mentioned earlier, increase in numbers during the process of digestion. Tape pellets are made up mainly of sloughed A-cells, and these pellets are released by lice under starvation, suggest that A-cells are involved in other process not directly related to digestion but probably possibly excretion or as part of the homeostatic machinery of the epithelium (see Chapter 3 and 7).

7. GENERAL DISCUSSION

An important aspect of the behaviour of an animal which is essential in the understanding of the function of the digestive system and the factors that may influence that function is the type or composition and frequency of the meals. Sea lice feed on the host's epithelium and blood. That had been suspected and/or proven by previous researchers (Scott 1901, Nylund *et al.* 1992, Bron *et al.* 1993, Kabata 1974, Boxshall 1985a). But, even though this is quite true, there seems to be an alternate preference for one or another. *Ergasilus sieboldi* is one of the few parasitic copepods in which the nature of the food ingested has been determined (Einzporn 1965b). Food comes from two sources, blood and gill epithelium, but both were found at the same time, mixed, meaning that they were probably not mutually exclusive. Blood in the gills is abundant and just one or two layers of cells deep. Feeding on the gill epithelium may inevitably cause the ingestion of red blood cells as well. In *L. salmonis*, in most instances, the occurrence of these two food materials seemed to be independent from each other. It is unlikely that the ingestion of blood in *L. salmonis* is a random event due to hitting a blood source by chance during feeding on epithelium. Lice that showed red guts continuously for several days were not stationary in one spot. They moved around and yet they kept on feeding on blood. This means that wherever they moved, they were able to find a source of blood in the skin, and hold on to it. In another member of the same genus, a striking similar feeding behaviour is known. But in this case the adult females of *L. thompsoni,* after spending their previous time on the surface of their host, move to the gill chambers after mating and there they move to the gill filaments and become haematophagous. How does *L. salmonis* find the blood source is not known. In the present study it was proposed that there are possibly chemoreceptors situated in the labrum. This has yet to be proved but chemoreceptors in this area certainly would be needed to guide the animal to the preferred source of food. Barnacles exhibit food selection prior to ingestion, rejecting particles at or near the mouth and the chemoreceptive basis for stimulating feeding activity has been shown (see Walker 1992).

Blood sucking is not an easy task. In any higher organism, fish included, there are efficient haemeostatic mechanisms that prevent the loss of blood. Against this, haematophagous

arthropods have developed a remarkable array of anticoagulants, antiplatelet and vasodilatory substances (Ribeiro 1995), which exist in the salivary glands. In larval stages of *L. salmonis* several glands that are apparently associated with the digestive system or with feeding activity have been reported (Bron *et al.* 1993). The nature of their secretions or the final point of delivery of the secretion has not been determined with certainty. Of interest is the "Gland 2", which appears to open into the oesophagus or oral cavity via a duct. Within the labium another glandular structure was observed, named the labial gland. Apparently there is a duct running onto the anterior intra-oral surface, nevertheless, no pores were seen in that part of the labium (Bron 1993) and neither in the present study. Blood feeding has evolved independently for almost every family containing haematophagous arthropods. Even within the same family different genera have different solutions to deal with the haemostasis barrier of the host. With this array of glands, *L. salmonis* certainly may have the tools needed to succeed as a haematophagous feeder. Certainly more studies with the goal to elucidate the role of these glands are necessary. On the other hand, the absence of a direct role in blood feeding by these glands should not be reason to discard the thesis that *L. salmonis* is a blood feeder, albeit intermittent. Recent work on the mechanisms of feeding by bloodsucking arthropods has altered the traditional view of the function of the saliva as necessary for haematophagy (Ribeiro 1995). The role of saliva is now understood to aid in probing rather than in blood digestion. By antagonising the components of haemostasis (vasoconstriction, platelet aggregation and/or clotting), haematophagous arthropods diminish the time needed to take a complete meal. Individuals in which the salivary glands have been surgically removed are perfectly capable of feeding but the duration of probing trying to find the blood source increased (Mellink & Van Den Bovenkamp 1981). Saliva, with its antihaemostatic properties, induces the formation of haematomas during probing, which in turn increases the probability of blood location. (Ribeiro 1987). This is important in organisms that have to reduce the host contact to a minimum for survival reasons. The longer they stay, the more chances of being killed by the host itself. *L. salmonis*, being a obligate ectoparasite on a fish apparently would not need this kind of protection, as the fish is unable to do much to get rid of lice. In a way, lice have all the time needed

to find the blood source when it is time to do so. Whether *L. salmonis* has or not the ability to counteract the haemostatic mechanisms of its host still has to be investigated in detail.

What is driving a sea louse to select between these two available food stuffs is not known, but from comparisons with other haematophagous arthropods some possible causes seem to come to light.

Due to the importance of haematophagous arthropods in both human and veterinary medicine the knowledge of the structure and function of their digestive system has advanced greatly in recent years (Billingsley 1990). Among the haematophagous insects, the Diptera are interesting for the present discussion because, during their life cycle, these organisms switch between two different and antagonistic feeding strategies. Sugar feeding is a cardinal characteristic of mosquito life. It is frequently ingested by both sexes and at all ages, and it is necessary to survive, to fly and to enhance reproduction (Foster 1995). Blood on the other hand is essential for egg development and apparently increases the survival as well (Almiron & Brewer 1996). Both types of feeding are antagonistic and mutually exclusive but share the same end goals.

Is the salmon skin epithelium the "sugar" of *L. salmonis*? There is not enough evidence from the present study to corroborate this, but it is enough to bring forth the possibility as a working hypothesis. From field observations during the samplings, blood feeding is apparently more common in adult females. This has to be tested properly with carefully designed field collections. As it is known that the reproductive output of *L. salmonis* varies seasonally (Ritchie *et al.* 1993) it would be worth comparing the blood feeding occurrence throughout the year, associated with the levels of reproductive investment in terms of both fertility and fecundity. The physiological age of the organisms involved could be an important factor to consider as well as it may be associated with the number of broods produced by the female. The reproductive system of *L. salmonis* is now well known due to the recent work of Ritchie, Mordue, Pike & Rae (1996) and this knowledge could be applied to investigate if the age of *L. salmonis* females could be calculated as it has been done in insects by analysis of the state of the ovaries. By knowing the state of the ovaries in insects, particularly mosquitoes, is possible to know if the female has laid eggs and how many times (Hoc & Schaub 1996, Hoc 1996). By applying this techniques to *L. salmonis*, the feeding strategies

could be then compared against physiological age or reproductive system maturity and investigate if there is any relationship between blood feeding and reproduction.

Experiments could be designed to follow the feeding behaviour of individual lice feeding on a fish, recording when they switch to blood feeding, the duration and a possible correlation of this behaviour with vitellogenesis or the output of eggs. De Meeüs *et al.* (1993a) have demonstrated that the haematophagous females of *L. thompsoni* are more fertile than the mucophagous females of *L. europaensis*.

Skin feeding probably provides enough nourishment for the survival and growth of *L. salmonis* and perhaps only when extra nourishment is needed is when it switches to a different source of food. For this, a different set of digestive enzymes are probably employed. In culicids, which alternate between sugar and blood feeding, the gut is divided in two regions. The anterior midgut is not involved in blood digestion, which is the role of the posterior midgut. The presence of glycosidases in the anterior midgut supports a saccharide-digestive role, while the posterior midgut digestion of blood is trypsin-based (Billingsley 1990). In *L. salmonis* it was evident the contrast between the incomplete digestion of the host' skin and the quick and thorough digestion of the blood meal. Moreover, in some lice that had fed on epithelium sometimes intact red blood cells were evident, with no sign of lysis or digestion. This suggests that two digestive strategies may be in use at different times. Perhaps *L. salmonis* does not need to digest thoroughly the epithelium ingested. Probably what is nourishing in the epithelium for the lice comes in small quantities and therefore lice need to ingest large quantities of "raw material" to get it. For this purpose, maybe *L. salmonis* is adapted to use less quantity of the same set of enzymes or to vary the proportions of them depending on the type of meal, resulting in a low efficiency to digest the bulk material from the epithelium. On the other hand, low digestive efficiency has also been observed in free living copepods suggesting that digestion happened to a limited extent only (Kiørboe *et al.* 1985). Ideally, less efficient assimilation may bring a greater benefit to the organism in an unlimited food supply situation (Penry & Jumars 1987), which happens to be the case with *L. salmonis*. Studies of enzymes occurrence and concentration on individuals at different feeding strategies and at different stages in the digestion process should give some answers to clarify this issue.

Food availability is not a problem for *L. salmonis*. In similar conditions of vast amounts of food, some planktonic copepods produce more and bigger pellets indicating that the ingestion rate increases as well (Butler & Dam 1994). Planktonic copepods do not evacuate pellets until they ingest sufficient food to build the smallest faecal pellets. Therefore, clearance time increases as food concentration decreases and vice versa (Tsuda & Nemoto 1990). Thus, gut clearance times in *L. salmonis* by definition would have to be very short. The production of faecal pellets observed in the present study does not seem to agree with fast gut clearance times. But then again, probably the comparison with planktonic forms is not adequate. Planktonic copepods during an algal bloom have to take advantage of the phenomenon and process as much food as they can, while it lasts but *L. salmonis*, with its food secured just below it, perhaps does not need to do that. In any case, the residence time of a faecal pellet is shorter than the time needed for a blood meal to be processed completely and this also may account for the poor digestion of a skin strips meal observed.

From the evidence gathered in the present study it seems that *L. salmonis* is not a continuous feeder. It appears that *L. salmonis* has a meal, processes it, discards the waste and then takes another one. When faecal pellets were found in the posterior midgut there never was evidence of a new meal being taken. This intermittent feeding has been observed also in *Lernaeocera branchialis* (Sproston & Hartley 1941). Indication of multiple meals would have been appreciated in the faecal pellets, as each meal probably would have had its own peritrophic envelope. This has been observed in mosquitoes, where up to 5 individual meals have been recognised in the posterior midgut, each one separated by a different peritrophic envelope (Scott, Clark, Lorenz, Amerasinghe, Reiter & Edman 1993).

The mode of feeding and feeding habits of *L. salmonis* described in the present study have important implications for several approaches that are currently being taken in search for a control of sea lice in the farmed fish.

Theoretically, vaccination of fish against lice would be the more efficacious way of reducing the parasite's infection on fish farms (Andrade-Salas *et al.* 1993, Reilly & Mulcahy 1992). Studies on the Australian cattle tick *Boophilus microplus*, has shown that gut-derived antigens can produce a

protective response in vaccinated cattle that reduces the burden of the parasite (Willadsen *et al.* 1988, Wong & Opdebeeck 1989). The success in cattle has led researchers to look for a way to immunologically disrupt the normal functioning of the louse gut, either by targeting an intrinsic functional protein or a structural constituent of the louse gut epithelium (Jenkins *et al.* 1993). Whereas the disruption of the gut function is of prime importance as it is one of the first tissues that the successful antibodies will contact, potential antibodies against other tissues are also important to take into account when considering the possibility for a "vaccine cocktail" of several protective antigens (Andrade-Salas *et al.* 1993).

Proteins are the most abundant nutrients of the blood meal. If *L. salmonis* is feeding on blood it must have the enzymatic machinery to deal with it and proteases must play an important part in the digestion, just as they do in other haematophagous arthropods (Billingsley 1990). To disrupt the functionality of the gut, targeting digestive enzymes like proteases for vaccine production seems to have its advantages because they are well understood group of molecules and this may aid in having a head start to produce a vaccine (Lehane 1994). Proteases and lipases separated from louse homogenates have been considered as possible immunogens (Jenkins *et al.* 1993) but Lehane (1994) is of the opinion that lipases may be a better candidate because proteases have the disadvantage of being poor antigens, possibly because, as proteases, they tend to destroy the antibody-making proteinaceous machinery which approaches them. In insects, it has been shown that reproduction and fecundity were affected in haematophagous flies fed on a lipid-deficient blood (Langley, Ogwal, Felton & Stafford 1987) and therefore lipases may be adequate targets for vaccine development.

Another possible candidate could be the haemolysins. Most of the nutrients in the blood are trapped in the blood cells, mainly in the erythrocytes. To have access to these nutrients haematophagous organisms have to break this barrier. In *L. salmonis*, it is not known how this is achieved but is done probably in a very short time scale. Two possibilities exist: mechanical or chemical. Fleas and some mosquitoes achieve this by physical means, but they have spines and armatures developed from the cuticular lining of the foregut, which punctures the cells as they pass through (Lehane 1994). *L. salmonis* does not seem to have such a mechanical refinement. It is

more likely that it is relying on a chemical means for haemolysis. The source of the haemolysins in *L. salmonis* could be in one of the glands associated with the mouth cone or in the gut itself. Very little is known about the nature of haemolysins, even in insects, but it could be worthy of investigation in the case of *L. salmonis*.

Other likely candidates for vaccine development could be the possible receptors that may trigger the enzymatic activity of *L. salmonis*. As was discussed in a previous chapter, two possible ways of modulation and control of digestive enzymes may exist, a secretagoge and a neural. Whichever is at play, there must be a receptor or set of receptors that detect a component in the food and start the digestive mechanism. But to arrive to this stage, a long way into the knowledge of the digestive physiology of *L. salmonis* is still awaited.

Testing the efficacy of a vaccine involves extremely expensive and labour intensive field trials. The ideal situation would be to have a way to culture the parasite or some means to deliver the vaccine into the gut of the louse for preliminary tests before engaging in field trials that may or may not give positive results. A successful parasite culture is faced in the first instance with the problem of feeding. Some attempts are being made to culture the larval stages of *L. salmonis*. Chand (pers. com.) have achieved to take *L. salmonis* larvae through three molts on a specially designed fish cell culture system that allows the larvae to fix onto and feed. The peculiar mode of feeding of lice represents a major challenge when trying to imitate the conditions on which a louse may feed properly. *L. salmonis*, in contrast with *Caligus elongatus*, is quite host specific (Kabata 1979) and it is yet not known what drives this specificity to salmon.

Manual force-feeding such as the one described in the present study has also been used to test vaccine candidates on a preliminary basis using haemolysed salmon blood (Reynard pers. com.). Manual feeding could be useful to test vaccines against structural constituents of the gut epithelium, because it would be possible to assess the physical damage that the vaccine may generate to it, *provided* that great attention is put on the fixation process. But this method would have strong limitations. The damage, if any, produced by the vaccine would have to be an immediate one, a one dose shot, which is unlikely to happen. There is evidence from the present

study that the feeding behaviour could be affected seriously when lice are taken off the host and handled. Repetitive handling could have unknown effects on the physiology of the louse that could prevent it from establishing normally again on its host and any follow up of the condition of the louse could be very unreliable.

If what a vaccine is targeting is the enzymatic machinery of the louse, then manual feeding would probably be inappropriate because the stimuli to engage in digestion seem to be lacking when lice are force to feed, and the target antigens may be simply unavailable. Moreover, the effect of a vaccine against the digestive enzymes is likely to be a long term one, which affects survival or fecundity.

In ideal conditions the vaccination regime should be applied orally to preferentially induce an immune response at the cutaneous surface of the fish (Jenkins *et al.* 1993). This is particularly applicable to *L. salmonis* as its main food source is the host's epithelium. But what must be taken into consideration is the poor digestion of the fish epithelium observed in the present study, which may not be sufficient to liberate enough quantities of the antigen. Therefore the titres would have to be quite high to produce a significant effect. If the set of enzymes that *L. salmonis* is using to process a skin meal is elucidated, then these enzymes could represent another candidate for a vaccine. If successful, it could affect the lice from the early days of development. In anopheline mosquitoes, engagement of the female in vitellogenesis depends much on the dietary history. If the energy reserves are low, the blood meals are used to restore the energy reserves and it is only then that energy from subsequent blood meals starts to be diverted to the production of yolk. If the reserves are very low, due to a poor larval nutrition in nature, a female may not develop follicles to a stage where they can accumulate yolk. Two or more non-vitellogenic blood meals are necessary to start the development of the follicles so that a subsequent blood meal causes egg maturation (Foster 1995). If lice feeding on these immunised fish are not capable of a proper nutrition during their life cycle, when they reach maturity their fecundity could be also impaired. As we do not know the extent of the importance of skin feeding to the physiology of the louse, this is merely speculative. If energies are low, lice may switch to blood feeding and take energy from this source.

A vaccine targeting the enzymes used in both feeding strategies, if different, probably would be more efficient.

The peritrophic membranes of haematophagous arthropods have also been considered as a target for immunological attack. Antimidgut antibodies have been shown to affect the formation of the peritrophic membranes in an anopheline mosquito (Ramasamy *et al.* 1996) but in this case the peritrophic membranes were not essential for its survival after a blood meal. Billingsley & Rudin (1992) also observed that peritrophic membranes in two anopheline mosquitoes were not essential for the digestion of a blood meal. On the other hand East & Eisemann (1993) vaccinated sheep with extracts of peritrophic membranes of the sheep blowfly *Lucilia cuprina* and showed retardation in growth and some mortality. Subsequent work showed that a single protein from the peritrophic membranes extracts could elicit an immune response that affected larval growth (Willadsen, Eisemann & Tellam 1993). Apparently the effect was that the permeability of the peritrophic membranes was affected. This approach would be useful only when *L. salmonis* is feeding on host' skin as peritrophic membranes were never seen during blood feeding. Whether the absence of the production of a peritrophic envelope in *L. salmonis* is deleterious for its digestive process remains to be investigated. The problems behind this are the possible difficulty in obtaining enough quantity of isolated material. A possible advantage is its relatively biochemical simplicity, facilitating the eventual isolation of protective antigens (Eisemann & Binnington 1994).

The cell types described in the present study require further study. Further characterisation and role of each cell type still has to be expanded with the use probably of more refined analytical techniques. Antibodies raised against digestive enzymes could be immunohistochemically useful to follow the ontogeny of certain cell types, particularly F- and B-cells. This approach was successfully used by Vogt *et al.* (1989), demonstrating the secretory role of astacin, a protease, by the F-cells in the crayfish *Astacus astacus.*

The exact fate of the B-cells, and its contents, after extrusion still has to be investigated as well as its contribution to absorption while it is still attached to the epithelium. The role of the esterases characteristic of these cells should also have to be investigated, mainly because they could be a potential target for vaccines as discussed before.

Here, it was proposed that R-cells that have finish their function die in an orderly way by means of apoptosis, and that the A-cells represent this apoptotic stage of the R-cells. Apoptosis is an important homeostatic mechanism that maintains a proper cell number in the body of animals and plants, balancing cell production and cell death (Strano & Blandino 1995). Obviously, more studies would be necessary to understand better the homeostasis of the gut epithelium of *L. salmonis*. Even though it is widely accepted that apoptosis is of prime importance for the development and homeostasis of metazoan animals (Steller 1995), to the best of my knowledge, programmed cell death has never been put as an homeostatic mechanism in crustaceans. The process is well known in insects and nematodes and by now the genetic pathway is well understood and it seems that there is a relatively well conserved mechanism throughout animal evolution. One of the first studies that should be carried out is to confirm that the A-cells are in fact apoptotic. For that purpose there are relatively simple techniques that could be applied. A characteristic of apoptotic cells is the fragmentation of DNA by nuclease forming "DNA ladders" (Ellis *et al.* 1991, Strano & Blandino 1995). These ladders can be recognised by in situ end labelling incorporating biotylinated nucleotides with polymerase. The method has the added advantage that can be applied on routinely fixed , paraffin embedded histological sections (Wijsman, Jonker, Keijzer, Van De Velde, Cornelisse & Van Dierendonck 1993).

The counter part of programmed cell death in the homeostatic system that maintains the correct cell number in a tissue is cell proliferation. The fine tuned balance between these two processes provides the precise control over tissue size (Ellis *et al.* 1991). Also, renewal of the intestinal epithelium fulfils the normal functions of maintaining the integrity of the organ, repairing possible cell injury, and replenishing the specialised cells of the epithelium (Eastwood 1995)

Cell proliferation in the epithelia of the digestive system and/or gills of marine invertebrates has been studied since the mid eighties in Russia. Unfortunately, most of the relevant papers are published in Russian except for two reviews (Leibson 1986, Epifanova & Brooks 1994). The study of the patterns of cell proliferation is of particular importance for the understanding of *L. salmonis* digestive system homeostasis. As stated before, the gut epithelium of *L. salmonis* seems to be in constant topographical change, alternating through states of "smooth gut" with no or little rugosity

and "rough gut", with more pronounced rugosity. These changes may be related to the interaction of cell proliferation and cell death. It is postulated here that a rough gut, with higher occurrence of ridges and crypts could be the result of both increased cell proliferation that pushes existing cells against each other forming these bulges, and increment in cell volume due to the increased number of organelles and products of absorption. When these cells have completed their functions, they transform into apoptotic cells which are finally shed from the epithelium, probably along with waste products accumulated and concentrated in them. When sufficient cells have been disposed of in this way, the bulges and crypts disappear and the gut appearance becomes smooth again. The rather constant 2:1 proportion of these gut types suggest that the phenomenon is not a stochastic event. Moreover, the regular production in starved organisms of tape pellets, with its associated A-cells , reinforces the hypothesis that A-cells might have a detoxification role as well.

The study of cell proliferation in *L. salmonis* is also important in terms of control of the parasite. It has been proposed that in mammals there are cell-derived inhibitors of intestinal cell proliferation. Nutritional, hormonal, neural and pharmacological factors are known to affect the intestinal cell synthesis indicating that a controlled process is at play rather than a simple extrusion of aged cells (Özgünes 1996). In eukaryotes, this control is accomplished by periodic transition of cells from proliferation to rest and vice versa as a result of a co-ordinated inter-relationship between intracellular growth inhibitors and extracellular growth stimulators or mitogens. There is now evidence that resting cells are able to produce molecules with antiproliferative activity, some of which behave as short-lived drepressor proteins (Epifanova & Brooks 1994). These molecules could also be regarded as potential targets for vaccines with the objective to disrupt the homeostasis of the gut epithelium.

Although it was not quantified, from personal observations it seems that different portions of the gut may have different topography at any one time. Also in the present study, some evidence of the possible regionalisation of some functions of the gut was put forward. What is co-ordinating this functions remains to be investigated but a strong candidate could be a humoral or neural control. The mesenteries associated with the gut and the gut itself could play an important part in this control as they do in other invertebrates, like insects and oligochaetes (Mulye & Davey 1995,

Polenov & Kulakovskii 1989, Cheli 1989, Rainbow & Walker 1977) and clearly further studies are necessary to reinforce this hypothesis. An even more careful look at the cells in the gut epithelium with refined analytical techniques should be performed to assess if there are cells that may have an alternative function as receptors or humoral mediators. If the transmission of control signals is done through the haemolymph, as it happens in insects and other crustaceans (Mulye & Davey 1995, Koulish 1976), a better knowledge of the circulation of the haemolymph would be necessary to be able to follow the path of these possible messenger molecules. Although a gross pattern of haemolymph circulation in copepods is known, the division of the body cavity in haemocoelic compartments through which the haemolymph circulates in a distinct pattern had not been described before. A better knowledge of this circulatory pattern could have positive implications in the knowledge of the overall physiology of *L. salmonis*.

The "discovery" of A-cells in the present study would have never happened if properly fixed material had not been used. These cells seem to be extremely vulnerable to osmotic damage and the slightest variation from the correct osmolarity of the fixative would cause the cell to burst leaving no trace or to show "artefactual necrosis" (see Chapter 2) depending on the degree of osmolarity shock. Unfortunately, "artefactual necrosis" seems to be a widespread problem in the field of morphology and ultrastructure of the digestive system in Crustacea, particularly marine crustaceans. Although the problem has been addressed superficially (Einzporn 1965a, Vogt 1994), it is the view of the author that this has been an underlying problem apparently unnoticed among researchers in the field. With the trial and error method devised in the present study it is possible to arrive at a correct fixation schedule with the required fixative or mixture of fixatives that can guarantee the best preservation possible for the morphology of the tissue. It is imperative that other researchers in this field realise the importance of securing the best possible preservation of the tissues under study, so that useful comparisons and extrapolations can be made with other systems. It is also important to realise that necrosis is a degenerative phenomenon which is always pathological (Searle *et al.* 1982) and in the future any sign of frothy cells, swelling, hypertrophied organelles, apical disintegration, "apocrine secretion", "merocrine secretion", blebbing, cytoplasm disintegration, sloughing or necrosis in a cell or groups of cells should be looked at with suspicion,

and consider fixation artefact as the first cause of that morphology. Only when fixation has been ruled out to be the cause, then, and only then, is when assumptions of a possible real physiological cause of the observed morphology or a real necrosis can begin to be considered. Probably several of the common discrepancies that exist in the literature on the digestive cell morphology and its functional interpretation between different groups of crustaceans could be conciliated by comparing equally well preserved material.

One last point that is worth addressing is that *L. salmonis*, in the context that is being studied, should be considered as a new domesticated animal. Aquaculture practices are far from being in the normal range of conditions that hosts and parasites usually live and therefore aquaculture could be considered as a form of artificial selection. In consequence, one has to be very cautious when trying to extrapolate the results of the studies made in organisms such as *L. salmonis* in artificial conditions to natural populations. It may not be a straight forward procedure. For example, De Meeüs *et al.* (1993a) found that in natural conditions, *L. europaensis* produced bigger clutches of eggs compared with the experimental females , which were significantly smaller. It is not known for instance if *L. salmonis* has adopted an haematophagous feeding strategy for reproduction when faced with farmed salmon or this strategy is also used by the wild populations as well. A study of the feeding habits in natural population would have to be conducted to clarify this issue.

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