Exocrine Glands of the Caligid Copepod *Lepeophtheirus salmonis* (Krøyer, 1837).

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By

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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 sources of information have been duly acknowledged.

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ABSTRACT

A variety of different functions have been attributed to the secretions of copepod exocrine glands. Such secretions have been suggested to possess, amongst other properties, hydrodynamic, predator deterrent, cuticle hardening and antifoulant activities. The nature of the secretion of a selected group of exocrine glands of the caligid copepod *Lepeophtheirus salmonis* was examined.

Firstly, the number and pattern of distribution of the defined glands of *L. salmonis* were identified in all life-stages. In comparison, several species of parasitic, commensal and free-living copepods and other crustaceans were examined to determine the extent of the distribution of such glands within the Crustacea.

Histochemical techniques and ultrastructural analysis of glandular tissues were utilised to suggest the probable biochemical characteristics of the gland contents and revealed that mucus and protein were components of the gland secretions. TEM analysis revealed a considerable mucus layer covering the body cuticle of *L. salmonis*. This layer was presumed to be derived from the exocrine glands of this species.

To characterise the secretion more precisely specific enzyme assays, selective staining procedures and high performance thin layer chromatography (HPTLC) were employed. These techniques indicated that the peroxidatic enzyme catalase was present in the glands at significantly higher levels than in the general body tissues. This enzyme was shown to be contained within the secretory vesicles of the glands.

Having positively identified an enzymatic component of the gland tissues, acrylamide gel electrophoresis was undertaken to specifically determine the molecular weight and quaternary structure of the enzyme. Further studies using this technique focused on identifying secreted enzymes of *L. salmonis* and correlated such proteins to those identified in samples of gland tissue. A four subunit, catalase of between 260 and 280kDa was demonstrated to be present in the secretions of *L. salmonis*.

The final stage of the work tested the hypothesised functions of the secretions of the identified glands of *L. salmonis*. The gland secretions of *L. salmonis* were demonstrated not to possess an antibacterial activity against some common species of marine bacteria whilst the deliberate removal of the external mucus layer significantly affected the hydrodynamic characteristics of *L. salmonis*.

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1. INTRODUCTION

1. GENERAL INTRODUCTION

1.1 INTRODUCTION

1.1.1 Caligid copepods and salmonid culture

Copepods of the Family Caligidae have long been known as ectoparasites of marine fish (Wilson 1905, Scott and Scott 1913). It is only with the relatively recent increase in Atlantic salmon (*Salmo salar* L.) culture in northern Europe and Canada that these parasites became pathogens of major economic importance and have therefore warranted a greater inclusion in the literature. The two main species of caligids in these regions which are commonly found in association with farmed salmon, and which have therefore been subject to greater scrutiny, are *Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* Nordmann 1832 (often referred to as 'sea-lice'). These modified copepods are adapted to feed on the skin, mucus (Kabata, 1974) and blood (Brandal, Egidius and Romslo, 1976) of the fish host and this feeding activity can be a cause of significant pathogenicity to the host (Kabata 1970, Wootten, Smith and Needham 1982, Jónsdóttir, Bron, Wootten and Turnbull 1992).

The high density conditions essential to viable salmon culture provide ideal conditions for epizootics of these species which, ordinarily, are present only in non-pathogenic numbers on wild fish (Bristow and Berland, 1991). Under culture conditions, however, numbers of sea lice on fish can reach such levels as to cause the death of the fish either directly through impaired osmoregulation (Wootten *et al.* 1982, Tully 1991), or indirectly by allowing secondary infection to occur across the damaged host integument (Paperna 1975, Håstein and Bergsjö 1976, Nylund, Wallace and Hovland 1993). Even at sub-lethal levels, the parasites can decrease the vitality of the host and cause cosmetic problems i.e. scarring, which may lead to a decrease in the market value of the fish (Costello, 1993).

These problems have lead for calls to provide novel, safe and effective treatments to control *L. salmonis* on farmed salmon. Fundamental to meeting these demands is a greater understanding of the biology of these copepods. In spite of the concerted efforts of scientists in recent years there still exist

large gaps in our knowledge of the biology, behaviour and population dynamics of even those species of caligid copepods of commercial interest. Entire aspects of the physiology of these animals remain unresolved despite the volume of research that has been conducted on them.

1.1.2 Life cycle and morphology of *L. salmonis*

Caligid copepods display a significant number of morphological adaptations compared to free-living copepods such as the calanoids. Kabata (1979) provides a comprehensive discussion of the morphological adaptations of caligid copepods. The most conspicuous adaptation is the flattened cephalothorax which is developed into a saucer-shaped structure and incorporates the third thoracic leg-bearing segment. Other morphological adaptations are apparent in the modification of several appendages to form hooks (antennae and maxillipeds) and the development of rugose pads (e.g. the post-antennary process) to facilitate the attachment of the parasite to the fish host. The structure of a typical caligid copepod is shown in **Figure 1.1**. Copepods of the genus *Lepeophtheirus* display similar body morphologies to that illustrated in **Figure 1.1** but do not possess the sucker-like lunules which are a feature of the genus *Caligus*.

The parasitic copepod *L. salmonis* is taxonomically placed as follows:

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

The general life-cycle of *L. salmonis*, in common with many other caligid copepods, is widely understood and involves five distinct phases and ten separate developmental stages which have been

comprehensively described by Johnson and Albright (1991a) and Schram (1993). The first phase consists of two lecithotrophic naupliar stages which are followed by the second phase, consisting of a free-swimming, host-seeking infective copepodid stage. Following attachment to a suitable fish host the copepodid moults into the first of four chalimus stages. Chalimus larvae represent the third phase in the life-cycle and are permanently attached to the host via a filamentous extension of their cuticle. Subsequent to this the preadult stage is attained. The two preadult stages display similar gross morphologies to their adult counterparts and are the stage at which the sexes become readily distinguishable. The final phase of the life-cycle is achieved with the terminal moult to the adult stage. Mating takes place between an adult male and a freshly moulted adult female. Multiple batches of eggs can be produced from a single fertilisation and several hundred eggs can produced in each batch by a female (Wootten *et al.* 1982, Johnson and Albright 1991b). The entire life-cycle can be completed within 49 - 90 days (Johnson and Albright, 1991b). This life-cycle is diagrammatically represented in **Figure 1.2.**

The aim of this project was to identify all of a previously identified (Bron, 1993) set of exocrine glands in *L. salmonis* and other parasitic, commensal and free-living crustacean species. The ultimate aim was to characterise the gland secretions and identify the role they play in the biology of these animals.

1.1.3 Glandular systems of caligid copepods

One particular aspect of sea louse biology that has only been briefly touched upon is their system of exocrine glands. Aside from two descriptions of the glands responsible for the secretion of the filament used for initial attachment to the host by the copepodid by Bron, Sommerville and Jones (1991) and Bron (1993), no other specific studies have been carried out in this area. This is surprising given the interest in mate location and pheromones that has been a feature of the literature of the less commercially important free-living copepods. Recent work by Ritchie, Mordue (Luntz), Pike and Rae (1996b) describes the presence of factors expressed by *Lepeophtheirus salmonis* that are attractive to potential mates although they give no indication whether they believe that these factors originate in exocrine gland systems or

elsewhere. The possibility of using analogues of such pheromones to trap sexually active males is one possible future avenue for control of theses species.

Bron (1993) first demonstrated the existence of a population of exocrine glands in *L. salmonis*, which stained positively with the compound 3'3-diaminobenzidine. This positive staining response was suggested to indicate the presence of endoperoxidases. The glands were observed close to the labrum and in the urosome and thoracic legs of the copepodid stages.

1.1.4 Overview of the current knowledge of crustacean exocrine glands

Descriptions of glandular structures in copepods appeared in early literature (Richards 1891, Yonge 1932), but it has only been in the last few decades, with the development of more refined microscopical techniques that such glands have been examined in detail. Unfortunately, the few histological studies that have been undertaken cover a wide range of genera and orders and, as such, the scarce details they have provided only serve to highlight our lack of understanding of these structures, their secretions and their significance to the biology and ecology of these animals.

Research on gland structure and function has been carried out along the two main paths of ultrastructural examination and cytochemical characterisation. The use of light microscopy and, more importantly TEM, has facilitated the study of these small structures, whilst the use of a wide range of histological stains has provided clues as to general types of compound produced by them. From here though, it is a long step to either specific chemical characterisation or proof of function. Proof of function can only really be determined through behavioural studies and is difficult to achieve satisfactorily so that almost all theories regarding gland function remain hypothetical, although often these hypotheses can be argued for convincingly.

Noirot and Quennedy (1974) published the first comprehensive survey of glandular structures, segregating them into three different classes depending on their cellular organisation. Despite the fact that their review described only insect glands, many crustacean researchers utilise their terminology and follow the classification they proposed. Although their work is excellent in many respects, the utility of

their scheme in the description of crustacean gland tissues seems limited, given that the multitude of different forms these glands take prohibits them from being easily placed within any one of their categories.

A review of the published work on crustacean gland systems readily demonstrates that it is more useful to segregate exocrine glands on the basis of their characterised secretion, or within homologous groups. Five main types of exocrine gland have been described from the crustacea and they are each described below, with a particular emphasis on those glands found in copepods.

1.1.4.1 Tegumental glands

Tegumental glands are not a clearly defined category of glands in terms of function but are instead defined solely by their location within the animal. The tegumental glands are those that lie close beneath, often in direct contact with, the body cuticle and have had a variety of functions proposed for them.

Functions so far attributed to these glands in the crustacea include: production of new epicuticle during ecdysis (Yonge, 1932), hardening of the cuticle (Stevenson, 1961), lubrication of the integument for maintenance and to decrease water resistance (Brunet, Cuoc, Arnaud and Mazza 1991, Bannister 1993a), anti-fouling (Boxshall 1982, Bannister 1993a) predator deterrence (Pochon-Masson, Renaud-Mornant and Cals 1975, Boxshall 1982), and sexual activity (Fleminger 1967, Gharagozlou-van-Ginneken 1979).

In addition to these varied hypotheses these glands have also been described under a range of pseudonyms and are variously present in the literature as tegumental, cuticular, sub-cuticular, dermal and epidermal glands.

Unicellular tegumental glands with a proposed role in cuticle formation were described for the copepod *Cucumaricola curvatus* Avdeev, 1977, a parasite of holothurians, by Rybakov and Dolmatov (1991). These glands were arranged either singly or in groups, often occurring over areas of considerable size. Dense areas of glands were especially prevalent in those regions of the female integument that underwent massive expansion during post-ecdysial growth. Unfortunately no detailed description of the

gland ultrastructure was given; neither was any attempt made to elaborate upon the type of material secreted by these glands.

Brunet *et al.* (1991), working on the free-living freshwater copepod *Hemidiaptomous ingens provinciae* (Petit and Schachter, 1943), found that the tegumental glands were distributed in an approximately bilaterally symmetrical pattern on both the dorsal and ventral surfaces; the greater profusion of glands occurring on the dorsal surface. *H. ingens* is a species of fresh water copepod, which suggests that such glands are not exclusive to marine copepod species.

One of the most comprehensive accounts of tegumental glands in any species is that of Bannister (1993). He studied the tegumental glands of the free-living calanoid *Temora longicornis* (Müller) and he suggested that the secretory products of those glands permitted a decrease in drag, either directly by being hydrophobic, or indirectly by reducing the numbers of bacteria adhering to the cuticle i.e. an anti-foulant. He also tried to correlate the position of the glands to his reduced drag hypothesis and suggested that, if the function of the secretion requires a constant coating over the animal, then secretion would be likely to occur slowly under an increased internal pressure caused by the production of more secretory product by the gland. Unfortunately no direct evidence was presented to support these hypotheses.

Von Vaupel Klein (1982a) states that there is a direct correlation between the number of pores in the integument and swimming speeds in the Copepoda. If this was true then it would suggest that the tegumental glands are responsible for secreting some compound which reduces the friction of water passing over the cuticle, either by covering the cuticle with a hydrophobic layer or by killing, or reducing, the number of epibionts known to occur on the cuticle surface (Gresty and Warren 1993, Dumontet, Krovacek, Baloda, Grotti and Vanucci 1996). Neither of these proposed roles to the gland secretion is unreasonable, as both of these phenomena have been demonstrated to occur in insects. It has been shown in whirligig beetles of the Family Gyrinidae (Vulinec, 1987) that the pygidial glands secrete a hydrophobic substance which causes water molecules to recoil. The beetles then ride on those water molecules that recoil away from the secretion (a technique known as 'expansion swimming') with specimens recorded swimming at speeds of 144cm s^{-1}.

Boxshall (1982) proposed that the tegumental gland secretions of the free-living misophrioid copepod *Benthomisophria palliata* Sars are involved in preventing the fouling of the body cuticle with epibionts. He identified unique glandular structures which he termed 'cone organs', that were found in great numbers in a patch on either side of the cephalosome. He believed that the secretion from these glands had a role in preventing the establishment and growth of microorganisms on the carapace of the copepod. This hypothesis was derived purely from morphological evidence i.e. the cone organs were located in a position where their secretions could be picked up on the antennae and mandibular setae and be smeared over the surface of the carapace which itself was devoid of tegumental glands although the chemical nature of the secretion was not ascertained.

A more detailed investigation of the tegumental glands of *Mytilicola intestinalis* Steuer, a copepod parasite of the haemocoel of mussels, was given by Poquet, Ribes, Gracia Bozzo and Durfort (1994). In this species, large number of unicellular tegumental glands were found distributed over the surface of the cephalothorax and the abdomen. It was concluded that the glands secreted either a proteinaceous or glycoproteinaceous material and that secretion was by an apocrine method as seen in the free-living calanoid *Centropages typicus* Krøyer, 1849 by Arnaud, Brunet and Mazza (1988a,b). There appeared to be a cyclical pattern to the secretory activity of the glands but this cycle was asynchronous between glands. However, no hypothesis as to the function of this secretion was given.

Other tegumental glands described include those found in the copepod *Notopterophorus papilio* Hesse (Gotto and Threadgold, 1980). This species, in-keeping with all five other members of the genus, live commensally inside ascidians such as *Ascidia* sp. *Phallusia* sp. and *Ciona* sp. Females of this copepod possess large 'wings' or 'alate processes' of uncertain function. Unicellular glands on these processes were commonly found to secrete material via pores found in close association with bulb-like sensillae on the cuticle surface. The apical regions of the glands were filled with secretory granules that appeared to be composed of muco- or glycoprotein. This cell was described as being morphologically similar to the goblet (mucous) cells of the vertebrate intestine. It was tentatively suggested by Gotto and Threadgold (1980) that the material secreted from these glands was used to form a layer over the bulb-like

sensillae, trapping a thin layer of electrolytically-produced hydrogen against their surface. The suggestion was that these then could function as devices sensitive to pressure changes – an ability presumed to be essential for the successful dispersal of the larvae of this species. It may also be possible that the secretions from these glands contributed to the 'fuzzy layer' which was seen to envelop the cuticle of this species. A similar 'surface fuzz' was described on the cuticle of *Paranthesius anemoniae* Claus (Briggs, 1978). The possibility that the secretions composing this surface fuzz may serve to inhibit or decrease the host defence mechanism should be considered. It has been stated (Gotto and Threadgold 1980, Poquet *et al.* 1994) that no visual sign of irritation of the host tissue could be detected in response to the presence of these animals. Although they do not feed on the host tissue, the presence of such sizeable objects (as much as 5mm total length in *N. papilio*) amidst the host tissues would be likely to be a source of some irritation which would be expected to elicit some form of host defence response, unless some form of immunosuppression or 'masking' was utilised.

Work by Hipeau-Jacquotte (1987) also linked the occurrence of small tegumental glands with the function of nearby sensory organs. The free-swimming sexually active stages of the copepod *Pachypygus gibber* (Thorell, 1859) that parasitises ascidians, have numerous pores leading from underlying glands in the pleural areas of the abdomen. These gland pores were found in close association with sensory pores. The secretion from these glands was again seen to form a surface fuzz over the surrounding cuticle. The structure of these glands was shown to vary through four successive stages, ultimately terminating in a phase of cellular autolysis, followed by secretion of all cellular contents. A temporal asynchrony was evident as regards the stages of gland cell development, discounting the theory that the glands were in some way related to the moult cycle. The glands secretory products were demonstrated to consist of acid-mucopolysaccharide. The author proposed that the secretion of *P. gibber* covered the sensory pores (and their exposed dendrites) and served to mechanically trap molecules thereby serving to concentrate 'sex pheromones' from the females, assisting in mate location. This 'secretion-trap' effect has been demonstrated in insects and in the olfactory mucosa of vertebrates.

There is some evidence that sexual attraction of crustacean males by females is mediated by the production of 'sex pheromones' (Kittredge, Terry and Takahashi 1971, Katona 1973, Griffiths and Frost 1976, Ritchie *et al.* 1996b) as has been shown to occur in insects. Although Dunham (1978), quite rightly, found fault with the experimental design of those studies, the evidence for pheromonal involvement in the mediation of mate recognition is convincing. Further evidence for pheromones being implicit in mate location has been provided by Snell and Nacionales (1990) and Snell,Morris and Cecchine (1993). Those authors found that surface-bound glycoproteins were responsible for mediating pre-copulatory behaviour by males of the rotifer *Brachionus plicatilis* (O.F. Muller). This phenomenon has subsequently been shown to be important in mate recognition in the copepod genera *Coullana* (Lonsdale, Snell and Frey, 1996). It is possible that tegumental glands are secreting these glycoproteins, and if this were true, we would expect to find an increased activity of these glands in females, especially during stages when they are 'receptive' to mating behaviour by males. It is also possible though that these glycoproteins are not part of a secreted coating but instead are an integral part of the cuticle and are produced during its formation. One obvious problem with this hypothesis is that, if substances acting as pheromones were expressed by the animal during all life stages, then it would receive attention from prospective mates during times when this will be undesirable i.e. when carrying egg sacs which could be dislodged by attaching males. It is possible that as males are usually attracted to females at specific times in their life-cycle (i.e. before the moult to adult stage in *L. salmonis*) that surface-bound pheromones only form part of the cuticle at that crucial life-stage.

Gharagozlou-van-Ginneken (1979) discovered, using transmission electron microscopy, that glycoprotein-secreting glands in the genital sternite of the harpacticoid copepod *Tigriopus brevicornis* (Sars, 1904) appeared to show increased activity during mating. From this evidence it was proposed that those glands were involved in secreting a substance which acted as a sex pheromone. It is difficult to imagine that all the tegumental glands are involved solely in producing a sex pheromone despite the vital importance of this to the continuation of the species. It is much more likely that only some of these glands are devoted to this function or that there is a functional dichotomy in the gland product i.e. what acts as a

pheromone at certain stages may have an altogether different function when mating is not an objective. Also, if the tegumental glands were solely responsible for pheromone production we might expect to see a sexual dimorphism between males and females as regards gland distribution. It has been shown in some copepod species that there is a very slight dimorphism in tegumental gland distribution but adult males still possess numbers of glands similar to that present in females.

1.1.4.2 Mucous glands

The crustacean gland structures which have received the most attention from investigators are those glands located in or near the buccal cavity – commonly referred to as 'labral glands' due to their frequent occurrence in the labrum, although they are also often found in the adjacent areas of the head. Evidence has frequently been provided to suggest that these glands are responsible for secreting mucous compounds (Hicks and Grahame 1979, Chandler and Fleeger 1984, Von-Vaupel-Klein and Koomen 1994, Williams-Howze 1996). They were first described by Richards (1891) in calanoid and freshwater cyclopoid copepods, since then their occurrence has been noted in many copepod species (Fahrenbach 1962, Park 1966, Arnaud *et al.* 1988a,b).

Similarly located glands have been described for a number of other crustacean orders. The exact structure, organisation and position of these glands varies between each of the species so far studied and there persists a great deal of confusion regarding the precise role of these glands although they have been shown to be responsible for the secretion of mucus e.g. *Euchirella* (Aetideidae) (Cahoon 1982, Von Vaupel Klein and Koomen 1994), *Centropages* (Centropagidae) (Arnaud *et al.* 1988a,b), *Talorchestia* (Talitridae) (Shyamasundari, 1979) and *Porcellidium* (Porcellidiidae) (Gharagolou-Van-Ginneken, 1977). However, arguments still remain regarding their functional significance, with some authors claiming a digestive role (Brunet *et al.*, 1991), others a food lubricating (Alexander, 1989) and others still a prey entangling role (Von Vaupel Klein and Koomen, 1994). It has even been suggested (Zaffagnini and Zeni, 1987) that the labral glands of *Daphnia obtusa* Kurz may be involved in the control of metabolism and ovary maturation. It is clear from these studies that mucus secretions have a crucial, although not fully

understood, role to play in the feeding mechanism of copepods. These glands, because of their position and structural similarity to the salivary glands of insects have frequently been referred to as 'salivary' glands in the crustacea (Gharagozlou-Van-Ginneken 1977, Arnaud *et al.* 1988b). The word salivary implies a digestive or enzymatic function and, as has been pointed out by Zeni and Zaffagnini (1992), these glands have not yet been proven to have such a function in the Crustacea.

Secreted mucus is also used in the production of tubes and capsules by various members of the copepod Order Harpacticoida. *Stenhelia palustris* Brady, 1862 and *Pseudostenhelia wellsi* (Coull and Fleeger) use mucus to construct dwelling-tubes in marine coastal muddy sediments, where they often constitute a significant proportion of the meiofauna (Chandler and Fleeger 1984, Nehring 1993). Under laboratory conditions both species were seen to rapidly construct mucus tubes, the mucus forming a conglomerate with silt-clay particles of the substratum. These tubes markedly altered the character of the upper layer of the sediment and it has been suggested that dense matrices of tubes may have a stabilising effect on the easily disturbed silt and clay particles of these habitats.

Whilst researching another species of harpacticoid (*Diarthrodes nobilis* (Baird)) Hicks and Grahame (1979) found that it, too, was capable of secreting copious quantities of mucus. *D. nobilis* exhibits an alga-associating behaviour involving the construction, by the adult, of a mucus capsule on the surface of the fronds of macroalgae species. The copepods use these capsules as a refuge from currents and predators and also as a place to rear their larvae. This phenomenon appears to be common amongst harpacticoids having also been described in *Diarthrodes cystoecus* Fahrenbach by Fahrenbach (1962).

Briggs (1978) revealed that the thin 'surface fuzz' present on the external surface of the integument of *P. anemoniae* was composed of acid mucopolysaccharide. He proposed that numerous unicellular sub-cuticular (tegumental) glands secreted this mucus. As this copepod is known to be an associate of the snakelocks anemone *Anemonia sulcata* (Pennant) it was suggested that the mucus conferred an immunity to the host nematocysts, allowing the copepod to live freely amongst its tentacles. A similar phenomenon has been reported in the damsel fish *Amphiprion percula* (Davenport and Norris, 1958) where mucus secreted by the fish acted to raise the discharge threshold of the host nematocysts,

enabling the fish to live safely amongst them. The structure of these glands of *P. anemoniae* was fully described and matches very closely the tricellular tegumental glands described by Talbot and Demers (1993).

1.1.4.3 Urosomal glands

Glands in the region of the anus in the urosome and caudal rami have been described for several species of copepod by Fahrenbach (1962), (Park, 1966), Boxshall (1982) and Bron (1993) and are present in most, if not all, species of copepod. Similar glands have been shown to be present in the crustacean Class Cephalocarida by Elofsson and Hessler (1998) which may suggest that such urosomal glands may not be a feature solely of the copepod Class. These urosomal glands are frequently very complex systems that stain for a variety of compounds and are drained via pores on the uropods and anal somite.

Fahrenbachs (1962) work on the harpacticoid copepod *D. cystoecus* Fahrenbach revealed that there were extensive glandular complexes present in the urosomal segments of that species. These complexes, lying on either side of the gut, consisted of three different cell types which stain for mucopolysaccharides and proteins, with ducts leading to pores in the caudal rami. That author provided no explanation as to their function. Boxshall (1982) also described a complex of three separate glands lying on either side of the gut in the anal somite and caudal rami of the misophrioid copepod *B. palliata*, but again no explanation was given as to their function.

Chapman (1981) showed that *Neocalanus plumchrus* (Marukawa) could actively remove dissolved radio-labelled glucose from seawater and incorporate it into glands in the midgut and urosome. The radio-labelled glucose was shown to have been incorporated into carbohydrates within those glands. Unfortunately the ultrastructure of these glands was not investigated nor was any information given concerning the precise location of these glands, but from the illustrations in the paper they appear to correspond to the urosomal glands described in other species. As *N. plumchrus* does not feed for the last seven months of its life it was proposed that the glucose had been taken up via the integument although it is possible that it was ingested orally.

1.1.4.4 Luminescent glands

Bioluminescence is a commonly occurring phenomenon amongst copepod species and especially so within the Calanoida (Herring, 1988). Various hypotheses have been put forward to explain the functional significance of luminescence, with the most plausible being that it serves as a means of predator avoidance. The light flash may serve to confuse or deter the predator and may also function as a means of disguising the animal thereby making capture more difficult (Ruppert and Barnes, 1994). It is generally believed that luminescence occurs through the simultaneous discharge of two secreted components (luciferin and luciferase) into the surrounding water (Barnes and Case, 1972). However, it has been shown (Clarke, Conover, David and Nicol, 1962) that luminescence can also occur without discharge, as in the copepod *Metridia pacifica* Brodsky where the luminescence was confined within the glands, as has also been shown for the sole luminescent poecilostomatoid copepod *Oncaea conifera* Giesbrecht (Herring 1988, Herring, Latz, Bannister and Widder, 1993).

1.1.4.5 Excretory glands

The antennary and maxillary glands are excretory organs, responsible for disposing of the products of metabolism, are common amongst crustaceans and have been studied in some detail (Fahrenbach 1962, Icely and Nott 1979, Mercade 1982, Ruppert and Barnes 1994). Amongst the Copepoda it is generally considered that a pair of these structures are present in the larval stages, where they are termed 'antennary glands', and correspond to the adult excretory organ or maxillary gland. The fundamental structure of the gland is generally similar in both larval and adult stages – the difference arising in the position of the exit pore; either on the antennae (in larvae) or near the bases of the maxillae in adults.

The fine structure and ontogeny of the antennal gland of larval *Mytilicola intestinalis* was described in an excellent paper by Mercade (1982). This paper provided the first comprehensive account of a larval copepod antennary gland. The same author also found that the three regions of the gland appear to undergo regression from the copepodid stage onwards. Parry (1960) claimed that in some

species of crustacean this functional ontogeny might be reversed with the maxillary gland being present in the larval stages but being replaced with antennary glands in the adult. Often the vestiges of the larval excretory organ can be discerned in the adult.

It is generally believed that in most crustaceans the antennary / maxillary gland complexes do not function as ammonia excreting units, this function being undertaken by the gills (Ruppert and Barnes, 1994). Instead the antennary / maxillary glands are believed to be responsible for the regulation of other metabolites and ions and maintaining internal fluid volume. It is possible that in the Copepoda, where gaseous exchange occurs across the integument, that such glands are responsible for metabolite excretion.

1.2 STUDY OBJECTIVES

The present research has been undertaken with the intention of contributing to the information that currently exists with respect to the exocrine gland system of *Lepeophtheirus salmonis*. Expressly, it is intended to identify, describe and characterise some, or all, of the glands that gave a positive reaction to the diaminobenzidine stain first described by Bron (1993) with the ultimate aim being to identify the role these glands play in the biology of this species, and by implication, in other species with similarly staining glands.

The study can be divided into five clearly distinguishable aspects:

I) Gland distribution. Detailed description of the distribution of stained glands in all life stages of *L. salmonis* and a comparison with other caligid species, commensal and free-living copepods as well as other, non-copepod, aquatic crustaceans.

II) Ultrastructure and histochemistry. The histochemical properties of the identified glands of *L. salmonis* are investigated and the ultrastructural characteristics of some of those glands are described.

III) Secretion characterisation. Various techniques are employed to assist in the identification of the enzymatic component of the gland tissues.

IV) Electrophoretic analysis of glandular proteins. Acrylamide gel electrophoresis was utilised in an attempt to isolate, and determine the mass and quaternary structure of the enzymatic component.

V) Determination of functional attributes. Some of the commonly proposed functions of copepod gland secretion are explored in *L. salmonis*

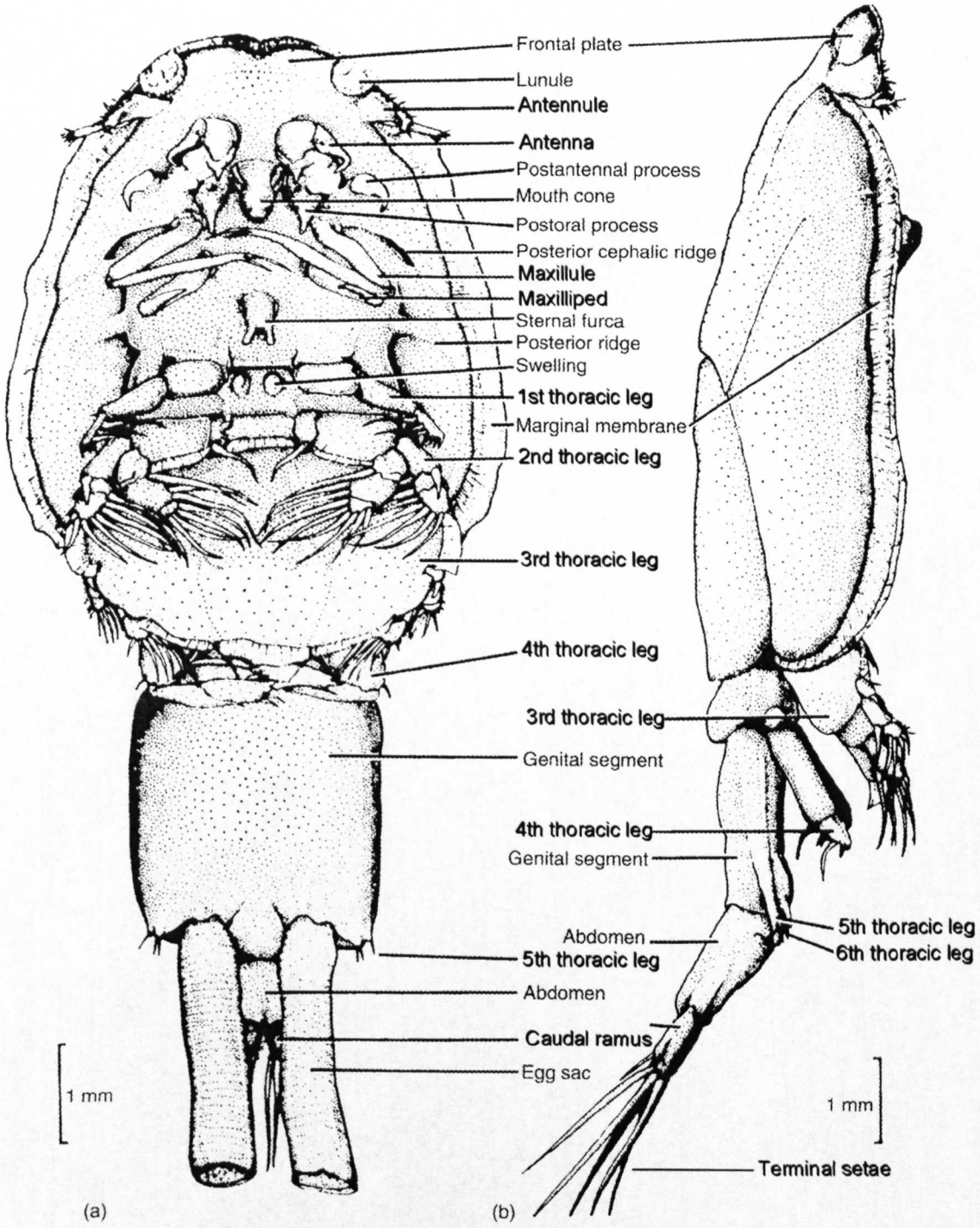


Figure 1.1 Diagram of the ventral surface (A) and lateral profile (B) of a caligid copepod. This image modified after Parker, Kabata, Margolis and Dean (1968).

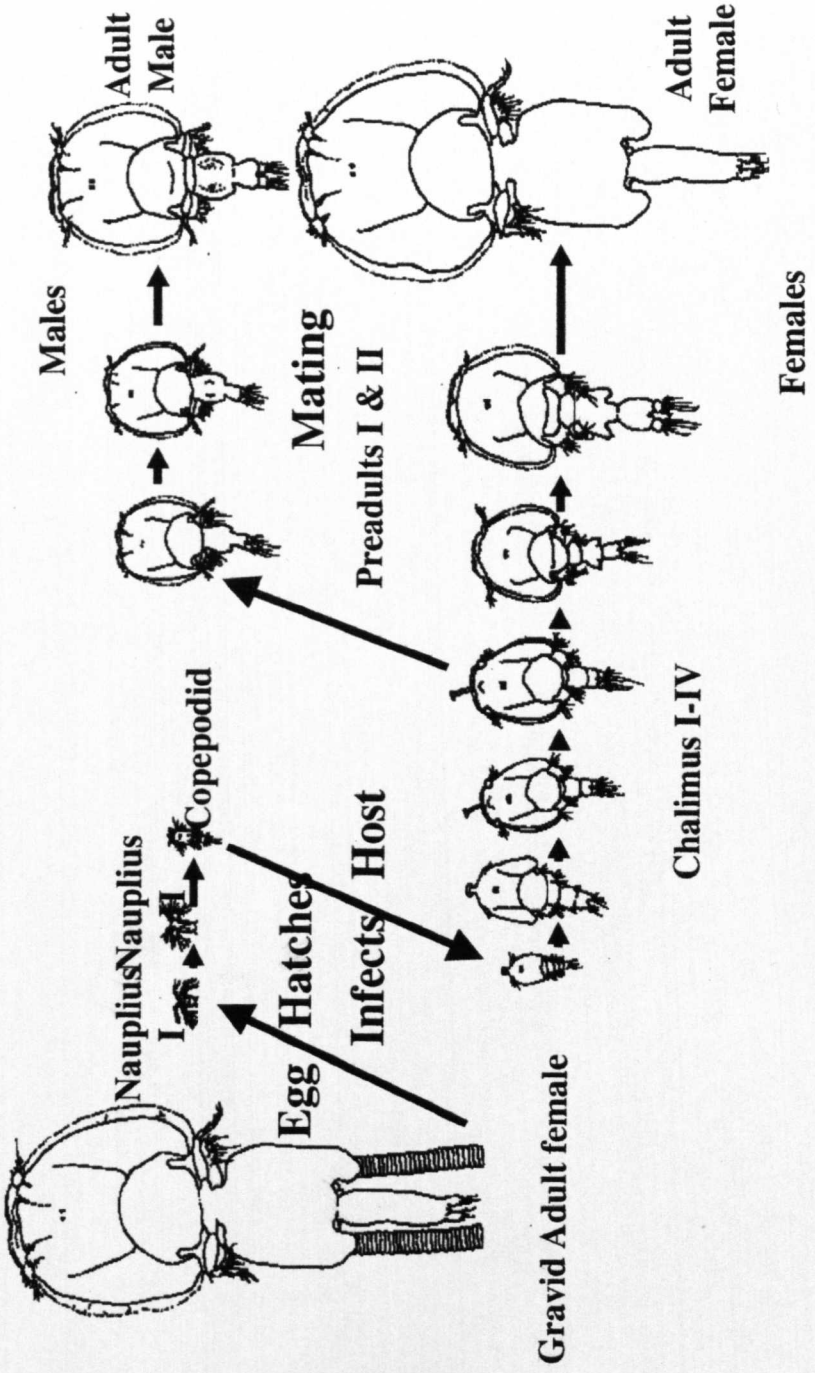


Figure 1.2 Diagrammatic representation of the life-cycle of *Lepeophtheirus salmonis* (Krøyer, 1837).

2. MATERIALS AND METHODS

2. MATERIALS AND METHODS

2.1 GENERAL ASPECTS

2.1.1 Louse collection and maintenance

Sea lice (*L. salmonis* and *C. elongatus*) were obtained from marine salmon farm sites on the West Coast of Scotland. Preadult and adult stages were carefully removed from anaesthetised or culled fish using fine forceps. Specimens were transferred to the laboratory in bags of fresh seawater (salinity approx. 35‰) in insulated cool boxes. Once in the laboratory the specimens could be maintained alive in small plastic aquaria for periods of several weeks if they were kept in clean, aerated seawater at 10°C. Every 24h dead and senescent lice were siphoned from the bottom of the aquaria before 75% of the water was replaced.

Larval stages were obtained by maintaining gravid adult females in separate containers until their egg strings hatched. Naupliar (both NI and NII) stages showed high survival rates *in vitro*. The first naupliar stage hatched immediately from dark egg strings and generally moulted to the second stage within 18h at 10°C. Larvae were sampled by pouring the culture water through a 50μm sieve then removing the larvae using a glass pipette.

Copepodid stage larvae were obtained by maintaining nauplii, obtained using the above procedure, in clean, aerated seawater at 10°C until they moulted to the next life stage (~36h post NII).

Chalimus stage larvae were obtained from cultured salmon at farm sites by removing them carefully by their attachment filament with fine forceps during the harvesting process.

2.2 MICROSCOPICAL TECHNIQUES

2.2.1 Light microscopy

Small specimens i.e. nauplius, copepodids and chalimus I and II were examined intact by placing them in a cavity slide and covering them with a cover slip. Lactic acid was used to clear the tissues to enable better observation of internal structures (Von Vaupel Klein and Koomen, 1994). Observations

were made using an Olympus BH-2 compound microscope. Photographs were taken using an Olympus C-35AD-2 camera mounted upon the microscope. Drawings were made using a drawing tube attached to the same microscope. Larger chalimus stages and mobile stages were best examined using an Olympus SZ40 dissecting microscope with the same photographic system.

2.2.1.1 Wax histology

Paraffin wax embedding was utilised for routine histology of animal tissues. Paraffin wax (Tissue Tek N°2, Bayer) permitted adequate tissue morphology retention for general use and histochemical analyses and was both straightforward and rapid.

Specimens for light microscopy wax (LMW) histology were fixed in 10% neutral buffered formalin (NBF) for 24h before processing in a Shandon Citadel automatic tissue processor. Sections of 3-4μm were cut on a Reichert-Jung Biocut microtome before being stretched on a water-bath at 45°C and floated onto slides. Slides were dried overnight in an oven at 60°C prior to staining.

2.2.1.2 Plastic / resin embedding for light microscopy

Plastics and resins were preferable to paraffin wax in terms of their ability to preserve tissue morphology and permit higher resolution of observation as thinner sections can be cut. These compounds involved a longer processing time over wax and sectioning was rather more difficult and therefore they were not used routinely but only when very good quality morphology was desired. Two different resin compounds were used: Historesin Plus and L.R (London Resin) White. Historesin Plus is a glycol methacrylate-based resin intended for light microscopy, which is suitable for immunohistochemistry as it retains sites of antigenicity normally lost with other embedding media. L.R White is a medium grade acrylic resin suitable for both light and electron microscopy.

Specimens were processed for light microscopy plastic (LMP) histology by fixing for 24h in 2.5% glutaraldehyde before being dehydrated through a graded ethanol series. Specimens were infiltrated in a 50:50 ethanol:Historesin Plus (Leica Cambridge) solution overnight on a rotator then 100% resin for 24h

before embedding in pure Historesin Plus embedding medium. Polymerisation was carried out anaerobically at 4°C to overcome the problems associated with the exothermic reaction caused by the addition of the hardening medium. Samples were mounted on wooden blocks using Araldite Rapide adhesive. Sections of 2-3μm were cut with glass knives on a Reichert-Jung Microtome 2050 Supercut at 6-20mm s^{-1} with a blade angle of 7°. Ribboning of sections could be achieved by lightly brushing the top and bottom of the block with a 50:50 mixture of infiltration medium and methanol. Sections were stretched on a water-bath at 50°C and floated onto poly-L-lysine (Sigma P8920) coated (1% in distilled water) slides.

Specimens for L.R White embedding were processed by fixing in Karnovsky's fixative (Karnovsky, 1965) at 4°C for 2-4h. This was followed by an overnight rinse in 0.1M sodium cacodylate buffer at 4°C then post-fixing in 1% osmium tetroxide in 1% borax for 1h. Specimens were dehydrated through an ethanol series; the second 100% ethanol wash containing 1% phosphotungstic acid. Specimens were infiltrated overnight on a rotator in a 50:50 100% ethanol : L.R White mix, followed by an 8h infiltration in 100% L.R White. This was followed by a further overnight infiltration on a rotator in 100% L.R White. Specimens were then embedded in Beem capsules sealed to preserve an anoxic environment. Capsules were polymerised at 60°C for 24h. Sections were cut using glass knives on either a Reichert-Jung Microtome 2050 Supercut for light microscopy or on a Reichert Ultracut E ultramicrotome for electron microscopy.

Slide preparations were examined using an Olympus BH-2 compound microscope and photographs taken using an attached Olympus C-35AD-2 camera. Whole mounts were examined using an Olympus SZ40 dissecting microscope, again with an attached Olympus C-35AD-2 camera.

2.2.1.3 Cryostat sections

Cryostat sections of 10μm were cut from fresh specimens embedded in Cryo-M-Bed embedding medium (Bright Instrument Co. Ltd., Huntingdon, England) using a Bright Model OTF (Bright Instrument Co. Ltd., Huntingdon, England) cryotome. Sections were cut at a temperature of -20°C and

placed onto poly-L-lysine (Sigma P8920) coated (1% in distilled water) glass slides. Sections were stored at -20°C until required for use.

2.2.2 Transmission electron microscopy (TEM)

Specimens for transmission electron microscopy were fixed following the method of Eisenman and Alfert (1982). Dissected regions of specimens were prefixed in 5ml of Karnovsky's fixative (Karnovsky, 1965) to which had been added 0.25ml of osmium tetroxide (1% in 1% borax solution). After 10min in this prefix solution, the samples were transferred to Karnovsky's fixative for 1h, then washed twice in cacodylate buffer (0.1M, pH7.2), before dehydration through a graded acetone series. After transferral to 100% acetone the samples were transferred to a 1:1 mix of Spurr resin (Spurr, 1963) and acetone for 1h on a rotator and then a 3:1 mix for a further 2h. Finally, the specimens were rotated for 24h in 100% Spurr resin before being embedded in Beem capsules and polymerised at 60°C for 48h. Gold sections were cut using glass knives on a Reichert Ultracut E ultramicrotome and were mounted on 200 mesh Formvar coated copper grids. Sections were stained with uranyl acetate and lead citrate according to the methods Bozzola and Russell (1992) and Reynolds (1963) respectively. Some specimens were stained *en bloc* with 2% uranyl acetate in 30% acetone for 2h in the dark immediately after the osmication step, following the protocol of Karnovsky (1967) and then stained with uranyl acetate and lead citrate post-sectioning according to the protocols given above. This technique provided a more superior resolution under the electron beam than post-sectioning staining alone. Sections were examined with a Philips 301 TEM operating at 80kV. Photographs were taken with a flat plate camera using black and white Kodak 4489 EM film.

2.2.3 Scanning electron microscopy (SEM)

Specimens to be examined with the scanning electron microscope were fixed at 4°C for 1h in a modified version of marine invertebrate egg fixative (MIF) of Eisenman and Alfert (1982). The fixative solution was composed of 1% glutaraldehyde and 0.1M sodium cacodylate. This initial fixation was

followed by 2-3 day immersion in 3% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C followed by rinsing in 0.1M sodium cacodylate buffer.

Specimens fixed in this manner were post-fixed in 1% osmium tetroxide (in 1% borax solution) for 2h before being dehydrated through an ethanol series. Once in 100% ethanol the specimens were transferred to a 50:50 mix of 100% ethanol and hexamethyldisilazane (HMDS) for 30min before transferral to 100% HMDS for a further 30min. Specimens were air-dried at room temperature in the fume cupboard before being mounted on aluminium stubs and coated with gold at 40mA for 90s using an Edwards S150B Sputter Coater.

Fine glass needles for use in microdissection of SEM specimens were made from Narishige GD-1 (1mm × 90mm) glass tubes using a PSU2-C pipette-puller (Research Instruments Ltd. Cornwall, England).

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

2.3 PHOTOGRAPHIC PROCESSING

Black and white photographs from light microscopes were processed by the author using darkroom facilities in the Institute of Aquaculture. SEM, TEM and other black and white photographs were processed and exposed in the first instance by the Department of Biological and Molecular Sciences, University of Stirling. Enlargements and further copies of such photographs were undertaken by the author. Colour photographs were processed by commercial companies.

3. GLAND DISTRIBUTION

3. GLAND DISTRIBUTION

3.1 INTRODUCTION

Studies concentrating solely on the distribution of glands and their associated pores, such as those by Brunet *et al.* (1991) and Bannister (1993a), are rare although both Fahrenbach (1962) and Park (1966) provided detailed information on the gland populations of individual species incidental to their descriptions of the biology of *Diarthrodes cystoecus* and *Epilabidocera amphitrites* McMurrish. Many studies have focused on the individual populations of particular types of exocrine glands of copepods i.e. luminescent glands (Clarke *et al.* 1962, Barnes and Case 1972, Herring 1988, Bannister and Herring 1989), mucous glands (Briggs 1978, Hicks and Grahame 1979, Chandler and Fleeger 1984, Von Vaupel Klein and Koomen 1994), labral glands (Arnaud *et al.* 1988a,b). These studies are significant because they represent the first detailed descriptions and analyses of the roles of some of the most commonly occurring copepod exocrine gland systems.

Integumental pore patterns, known as pore signature patterns or PSP, however, have been described for many species of calanoid copepod and these results have shown that these patterns may be used as a taxonomic guide for the identification of individual species (Fleminger 1973, Mauchline 1977, Mauchline and Nemoto 1977, Mauchline 1987). Fleminger found that no more than 60% of the sites present in a genus are ever occupied in any one species but about 80% of sites in each species was observed in all specimens. The later studies of Hulsemann and Fleminger (1990) and Park and Mauchline (1994) showed that the PSP particular to individual species could be distinguished sufficiently in the genital segment or urosome alone, thereby considerably increasing the rapidity of analysis. In these studies there is some disagreement as to whether the full species-specific PSP is present in copepodid stages or whether this only occurs in adults. Mauchline and Nemoto (1977) provided evidence that suggests that copepodids of calanoid species have PSPs with components identifiable in the adults but that the PSP only reaches full development in the adult stages. PSPs have been implicated as having a role in mating behaviour as sexual differences in the pore pattern are frequently apparent (Mauchline and

Nemoto, 1977) and also because differences in PSP between species are most apparent where phylogenetically close genera occur in the same geographic location. Boxshall (1982) claims that PSP's can be utilised in the taxonomic discrimination of poecilostomatiod copepods, providing the only information relating to PSP's that considers copepods outwith the Calanoida.

It appears that, in these studies of integumental pore pattern, the term pore encompasses not only 'true' pores associated with underlying glandular structures, but also the holes left in the integument where the cuticular sensilla had been dissolved by alkali during processing. The pore patterns described for those species therefore give no indication of the extent of the full population of exocrine glands present in each species. Mauchline (1977) suggested that the size of the cuticular pores of copepods could be used to infer the function of the underlying structures but he provides no evidence to support this hypothesis.

Exocrine glands within the Copepoda can be roughly divided into two categories: the tegumental glands which lie close to the body cuticle, and those others not located intimately with the cuticle. The tegumental glands are the most interesting of these groups because they are the least well understood and because of the numerous functions that have been attributed to them. The title of tegumental gland is generally applied to the small, uni-/ bi-/ tricellular glands (for details of classification see Noirot and Quennedey, 1974) located closely beneath the cuticle and which are believed to be derived from epidermal cells. Tegumental glands have been widely reported in the Copepoda, although they are widespread throughout the Crustacea, and appear to be present in all species studied, although they have been variously termed cutaneous glands (Park, 1966), hypodermal (Briggs, 1978), dermal (Hipeau-Jacquotte, 1987) and sub-cuticular glands (Bannister 1993a). The functions of the tegumental glands remain unknown, although theories abound as to the roles they may fulfil in crustaceans. The physical location of the gland, and its closely associated integumental pore has often been used as a basis to infer different roles for the secretions of these glands. However, few behavioural studies have been undertaken to support the theories given, and although in many cases the hypotheses of gland function are convincing, they must remain, without further evidence, mere speculation.

Despite the numerous studies undertaken into the biology of the sea lice species *Lepeophtheirus salmonis* and *Caligus elongatus* very little mention of glandular systems has been made with the exception of Bron *et al.* (1991) and Bron (1993). Studies of the glandular systems and secretions of parasitic crustaceans have never been undertaken but detailed studies of many free-living species have been carried out over the last two centuries (Richards 1891, Fahrenbach 1962, Gharagozlou-van-Ginneken 1979, Boxshall 1982, Brunet *et al.* 1991, Bannister 1993a and b).

Bron (1993) showed that copepodid larvae of *L. salmonis* possessed glands which gave a positive reaction with the chromogenic substrate 3',3-diaminobenzidine tetrahydrochloride (DAB) when the animals were stained as whole, fresh specimens. A positive reaction to this DAB stain is generally accepted to indicate the presence of endogenous peroxidase enzymes such as glutathione peroxidase, catalase and cytochrome oxidase (Angermüller and Fahimi, 1981) although other compounds such as sulphated mucosubstances (Bussolati, 1971), are also known to stain positively with this compound.

No information suggesting peroxidases to be present as gland products in copepods can be found in the literature, although the widespread presence of peroxidase enzymes in animal tissues has been well established (Halliwell and Gutteridge, 1985). Peroxidase enzymes have been shown to be involved in many different functions within animal tissues. Such functions include free radical neutralisation (Felton and Duffey 1991, Bell and Smith 1994, Gamble, Goldfarb, Porte, Livingstone, Forlin and Andersson 1994), prostaglandin production (Morita, Schindler, Regier, Otto, Hori, DeWitt and Smith 1995, Rowley, Knight, Lloyd-Evans, Holland and Vickers 1995) prevention of lipid peroxidation (Halliwell and Gutteridge 1985, DeRobertis and DeRobertis 1987) and protothyroid activity in tunicates (Fredriksson, Öfverholm and Ericson, 1988).

3.2 OBJECTIVES

The objective of this work was to determine the extent of the development of exocrine glands that stained positively with DAB in the sea louse species *L. salmonis*, *C. elongatus*, *L. pectoralis* (Müller, 1777) and *L. hippoglossi* (Krøyer, 1837). Patterns of gland populations specific to life stages and

ontogenetic patterns were recorded. Comparative studies with commensal and free-living copepod species were undertaken and the possible evolutionary significance of such glands to parasitic species were discussed.

Scanning electron microscopy was utilised to determine whether number of glands identified using DAB corresponded with the number of integumental pores. The aim was to determine whether stained regions did actually correspond to the positions exocrine glands and also to assess the suitability of these stains to function as taxonomic devices to facilitate the distinguishing of caligid copepod species based on their integumental pore pattern.

3.3 METHODS

3.3.1 DAB (3,3'-Diaminobenzidine) stain

The evidence from Bron (1993) suggested that DAB was successful at staining the exocrine glands of *L. salmonis* but as many different formulations of this compound were available, an analysis of the suitability of two of the most commonly available forms was undertaken. The first method utilised tablets of DAB that had the substrate H_2O_2 already incorporated into them (Sigma D4293). These were dissolved in 5ml distilled water to give an immediate working solution of DAB to which specimens were added for varying time periods. The second method involved the use of 3,3'-diaminobenzidine tetrahydrochloride 10mg tablets (Sigma D5905) prepared as follows. One 10mg DAB tablet was added to 15ml of Tris-HCl buffer (pH 7.2) to give a 0.66% w/v solution to which 12µl of 30% H_2O_2 was added (0.026%) to provide a substrate for the reaction. This formed the staining solution utilised in all further steps.

Preliminary trials using *L. salmonis* as an experimental animal indicated that the DAB tablets incorporating H_2O_2 were less suited to the staining of exocrine glands. This lack of suitability manifested itself as a greater propensity to disrupt body tissues and as a less distinct staining of the appropriate tissues. In all staining trials described in this work the second method of DAB preparation, incorporating the addition of fresh H_2O_2, was followed.

3.3.2 DAB-staining technique applied to *L. salmonis* and *C. elongatus*

A range of staining times was tested for each different developmental stage of *L. salmonis*.

3.3.2.1 Nauplius

Whole nauplius larvae were added to the freshly prepared DAB solution and left to stain for 5, 10 and 15 minutes at room temperature. After these periods had elapsed the reaction was halted by carefully pipetting off the stain solution and replacing it with a 70% ethanol solution. In an attempt to minimise disruption of tissues due to osmolarity differences, sucrose was added, at a final concentration of 0.35M,

to the staining solution following the method of Eisenman and Alfert (1982) for one of each set of staining trials. For observational purposes lactic acid (87.5%) was used to aid in the clearing of the body tissues of all larval stages to enhance visualisation of gland structures without affecting the quality of staining.

3.3.2.2 Copepodid

Copepodid larvae were stained for times ranging between 1min and 6h with the optimal being found to be between 5min and 10min. No attempt to alter the osmolarity of the solution was made as preliminary studies had shown that copepodids were resistant to the distortion of body shape observed for the nauplii.

3.3.2.3 Chalimus

Chalimus larvae were stained in a similar manner except that, on account of their greater size, they had to be stained for 6h to allow optimal visualisation of positive tissues i.e. glands highlighted but body tissues not overstained. The optimal period of 6h for staining was determined through the use of an experimental time series (ranging from 1min to 10h) and subsequent observations.

3.3.2.4 Preadults and adults

Preadult and adult stages were stained within 24h of removal from the host to minimise any effects on gland activity that being removed from the salmon host might have incurred. The optimal staining period for these stages was found to vary greatly but was generally between 4-10h. The basis for this variability was not apparent. As a result of this variability, over-staining could occur if samples were not monitored whilst in the staining solution.

3.3.3 TMB (tetramethylbenzidine) stain

TMB is a compound with similar staining affinities to DAB but it is considered to be less hazardous to the user than DAB. It is simple to use and has an alleged 50-fold greater sensitivity than DAB, although it had the drawback of not forming a stable reaction product. Preliminary studies using

adult *L. salmonis* indicated that the blue TMB stain diffused very rapidly into surrounding tissues such that observations had to be made immediately. The vivid blue colour permitted easier observation of positively staining tissues than DAB. TMB is alleged to react with 'any and all peroxidases and will not differentiate between different sources of the enzyme' (Dr. Ann Brighty, Vector Laboratories, U.K. pers. comm).

The staining solution was prepared as follows. The different solutions in a Peroxidase Substrate Kit (SK 4400, Vector Laboratories, England) were added in the proportions stipulated in the manufacturers instructions to 5ml of distilled water. This solution was prepared immediately prior to use in order to maximise the efficacy of staining.

A series of staining trials with *L. salmonis* and *C. elongatus* identical to that used for DAB was carried out using TMB to test whether the greater sensitivity would reveal details not distinguished by the DAB technique alone. Staining times were markedly shorter using TMB: adult *L. salmonis* could be stained optimally in approx. 20min, whilst all other stages were successfully stained after shorter periods than those used for the DAB stain. The nature of the staining achieved with both stains was identical and therefore the results of both the DAB and TMB staining trials will be considered together.

3.3.4 Staining of other species

Adult stages of three other species of parasitic copepod were stained in the same manner and for the same time periods as optimised for *L. salmonis* and described in Sections 3.2.2 and 3.2.3. These were *Lepeophtheirus* hippoglossi, *Lepeophtheirus pectoralis* and *Clavella adunca* (Strøm, 1762). The staining times were of the same order as described for adult *L. salmonis* in that section.

The calanoid copepods *Eurytemora affinis* (Poppe, 1880), *Acartia tonsa* Dana, 1849 were also stained with the DAB stain prepared as outlined in Section 3.2.2. *E. affinis* were obtained as a pure culture from Otter Ferry Salmon Farm (Loch Fyne, Scotland) whilst *A. tonsa* were obtained as pure culture from Guernsey Sea Farms Ltd. The staining method utilised was as described for *L. salmonis*

(Section 3.2.2), with some minor adjustments of the staining period for each species to allow optimal staining to occur.

Several species of algal-associated copepods (Order Harpacticoida) were obtained from a variety of seaweed species at West coast marine salmon farm sites in Scotland. These were identified, with the assistance of Dr. R. Huys of The Natural History Museum, London, *Parathalestris hibernica* (Brady and Robertson, 1873) (family Thalestridae), *Harpacticus chelifer* (O.F. Müller, 1776) (family Harpactidae), *Dactylopusia vulgaris* Sars, 1905 and *Diosaccus tenuicornis* (Claus, 1863) (family Diosaccidae). These small copepods were stained successfully after relatively short periods, approx. 15-20min.

Specimens of the branchiuran fish parasite *Argulus foliaceus* (L.) were stained with DAB and the position of positively staining regions recorded. The same staining protocol was used to stain larvae of the malacostracan *Macrobrachium rosenbergii* (de Man), larval stages of the branchiopod *Artemia salina* (L.) and adults of the branchiopod *Daphnia magna* Straus, 1820. Staining times varied markedly between species and were determined by similar time trials to those conducted for *L. salmonis*.

3.3.5 SEM determination of integumental pore pattern

A SEM examination was undertaken to establish the pore pattern of the cephalothorax of adult male and female *L. salmonis* to ascertain whether the pore pattern could be correlated with the sites of glands located using the DAB / TMB staining techniques. SEM techniques were also employed to define the pore signature patterns (PSP) of the urosomes of adult male and female *C. elongatus*, *L. hippoglossi* and *L. pectoralis* to ascertain whether the PSP matched the pattern of stained glands in this body segment. The methodology for SEM processing outlined in Chapter 2 was followed.

3.4 RESULTS

3.4.1 DAB / TMB-staining of *L. salmonis* nauplius larvae

It was found that the DAB / TMB stains were successful in highlighting gland structures within the nauplii when stained over all three time periods, 5, 10 and 15min. Observations showed that larvae stained with added sucrose showed less severe tissue disruption than those which were stained without it, but the body tissues were generally much darker and the stained areas were less distinct than in those specimens stained without sucrose. It was possible that the tissue disruption seen in the non-sucrose nauplii allowed greater staining to occur by permitting entry of the stain solution to the body cavity. To overcome this disruption, maps of the glands had to be constructed from several different specimens to provide composite images. After clearing in lactic acid, the gland structures of the nauplii were clearly discernible from both the ventral and dorsal surfaces.

The regions of positive staining seen in nauplius stages, shown in **Figure 3.1**, can be identified as glandular structures on account of two features: 1. they have ducts associated with them and 2. those ducts can be followed to exit via a pore in the cuticle surface, often with stained secreted material adhering to the cuticle close to the pore. These two criteria were used to distinguish gland tissues from other positively staining tissues at this stage of the study.

Both first and second stage nauplii had the same general pattern of peroxidase-positive staining regions although some slight differences were evident as noted below. The gland distribution had a bilaterally symmetrical pattern and was as follows:

3.4.1.1 Posterior glands

A pair of large glandular complexes was evident in all specimens at the most posterior region of the body cavity lying close to the bases of the paired posterior balancers (**Figure 3.1**). Under high magnification it was possible to discern ducts extending anteriorly from these glands into the body cavity.

These ducts could be detected as far as 200μm from the gland into the body, a distance equivalent to 35% of the total body length. The point at which these ducts arise or discharge has yet to be located.

A further duct was evident from the posterior margin of each gland extending the short distance to the posterior cuticle of the animal where it was seen to discharge close to the bases of the balancers. This discharge point was visible as a small cluster of stained secreted granules adhering to the external surface of the body cuticle.

3.4.1.2 Lateral glands

Evident in all specimens examined were a pair of gland structures lying in close proximity to the body cuticle on the lateral margins of the animal, approximately half way down the body length from the anterior edge (**Figure 3.1**). From each of these glands a single duct was seen to extend anteriorly in a medioventral direction although the origin of this duct could never be discerned.

In some late NII larvae it was possible to see a small stained duct arising from the posterior margin of these glands and passing the short distance to the cuticle of the lateral margin. In some specimens, clusters of DAB-positive grains were visible adhering to the outside of the cuticle adjacent to these glands in close proximity to the exit point of these ducts.

3.4.1.3 Median glands

In the centre of both naupliar stages, at the anterior end, lay two large spherical glands that are termed here the 'median glands' (**Figures 3.1, 3.2**). These glands, although apparently lying adjacent to one another in a lateral plane actually overlaid one another in a dorsoventral direction. The displacement observed in these glands probably arose as a result of the tissue disruption common at the anterior end. Two ducts arose from the dorsal median gland, one of which lead from the anterior region of the gland to exit via a pore (**Figure 3.2**) in the cuticle at the most anterior tip of the nauplius, whilst another longer duct connected with the posterior region of the gland and extended posteriorly into the body cavity. The exit / destination of this posterior duct was not located.

The ventral median gland lay closer to the ventral surface of the animal and gave rise to a single duct from its posterior region which lead in a posterior direction for a short distance before splitting into two separate ducts. These ducts continued to run parallel to each other in a posterior direction. In all specimens examined these ducts never stained fully enough to allow the observation of its source / destination.

3.4.1.4 Anterior gland complexes (denoted AGC in text and agc in figures)

A pair of gland complexes, each composed of three distinct regions, was evident in all specimens examined. These were located in the lateral margins of the anterior region of the animal (**Figure 3.1**) and were evident when viewed from either the dorsal or ventral surface. The most posterior regions of the glands were located close to the bases of the mandibles. This region, the largest of the three, had darkly staining finger-like extensions (**Figure 3.1**) arising from its posterior margin extending into the body cavity of the animal. From this posterior region a connecting duct led the short distance anteriorly to the second region of the gland complex. This mid-region was smaller than the posterior region and appeared to be connected anteriorly to the third and smallest region of the complex which was seen to discharge via a duct, the exit point of which was never discernible. This exit point may lie on the anterior margin of the body in close proximity to the bases of the antennules, the bases of which were level with the most anterior portion of the gland complex. DAB-positive granules were often seen adhering to the cuticle in this area.

The gland complex had a stepped orientation with the posterior region lying closest to the dorsal surface and the anterior region lying ventral-most with the mid-region lying between the two.

3.4.1.5 Other DAB / TMB-positive areas

Further DAB / TMB-positive regions were frequently visible in late NII larvae (**Figure 3.3**). There were 10 small, discrete glandular areas arranged in a bilaterally symmetrical pattern visible under the cuticle of the ventral surface in the posterior half of the animal. The occurrence and position of these

glands were consistent amongst specimens of the same age and they appeared to correspond to the thoracic leg glands of the following copepodid stage.

All the DAB-positive glands seen in the NI larvae were also evident in unhatched nauplii (**Figure 3.4**).

3.4.2 DAB / TMB-staining of *L. salmonis* copepodid larvae

All of the copepodid larvae examined consistently showed the same pattern of DAB-positive glands (**Figure 3.5**). The pattern of gland distribution was as follows.

3.4.2.1 Posterior (urosomal) glands

A pair of cigar shaped glands were always present in the elongate, unsegmented genital region and abdomen of the copepodids, one lying on either side of the gut in close proximity to the anal aperture as shown in **Figures 3.5 and 3.6**. The glands were of a substantial size and filled nearly the entire segment, each extending posteriorly almost to the caudal rami. A short duct can be seen in **Figure 3.6** exiting from the posterodorsal region of the urosomal gland and leading to the posterior margin of the caudal rami where the duct is presumed to exit onto the cuticle surface. The glands each displayed a long, fine duct extending from the anteroventral region of the gland into the body cavity (**Figure 3.6**) of the animal. These ducts could be seen to extend at least as far as the third thoracic segment, approximately 24% of the larvae's total body length.

The glands were apparent when viewed either from the dorsal or ventral surface and appeared to correspond to the posterior glands of the nauplius as described earlier. As copepodid larvae possess a urosome incorporating the genital segment and the abdomen, in contrast to the naupliar stage, it seems reasonable to rename the posterior glands the 'urosomal glands' at this stage. This follows the terminology of Boxshall (1982).

3.4.2.2 Lateral glands

A large gland was apparent at either lateral margin (apparent in **Figures 3.5, 3.6, 3.7** and **3.8**) of the cephalothorax, each lying slightly posterior to the bases of the maxillae when viewed either from the dorsal or ventral surface. Both these glands had two ducts associated with them, one passing in an anterior direction from the anterior margin of the gland (shown clearly in **Figure 3.7**) and the other passing the short distance from the posterolateral margin of the gland to the surface of the cuticle at the lateral margin of the cephalothorax. The exit pore of this duct was presumed to be located there, as there was frequently an area of stained granular material adhering to the outside of the cuticle at the point where the duct came into contact with the cuticle. These glands seem certain to be homologous to the lateral glands described for the nauplius stages.

3.4.2.3 Median glands

There were two large, spherical DAB-positive glands in the cephalothorax immediately anterior to the eyes (**Figures 3.7** and **3.8**). These glands overlay each other in a dorsoventral aspect. Several ducts arose from these glands but the relationship between the glands and ducts was too complex to be fully realised using this technique. However, some tentative hypotheses concerning their interconnections can be put forward. The dorsal median gland had one duct which passed directly from the centre of the gland to traverse the short distance to the cuticle immediately overlying it where it exited via a pore. This exit pore could frequently be discerned in stained specimens as an area of densely packed DAB-positive granules on the dorsal cuticle. This gland often had a large area of diffuse DAB-positive granules with no apparent structure lying close to its anteroventral margin as seen in **Figure 3.8**. A further duct arose from the dorsal median gland and passed in an anterodorsal direction to extend into the developing filament. This duct stained clearly and stained material could be seen to extend as far as the distal point of the filament duct as it appeared at this stage.

The ventral median gland was a large, clearly demarcated gland located within the body cavity immediately at the base of the labrum. This gland appeared to have several ducts associated with it, one

of which extended ventrally into the labrum. The duct passed close to the anterior cuticle of the labrum to exit via a pore on the anterior margin approximately half way down its length. There appeared to be at least three other ducts associated with this gland. One of these ducts passed directly posteriorly from the gland and could be followed for some distance within the body cavity. Both the duct entering the labrum and the posterior duct are evident in **Figure 3.8**. Smaller ducts appeared to arise from each lateral margin of the gland and passed for a short distance in a lateral direction. The exit / distal points of these ducts were never visualised.

3.4.2.4 Anterior gland complexes (AGC)

In the anterior region of the cephalothorax a pair of glandular complexes lay close to the lateral margins of the body near to the bases of the antennules. Each of these complexes was composed of three distinct, but connected, regions (**Figure 3.5** and **3.7**) and was presumed, on account of their location and similar morphology, to be the equivalent of the AGC of the nauplius stage.

The largest, most posterior region stained heavily in all specimens. Immediately anteroventral to this region was the second region of the gland which did not stain as heavily, and could be seen to be composed of closely packed, stained granules. Immediately anteroventral to this second region, there was a further small, DAB-positive region which appeared only faintly as a collection of loosely packed granules. This region of the gland lay closest to the antennules, and frequently a duct could be seen to arise from this region and pass a short distance anteriorly to its exit pore which was presumed to be located close to the bases of the antennules. DAB-positive granules were often observed adhering to the outside of the cuticle on the posterior surfaces of the antennules. This material was presumed to have originated in the AGC.

A further duct could also be discerned arising from each AGC which appeared to lead posteriorly in a medioventral direction where it could be followed as far as the point of fusion of the first and second thoracic segments, although the distal point of this duct was not determined.

3.4.2.5 Thoracic leg glands

At this life-stage the first, second and third thoracic legs were present. DAB / TMB-positive glands were always present in both the first and second thoracic swimming legs. In total, 10 glands were present in the thoracic legs: 4 in the first thoracic legs (th_I) and 6 in the second thoracic legs (th_{II}). Glands were present in all segments of both pairs of legs (**Figure 3.9**) with the exception of the exopods of the first thoracic legs that did not appear to contain any glands which stained with DAB. The glands present in the basal region of the sympods were the largest of this group of glands, the glands of the exopods and endopods were smaller but of a similar size to each other.

Ducts associated with these glands were frequently visible. Ducts in contact with the glands of the sympods could be followed from the basal region of the gland and from there extending a short way into the cephalothorax. The glands of the endopods and exopods were also located in the basal region of the segments but had ducts leading from them which passed through the length of the segment and which appeared to exit close to the base of the natatory setae at the distal margin of the legs. No interconnections between the glands of the thoracic legs were ever observed.

3.4.2.6 Other DAB-positive areas

In addition to structures that fitted the criteria given for gland structures there were at least 10 other, smaller regions, present in each specimen which stained positively with DAB and TMB. All these regions had the same outward physical appearance i.e. small conglomerations of stained material that recurred in a similar pattern in all specimens. This pattern was as follows.

(I) Eye-Spot group (ESG): A group of four stained tissues (two of which are highlighted in **Figure 3.10**) was found close to the dorsal cuticle and formed a symmetrical pattern around the eyes of the copepodid. Two of these were located anterolaterally to each eye, and the remaining two at the most postero-lateral point of each eye. A fifth stained structure was located immediately in front of and between the eyes lying directly above and slightly posterior to the dorsal median gland but is not visible in **Figure 3.10**.

(II) Thoracic Segment Group (TSG): Three regions with a similar appearance to those of the Eye-Spot Group were found on the third thoracic segment (**Figure 3.10**). One of the regions lay almost exactly on the mid-line of the animal and the remaining two were located slightly forward of this one, an equal distance apart on either side of it.

There were two, small, stained structures present in the fifth thoracic segment. These were located near the anterior edge of the segment, close to the junction with the fourth thoracic segment. They were in a lateral position, one on each side close to the dorsal cuticle. These structures appeared to have a different morphology from those of the ESG, forming denser, compact units. Additionally, there was a single gland in the centre of the fourth thoracic segment.

3.4.3 DAB / TMB-staining of *L. salmonis* chalimus larvae

All four chalimus stages of *L. salmonis* displayed a similar pattern of DAB-positive gland distribution, although some slight differences were evident between the stages. **Figures 3.11** and **3.12** demonstrate the extent of the gland distribution in chalimus larvae.

By the chalimus III stage some specimens showed the characteristic 'winged' genital segment of females whilst other specimens of the same stage did not. However, as a result of inconsistent and variable staining between specimens it was not possible to determine whether there was a sexual dimorphism in regard to gland distributions at this stage. It was apparent, though, that if there was a dimorphism in gland distribution it was limited, all specimens of the same stage showed similar patterns of glands.

3.4.3.1 Urosomal glands

All stages of chalimus larvae possessed a large paired gland complex in the urosome one lying on either side of the gut close to the anal aperture each of which extended into the caudal rami. These glands stained positively, but weakly with DAB and TMB and are evident in both **Figures 3.11** and **3.12**.

This gland complex developed and became more complex during the progression of the larvae through subsequent chalimus stages. Chalimus I larvae had only a single region to the gland, but by chalimus II a second, smaller region had developed in association with each of the large, original, components of the gland. The gland at this stage, homologous with the urosomal and posterior glands of the copepodid and nauplius stages, still possessed the long anteriorly extending stained duct, as can be seen in **Figure 3.13**, leading from the anteroventral region. In chalimus III and IV larvae each urosomal gland appeared to be composed of three regions: the large original region and two smaller regions which were closely associated with it, indicating that the gland is becoming a more complex structure over those of the preceding stages.

It was presumed that these glands discharged via pores on the posterior margins of the caudal rami, as described for the urosomal glands of the copepodid stage and the posterior glands of the nauplius, as very faintly staining ducts were sometimes visible extending from the posterior margin of the gland complex (these are not visible in **Figure 3.13**), although little staining was evident on the external cuticle.

3.4.3.2 Lateral glands

Glands corresponding to the lateral glands of the nauplius and copepodid stages were observed in the chalimus stages but were not present consistently in any one developmental stage. Stained glands were apparent in some specimens but not in others of the same stage. These glands, located near the lateral margin of the cephalothorax (one on either side) lay close to the ventral cuticle within the cephalothorax, directly above the maxillipeds (**Figure 3.13**). The glands appeared, superficially, to be composed of one unit but when viewed under higher power after clearing in lactic acid, areas of diffuse staining located around the gland were apparent, although no definite structure could be defined.

3.4.3.3 Frontal gland complex (FGC)

The FGC was visible from the dorsal surface of the chalimus larvae, immediately anterior to the eyes. The complex was present, but too faint to be distinguished clearly in chalimus I, however in all

chalimus stages from chalimus II it appeared to consist of three regions lying in close proximity to one another. When these glands were studied under higher power in lactic acid it became apparent that the filament duct arose from this complex. The filament duct was DAB-positive and could be clearly seen to arise from the central region of the gland and pass in an anterior direction through the filament to the point where it had been detached from the fish host. In **Figure 3.14** only the duct and the internally developing filament are apparent, as the frontal gland complex is not evident from the ventral aspect in this specimen. The filament duct was not apparent in all specimens. However, those specimens which did have a stained filament duct, also had a developing filament present internally (**Figure 3.14**) within the anterior end of the cephalothorax, ready to be extruded prior to the next moult.

3.4.3.4 Anterior gland complexes (AGC)

A large conspicuous group of positively staining glands at each anterolateral margin of the cephalothorax, close to the bases of the antennae was always present in early stage chalimus larvae. In chalimus I and II the complex appeared to consist of two regions only, as seen in **Figures 3.12** and **3.13**, as opposed to three regions in the preceding nauplius and copepodid stages. It was never possible to determine how, or whether, these two regions were connected. This complex became larger and more complex as the animal progressed through further moults: in chalimus III larvae it consisted of approximately 5 regions on either side and six or more in chalimus IV larvae. Given their similarity, in terms of location, to the AGC of the nauplius and copepodid stages the title of AGC has been ascribed to them, although ultrastructural studies will be required to resolve whether these are the same complexes.

A fine duct was always present leading from the most anterior region of the complex and could be followed for a short distance to where it appeared to exit via a pore on the anterior margin of the cephalothorax, facing the posterior margin of the antennules, as secreted stained material was often seen in that area.

In chalimus III and chalimus IV, this group of glands, or gland regions, was large and loosely associated making it difficult to ascribe a particular orientation or structure to them. However, the same

approximate grouping of glands occurred in all specimens of each stage and constituted a conspicuous feature of the gland population.

3.4.3.5 Postoral and circum-oral glands

A complex grouping of DAB stained glands were identified in association with the mouth tube. These were separated into two groups entitled the 'circum-oral' and 'postoral' glands.

Apparent in some stages of chalimus larvae (II, III, and IV at least) were four DAB-positive glands, which approached the ventral cuticle close to the base of the labium. These glands were visible from both the dorsal and ventral aspects but were seen more clearly from the ventral surface (**Figure 3.14**). No ducts were seen in association with these glands. The title of circum-oral gland was ascribed purely on a positional basis and was not meant to imply any functional role for these glands in relation to either the mouth-tube or feeding. The glands were arranged around the base of the mouth-tube, each forming a corner of this approximately rectangle shape gland grouping; one pair at the posterior margin of the mouth-tube and one pair at its anterior margin. These circum-oral glands occur in a similar position to the 'lateral' glands of adult *L. salmonis* as described by Bron (1993).

No glands in the labrum, corresponding to the ventral median gland of the copepodid stage, were observed in any chalimus larvae.

A pair of positively staining glands could be seen under the ventral cuticle of all chalimus larvae with the exception of chalimus I. These V-shaped glands lay slightly anterior to the 'first cuticular ridge' (CR1) close to the bases of the maxillules and maxillae (**Figure 3.15**). These glands were only visible from the ventral aspect. A fine, stained duct could be seen leading in a medioventral direction and was presumed to exit close to the bases of the maxillules / maxillae. The evidence for this conclusion was the presence of quantities of stained material adhering to the cuticle close to the base of the maxillules. This material was clearly granular in nature and could often be seen to coat large areas of cuticle with the focus (and presumed source) always being around the bases of the maxillules / maxillae at the suspected end point of the duct.

3.4.3.6 Thoracic leg glands

Few glands were apparent in the thoracic legs at this stage and those present were much less obvious than the thoracic leg glands of the copepodids. Due to the indistinct segmentation of the thoracic legs and their indistinct segmentation in the chalimus stages, it was very difficult to precisely determine where these glands were located within the leg.

It was apparent, though, that glands in the thoracic legs were more conspicuous in the early chalimus stages (chalimus I and II) than the later ones. This may, in part, be due to the variable staining to which chalimus larvae were prone. Both chalimus I and chalimus II larvae appeared to have a pattern of thoracic leg glands matching exactly that of the preceding copepodid stage i.e. a gland in each sympod and endopod of the th_I legs and one in each sympod, endopod and exopod of the th_{II} legs.

Chalimus III and IV larvae may have possessed thoracic leg glands but staining was not conclusive. In some specimens very small regions of faint colouration were seen but these were so small as to be almost indistinguishable.

3.4.3.7 Suture line glands

A recurring group of DAB-positive glands was found in all specimens of chalimus II, III and IV. These glands lay close to the dorsal cuticle immediately anterior to, and at each lateral margin of, the transverse cuticular suture (**Figure 3.11**). A pair of these regions, one at either lateral margin of the transverse cuticular suture, were present in chalimus II larvae. This gland group developed with succeeding moults through the chalimus stages: two regions in chalimus III and three in chalimus IV larvae. The secretory pores of these glands could not be located using SEM, but they may be situated within the fold of cuticle that comprises the transverse suture line.

3.4.3.8 Other DAB-positive areas

Two chalimus larvae examined showed a considerable number of small DAB-positive areas present throughout all regions of the gut, with the exception of the hindgut. These stained areas appeared

too small to be glandular complexes, and from their position (**Figure 3.13**) they appeared to correspond to secretory cells lining the lumen of the gut. However, similar staining was never observed in any other specimens of this stage so the validity of this reaction must be questioned in case this result is artifactual.

Three regions of positive staining were always seen in the free thoracic segment of all chalimus stages and were presumed to correspond to the TSG of the copepodid stage.

A further set of stained areas was seen on the ventral surface of many specimens of all stages. These took the form of two small spots lying on either side of the sternal furca in chalimus IV larvae. These regions did not appear to be associated with any particular body structure that could be seen at this level using light microscopy. Body musculature was seen to give a slight positive response to the DAB stain. Two small regions of positive DAB staining were frequently observed on the dorsal surface lying on the longitudinal midline of the posterior half of the cephalothorax.

Some small areas of DAB staining were observed close to the dorsal cuticle of some later stage chalimus larvae. These regions were irregularly spaced with no apparent pattern to their distribution and appeared to be absent in the chalimus II stage.

3.4.4 DAB / TMB-staining of *L. salmonis* preadults

The preadult stage of *L. salmonis* is the first developmental stage with a strong morphological resemblance to the adult stages and is also the first clearly sexually dimorphic stage.

3.4.4.1 Urosomal glands

Large, bilaterally symmetrical paired complexes of glands were present at the posterior tip of the abdomen, extending into the caudal rami, in all specimens examined (**Figure 3.16**). These complexes lay one on either side of, and in close contact with, the gut. The glands appeared to consist of either several lobes of the same gland, or were perhaps separate gland units. The same pattern of urosomal gland distribution was seen in both preadult stages of both sexes, and consisted of a pair of large, elongate glands on either side of the gut close to the anal aperture with three symmetrical pairs of smaller glands

overlying them ventrally and a further three dorsally. These glands undoubtedly represent a further development of the same gland group as described for the chalimus stages.

In addition to the positive staining of these glands there were often large quantities of positively stained material adhering to the long terminal setae of the caudal rami. This material was presumed to have been secreted by these glands as the secretory ducts were seen to lead to the posterior margins of each ramus where they were presumed to discharge via pores in the cuticle. It was noted that males were more frequently observed to have this positively staining material adhering to their setae.

3.4.4.2 Frontal gland complexes (FGC)

The gland complex corresponding to the FGC of the chalimus stage larvae could be clearly seen in all preadult specimens regardless of sex or developmental stage.

At this stage, this complex appeared to consist of four pairs of glands plus one single central glandular tissue. Three of the pairs of glands lay close together and gave the impression of being, if not lobes of one gland, then at least several intimately associated glands. The arrangement of this complex can be seen in **Figure 3.17**. The central region and one of the pairs of glands appeared similar to those seen, and described as the FGC of the chalimus stages.

The three closely associated pairs of glands and the central gland appeared to form the complex first described by Bron *et al.* (1991) as the 'filament producing glands' for *L. salmonis*, and described in Section 3.4.3.3 of this chapter for chalimus larvae. The three pairs of glands seen here could be correlated to Bron's description of the A, B and C glands of that organ.

The fourth pair of gland regions lay apart from the main complex close to the point of fusion between the frontal plate (**Figure 3.17**) and the cephalothorax. Each of these glands was seen to have a fine duct leading to a pore in the cuticle close to the point of fusion of the frontal plate and the cephalothorax. This pair of glands was not believed to be involved in filament production, as they were remote from the recognised structures of the filament-producing glands, as indicated by an arrow in Figure **3.17**. Additionally, these glands were clearly secreting their product onto the dorsal cuticle of the

cephalothorax, further precluding their role in filament production. As such the FGC cannot be looked upon as a contiguous gland system with a common function, rather it is a title to describe a group of glands that are visually distinct and can be readily grouped together on a purely locational basis. No suggestion of similarity in terms of secretory product, or function, is intended.

Several specimens were found to have large pieces of strongly DAB-positive, adhesive material attached to the ventral surface of the cephalothorax. This material appeared to have been extruded from the 'filament organ' on the anterior margin of the ventral surface.

3.4.4.3 Anterior gland complexes (AGC)

In all preadult stages there was a closely grouped region of DAB-positive structures in the anterolateral region of the cephalothorax (in the same position as the AGC of the chalimus stages) visible from both the dorsal and ventral surfaces. In both males and females the complex became more developed from preadult I to preadult II with an increase in the number of glands, or regions of the same gland.

In preadult I males there were approximately 5 regions on either side, developing to 6 regions at preadult II. In contrast, preadult I females had approximately 6 regions which had increased to 8 or more on either side by preadult II. When viewed from the ventral surface those glands appeared to lie within the cephalothorax immediately lateral to the antennae and directly below the post antennary process. Immediately bordering the posterior margin of this gland lay the 'first cuticular ridge' (CR1). The space that this complex occupies has here been termed the 'lateral sinus'.

It was impossible to say whether there was a functional association between these glands or even whether they were connected physically so the title of AGC was applied purely on a locational basis i.e. it lay in the anterior region of the body. This structure may, however, represent a further development of the AGC of the chalimus stages, as it formed a conspicuous grouping of stained gland tissues in approximately the same region of the body as the AGC of the chalimus, copepodid and naupliar stages.

3.4.4.4 Postoral and circum-oral glands

The grouping of the DAB-positive glands around the mouth tube observed in the chalimus larvae was also present in the preadult stages.

A small very faintly staining DAB-positive gland was present at the proximal end of the labrum in most, but not all specimens, and probably corresponded to the ventral median gland of the copepodid stage. In addition, a pair of other glands were frequently seen in conjunction with this gland and appeared, at a light microscope level, to be connected to it. These glands lay immediately on both sides and anterolaterally to, the labral gland. There appeared to be a fine duct extending from each of these glands towards the labral gland. A further pair of small glands were visible under the ventral cuticle at the base of the labium. These glands were only visible when the mouth-tube was elevated perpendicularly to the ventral surface of the animal. Together these glands probably represent the circum-oral gland complex at this stage.

In all preadults a pair of large, heavily staining glands were visible, level with the mouth-tube on the ventral surface. The glands, one on either side of the mouth-tube, lay immediately anterior to the maxillules within the cephalothorax. These glands had a characteristic kidney, or bean shape and are labelled 'po' in **Figure 3.18**. The glands were commonly observed to have a clearly stained duct leading a short distance medioventrally from them, which could be seen to exit onto the ventral surface of the animal, close to the lateral margins of the bases of the maxillules. These glands appeared to correspond to the glands described as 'postoral glands' for chalimus larvae.

It was common to find a great deal of positively stained material adhering to the cuticle of, and around, the maxillules. This material was granular in nature and often covered large areas of the surrounding cuticle. This granular appearance of the stained material may not be representative of the secretion itself but may be a characteristic of the DAB stain.

3.4.4.5 Thoracic leg glands

The thoracic legs of the preadults were seen to possess very few DAB-positive glands although staining was never consistent between specimens and those stained regions that were present were faint and difficult to visualise.

Preadult I males had no positively staining glands in either the th_I or th_{IV}. One gland was present in the distal sympod of the th_{II} whilst the th_{III} contained three positively staining glands, two in the sympod and one in the exopod. Preadult II males had no glands in the th_I but two small glands were present in the basal segment of the th_{II}. Preadult I males never showed any positively staining material on the setae of their thoracic legs and only small amounts were observed on the setae of the th_I and th_{II} preadult II males.

Preadult I females had very few such glands in their thoracic legs. The th_I and th_{II} appeared not to have any DAB-positive gland, although there was one small gland present in the posterolateral margin of the th_{III}. The th_{IV} of preadult I females contained four glands, three in the sympod and one in the exopod. In general, few glands were evident in the legs at this stage. By the time the preadult II stage had been reached more glands had developed in the thoracic legs. Two were present in the th_I, one in the sympod and one in the proximal segment of the exopod. The th_{II} had one gland present in the distal segment of the endopod. No glands were present in the th_{III}. The th_{IV} had several positively staining glands, although the precise number was difficult to determine. At least three were present in the sympod and at least one was observed in the exopod. This uncertainty was caused by inconsistent and variable staining between specimens.

3.4.4.6 Suture line glands

The suture line glands seen in the chalimus larvae were present, but difficult to distinguish, in the preadult stages. Preadult female I stage *L. salmonis* possessed two glands on either side of the longitudinal midline, close to the transverse dorsal suture whilst preadult II females often appeared to have three such glands on either side. In both stages the glands were grouped close together but were not apparently in connection with one another (**Figure 3.18**). The staining of these glands was subject to

considerable variability between specimens. Similarly positioned glands were apparent in some preadult males but staining was faint and inconsistent between specimens.

3.4.4.7 Dorsal surface glands (DSG)

A large number of DAB-positive glands were distributed across the dorsal surface of all specimens (**Figures 3.16** and **3.17**) and were present in all body segments present at this stage; cephalothorax, free thoracic segment, genital segment and abdomen. These glands were very small and appeared to lie immediately beneath the cuticle and appeared similar, in their gross morphology, to the small glands observed under the dorsal cephalothorax cuticle of late stage chalimus larvae.

The greater sensitivity of TMB meant that it stained these glands, and their ducts, more completely than DAB. TMB frequently permitted the cuticular pore associated with each gland to be visualised, something which DAB failed to do. The use of TMB therefore provided strong evidence that the regions of positive staining were indeed glands.

Although there was variable staining between specimens a picture of the distribution of these glands could be built by examining several specimens and creating a composite image.

Preadult I females possessed around 50 of these glands arranged in a bilaterally symmetrical pattern with approximately 4 unpaired glands along the longitudinal mid-line. A substantial proportion of the total number of the DSG was accounted for by glands located around the margins of the cephalothorax, close to the marginal membrane. After the moult to preadult II the number of DSG increased to between 70 - 80 per specimen with approximately 5 unpaired glands found along the longitudinal mid-line. Again, at this stage, the greatest majority of the DSG were located around the margins of the cephalothorax, close to the marginal membrane.

The genital segments of both preadult I and II females possessed several DAB-positive glands, along their margins and dorsal surface as well as on their ventral surfaces. No such glands were observed on the ventral surface of the cephalothorax.

Preadult males had fewer DSG than their female counterparts. The total number of DSG found in preadult I males was approximately 40 per specimen, most of which were again distributed principally around the margins of the cephalothorax, close to the marginal membrane. Few such glands were present in either the genital segment, free thoracic segment or on the ventral surface of the cephalothorax. Preadult II males showed an increase in DSG number over the preceding stage. At this stage approximately 50 - 60 glands could be distinguished.

3.4.4.8 Other DAB-positive areas

Other DAB-positive areas were observed in preadults. These included, the structure variously termed the median sucker (Kabata, 1981) and frontal organ (Anstensrud, 1990) which usually stained a light brown colour, whilst within the genital segment of preadult males the spermatophore sacs stained very darkly with DAB and TMB suggesting that high levels of peroxidase enzymes may be present there. The retinal cells and the protocerebrum also showed a slight positive response to the DAB stain as did points of muscle attachment to the cuticle.

3.4.5 DAB / TMB-staining of *L. salmonis* adults

Adult *L. salmonis* showed the greatest total number of DAB-positive regions of all life-stages. Both males and females had large numbers of glands, although a slight sexual dimorphism in gland distribution was apparent.

3.4.5.1 Urosomal glands

Glands corresponding to the urosomal glands of the preceding stages were always seen in stained adult specimens of *L. salmonis*. There was a pair of large, strongly positive gland complexes lying immediately on either side of the gut close to the anal aperture. In some specimens the main regions of these complexes could be seen to extend into the caudal rami. In addition to these large regions there were also several associated smaller glands or further lobes of the main gland region. These regions

comprised six pairs of bilaterally symmetrical glands overlying the main regions, three dorsally and three ventrally. These gland systems had clearly reached their greatest development in these adult stages.

In adult males it was common to find considerable quantities of DAB (or TMB) -positive material adhering to the long terminal setae of the caudal rami (**Figure 3.19**). The urosomal glands of adult females appeared to be much smaller than those of the males and stained less intensely. The terminal setae of the caudal rami of adult females were never seen to be coated with stained material.

3.4.5.2 Frontal gland complex (FGC)

The FGC, with essentially the same structure as described for previous stages, was also seen to be present in the adult stages. In both adult males and females (**Figure 3.20**) it appeared to consist of four paired (symmetrical) regions and one central gland. These gland regions were seen to form the same arrangement in all specimens examined. Some elements of this gland complex were visible from the ventral surface of the animal, immediately anterior to the mouth-tube. The regions of this gland could be matched with those described as the filament-producing glands by Bron *et al.* (1991).

The FGC described here matches that described earlier for the preadult stages and includes all of the regions described by Bron *et al.* (1991) and Bron (1993) for this structure in *L. salmonis*.

A single duct was seen to lead from each of the most anterior glands to pass the short distance to exit on the dorsal surface of the cephalothorax close to the point of articulation between the frontal plates and the cephalothorax, as observed for the preadult stages. No direct association between these glands was witnessed nor is it implied at this stage, they were grouped together only by their close physical association.

3.4.5.3 Anterior gland complexes (AGC)

These gland complexes in the adult stages were similar, in size and number of regions, to those described for the preadult stages. These complexes were easily distinguishable from the DSG on account of their much greater size of the individual components of this complex (**Figure 3.20**).

3.4.5.4 Postoral and circum-oral glands

There was at least one DAB-positive gland present in the proximal region of the labrum, which was presumed to be the labral gland described for earlier stages. In some specimens (both males and females) there appeared to be a pair of positively staining glands which lay slightly anterodorsally to the labral gland. These glands were large, oblong in shape, and appeared to be connected to the labral gland via fine ducts, one emerging from either of their medial margins. These glands were located medial to the bases of the antennae and appeared similar, in location at least, to the anterior most pair of circum-oral glands of the preadult and chalimus larvae. It was not possible to determine conclusively whether they were physically connected to the labral gland or whether their ducts merely passed into the mouth-tube close to the labral gland as a pair of mucous glands have been shown to (Bron, 1993). It appears likely that these glands do correspond to the 'lateral glands' described by that author.

The possibility that the labral (ventral median) gland of the copepodid with its two lateral ducts (**Figure 3.7**) may develop into this three-lobed structure should be considered.

A pair of glands similar in location to those described as the postoral glands for chalimus and preadults was seen in the majority of adult specimens examined. Although these glands were not observed in all specimens it may be presumed that this lack of consistency in staining could be accounted for by staining variation between animals, cyclical nature of glandular activity etc. and that such glands were present in all specimens even if not observed.

3.4.5.5 Thoracic leg glands

The results of the staining trial revealed that the thoracic legs of the adults were well supplied with DAB-positive glands. Glands were present in thoracic legs one (th_I), two (th_{II}), three (th_{III}) and four (th_{IV}). The pattern of gland distribution in the thoracic legs (**Figure 3.21**) was found to be the same in both male and female specimens.

The th_I legs always had a small gland in the distal segment of the sympod and a further gland was present in the distal region of each proximal exopod segment. A total of eight glands were present in the

th_{II} legs: one in each of both the proximal and distal sympods, one in each proximal segment of the endopod as well as one in the distal segment of each exopod. The th_{III} legs had several (≥8) glands present in the broad interpodal bar close to the bases of the exopods, although staining was variable between specimens. No glands were seen to be present in either the exopods or endopods of the th_{III}. The th_{IV} legs contained several DAB-positive glands in both the sympod and exopod.

The natatory setae of the th_{I} and th_{II} were often seen to be covered with large quantities of positively staining material. This phenomenon was observed in both males and females.

The thoracic leg glands in adult specimens always stained heavily, were very conspicuous and showed a consistent pattern between specimens compared to those of the preadult and chalimus stages.

3.4.5.6 Suture line glands

Stained glands were present close to the lateral suture in the dorsal cephalothorax. These glands were present in both adult males and females as is evident from **Figures 3.20** and **3.22.**

3.4.5.7 Dorsal surface glands (DSG)

Small DAB-positive glands located immediately below the cuticle were prevalent across the dorsal surface of the cephalothorax of both males and females. Adult female specimens had between 100-140 (**Figure 3.20**) of these glands, arranged in a bilaterally symmetrical pattern with an additional small number of unpaired glands arranged along the longitudinal mid-line. Adult males, on the other hand, possessed around 60 DSG with a bilaterally symmetrical pattern (**Figure 3.22**) again with a few additional unpaired glands arranged along the mid-line. No single specimen displayed all of these glands, as staining varied between animals and so no theoretical maximum number of DSG could ever be determined. No similarly staining glands were found on the ventral surface of the cephalothorax although the genital segments of both males and females did have small DAB-positive glands on their ventral surfaces.

Despite the difference in number of DSG found between the sexes, both adult males and females possessed approximately similar numbers in the cephalothorax. The difference in the total number of

DSG between the two sexes was restricted primarily to the genital segment where gland numbers in females greatly outnumbered those of males.

3.4.5.8 Other DAB-positive areas

In addition to the areas described here as glands there were often other regions of the animal which appeared to stain slightly with DAB. These sites included the attachment points of muscles to the cuticle, especially where they attach to the dorsal cuticle (**Figures 3.20** and **3.22**), some of the muscles as a whole e.g. thoracic leg muscles, labral muscles, cells of the retina, the protocerebrum and the egg strings of the females. Sites of injuries and cuts to the cuticle were also observed to stain intensely with DAB such as the sternal furca of the specimen in **Figure 3.21**.

3.4.6 DAB-staining of *Caligus elongatus*

Nauplii, copepodids and adults of *C. elongatus* all displayed a considerable number of DAB / TMB-positive glands. Staining in *C. elongatus* was generally much more consistent than in *L. salmonis*, and revealed that both male and female adult specimens possessed similar distributions of such glands, although there were some slight differences in gland distribution between the two species.

Nauplius stages displayed an identical pattern of DAB-positive gland distribution to that found in *L. salmonis* nauplii. Posterior, lateral, anterior and median glands were (**Figure 3.23**) all present in the same positions with no apparent differences in gross morphology.

Copepodids of *C. elongatus* appeared to have a similar pattern of DAB-positive regions to their *L. salmonis* counterparts. Minor differences were observed in the thoracic leg glands. In *C. elongatus* a total of only 3 pairs of glands were found in the two pairs of thoracic legs present at that stage compared to 5 pairs in *L. salmonis*. The th_I of *C. elongatus* copepodids appeared to contain only one gland in the basal region of each sympod, whilst the th_{II} had one in each sympod and one only in the basal region of either endopod. The thoracic legs of adult specimens of *C. elongatus* were found to contain many DAB-

positive glands within them, which appeared to be arranged in a distribution identical to that observed for *L. salmonis* adults.

Differences were apparent in the composition and structure of the FGC which was more compact but appeared to be composed of more regions than that seen in *L. salmonis*: 6 c.f. 5. The two most anterior regions of the FGC (those not related to filament production), adjacent to the posterior margin of the lunules, appeared to discharge to the dorsal surface of the animal at the point where the lunules and cephalothorax meet. These exit points were visible as small deposits of DAB-positive material on the dorsal cuticle. Two further exit points from these glands were visible as the distal points of two ducts, one running from each anterior-most region of the complex to cross the lunules and exit on the very anterior margin of them. The AGC of adult *C. elongatus* were similar in both male and female specimens, being composed of approximately 7 distinct regions, the most anterior of which appeared to exit via a pore on the anterior face of the cephalothorax at the base of the antennule. The urosomal glands of *C. elongatus* appeared to have a slightly different morphology to those of *L. salmonis* (**Figure 3.24**) and they also appeared to stain more intensely.

The most obvious difference, in terms of DAB-positive glands, between *L. salmonis* and *C. elongatus* was the greater number of DSG present in *C. elongatus*. Both adult males and females had comparable numbers of DSG (**Figure 3.25**) with around 150-200 of these regions distributed in a bilaterally symmetrical pattern across the cephalothorax, genital segment and abdomen. The pattern of distribution on the genital segments and abdomen differed greatly between males and females. Suture line glands were large and readily distinguished in adult *C. elongatus*. Three pairs of suture line glands were present on either side of the longitudinal midline in both adult males and females.

Both males and females had a pair of heavy spots of DAB-positive-staining on the ventral cuticle, immediately posterior to the th_I legs. Adult specimens also had a pair of glands corresponding to the postoral glands seen in *L. salmonis*, as well as a DAB-positive gland located at the base of the labrum which appeared to correspond to the labral gland of *L. salmonis*.

3.4.7 Other copepod species

3.4.7.1 Order Siphonostomatoida

3.4.7.1.1 *Lepeophtheirus pectoralis*

This species is predominantly found parasitising pleuronectid flatfish along the Atlantic coast of Europe (Kabata, 1979). The adult stages of this parasite tend to be more sedentary than species such as *L. salmonis* and *C. elongatus* and are generally located under the pectoral fins of the host fish. Adult stages of this species also displayed the same main groups of stained glands observed in the other caligid species (**Figure 3.26**). Glands corresponding to the AGC, FGC, DSG, labral, circum-oral, postoral, thoracic leg, urosomal and suture line glands were all evident. No conspicuous differences in the structure of these gland complexes was apparent.

3.4.7.1.2 *Lepeophtheirus hippoglossi*

L. hippoglossi is a morphologically similar copepod to *L. salmonis* although it is slightly larger and more robust and is generally only found associated with Atlantic halibut (*Hippoglossus hippoglossus*). Adult specimens showed an essentially identical staining pattern to that observed for *L. salmonis* and *C. elongatus* when stained with DAB and TMB. The same major gland groupings were detected including the FGC, AGC, DSG, urosomal glands (**Figure 3.27**), thoracic leg glands, suture line, labral, circum-oral and postoral glands. The primary difference in the pattern of gland distribution was restricted to the DSG, of which large numbers were observed across the dorsal surface of the cephalothorax, genital segment and abdomen. The number of these glands and the bilateral pattern they formed differed from the numbers and patterns observed in *L. salmonis* and *C. elongatus*. These glands however were far more numerous in both adult male and female stages of this species than in the equivalent stages of both *L. salmonis* and *C. elongatus*.

3.4.7.1.3 *Clavella adunca*

Live adult female specimens of the lernaeopodid parasite *C. adunca* were taken from freshly caught cod (*Gadus morhua* Linnaeus, 1758) from the North Sea. This species is significantly more adapted to the parasitic lifestyle than caligid species, evident in its loss of body segmentation and legs and specimens were removed from the gill filaments of infected fish. Specimens of this species when stained with TMB showed conspicuous spots of discrete staining which stood out from the pale background of the general body tissues. No definite statement can be made here regarding their status as gland tissues as they never displayed any features which were deemed as essential criteria for the identification of glands in this trial. The stained areas observed did not show any pronounced similarity to any of the recognised gland complexes described for *L. salmonis*. The stained areas were apparent as small individual regions close to the body cuticle and resembled, superficially, the DSG of the caligid species. Without histological evidence the status of these staining areas in this species remains unresolved but the possibility that these stained areas represent gland tissues with similar characteristics to those identified in caligid species should be considered.

3.4.7.2 Order Calanoida

3.4.7.2.1 *Acartia tonsa*

Adult specimens (**Figure 3.28**) and some nauplii were stained with DAB and were found to possess few positively staining glands, however a consistent pattern of gland distribution was evident between specimens. A pair of distinct, DAB-positive regions were located on the ventral surface of all specimens examined. Although they were difficult to discern fully, it appeared that they were located in the region of the buccal cavity.

Also present in all specimens was an area of DAB-positive staining within the body cavity at the area of fusion between the cephalosome and the post-cephalic trunk. A small region of granular DAB-positive material was seen, in most specimens, in the centre of the anterodorsal face of the cephalosome.

No glands corresponding in size, shape or location to the DSG of the caligid species examined were observed in this species.

Nauplius larvae showed only two DAB-positive areas. These regions were located in the anterior region on the ventral surface of the animal and appeared to consist of a pair of discrete regions, one on either side of the buccal cavity. Fine ducts could be seen in association with these regions lending weight to the theory that they were glandular structures.

3.4.7.2.2 *Eurytemora affinis*

Adult specimens of *E. affinis* also showed little DAB-positive staining. Body pigmentation partially obscured what little staining did occur (**Figure 3.29**) but some areas of staining could be visualised after clearing in lactic acid. In particular, egg clusters and oviducts of females stained heavily with DAB, allowing visualisation of the oviducts within the animal. Two other regions of staining were consistently found in both adult males and females. These were a small cluster of at least six, discrete vesicles in the region of the buccal cavity. Three of these regions were located in a patch either side of the longitudinal mid-line, presumably in the paragnaths.

Within the body cavity of most specimens a dense area of DAB-positive vesicles was located immediately posterior to the buccal cavity region and appeared to be located within the gut of the animal. These vesicles superficially appeared similar to the DAB-positive vesicles seen in the gut of *L. salmonis* chalimus larvae. This region was restricted to a small area in the anterior midgut.

In adult females two further regions of staining were found, one on either posterolateral margin of the cephalosome. These granular regions had a U-shaped appearance with the tapering posterior point passing in a medioventral direction to come close to the point where the eggs emerged from the genital aperture and may correspond to the spermatophores.

No glands in close association with the body cuticle were observed in any of the specimens examined.

3.4.7.3 Order Harpacticoida

Staining revealed that all four species of harpacticoid copepod possessed numerous exocrine glands the contents of which had a positive reactivity with DAB. These species possessed fewer such glands than caligid copepods but many more than the calanoid species stained. Only the adult female stage of these species could be identified and this was therefore the only stage stained with DAB but the results can be used to gain an insight into the general biology of the species as a whole.

3.4.7.3.1 Parathalestris hibernica

Adult female *P. hibernica* possessed many clearly distinguishable DAB-positive glands. The most prominent of these glands were in the cephalosome (**Figure 3.30**). One pair of glands was present ventrally close to the bases of the maxillipeds, another pair anteroventrally close to the bases of the antennae whilst two pairs of glands were present dorsally. There was a gland present within the body cavity immediately at the base of each of the third and fourth swimming legs and a smaller pair at the base of the fifth legs as well as glands being present in most segments of each swimming leg. Glands were present in both the dorsal and ventral regions of each urosomal segment (**Figure 3.31**). Secretory ducts leading to exit on the ventral surface were observed in association with the ventral glands. A particularly large and prominent gland complex occupied a substantial portion of the posterior anal segment and is evident in **Figure 3.31**. Quantities of DAB-positive material were frequently seen adhering to the terminal setae of the urosome.

3.4.7.3.2 *Harpacticus chelifer*

DAB-positive glands were also prominent in adult female specimens of *H. chelifer*. Many small glands were evident along the ventral margin of the cephalosome but in addition, several larger, darkly staining glands were also present as can be seen in **Figure 3.32**. A large gland was present in the ventral region of the cephalosome and appeared to extend into the region of the buccal cavity as shown in **Figure 3.32**. In contrast to *P. hibernica* there were no stained glands evident within the cephalosome at the bases

of legs two and four. Instead there were DAB-positive glands present within either the endo- or exopods of the three pairs of legs. A pair of large, positively staining glands was present in each segment of the urosome and the secretory ducts of these glands could be seen to lead to a pore on the lateroventral aspect of each segment. A large stained gland was also evident in the anal segment close to the anal aperture and the terminal setae **Figure 3.33**.

3.4.7.3.3 *Diosaccus tenuicornis*

Many small DAB-positive glands were also evident in adult female *D. tenuicornis* although they were more difficult to discern than in either *P. hibernica* or *H. chelifer* due to the fact that the general body tissues of *D. tenuicornis* stained more darkly with the DAB stain. Positively staining glands were however, observed in the urosome and swimming legs. Large stained glands filled the ventral portions of each urosomal segment. A large gland was observed in the anterodorsal region of the cephalosome and other, smaller glands, were observed scattered across the surface of the cepahlosome. A duct was observed to extend from one of these anterior glands and pass into the short cuticular process on the anterior face of the cephalosome (**Figure 3.34**).

3.4.7.3.4 *Dactylopusia vulgaris*

Dactylopusia vulgaris stained very darkly with DAB over the same staining period as the three other harpacticoid species. Despite prolonged clearing in lactic acid it was never possible to clearly discern all regions of positive staining in this species. Glands were positively identified in the swimming legs, the ventral regions of the abdominal segments as well as some others across the dorsal surface of the cephalosome. The ventral region of the cephalosome remained too darkly stained to observe the mouth area.

3.4.8 DAB-staining of non-copepod species

3.4.8.1 Argulus foliaceus

Adult specimens of this branchiuran parasite of freshwater fish were obtained from pond-reared carp (*Cyprinus carpio* Linnaeus, 1758) from various locations in Scotland. Staining with DAB failed to reveal any positive regions of activity.

3.4.8.2 Macrobrachium rosenbergii

Zoea II stage larvae of *Macrobrachium rosenbergii*, were stained over a wide range of time periods but no positive staining was ever observed in any of the body tissues.

3.4.8.3 Artemia salina

Larval stages (approx. 48h post-hatch) of *Artemia salina* showed very little reaction to the DAB stain (**Figure 3.35**). Some inconclusive staining was observed within the digestive tract of some specimens but this was faint and ill-defined suggesting that it was a response to gut content material rather than a specific reaction to any endogenous components.

3.4.8.4 Daphnia magna

Adult stages of *Daphnia magna* also showed very little positive response to the DAB stain as can be seen in **Figure 3.36**. In most specimens though two small regions of staining were present within the body cavity immediately posterior to the buccal cavity. These two spots of staining lay close beneath the ventral cuticle of the animal.

3.5 SEM examination of caligid species and PSP evaluation

The greater sensitivity of the TMB stain made it an ideal tool for highlighting the cuticular pores through which the glands were presumed to secrete their product (**Figure 3.19**). A distribution map of

integumental pores in the cephalothorax was constructed from TMB-stained adult male *L. salmonis* as shown in **Figure 3.37**.

Comparison of the pattern of dorsal surface pores found using the TMB stain with that obtained from SEM examination clearly demonstrated that the suspected glandular pores highlighted by the stain were indeed pores in the cuticle that closely matched the distribution of the DSG. Approximately 60 DSG's were identified in adult male *L. salmonis* using the TMB stain (see Section 3.4.5.7) and the SEM study showed there to be 59 pores in the dorsal cuticle. No pores were located which had not been found using the staining technique suggesting that the pores of the DSG constituted the total number of dorsal surface pores. The slight discrepancy between the two values could be accounted for variously as either an oversight in the SEM analysis due to physical obstruction of pores by wrinkles and folds in the cuticle or because of slight variability in staining between specimens. Adult female *L. salmonis* have been shown to possess approximately between 90 and 140 DSG (Section 3.4.5.7) and this number correlated well with the number of cuticular pores identified using SEM. No secreted material was ever observed either in or close to the secretory pores of *L. salmonis* using SEM.

With the confirmation that the stains used here were highlighting exocrine glands, and their intimately associated pores, some additional observations were made of the urosomal area using SEM. It was hoped that any differences between species in the pattern of pores could be characterised and used as a further taxonomical discriminant. A brief analysis of the integumental pore pattern of the urosome of *L. salmonis*, *C. elongatus*, *L. pectoralis* and *L. hippoglossi* however indicated that the DAB stain was not sensitive enough to highlight the cuticular pores associated with underlying glandular structures. Despite the fact that gland tissues stained positively their associated pore stained infrequently and inconsistently. It was also apparent that the pores draining such glands were not necessarily intimately located with them and so the pattern of integumental pores often did not match the pattern of glands as revealed by the DAB stain. As the evidence in this study has shown that TMB was sufficiently sensitive enough to highlight the integumental pores associated with glands it may be of greater utility as a stain to specifically highlight cuticular pores thereby making the analysis of a pore pattern more straightforward. It was apparent

though, that the identification of a urosomal pore pattern from SEM analysis was more problematic, the pores were frequently obscured by matter, possibly secreted material, on the cuticle surface especially in *L. hippoglossi* and *C. elongatus*. The cuticle of the urosome was also prone to distortion and wrinkling during SEM processing which further added to the uncertainty of the correct identification of the pore pattern. No reliable pore pattern of the urosome of either *L. hippoglossi* or *C. elongatus* were constructed in this study using either the TMB stain or SEM analysis.

3.5 DISCUSSION

This is the first comprehensive description of exocrine glands in caligid copepods. No comparably staining glands have previously been described in other parasitic marine copepods, freshwater copepods or free-living copepod species. It is clear from the results presented here that a population of glands, whose contents appear to have some peroxidatic properties, are a conspicuous feature of the caligid species *L. salmonis*, being present in all life-stages. Due to the large numbers of these glands present in individuals of all life-stages it can be presumed that they play a significant role in the biology of these animals. Similar glands, with similar distributions, are also present in the related caligid species *C. elongatus*, *L. hippoglossi* and *L. pectoralis*. The DAB and TMB stains revealed the same population of glands in all species.

All the DAB-staining glands located in both the first and second nauplius stages fulfil the criteria dictated earlier for glandular structures, and can therefore be described as glands. However, only the pores associated with the posterior glands could be located using SEM. This failure to locate the exit pores of the other gland groups was attributable to the shrivelling to which these stages were prone during processing for SEM examination. However, the results described here clearly suggest that all of the remaining gland groups do exit via integumental pores and they can therefore all be described as 'exocrine' glands.

3.5.1 Pattern, distribution and ontogeny of stained glands of *L. salmonis*

The results of this study demonstrate that some of the DAB-staining glands found in the nauplius stages of *L. salmonis* can be followed through all the subsequent developmental stages, and that some of those glands which first become evident in later stages i.e. chalimus, also persist throughout the remaining life-stages.

All glands located in nauplius larvae (anterior, lateral, median and posterior) were clearly distinguishable in the subsequent copepodid stage. The fact that these glands are present and are apparently actively secreting material in these free-swimming stages suggests that the role of the

secretions of these glands is of fundamental importance to the biology of the animals during these stages and does not necessarily represent a specific adaptation to parasitism. All the DAB-positive glands located in the NI larvae were also observed in unhatched nauplii within the egg capsule suggesting that the glands are functional even at this early stage but it is unlikely that they will be actively secreting material prior to hatching. Although, the ventral median gland of the nauplius stages had no obvious secretory pore associated with it (unlike the subsequent copepodid stage), as would be expected in the nauplius where the oral cone is not developed, the gland displayed a positive reaction to the DAB stain. This may indicate that a positive reaction of a gland structure to the DAB stain does not necessarily suggest that the gland is functionally active. Glands may be developed in stages prior to that in which the secretion is utilised and it is therefore possible that the DAB-positive material observed close to secretory pores of nauplius and copepodid stages may have been ejected from those glands as a result of immersion in the staining solution and may not be representative of the condition *in vivo*.

The filament-producing glands of *L. salmonis* larvae have been described previously (Bron *et al.* 1991, Bron 1993). Those studies described how the copepodid stage larvae attach to the fish host via a filament that was secreted by a complex of glands in the anterior region of the cephalothorax. The dorsal median gland observed here appeared to be secreting peroxidase-positive material into the filament duct of copepodid larvae (**Figure 3.8**) a function that has not before been described for a component of the filament-producing apparatus. The dorsal exit pore of the dorsal median gland of the copepodid found here has already been found by Bron *et al.* (1993), although those researchers restricted themselves to mapping the pores of the dorsal integument and made no comments on possible underlying gland structures. It seems reasonable to presume that the dorsal median gland of the nauplius stage is the precursor of the dorsal median gland of the copepodid as the dorsal median glands of both NI and NII larvae were seen to have DAB-positive ducts leading to and secreting onto their anterior surfaces. If this is the case then it suggests that the filament duct is already present internally in the nauplius stages although the function of the gland at this stage and whether it is actively secreting material is unknown.

The ventral median gland of this study appears to correspond to the median gland described for the copepodid stage of *L. salmonis* by Bron (1993). That author also stated that the median gland in the proximal region of the labrum stained positively with DAB. This ventral median gland appears, from its similar location, to be equivalent to the 'labral gland' described before for other species of copepod (Fahrenbach 1962, Boxshall 1982, Arnaud *et al.* 1988a,b, Nishida and Ohtsuka 1996). Possible functions of such glands are discussed in Section 3.5.4. The labral gland appeared to be absent, or at least did not contain a component reactive with DAB in the chalimus stages of *L. salmonis*. A prominent labral gland was observed in all other stages, including the non-feeding copepodid stage. Given the similar nature of the food material of all attached life stages it is difficult to account for this apparent absence of the labral gland in the chalimus stages. Perhaps, as chalimus were the only stage observed to have DAB-positive vesicles within their guts, they do not require an active secretion within the mouth-tube; the activity of the secretions being restricted within, and produced by, the gut itself.

The two stained glands identified at the base of the labium in chalimus stages and described as circum-oral glands appear, from their location, to match glands of *L. salmonis* described by Bron *et al.* (1993) and suggested by those authors to secrete mucus via pores located on either side of the oral cone. Labial glands in copepods have been described by Fahrenbach (1962), Gharagozlou-van-Ginneken (1977) and Miller, Nelson, Weiss and Soeldner (1990) and are likely to be present in many other species but have yet to be described. No stained glands however, were observed actually within the labium of *L. salmonis* in this study.

The AGC of the copepodid appeared structurally similar to that of the nauplius i.e. three connected units arranged in a stepwise orientation exiting via a pore, which was presumed to be present on the anterior margin of the cephalothorax close to the bases of the antennules. Bron *et al.* (1993) described two pores in the copepodid stage at the anterolateral margins of the cephalothorax which are possibly the exit pores of the AGC. The role of these large gland complexes is unclear. Few accounts of gland structures in larval copepods are available except those of Fahrenbach (1962) and Mercade (1982) who examined the antennal glands of larval *Diarthrodes cystoecus* and *Mytilicola intestinalis* respectively.

However, the antennal glands cannot be looked upon as true secretory systems but instead are attributed with an excretory function. This does not preclude the possibility that material excreted from such glands is not adapted to function in other ways beyond that of a waste material, such as the presumed pheromonal role of the urine of some female decapods (Kittredge *et al.* 1971). There does exist the possibility that the AGC are antennary glands but with no information regarding the ultrastructure of the AGC we cannot determine whether they are secretory glands or excretory units. Further detailed histology of these stages will be required to determine the function of these glands. The AGC remain present and active throughout the chalimus stages and gland structures that may represent these can also be discerned in the preadult and adult stages.

Glands in the thoracic legs are a prominent feature of copepodid larvae, the precursors of which are present, and stain positively, in the NII larvae. Ducts were frequently seen extending from these glands into the body cavity, and DAB-positive secretion was often seen adhering to the setae of the legs suggesting that the exit pores glands are located on the margins of the legs, a hypothesis confirmed here by SEM examination. Thoracic leg glands are a conspicuous feature of copepods that have been described for many other species (Fahrenbach 1962, Park 1966, Von Vaupel Klein 1982b, Bannister 1993a). Proposed functions for these glands are rarer than accounts of their morphology and have included pheromone secretion by Von Vaupel Klein (1982a) and secretion of compounds with drag-reducing properties (Bannister 1993a). Such glands have also been described in other crustacean Classes such as the Cephalocarida (Elofsson and Hessler, 1998) and the Branchiopoda (Dumont and Silva-Briano, 1997). Non-caligid species in this study generally displayed few DAB-positive glands in their thoracic legs, the exception being the harpacticoid species which possessed conspicuous staining glands. The presence of these glands in commensal and parasitic species suggests that their secretions are involved in processes not found, or are reduced, in free-living species. Despite the widespread occurrence of glands associated with the swimming legs in crustaceans their functions remain undetermined. It is possible that the thoracic legs could in some way serve to assist in the respiration of the animal, especially in host-attached species such as *L. salmonis* where water flow around the body is likely to be restricted. The movements

of the legs may circulate oxygenated water to those regions of cuticle where respiration is likely to occur although this must be countered against the possibility that any activity of the legs of attached specimens may jeopardise the security of attachment.

One interesting aspect of the glands present in nauplius and copepodid stages was the long ducts associated with them that extended into the body cavity. These ducts became less prominent as they narrowed distally. Whether these ducts were draining material from the body cavity into the glands prior to secretion cannot be stated without further detailed study of these stages. Chapman (1981) has shown that dissolved glucose in seawater was incorporated into the carbohydrate-rich secretion of glands in the urosome of *Neocalanus plumchrus*. However, he suggested that the glucose was taken up across the cuticle and not via the gut. The presence of internally directed ducts from glands in *L. salmonis* may suggest that those ducts are bringing compounds, absorbed across the gut, to these glands where they may then be assimilated into the secretion. These ducts may extend from the gut itself or from the haemolymph and further study is required to determine the true origin of such ducts.

With the moult to the first chalimus stage the specific pattern of DAB-positive gland distribution becomes less clear, and the identification of the glands present in the preceding larval stages becomes more difficult. In chalimus stage larvae the observed gland regions appeared to stain less intensely than in the preceding stages and there was great variability in staining between specimens of the same stage. Glands present in nauplius and copepodid stages that could be identified in the chalimus stage included the urosomal, anterior, lateral and thoracic leg glands. Notable differences occurred in the urosomal glands which now appeared to be comprised of two regions, c.f. one in nauplii and copepodids. The lateral glands of the chalimus appeared to be undergoing regression, in that they were only infrequently observed and did not appear to be present in all specimens. In addition to this the AGC of the chalimus larvae appeared to consist of only two, less clearly connected, regions c.f. three in earlier stages. The thoracic leg glands of chalimus larvae stained so faintly as to be almost impossible to discern. A possible reason for this may be that these legs are not essential for mobility at this stage where active swimming does not occur and where host attachment is achieved principally by the filament. This is in contrast to

the clearly discernible glands of the thoracic legs of those stages that are capable of active swimming such as the copepodid and adult stages and supports the hypothesis that these glands play a fundamental role in the swimming and mobility of these animals.

In addition to those glands which were described in both nauplius and copepodid stages, other DAB-positive glands were apparent in the chalimus larvae, most notable of which was the frontal gland complex. This structure, here termed the FGC, without doubt consists of some of the components of the filament-producing glands described by Bron *et al.* (1991) who termed them the 'filament producing glands'. They proposed that this gland system was responsible for the production of the attachment filament although the evidence suggested that the system was a complex structure secreting multiple compounds. The structure of the gland complex described by those authors corresponds to that of the structure seen here. The complex consisted of at least three distinct regions, which corresponded to the A, B and C glands as described by Bron *et al.* (1991) and Bron (1993). From the observations made here it seems possible that this gland complex incorporates the dorsal median gland of the nauplius and copepodid stages. Bron *et al.* (1991) did state that the filament-producing glands are present in the copepodid stage and are similar in structure but smaller and less well developed than in the subsequent chalimus stages. It is interesting to consider why the filament glands of adults appear to have an actively staining component within them when they would appear to be functionally redundant, as adults are not known to retain the ability to attach to the host via a filament, as preadults have been shown to do (Anstensrud, 1990), although very occasionally adult specimens *in vivo* are found attached via a filament (author's unpublished observations). These filament-attached adults have probably only recently undergone the final moult to the adult stage and will detach as soon as the new cuticle has hardened but whether functionally active filament glands are required during this brief phase is unknown.

The circum-oral glands that first become apparent in the chalimus stages may be involved in some way in the feeding activity of the larvae. No evidence suggesting that these glands secrete into the mouth-cavity was found, although their close physical proximity to the mouth-tube suggests that if the glands are not opening into the mouth cavity, then they may at least be opening close to it. The circum-oral glands

described here for the preadults glands appear to match the description of the 'lateral' glands described for *L. salmonis* by Bron (1993), although that author stated that those glands had a negative reactivity with DAB. This inconsistency in the results of the staining between this study and that of Bron (1993) may be explained in terms of the variability in staining between specimens and the limited use of DAB staining in the study of Bron (1993).

After the moult to the chalimus II stage, a further pair of large DAB-positive glands became apparent. These glands, here termed the postoral glands, were a conspicuous feature of the gland population, being some of the few glands on the ventral surface, and were present in all further chalimus stages. Glands opening on the maxillary somite have been recorded for several species of copepod (Lowe 1935, Park 1966, Boxshall 1982) where they were termed the 'maxillary glands' and were attributed with an excretory function c.f. a secretory one. The maxillary glands are thought to function as filtration units and are responsible for the removal of metabolites from within the body. The postoral glands may undertake this function in *L. salmonis* but the only evidence for this hypothesis is the similarity in their location to those glands in other species and the fact that the postoral glands of *L. salmonis* appear to discharge via pores located near the bases of the maxillules / maxillae.

Preadult stages of *L. salmonis* were found to possess all the glands described for the preceding chalimus larvae, although differences in the structure of some of the glands were evident. In particular, the AGC and the urosomal glands appeared to have become more complex structures by this stage. The urosomal glands now consist of, what appears superficially, to be several lobes, or distinct regions of the gland. The urosomal glands reach their fullest development in the adult stages where they consist of six pairs of bilaterally symmetrical lobes or regions.

The lateral glands that were a consistent feature of the gland population of the nauplius, copepodid and chalimus stages are no longer apparent in the preadult and adult stages. These glands had become much less conspicuous by the chalimus stages and were apparently undergoing some form of regression as the chalimus larvae progressed through the moults of that stage. This regression suggests

that the lateral glands may have become defunct by the preadult stage, their role either no longer required or undertaken by other glands.

The dorsal surface glands (DSG) begin to become apparent in the chalimus stages but they do not form a conspicuous and recognisable component of the gland population until the preadult stage has been reached. These glands become more prolific in number as the copepods progress through the subsequent moult to the preadult II stage and this progressive development of the DSG continues into the adult stages where the full compliment of glands is found. As the DSG do not reach their fullest development until the adult stage it can be suggested that their primary role is not associated with the sexual behaviour of the animal as mating takes place before the female moult to the adult stage (Wootten *et al.* 1982). Mauchline and Nemoto (1977) have also described ontogenetic increases in numbers of integumental pores in calanoid copepods and state that pore patterns do not represent secondary sexual characteristics although components of the pore system may be utilised in the recognition of conspecifics.

To summarise, the identified population of DAB-positive glands of *L. salmonis* consist of a definable and characteristic set of complexes, some of which can be observed in all life-stages whilst others develop sequentially and reach their full development only in the adult stages.

3.5.2 Pore signature pattern evaluation

Use of TMB and SEM in conjunction served to confirm that the DAB and TMB stains are selectively highlighting exocrine glands in the species studied. Examination of both preadults and adult *L. salmonis* using SEM revealed a pattern of integumental pores matching that of the DSG identified using the DAB and TMB stains indicating that the regions of positive staining observed represented glandular structures underlying cuticular pores.

The intention of this part of the study was to assess the suitability of DAB to facilitate the rapid identification, and thereby discrimination, of caligid species. The evidence of this trial suggests that the DAB stain, as used in this study, is not sufficiently sensitive to resolve the PSP of the caligid species examined. The PSP of the urosome was never elucidated using DAB and its description relied solely

upon the use of SEM. SEM was also not straightforward as several specimens needed to be examined as not all the pores were visible in any one specimen due to abundant bacterial growth and, in the case of *L. hippoglossi*, a thick layer of secreted material obscuring the surface of the cuticle. TMB may be more suitable for this purpose as its greater sensitivity, with respect to DAB, meant that integumental pores were frequently observed due to secreted material within them being stained. However, its utility as a means of rapid identification is limited because of the unstable nature of the resultant coloured reaction product.

One interesting aspect of pore morphology revealed by the SEM study was the failure to locate any material either inside or closely associated with the secretory pores of *L. salmonis*. Previous researchers (Briggs 1978, Hipeau-Jacquotte 1987, Boxshall 1982, Bannister 1993, Williams-Howze 1996) have found, using SEM, what they presumed to be secreted material in and around secretory pores. It is possible that secreted material which may be present close to the pores in this *L. salmonis* may either be washed away during the SEM processing or fails to be fixed during the process. The presence of positively staining material identified on the cuticle during the DAB / TMB staining process may have occurred as a result of the glands being evacuated, either deliberately by the louse since they are stained alive or, as is more likely, as a consequence of immersion in a staining solution of differing osmolarity. Work presented in Chapter 7 examines the presence of surface adhering material more closely.

3.5.3 Comparison of gland distribution to other species

The data obtained from the comparative staining trials with other copepod species provides information that may have implications for the understanding of the evolutionary development of copepod species, or their adaptation to parasitism. The related caligid species *C. elongatus*, *L. hippoglossi* and *L. pectoralis* all displayed essentially similar populations of glands to each other and to that of *L. salmonis*. Although these four examples constitute only a fraction of the species of these two parasitic genera the implication is that such glands may be a widespread feature of these copepods. Slight differences were apparent between these closely related species in terms of their DAB-stained gland populations, but these

were essentially superficial and were restricted primarily to the actual numbers of the DSG population. These differences may be attributable solely to the marked differences in size between these species.

Free-living calanoid copepods possessed very few glands with such staining characteristics. Both species examined in this study showed only a very restricted population of glands that stained with DAB, most notable of which were those in the buccal cavity region that may correspond to the labral glands described for other free-living copepods and aquatic crustacea, and described here for *L. salmonis*. Both *E. affinis* and *A. tonsa* have been shown (Hoffmeyer and Prado Figueroa, 1997) to have pores in the integument of both the labrum and labium suggesting that exocrine glands secreting into the mouth are present in these species.

Algal-associated commensal copepod species (*P. hibernica*, *H. chelifer*, *D. tenuicornis* and *D. vulgaris*), in keeping with the caligid species, displayed significant positive staining with the DAB stain. All species examined showed numerous glandular regions that gave a positive response to the stain. The glands of the anal segment and legs of the harpacticoid species are almost certainly homologous with the urosomal glands and thoracic leg glands identified in all the caligid species examined. Williams-Howze (1996) described how the glands in the urosome of *H. nunni* were composed of two structurally distinct regions with distinct histochemical attributes, a dorsal sac containing a homogenous mass and a ventral sac containing globular secretion. These tissues were shown to secrete via separate pores in the urosome. Large integumental pores in the antepenultimate segment of the urosome of the harpacticoid species *Diarthrodes nobilis* have been described by Hicks and Grahame (1979) and probably represent the secretory pores of the urosomal glands identified in the species examined here. Those authors also described how mucus glands present in the cephalothorax of *D. nobilis* secrete via vents located at the bases of the first pereiopods, which might suggest that the ventral cephalothorax glands observed in *P. hibernica* and *H. chelifer* are in fact mucus glands. The harpacticoid copepod *Pseudostenhelia wellsi* was shown by Chandler and Fleeger (1984) to secrete mucus from glands present in the ventral regions of the cephalothorax. This mucus was demonstrated to be used to aid in the construction of mucus tubes that the copepods used as a refuge. The natural behaviour of algal-associated harpacticoid species is poorly

understood so it cannot be stated whether the species examined in this study build mucus capsules as other species of this family are known to (Fahrenbach 1962, Hicks and Grahame 1979, Williams-Howze *et al.* 1987). Hicks and Grahame (1979) suggest that nauplii of members of the Thalestridae family directly rely on host tissue for their sustenance and that adults remain associated with macroalgae for the duration of their life-cycle but data pertaining to the behaviour and source of nutrition of adults is scarce. Fahrenbach (1962) makes particular mention of the gland systems of the harpacticoid species *Diarthtrodes cystoecus*. That author describes glands to be present in the cephalothorax, all legs and the urosome as well. The description of exocrine glands in harpacticoid copepods in the present study is therefore not unique, but the staining characteristics of those glands described in this study represents a unique addition to our knowledge of these glands, the significance of which needs to be ascertained.

The positions of the staining glands of the ventral cephalothorax of these harpacticoid species also corresponds closely to the podocytic excretory glands of the cephalocarid *Hutchinsoniella macracantha* Sanders as described by Hessler and Elofsson (1995). Those authors described how these glands, located near the base of each protopod, possessed morphological characteristics indistinguishable from that of maxillary and antennal glands and they proposed that the glands were responsible for storage of metabolic waste products. The DAB-staining glands observed in the cuticular projection of the anterior cephalothorax of *D. tenuicornis* appear to correspond to the 'frontal glands' of the misophrioid copepod *B. palliata* described by Boxshall (1982). *B. palliata* was described as having five pairs of glands beneath the cuticle of the anterior surface of the head that are suspected of secreting their contents onto the anterior body surface. Boxshall (1982) describes one pair of frontal glands of *B. palliata* as being homologous to the organ of Gicklhorn (see Elofsson, 1966) whilst the remaining four pairs are described as being secretory rather than sensory. The role of this gland in *D. tenuicornis*, in keeping with the other identified glands, remains unresolved.

The DAB stain failed to reveal the presence of similarly staining exocrine glands in *D. magna* in this study. However, Mauchline (1977) demonstrated, using alkaline digestion of the integumental organs, the presence of numerous pores in the body cuticle of *Daphnia* sp. suggesting that species of this

genus possess abundant exocrine glands. The evidence from this study and that of Mauchline indicates therefore that *Daphnia* possess exocrine glands but have not evolved secretions with staining characteristics similar to those of caligid species.

In contrast to the species of caligid copepod studied, all other crustacean specimens examined showed negligible and inconclusive staining with the DAB and TMB stains suggesting that exocrine glands with peroxidatic components are a feature restricted to copepod species, but particularly to commensal harpacticoid species and parasitic caligid species. Failure to detect any regions of positive staining in *A. foliaceus*, *M. rosenbergii*, *A. salina* and *D. magna* suggests that either no exocrine glands are present or that they do not contain the same active components of those identified in caligids, harpacticoids and to some extent calanoids. The latter hypothesis seems the more reasonable presumption as cladocerans have been shown to possess numerous cuticular pores (Mauchline, 1977), presumably some of which are associated with underlying glands, whilst branchiurans of the genus *Argulus* have been shown to possess at least one exocrine gland closely associated with the mouth parts (Shimura and Inoue, 1984).

TMB was not tested, but not utilised in full for any species other than *L. salmonis*, but its application in further studies may resolve details of gland and duct morphology not revealed using DAB in this study.

3.5.4 Hypothetical functions

The paucity of specific data regarding the composition of the secretions of these glands at this point precludes the accurate prediction of their function, but from the data obtained from this study of their distribution, and our knowledge of the staining characteristics of DAB, some tentative hypotheses can be put forward as to their role.

As all of the gland groups stain similarly this suggests either that all the glands have the same function or that some component of the secretion is a ubiquitous component of secretions with different functions. Bussolati (1971) demonstrated that sulphated mucus groups display a positive reaction with

the DAB stain which might suggest that the positive responses observed by species stained in this study are being caused by a mucus component of the secretions. Mucus has been identified as a component of the secretions in most copepod gland systems where histochemical analyses were made. Further analysis is required to determine whether the stain here is reacting merely with sulphated mucus groups.

The presence of defence factors, such as lysozymes, Ig, proteases, complement etc., in fish mucus has been previously demonstrated by Hjelmeland (1983), Woo (1991) Shephard (1994) and Yano (1996) and such compounds will be substantial barriers to be overcome by attaching copepodids and subsequent host-associated stages. It may be possible that the exocrine glands of caligids secrete factors to counteract these defences. If this is true then it is likely that these factors must continue to be secreted by all subsequent life-stages. Why then are there fewer staining glands in the chalimus and preadult stages? Recent research by Ross *et al.* (2000) has demonstrated an increased protease activity in the epidermal mucus of salmon parasitised by *L. salmonis*. Those authors also suggested that *L. salmonis* itself may secrete trypsin-like proteases that may serve to disrupt the cell membranes of the host fish, facilitating feeding by the parasites.

Other hypothetical considerations can be made at this stage in the absence of histochemical proof regarding the presence of DAB-staining mucus in the gland tissues. Peroxidase enzymes are known to be indirectly linked to host immunosuppression as enzymes with a peroxidatic function are known to be implicit in prostaglandin synthesis as part of the cyclooxygenase pathway (Gurr and Harwood 1991, Rowley *et al.* 1995). Prostaglandins are derivatives of polyunsaturated fatty acids that display a variety of physiological and pharmacological properties. They have been shown to be important components of the saliva of haematophagous parasites where they are postulated to function in several ways to mediate the host immune response, including; blood coagulation inhibition, vasodilation, immunosuppression, and inflammation reduction (Bowman *et al.* 1996). It does not seem unreasonable to presume that, as infective larvae attach to the host fish solely via their filament (following initial attachment and moult to chalimus), that modulation (reduction), of the host immune response would be beneficial to the parasite. The fact that a DAB-positive secretion appears to be present in the filament duct of the copepodid, the

filament of the chalimus and the FGC of the preadult stages may also suggest that the secretions serve some role in host immune response modulation. Such a function of these gland secretions might also be of benefit in the labral gland as its exit duct, which passes into and secretes onto the anterior surface of the labrum, (**Figure 3.8**) would be in close contact with the host tissue during feeding of the host-associated stages (Kabata 1974, Jones *et al.* 1990, Jónsdóttir *et al.* 1992, Andrade-Salas 1997). A secretion which served to reduce the host immune response, would be beneficial in such an area of presumed high host immune activity.

It may be that, as these parasites remain highly mobile during their preadult and adult stages that they are never subjected to a specific host immune response as an immediate and direct effect of their attachment and feeding activities. Immune response chemicals are likely to be widespread within the host blood, tissue and epidermal mucus as a result of infection by these parasites. Modulation of the host immune response via peroxidase-derived prostaglandins would still be beneficial if effected by prostaglandins released 'non-specifically' from the parasite. The prostaglandins may be able to enter the fish host via its damaged integument and bring about a resultant decrease in parasite inhibitory compounds. This suggests that the parasites are not trying specifically to protect themselves from host immune factors, but instead maintain a tolerable environment which is likely to be of benefit to themselves and conspecifics and which will therefore maximise reproductive success. This is in contrast to the injection of prostaglandins from the salivary glands of ticks with a prolonged host-attachment phase at one site (Bowman *et al.* 1996).

The frequent observations of internally extending DAB-stained ducts associated with the exocrine (e.g. lateral, thoracic leg and urosomal) glands of *L. salmonis* in this study might suggest that such ducts are transporting compounds from some undisclosed source to the gland tissues. Radio-labelled isotopes were shown to be sequestered in the glands of the urosome of the calanoid copepod *Neocalanus plumchrus* by Chapman (1981), who proposed that the sequestered material was taken up across the integument. The possibility that such large, active secretory units, as the glands of *L. salmonis* can be presumed to be, require a direct supply of nutrients from the gut, or the haemolymph, should be

considered. The distal point of the gland ducts of *L. salmonis* may indeed be the gut, as all of the internally extending ducts were observed to be passing into the cephalothorax close to where the gut is located. Stained, internally extending ducts were observed in the nauplius stages too which may preclude the theory that these ducts are transporting nutrients from the gut to the glands, although it is possible that the ducts transport nutrients from the stored yolk reserves of these lecithotrophic stages. Why these ducts should give a positive reaction with the DAB stain is difficult to explain. The positive reaction suggests either that the nutrients acquired by these ducts have some peroxidatic properties or that the glands may secrete in two directions, both internally and externally. A further possibility is that some DAB-positive compound is involved in transporting the material through these ducts. Further research should focus on the nutrient supply of these glands.

The secretions from the identified glands might also be hypothesised to be involved in neutralising free radicals which are likely to occur as a result of host tissue damage caused by feeding. This phenomenon has been witnessed in caterpillars (Felton and Duffey, 1991) where peroxidases were found to be responsible for neutralising free radicals which occur as a result of plant tissue damage. Neutralisation of such compounds was proposed to be necessary to protect the lining of the gut from the harmful effects of such compounds. This may also explain why DAB-positive regions were present in the mouthparts of all caligid copepods examined and also in the calanoid species examined, as all of these species are likely to encounter free radicals as a result of tissue disruption of host or prey items caused by feeding. DAB-stained specimens of *A. tonsa* and *E. affinis* also showed positive staining within their guts, but whether this positive reaction represented secretions from the buccal cavity glands or was released from ingested food cannot be stated on the basis of the study undertaken here. As *A. tonsa* and *E. affinis* are omnivorous species they will be exposed to the products of uncontrolled degradation from both plant and animal tissues as a by-product of their feeding activities and may therefore require some form of protection from free-radicals. Some specimens of *L. salmonis* chalimus stages also showed distinct staining of cells in their midgut region suggesting that the enzymes have some role to play either in digestion or in protecting the cells of the gut from harmful chemical species. This staining was only seen

in a very few specimens and was never observed in any stages other than chalimus. These hypotheses though fail to explain why such glands are present, active and apparently so well developed in non host-associated larval stages as seen in this study, although glands may develop in advance of the stage in which they are functional.

It seems unusual that the labral gland of *L. salmonis* exits via a pore on the external, anterior surface of the labrum because, if free radicals are released from damaged tissue, it would seem more logical to release the secretion inside the buccal cavity where it will be able to interact directly with ingested food material. Light microscope and SEM observations showed the existence of a pore in the centre of the anterior face of the labrum but there remains the possibility of there also being an internally-opening pore to deliver the secretion directly into the mouth-tube where it can interact with food material as in *C. typicus* (Arnaud *et al.* 1988b). It may still be possible that secretions released from the anterior face of the labrum will pass backwards and bathe the feeding wound in secretion, and, as prostaglandins are known to be effective at very low levels, then the potential for the labral gland to be secreting such immunomodulatory compounds still exists.

Roles suggested for labral glands of copepods include lubrication (Arnaud *et al.* 1988b), entangling of food matter (Von-Vaupel-Klein and Koomen, 1994), and salivary activities (Gharagozlou-van-Ginneken 1977, Arnaud *et al.* 1988b), implying an active role in the digestion of food material. It may be possible that the labral and circum-oral glands, either in conjunction or separately, serve such functions in *L. salmonis*. These glands appear to correspond to glands previously described by Bron (1993). Using a polychrome stain, that author found a set of two pairs of glands with a similar distribution near the mouth-tube of this species. If this is indeed true then the most anterior pair of glands found in this study correspond to Brons lateral glands and the posterior pair of glands, at the base of the labium, to his mucous glands (Bron *et al.* 1993). A definite statement regarding the classification of these glands cannot be made until ultrastructural studies have been undertaken. Nishida and Ohtsuka (1996) suggest that labral glands of the calanoid family Heterorhabdidae secrete toxic or anaesthetic substances to help these small copepods maximise their feeding potential in the sparsely populated deep sea. Secretions with

toxic / anaesthetic actions appear to be an adaptation restricted to members of the Heterorhabdid family and such a role for the secretions of the labral gland of *L. salmonis* is extremely unlikely.

The functional significance of the posterior / urosomal glands remains unclear although glands in the urosome have been described for several species of copepod (Fahrenbach 1962, Park 1966, Gharagozlou-van-Ginneken 1979, Boxshall 1982, Williams-Howze 1996). These studies suggested that the urosomal glands of marine copepods produced multi-component secretions consisting of, at least, a mucopolysaccharide fraction and a proteinaceous fraction. These different glandular products were produced either by separate glands within the complex or by regions of the same gland in the urosome. The urosomal glands of all stages subsequent to the copepodid in *L. salmonis*, were apparently composed of multiple units, or regions, which might suggest that separate products are secreted by each unit. The functions of these glands in other species still remains undefined although Gharagozlou-van-Ginneken (1979), using TEM, noted a cyclical nature to their activity which leads to the assumption that the glands were in some way involved in sexual activity. The secretions of the urosomal glands of the harpacticoid species *Heteropsyllus nunni* Coull were suggested by Williams-Howze (1996) to produce the secretion required for cementing together sand grains to form the cyst in which the animal lives during its diapause period. Urosomal glands were also described in the harpacticoid *D. cystoecus* by Fahrenbach (1962), but that author made no statement regarding the functional significance of those glands.

No structures homologous with the lateral glands described in this study have been described for any other species of copepod. As such it is difficult to ascribe a role to these glands that are prominent in all life-stages up to the preadult stage. It is possible that these glands have some role in excretion of metabolites similar to the widely described antennary glands. However, the fact that in the larval stages where they occur they are located far from the antennules, seems to argue against this theory, as nearly all records of larval crustacean excretory organs place them close to the antennules. Detailed histological analysis of this gland complex is required to resolve its true structure and its possible function.

It is interesting to note that the majority of the DSG are located around the peripheral regions of the animal i.e. close to the marginal membrane and around the genital segment, areas which SEM

examination revealed to possess large numbers of sensilla. It is possible that some, or all, of these sensilla are innervated as has been shown by Bron (1993) for copepodid larvae of *L. salmonis*. It is known that peroxidases are present in neurons (Rainbow, 1996), a fact which would be consistent with this theory if the point of innervation of the sensilla were showing the positive reaction to the DAB stain. It would be logical for the sensilla of the animal to be innervated as these regions of the animal will first come into contact with its external surroundings, and an ability to detect objects coming into contact with these regions would be beneficial in terms of the animal being 'aware' of its environment. However, there were often quantities of DAB-positive material adhering to the marginal membrane itself and we should consider the possibility that this material may have originated in the DSG located intimately with the marginal membrane. Epibionts such as the ciliate genera *Ephelota* and *Epistylis* and algal and fungal species were also seen to give a positive reaction to the DAB stain although this may have occurred if these epibionts were covered with the secretion from the glands. The evidence from the SEM study would suggest that it is not the sensilla that give a positive reaction to the DAB stain but tegumental glands close to the cuticle.

It was noted that the thoracic leg glands, which were conspicuous in the copepodid stages of *L. salmonis*, became almost indistinguishable in the chalimus and preadult stages, yet were again prominent in the adult stages, where large quantities of DAB-positive material was frequently found adhering to their setae. This apparent reduction in the activity of those glands in the chalimus and preadult stages may suggest that the secretion from those glands is involved in some way in the maintenance of the setae in the stages where leg function is most important. We can presume that in the free-swimming copepodid stages (which can survive in the water column for as long as 13 days (Wootten *et al.* 1982)) before attaching to a host, the thoracic legs are vital to the success of the larvae as they represent their sole means of propulsion for seeking out a suitable host (Bron *et al.* 1991). Again, in the adult stages, where there are no further moults to renew the setae, the glands are again prominent and apparently very active. This could suggest that in the chalimus stages which are attached to the host by a filament, and therefore do not have any requirement for natatory propulsion by the thoracic legs, and in preadults where there is a relatively short

period between moults, the gland secretions may not be necessary. This would explain the apparent regression of the glands, or at least their reduced activity, in these stages. A further possible function of these glands that would be especially beneficial on the thoracic legs is if the secretions were to serve to prevent the epidermal mucus of the host fish from clogging the natatory setae of the legs. Such a function would also be of utility for those glands that secrete onto the terminal setae of the urosome and the marginal membrane. If the body and appendages of a louse were to become clogged with host mucus then its ability to attach to the fish and to move, either on the host or off it, would be seriously impeded with all the attendant risks that such impairment would bring. The gland secretion could act either directly by reacting with the host mucus or indirectly by preventing the host mucus from adhering to the animal in the first place.

The secretions of the thoracic leg and urosomal glands may also serve, in some way, to maintain the setae of those structures. One possible way this could be accomplished is if the secretions possess anti-bacterial properties. Anti-bacterial gland secretions have been isolated from several species of aquatic insect, where the secretions served to prevent the build-up of bacteria on essential setae (Kovac and Maschwitz, 1991). Chitinophagic bacteria are commonly found attached to the cuticle of marine copepods (Sochard *et al.* 1979, Dumontet *et al.* 1996), where presumably they derive their nourishment to the detriment of the animal. Bacteria were commonly found adhering to the cuticle of *L. salmonis* and *C. elongatus* in this study as has been shown before for these species (Nylund *et al.* 1991, Nese and Enger 1993, Nylund *et al.* 1993). The fact that bacteria are commonly found attached to copepod cuticles does not necessarily preclude an anti-bacterial function to gland secretions, the numbers of bacteria present may represent a reduced burden compared to that which would occur if no secretions were active in either killing the bacteria or in preventing their initial establishment. The greatest proliferation of the DSG in caligid species examined in this study occurs around the periphery of the cephalothorax close to the marginal membrane. As the marginal membrane is a cuticular structure that will not be renewed after the final moult to the adult stage it, also, may benefit from a secretion that serves to protect it from bacterial, and other, infestation.

Anti-bacterial / anti-foulant secretions may also act to prevent smothering of cuticle resulting in impaired respiration. Either the direct or secondary effect of this reduced epibiont burden may be to decrease hydrodynamic drag thereby enhancing streamlining. This seems a more likely proposal than cuticle repair / maintenance as presumably the ventral surface cuticle will require as much maintenance as the dorsal surface yet it has no regions equivalent to the DSG. This disparity may be due to variations in cuticle thickness between the dorsal and ventral surfaces; the dorsal cuticle of *L. salmonis* being substantially thicker than the ventral (author's unpublished observations).

A lubricatory action of the gland secretions is similar in that it may actually promote the smoother passage of water over the cuticle. Von Vaupel Klein (1982a) states that there is a direct correlation between the numbers of secretory gland pores in the integument and swimming speed in copepods although larger copepods are likely to swim faster than smaller ones and Von Vaupel Klein makes no allowance for this factor. This statement suggests that the secretion from these pores directly increases the ability of the copepod to swim rapidly. What is unclear from this statement is how this increased swimming ability is effected: decreased epibiotic burden and resultant decreased drag or lubrication with a hydrophobic layer such as that seen in whirligig beetles (Vulinec, 1987). It is possible that the long setae of the caudal rami and the thoracic legs are areas of high hydrodynamic drag and therefore a secretion that allowed water to move across them with less resistance may be beneficial. It is noteworthy that in *L. salmonis* and *C. elongatus* those areas with long setae tend to have large, conspicuous gland complexes closely associated with them. Research suggests that adult male *L. salmonis* are more likely to swim freely off the host fish, presumably to seek out potential reproductive partners (Ritchie, Mordue (Luntz), Pike and Rae 1996, Hull, Pike, Mordue (Luntz) and Rae 1998) and it is possible therefore that they may display greater adaptations to swimming than their female counterparts; a hypothesis that is more fully investigated in Chapter 7 of this study.

DAB-positive material, presumably originating in the urosomal glands, was more frequently found adhering to the terminal setae of the urosome of male preadults and adults whilst females very rarely had such material adhering to their setae. The reasons for this phenomenon remain unclear. Both

males and females appear to possess morphologically similar urosomal glands, which might suggest that they serve a similar function in both sexes, although this is by no means certain. The fact that the urosomal glands appeared to be more active (suggested only by their more intense staining reaction) in males may indicate that the secretions from these glands have a sexual function. Gharagozlou-van-Ginneken (1979) has suggested that the urosomal glands of the copepod *Tigriopus brevicornis* are involved in sexual activity, and pheromonal attraction between conspecific crustacea has been widely suggested (Kittredge *et al.* 1971, Katona 1973, Dunham 1978, Snell and Carmona 1994, Lonsdale *et al.* 1996, Kelly *et al.* 1998). In all instances where pheromonal involvement in sexual activity has been suggested, it has been the female that has been proposed to be the producer of the pheromone, an occurrence widely noted amongst insects. It would appear either that female *L. salmonis* do not produce as much secretion from the urosomal glands as males or that the secretion from their glands behaves in a different manner to that of the males. It could also be that the secretions of the male urosomal glands serve to deter other males from attempting to copulate with females already attended by males.

Areas of damage to the cuticle frequently showed DAB-positive staining that appeared not to be associated with any identified glandular structures. This staining may have arisen either as a result of interaction of the DAB stain with internally-expressed components of the cuticle or underlying tissues, or possibly also as a result of secreted material pooling in areas of damage. Damage may also have disrupted underlying gland tissues or ducts and released positively staining secretion into the area of the damage. Experiments could easily be designed to test these various hypotheses.

3.5.5 Evolutionary significance

From the small number of species examined in this study it appears that there may be a correlation between the adoption of a commensal / parasitic lifestyle and numbers of glands with such characteristics described here. It is apparent that copepods of the Family Caligidae possess the greatest number of such staining glands of all the species examined here and that commensal species have fewer such glands than caligids but more than copepods of the free-living Order Calanoida. Whether this apparent trend holds

true over the separate orders of parasitic, commensal and free-living species is yet to be resolved. Importantly, whether this apparent trend reflects specific adaptation in terms of gland development or represents pre-adaptation cannot be stated with the data derived from the staining trial alone. It is essential to remember that not all characters found in the evolutionary descendants of a group of animals (such as the caligids, derived from harpacticoid ancestors) represent specific adaptations, they may be pre-adaptive characters or may only be 'evolutionary artifacts'.

It is known that the epidermal mucus of fish contains many components designed to defend against infection and it may be presumed that animal's parasitising fish will face an array of host-origin defence mechanisms aimed at removing the parasite. Woo (1991) and Shephard (1994) provide comprehensive reviews of the defensive components of fish epidermal mucus. Various defence systems have been evolved in parasites to help overcome these difficulties and the DAB-positive glands of *L. salmonis* and *C. elongatus* may represent some such development and may explain the absence of such glands in non-parasitic copepods.

Some of the possible ways that gland secretions, and in particular peroxidase secretions, could be of benefit to a parasitic species have been discussed here. However, little information exists pertaining to the behaviour and life-history of the copepod Order Harpacticoida. The presence of similarly staining glands in these commensal harpacticoid species may be a reflection of the environment that these species inhabit. Although they are not parasitic, their close association with their algal hosts means that they are likely to be subjected to various chemical defences expressed either internally or externally by the host species. This intermediate stage between free-living and parasitic copepod species may have first developed the gland secretions that reach their greatest development in parasitic species, as they were 'pre-adapted' to the parasitic mode of existence.

Glands in the thoracic legs of marine copepods have been described by many authors (Fahrenbach 1962, Park, 1966, Von Vaupel Klein 1982, Bannister 1993) and can be presumed to be a widespread feature amongst these animals. This study failed to show the existence of DAB-positive glands in the thoracic legs of the free-living copepod species examined, but we should not discount the likelihood that

glands were present in these species but did not react with the stains used here. As the cephalocarida represent the primitive benthic ancestor of the free-living copepods it is interesting to consider whether the podocytic excretory glands observed in *H. macracantha* by Hessler and Elofsson (1995) correspond to the thoracic leg glands of caligid and free-living species.

Many of the DAB-positive glands located in *L. salmonis* secrete via integumental pores located close to the periphery of the copepods body i.e. close to the point of contact between the parasite and the host. Such positioning would serve to bring the secretion from these glands into close contact with the host body surface, which must be fundamental if these chemicals are to have an effect on host physiology. There was a pronounced dichotomy in DAB-positive gland numbers between the dorsal and ventral surfaces of the cephalothorax. The hypothesis of prostaglandin production proposed earlier to explain the DAB staining of the median glands of the copepodid and the labral and circum-oral glands of the later stages may also serve to explain the role of the other glands present in the caligid species. If prostaglandin synthesis is one of the functions of the peroxidase in these glands then it may also be beneficial to have prostaglandins produced / secreted in areas other than the feeding appendages.

Legs such as the maxillipeds and the antennae are known to cause damage to the host integument through their grappling actions (Kabata and Hewitt, 1971). Such damage to the host would be likely to elicit host defence responses and if the gland secretions have 'host-active' properties we might therefore expect to find DAB-positive glands associated with these legs. However, it is highly improbable that a species will evolve entirely new glands in previously gland-free regions to accommodate new behavioural traits such as the establishment of a parasitic mode of life. Secretions of extant gland systems may evolve new functions in response to such a change in behaviour and as such we would expect caligid copepods to display similar sets of glands to those found in their free-living relatives i.e. the free-living ancestor of caligids was pre-adapted to a commensal, or parasitic life-style. This indeed appears to be the case. With the exception of the filament-producing glands (FGC) all the glands identified in the caligid species studied here can be correlated to gland groups identified in free-living copepods. Urosomal, thoracic leg glands, tegumental glands and antennary and maxillary glands have been widely reported in many

different groups of copepod and can be presumed to be present in most, if not all, species. Slight modifications, which probably represent habitat and behavioural differences, in these glands are apparent but there is an obvious homology between these glands in all species where they have been described. The striking disparity between numbers of glands found on the dorsal and ventral surfaces of the cephalothorax of caligids is not so surprising when it is considered how their morphology is derived from that of a typical copepod body form. The dorsoventral compression of the cephalosome and associated pedigerous tagmata has resulted in a greatly increased ventral surface area as the lateral regions of the cephalosome were elevated dorsally. The net result of this is that, what was originally a very narrow ventral groove with closely packed appendages has become a large shield of expanded cuticle.

3.6 CONCLUSIONS

It is clear that both *L. salmonis*, *C. elongatus* and other caligids display many exocrine glands that may contain either some form of peroxidase enzyme or sulphated mucus as evidenced by stained material adhering to setae. These glands occur in identifiable localities through all life-stages. However, the precise nature of this secretion and its function at this time remain unknown. It is impossible at this stage to say whether the secretions from all of these glands have the same function, or even have the same composition. The evidence here suggests that one or more enzymes with peroxidatic properties may be a component of the gland secretion, but this does not preclude the possibility that there are other components to the gland secretion. It is possible that peroxidases are present in all gland secretions regardless of their function i.e. peroxidases may be a ubiquitous component of copepod gland secretions. The variation in staining between specimens of the same stage may result from a cyclical changes in gland activity, a phenomenon noted for gland systems in several species of copepod (Gharagozlou-van-Ginneken 1979, Hipeau-Jacquotte 1987).

It has been shown here that many of the gland systems of the larval stages of *L. salmonis* are also present in the adult stages indicating that these glands are probably an essential component of the physiological system of the animal and are not likely to be stage-specific adaptations. Some of these

gland systems can be seen to increase in complexity as the animal develops. As the DSG do not reach their full development until the adult stages it may be surmised that they fulfil some role which is essential to the physiology or behaviour of the adults that was not necessary (or was undertaken by other means) in earlier life-stages. A role in the sexual behaviour of these animals for the DSG can probably be ruled out as mating takes place before the females undergo the final moult to the adult stage. No evidence of a sexual dimorphism in the main gland populations was found, as would be expected if these glands are not involved in sexual activity. It is possible though that some form of pheromonal attraction is mediated by secretions of these glands in the absence of a dimorphism.

No evidence for a pronounced and consistent sexual dimorphism of the DSG of the cephalothorax was ever found but, because of the inconsistent staining apparent between specimens, it is difficult to make any firm statements regarding this. It is certain though that the pattern of DSG distribution of the genital segment is markedly different between adult males and females of both *L. salmonis* and *C. elongatus*. Glandular pores in the female genital segment may function either as a physical pattern for males to feel during pre-copulatory attachment (as suggested for calanoids by Mauchline and Nemoto 1977, Park and Mauchline 1994) or may be responsible for secretion of pheromonal substances. As mating has taken place before adulthood, this pattern must be evident before adulthood for this theory to work.

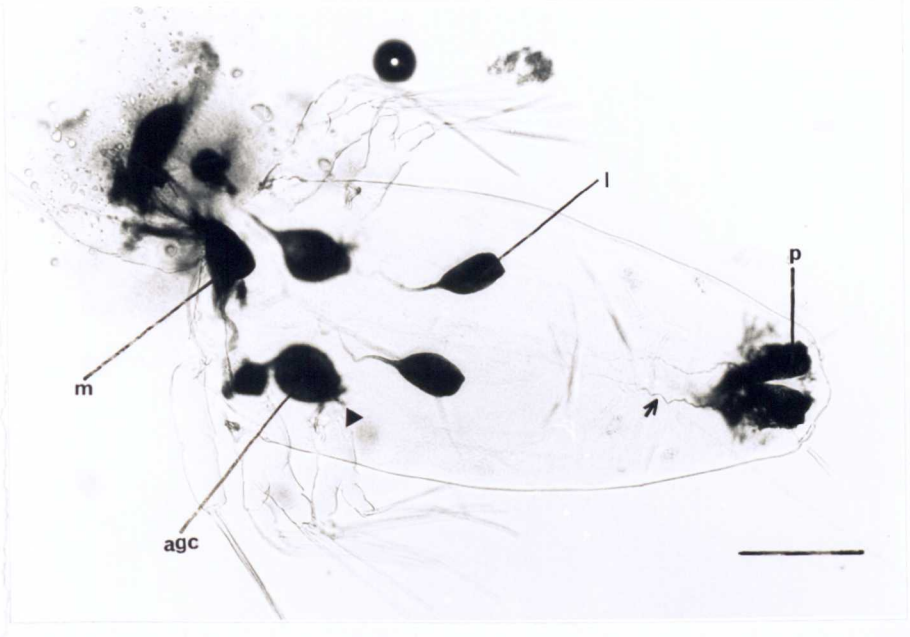
The attempted analysis of the PSP of the genital segment and urosome of caligid species was not successful because the DAB stain was of insufficient sensitivity to distinguish with clarity the integumental pores associated with stained gland structures. A repeat experiment utilising TMB alone may be more successful but some method of halting and preserving the TMB reaction and its coloured products will first need to be developed before this can be of any potential use to the taxonomist. The utility of a successful species-specific identifier in the PSP of caligid copepods is however limited, as there are clearly distinguishable morphological differences between species.

Due to the large numbers of glands located in caligid species, albeit with similar staining properties, the conclusion that they all serve the same function should not be made. Gland systems that

we suspect to have very different functions i.e. those of the thoracic legs and those of the frontal gland complex, show similar staining properties with DAB and TMB but on account of their different functions it is likely that the composition of their secretions will differ. Further work must be undertaken to specifically detail the ultrastructure of these glands and their secretions before any definite statements can be made regarding their function.

A more detailed examination of other species within the Siphonstomatoida should be undertaken to determine the extent of the development of such glands in this Order.

To gain a greater understanding of the composition of the secretions of the identified glands of *L. salmonis* histochemical analyses, and ultrastructural examination of selected glands, will be pursued in the following chapter.



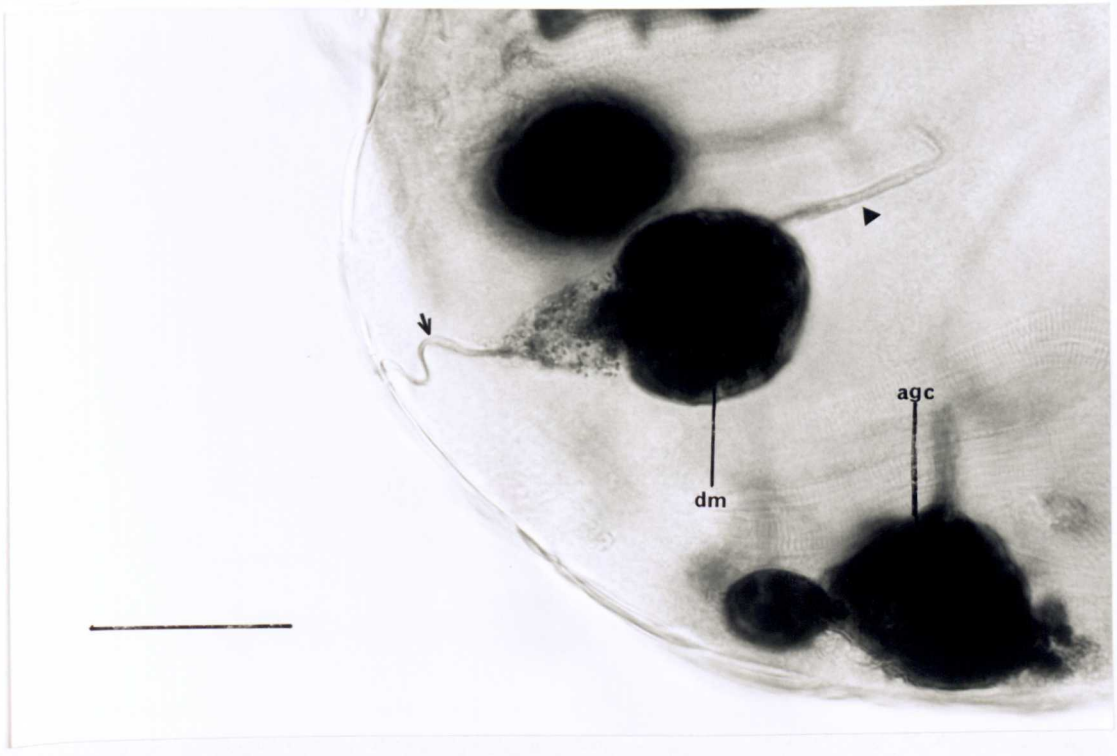
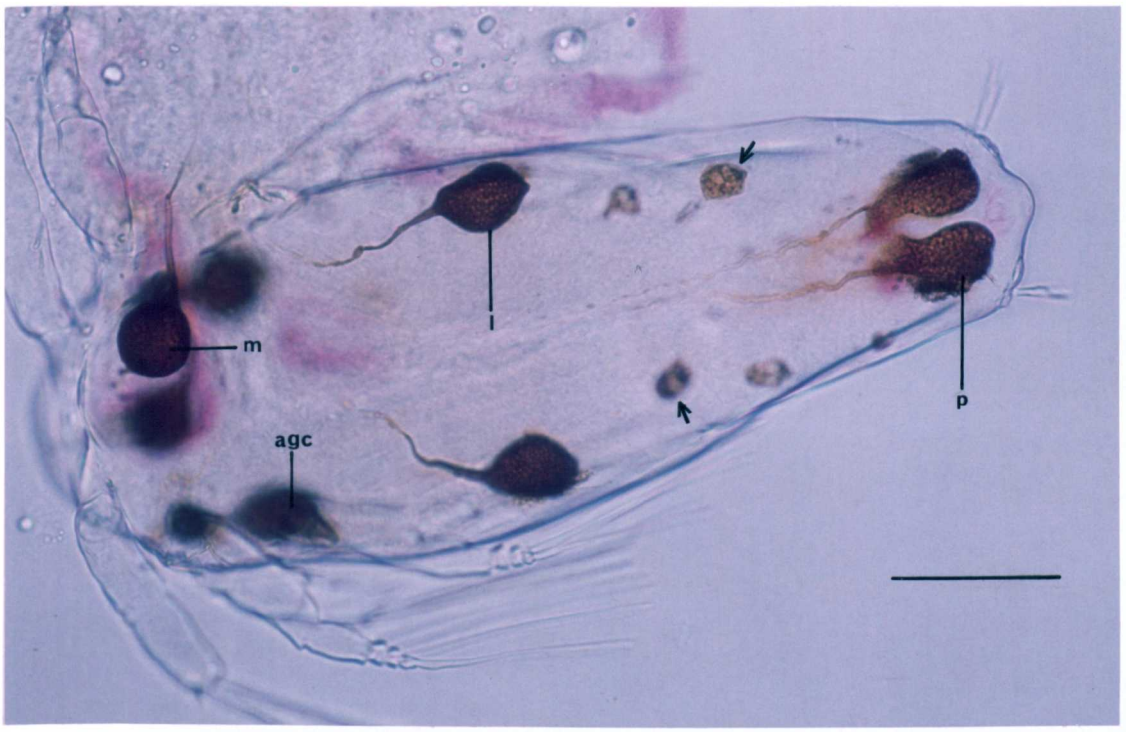


Figure 3.1 A DAB-stained *L. salmonis* nauplius I larva. The four paired gland groups are clearly evident. Note internally extending ducts (arrowed) associated with the posterior glands and finger-like extensions of the AGC (arrowheads). m median glands; agc anterior gland complex; l lateral gland; p posterior gland. Scale bar = 100μm.

Figure 3.2 Dorsal view of a DAB-stained *L. salmonis* nauplius I larva. The anterior duct from the dorsal median gland (arrowed) can be seen leading to its exit pore on the anterior cuticle. The posterior duct of the dorsal median gland is also visible (arrowhead). Two regions of one AGC are visible below the dorsal median gland. dm dorsal median gland; agc anterior gland complex. Scale bar = 50μm.



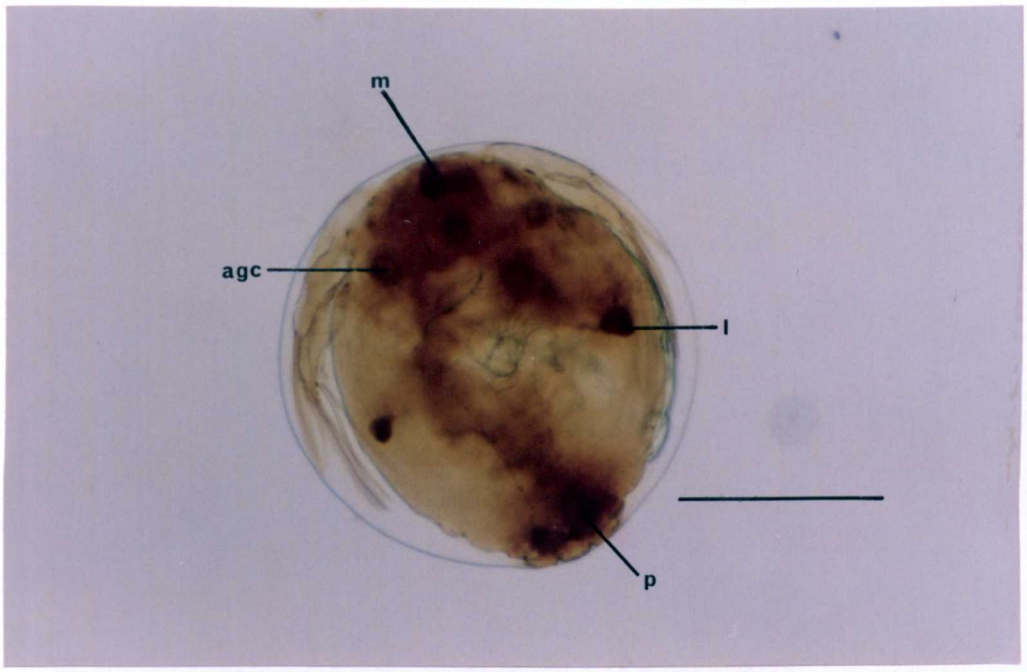
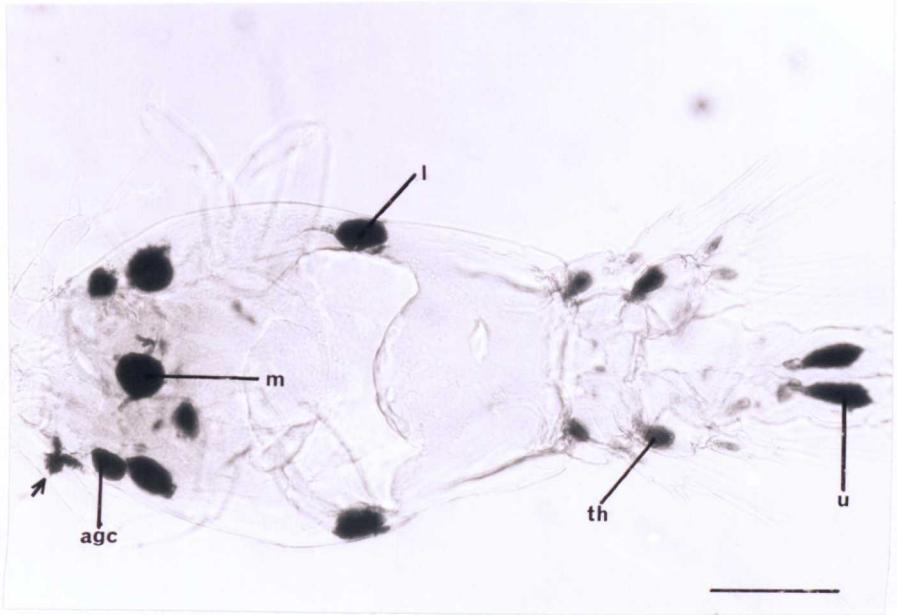


Figure 3.3 A late stage *L. salmonis* nauplius II larva stained with DAB. All the described gland groups are visible, the posterior glands with their anterior and posterior ducts, the lateral glands and the AGC. The median glands are displaced in this specimen due to the distortion of the body contents caused by the body cuticle rupturing in the staining solution. The precursors of the thoracic leg glands (arrowed) of the copepodid stage are visible beneath the ventral cuticle. agc anterior gland complex; m median gland; l lateral gland; p posterior gland. Scale bar = 100μm.

Figure 3.4 An unhatched *L. salmonis* nauplius stained with DAB whilst still within the egg. Note how the gland structures give a positive reaction to the stain even at this early stage. All of the gland structures identified in hatched NI larvae are visible. agc anterior gland complex; m median gland; l lateral gland; p posterior gland. Scale bar = 200μm.



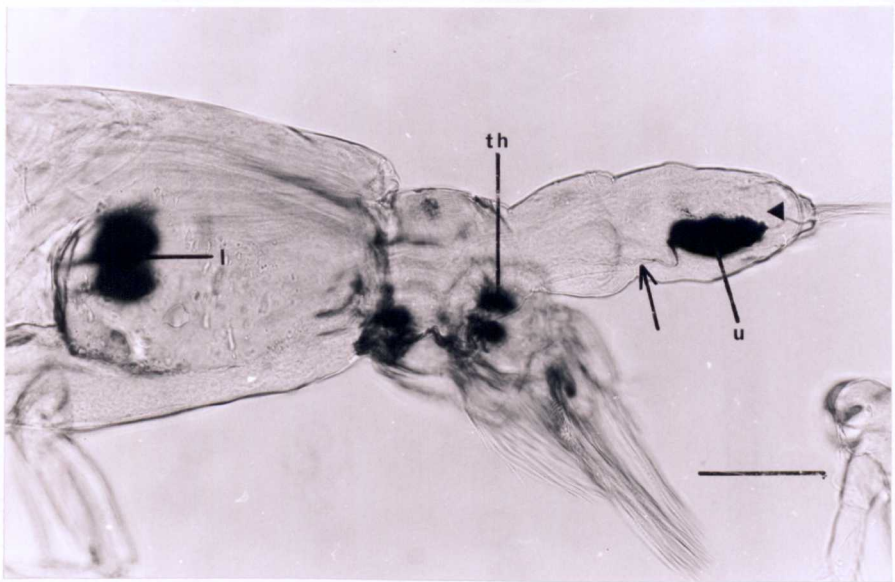
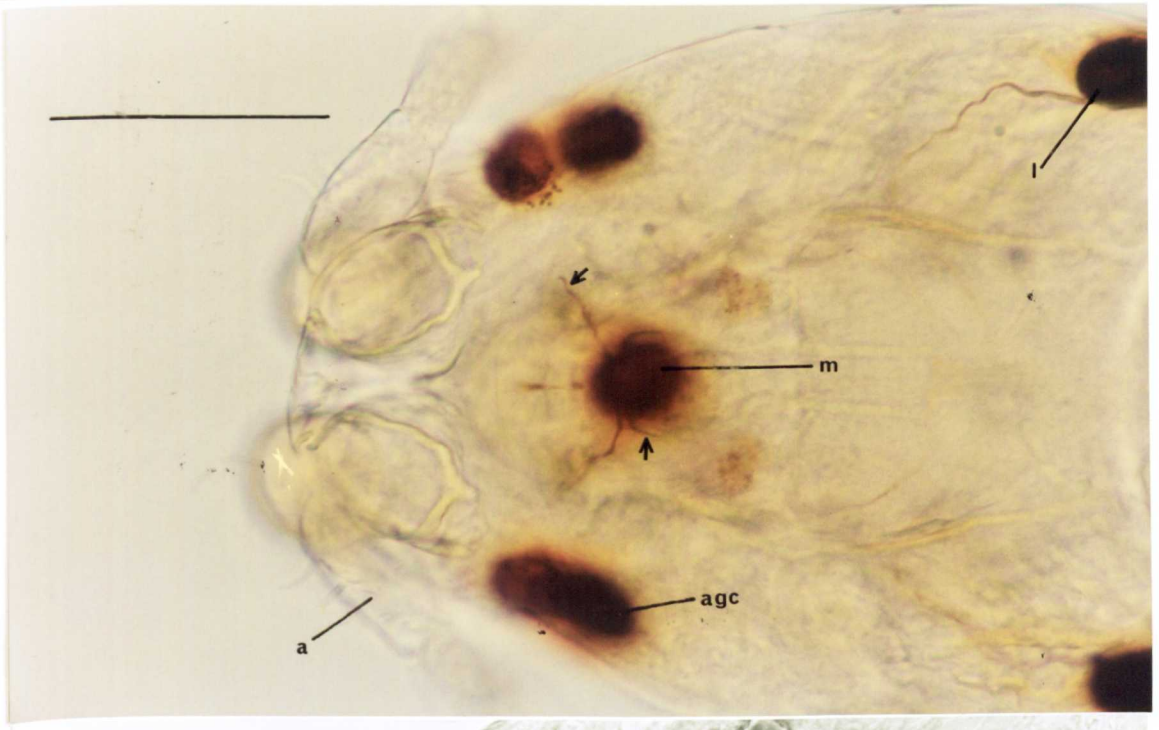


Figure 3.5 Ventral view of a DAB-stained *L. salmonis* copepodid larva. All of the positively staining glands are visible. The anterior ducts of the urosomal glands are evident, as is some stained material adhering to the external cuticle close to the bases of the antennules (arrowed). m median glands; agc anterior gland complex; l lateral gland; th thoracic leg glands; u urosomal glands. Scale bar = 100μm.

Figure 3.6 Lateral view of the posterior cephalothorax and urosome of a DAB-stained *L. salmonis* copepodid. The lateral glands, thoracic leg glands and the urosomal glands are visible in this specimen. Note the long, anterior (arrowed) and the smaller posterior ducts (arrowhead) of the urosomal glands. l lateral gland; th thoracic leg glands; u urosomal gland. Scale bar = 100μm.



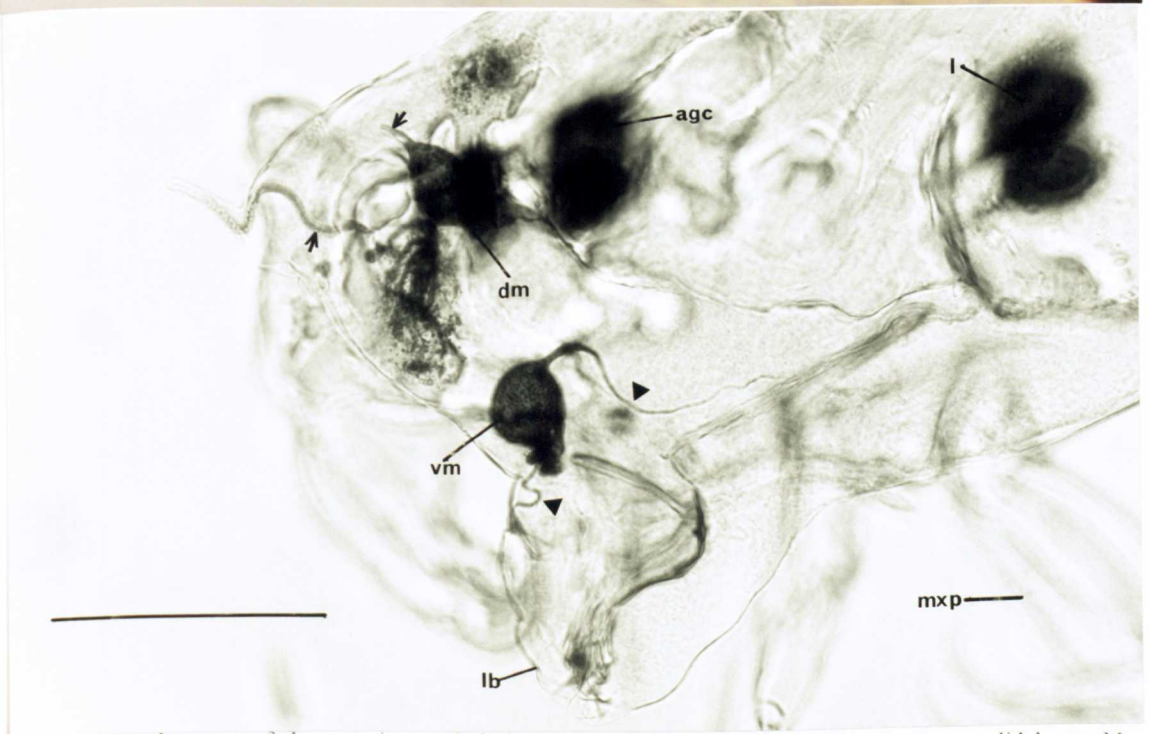
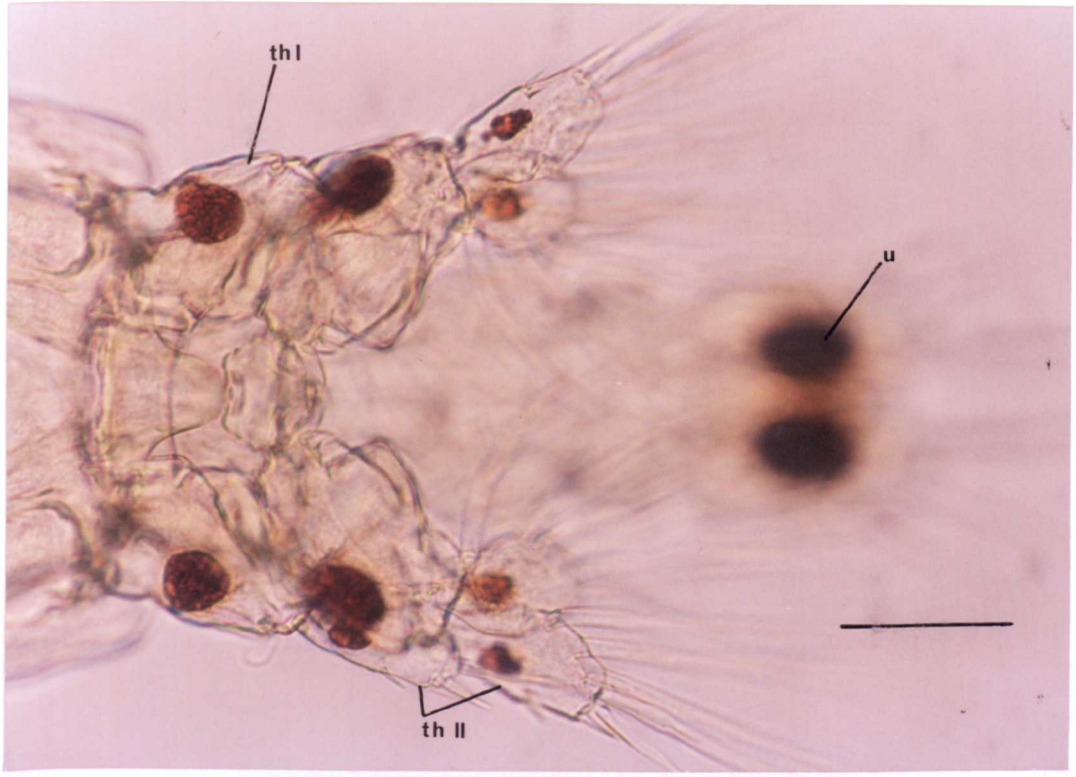


Figure 3.7 Dorsal aspect of the anterior cephalothorax of a DAB-stained *L. salmonis* copepodid larva. Note the numerous stained ducts arising from the centrally placed median glands (arrowed). The two dorsal most regions of the AGC are also visible, as are the lateral glands and their anterior ducts. m median glands; agc anterior gland complex; l lateral gland; a antennule. Scale bar = 100μm.

Figure 3.8 A lateral aspect of a DAB-stained *L. salmonis* copepodid larva emphasising the vertical separation between the two median glands. Both the anterior and posterior ducts of the ventral median gland are clearly visible (arrowheads). The ducts of the dorsal median gland are also visible: one entering the filament duct and one leading vertically towards the dorsal cuticle (arrows). vm ventral median gland; dm dorsal median gland; l lateral gland; agc anterior gland complex; lb labrum; mxp maxilliped. Scale bar = 100μm.



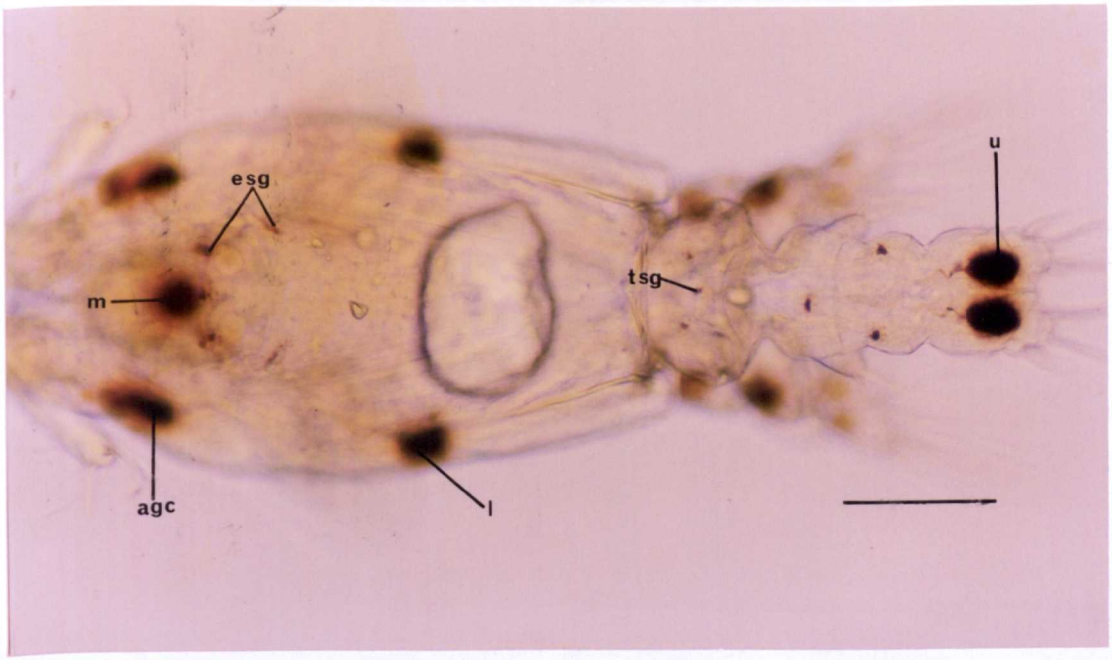
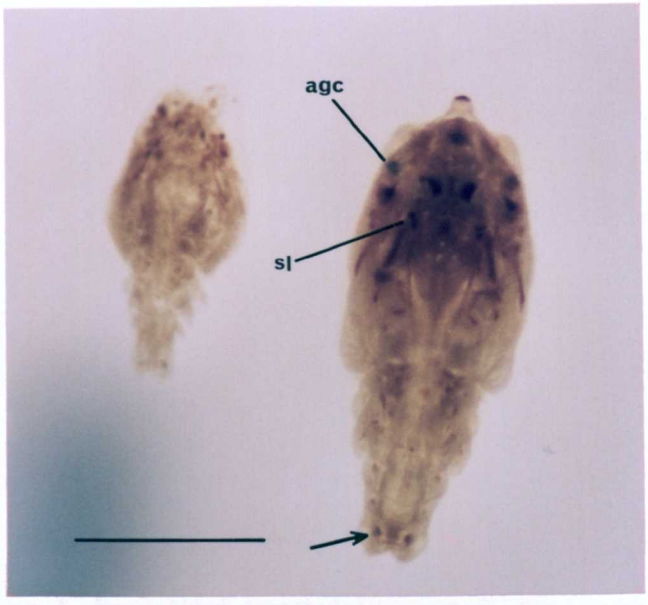


Figure 3.9 Ventral aspect of a DAB-stained *L. salmonis* copepodid larva. The distinctive pattern of the thoracic leg glands can be seen. th_I first thoracic leg; th_{II} second thoracic legs; u urosomal glands. Scale bar = 50µm.

Figure 3.10 Dorsal view of a DAB-stained *L. salmonis* copepodid larva. The three glands of the Thoracic Segment Group (tsg) are visible as are the four glands of the Eye-Spot Group (esg). m median glands; agc anterior gland complex; l lateral gland; u urosomal gland. Scale bar = 100µm.



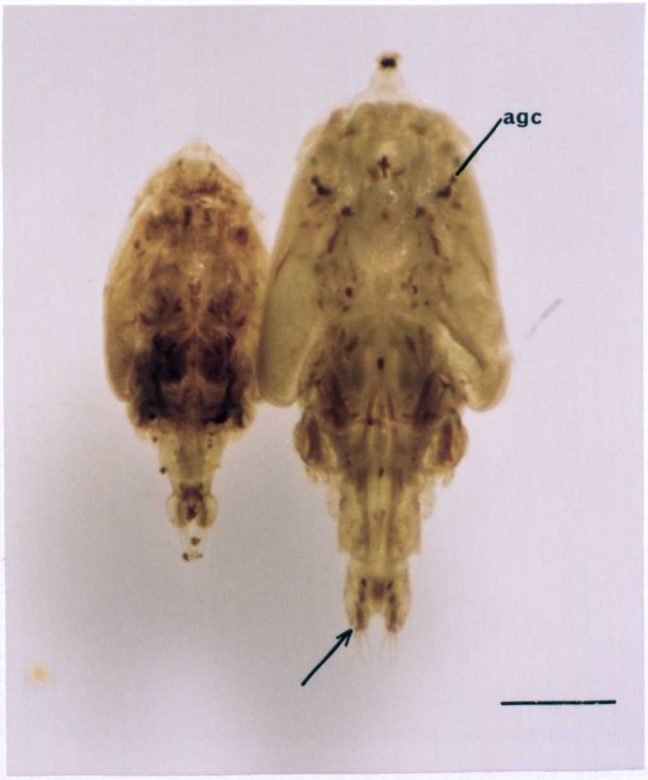


Figure 3.11 Dorsal view of DAB-stained *L. salmonis* chalimus larvae. Chalimus stages I (A) and II (B) are shown. The AGC (agc), suture line glands (sl) and the urosomal glands (arrowed) are all visible. Note the two distinct regions to the AGC of the chalimus II larva. Scale bar = 600µm.

Figure 3.12 Ventral view of DAB-stained *L. salmonis* chalimus larvae. Chalimus stages III (A) and IV (B) are shown. The AGC (agc) and urosomal glands (arrowed) are evident from this perspective. Scale bar = 500µm.

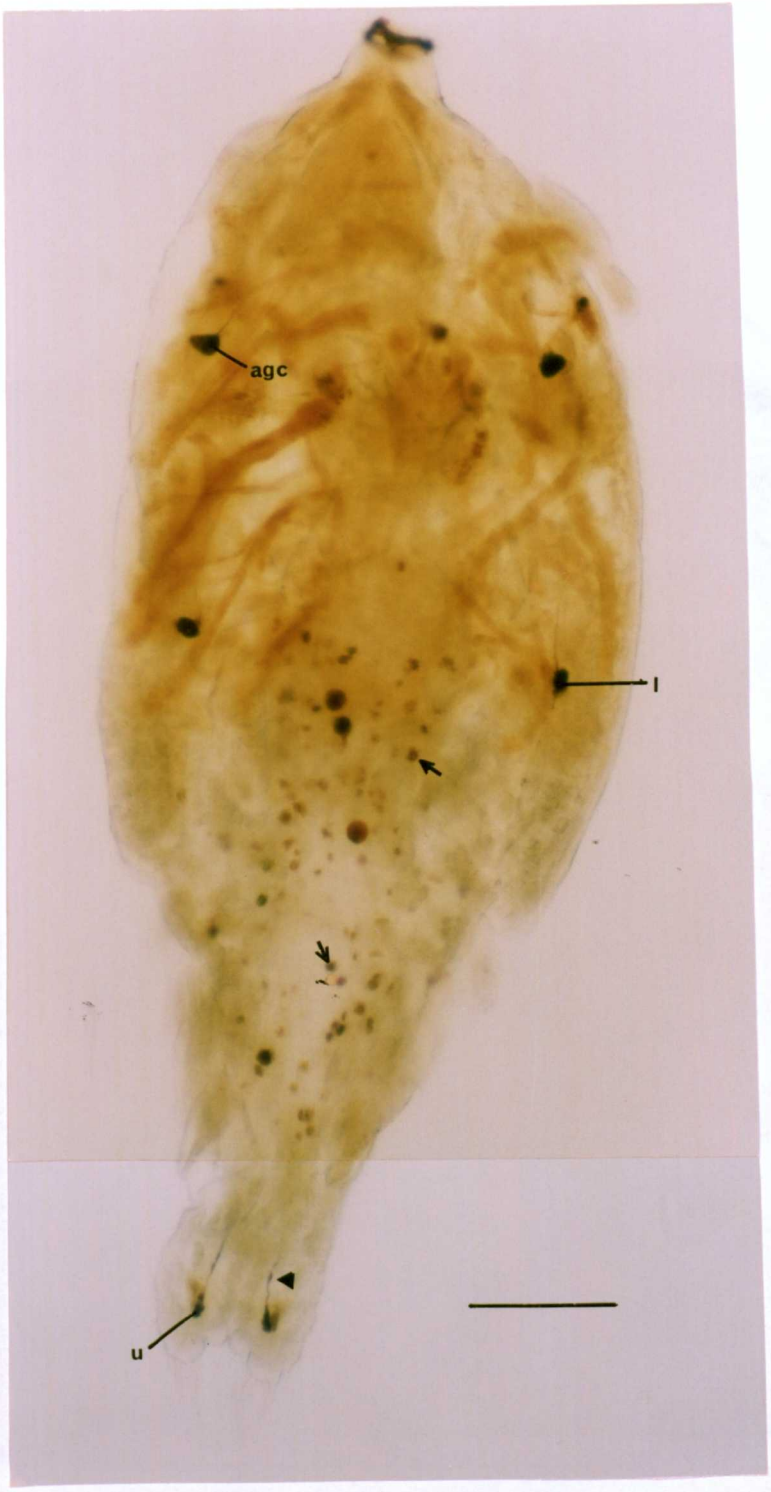
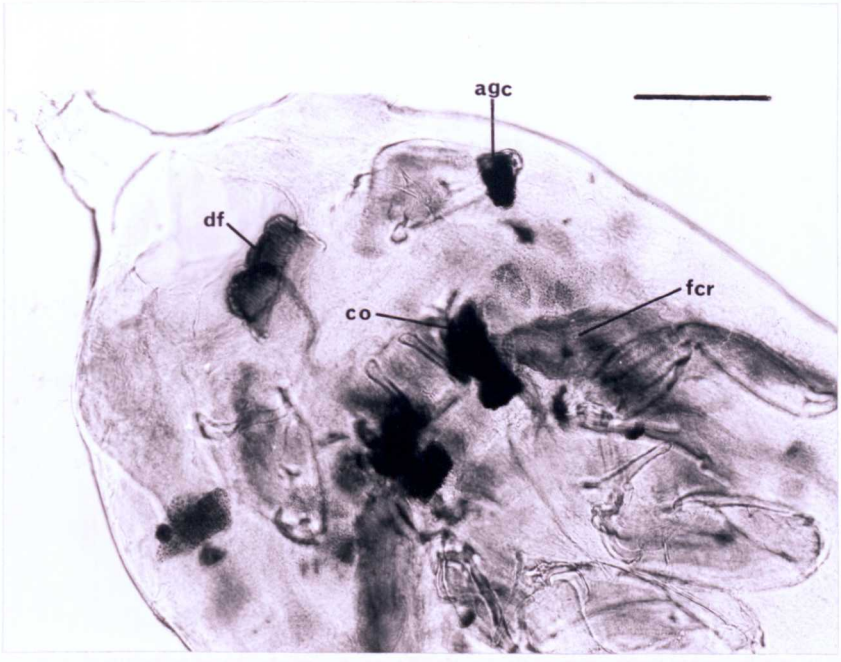


Figure 3.13 DAB-stained second stage *L. salmonis* chalimus larva – cleared in lactic acid. Visible in this specimen are some stained regions within the gut (arrowed). Ducts extending anteriorly from the urosomal glands are evident (arrowhead). agc anterior gland complex; l lateral gland; u urosomal gland. Scale bar = 20µm.



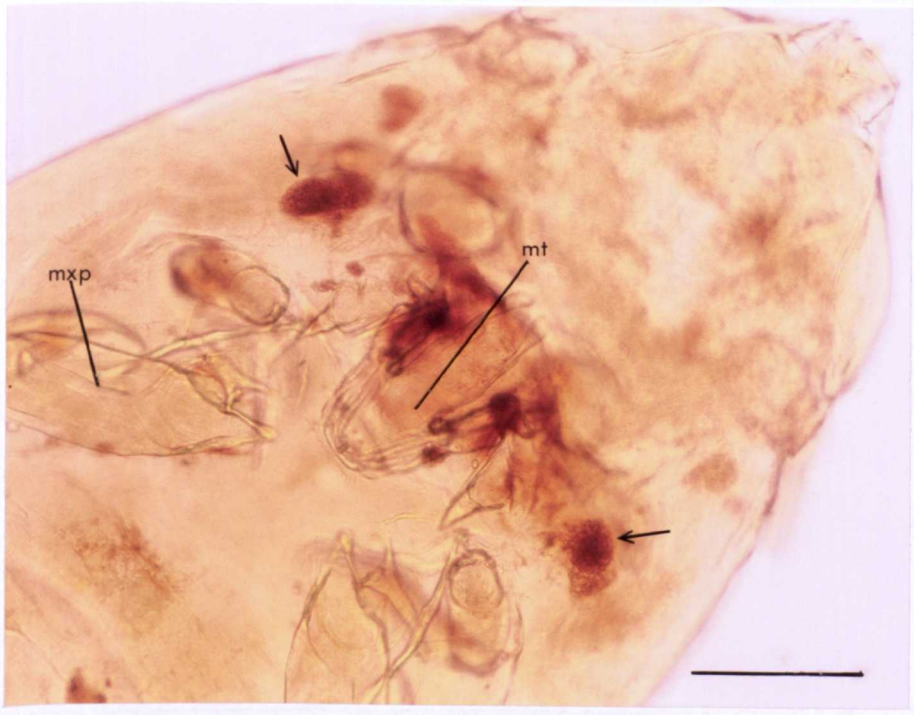
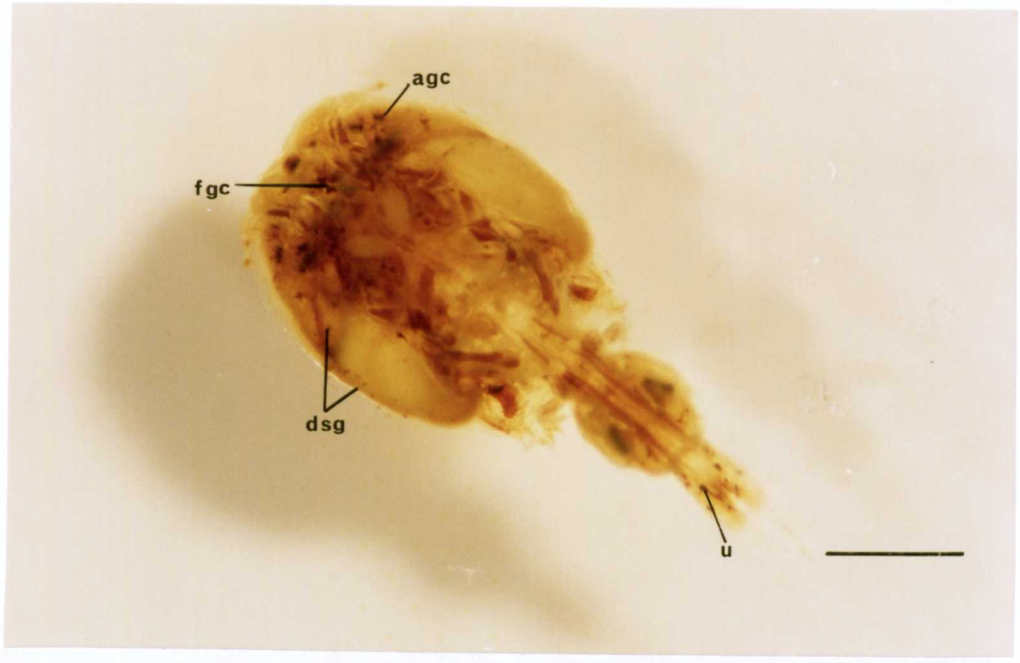


Figure 3.14 Ventral view of a DAB-stained *L. salmonis* chalimus III larva. The developing filament (df) is visible within the anterior cephalothorax. agc anterior gland complex; co circum-oral glands. The thickened cuticle of the first cuticular ridge is also evident (fcr). Scale bar = 200μm.

Figure 3.15 DAB-stained *L. salmonis* chalimus larva showing the positions of the postoral glands (arrowed). Mxp maxilliped; mt mouth-tube. Scale bar = 250μm.



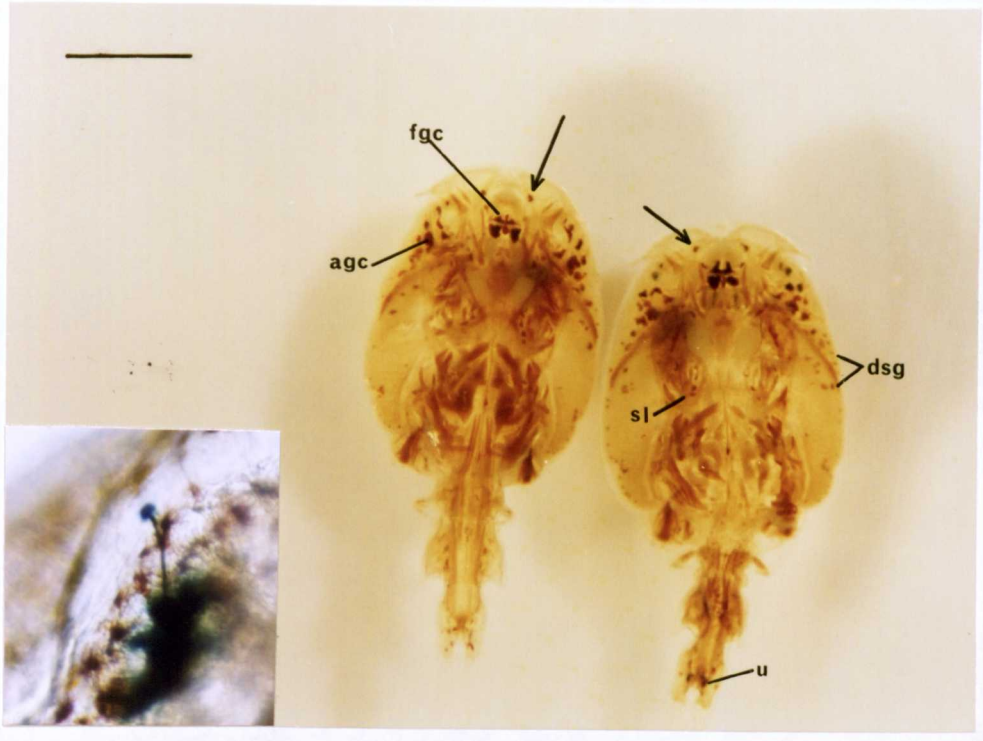
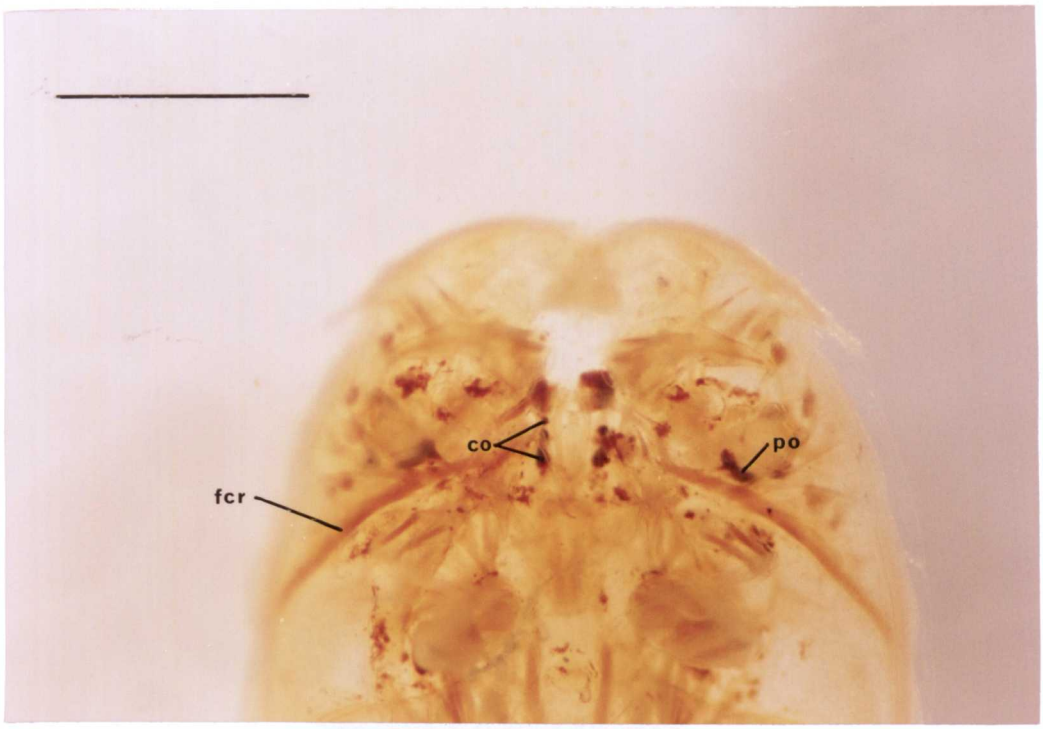


Figure 3.16 DAB-stained preadult *L. salmonis*. The urosomal glands are clearly visible (u) as are the glands of the frontal gland complex (fgc), anterior gland complex (agc) and some dorsal surface glands (dsg). Scale bar = 100μm.

Figure 3.17 Dorsal aspect of two DAB-stained *L. salmonis* preadult I females. All the glands developed by this stage are evident in these two specimens. The filament-producing glands (fgc) lie centrally at the anterior end of the cephalothorax with the two remote components of this complex being obvious at the margin of the frontal plates and the cephalothorax (arrows and inset). The FGC are flanked by the loose groupings of the anterior gland complexes (agc). The few dorsal surface glands (dsg) as they appear at this stage are apparent close to the first cuticular ridge. sl suture line glands; u urosomal glands. Scale bar = 100μm.



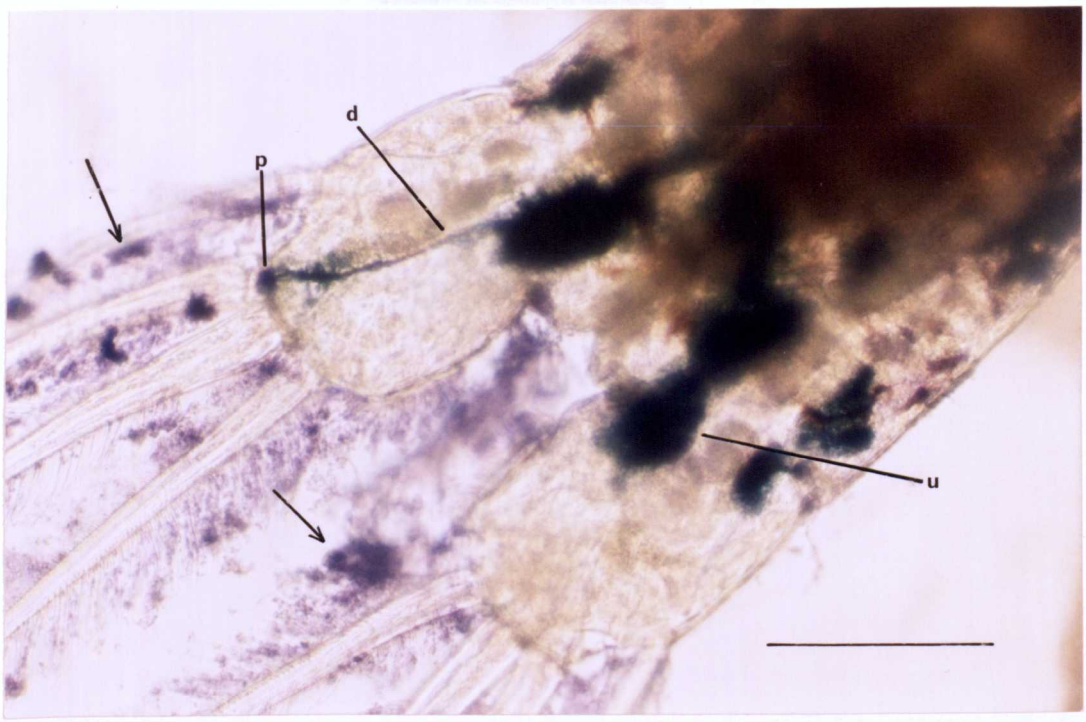


Figure 3.18 DAB-stained *L. salmonis* male preadult II. The bean shaped post oral (po) and the circum-oral (co) glands are evident, as is the first cuticular ridge (fcr). Scale bar = 100μm.

Figure 3.19 The terminal setae of a TMB-stained *L. salmonis* adult male showing a stained duct (d) and pore (p) associated with the urosomal gland complex and quantities of stained material adhering to the terminal setae and their setules (arrows). The overstained urosomal glands are also evident in the urosome and caudal rami (u). Scale bar = 200μm.

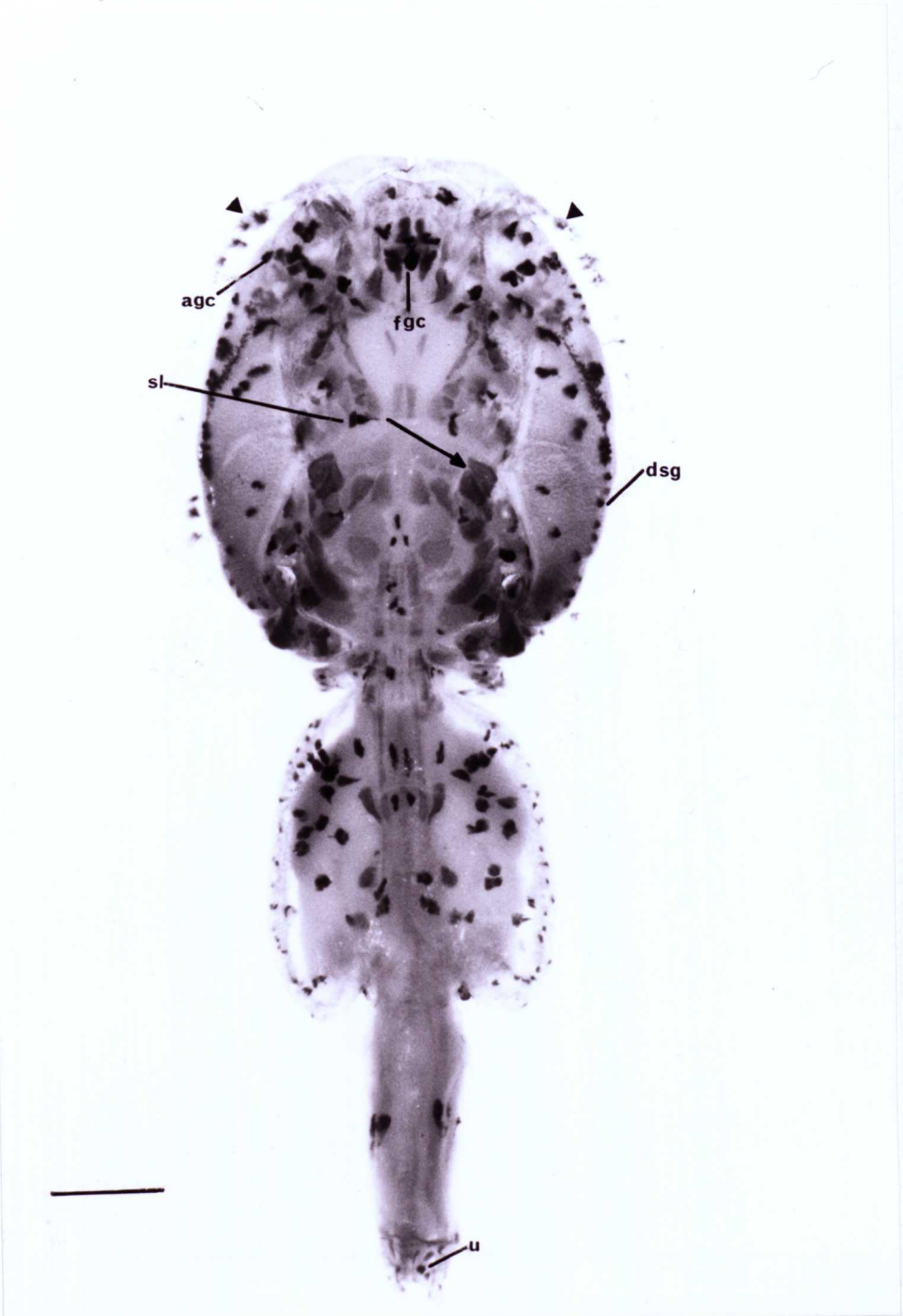
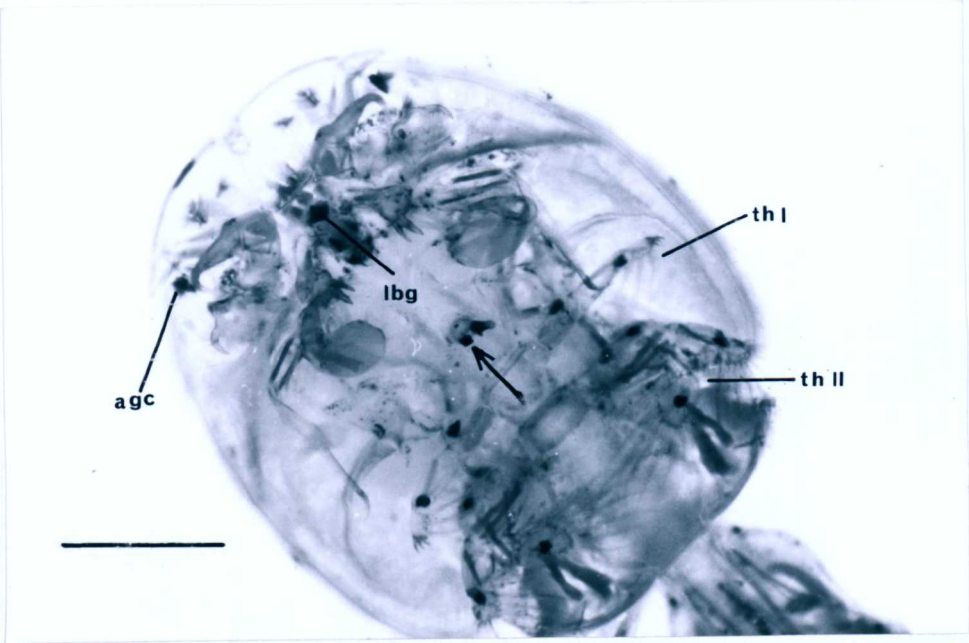


Figure 3.20 DAB-stained adult female *L. salmonis* showing the full complement of positively staining glands of the dorsal surface. Note also the regions of staining associated with the muscle attachments to the cuticle (arrowed) and the stained material adhering to the marginal membrane (arrowheads). fgc frontal gland complex; agc anterior gland complex; dsg dorsal surface gland; sl suture line glands; u urosomal glands. Scale bar = 100μm.



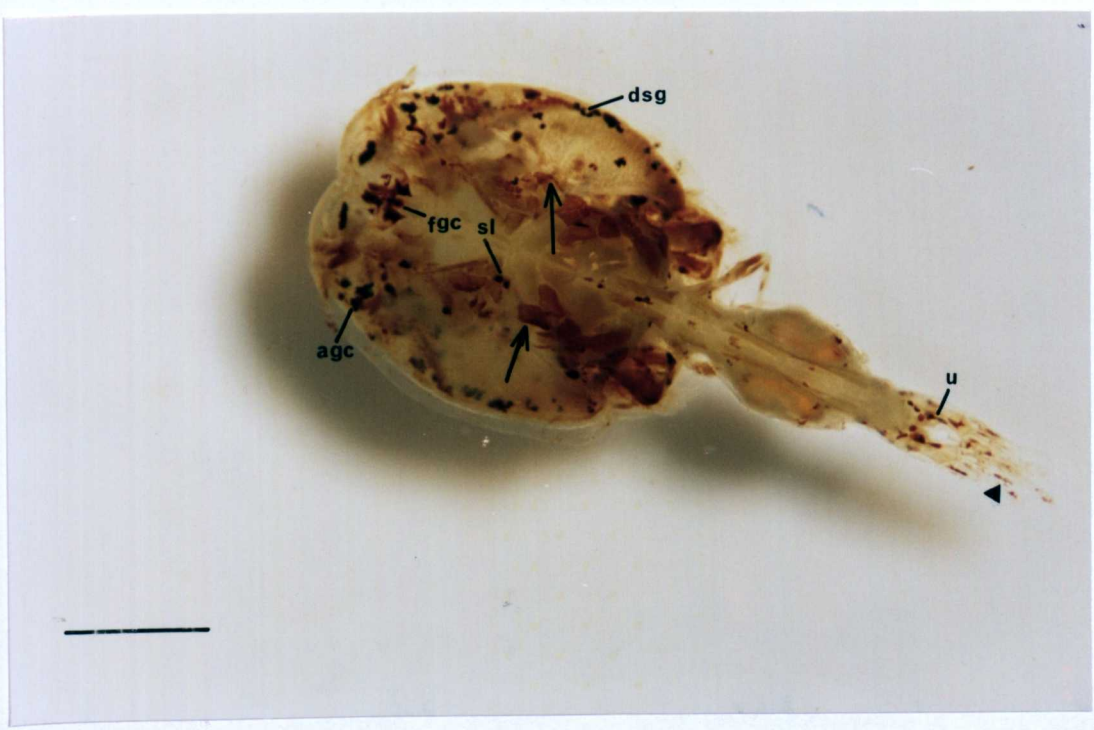
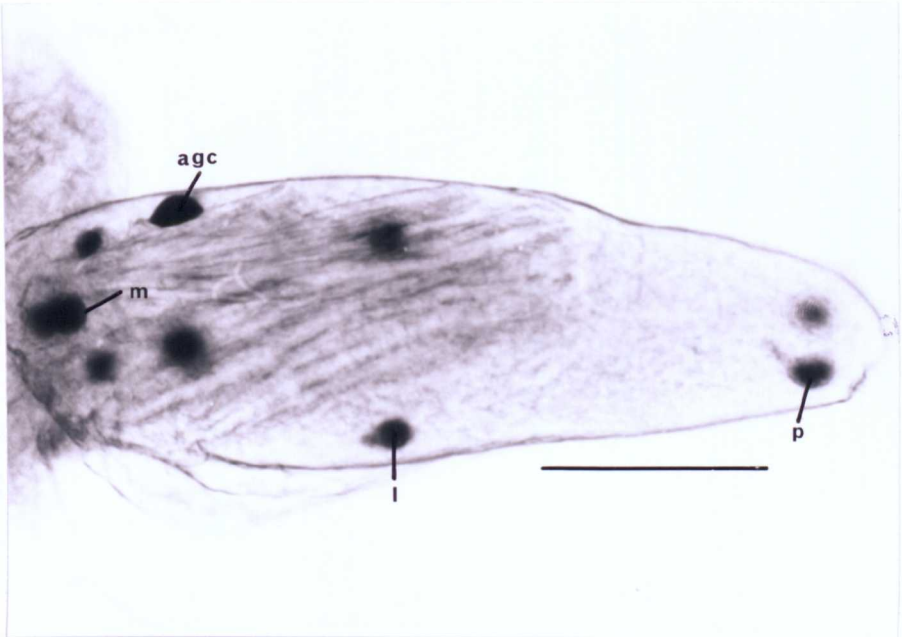


Figure 3.21 DAB-staining of the thoracic leg glands in an adult female *L. salmonis*. Note also the positive staining of the broken tines of the sternal furca (arrow). th_I glands thI; th_{II} glands thII; lbg labral gland; agc anterior gland complex. Scale bar = 100µm.

Figure 3.22 Dorsal aspect of a DAB-stained adult male *L. salmonis*. The full complement of gland structures has been developed by this stage and are a conspicuous feature of the specimen. Stained muscle attachments (arrows) and stained material adhering to the terminal setae are also visible (arrowhead). agc anterior gland complex; fgc frontal gland complex; sl suture line glands; dsg dorsal surface glands; u urosomal glands. Scale bar = 100µm.



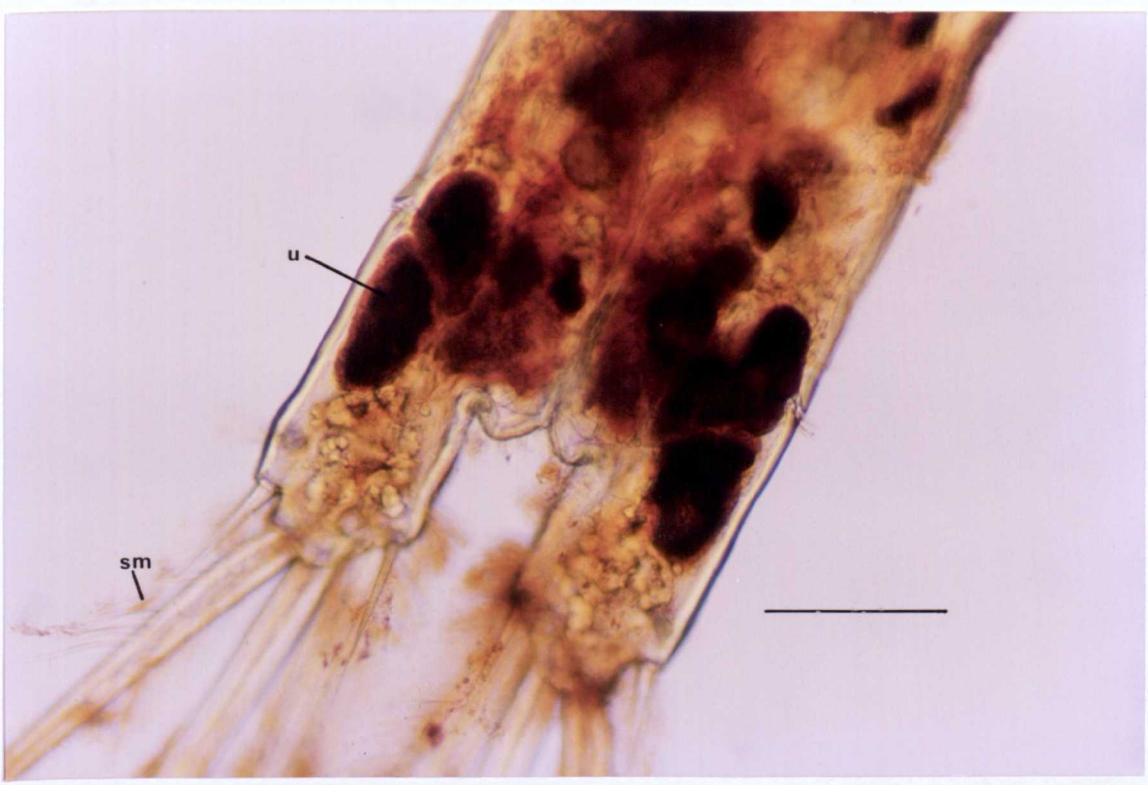
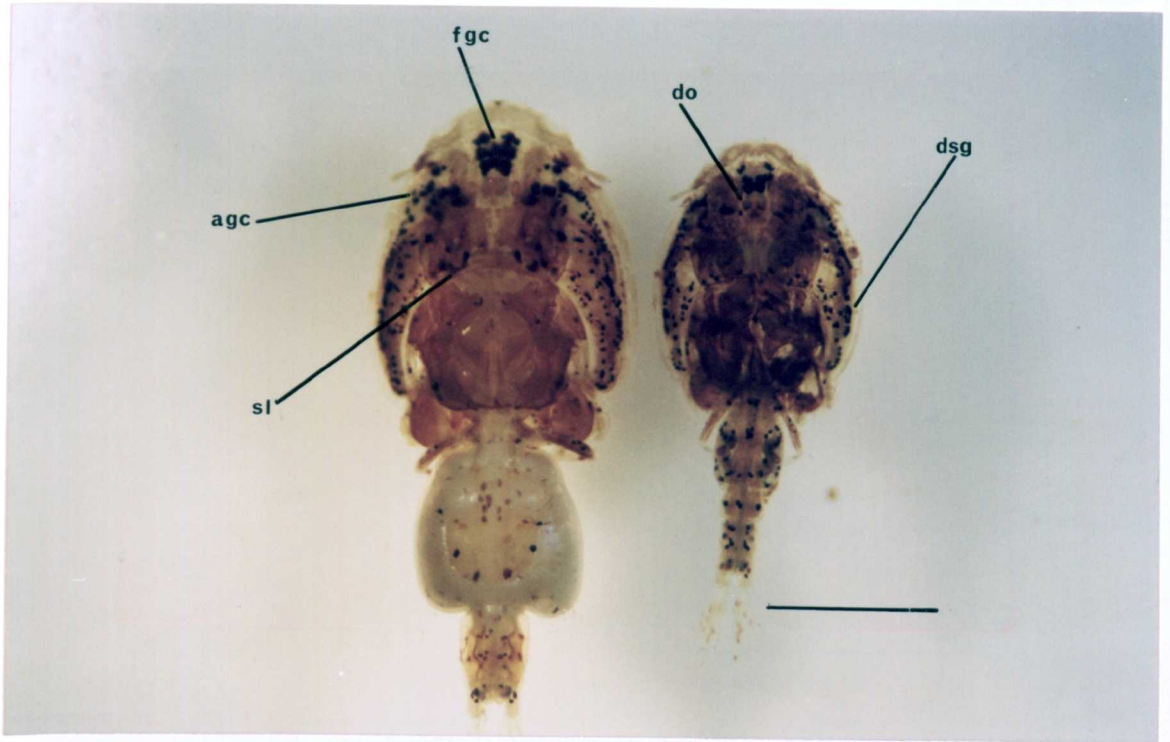
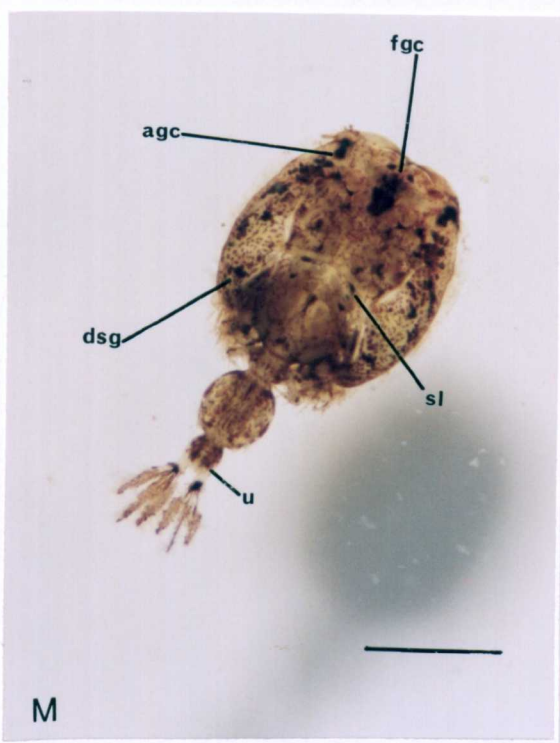


Figure 3.23 DAB-stained nauplius larva of *C. elongatus* showing all of the positively staining gland systems. agc anterior gland complex; m median gland; l lateral gland; p posterior glands. The disruption of the body tissues caused by immersion in the staining solution is evident at the anterior margin of the animal. Scale bar = 100µm.

Figure 3.24 The urosome of a DAB-stained male specimen of *C. elongatus*. The strongly staining paired urosomal glands are evident through the cuticle (u) and positively staining material (sm) can also be seen on the terminal setae of the caudal rami. Scale bar = 100µm.





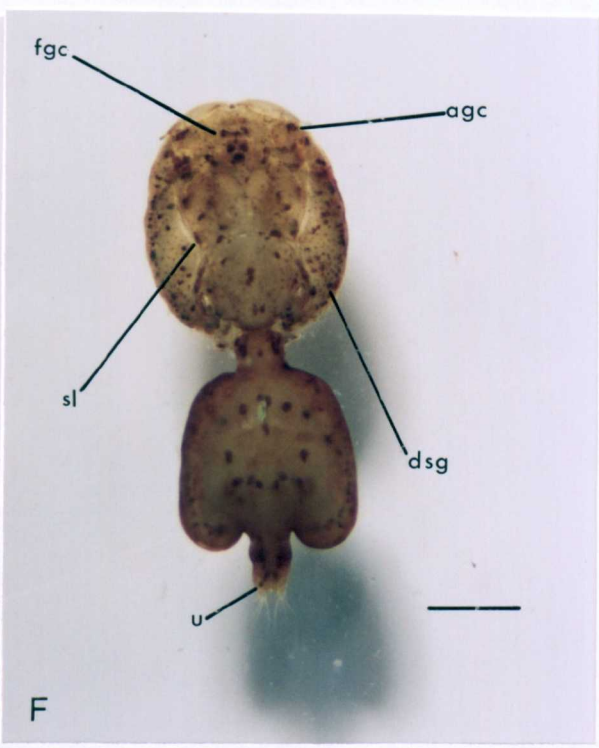
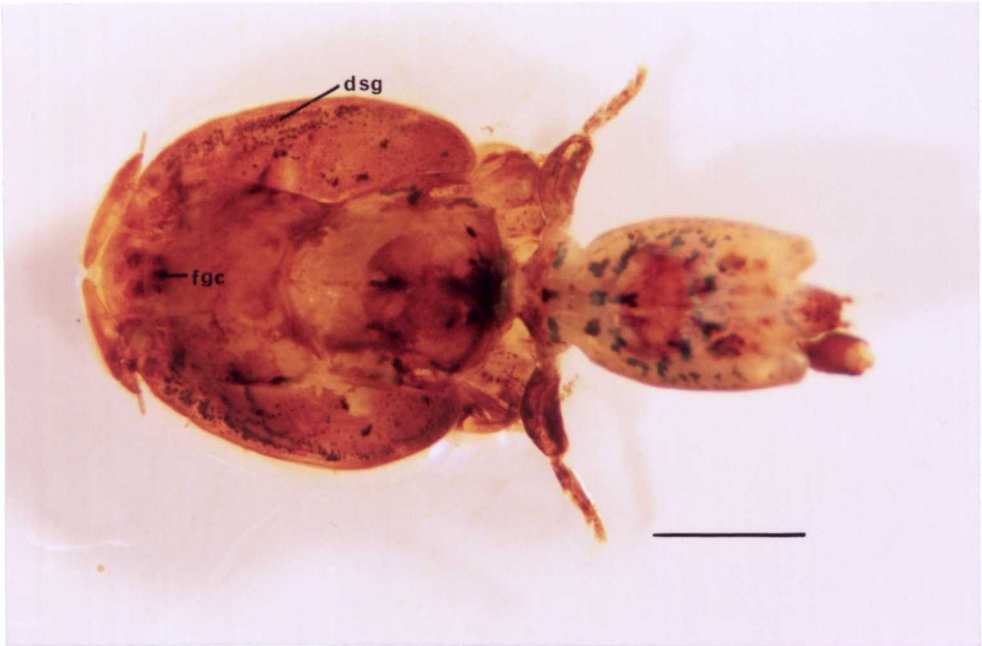


Figure 3.25 Dorsal view of the cephalothoraxes of DAB-stained adult male and female *C. elongatus*. The numerous dorsal surface glands (dsg) can be seen in their characteristic bilaterally symmetrical pattern. fgc frontal gland complex; agc anterior gland complex; sl suture line glands; do dorsal ocelli. Scale bar = 1.5mm.

Figure 3.26 Adult male (M) and female (F) *L. pectoralis* showing the exocrine gland population revealed by the DAB stain. fgc frontal gland complex; agc anterior gland complex; dsg dorsal surface glands; sl suture line glands; u urosomal glands. Scale bar = 2mm.



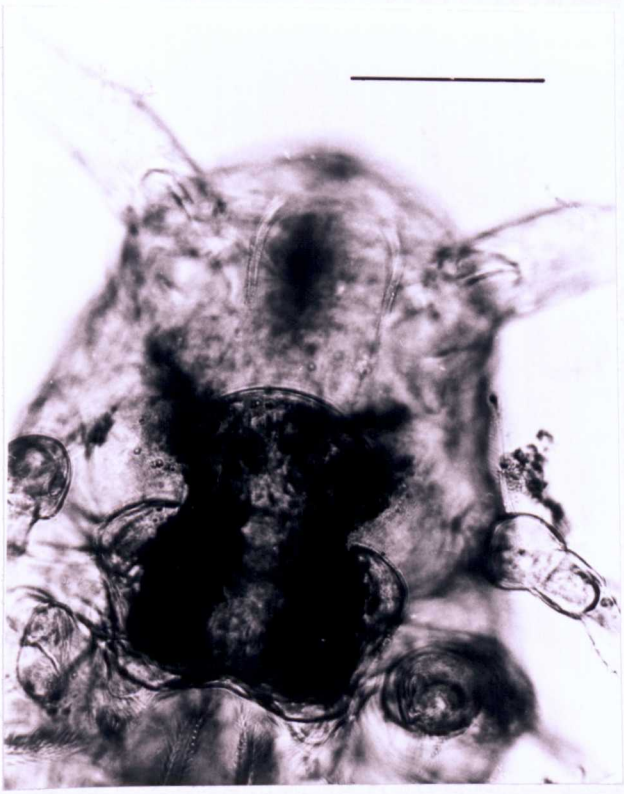
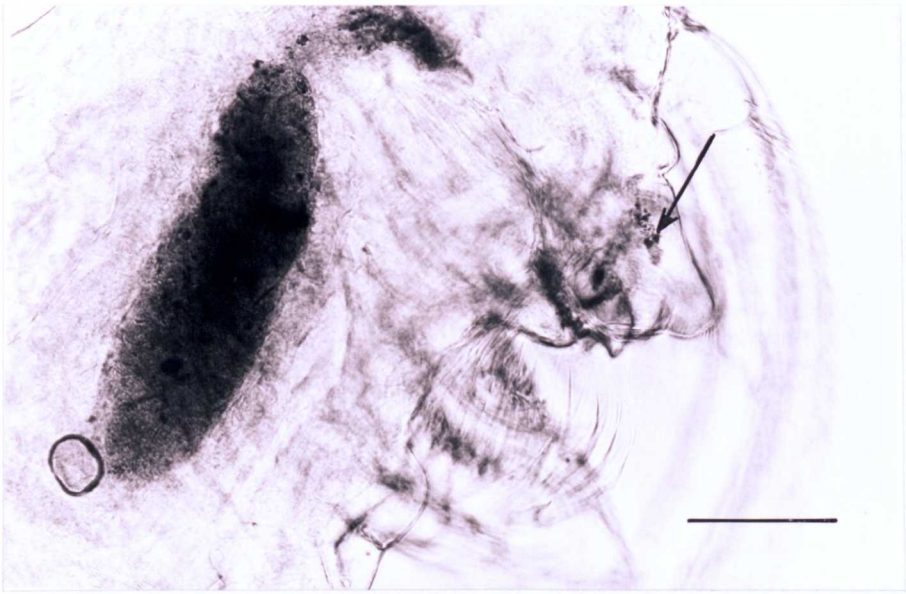


Figure 3.27 DAB-stained *L. hippoglossi* female showing all of the DAB-stained gland groups described for *L. salmonis*. The most conspicuous feature of this specimen is the large number of dorsal surface glands present across the lateral margins of the cephalothorax (dsg). The other gland group evident in this specimen is the frontal gland complex (fgc). Scale bar = 2.5mm.

Figure 3.28 The calanoid copepod *Acartia tonsa* stained with DAB. Very few regions of positive gland staining are evident and these are restricted to the obvious staining of the buccal cavity region. Scale bar = 50μm.



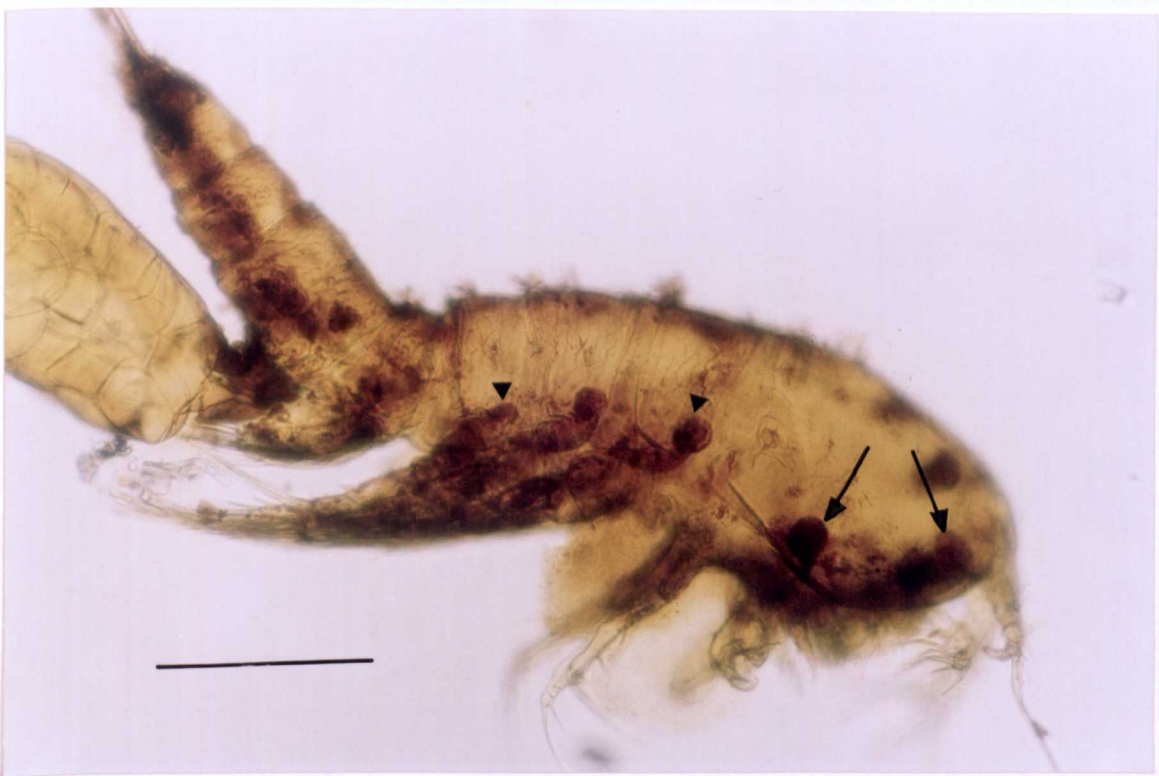


Figure 3.29 DAB-stained specimen of the calanoid copepod *Eurytemora affinis*. Very little positive staining is evident in this specimen apart from the non-specific staining of the gut contents and a small amount of granular staining in the mandibles (arrow). Scale bar = 100µm.

Figure 3.30 Adult female *Parathalestris hibernica* stained with DAB showing positively staining exocrine glands. Conspicuous glands are present in the cephalosome (arrows) and in the ventral regions of each thoracic segment (arrowheads). Scale bar = 200µm.

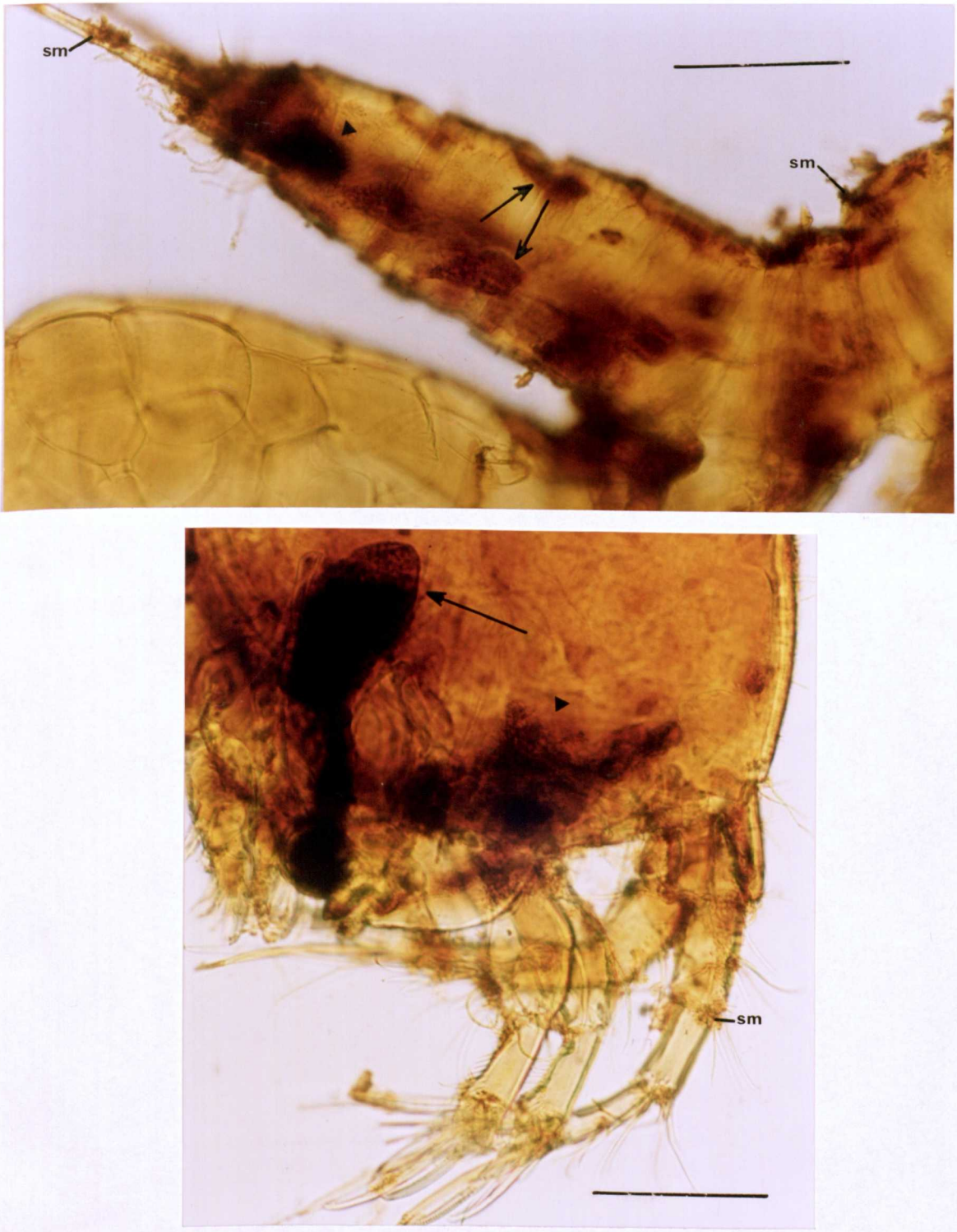


Figure 3.31 Higher magnification of the urosomal segment of a DAB-stained adult female *Parathelestris hibernica*. The ventral and dorsal glands are evident (arrows) as is the large gland complex in the anal segment (arrowhead). Note also the stained material adhering to the terminal setae and body cuticle (sm). Scale bar = 100µm.

Figure 3.32 Anterior cephalosome of a DAB-stained adult female *Harpacticus chelifer*. Note the large stained gland within the body cavity (arrowed) extending ventrally into the area of the buccal cavity. Note also the less well defined stained mass anterior to the large gland (arrowhead) and the stained material (sm) adhering to the body cuticle and setae of the limbs. Scale bar = 100µm.

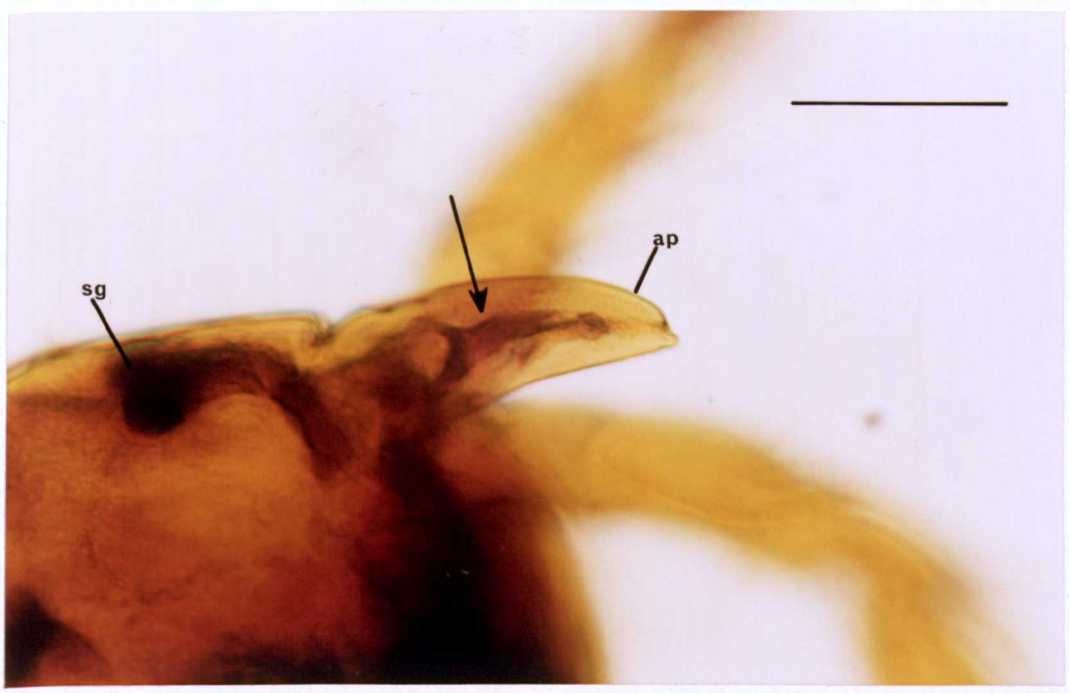


Figure 3.33 Urosome of a DAB-stained adult female *Harpacticus chelifer*. Note the large multi-component stained gland in the anal segment (arrowed) and the smaller glands of the individual segments (arrowheads). Scale bar = 100μm.

Figure 3.34 Anterior cephalosome of a DAB-stained adult female *Diosaccus tenuicornis*. Note the stained gland (sg) and the duct (arrowed) passing from it into, and extending to the tip of, the anteriorly projecting process (ap). Scale bar = 50μm.

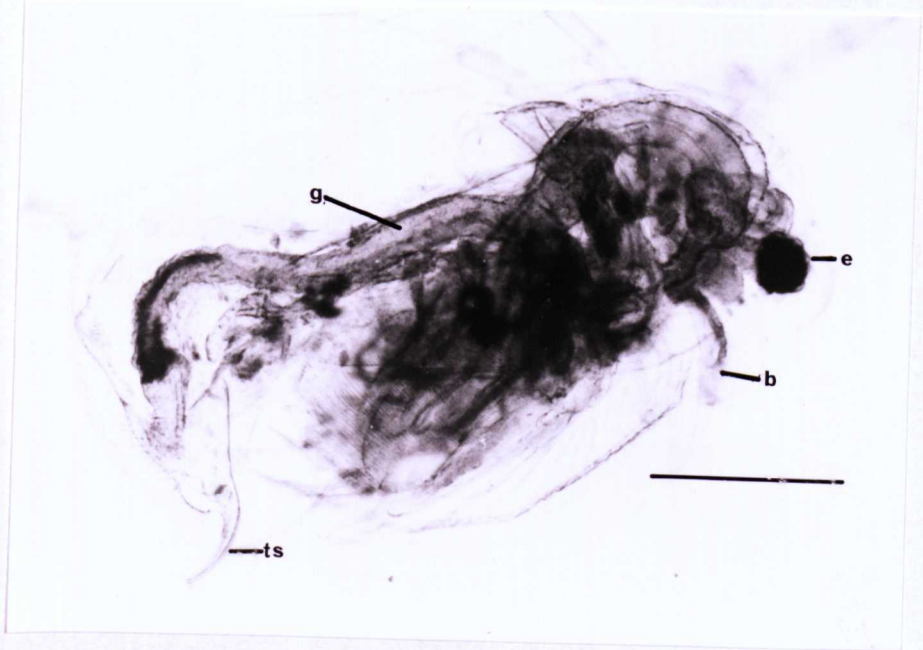


Figure 3.35 A 48h larval *Artemia salina* stained with DAB. No regions of positive staining are evident. The dark region present anteriorly are the eyes and not specific staining. Scale bar = 200μm.

Figure 3.36 An adult specimen of the branchiopod *Daphnia magna* stained with DAB. No glandular regions are evident. es eye; ts tail spine; g gut; b beak. Scale bar = 500μm.

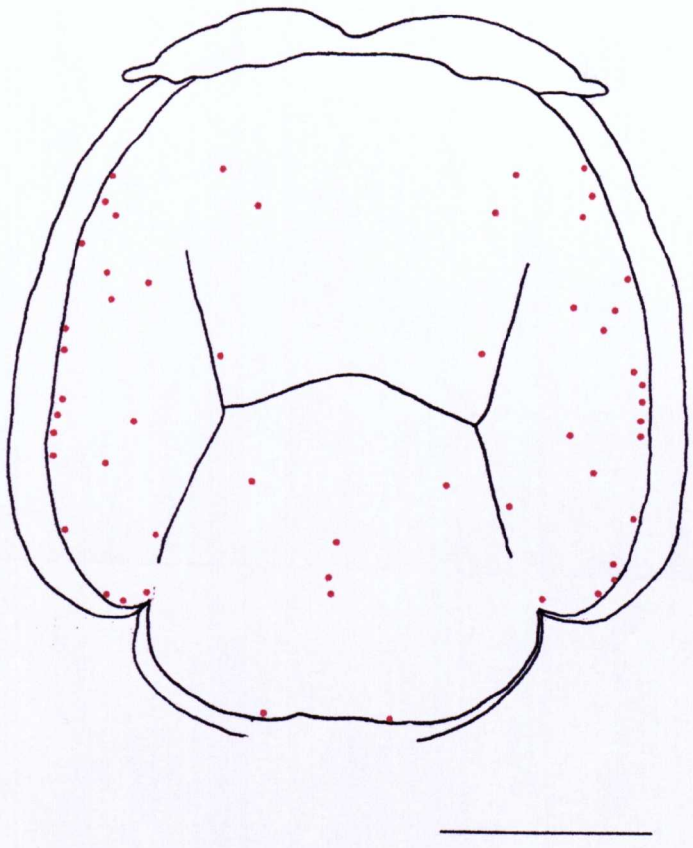


Figure 3.37 Pore signature pattern of the dorsal cephalothorax of an adult male *L. salmonis* as identified using TMB staining and SEM analysis in combination. Scale bar = 1mm.

4. GLAND HISTOCHEMISTRY AND ULTRASTRUCTURE

4. GLAND HISTOCHEMISTRY AND ULTRASTRUCTURE

4.1 INTRODUCTION

Histochemical and ultrastructural analyses are the first, and fundamental, steps to be taken when trying to identify the components and secretions of gland tissues. All further, specific analyses depend on some knowledge of the types of compounds present. The ultrastructural features of crustacean glands have begun to be described in recent years with the increase in technological ability allowing visualisation of microscopic cell components. The ultrastructure of insect glands has received more attention largely, because of the great tradition of entomological study and the continued importance of insects as vectors of disease and as pests of crops. The ultrastructure of insect glands was comprehensively reviewed by Noirot and Quennedy (1974).

4.1.1 Histochemistry of copepod glands

One of the commonest components of copepod gland secretion identified using histochemical techniques is mucus (Fahrenbach 1962, Gharagozlou-van-Ginneken 1977, Briggs 1978, Chandler and Fleeger 1984, Hipeau-Jacquotte 1987, Brunet *et al.* 1991, Rybakov and Dolmatov 1991, Bron *et al.* 1993). Mucus has been identified in the glands of the majority of species where specific attempts to identify gland contents have been made. Furthermore, it is a commonly found component in the exocrine glands of other crustacea, having been shown to constitute at least a part of the secretion of the exocrine glands of isopods (Stevenson and Murphy, 1967), decapods (Shyamasundari and Hanumantha Rao, 1978) and amphipods (Shyamasundari, 1979).

Many histochemical techniques of varying degrees of refinement and sensitivity have been developed over the last two centuries and these allow the discrimination of a wide variety of different compounds and specific chemicals within animal and plant tissues. These techniques can be applied to fresh, frozen or fixed tissues to provide information at a cellular and subcellular level regarding the chemical composition of those tissues. The application of such techniques in studies of copepod gland

research has revealed the frequent presence of two main groups of mucus compound, namely acidic (sulphated) mucus and neutral mucus. The functional significance of these two types of mucus remains to be discovered. Since mucus compounds have always been demonstrated or implicated in the glands and secretions of copepods (Fahrenbach 1962, Park 1966, Briggs 1978, Boxshall 1982, Hipeau-Jacquotte 1987, Brunet *et al.* 1991, Bron *et al.* 1993) it suggests that they are of fundamental importance in gland activity. Mucus components of copepod glands have been suggested to be involved in a variety of functions including food capture (Von Vaupel Klein and Koomen, 1994), food agglutination (Arnaud *et al.* 1988b), protective encapsulation (Hicks and Grahame, 1979), sensory-glandular interaction (Hipeau-Jacquotte, 1987) and protection from host nematocysts (Briggs, 1978). From these studies it seems that mucus is a widespread feature that is adapted to fit particular needs in different species.

Snell and Nacionales (1990) and Snell *et al.* (1993) found that surface-bound glycoproteins (a group to which mucus compounds belongs) of females were responsible for stimulating pre-copulatory behaviour by males of the rotifer *Brachionus plicatilis*. This phenomenon has subsequently been shown to occur in the copepod genera *Coullana* (Lonsdale *et al.* 1996, Frey *et al.* 1998) and *Tigriopus* (Kelly *et al.* 1998). It is possible that exocrine glands secrete these glycoproteins, as they were particularly prominent on the caudal rami and the urosome and, if this were true, we would expect to find an increased activity of these glands in females, especially during stages when they are 'receptive' to mating behaviour by males. It is also possible, though, that these glycoproteins are not part of a secreted coating but instead are an integral part of the cuticle and are produced during its formation. Gharagozlou-van-Ginneken (1979) discovered that glycoprotein-secreting glands in the genital segment of the harpacticoid copepod *Tigriopus brevicornis* appeared to show increased activity during mating and, from this, proposed that those glands were involved in secreting a substance which acted as a sex pheromone.

The other, less commonly occurring component of copepod glands that has been identified histologically is protein. Proteins have been identified in the glandular secretions of *Diarthrodes cystoecus* by Fahrenbach (1962), *Porcellidium* sp. by Garagozlou-van-Ginneken (1977) and for *Temora longicornis* by Bannister (1993). Protein production in gland cells has been demonstrated mainly by the

analysis of ultrastructural components (Arnaud *et al.* 1988a,b, Bannister 1993a). Where proteins have identified in copepod gland secretions, they have been generally considered to indicate the presence of enzymatic fractions in those secretions.

Histochemically, mucus is relatively easily distinguished but no real information regarding the specific biochemical attributes of the mucus can be ascertained without rigorous biochemical techniques being applied. Much confusion persists regarding the nomenclature of different types of mucus and the terminology is not uniform throughout the literature with histologists, biochemists and physicians all employing a confusing legion of terms. This problem is not restricted to historical studies but remains a source of consternation in recent research publications. Mucin, mucopolysaccharide, mucoprotein and glycoprotein are terms frequently used to describe substances with mucus-like properties but, at the histochemical level, it is largely immaterial which term is applied. Cook (1990, pg 179, para 4) rightly points out that, in any case, all these terms refer to '*hexosamine containing polysaccharides covalently bound to varying amounts of protein*' and are therefore all are as equally justified as they are vague. In the current work the term 'mucus' shall be applied whenever a substance with mucus-like properties is discussed. The term 'mucus' shall be used in the present study to denote both the intracellular gland products prior to secretion as well as the secreted form of those mucus components and encompasses all other chemical components that mucus may contain regardless of their biochemical attributes. Following secretion, the individual mucus granules become hydrated and form a seamless mucus gel but it is unclear whether their biochemical characteristics are altered in the post-secretion state (Silberberg, 1989). Reid and Clamp (1978) state that secreted mucus has the same staining characteristics as the intracellular material prior to secretion.

Mucus compounds generally possess high molecular weights and, although their precise structural composition varies, they are all generally very similar in their gross structure (Silberberg, 1989). The high molecular weight and high carbohydrate content of mucus compounds confer upon them the properties of a high viscosity in solution. Mucus secretions are very common amongst terrestrial invertebrates but are most widely utilised by those invertebrates of the marine environment (Astley and Ratcliffe, 1989).

Marine invertebrates exploit secreted mucus gels in a wide variety of ways including locomotion, desiccation resistance, as a food source and as an anti-foulant factor to prevent shell infestations (Jones, 1984) and can account for up to 80% of the total energy budget of some species (Denny, 1989). Mucus compounds are also commonly found in vertebrates where they serve to lubricate and protect epithelial surfaces (Allen, 1983). This protective action consists of two distinct properties: a physical one, preventing abrasive damage to surfaces but also a chemical one where enzymes within the mucus serve to protect the surfaces from chemical damage i.e. proteolytic enzymes in the digestive fluids of mammals. Mucus is also central to the protective ciliary action of bronchial passages. A further mechanism of protection by mucus has also been suggested (Parsons and Mulholland 1978 cited by Bancroft and Stevens (1990)) where mucus serves as an 'anti-adhesive', preventing bacteria from attaching to human urinary bladder epithelium.

4.1.2 Ultrastructural characteristics of copepod glands

Exocrine gland ultrastructure has been examined in copepods, cephalocarids, branchiopods and many malacostracans as well as in some terrestrial arthropods, with many similarities between structure becoming apparent. In the Copepoda three main types of gland (labral, luminescent and tegumental) have been examined in detail and, with the exception of the luminescent glands, these gland types have frequently been found to have very similar ultrastructural characteristics despite their differences in terms of the proposed functions of their secretions. This close similarity of ultrastructure is not surprising given the frequent occurrence of mucus compounds within gland tissues and secretions and the same basic pathway of synthesis recognised for such compounds.

In general, the presence of three particular features; rough endoplasmic reticulum (RER), Golgi bodies and secretory vesicles typify secretory gland cells, in particular those secreting mucus compounds. The types, and relative abundance, of these features present in glandular tissues can be used to infer the class of compound that is being produced and whether that material is intended for secretion or use within the cell. RER plays a central role in the synthesis of secretory proteins, membrane proteins and proteins

destined for the lumen of the endoplasmic reticulum. The degree of development of RER is frequently used as an indication of the level of activity of protein synthesis (Arnaud *et al.* 1988a). The Golgi apparatus (also referred to in the singular as a dictyosome) is a system of flattened membranous sacs that are continuous with the envelope of the cell nucleus. One of the known functions of Golgi complexes is their role in the addition of carbohydrate groups to proteins to form glycoproteins through the action of various glycosyl transferase enzymes. Golgi complexes and rough endoplasmic reticulum are often found in close association with one another and together form the main pathway of synthesis of glycosylated proteins, or carbohydrates (Leblond and Bennett, 1977). The peptide cores of these glycoproteins are synthesized on the membrane-bound ribosomes of the RER. Proteins synthesized by the attached ribosomes of the RER are segregated in the lumen of the ER and from there can be transported to the Golgi complex by shuttle vesicles that pass back and forth between the two structures. Smooth endoplasmic reticulum (SER), without attached ribosomes, is less typical of copepod gland cells and is generally thought to be involved in lipid synthesis or detoxification processes as opposed to protein synthesis. Free ribosomes (those not associated with ER) are central in the synthesis of proteins for use within the cell. These proteins are utilised in a number of ways, being involved in the Krebs cycle in the mitochondria, are essential as components of the membranes of organelles and as intracellular enzymes (DeRobertis and DeRobertis, 1987).

Golgi bodies, associated with RER and derived secretory vesicles are features reported in all ultrastructural studies of copepod exocrine glands (Briggs 1978, Gharagozlou-van-Ginneken 1979, Gotto and Threadgold 1980, Hipeau-Jacquotte 1987, Arnaud *et al.* 1988a,b, Bannister 1993a,). Arnaud *et al.* (1988a,b), provide the most comprehensive description of the ultrastructure of copepod glands and the detail of their analysis of one particular set of glands has never been replicated since. Their analysis of the labral gland complex of *Centropages typicus* indicated that the complex was composed of multiple gland cells responsible for secreting different compounds into the buccal cavity. From the organisation of the gland cell organelles they proposed that mucus compounds and digestive enzymes were produced by the labral gland complex.

The frequently identified urosomal glands in copepods have only been examined at a light microscope level and, as such, their ultrastructural characteristics remain largely unresolved.

One of the distinguishing aspects of gland ultrastructure is the appearance under EM of the secretory vesicles. Substantial differences in the morphology of these structures are apparent between species, although these differences may reflect different stages in the development of the vesicles or different fixation procedures. A further characteristic is the pattern formed by the vesicles as a whole within the gland cell. Discrete, separate vesicles have been described (Briggs 1978, Gotto and Threadgold 1980), as have large homogeneous masses of secretion (Arnaud *et al.* 1988a, b) as well as a combination of the two conditions where discrete vesicles in the base of the cell progressively merge in the distal region of the cell to form a large mass, whilst vesicles retain their individual form in the basal regions (Gharagozlou-van-Ginneken 1979, Bannister 1993a, Nishida and Ohtsuka 1996). The secretory vesicles of copepod gland cells have sometimes been described as being membrane-bound (Briggs 1978, Hipeau-Jacquotte 1987, Gotto and Threadgold 1980) although this feature is one that is often overlooked by researchers. The actual morphological appearance of the secretory vesicles may be of utility in identifying the biochemical characteristics of their contents but no work has ever attempted to correlate morphology with histochemical attributes across a range of copepod species and gland types.

4.1.3 Control of secretion

An aspect of major importance to be considered when characterising gland function is that of the method of control of secretion. The actual secretion of gland products may be instigated in a number of ways. It may be a continuous 'trickle' flow (Bannister, 1993a) with or without a mechanism of control over the rate of secretion or it may be under neural control in situations where secretion occurs as a specific reaction to certain stimuli (i.e. luminescence in copepods). Direct innervation of exocrine glands has previously been demonstrated by Bannister (1993b) in copepods and by Pochon-Masson *et al.* (1975) in mystacocarids. The utilisation of increased internal hydrostatic pressure for the expulsion of gland

secretions has also been widely suggested (Clarke *et al.* 1962, Hipeau-Jacquotte 1987, Bannister 1993a, Von Vaupel Klein and Koomen 1994) but currently remains hypothetical.

4.2 OBJECTIVES

The objectives of the work presented in this chapter were twofold, aiming to determine, as far as possible, the histochemical properties of the secretions and the ultrastructural characteristics of the glands identified with DAB and TMB in Chapter 3. It was of particular interest to discover whether the gland secretions had an acidic (sulphated) mucus component as such a substance could have caused the observed reaction with the DAB and TMB stains and to discover what other compounds were present in the gland secretions. To this end, a variety of histochemical tests were used to elucidate the constituent parts of the gland contents of *L. salmonis*. A specific stain to highlight sites of cholinesterase activity (cholinergic nerves) was employed to determine whether any of the glands identified were under direct neural control.

The morphology and ultrastructure of the urosomal, labral and anterior gland complexes were examined using electron microscope techniques. The urosomal and labral glands were selected because of the occurrence of apparently homologous glands in other species, whilst the AGC was chosen because it represented a gland structure that appeared not to have been recorded in other species. It was hoped that this ultrastructural data would contribute to the information provided by the histochemical analyses.

Finally, a 3-D reconstruction of a selected glandular complex was also undertaken to assist in understanding how the various components of the complex were related, an objective not realised using serial sections alone.

4.3 MATERIALS AND METHODS

4.3.1 Histochemistry

Adult male *L. salmonis* was the preferred stage for ultrastructural and histochemical studies owing to their smaller size compared to females. This facilitated sectioning and optimised the likelihood of finding glands in any one section, however, adult females were also utilised for histochemistry.

Wax, plastic, resin and cryostat sections were subjected to a variety of histochemical tests (**Table 4.1**) to assess the types of material present within the glandular tissues. Sections for histochemical analyses were prepared following the protocol outlined in Section 2.2.1 The general tissue stains haematoxylin and eosin (H&E), used in conjunction, were used with other, specific histochemical tests where a counterstain was desired. The staining properties of haematoxylin meant that it could also be utilised alone to provide evidence regarding the cellular morphology of identified gland tissues.

Table 4.1 Histological stains utilised and target compounds.

Stain	Compound	Source
Alcian Blue (pH 1.0)	Strongly acidic (sulphated) mucus	Drury and Wallington (1980)
Alcian Blue (pH 2.5)	Acidic (sulphated) mucus	Drury and Wallington (1980)
Bromophenol Blue	Proteins	Mazia *et al.* (1953)
Catechol	Phenoloxidases	Martoja and Martoja (1967)
Diaminobenzidine (DAB)	Peroxidases	Graham and Karnovsky (1966)
Haematoxylin and Eosin	Cytoplasmic and nuclear material	Drury and Wallington (1980)
Periodic Acid-Schiff (PAS)	Neutral mucus and glycogen	M^{c}Manus (1946)
PAS / Diastase	Neutral mucus	Bancroft and Stevens (1990)
Sudan Black	Lipids	Lison and Dagnelie (1935)
Toluidine Blue	General tissues	Kiernan (1990)

The specific tests were carried out against a number of commonly occurring tissue components i.e. protein, mucus and lipid.

To test whether the histochemical properties of the secretions of the urosomal glands were altered after secretion fresh lice had their urosomal glands cut open using a fine scalpel, exposing the secretory granules to the surrounding water. Following this process the specimens were processed as described in Section 2.2.1.1 and stained with Alcian Blue or PAS.

Diastase (combined α- and β-amylase activity) blocks any reactivity which may be attributable to glycogen deposits and therefore, when used in conjunction with PAS, any positive PAS staining can be accounted for largely by the presence of mucus compounds. The malt diastase digestion used in conjunction with the PAS technique adequately discriminates between the reaction of glycogen and neutral mucus compounds.

4.3.1.1 Cholinesterase staining

The method of control of secretion may have implications as to the role that these secretions play. To this end attempts were made to identify sites of neural activity, in this case through the presence of acetylcholinesterase, the hydrolytic enzyme present in nerve membranes responsible for degrading the neurotransmitter acetylcholine. The procedure of Cantwell (1981) to stain sites of acetylcholinesterase activity was modified by fixing the specimens for at least 24h (c.f. 1 – 12h) followed by immersion in the incubation medium for approximately.5 days (c.f. 2 – 12h).

Nauplii, copepodids and adult specimens of *L. salmonis* and adult *C. elongatus* were stained and examined whole, although some were first stained and then processed for wax histology to locate the sites of activity at the cellular level.

4.3.1.2 3-D reconstruction techniques

Because of the complexity of the urosomal gland complex it was deemed necessary to utilise electronic 3-D reconstruction to visualise the interactions of the components. This complex was chosen largely because of its apparent homology with the gland complexes found in the urosome of many free-living species of copepod and therefore its potential for revealing differences in the free-living and

parasitic function of the gland. Its more complicated structure, as compared to the other glands identified in this study also meant that it was the gland whose function and structure were least well understood.

Specimens embedded in Spurr resin (as for electron microscopy, Section 2.2.2) were serially sectioned at 1μm and stained with toluidine blue. Sections were drawn using a drawing tube attached to an Olympus BH-2 compound microscope. Images of alternate sections were digitised and assimilated into two different 3-D reconstruction computer packages (Zeiss KS 300 and VIDAS) to provide a three-dimensional rendering of the gland complex and its associated ducts.

The Zeiss KS 300 system was also exploited to automatically deconvolve stained gland complexes and like structures to allow their clearer visualisation. Smaller stages such as nauplius and copepodid were used for this study as their transparency and limited glandular development contributed to the easier development of a system suitable for the purposes of deconvolving. It was relatively straightforward to design a program to pick out allotted regions of a desired colour and then form them into whole inter-connected systems whilst removing specified background colours or objects. Briefly, the computer was set up to stack sequential images of a specified vertical separation through a whole mount specimen of *L. salmonis*. This stack of images was then compressed into one entire image from which all regions of specified tones, colours or contrasts could be excluded to leave an image consisting solely of the features under investigation.

The Zeiss KS 300 program was the most modern and powerful of the two systems but was the most difficult of the systems to use. The system was designed as an open package for which the operator had to write macros to suit the specific needs of each technique. This was labour intensive, time-consuming and difficult to achieve without extensive knowledge of computer programming. It was more successful at deconvolving images, either from whole specimens or sectioned material. Deconvolving is the process whereby intricate systems can be simplified by effectively disregarding all extraneous information allowing previously obscure relationships to be revealed.

4.3.2 TEM

Transmission electron microscopy was used to identify the ultrastructural characteristics of the urosomal glands, the labral gland and the AGC. These glands were chosen as being representative of the whole gland population subsequent to the histochemical analyses and the conclusions drawn from those analyses. Specimens were prepared for TEM examination following the process described in Section 2.4.

In order to identify fully the relationship between the multiple regions of this complex, transverse sections were taken at various points on the abdomen / urosome, and the gland structures contained within were examined.

4.3.3 SEM

Internal structures of *L. salmonis* were examined with an SEM using several different techniques designed to expose underlying tissues. A wax-carving technique, which was a modification of the method of Oshel (1985), was first tried. Specimens were processed in the same manner as for wax histology (Section 2.2.1.1). Wax-embedded specimens were sectioned at 5μm on a Jung Biocut microtome. Sections were examined under a light microscope until the desired internal structures had been revealed at which point the specimens were de-waxed in xylene (4 changes over 1h at 60°C). The fourth xylene rinse was replaced with 100% ethanol (rinsed 3 times) prior to rotation overnight in 100% ethanol to ensure all xylene had been removed. Specimens were again rotated overnight in a 50:50 solution of ethanol : HMDS before transferal to 100% HMDS (2h minimum) and a final change of HMDS which was left to evaporate overnight in a fume-hood. Dried specimens were mounted on aluminium stubs using double-sided sticky tape and coated with gold using an Edwards Sputter Coater S150B.

Other methods used included the Dry Fracturing technique (Toda, Suh and Nemoto, 1989) and a more controlled Dry Dissection described by Andrade-Salas (1997). The Dry Fracturing Technique was used on both nauplius and copepodid stages with mixed results. The process involved preparing a SEM stub by covering its surface with a uniformly thin layer of Araldite Rapide Adhesive (Ciba-Geigy Co.). To this stub two narrow strips of aluminium cooking foil were attached along opposite edges leaving a

strip of adhesive coating uncovered in the centre of the stub. Care was taken to ensure that the foil strips overlapped the edge of the stub. Dried, processed specimens were carefully placed on the adhesive-covered strip and left until they were securely attached to the surface. A further stub, coated with a fine layer of fresh adhesive was then placed directly onto the specimen-carrying stub. After being left to harden overnight, the two stubs were pulled apart, fracturing the specimen in the process. The foil strips acted to prevent the two opposing stubs coming together completely and crushing the specimen. Following fracturing, the specimens were coated as usual before examination. This process, although to some extent successful, was largely hit and miss – very little control could be exerted and specimens were frequently severely damaged.

A more successful technique, especially for the larger stages was the Dry Dissection technique. Dried, processed specimens were attached to stubs using either Araldite Rapide adhesive or double-sided specimen-mounting tape. After the specimens were mounted in the desired orientation they could be crudely dissected under a binocular microscope using either very fine dissection needles or specially prepared glass needles. Very fine glass needles were prepared using a pipette-puller, which could be used to provide needles which were sufficiently fine for the required purposes but were strong enough to penetrate the cuticle without breaking or flexing excessively. These needles were used to gently remove flaps of cuticle to expose desired tissues. Due to the lack of finesse afforded even with this technique, only large gland complexes such as the AGC and the urosomal glands could be exposed, as smaller glands such as dorsal surface glands were destroyed whilst trying to penetrate the brittle cuticle. Again, after the dissection was complete the specimen was coated with gold prior to observation.

4.4 RESULTS

4.4.1 Histochemistry of gland complexes

All the glands located using DAB and TMB (Chapter 3) were found to share the same broad histochemical characteristics. A summary of these results can be seen in **Table 4.2**. All glands stained negatively with Alcian Blue suggesting that the positive reactions of the glands to TMB and DAB indicate that at least a component of the secretion is a peroxidatic enzyme. **Table 4.2** clearly indicates that none of the gland tissues examined gave a positive reaction to the Alcian blue stain, whereas they all showed a positive response to the PAS stain. Proteins and lipids were both identified in all gland tissues, where observations were made. A full description of the results of each of the stains follows below.

Table 4.2 Results of histochemical analyses of *L. salmonis* tissues.

	PAS	AB (1.0)	AB (2.5)	BPB	CH	SB
Urosomal	+	-	-	+	-	+
Circum-oral	+	-	-	+	-	+
Labral	+	-	-	+	-	+
Thoracic leg	+	-	-	+	-	+
FGC	+	-	-	+	+	+
Suture	n/o	-	-	+	-	n/o
AGC	+	-	-	+	-	+
DSG	+	-	-	+	-	n/o
Gut	+/-	-	-	+	-	-

FGC, filament gland complex; Suture, suture line glands; AGC, anterior gland complex; DSG, dorsal surface glands. Stains used: PAS, Periodic Acid Schiff; AB, alcian blue; BPB, bromophenol blue; CH, catechol; SB, sudan black. n/o no observation.

Formalin, or the automated process associated with its use for paraffin wax histology, was found to cause excessive tissue shrinkage making it of limited value for preserving the precise structure of the glands, although morphology was retained sufficiently for histochemical purposes. However, glutaraldehyde fixatives such as Karnovsky's (Karnovsky, 1965) and Marine Invertebrate Fixative (Eisenman and Alfert, 1982) and the picric acid-based fixative Bouin's fluid were found to adequately

retain the morphology of the glands when used with plastic or synthetic resin embedding media, and were the fixatives of choice with these media.

4.4.2 Specific results of histochemical trials

4.4.2.1 Alcian Blue

Staining, or more correctly dyeing, with Alcian Blue gave no positive results with either the vesicle contents or cytoplasm of any exocrine glands at both pH 1.0 and pH 2.5, suggesting that acidic (sulphated) glycoproteins are not a component of the gland product. Amorphous material adhering to the setae of the thoracic legs (**Figure 4.1**) and urosome was frequently found to give positive reactions to the Alcian Blue stain despite the negative reaction of the glands and their contents. The glands of those lice which had had their urosome incised and their gland contents exposed to water still showed a negative response to Alcian Blue at both pH's, suggesting that the secretion does not alter in its biochemical and histochemical characteristics post-secretion or as a result of the hydration associated with this process. Positive Alcian Blue staining was frequently seen within the guts of lice, as either a fine layer coating the surface of the cells of the gut lumen or as a dispersed, flocculent substance, throughout the whole of the gut lumen.

Occasional, positive Alcian Blue staining was also observed in a thin layer located immediately below the cuticle. This staining was inconsistent, sometimes appearing as a strongly staining band and sometimes not at all and may correspond to the epidermis. There was no apparent order to this staining of the cuticle, with differential staining often occurring within specimens. It seems likely that the staining material was located between the epidermis and the cuticle. The lumen of the natatory setae of the thoracic legs and of the terminal setae of the caudal rami also showed a slight positive response to this stain suggesting that acidic glycoproteins are present there again either within, or on top of, the layer of epidermis immediately underlying the cuticle. There was no evidence to suggest that this positively staining material originated in any of the glands identified in this study.

4.4.2.2 Bromophenol Blue

Despite being a broad-spectrum protein stain, some useful histochemical information was acquired from sections stained with this compound. All the previously identified glands stained positively with bromophenol blue, although a response to general structural proteins in most body tissues would be expected.

Typical Bromophenol Blue staining (of the AGC) is shown in **Figure 4.2.** All regions of the AGC showed a positive reaction to this stain. Examination under higher magnification revealed that the honeycomb appearance was due to the secretory vesicles giving only a slight positive reaction to this stain, suggesting that the secretion from this gland complex does not have a particularly high protein content, although the sparseness of material in a section of vesicle may also have some bearing on the intensity of staining observed.

All regions of the urosomal glands showed similar strength staining with the exception of one region which stained with a markedly greater intensity. This region of strong staining appeared to correspond to the large vesicular region of this complex. All other regions of this gland stained weakly although several slightly darker staining spots were evident within the gland syncitium. Whether these spots represent glandular ducts or were merely nuclei taking up the stain could not be determined at the level of resolution of this technique.

The DSG stained weakly with bromophenol blue although their nuclei stained strongly. Frequently evident in the DSG were fine, strongly staining thread-like seams of material which were presumed to be the secretory ducts. The walls of the duct, as opposed to the secretion, may have caused the intense staining of the ducts itself as suggested by the weak response of the gland tissues themselves to this stain, although concentrated secretory material within the duct could also have caused this apparent difference.

Most sea louse tissues gave a positive reaction to this stain. In particular, strong reactions were observed in regions of thick cuticle i.e. the sternal furca and the cuticle of the interpodal bars of the thoracic legs. The outer cuticles of the spermatophores also stained very strongly.

4.4.2.3 Catechol

Phenoloxidase was identified in large amounts in certain regions of the cuticle using the catechol staining technique. Positive staining of tissues with catechol arises due to polyphenol oxidases in tissues reacting with catechol to form quinone that reacts with adjacent protein molecules and 'tans' them to a brown / orange colour. There was no evidence to suggest that this compound was being actively secreted by any of the glandular complexes identified in this study. No positive reaction to catechol was seen in any of the glands except the filament-producing glands (FGC), and even there only a very weak reactivity to catechol was observed. Most regions of the cuticle gave a weak response to catechol suggesting that phenoloxidases are a ubiquitous presence in the cuticle. Regions of what are known to be thicker than average cuticle showed a marked amber colouration indicating higher levels of phenoloxidases. These regions included the tips of the antennae and maxillipeds, the post-antennal processes, maxillules, sternal furca and the interpodal bars of the thoracic legs.

4.4.2.4 Diaminobenzidine staining

Sea lice stained with DAB according to the protocol given in Chapter 3 were also processed for paraffin wax processing. Sections of such tissues were counterstained with H&E to demonstrate the specific location of the DAB staining.

All the suspected gland structures identified in Chapter 3 were observed in sections, such as that shown in **Figure 4.3**, to be stained to varying degrees with the DAB stain. Generally all regions of the gland tissues stained positively although considerable variation in the intensity of the DAB staining was seen to occur between specimens. DAB-positive staining of the glands was seen to occur throughout the whole gland tissue and was not restricted to any particular region or pole. The effect of either the DAB

stain or the hydrogen peroxide used in the staining process frequently caused substantial damage to those tissues that gave a positive response to the stain. This damage however did not prevent the discrimination of stained tissues from non-stained ones.

4.4.2.5 Haematoxylin and Eosin (H&E) staining

H&E staining gave an insight into the ultrastructural assemblage of the gland tissues. The purple staining of the haematoxylin component revealed the nuclei of gland syncytia and, in the AGC, labral, thoracic and urosomal glands, clearly showed that there was a structural dichotomy to the gland tissues. This dichotomy was evident as a peripheral region of concentrated nuclei distinct from a nucleus-free eosinophilic central region. **Figure 4.4** clearly demonstrates the ultrastructural details typically revealed in gland tissues stained with H&E.

The remaining central tissue of the gland syncytium stained pink with the eosin component indicating that the remainder of the syncytium was a nucleus-free zone.

4.4.2.6 Periodic acid-Schiff (PAS)

All regions of the urosomal glands stained positively with PAS although some differences in the intensity of staining were evident between the different regions of the gland. All regions of the complex, except one, showed an intense positive response to PAS (**Figure 4.5**). The gland region that displayed the weak response was the region of this complex which had a honeycomb-like appearance when viewed under the light microscope and is clearly visible in **Figure 4.5**. The positive reaction to PAS was visualised throughout the whole of the gland cytoplasm and did not appear to be confined solely within vesicles. Many small areas of strongly positive staining were observed in the cytoplasm of the urosomal glands as seen in **Figure 4.5**. Due to the dendritic pattern of these intensely stained thread-like structures, it was presumed that they were the ductules and ducts of the gland and their intense reaction with the stain arose due to the concentration of gland product in their lumen. The ducts were evident either as bright

spots or as long, meandering threads of magenta colouration which was dependent on the angle of sectioning.

All the DAB-positive thoracic leg glands gave positive responses with the PAS stain. The glands displayed staining characteristics similar to that of the urosomal glands, staining a uniformly dark pink colour with the main ducts and collecting ductules appearing as intense magenta coloured spots, or streaks, within the gland syncytium. In the larger glands, such as those of the endopods of the th_{II}, these spots could be seen to converge on larger, more intensely staining, ducts indicating that the secretion is drained from the gland via many smaller ductules which converge on larger, less numerous ducts.

The labral gland was composed of two structurally, and histochemically, different regions, one appearing to be a zone of accumulated vesicles characterised by numerous closely packed vesicles, and a region peripheral to this of cytoplasmic material with no obvious vesicles. The non-vesicular zone showed a clear positive reaction to the PAS stain with the ductules staining more intensely still. The zone of vesicles stained weakly positive whilst the remaining region of the gland tissue stained more intensely **(Figure 4.5)**, a phenomenon which may be attributable to the density of material in the section at specific points and not with any specific histochemical / cytochemical properties. The PAS stain also clearly showed how the ducts of the circum-oral glands (lateral glands as identified by Bron (1993)) merge with the labral gland. The collecting ducts of the circum-oral glands, where they pass into the labral gland, showed a strong reaction to the PAS stain indicating that neutral mucus forms at least one component of their secretion.

Other, non-glandular regions, also showed strong positive reactions to the stain. In particular, the outer cuticle of the spermatophores stained a very deep magenta indicating that the tissue contains a high proportion of glycoproteinaceous material. Similarly, the outer membranes of the accessory glands / cement glands showed a very strong positive response to PAS. A thin layer of the epicuticle also stained weakly positive but, this layer was never very distinct and appeared to lack uniformity, being present in some areas and not in others. It cannot be said with certainty whether this staining represented a facet of the cuticle itself, was caused by gland secretions on the external surface of the cuticle or was artifactual.

Comparison of sections stained with PAS and sections stained with PAS following malt diastase digestion, showed no apparent differences in the staining profiles of the gland tissues. This positive reactivity to PAS following diastase treatment suggests that mucus, and not glycogen, was giving rise to the positive response to the PAS stain seen in this species.

4.4.2.7 Combined PAS and Alcian Blue staining

PAS and Alcian Blue used in combination, and utilising two different pH's of Alcian Blue to discriminate between mucus compounds of differing acidity, provided little further histochemical evidence over that found using the stains independently of each other. The combined stains indicated that none of the PAS staining observed in gland tissues was attributable to Alcian Blue-positive components. **Figure 4.6** shows the urosomal region of an adult male *L. salmonis* stained with PAS and Alcian Blue in combination and clearly indicates that the urosomal glands stain positively with PAS, and that the Alcian blue staining is restricted primarily to material adhering to the external surface of the cuticle. Details of histological importance are still evident although not with the same integrity as in specimens processed for plastic embedding such as the urosomal gland stained with PAS of **Figure 4.5**.

4.4.2.8 Sudan Black B

Sudan Black B is a fat-soluble dye which stains neutral hydrophobic fats and lipids (triglycerides), liquid cholesterol esters and some phospholipids in tissue sections but can also be utilised as an immersion stain for whole, unfixed specimens. There is no chemical linking involved in Sudan Black staining, staining is based purely on a difference in solubility between the substrate and the stain solution: Sudan Black is more soluble in lipids than in its solvent.

Whole nauplius larvae did not readily stain with Sudan Black, however, whole copepodids did stain easily, revealing a consistent pattern of densely staining, presumably glandular regions. These regions were largely confined to the thoracic legs and occurred in the same pattern found for DAB-

positive glands in these limbs. The lipid vesicles prominent at this stage also stained heavily and could be seen as a large reservoir of globules within the protogut within the cephalothorax.

The general body tissues of preadult and adult specimens of *L. salmonis* stained very heavily although several areas of denser staining stood out from the background staining. These darker areas persisted after the background staining had been decreased by rinsing the specimens in 70% ethanol. It was presumed that these densely staining regions represented deposits, or accumulations of lipids. The thoracic leg glands of the adult stages stained positively with Sudan Black suggesting that there was a strong lipid component within those glands. The pattern of lipid-containing glands found in the thoracic legs matched exactly that of DAB-positive glands in the adult thoracic legs. The thoracic legs of the preadult stages showed no positive staining with Sudan Black.

Other lipid-rich regions were found in both preadults and adults of both sexes. A pair of gland complexes corresponding to the urosomal glands of the DAB-staining trial was found in all specimens. In adult males considerable quantities of material adhering to the terminal setae of the caudal rami stained positively with Sudan Black, in contrast to preadult and adult females which never appeared to have any such material on their setae. There was always at least one lipid-containing gland, and sometimes as many as three, found at the most anterior edge of the cephalothorax, on either lateral margin, immediately posterior to where the antennules contact the cephalothorax as evident in **Figure 4.7**. These glands were large and were visible from both the dorsal and ventral surface and corresponded to the AGC of the DAB-staining trial. The postoral glands were also seen to stain positively with Sudan Black. This staining was always most conspicuous in preadult I females although the glands stained positively in all preadult and adult stages.

A further pair of positively staining glands was found in most specimens examined and were located on the ventral surface of the animal close to the base of the labium and could only be seen by lifting the mouth-tube into a position perpendicular to the ventral surface. These glands corresponded to the DAB-positive circum-oral glands.

Staining of cryostat sections with Sudan Black B was problematic initially, but after some refinement of various protocols a successful system was developed. It was found that sections post-fixed for 1h in formal-calcium and stained in Sudan Black B for 30min gave the best results. De-lipidised sections were used as negative controls and rainbow trout (*Oncorhynchus mykiss*) brain and liver were used as positive controls in all trials. Effective counterstaining of Sudan Black stained sections was never fully achieved in this study. It was found that the use of a nuclear stain (Mayers Haematoxylin) caused much consternation as the resulting purple colour served only to complicate the pattern of staining as Sudan Black can give rise to a variety of colours of which purple is one.

The labral and AGC glands showed distinct sudanophilia. The entirety of the labral gland showed positive, but uneven, staining with Sudan Black as shown in **Figure 4.8** and, in one specimen, the ducts of the lateral glands entering the labral gland could be clearly seen to give a positive reaction to the stain. The labral gland displayed a dichotomy in intensity of staining and this is evident in **Figure 4.8** where the peripheral regions of the gland stain less intensely than the internal regions. Such lighter staining however may occur in thinner sections of the gland tissue. This positive staining had the appearance of small oil droplets with obvious refractive properties. This would suggest that the stain is reacting with a component of the secretion as opposed to membrane lipids, which may be present in high concentrations in gland tissues with their high levels of ER, and Golgi bodies c.f. muscle tissue.

The thoracic leg glands all stained positively but a duality of staining intensity within each gland was evident in stained sections that was not observed using whole animals. The whole of each gland could be visualised as the outer zone of production stained a distinct grey colour while the inner zone of accumulation stained very darkly and could be seen to be composed of a multitude of small black spheres which were presumed to be the secretory vesicles. Whether the contents of the vesicles or their membranes were reacting with the stain could not be determined.

No other tissues of *L. salmonis* gave a positive response to this stain in cryostat sections suggesting that lipids formed a significant component of glandular tissue in this species, although other lipids will undoubtedly be present, perhaps at levels beyond the limit of this technique.

Some small regions of positive staining in the cuticle caused some confusion. This staining was witnessed in all sections except those treated with the de-lipidising solution (50:50 chloroform:methanol) suggesting that the response was being generated by some form of lipid material. This staining was evident in all body segments including the genital segment and the abdomen. The staining took the form of many small regions of purplish colour, with a granular nature, lying either immediately below the cuticle but frequently appeared to extend into the cuticle, and sometimes extended through the full thickness of the cuticle. Examination of cryostat sections which had not been fixed or stained proved conclusively that these sites of staining were actually melanophores.

4.4.2.9 Toluidine Blue

Nearly all tissues of *L. salmonis* stained with toluidine blue. Its utility as a stain lay in its ability to emphasize structural facets, which were less visible with routinely applied stains. Toluidine blue is routinely used to stain semi-thin sections for TEM analysis and reacts with a wide variety of different tissues.

In this study only the urosomal, labral and AGC complexes were stained. Both the urosomal glands and the labral gland, when stained with toluidine blue showed different intensities of staining which corresponded to the two superficially different structural regions of the glands observable at a light microscope level. The non-vesicular regions stained a uniform dark blue colour whilst the zones of vesicle accumulation displayed a weak grey-blue translucency (**Figure 4.9**). This lighter colour of the vesicular zone may have arisen as a result of the lower density of material in the vesicles compared with that of the cytoplasm of the gland syncytium itself.

4.4.2.10 Cholinesterase staining

This technique for demonstrating nerve tissue proved to be of limited utility in this study. The results were inconsistent and staining was difficult to achieve to any degree of satisfaction. The failure of stained nerves to persist through the processing for wax histology also meant that little detailed

information could be derived from the staining trials. Some useful information however was obtained regarding the general pattern of nerve axons in *L. salmonis*. Adult *L. salmonis* showed the clearest staining of all the life stages examined. The results for the larval stages were inconclusive. The same was also true for *C. elongatus*, where again, their small size may mean that fixation was difficult to achieve satisfactorily. The adult stages of *L. salmonis*, however, showed very clear staining of the main ganglion and the main nerves associated with it, including nerves innervating the limbs, antennules, frontal plate, mouth-tube as well as some leading to, what was presumed to be, the terminal setae of the caudal rami. Several were also seen to lead to the lateral margins of the cephalothorax, particularly towards the region where the AGC were located. Macroscopic observation of whole animals failed to elucidate whether any of these nerves were innervating any of the exocrine glands identified. There were too few stained nerves present to innervate all the DSR but the presence of nerves, which were too small to be distinguished by this method, should be considered.

To determine whether any of these nerves did innervate the exocrine glands some acetylthiocholine-stained specimens were processed for wax histology. After this processing however, and sectioning as described above, the stained nerves were not apparent and could only be detected vaguely in certain areas such as the bases of the antennules. The question of whether or not the glands are innervated remains unresolved using this technique.

4.4.3 3-D reconstruction techniques

Figure 4.10 gives an example of how the 3-D reconstruction technique can be applied to routine analysis. This image of the gland system of a DAB-stained *L. salmonis* nauplius was constructed automatically from a whole specimen using the Zeiss KS 300 system and clearly shows the internally leading ducts of the posterior and lateral glands. **Figure 4.11** shows a full sequence of images of the specimen depicted in **Figure 4.10** as it rotates through a 180° path through its longitudinal axis and illustrates the utility of this system in allowing the complex interactions of the gland complexes to be realised in three dimensions.

The VIDAS system, although older and less powerful than the Zeiss KS 300 system was significantly easier to use and provided better 3-D reconstruction capabilities. The urosomal gland complex was used as a test subject as it was the most complicated exocrine gland system in *L. salmonis* whose structure had not been fully resolved. Light microscopy could only go so far in allowing the visualisation of the intricate associations of the different gland tissues identified within this structure. **Figure 4.12** shows a 3-D reconstruction of the urosomal gland complex of a single caudal ramus of an adult male *L. salmonis* achieved using the VIDAS system. Although providing a less refined image than the Zeiss system, images produced by the VIDAS system were significantly easier to interpret and yielded more information than those produced by the Zeiss KS 300 system. **Figure 4.12** shows the final result of the 3-D reconstruction of the urosomal gland complex. It clearly shows the complicated interrelationship of the gland tissues and was used as an aid to interpret the 2-D images described in the following section.

4.4.4 Ultrastructural characteristics of the urosomal gland complex

Light microscope measurements of the urosomal gland complex demonstrated that it was a large system of approximately 290μm length, 90μm height and 110μm width, as a whole mass. At the light microscope level, the complex appeared to consist of three or four intimately associated, but cytologically separate, glandular units. TEM helped to resolve the inadequacies of light microscopy and revealed the true nature of the complex and served to reaffirm the evidence of the 3-D reconstruction. Transverse sections for TEM examination were taken at various points across the urosome and caudal rami, the approximate positions of which are shown in **Figure 4.13.**

4.4.4.1 Gross morphology of the urosomal gland complex

The most anterior section (I) through the gland complex revealed a variety of different syncytial glands to be present at this point. For simplicity these glands were appointed alphabetic titles (**Figure 4.14**). Two glands were present dorsal to the hindgut and were termed the 'A' glands, whilst the two large glands present in the ventral half of the urosome were termed the 'B ' glands. A third gland was also

present at the outer lateral margin of one side of the urosome and was termed the 'C' gland. Section II showed that four gland tissue types were present at this point of the urosome, as shown in **Figure 4.15.** Gland B was closely associated with gland A and a further large gland termed 'D'. The D gland had a conspicuous central region that had a honeycombed appearance under the light microscope. Where the gland components were seen to come into contact, distinct membranes were evident between them suggesting that they were either separate glands or at least different lobes of the same tissue.

In section III glands only B, C and D are present, as the posterior margins of the A gland have been passed. In the left caudal ramus **(Figure 4.16)** gland B presents two lobes that form an L-shape that are clearly separated by a membrane giving the appearance of two separate glands. This L-shaped tissue closely adjoins gland D on its right lateral and basal faces. Gland C reaches its largest size at this point and fills the bulk of the lateral margin of the urosome.

Section IV shows a similar pattern of glands as identified in section III with the bulk of the gland material present being accounted for by glands B and D. At this point the urosome divides into two parts **(Figure 4.17)** indicating that the section was taken posterior to the anus and now shows the extent of the complex within the caudal rami. Due to the sections not being made at perfectly perpendicular orientations across the urosome there were slight differences between the left and right caudal rami. Rather than being a hindrance this was in fact beneficial as, in effect, it allowed the visualisation of two planes of sectioning of the complex within each section of the specimen. The right caudal ramus contains only one gland tissue at this point and it appears to correspond to gland B. If this area of section IV is compared with section III **(Figure 4.16)** we can develop a more detailed understanding of the close spatial relationship between these two tissues. Comparison of the two sections clearly indicates that gland B enlarges posteriorly to envelop gland D. Whether one or both lobes of gland B are responsible for this is unclear. Certainly the remnant of gland B that is visible posterior to gland D is a single tissue that is not divided by a membrane suggesting that only one lobe of B persists here. This evidence supports that of the VIDAS reconstruction of the complex **(Figure 4.12)** where gland B is seen to surround the posterior margin of gland D.

The posterior margin of gland C is still evident in the left half of the urosome although it is much smaller than in previous sections as it tapers away posteriorly. The left caudal ramus (sectioned slightly more anteriorly than the right) again shows gland D with its conspicuous zone of secretory vesicles although it is at this point diminishing in size.

In the final and most posterior section (V) through the urosomal gland complex the tissues present (**Figure 4.18**) could be easily correlated with the glands identified in section IV in **Figure 4.17**. The remote gland (C) was present in the left caudal ramus, as was the posterior-most vestige of gland D along with two smaller lobes of gland B. A similar situation appears to be occurring in this section as occurred in the right caudal ramus of section IV where gland B was about to engulf the posterior-most margin of gland D. The right caudal ramus contained only a small portion of gland B with a conspicuous central duct.

4.4.4.2 Ultrastructure of gland A

Gland type A lay immediately dorsal and dorsolaterally to the hindgut and was particularly prominent at this point. In some sections this gland was seen to be bisected by a membrane running in a dorsoventral direction and appeared therefore to consist either of two lobes, or possibly two separate glands. Both lobes, or glands, had similar ultrastructural characteristics. Many nuclei were present throughout the gland tissue indicating that the gland was syncytial. This tissue was characterised by its prolific ER with the very swollen electron-lucent lumen apparent in **Figure 4.19**. It was difficult to determine the exact nature of this ER but it did not have any conspicuous attached ribosomes, which suggests that it was SER. Very little typically shaped RER was present but the general cytoplasm of the gland contained large numbers of free ribosomes (approximately 20nm diameter). Golgi complexes and mitochondria were also abundant in the tissue and are both evident in **Figure 4.19**. Mitochondria were generally spherical in shape with lamellar electron-lucent cristae. Transitional vesicles (530nm – 0.11μm diameter) between the RER and the Golgi complexes are evident in **Figures 4.20** and **4.21** and the trans faces of the Golgis can be seen to have swollen cisternae where secretion production is underway. Shuttle

vesicles (0.11μm – 0.17μm diameter) moving between the cisternae of the Golgis were also frequently observed and are also evident in **Figures 4.20** and **4.21**. The Golgi complexes of this region often had large secretory vesicles (approximately 0.80μm diameter) associated with their trans, or maturing, faces.

Such secretory vesicles (**Figure 4.20**) were present, although not abundant, in small clusters throughout the cytosol. The physical appearance of the vesicles was not uniform with some containing a homogenous electron-lucent material whilst the majority of vesicles had a very distinctive structure, each vesicle appearing to be composed of two separate constituents: the bulk of the vesicle was relatively electron dense but contained within it a smaller, electron lucent region. The Golgi complexes were generally observed close to the sparse RER present within the gland cytoplasm (**Figure 4.21**)

Irregularly shaped structures, all with similar gross morphologies were frequently observed within the cytoplasm of the gland. These structures appeared to be membrane bound and internally had what appeared to be microvillous projections (**Figure 4.22**) arising in many directions. The dimensions of the microvilli varied considerably according to the plane of sectioning and in **Figure 4.22**, but in cross section they varied between 0.11μm and 0.17μm diameter. In gland A these structures appeared to be present throughout the cytoplasm with no evident order to their distribution.

4.4.4.3 Ultrastructure of gland B

Glands of type B occupied the bulk of the ventral half of the urosome, one being present in the anterior reaches of each caudal ramus (**Figure 4.14**). The cytoplasm of gland B consisted of a dense mass of very electron-dense granules (**Figure 4.23**) that, from their dimensions (20 – 25nm diameter), are presumed to be ribosomes. The ER of this gland appeared discontinuous, possibly due only to its orientation within the tissue section. Due to this fragmented nature, it was difficult to determine whether the ER was of the rough or smooth type. The mitochondria of this region tended to be rather irregularly shaped but with conspicuous electron-lucent cristae, as apparent in **Figure 4.23**. Very few secretory vesicles were present in gland B and these were often observed close to the small microvillous regions (similar to those described for gland A) that were evident within the gland cytoplasm and shown in **Figure**

4.24. These vesicles were generally small (0.13 - 0.19μm) with very electron-lucent flocculent material within them (**Figure 4.25**). Golgi bodies were also evident but not abundant. Those Golgis observed were seen to be very active with transitional vesicles entering them on their cis faces. These vesicles were presumably derived from the closely associated ER. Nuclei were present primarily in the peripheral margins of the gland tissue indicating that gland B was also a syncytial tissue.

4.4.4.4 Ultrastructure of gland C

Gland C was remote from the main body of the urosomal gland, lying close beneath the dorsolateral cuticle at the point where the urosome separates into the caudal rami (**Figures 4.14** and **4.15**). However, its spatial proximity to the urosomal gland complex, and its similar staining qualities, may indicate a commonality in respect to function and so has been included within the urosomal gland group. This gland also appears homologous with components of the 'perianal' glands of Boxshall's (1982) description of *B. palliata*. As with the other gland regions, gland C was also syncytial but nuclei appeared to be less prevalent than in other regions. The matrix of the gland was homogenous and there were no demarcated zones of production / accumulation etc. Gland C had a similar ultrastructure to gland B. The cytoplasm was very electron-dense and had a granular appearance with the bulk of the cytoplasm being taken up large quantities of very full ER (**Figure 4.26**) of an undetermined nature. This organelle was so prevalent and so well developed that it gave the gland a superficial honeycomb appearance at low magnification due to the presence of large areas of lumen of low electron density and was the most significant feature of this tissue. Golgi complexes with numerous tightly packed, narrow cisternae were frequently seen in association with this ER. Some vesicles closely resembling the previously described heterogeneous vesicles of gland A (**Figure 4.20**) were observed in the gland cytoplasm but were never present in large numbers. Many ductules were present in the main body of the gland but very few vesicles were apparent at this point. The ductules appeared to be concentrated in the dorsal region of the gland with very few found elsewhere. A large duct was located running in an anterior to posterior direction on

the dorsal surface of the gland at this point. Internally the duct displayed numerous projections, which presumably served to strengthen the duct.

4.4.4.5 Ultrastructure of gland D

Gland D, along with gland B form the bulk of the gland tissue of the urosomal gland complex, (**Figures 4.15, 4.16** and **4.17**) had the most striking appearance of all the glands, being easily identified as a result of the distinctively numerous membrane-bound secretory vesicles which formed the bulk of the internal zone of the syncytium. The characteristic central core of secretory vesicles is evident even at a light microscope level. A further distinctive difference between glands B and D was the electron-dense cytoplasm of the former in contrast to the much more electron-lucent cytoplasm of gland D. The contents of these vesicles showed some variation in their apparent textures and electron densities but generally they contained a moderately electron dense, flocculent, or fibrillogranular, material. No apparent order was evident in the position of the superficially different vesicles within the gland syncytium nor was there any apparent correlation between the size of the vesicles and their electron densities. Immediately within the vesicle membrane there was frequently a ring around the circumference where no material was found. The vesicles were generally spherical or slightly ovoid in shape and varied in size from 1 – 1.5μm.

The vesicle-free cytoplasm of this gland was restricted to a thin layer around the peripheral margins of the tissue as seen in **Figure 4.27.** The matrix of this cytoplasm was relatively electron-lucent (in contrast to that of Gland B) so that it appeared as a quite distinct layer even under low magnification. The thin layer of cytoplasm of the vesicle-free peripheral regions of region D contained many nuclei and a great amount of ER with an electron-dense lumen. It was difficult to distinguish the type of ER but it was possibly SER.

The vesicle-free cytoplasm present contained many free ribosomes (17 – 25nm diameter), lots of ER, the lumen of which was very full and some Golgi complexes and mitochondria. RER was not abundant. Gland D contained a great number of the unidentified microvillous structures, as shown in **Figure 4.28** and **Figure 4.29,** first observed in gland A. There was no obvious order or symmetry to the

location of these structures within the gland tissue as they were observed randomly throughout the gland cytoplasm. Small ductules were also evident throughout the gland at this point. These ductules appeared to contain a flocculent material similar to that found within the secretory vesicles. The ductules were often seen to contain empty spherical, or ovoid, membranes that presumably bounded the secretory vesicles prior to their coalescence with the contents of the ductule.

Many of the vesicles present in the cytoplasm appeared to be fusing together. Both vesicles with electron-dense and electron-lucent contents were observed fusing together (**Figure 4.29**), as were vesicles of the same morphological type. This phenomenon, and the lack of segregation of the different vesicle types, suggests that the contents of the secretory vesicles is the same that the relative electron-densities of the material within the vesicles may be misleading in terms of components of the secretion.

A nerve bundle was also found in close proximity to gland D. The bundle (**Figure 4.30**) consisted of 3 axons: membrane-bound and round or oval in shape in transverse section, the contents of which were only slightly electron-dense but contained within them many small dark, spherical spots of a much more electron-dense material each of diameter approximately 260Å. These electron-dense spots may be microtubules running longitudinally within the nerve tissues. The individual axons ranged from 0.47μm to 1.09μm at their widest point, and were contained within a single tissue (possibly a Schwann cell) of approximately 1.80μm diameter. The axons in this case were unmyelinated. This bundle was seen to lie within the membrane enclosing the gland. Whether this nerve innervates the gland was not established in this study but no synapses between the neuronal bundle and the gland were ever seen in any section examined.

Figure 4.31 shows a composite TEM image of gland D clearly demonstrating the main features of this tissue. The demarcation between the zone of production and the zone of accumulation is obvious, as are the collecting ductules and the tissue of the main duct. A nerve bundle was also present close to the ventral surface of the gland. Gland B borders the ventral and medial margins of D as shown in **Figure 4.16**. There was also present at this point a cluster of ducts close to the ventral face of region D. This

group of ducts appeared to be contained within a membrane which ran between region B and region D and appeared to be separate from the gland tissue. The membrane was very electron-dense and contained many nuclei and a lot of RER. Numerous Golgi bodies and mitochondria were also observed in this tissue.

4.4.4.6 Secretory ducts and pores of the urosomal gland complex

The secretory ducts of the complex, once they had passed out of the gland tissue itself were never located using TEM. They were however frequently visualised in semi-thin sections. The lumen of the main ducts of the urosomal glands (one of which is visible in **Figure 3.19**), when sectioned along their long axes, had evident ribs within them. The ducts, when viewed under the light microscope appeared roughly ovoid in transverse section with their widest point being approximately 1.9μm. The evidence for where the gland ducts discharge their secretion has been derived from a comparison of the location of the components of the gland complex with the stained ducts observed in Chapter 3 using DAB and TMB and from the pattern of cuticular pores observed on the urosome using SEM.

The pores through which the ducts of various components of the urosomal glands are presumed to pass their secretion are primarily located on the ventral surfaces of the caudal rami close to the bases of the large terminal setae. Pores were numbered according to their location relative to the terminal setae which were numbered after the definition of Huys (1988). Three glandular pores were located in this region (two of which are visible in **Figure 4.32**) using SEM, whilst a fourth ventral pore was located on the medial margin of each ramus. A further pore was also located in the central region of the dorsal surface of each caudal ramus. The relative locations of these pores are shown in **Figure 4.33**. The pores identified were all either spherical or slightly ovoid in shape and none were observed to have any modifications such as closing flaps.

As the A glands were present only in the dorsal region of examined sections and their ducts were never visualised beyond their posterior margins, it must be assumed that they secrete their product onto the dorsal cuticle of the caudal rami via the single cuticular pore observed (pore V in **Figure 4.33**). The B

and D glands are proposed to secrete their products separately via the pores on the posterior margin of the caudal rami (pores II and IV in **Figure 4.33**), at the bases of the terminal setae, although which gland is associated with which of the pore has not been determined in this study. The C gland is certain to secrete via the single pore on the lateral margin of each caudal ramus, numbered I in **Figure 4.33**, on account of its location and from the staining of the duct observed with the TMB staining in Chapter 3. The association of pore III (**Figure 4.33**) with an element of the urosomal gland complex has not been achieved satisfactorily in this study. No stained ducts were observed leading to it and no gland was observed close to it but it is possible that it is associated with a further non-staining component of this gland which has not been observed in this study. A duct leading from either the B or D gland is stained in the left caudal ramus but is not in the left one. Such inconsistent staining was frequently evident in specimens and was the cause of some analytical difficulties and was the main cause of the failure to utilise the DAB and TMB stains as diagnostic tools in pore signature pattern analysis.

4.4.5 Ultrastructural characteristics of the labral gland

The labral gland lay entirely within the cephalothorax with its ventral margin closely apposed to the proximal end of the labrum. The gland had a distinctive 'heart shape' with the tapered end extending ventrally into the proximal end of the labrum. When examined using TEM at low magnification as in **Figure 4.34,** it became apparent that the gland was composed of two ultrastructurally distinct regions, a peripheral zone of production and a large irregularly shaped central reservoir of secretory vesicles. The zone of production was characterised by large quantities of RER, and large numbers of Golgi complexes, as well as numerous mitochondria and many nuclei. The secretory vesicles of the labral gland showed varying electron densities again, with no apparent order to their distribution within the zones of accumulation. The gland drained via a single, ventrally leading duct (**Figures 4.34** and **4.35**) that passed into the labrum and was presumed to empty via a pore on the anterior surface of that structure. Examination of this duct revealed the presence of numerous internal filaments (evident in **Figure 4.35**)

running parallel with the long axis of the duct. The ultrastructural characteristics of the labral gland closely resembled that of gland region D of the urosomal gland complex.

The ducts of a further pair of glands were seen to enter, and merge with, the labral gland and are apparent in **Figure 4.34**. These glands lay close to the labral gland, one immediately to either lateral margin, with their ducts extending into the dorsal region of the labral gland. These glands correspond to the circum-oral glands described in this study (Chapter 3) which are believed to correspond to the 'lateral glands' described by Bron (1993). **Figure 4.36** shows the point of fusion of the circum-oral gland ducts with the labral gland. Where the ducts of the circum-oral glands interdigitate with the lumen of the labral gland numerous desmosomes were evident (**Figure 4.36**). The merging ducts from the circum-oral glands contain large areas of tissue with microvillous structures of the type described for the urosomal glands. Brief examination of the circum-oral glands showed them also to be syncytial tissues with well developed regions of microvillous inclusions (as evident in **Figures 4.36** and **4.37**) with many widely dispersed nuclei and a very well developed system of SER. The lumen of this SER, as shown in **Figure 4.38** was very swollen suggesting that it is very active. SER mediates a diverse range of biochemical reactions including steroid synthesis and detoxification.

4.4.6 Ultrastructural characteristics of the anterior gland complex (AGC)

Only a brief ultrastructural study was made of the AGC. This brief study though did reveal some important details regarding the structure and ultrastructure of this complicated system.

The AGC was a complex system of an undefined number of superficially different regions or lobes. The complex appeared to be composed of several closely associated glandular components although their precise relationship could never be deduced from sections alone, however at least two main glandular regions are present.

From TEM analysis the details of the ultracellular composition of the AGC became apparent. From the few sections that were observed it was obvious that the AGC had some very different morphological features from the urosomal and labral glands of *L. salmonis*. The tissue was large and

obviously syncytial with many nuclei (**Figure 4.39**) being apparent throughout the cytoplasm. The gland filled the full depth of the cephalothorax at this point, extending from immediately below the dorsal cuticle to immediately above the ventral cuticle and epidermis. The greatest majority of the gland tissue in the sections observed was composed of enormous numbers of large membrane-bound vesicles (**Figures 4.39** and **4.40**) all with similar electron densities. There was considerable variation in vesicle size within the gland tissue (0.5 – 1.1μm) but no order to this size variation was evident. The vesicles generally displayed heterogeneous electron densities with a ring around the internal circumference having slightly denser staining characteristics than the rest of the contents. The remaining, lighter, contents of the vesicles were generally not of a constant density but instead there was some mottling effect apparent (**Figure 4.40**), the significance of which was unclear. Within this region of secretory vesicles there were many small ductules (labelled 'mi' in **Figure 4.40**), of varying sizes of a similar morphology to the ductules observed in both the labral gland and the different regions of the urosomal gland. This region of prolific secretory vesicles probably explains the 'honeycomb' appearance of this gland observed in wax sections during histochemical studies (Section 4.4.2.2).

The ventral region of the gland appeared to be the zone of production and also contained the majority of the nuclei (**Figure 4.39**). This zone of production had a very distinct morphology possessing large quantities of ER with extremely full electron lucent lumens as shown in **Figure 4.41**. The ventral region of the gland was also bordered by a thick membrane that was compressed at one point where a nerve came into close contact with the gland. The nerve was never observed to come into direct contact with the gland tissue, as shown in **Figure 4.39**, in any of the sections examined and no synaptic vesicles were ever observed in the nerve close to the gland tissue.

Light microscope analysis of semi-thin resin sections of this gland complex revealed the presence of internal ducts that appeared to have internal thickening ribs, visible as finger like extensions of the duct cuticle, on their internal walls. Given the complexity of this gland tissue no in depth study was undertaken, this having been reserved for the urosomal gland only.

4.4.7 SEM analysis of exocrine gland tissues

The application of SEM to provide information regarding the structure of exocrine gland tissues of *L. salmonis* was only moderately successful in this study. The techniques applied to larval stages to expose the internal gland systems were of insufficient delicacy to provide any reliable information. The Dry Fracturing Technique of Toda, Suh and Nemoto (1989) caused extensive and unpredictable damage to the larval stages to which it was applied. Due to the small size of the nauplius and copepodid stages the damage was so severe that it was almost impossible to identify exposed tissues and the damage caused almost certainly meant that the tissues observed were so out of natural alignment as to be almost of no utility for structural analyses.

The Dry Dissection Technique (Andrade-Salas, 1997) was of more utility than the Dry Fracturing method but again caused severe damage as a consequence of the brittleness of the processed specimens. This technique though could not be applied with any great degree of precision and desired structures were frequently destroyed, misaligned or damaged beyond recognition.

The most suitable method for precisely revealing internal structures was the Wax Carving technique of Oshel (1985). This, despite being the most time-consuming of the techniques, provided the greatest degree of control of the three techniques and retained the tissues *in situ* allowing spatial relationships to be more clearly visualised. In this study SEM analysis formed only a small portion of the ultrastructural analysis but its use did however emphasize the close spatial relationship of tegumental glands (DSG) with the overlying dorsal cuticle (**Figure 4.42**) and clearly demonstrated the extent to which the gland cytoplasm was dominated by secretory vesicles.

4.5 DISCUSSION

4.5.1 Histochemical analyses

Evidence obtained from microscopical and histochemical analysis serves to confirm the hypothesis that the labral, anterior, urosomal, thoracic leg and dorsal surface glands of *L. salmonis* are active secretory units. The histochemical tests, the most revealing of which were the PAS and Sudan Black B stains, provided a great deal of information regarding the composition of the glandular secretions. The reactions to these stains suggest that the secretions of these glands have multiple components other than the already identified DAB-reactive component, possibly either a peroxidase enzyme or an acidic mucus (Chapter 3). The PAS stain clearly indicates that neutral mucus forms a substantial component of the secretion of all the glands identified in this study as evidenced by the strong positive response to this stain observed in all glandular tissues. The fact that the glands of *L. salmonis* appear to be secreting a PAS-positive mucus but no material with such staining characteristics was ever observed adhering to the external surfaces of stained specimens can be explained by the fact that mucus compounds that stain with PAS only do so in their unhydrated state (Wiebkin, 1996). Staining therefore will not be apparent in secreted and hydrated mucus secretion present on the external surfaces of *L. salmonis*.

As no distinction between specific biochemical categories of neutral mucus compounds can be made using the PAS stain (the subtle differences between classes of compound being beyond the scope of the technique) a positive response to this stain was here deemed only to indicate the presence of neutral mucus. This apparent liberty with the terminology should be acceptable as Wiebkin (1996, part 6.6-2, para 2) stated that '*the close spatial and structural relationships of mucosubstances preclude strict comparmentalization*' and Cook (1990) concluded that the multiplicity of commonly used terms allows the use of any one term as long as it used consistently throughout a work.

In *L. salmonis* glands, staining intensity varied between different regions of the same gland and can probably be explained in terms of the relative concentration of the secretion present in the different areas. Secretory vesicles showed a weak response to PAS in the urosomal, labral and AGC possibly

because the contents of vesicles will only be present as a thin layer of dehydrated material. The ducts and ductules of those glands however, showed a very intense reaction to the stain, more so than the gland syncytia themselves presumably, as the secretory product of the glands was concentrated within them.

The use of PAS following diastase digestion indicated that glycogen was not causing the observed positive reaction with the PAS stain and substantiates the finding that neutral mucus is the glandular component giving rise to the positive reaction with this stain.

Neutral mucus has been shown to be present in the glands of the copepods *Paranthessius anemoniae* (Briggs, 1978), *Diarthrodes nobilis* (Hicks and Grahame, 1979) and *Hemidiaptomus ingens* (Brunet *et al.* 1991) as well as in exocrine glands of isopods (Stevenson and Murphy, 1967), decapods (Shyamasundari and Hanumantha Rao, 1978) and amphipods (Shyamasundari, 1979).

Despite giving a negative reaction in gland tissues in this study, the results of the Alcian Blue histochemical test are of great significance as they are essential in helping to discriminate between sulphated mucus compounds and the peroxidatic component postulated here to be present within the glands of *L. salmonis*. The evidence from this study indicates that acidic (sulphated) mucus is not a feature of the secretory material. The presence of an acidic mucus component within the epidermal mucus of salmonid fish has been demonstrated using Alcian Blue by both Fletcher, Jones and Reid (1976) and Pottinger, Pickering and Blackstock (1984). The positively stained material observed in the guts of some specimens of *L. salmonis* in this study probably represents host tissue and mucus, as similar positive staining was never detected within the secretory cells of the gut lumen, suggesting that the material was of an exogenous source. This conclusion is supported by the work of Bron (1993) who demonstrated a positive response to Alcian Blue in the midguts of attached copepodid and chalimus stages of *L. salmonis* as Alcian Blue staining was observed in the guts of adults in this study. That author also showed that goblet cells of the epidermis of the salmon host gave a positive response to the stain indicating the presence of acidic glycoproteins (sulphated mucus). These findings then, lead to the conclusion that the positive Alcian Blue staining sometimes observed in material attached to the setae of the thoracic legs and the terminal setae of the urosome of *L. salmonis* may be of host origin.

The absence of an acidic mucus component in the exocrine glands of *L. salmonis* supports the hypothesis that the DAB stain was reacting with peroxidase enzymes and not with sulphated mucus compounds as has also been shown to occur by Bussolati (1971). This convincing discrimination between these two compounds with similar staining propensities is of central importance in this work. The identification of exocrine glands with acidic mucus secretions in copepod species has previously been demonstrated for *P. anemoniae* by Briggs (1978), Hicks and Grahame (1979) for *D. nobilis* and by Brunet *et al.* (1981) for *H. ingens*. The significance of the different types of mucus (acidic and neutral) identified in copepod glands is a subject area that is entirely unresearched. The physical attributes and physiological significance of the two types of mucus are unknown. It may be that they have similar attributes and serve similar functions or that they have entirely different roles. It is more likely, given the generalised structure of mucus compounds, that they have similar attributes but have evolved from different paths to fill the same role. Frequently both neutral and acidic mucus (Briggs 1978, Hicks and Graham 1979, Brunet *et al.* 1991) are identified in copepod glands which may suggest that the two secretions interact in some way to form a single, active secretion or the two secretions may act separately following secretion.

The distinction here then is that the exocrine glands of *L. salmonis* appear to contain a peroxidase enzyme component that has never been described before and which appears, from this study, to be largely absent and certainly greatly reduced in free-living copepod species. The implications of this are far reaching. The evolution of gland systems within the Copepoda probably represent life-style specific adaptations such that further analysis of these systems may provide important insights into, and thereby extend, our knowledge of the behaviour of these animals.

Mucus components of gland secretions in copepods have been widely described and have been shown to be a component in all glands where histochemical tests were performed. Both neutral and acidic mucus have been identified in the glands of different copepod species but as yet no hypotheses regarding the functional significance of differentially staining mucus compounds have been proposed by researchers in this field. Whether different mucus types in copepod glands have different functions or merely

represent different convergent evolutionary paths remains to be resolved using appropriate techniques such as molecular analysis.

The fact that mucus appears to be a ubiquitous secretion component suggests a fundamental role for this material. It is likely that the many different glands described in all the species studied do not have identical roles as the actions of secretions are bound to reflect peculiarities of habit or behaviour. The uniformity of the presence of this mucus component however would suggest either a common ancestral role to these glands (i.e. pre-adaptation) which have become adapted to suit behavioural / environmental changes or that mucus in some way represents the 'ideal' secretion which may be reflected in a convergent evolution. It is more likely though that such glands are ancestral and have become variously modified to suit particular needs and therefore specific mucus compounds have become evolved to suit particular needs which in itself suggests that mucus compounds have separate and specific modes of action.

Lonsdale *et al.* (1996) showed high levels of lectins binding to the urosome and caudal furca of copepods of the harpacticoid genus *Coullana*, indicating the presence of surface glycoproteins in those areas. Those authors made no mention, however of glandular systems in those species and believed that the glycoproteins were an intrinsic part of the cuticle. Kelly, Snell and Lonsdale (1998) showed that adult females of the harpacticoid species *Tigriopus japonicus* (Nori) expressed glycoproteins, which served to attract male conspecifics, but copepodids did not release such chemicals. Snell and Carmona (1994) also demonstrated the potential for surface glycoproteins to be involved in mate recognition of marine calanoid and harpacticoid copepods as well as in freshwater calanoid and cyclopoid copepods. In these species the highest levels of surface glycoprotein were identified around the margins of the cephalothorax and the urosome area. All these studies suggest that surface expressed glycoprotein (mucus) compounds are implicit in mate attraction and represent a secondary sexual feature in the copepod species in which they have been found. The evidence from this study (Chapter 3) has shown that several harpacticoid copepod species possess urosomal glands with similar DAB and TMB staining characteristics to *L. salmonis*. Williams-Howze (1996) demonstrated that the urosomal glands of some harpacticoid species also secrete mucus compounds, as those of *L. salmonis* have been shown to here. Such a condition could explain the

high levels of glycoprotein observed on the urosome and caudal furca of those species of harpacticoid where such chemicals have been suggested to mediate mate attraction / recognition. It should be emphasised that the absence of acidic mucus compounds in the exocrine glands of *L. salmonis* and the assumption from that that the positive DAB staining is therefore caused by peroxidase enzymes does not necessarily indicate that the DAB staining glands of the harpacticoid and calanoid species is not caused by acidic mucus secretions. In the absence of specific histochemical analyses of these other species the DAB staining of their glands must be interpreted in the context that either or both peroxidase enzymes and acidic mucus compounds may be present.

Staining with bromophenol blue demonstrated that the secretions of the dorsal surface glands, urosomal glands and AGC are not strongly proteinaceous. The observed response with this stain, where the vesicle-free region of the gland tissue stains strongly but the secretion itself does not (reminiscent of the PAS stain) can be explained easily if relative quantities of protein in different gland regions are considered. It is not surprising that the general cytoplasm of gland structures contain considerable protein, as this is where the bulk of the organelles with their proteinaceous membranes are located. The secretion itself, even if it contains a strongly reactive enzyme, will not necessarily contain large quantities of protein, as small amounts of enzyme, particularly catalase, are known to be strongly reactive and may cause a significant observable reaction with the DAB stain despite being present in small amounts. It is also possible that the secretion is some form of mucus / protein complex, often termed glycoprotein or proteoglycan, depending on the relative proportions of the two constituent parts. Wiebkin (1996) describes how glycoproteins (proteins with attached polysaccharide chains) give a positive reaction with PAS and such a compound may be causing the reaction observed here. The bromophenol blue staining of the secretion within glands of *L. salmonis* may therefore indicate the presence of an enzyme fraction, the protein part of glycoprotein (mucus) or both compounds together. Bannister (1993a) has demonstrated the proteinaceous nature of the gland secretions of the calanoid copepod *Temora longicornis* with bromophenol blue but failed to identify a mucus component to the secretory material. He concluded that

the secretion may serve as an anti-fouling agent preventing the establishment of fouling organisms, such as bacteria, on the cuticle.

The conclusions that can be drawn from the lipid staining trial are less certain than for the other stains utilised. For example, the failure to identify the presence of lipids in whole nauplius larvae with the Sudan Black stain is in contradiction to the findings of Tucker (1998) who demonstrated the presence of substantial lipid reserves in the nauplius stages of *L. salmonis*.

Sudan Black has been reported to give a positive reaction with Golgi bodies (Humason, 1979) as a result of the presence of phospholipids in their membranes and such an action may explain the areas of lightly positive Sudan Black staining frequently observed in the regions of the glands which were later identified with TEM to be the zones of production that contained large numbers of Golgi complexes. The strong positive response to Sudan Black seen in the regions of vesicle accumulation in the glands cannot be attributed solely to a reaction with Golgi complexes. It is interesting that the pattern of lipid-positive gland distribution found using the Sudan Black stain does not match exactly the pattern found using the DAB stain although all of the lipid-positive glands located also stained positively with DAB. It is not possible to account for this variation except to suggest that either the staining technique was prone to variation, or that gland lipid content may vary temporally or that not all the DAB-staining glands contain both peroxidase enzymes and lipids. Lipid staining of copepod exocrine glands using Sudan Black has never been reported in the literature so there is no information with which to compare these results, but Stevenson and Murphy (1967) obtained positive results when staining 'mucopolysaccharide' glands of the isopod *A. vulgare* with Sudan Black. Those authors concluded that it was the membranes of the secretory droplets that stained rather than their contents. It is possible in this study that the positive reaction to Sudan Black arose either as a response to the contents of the secretory vesicles or in response to the lipids present in the membranes bounding them. The implications of a lipid secretion are interesting to consider. Lipids would be an unusual component of a secretion to be discharged externally, not least because of its high metabolic cost although such a factor may not be of importance to obligate parasite species such as *L. salmonis* where fatty acids will be readily available from their salmon host diet. The presence of Sudan

Black staining on the terminal setae of the urosome along with the observed DAB staining of such material suggests that the material on the setae has both peroxidatic and lipid components in addition to the mucus already identified. Further detailed, specific analysis will be required to determine whether lipids do form a component of the glandular secretion of *L. salmonis* as the histochemical stain utilised here has provided incomplete information with this regard.

The initial confusion caused by the presence of melanophores in cryostat sections of control tissues suggests that de-lipidisation causes the removal of the melanophores of *L. salmonis*, or renders them impervious to Sudan Black. This in itself suggests that melanophores have a strong lipid component. Bron and Sommerville (1998), in their description of the photoreceptors of *L. salmonis* larvae, observed that the pigment cells surrounding the paired ocelli contained large numbers of vesicles, some of which they propose contain lipids. The data of Bron and Sommerville (1998) supports the proposal that melanophores have a significant lipid component. Although melanophores in *L. salmonis* have been described previously (Bron, 1993), they were described as being located within the epidermis and not the cuticle. Their apparent observation in the cuticle here may have possibly occurred if the melanophores were 'smeared' across the cut face of the sectioned cuticle as a consequence of the sectioning technique. It is therefore likely that their presence in the cuticle is artifactual.

Catechol staining of *L. salmonis* to highlight peroxidases discounted the possibility that exocrine glands are involved in producing phenoloxidases for use in cuticle sclerotisation. No positive staining was observed in any glandular tissue at any life stage with the exception of a generalised weak response in the FGC, the source of the attachment filament. This confirms the results of Bron (1993) who found that the filament of chalimus stages of *L. salmonis* contains phenoloxidases. The precise role these chemicals play in the action, or production of the filament is unclear, but as phenoloxidases are believed to be involved in the hardening of the crustacean cuticle (Dennell 1947, Stevenson 1961) it is possible that they serve to cause the newly secreted filament to harden swiftly before the newly moulted parasite can be dislodged. Other, general responses to the catechol stain suggest that phenoloxidases are widespread throughout the cuticle but are especially prevalent in areas where the cuticle is thickest, again probably as a function of

their cuticle hardening properties. Importantly though, no specific source of these compounds was ever visualised which may suggest that they are secreted by the cells of the epidermis and are not derived from specific glands as has been suggested for other species. Stevenson (1961) found that tegumental glands in the legs of the isopod *A. vulgare* contain droplets of polyphenoloxidase secretion and proposed that the enzyme served to catalyse the sclerotisation of the cuticle as the contents of the glands showed a cyclical flux, peaking immediately prior to moulting. That author did accept though, that it was likely that not all tegumental glands in a single species would serve the same purpose.

The cholinesterase staining of *L. salmonis* did not reveal evidence that would support evidence of direct innervation of the identified exocrine glands.

4.5.2 3-D reconstruction

The 3-D reconstruction techniques employed to assist in the deconvolution of gland structures were beneficial in aiding the comprehension of the gland systems but their utility did rely to some extent on a substantial understanding of the structure prior to analysis. Once the structure had been reconstructed though, the VIDAS system especially, proved very powerful in providing further insights into the spatial interactions of the various gland regions. In particular, the computer generation of sections taken at any plane specified by the user allowed the intricate tissue associations of multi-lobed glands to be fully appreciated. The VIDAS system was of particular utility when used in conjunction with TEM analysis as it allowed the 3-D visualisation of the 2-D information provided by the light microscope semi-thin sections helping to resolve intricate associations of closely associated glands. The utility of the deconvolution system in general image analysis and specifically, the rapid and automated identification of species is clearly apparent in **Figures 4.10** and **4.11.**

The Zeiss KS 300 system, on the other hand, had a more limited utility. The system, with the set-up used in this study, provided little detail over that which could be obtained using conventional microscopic techniques but it did allow for images consisting solely of selected tissues to be constructed. The systems greatest potential lay in its ability to discard extraneous information leaving only objects of

interest in view, with no other structures obscuring the image. This system could prove beneficial in future studies of gland and duct interrelationships but will need some refinement.

4.5.3 Ultrastructural analyses

The ultrastructural evidence from the TEM analysis corroborates the results of the histochemical tests. Ultrastructural components identified in gland tissues of *L. salmonis* are consistent with mucus, protein and, possibly, lipid-secreting tissues. The identification of large numbers of Golgi bodies, in association with RER is indicative of mucus production, whilst RER in itself suggests that protein synthesis is a feature of the gland tissue. It is impossible to say which process is dominant in a tissue where both organelles are prevalent, as is the case in the urosomal gland tissues of *L. salmonis*. The substantial amounts of SER observed in the tissue of the circum-oral glands suggests that lipid production takes place in those glands. SER is recognised to be implicit in the synthesis of lipids.

All glands examined using TEM were syncytial as evidenced by the large numbers of nuclei contained within each membrane-bound tissue. Glands with such characteristics do not correspond to any of the gland types classified for insects by Noirot and Quennedy (1974), but syncytial glands have been described in copepods by Park (1966), Arnaud *et al.* (1988a,b) and Brunet *et al.* (1991) and have been characterised in a review by Talbot and Demers (1993). The ultrastructure of the glands described in these studies varies in terms of the relative proportions of organelles, vesicle structure and the overall configuration of the tissue as a whole i.e. with regard to the development of secretion reservoirs, duct morphology etc.

4.5.3.1 Urosomal gland complex

TEM sections taken at various points across the urosome of *L. salmonis* to elucidate the structure of the urosomal glands assisted in resolving the complex relationship of the different regions of these structures. The urosomal glands consist principally of four distinct secretory tissues. The two main tissues (B and D) are intimately associated with each other but are relatively easy to distinguish at an

ultrastructural level on account of their markedly different ultrastructural characteristics. The cytoplasm of gland A is similar in structure to gland B and was frequently mistaken for the latter in some sections where orientation was difficult to determine, although differences in the morphology of their secretory vesicles helped to distinguish the two at an ultrastructural level. Gland C is physically separate from glands A, B and D and as such is easily distinguished in tissue sections.

The different gland tissues of the complex, although displaying some notable differences in their ultrastructural characteristics also display marked similarities in some important respects. Some elements of ultrastructural makeup were constant between the different tissues and this was principally concerned with the presence of highly active Golgi complexes frequently found in association with RER. Transitional vesicles were also frequently observed in the cytoplasm between the RER and the Golgi complexes. These transitional vesicles arose from the RER and transfer proteins, synthesized by the attached ribosomes of that structure, to the cis, (forming) face of the Golgi complex where they are further modified by the addition of carbohydrate moieties. These carbohydrate components are added via the action of glycosyltransferases located within the cisternae of the Golgi Following this addition they emerge from the trans (maturing) face, (**Figures 4.20** and **4.21**) and coalesce to form the observed secretory vesicles. This synthetic pathway has long been understood and is known to be the principal route of mucus (glycoprotein) synthesis (Wolfe, 1993). Whilst the specific composition of mucus may vary, the basic structure and paths of synthesis and secretion are similar for all mucus classes.

This active process of secretion production was frequently and clearly visualised, in glands A, B and D of the urosomal gland complex. Such a discovery correlates well with the results of the histochemical tests employed in this study, confirming that mucus and probably enzyme production are taking place within these glands. The morphology of the secretory vesicles of region D were markedly different both from those of regions A and B, but the significance of the different morphologies of the secretory vesicles remains to be discovered. No vesicles were observed merging with the collecting ducts of the glands of *L. salmonis*. From the evidence collected, the following mechanism can be proposed: vesicles are formed in the zone of production and are secreted into the central core of vesicle

accumulation. The continuous supply of new vesicles gradually push older vesicles close to the collecting ducts where some may coalesce prior to entry to the duct. The entire vesicle appears to enter the duct, although how this is effected has not been realised in the sections examined. The presence of almost intact membranes, of an appropriate size, within the ducts and ductules of the glands confirms that the vesicles enter the ducts intact and that the vesicle membranes are not retained within the gland syncytium. The membranes of the secretory vesicles are probably derived from those of the Golgi cisternae, as described by Silberberg (1989) and as such will be composed of lipids, as suggested in this study by the positive response of gland tissues with the Sudan Black stain. The apparently greater intensity of this staining reaction in the zone of accumulation of the labral gland does suggest that the vesicle membranes are composed of lipids, given the positive PAS response of the vesicles and the ultrastructural evidence suggestive of mucus production. What stimulus, or force, causes the vesicles to rupture is unknown but in terrestrial slugs various stimuli have been proposed: increased pH, mechanical force and increased levels of ATP (Kapeleta, Jimenez-Mallabrera, Carnicer-Rodriguez, Cook and Shephard, 1996).

The electron dense granules conspicuous throughout the cytoplasm of Regions A and B are, probably free ribosomes based on their size, but definitely not glycogen granules as demonstrated by the persistence of PAS staining following diastase digestion. Free ribosomes are widely accepted to be responsible for production of proteins for use within the cell whereas those associated with ER are attributed with synthesis of proteins and enzymes for secretion. All the different gland regions of the urosomal complex contained abundant free ribosomes in their cytoplasm. Intracellular proteins are required for a multitude of purposes. In addition to those synthesised on RER and passed to the Golgi others remain in the cytoplasm whilst others migrate to the nucleus or mitochondria (DeRobertis and DeRobertis, 1987). Very active cells such as those of glandular tissues will require more such proteins, reflected in the abundance of free ribosomes in the cytoplasm.

The unidentified microvillous inclusions shown in **Figures 4.22, 4.24 and 4.28** were observed in all gland tissues of the urosomal glands but did not appear to be present in other, non-glandular, tissues examined. No similar structures could be found in any studies of crustacean or insect ultrastructure but

they did appear markedly similar to the structure of the lumen of acinar cells of the mammalian pancreas (Jamieson and Palade, 1977). This suggests that these unidentified structures in the gland tissue of *L. salmonis* are in fact ductules that serve to collect secretory material from throughout the gland cytoplasm and transport it towards the main secretory ducts. This would serve to explain the dendritic pattern of PAS stained ductules observed in the urosomal glands and AGC of this species (Section 4.3.2.6). The lumen of these ductule structures were frequently seen to contain spherical membranes, as evident in **Figure 4.28,** which are presumed to be the remains of the secretory vesicle membranes after they have entered the duct. The membrane in the duct shown in **Figure 4.28** is appropriately sized to be a membrane of a secretory vesicle. Also, the lumens of the ductules in the micrograph contain a material of similar electron density and texture to the material within the secretory vesicles of the tissue. This evidence suggests that the whole of the secretory vesicle, including the external membrane, enters the ductules rather than the contents being disgorged into the lumen of the duct and the membrane being retained within the cell.

Only gland D of the urosomal glands contained a prominent region of vesicle accumulation, in contrast to the other three regions where secretory vesicles were dispersed throughout the gland cytoplasm. The region of accumulation was present as a core of the gland tissue, surrounded by a region of non-vesicular tissue that appeared to be the zone of production. This may suggest that gland D is more active than the other gland units as it seems certain that it contains a far greater volume of secretory material. Conspicuous poles of vesicle accumulation or entire reservoirs of secretory product are frequently described features of copepod exocrine glands (Hipeau-Jacquotte 1987, Arnaud *et al.* 1988a,and b, Brunet *et al.* 1991). Such poles of accumulation though are more usually found concentrated in one end of the gland cell, usually the end closest to the point of secretion. Such a phenomenon was never observed in any of the regions of the urosomal gland complex of *L. salmonis*.

Whether the granular / flocculent appearance of the secretory vesicle contents of gland D is artifactual or is representative of the situation *in vivo*, is disputable but it is known that mucus compounds often suffer partial extraction during processing for TEM leaving only a precipitate (Gotto and Threadgold

1980). It is possible therefore that the contents of the vesicles form a precipitate during processing, although in human goblet cells mucus is known to be stored in secretory vesicles in its unhydrated form (Cross and Mercer 1993, Davies and Hawkins 1998). The hydration process that occurs after secretion is only partially understood probably because it is likely to vary between species and between the types of mucus secreted. Cross and Mercer (1993) suggest that a 600-fold increase in volume occurs after hydration of human epithelial mucus whilst Aitken and Verdugo (1989) claim an 8 to 10-fold increase following hydration of rabbit tracheal mucus. Other research on the extent of secretion volume increase variously propose 300-fold for the terrestrial snail *Arion ater* L. (Kapeleta *et al.* 1996) and 600-fold for the terrestrial slug *Ariolimax columbianus* (Gould) (Verdugo, 1991). Regardless of the expansion factor a similar mechanism is likely to exist in invertebrate classes such as the Copepoda given the apparent similarity between the general ultrastructural characteristics of mucous glands as widely described for vertebrates, and those shown here for *L. salmonis*.

It is also possible that the apparent differences in vesicle density were caused by cross-sections being taken through the vesicles at different points i.e. a thin section from the outer layer of a vesicle may appear less dense than a section taken through the middle of a vesicle. A population of cross-sections should show all possible morphologies. It is unlikely that the apparent differences are due to the vesicles containing different types of secretion or even represent different stages in the development of the vesicles as it is unlikely that any of the identified gland regions are responsible for producing more than one product.

Despite the apparent morphological differences between the secretory vesicles of glands A, B and D the organisation of their organelles and their relative proportions are similar. Presumably the differences in vesicle structure between glands A, B and D represent chemical differences in their contents, but what these differences are cannot be determined using TEM and histochemistry alone. Gland C on the other hand was more difficult to categorise although its ultrastructural characteristics were grossly similar to those of region B.

This multi-component structure to exocrine glands in the urosome of copepods has also been described by Park (1962), Fahrenbach (1966), Boxshall (1982) and Williams-Howze (1996). These studies examined the general morphology of the complex and gave some indications to the staining characteristics of the tissues but gave no information regarding their ultrastructural characteristics.

Variations in the precise composition of this complex are apparent between species although the whole unit is generally described as being composed of two distinct glands systems: the urosomal being the large glands on either side of the gut which open on each ramus and the perianal glands (*sensu* Boxshall 1982) being the smaller glands contained within the anal somite and caudal rami which discharge their secretions on the urosomal somite away from the tips of the caudal rami. Boxshall (1982) describes the urosomal glands of *B. palliata* as being present only in females. Similar glands have also been described in the cephalocarid *H. macracantha* by Elofsson and Hessler (1998). These studies suggest that urosomal glands, with similar gross morphologies, are widespread in the Copepoda and Cephalocarida. The functional significance of these glands still remains unresolved but presumably they must serve some purpose essential to the life of these animals with diverse habitats ranging from free living (Park, 1962), algal associated (Fahrenbach 1966, Williams-Howze 1996), interstitial (Elofsson and Hessler, 1998) and obligate parasitic species such as *L. salmonis*. Their possible role in secreting compounds responsible for mediating mate attraction or recognition, as discussed earlier should be more fully investigated. Ritchie *et al.* (1996b) suggested that some water-borne factor mediated the mutual attraction of adult male and female *L. salmonis* suggesting a pheromonal involvement in the mating of this species.

4.5.3.2 Labral gland

The labral gland of *L. salmonis* has similar staining characteristics to the urosomal and AGC glands in that the presence of neutral mucus, protein and possibly a lipid component were demonstrated using the histochemical tests utilised. Such results suggest that the gland is secreting a mucoid secretion, possibly with a protein moiety attached, and that the secretory vesicles of the gland are bounded by

membranes rich in lipid. The results from this gland may shed light on the functions of other glands such as gland D of the urosomal complex. The labral gland possessed only one type of secretory vesicle (similar to those of gland D) yet stained positively with DAB and PAS. This might suggest that these glands are secreting a mucus compound with an attached, or associated, enzymic fraction. Mucus and enzymes have been proposed to form the principal components of labral gland secretions in other species studied (Garagozlou-van-Ginneken 1977, Arnaud *et al.* 1988a,b, Brunet *et al.* 1991)

Ultrastructural analysis using TEM indicated that the labral glands of *L. salmonis* were relatively simple syncytial structures consisting of only two distinct zones, one of production and one of vesicle accumulation. The organelles present in the zone of production (RER, Golgis etc.) and the morphology of the vesicle contents in the zone of accumulation suggest that these glands are involved in mucus production as described in Section 4.4.4 for components of the urosomal glands.

The proximal end of the secretory duct as it emerges from the gland has many internal filaments that presumably serve to prevent the duct from collapsing at this point. Although no data was obtained regarding the internal structure of this duct as it passes further into the labrum it may be presumed that internal ribs of thickened cuticle may serve this function in these regions, either with or without the assistance of the filaments observed. The evidence for this hypothesis is the presence of strengthening cuticular ribs in the ducts of other glands examined e.g. AGC and urosomal. The precise nature of the filaments within the gland tissue is unknown, although it is possible that they may be either actin filaments or microtubules. Such filaments have been described within auxiliary cells encasing the ducts of glands in other crustaceans (Arnaud *et al.* 1988b, Elofsson and Hessler 1998) and were credited with maintaining the shape of the secretory duct. Elofsson and Hessler (1998) also suggested that such filaments may provide the capability of controlling release of glandular secretions. The main secretory ducts of the urosomal glands were never encountered in sections so data regarding the presence or absence of such filaments is lacking.

These ultrastructural characteristics of the labral gland of *L. salmonis* are almost identical to those of gland D of the urosomal gland complex. The proportions of the cellular organelles are similar, as are

the electron density of the gland cytoplasm and the structures of the secretory vesicles. The physical location of the vesicles also reflects that of gland D where the vesicles are contained centrally within the gland, contained by a zone of production. In keeping with gland D of the urosomal glands of *L. salmonis* it appears that the labral gland is also involved in the production of mucus secretions as evidenced by the characteristic ultrastructural components of the pathway of glycosylation and the large secretory vesicles with their fibrillogranular granular contents.

The secretory ducts of the circum-oral glands ('lateral glands' of Bron (1993)) were observed to pass into the dorsal zone of accumulation of the labral gland and these ducts then merged ventrally with the labral gland tissue (**Figure 4.36**). This observation suggests that the secretions of the circum-oral glands are secreted together with the secretions of the labral gland via a common duct although a more thorough examination of this gland complex is recommended if the true nature of the interaction of the two glands is to be revealed. In spite of this however, no vesicles, or material, with similar properties to that of the circum-oral glands was ever observed within the tissue of the labral gland. It may be that the circum-oral glands pass into a centrally located duct in the labral gland that was never encountered in the sections examined in this study. The circum-oral glands displayed cellular structures characteristic of lipid production. The presence of SER within the gland suggests that lipid synthesis may be a substantial role of this tissue. The secretory vesicles were large and very electron lucent, appearing as empty vacuoles under TEM, a morphological characteristic of unsaturated lipid compounds: saturated lipids appearing electron-dense. The results of the TEM analysis concur with the findings of the Sudan Black histochemistry of these glands where material with apparent lipid attributes was observed in the collecting ducts that merged with the labral gland. How a lipid, or lipid / mucus mix secretion would function in the mouth area is unclear.

Mucus secretions have frequently been described from the labral glands of copepods, and other aquatic crustacea (Fahrenbach 1962, Park 1966, Boxshall 1982, Arnaud *et al.* 1988a,b, Zaffagnini and Zeni 1987, Zeni and Zaffagnini 1992, Von-Vaupel-Klein and Koomen 1994), where they were proposed to function to trap or bind food particles. This could conceivably be the function of the mucus component

of the labral gland of *L. salmonis*. Mucus might serve to bind the chunks of salmon epidermis (as shown by Andrade-Salas, 1997) as they are scraped off by the strigil. The labral glands of other species have been shown to secrete directly into the buccal cavity and their secretions are presumed to be implicit in the digestion of prey items, whereas the labral gland of *L. salmonis* appears only to secrete onto the external surface (anterior face) of the labrum. Without detailed knowledge of the proportional roles played by the digestive enzymes of the guts in these different species it is difficult to account for this disparity in labral gland development. Some species may rely heavily on labral gland secretions for digestion whereas this function may be undertaken to a greater degree by enzymes in the gut in other species.

The structure of the labral gland in *L. salmonis* is much simpler than the equivalent structure described for other species of aquatic crustacean. The labral glands of the calanoids *C. typicus* (Arnaud *et al.* 1988a) and *Heterorhabdus* sp. (Nishida and Ohtsuka, 1996), the harpacticoid genus *Porcellidium* (Gharagozlou-van-Ginneken, 1977) the branchiopod *Leptestheria dahalacensis* (Rüppel, 1834) (Zeni and Zaffagnini, 1992) and the cladoceran *D. obtusa* (Zaffagnini and Zeni, 1987) are all complex structures, involving several large and structurally varied, but closely associated gland tissues. In these detailed studies of crustacean labral glands almost the entire volume of the labrum was filled with secretory tissues, and these tissues were variously described as secreting mucus and / or proteins as evidenced by their ultracellular characteristics: highly developed ER, plentiful active Golgi complexes and large secretory granules. The secretions from such glands have been apportioned both an enzymatic role and a food entanglement role, as described in Chapter 3. Both such proposals are valid as there is likely to be differences between species regarding the role of secretions depending on their life history and food source. A conspicuous feature of the labral glands described by other researchers is the presence of large reservoirs of secreted material within the gland tissue. This is in contrast to the condition identified in *L. salmonis*. The reasons for *L. salmonis* having such a relatively basic gland in this area may be attributable to its adaptation to one particular dietary component i.e. salmon tissues. Such an adaptation may require only a small repertoire of enzymes to be secreted prior to ingestion, and similarly, the louse will not have a requirement to trap food with secretions as it is so closely associated with its host its food does not need to

be trapped or restrained. Even if some food material is lost, or washed away the louse is not disadvantaged as it has a close and permanent supply unlike the pelagic species cited, where loss of food particles is likely to pose a more serious threat to their survival.

4.5.3.3 Anterior gland complex

The anterior gland complex (AGC) of the adult stages was only briefly studied in this trial, as the main focus of the TEM analysis was the structure of the urosomal gland complex. The brief analysis undertaken demonstrated that the AGC was a strikingly large tissue with an abundance of vesicles with a distinctive morphology and suggests that at least one tissue of the multi-lobed AGC consists of a great abundance of secretory vesicles that had a different morphology to those of the different regions of the urosomal glands or the labral glands. The vesicles differ in their ultrastructural characteristics but appear to have similar staining characteristics in terms of their general histochemistry and their response to the DAB and TMB stains. The small collecting ductules of the AGC are similar in their structure to those identified in both the urosomal and labral glands. The composition of the organelles of the zone of production of the AGC was unique and did not correlate with any other gland tissues identified in this study. The greatly developed ER apparent in **Figure 4.41** filled the bulk of the remaining cytoplasm not filled with secretory vesicles. This ER was the dominant component of the gland cytoplasm with other components (Golgi complexes and mitochondria) being much more sparsely distributed. From the data obtained pertaining to the synthetic pathway leading to the formation of vesicles in the AGC the following suggestions can be made: the abundant ER but the low numbers of Golgi complexes suggest protein synthesis is a predominant activity of this tissue, as evidenced also by the strong reaction with bromophenol blue. The positive reaction to PAS however also suggests that a fraction of this protein has been glycosylated by their passage through Golgi complexes. The conclusion is therefore that the secretion of the AGC is a mucus compound with a peroxidase enzyme component although the mucus component may differ from that of the labral and urosomal glands. The implications of this cellular organisation on the type of secretion being produced are not clear. The AGC showed similar staining

attributes to other glands identified in this study and might be expected to show similar characteristics under TEM but the ultrastructural data amply demonstrates the danger of relying solely on non-specific histochemical tests.

Without more detailed examination of the AGC than were observed in this tissue it is impossible to say whether all the different lobes of the gland, apparent at a light microscope level, have the same ultrastructural features. What is apparent though is that the AGC of adult *L. salmonis* are definitely not excretory units. The suggestion in Chapter 3 was that the AGC of the larval stages might be equivalent to the antennary gland, the excretory organ described for larval crustaceans (Icely and Nott 1979, Mercade 1982) that does not contain secretory vesicles. The presence of large secretory vesicles in the AGC of the adults however, leads to the conclusion that either the AGC of the larval stages of *L. salmonis* are not the equivalent of the antennal excretory gland or that the structure described as the anterior gland complex (AGC) of adult stages is not homologous with the AGC of larval stages.

Unfortunately no ultrastructural observations were made of the DSG in this study. Their histochemical attributes suggest a commonality of secretion type with the other glands described in this study but caution should be exercised in such a proposal given the apparent similarity, from their histochemistry, of the AGC to the urosomal and labral glands but their markedly different ultrastructure.

4.5.4 Mechanism of secretion

From the evidence obtained from TEM, the mechanism of secretion may be as follows: the membranes of the secretory granules fuse to the membrane of the secretory ducts thereby releasing their contents into the duct lumen i.e. merocrine secretion. TEM examination of the lumen of the urosomal gland ducts showed the presence of a flocculent material with similar characteristics to that present in the secretory vesicles of the glands. This material was free within the lumen of the duct along with misshapen, but essentially spherical membranes, which presumably had bounded the vesicles prior to their coalescence with the duct. The TEM evidence that vesicles in gland D coalesce into one another suggests that exocytosis may occur rapidly as a result of this fusion, a process known as 'compound exocytosis'.

The stimulus for secretion also remains unknown, and an understanding of such stimuli would assist the determination of gland function. The fact that the glands always contain full reservoirs of vesicles would suggest either that recovery (in terms of increased secretion production) is rapid following secretion or that secretion is a continuous process caused by an increase in internal pressure as a result of constant production of secretory material.

In no specimen examined was a depleted reservoir of secretory vesicles ever found in either the labral, AGC or region D of the urosomal complex, suggesting that these glands persist in a constant state of secretory 'readiness' and are apparently not subject to cycles of production / secretion. This is in contrast to the cyclical nature of gland activity described for some species i.e. *Tigriopus brevicornis* (Gharagozlou-van-Ginneken, 1979) and leads us to the conclusion that these glands are not involved in an aspect of the louse's biology / physiology which has a cyclical nature i.e. oogenesis, moulting etc. Zaffagnini and Zeni (1987) claimed that the ultrastructure of the labral glands of *Daphnia obtusa* changed with the moult cycle and may be hormonally involved with oogenesis.

An aspect of major importance regarding gland function is that of the method of control of secretion. The actual secretion of gland products may be instigated in a number of ways. It may be a continuous 'trickle' flow with or without a mechanism of control over the increase / decrease of rate of secretion. It may be under neural control suggesting that secretion occurs as a specific reaction to certain unknown stimuli. These stimuli could be as diverse as approach or contact of a mate, conspecific, and predator or prey items. Bron (1993) provided evidence of direct innervation of sensilla on the cephalothorax of *L. salmonis* copepodids using this technique, and in this study, several of the nerves pass close to the points where glands are found. Neural control would suggest that temporally discrete circumstances cause release of gland secretions. Trickle flow and neural control could also exist together where neural control is used to synergistically enhance secretion rate in response to certain stimuli. TEM examination of the urosomal gland of *L. salmonis* demonstrated the presence of nerve axons in close contact with the gland complex, as the histochemical test for acetylcholine had also indicated. These nerve bundles run along the longitudinal length of the glands and appear to be contained within the

membrane that encloses the gland syncytium as a whole. No evidence, in the form of synapses, that these nerves innervated the glands was ever observed in this study, however this possibility cannot be precluded. Direct innervation of exocrine (luminescent) glands in the Copepoda has been demonstrated previously by Bannister (1993b) and suggested by Bowlby and Case (1991) whilst Pochon-Masson *et al.* (1975) demonstrated the innervation of tegumental glands of the mystacocarid *Derocheilocaris remanei* Delamare-Debouttville and Chappuis, 1951 and Zeni and Zaffagnini (1988) demonstrated such control in the labral glands of the cladoceran *Daphnia obtusa*. The nerves observed in this study, in association with glands in *L. salmonis* were unmyelinated, as were those nerves observed in other studies cited, but myelin-like sheaths around copepod nerves have been recently discovered (Davis, Weatherby, Harline and Lenz, 1999). Myelin sheaths around nerves have never been described in invertebrates prior to this study. Davis *et al.* (1999) describe myelin sheaths around nerve axons of several calanoid genera and relate their findings to fast escape responses in the pelagic species in which they are found. The absence of myelin sheaths around the nerves of *L. salmonis* in this study does not neccessarily preclude their existence in this species. Such adaptations may only be present in those axons involved in fast response behaviour such as those innervating the antennules, swimming legs etc. – tissues that were not examined in this study.

The possibility of a mechanism of secretion control other than nervous should also be considered. Hormonal control of secretion in insect glands is widely recognised, as is the fact that crustacea are not so advanced with respect to the insects to have evolved such systems. A mechanism to assist in the expulsion of gland secretions involving increased internal hydrostatic pressure has been postulated by Hicks and Grahame (1979), Bannister and Herring (1989) and Von Vaupel Klein and Koomen (1994). The urosomal gland complex of *L. salmonis* lies amongst a number of anal dilator muscles that dilate the lumen of the hindgut during defaecation and anal drinking as well as the sphincter muscles which open the midgut to expel faecal material into the hindgut (Boxshall, 1990). These muscles are highly active (author's unpublished observations) and can cause considerable contraction of the anal somite of the animal and such contractions could theoretically help expel the contents of the urosomal glands. Direct muscular control of emission of gland contents has been suggested for the labral glands of *C. typicus* by

Arnaud *et al.* (1988b) and also for the accessory glands of male insects (Happ, 1984) although the dynamics of ejaculation are likely to be very different from the dynamics of other exocrine gland systems. No evidence of muscle groups specifically associated with any gland systems were noted in *L. salmonis*.

Repeated observations of experimental animals in this study failed to reveal any observable secretory activity *in vivo*. This failure to observe the expulsion of gland contents could be caused by many factors not least the fact that the secretion is probably colourless in its natural state as evidenced by the failure to observe secreted material adhering to unstained specimens. TMB staining of *L. salmonis* nauplii did allow the observation of plumes of stained material emanating from the region of the posterior gland ducts but the rapidity and extent of this secretion suggested that the process was artifactual, arising as a consequence of immersion in an incompatible solution, although active secretion in response to a stimuli (the TMB solution) may also have caused this reaction. TMB also revealed the presence of a, normally colourless, layer on the external surfaces of *L. salmonis* adults, especially on the dorsal cuticle and the setae of the thoracic legs and caudal rami. This layer was particularly prominent in specimens of *L. hippoglossi*.

4.6 CONCLUSIONS

The histochemical analyses performed in this study indicate that neutral mucus and protein form the principal components of the secretions of the greatest majority of exocrine glands identified using the DAB and TMB stains.

The exocrine glands of *L. salmonis* described here and, by extrapolation other caligids, are actively secreting, either separately, or in combination, mucus and some form of protein, possibly peroxidatic enzyme. Evidence also suggests that some glands contain some form of lipid material but whether this is actively secreted or is present only in the membranes of the gland tissue, or those that contain the secretory vesicles, has yet to be determined. The mucus component of the secretions may serve as a carrier, or vehicle, to render other components of the secretion relatively immobile and prevent

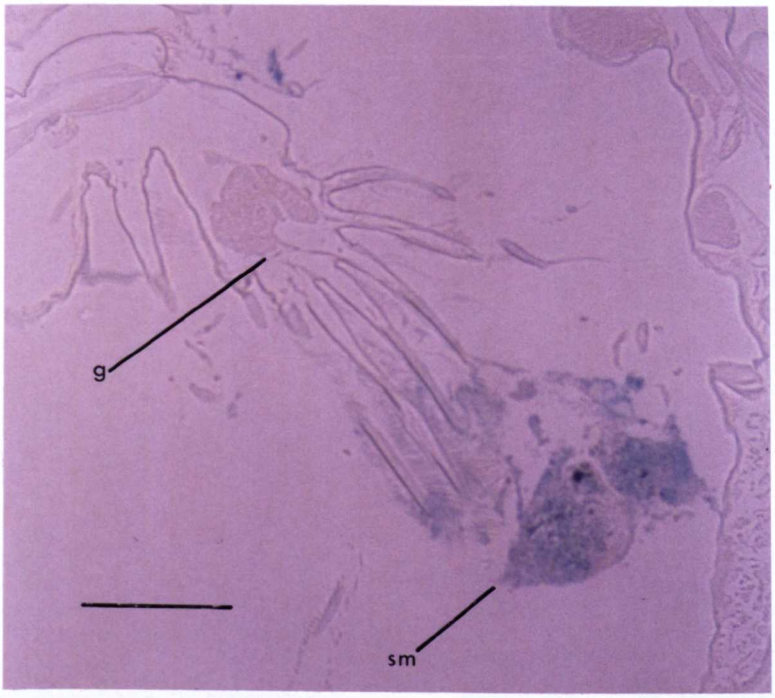
them being washed away from their site of action, but the roles of these other components need to be determined.

Despite the frequent identification of mucus compounds in crustacean glands very little explanation has been made as to the specific role that such compounds fill and how the chemistry or dynamics of those compounds are suited to their purpose. The utilisation of mucus secretions is widespread in the marine environment where they serve a variety of functions. In addition to the already described suggestions made by various researchers for mucus secretions in copepods (Briggs 1978, Gharagozlou-van-Ginneken 1979, Hicks and Grahame 1979, Von Vaupel Klein 1982a, Williams-Howze, Silverman and Fleeger 1987, Bron *et al.* 1993, Von Vaupel Klein and Koomen 1994), mucus is involved in the locomotory mechanism of marine gastropods (Davies and Hawkins, 1998), the feeding mechanism of marine bivalves and performs a variety of functions as a component of an epidermal layer in fish (Shephard, 1994). Given the general lack of information regarding the precise functions of secreted mucus in copepod species, and the limited biochemical information concerning those compounds, no functional attributes can yet be accorded to the secretions based on its general staining characteristics.

It is apparent from a comparison of the histochemical and ultrastructural data that despite the fact that the population of glands identified showed similar staining properties with the DAB and TMB stains their ultrastructural characteristics varied markedly. Given the similar staining qualities of all the glands this variation in ultrastructure is difficult to account for. The evidence suggests that the DAB-positive staining of the glands occurs regardless of their ultrastructural (and therefore secretory) attributes which, in itself, suggests that the DAB staining arises as a result of some common component of the gland tissue and has no reflection on the secretory product of those glands.

No ultrastructural evidence was obtained for several of the identified exocrine gland groups of *L. salmonis*. The DSG, thoracic leg glands and suture line glands were not examined at an ultrastructural level although their histochemistry was identical to that of the large gland complexes i.e. urosomal, AGC, labral. The DAB and TMB stains and SEM analysis did however demonstrate that all these gland systems were exocrine in nature.

Given the identification of a proteinaceous component within the identified gland tissues of *L. salmonis* and the discovery that the DAB stain appears to be reacting with some form of peroxidase enzyme the next step is to identify that enzymatic component. The following chapter shall attempt such an identification.



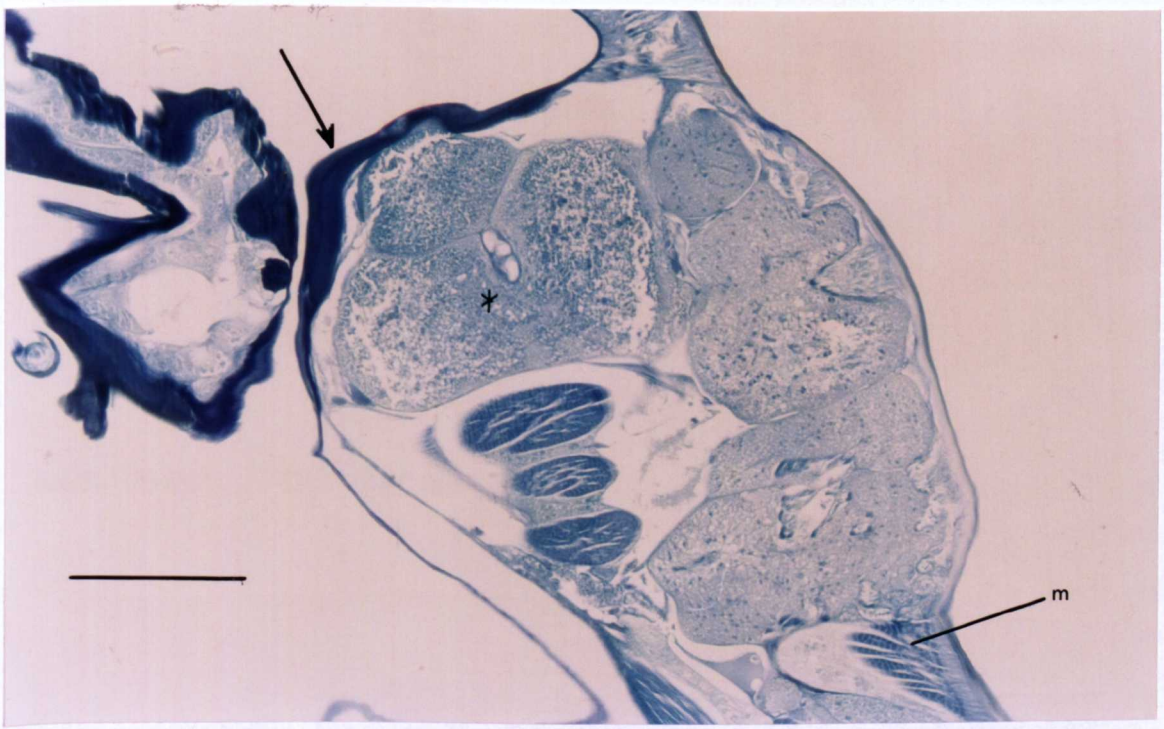
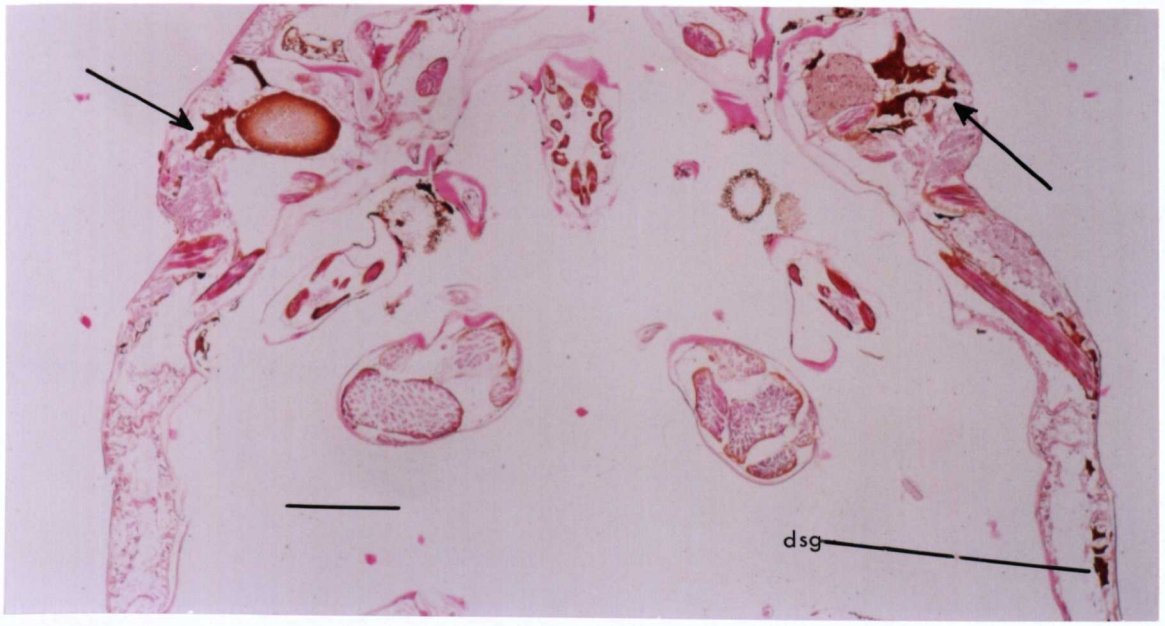


Figure 4.1 Wax section of an adult male *L. salmonis* with Alcian Blue stained material (sm) adhering to the setae of the th_{II}. Note how the lumen of the gland (g) in the distal segment of the endopod of the th_{II} has not stained. Scale bar = 200µm.

Figure 4.2 Typical Bromophenol Blue staining of a wax section of the AGC (*) of an adult female *L. salmonis*. Note the strong staining reaction of the muscles attached to the cuticle of the cephalothorax (m) as well as the cuticle itself (arrowed). Scale bar = 50µm.



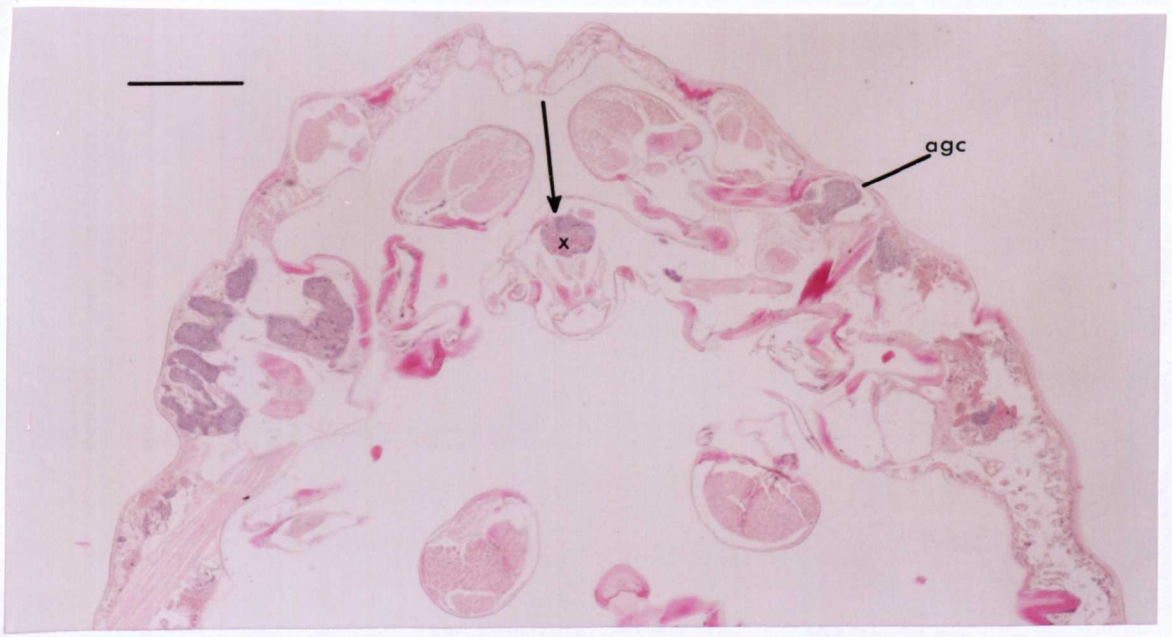
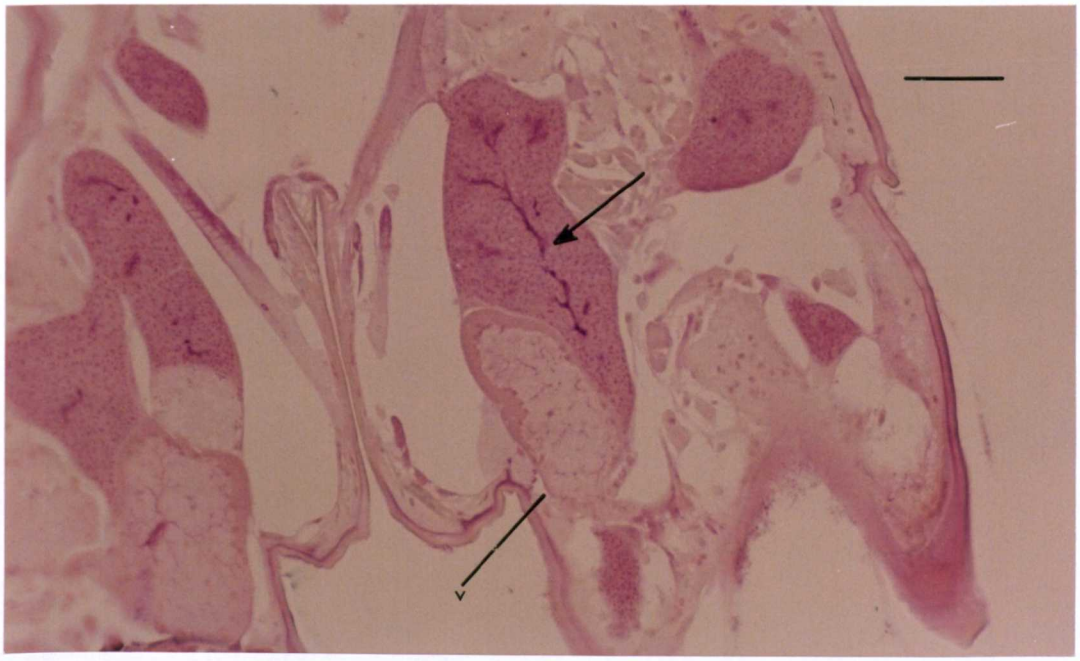


Figure 4.3 DAB-staining glands in a plane section of the cephalothorax of an adult female *L. salmonis*. Note positive staining of the AGC (arrowed) and some DSG (dsg). Scale bar = 150μm.

Figure 4.4 Haematoxylin and Eosin staining of a plane section of the cephalothorax of an adult female *L. salmonis*. Note the eosinophilic staining of the vesicular region (x) of the labral gland (arrowed). agc anterior gland complex Scale bar = 150μm.



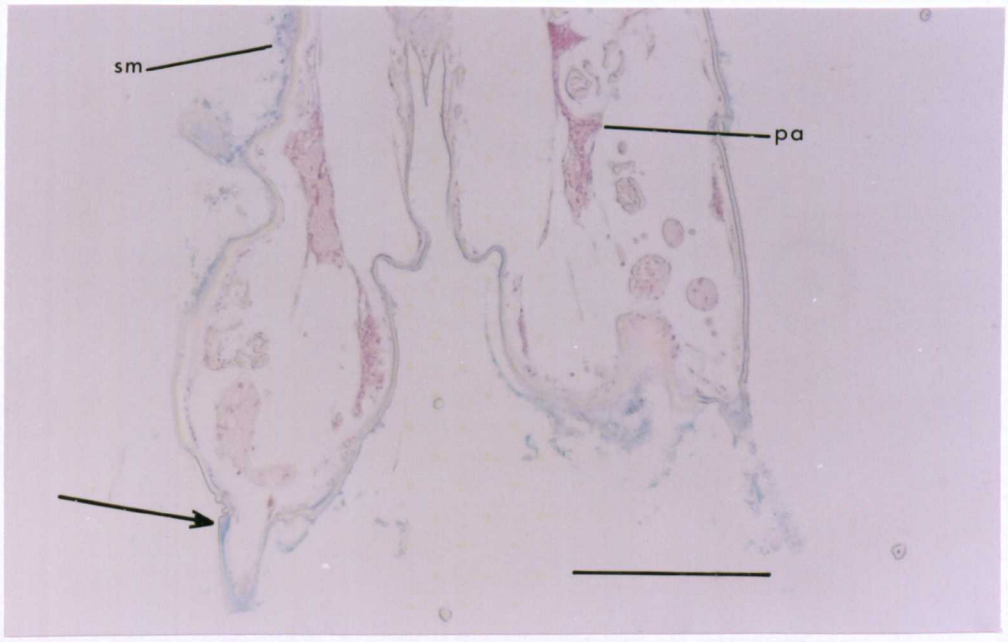


Figure 4.5 Plane section of the urosomal gland complex of an adult male *L. salmonis* (plastic embedding medium) demonstrating the differential PAS staining observed between gland regions (v denoting vesicular region). The dendritic pattern of the ductules is also apparent (arrow). Scale bar = 50μm.

Figure 4.6 Combined PAS and Alcian Blue staining of the urosomal gland complex fixed with 10% neutral buffered formalin and embedded in paraffin wax. The PAS-positive response to regions of the complex (pa) are evident as is Alcian Blue staining of material adhering to the external cuticle (sm) and the internal cuticle of the terminal setae (arrows). Scale bar = 200μm.

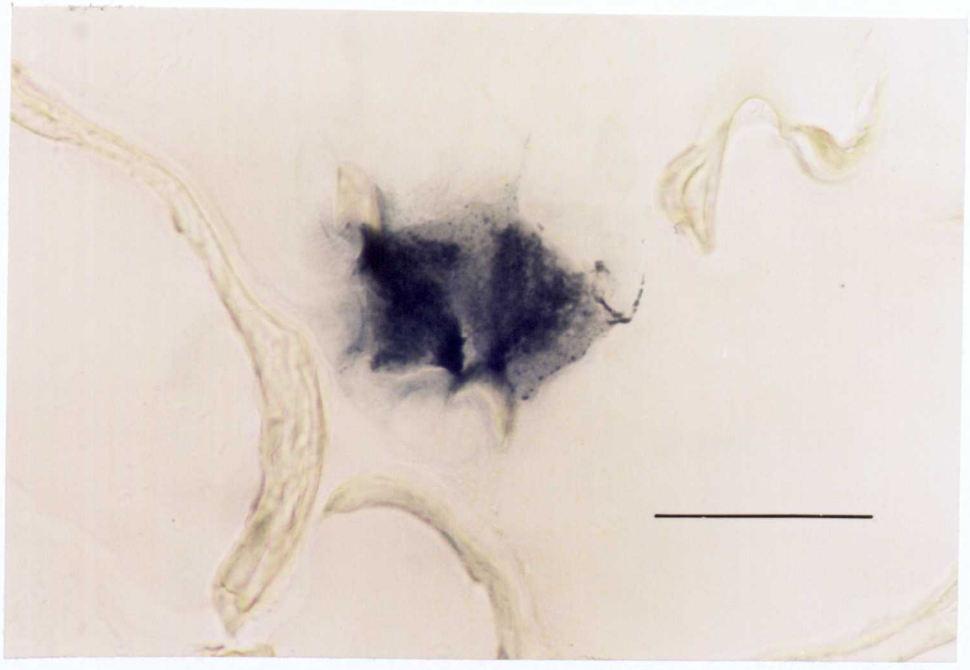


Figure 4.7 Ventral surface of the cephalothorax of a Sudan Black stained whole *L. salmonis* adult male. In this partially cleared specimen positive staining is evident in two glands present at either anterior margin of the cephalothorax as well as one in either th_{II}. Positively stained urosomal glands are also visible in the urosome of a second specimen at the bottom right hand corner of the figure. Scale bar = 1mm.

Figure 4.8 Cryostat plane section of the mouth-tube region of an adult male *L. salmonis* stained with Sudan Black. The duality of the staining of the labral gland is apparent with lighter grey staining areas occurring around the peripheral margins of the gland. Scale bar = 50μm.

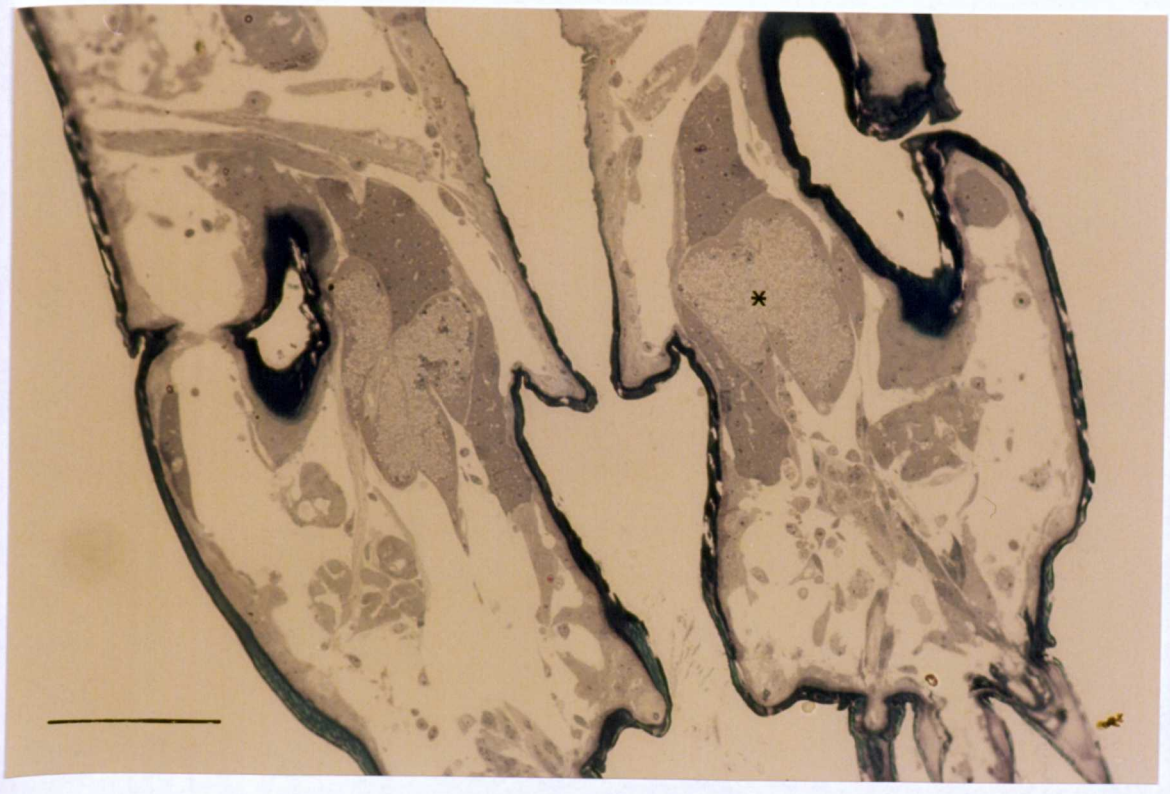


Figure 4.9 A semi-thin resin section of the urosomal gland of an adult male *L. salmonis* stained with toluidine blue showing the structurally distinct regions of this complex including the prominent zone of vesicle accumulation (asterisk). Scale bar = 100µm.

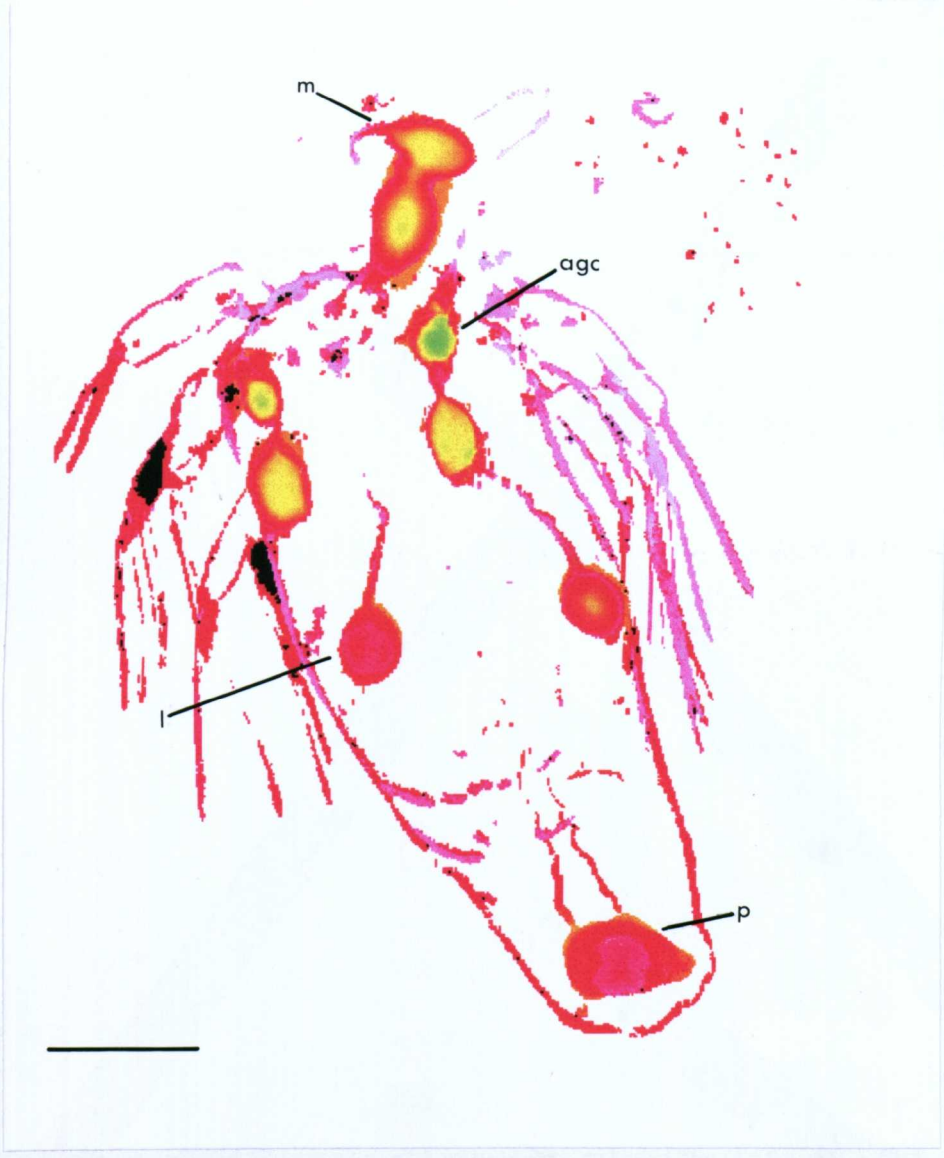


Figure 4.10 Deconvolved 3-D reconstruction of a DAB-stained *L. salmonis* nauplius I larva. The cuticle has also been included to provide a frame of reference. m median glands; agc anterior glsnd complex; l lateral glands; p posterior glands. Scale bar = 100μm.

Figure 4.11 Full sequence of a computer generated 3-D reconstruction of a DAB-stained *L. salmonis* larva.

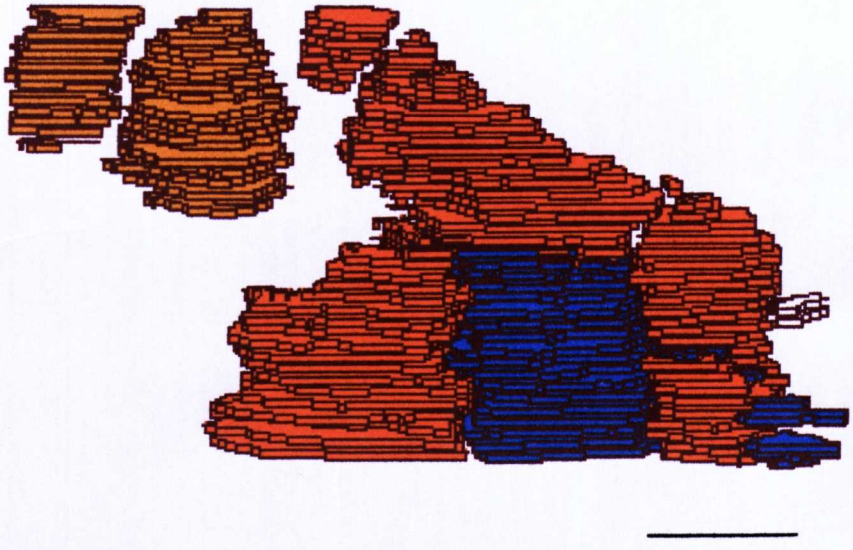


Figure 4.12 Reconstructed urosomal gland complex of an adult male *L. salmonis* made using the VIDAS image analysis system. Scale bar = 50μm.

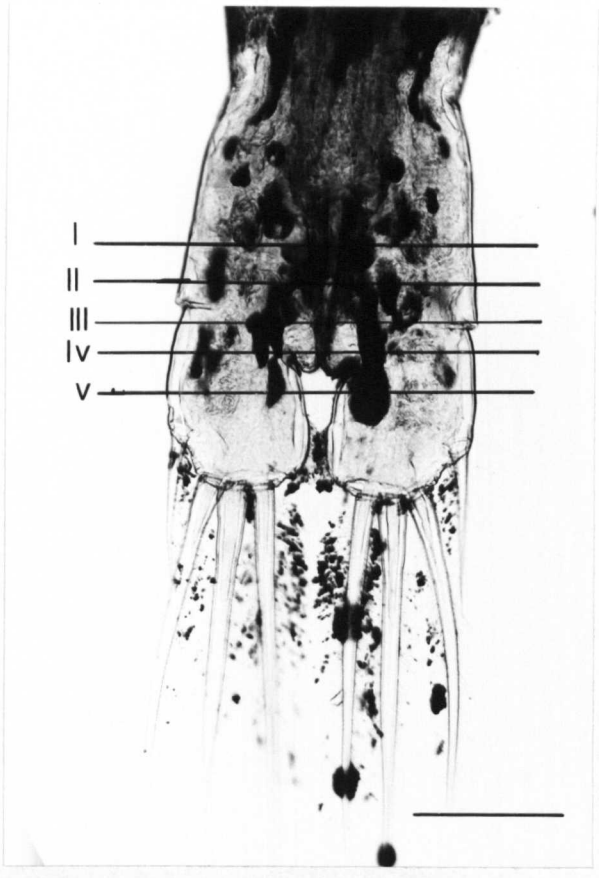
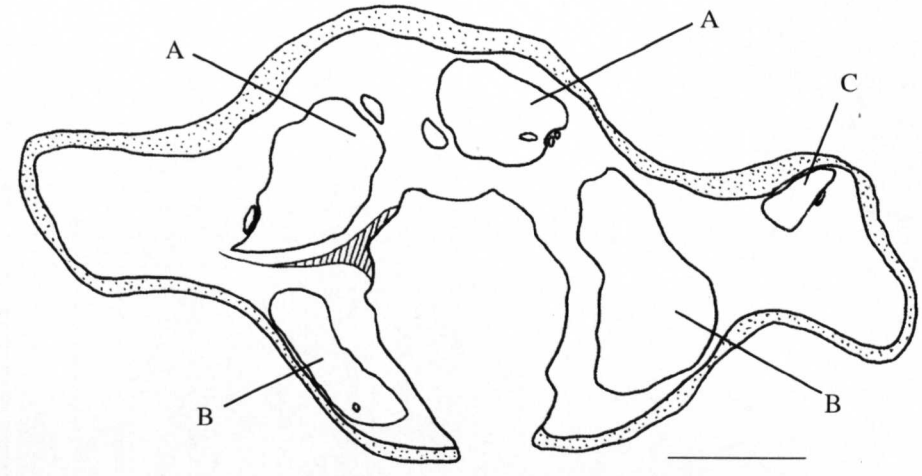


Figure 4.13 The urosome of an adult male *L. salmonis* showing the components of the urosomal gland complex and the approximate plane of sections examined using TEM. Lines I –V correspond to section planes. Scale bar = 200μm.



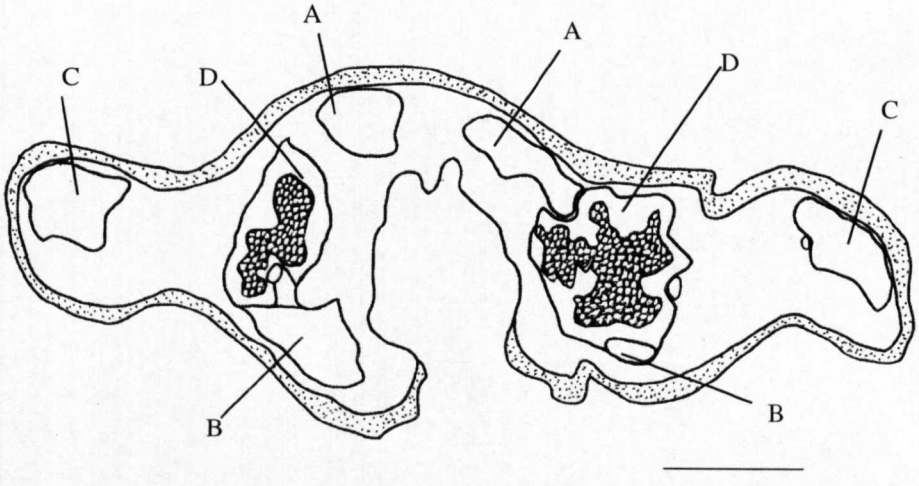
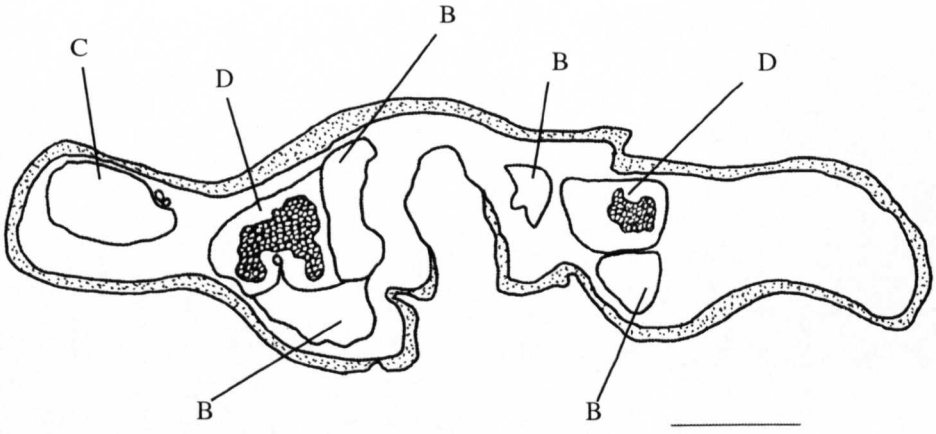


Figure 4.14 Transverse Section I through the urosome of an adult male *L. salmonis*. This image of a semi-thin section, made with the aid of a drawing tube demonstrates the positions of gland tissues of the urosomal gland complex. Scale bar = 100μm.

Figure 4.15 Transverse Section II through the urosome of an adult male *L. salmonis*. This image of a semi-thin section, made with the aid of a drawing tube demonstrates the positions of gland tissues of the urosomal gland complex. Scale bar = 100μm.



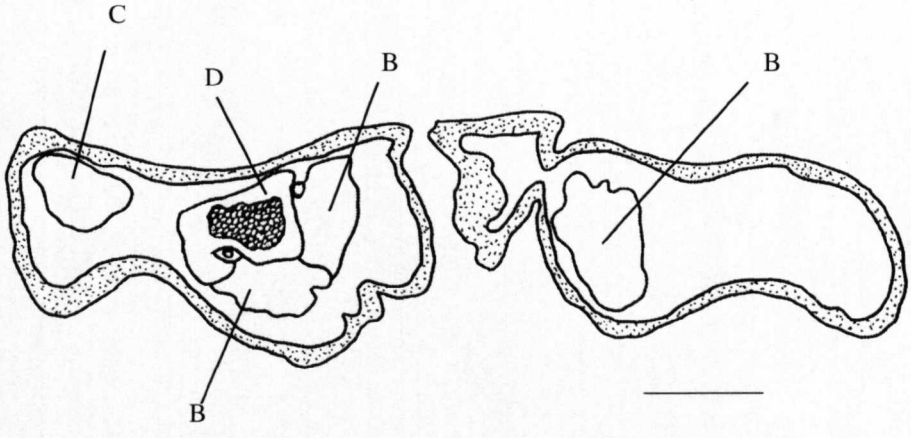


Figure 4.16 Transverse Section III through the urosome of an adult male *L. salmonis*. This image of a semi-thin section, made with the aid of a drawing tube demonstrates the positions of gland tissues of the urosomal gland complex. Scale bar = 100μm.

Figure 4.17 Transverse Section IV through the urosome of an adult male *L. salmonis*. This image of a semi-thin section, made with the aid of a drawing tube demonstrates the positions of gland tissues of the urosomal gland complex. Scale bar = 100μm.

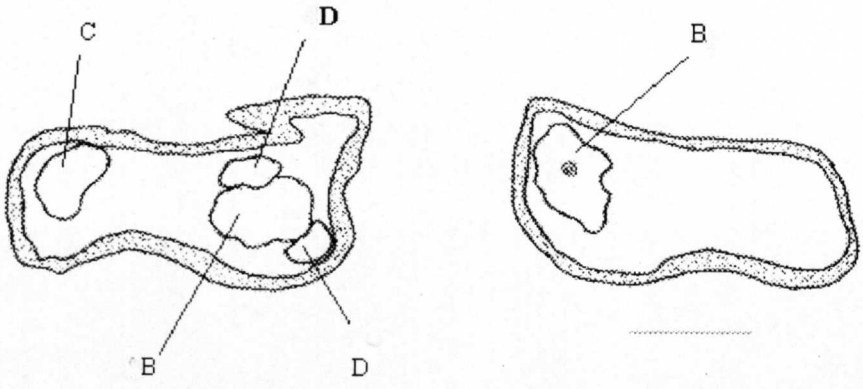
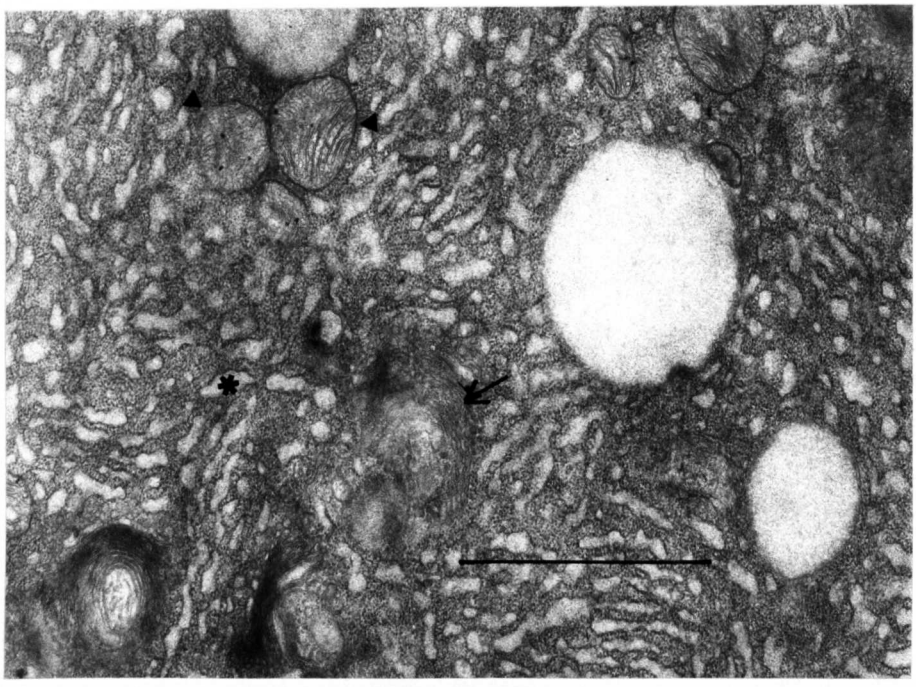


Figure 4.18 Transverse Section V through the urosome of an adult male *L. salmonis*. This image of a semi-thin section, made with the aid of a drawing tube demonstrates the positions of gland tissues of the urosomal gland complex. Scale bar = 100μm.



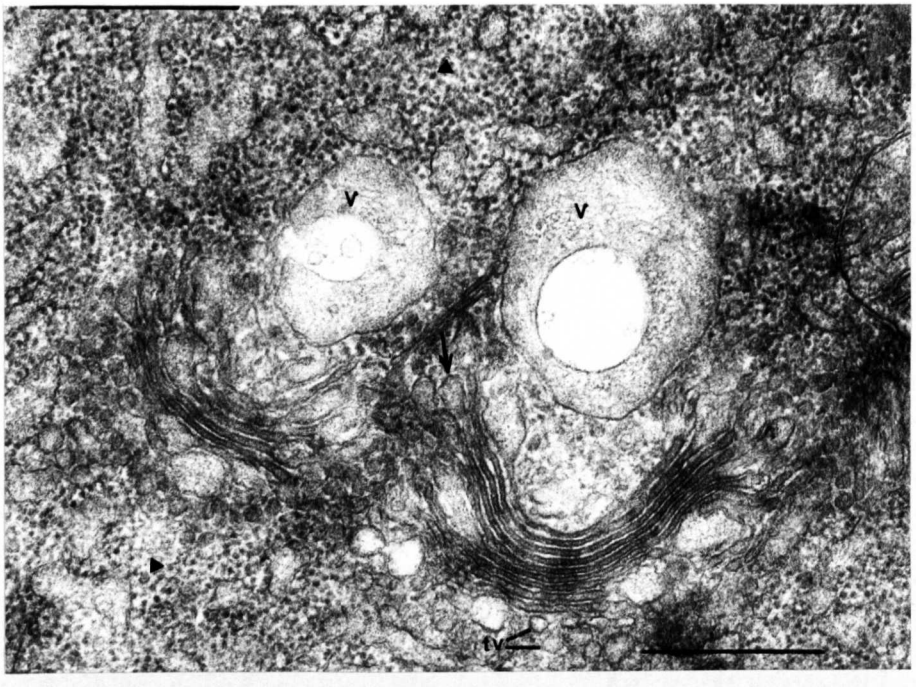
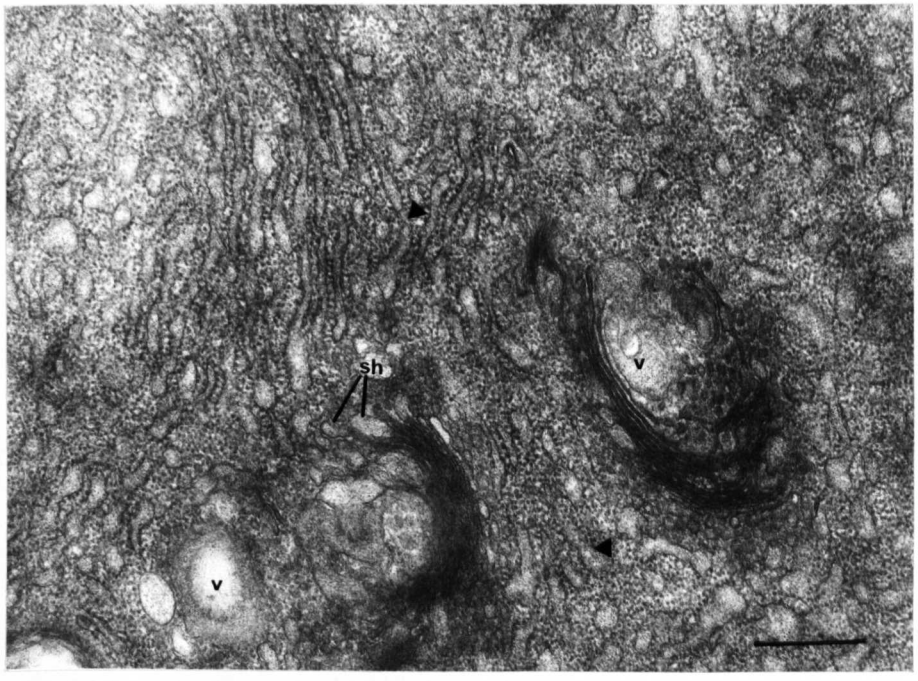


Figure 4.19 TEM image of the general cytoplasmic material of gland A showing the abundant ER with swollen lumen (*), as well as Golgi complexes (arrowed) and mitochondria (arrowheads). Scale bar = 2μm.

Figure 4.20 TEM micrograph of two Golgi complexes of gland A of the urosomal complex. The transitional vesicles (tv) on the cis face are evident as are secretory vesicles (v) blebbing off from the trans faces. Note the shuttle vesicles (arrowed) passing between the cisternae of the Golgi complexes. Large numbers of free ribosomes are evident throughout the cytoplasm (arrowheads). Scale bar = 0.5μm.



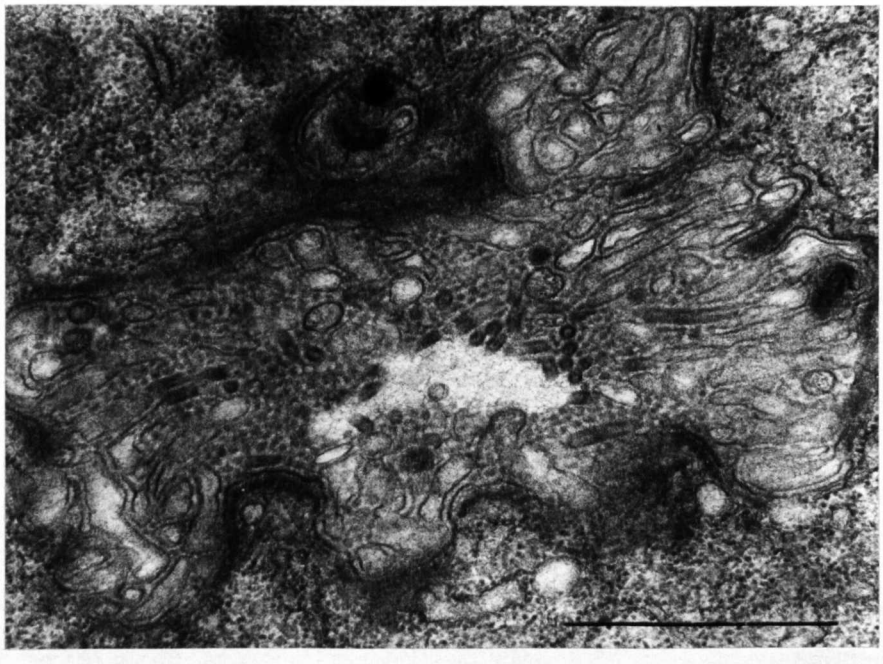
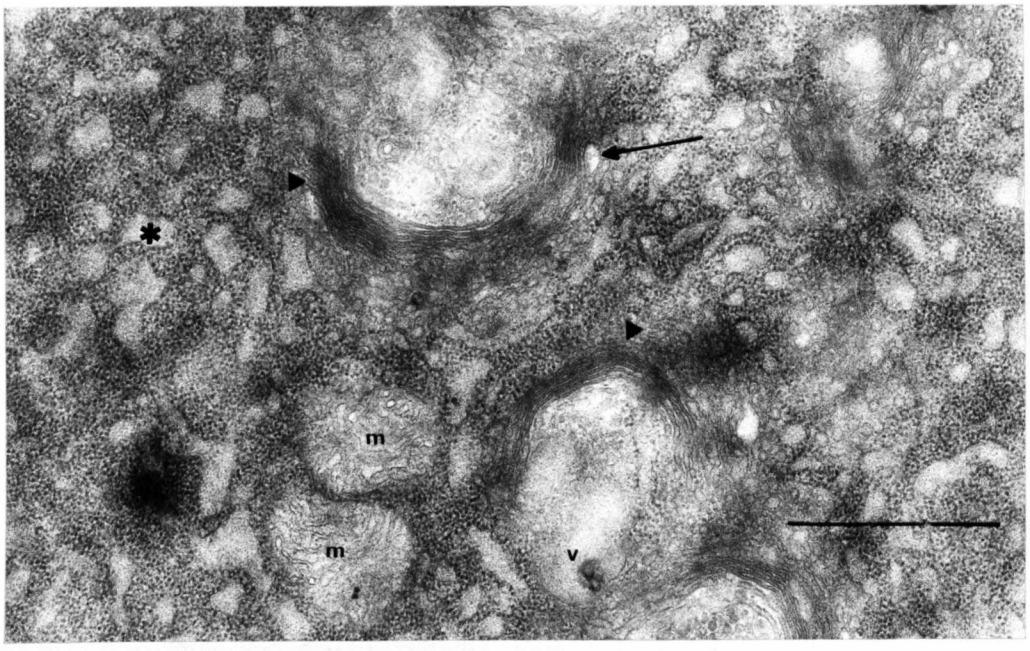


Figure 4.21 TEM micrograph of the cytoplasm of gland A of the urosomal gland. Note the RER (arrowheads) closely associated with the Golgi complexes. Shuttle (sh) and secretory vesicles (v) are apparent in association with the Golgi complexes. Scale bar = 0.5μm.

Figure 4.22 Irregularly shaped microvillous inclusion within gland A of the urosomal gland complex. Scale bar = 1μm.



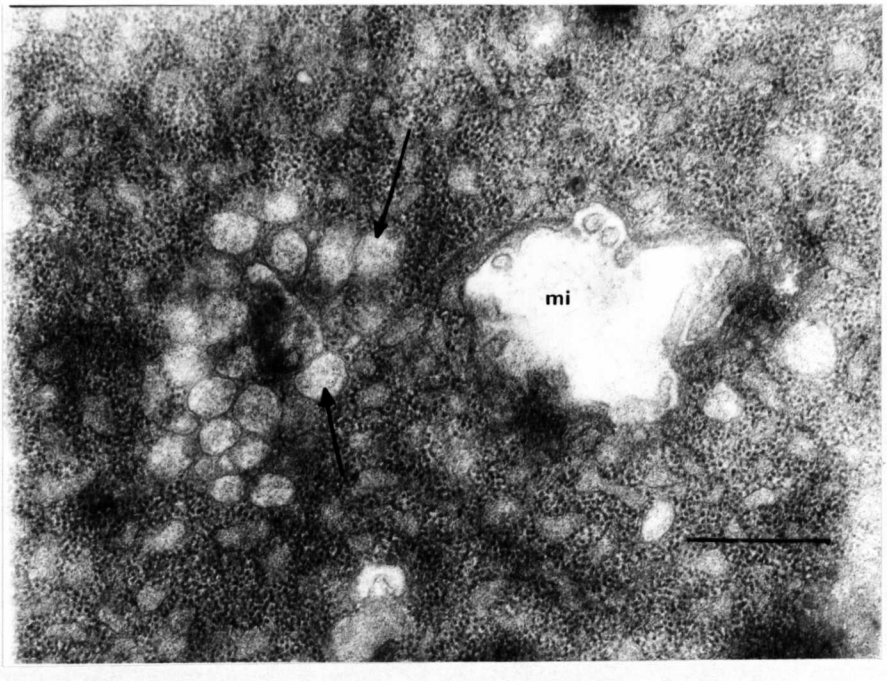
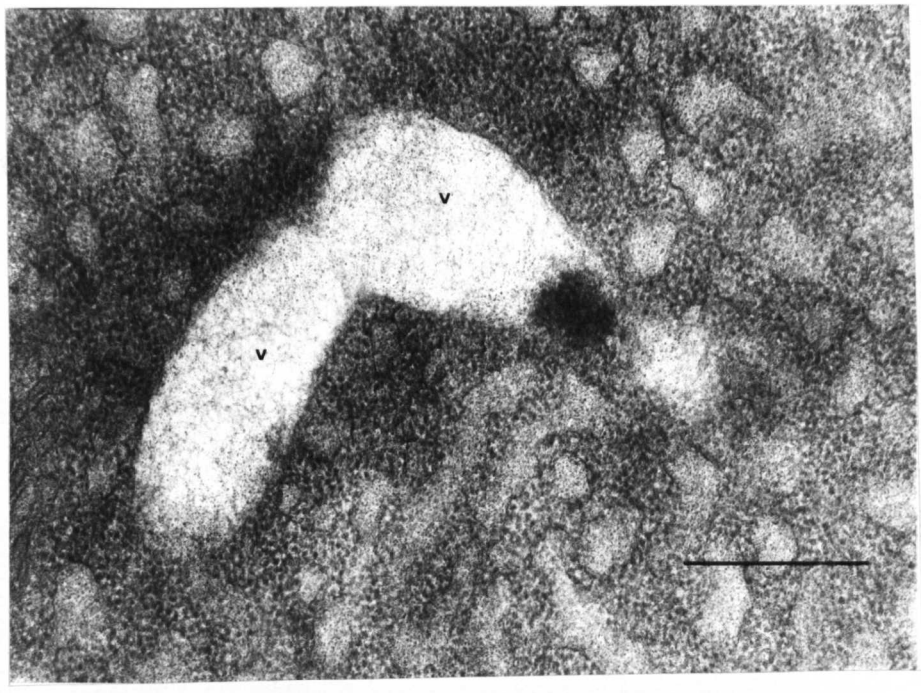


Figure 4.23 TEM micrograph of a typical region of the cytoplasm of urosomal gland B. Note the numerous Golgi complexes (arrowheads) with their associated shuttle (arrowed) and secretory (v) vesicles. Also evident are fragments of ER (*) and irregularly shaped mitochondria (m). Scale bar = 1μm.

Figure 4.24 Microvillous (mi) inclusion and secretory vesicles (arrowed) within the cytoplasm of urosomal gland B. Scale bar = 0.5μm.



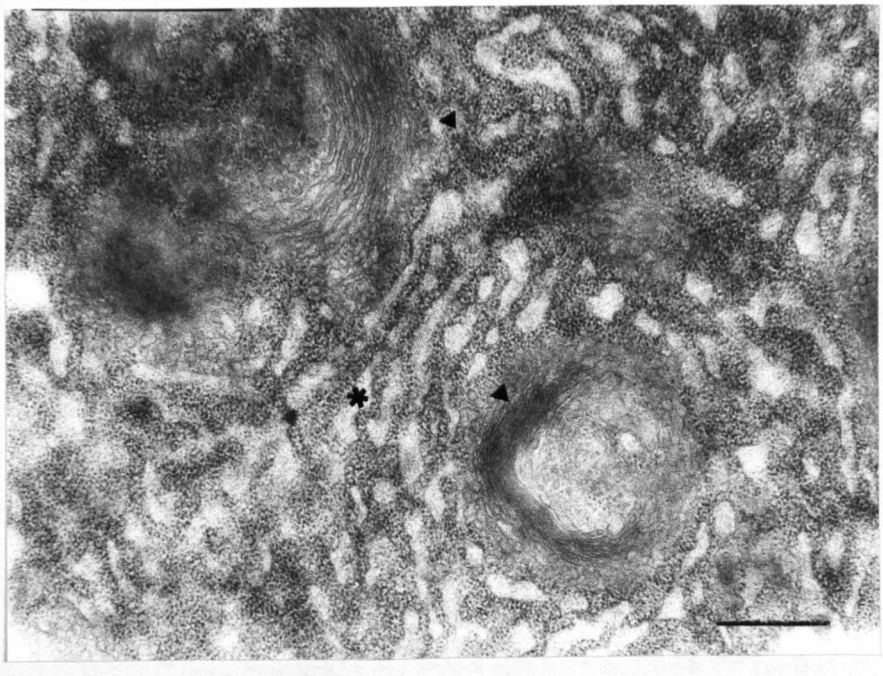
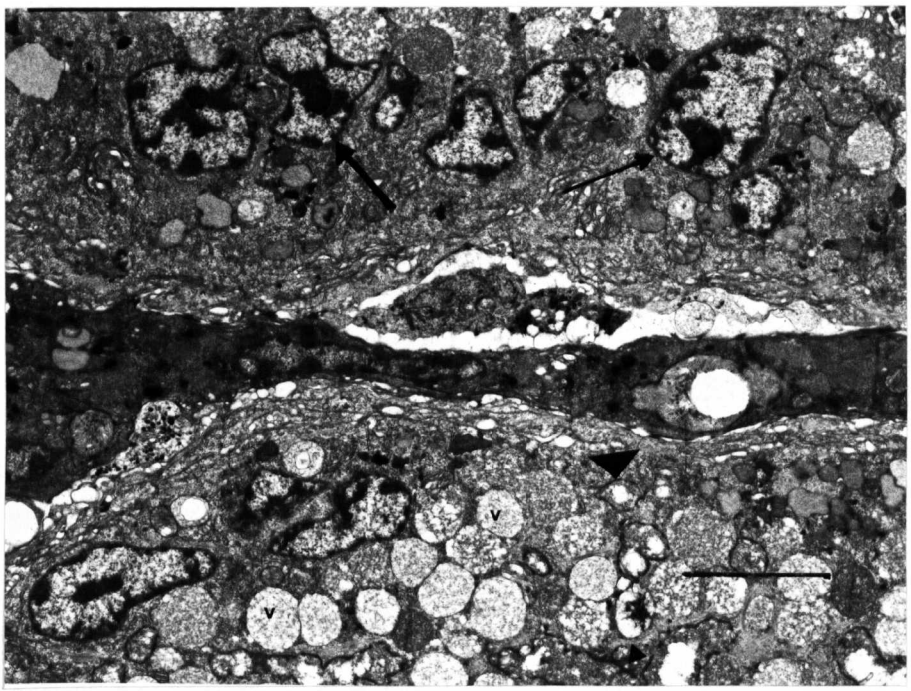


Figure 4.25 TEM micrograph of secretory vesicles (v) of urosomal gland B. Note the profusion of ribosomes in the gland cytoplasm. Scale bar = 0.5μm.

Figure 4.26 TEM micrograph of a typical region of gland C of the urosomal gland complex. The ER with its very swollen, electron-lucent lumen is obvious (*), as are two Golgi complexes (arrowheads) with very closely packed cisternae. Scale bar = 0.5μm.



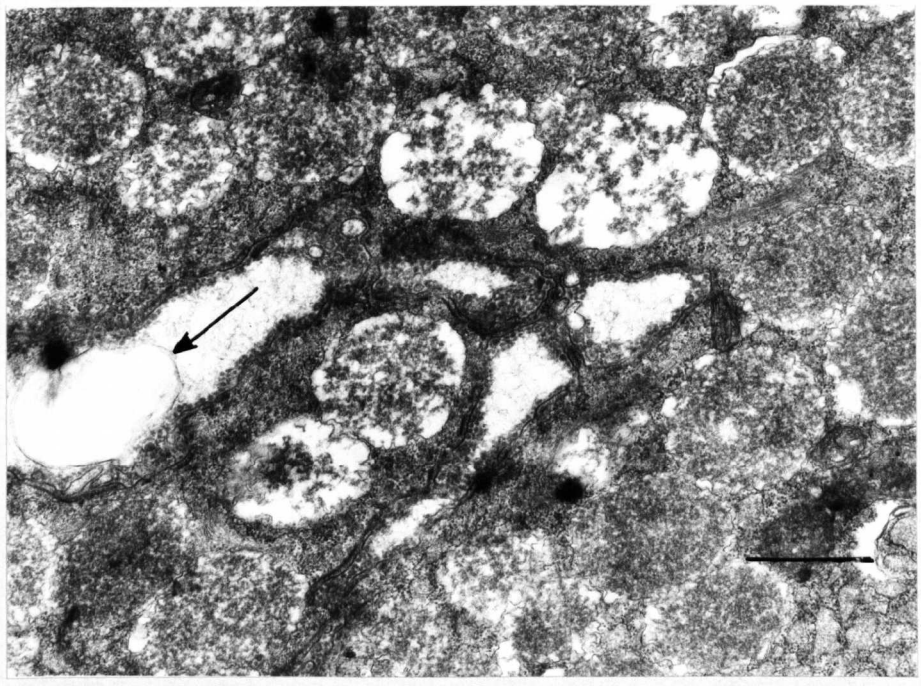
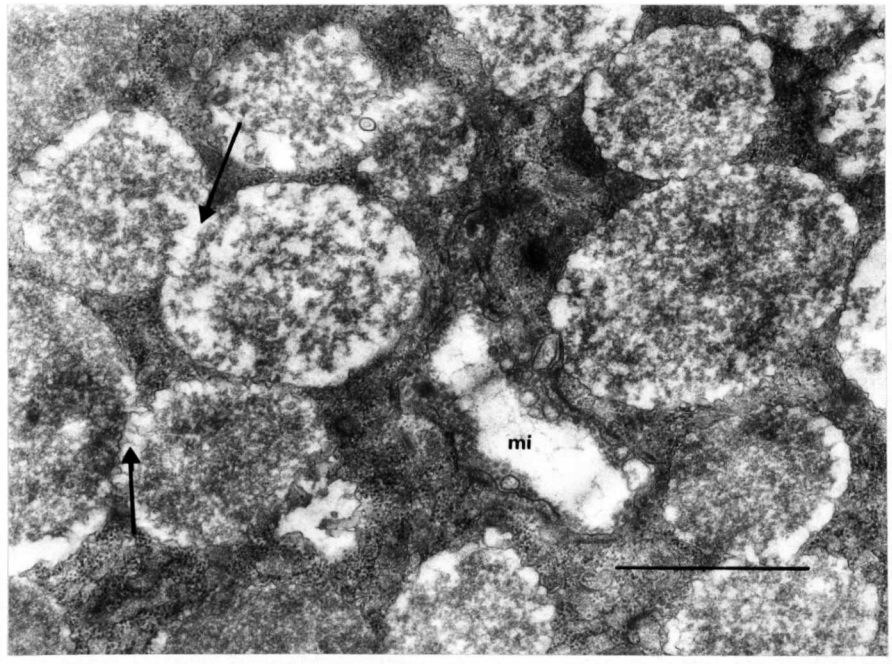


Figure 4.27 TEM micrograph of two closely apposed lobes of urosomal gland D. Note the proliferation of nucleii in the peripheral regions (arrows). Numerous vesicles (v) are also evident. Some small microvillous structures (arrowheads) are also scattered throughout the gland tissue. Scale bar = 4μm.

Figure 4.28 TEM micrograph of the zone of vesicle accumulation of urosomal gland D. The varying electron densities of the vesicles is apparent in this image as is the presence of a membranous structure (arrowed) within the lumen of a microvillous structure. Scale bar = 1μm.



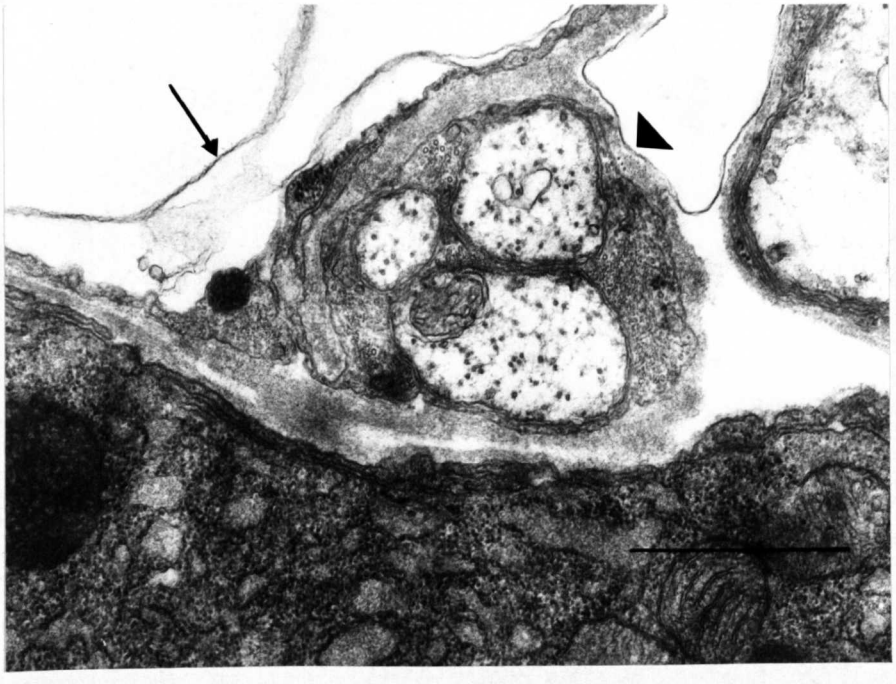


Figure 4.29 Secretory vesicles of urosomal gland D merging with one another (arrows). A microvillous inclusion (mi) is also evident in this micrograph. Scale bar = 1μm.

Figure 4.30 A transverse section of a nerve bundle in association with gland D of the urosomal complex. Note the membrane (arrow) that contains the gland syncytium that also encloses the nerve bundle (arrowhead), although it is slightly lifted off in this section. Scale bar = 1μm.

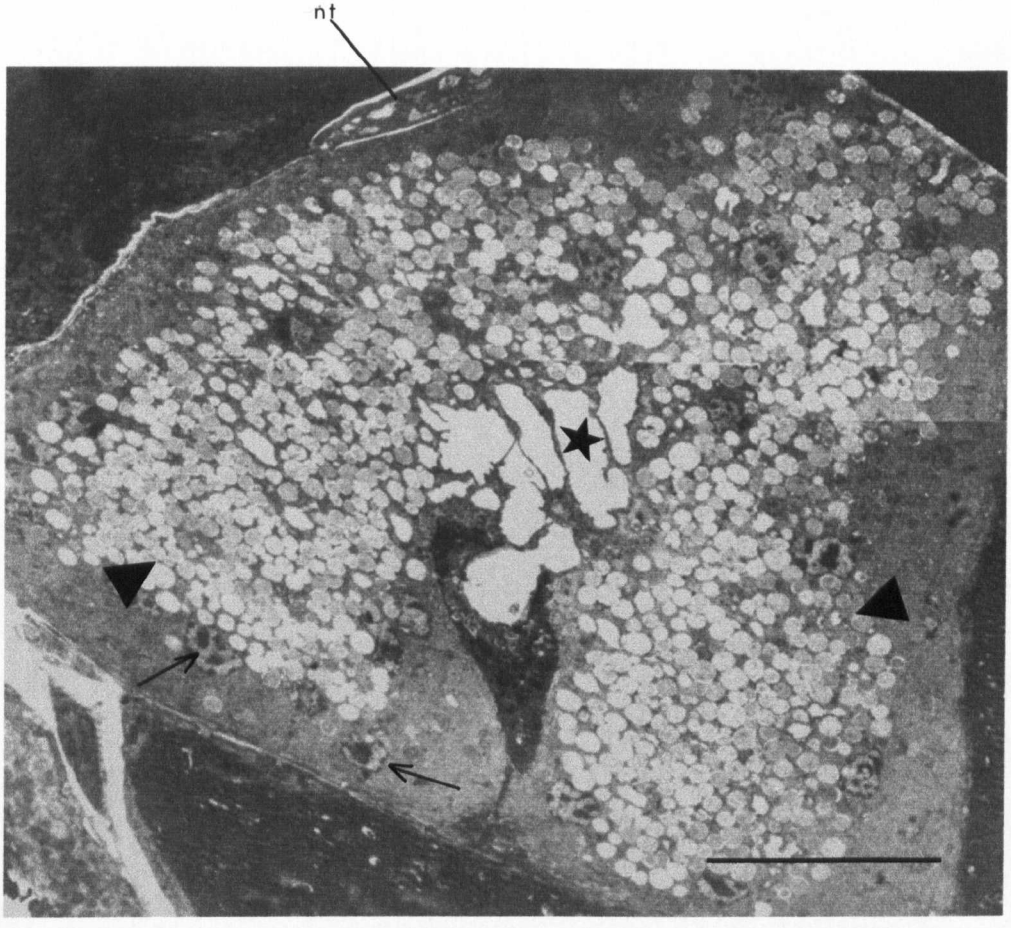
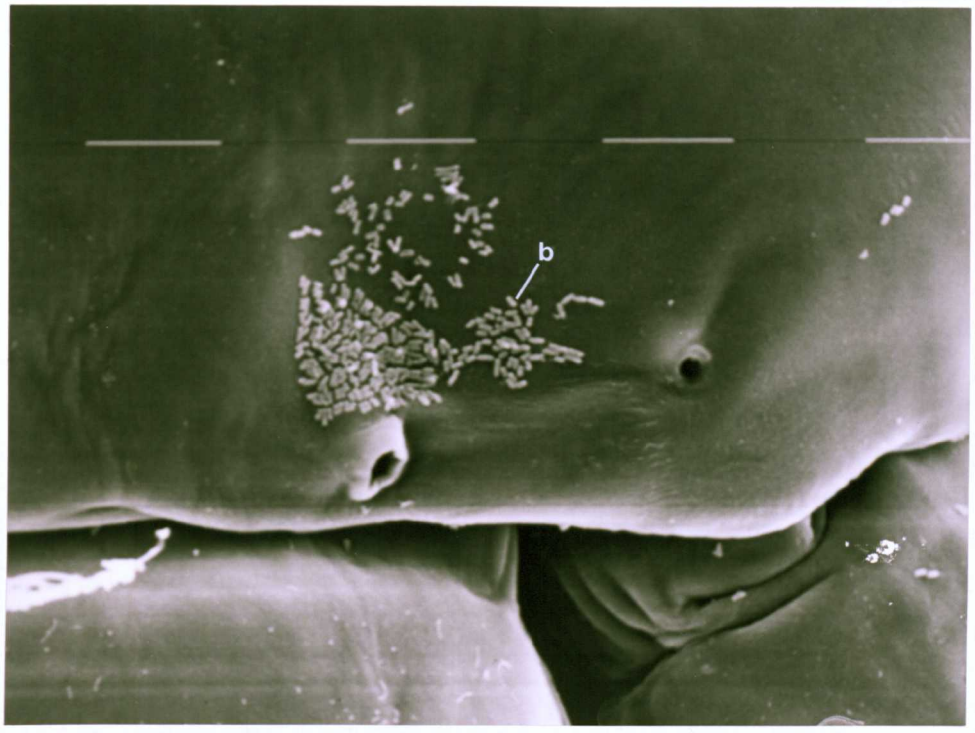


Figure 4.31 Composite TEM micrograph of the general cytoplasm of urosomal gland D. The central zone of vesicle accumulation (arrowheads) is clearly revealed by this transverse section image of the entire gland. The peripheral vesicle-free zone with its abundant nucleii is also evident (arrowed). The central duct and smaller ductules (*) associated with it are apparent in the centre of the accumulation zone. A portion of nerve tissue is visible at the dorsal margin of the tissue (nt). Scale bar = 25μm.



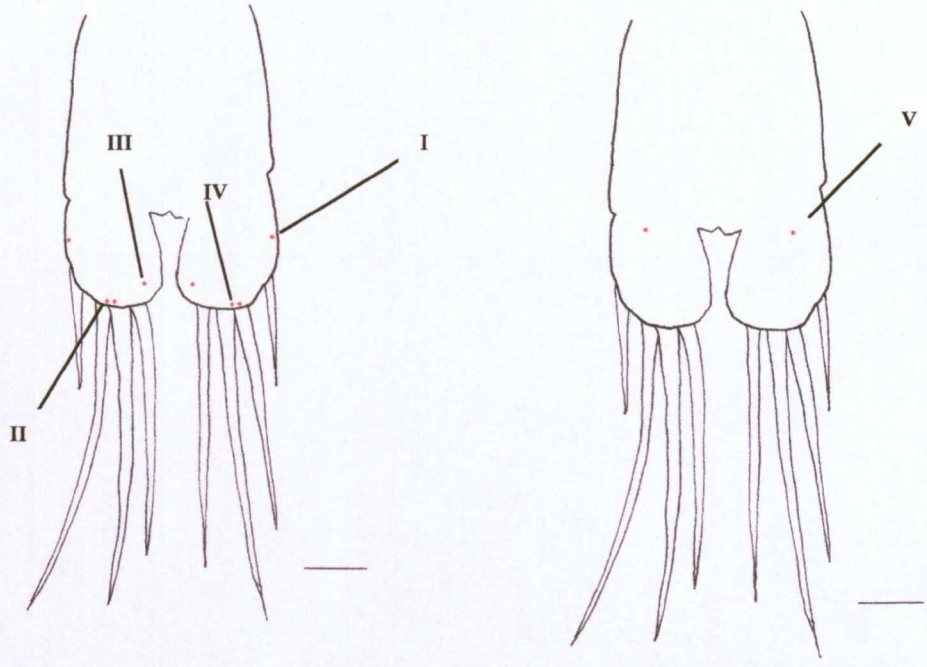


Figure 4.32 SEM micrograph of cuticular pores II and IV of the urosomal gland complex close to the bases of the terminal setae on the ventral margin of the caudal ramus of an adult male *L. salmonis*. Note the bacteria attached to the cuticle (b). Scale bar = 1μm.

Figure 4.33 Diagrammatic representations of the urosome of an adult male *L. salmonis* showing the locations of the cuticular pores on both the ventral (A) and dorsal (B) surfaces. Scale bar = 100μm.

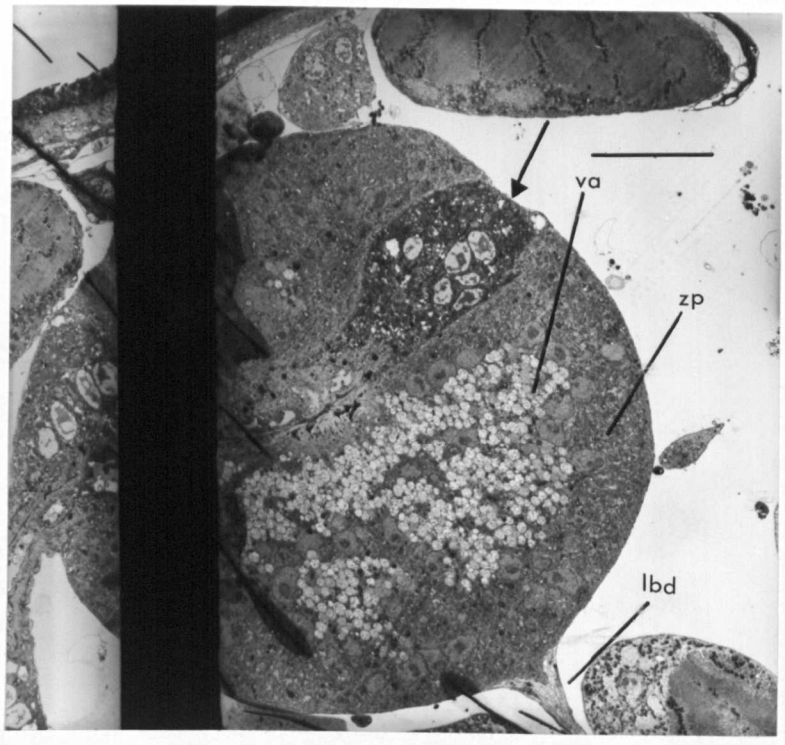


Figure 4.34 Low magnification TEM micrograph of the labral gland of *L. salmonis*. The zones of production (zp) and vesicle accumulation (va) are evident at this magnification as are the ducts of the circum-oral glands (arrowed) which traverse the tissue immediately dorsal to the zone of vesicle accumulation. Note also the labral gland duct (lbd) emerging from the ventral margin of the gland to pass into the labrum. Scale bar = 20μm.

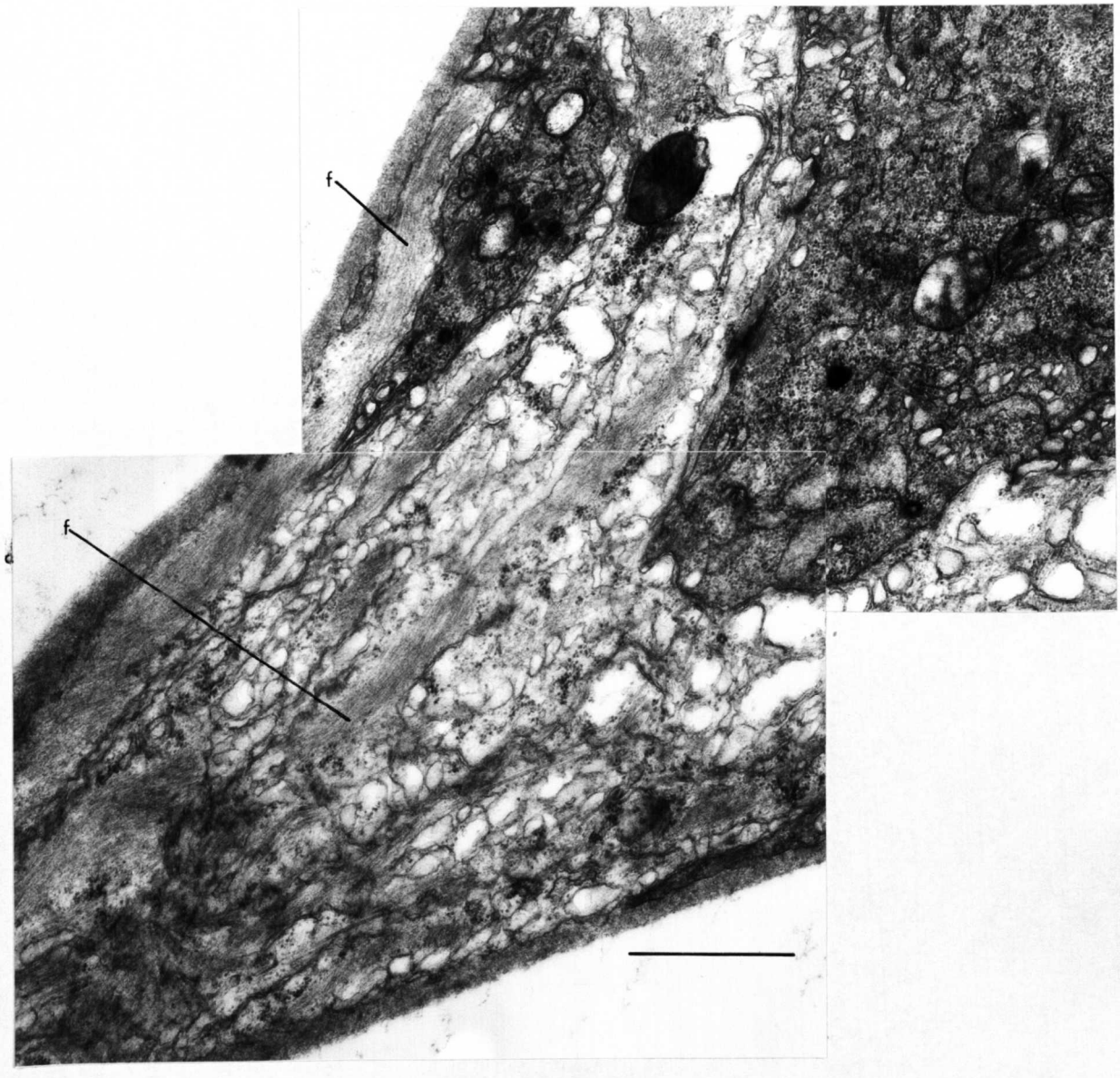
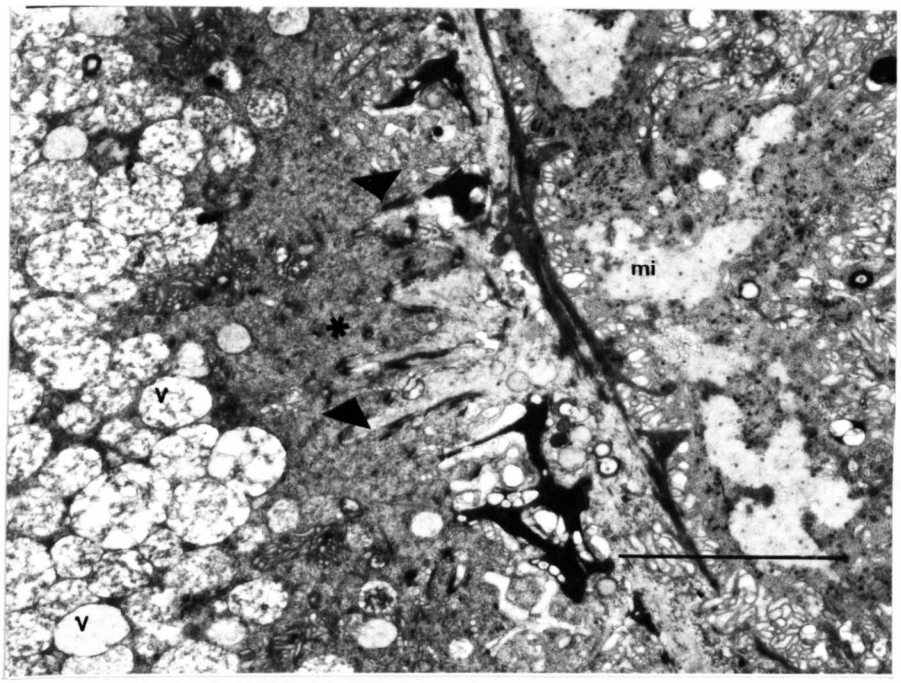


Figure 4.35 Composite TEM micrograph of the labral gland duct at the point where it emerges from the gland syncytium to pass into the labrum. f internal filaments. Scale bar = 1μm.



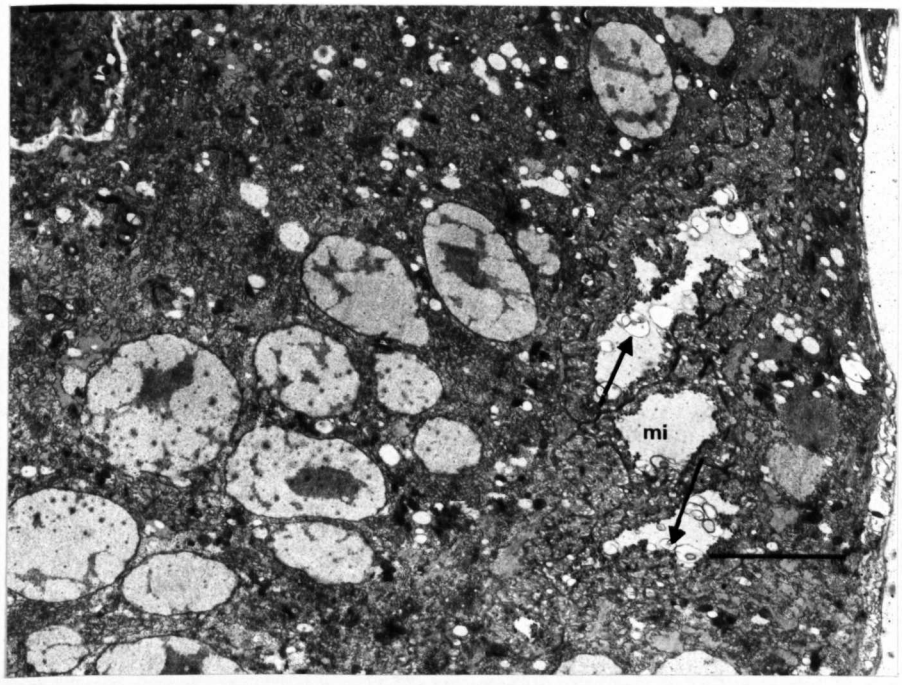
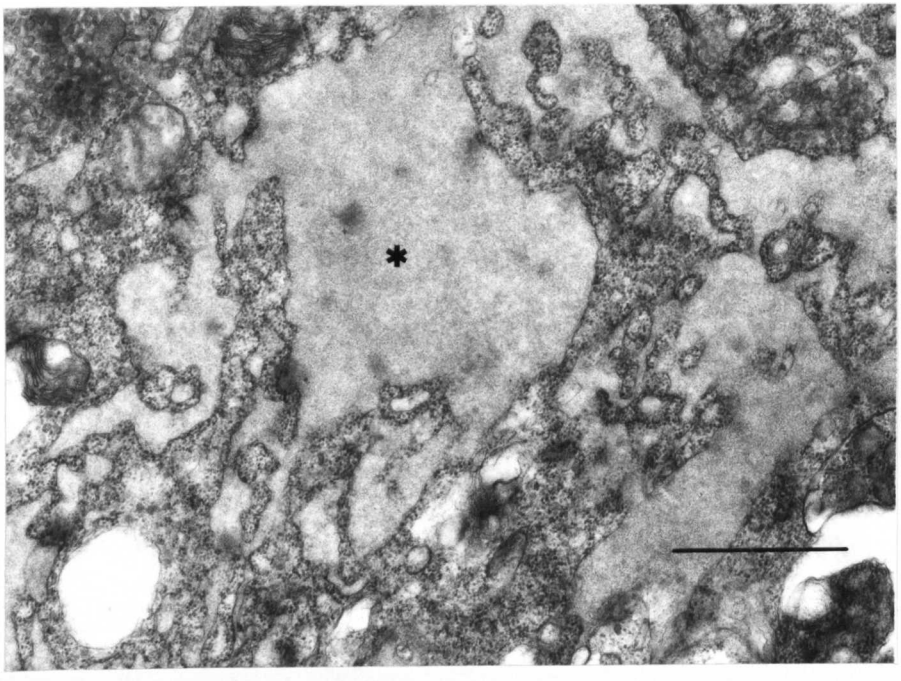


Figure 4.36 TEM micrograph of the point of fusion of the circum-oral gland ducts with the labral gland. The labral gland tissue is to the left of the dark line that runs diagonally to the bottom right of the picture. The secretory vesicles (v) and a region of the peripheral zone (*) are evident in the labral gland. Finger-like inclusions extending into the labral gland from the ducts of the circum-oral gland are clearly visible (arrowheads). A great profusion of microvillous (mi) structures are apparent in the ducts of the circum-oral glands. Scale bar = 4μm.

Figure 4.37 TEM micrograph of the cytoplasm of the circum-oral glands. Extensive regions of microvillous structures (mi) are evident in the lower right hand corner of the figure, some of which appear to contain spherical membranes (arrows) with no obvious contents. Scale bar = 5μm.



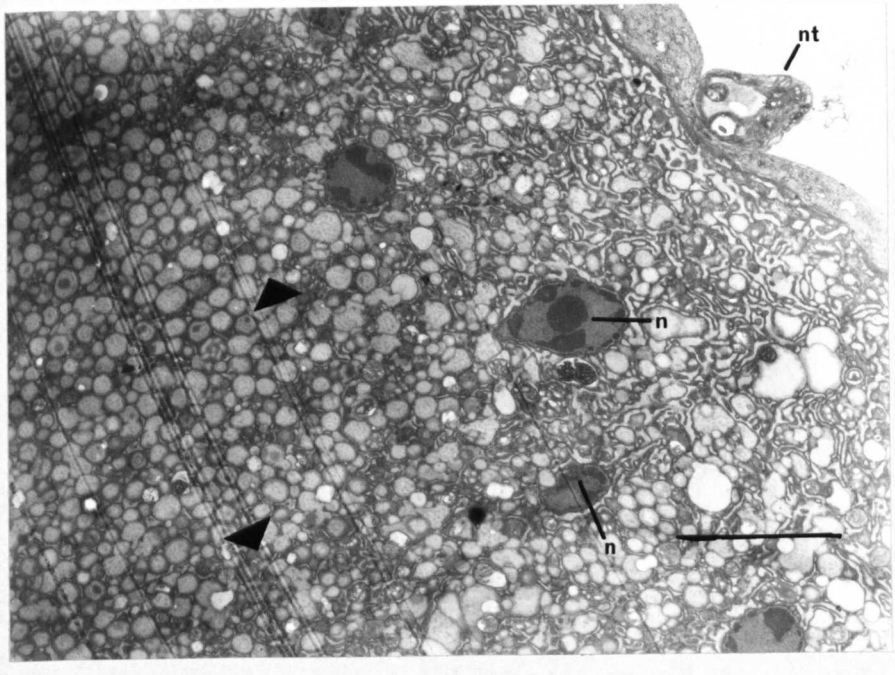
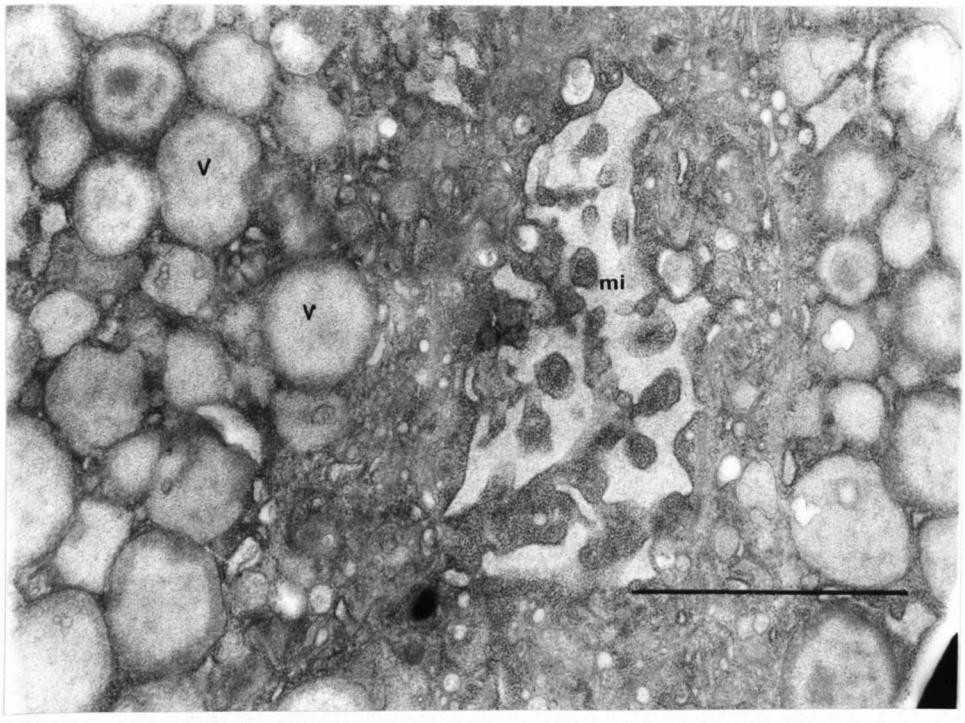


Figure 4.38 SER (*) typical of the circum-oral glands of *L. salmonis*. Scale bar = 1μm.

Figure 4.39 TEM micrograph of the basal region of the AGC of *L. salmonis*. Note the numerous nuclei (n) and prolific ER in the peripheral zone and the large numbers of secretory vesicles(arrowheads) in the central zone of accumulation. A bundle of nerve tissue (nt) is apparent at the basal margin of the tissue within the membrane that encases the syncytium. Scale bar = 5μm.



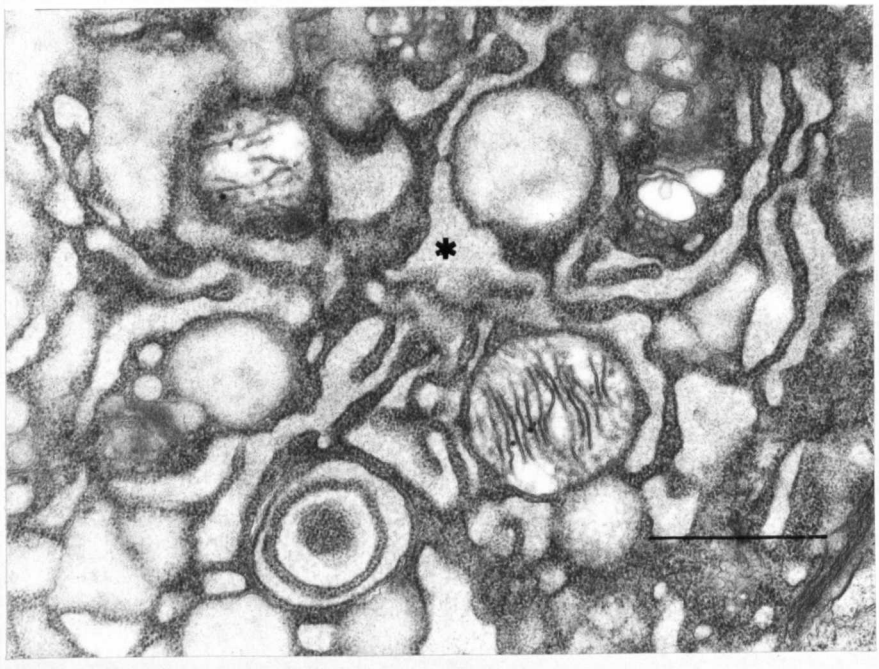


Figure 4.40 A microvillous region (mi), possibly a ductule, in the cytoplasm of the AGC with closely associated secretory vesicles (v). Scale bar = 2μm.

Figure 4.41 TEM micrograph of a typical region of the zone of production of the AGC of an adult male *L. salmonis*. The very full ER (*) of this region is clearly apparent in this image. Scale bar = 1μm.

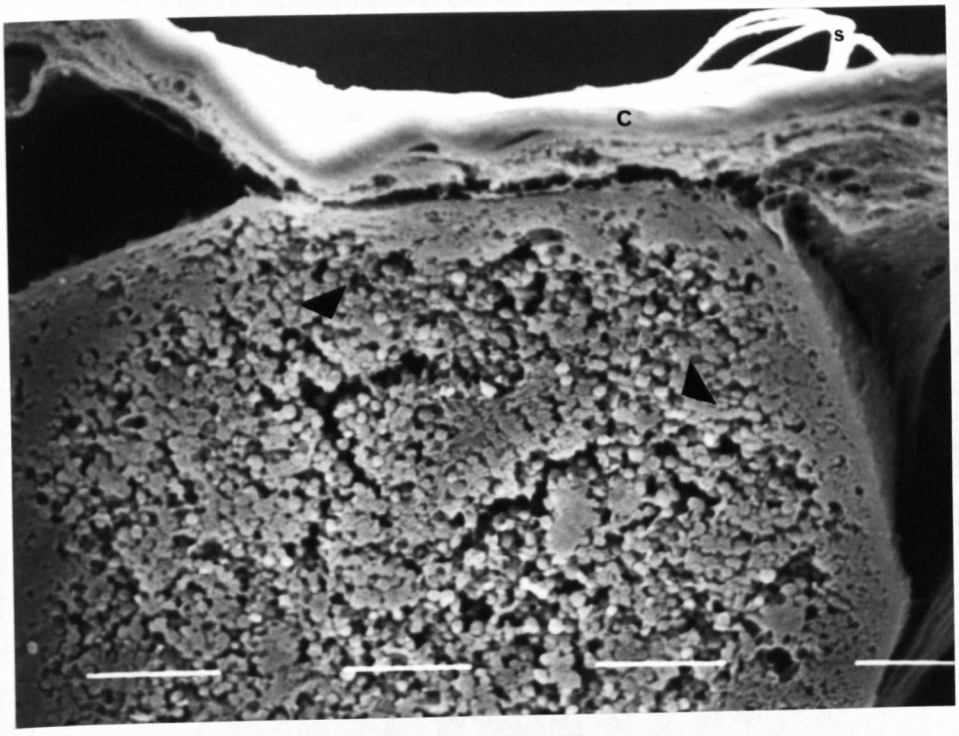


Figure 4.42 SEM micrograph of a DSG of the lateral margin of the cephalothorax of *L. salmonis*. Note the mass of vesicles (arrowheads) that comprise the bulk of the cytoplasm and the vesicle-free peripheral zone. A setule is also visible on the external cuticle (c). Scale bar = 1μm.

5. SECRETION CHARACTERISATION

5. SECRETION CHARACTERISATION

5.1 INTRODUCTION

5.1.1 Copepod enzymes

Many different enzymes have been demonstrated to be present within the tissues of many different copepod species from many different genera but research has tended to focus on their involvement in the digestion of food (Gurein and Kerambrun 1982, Mayzaud 1986, Mayzaud, Biggs and Roche-Mayzaud 1994, Kumlu 1997). Very little research has considered the possible presence of secreted copepod enzymes that may have functions not related to digestion, although in recent years, other, apparently ubiquitous copepod enzymes, such as cytochrome c oxidase have been identified in many copepod genera as their role as evolutionary markers has been utilised in population studies (Einsle 1994, Edmands and Burton 1998, Rawson, Brazeau and Burton 2000). Studies of enzymes of parasitic copepods are less common but again have largely concentrated on their roles in feeding although some reference has been made to their possible roles as virulence factors assisting in host invasion (Perkins, Haley and Rosenblatt, 1997) as has also been demonstrated in many different forms of parasites of vertebrates (McKerrow 1989, Knox, Redmond and Jones, 1993).

Little detailed research on the enzyme profile of *L. salmonis* has been undertaken although several specific enzymes have been detected by various researchers, all of which have been presumed to be involved in some mechanism related to the feeding of the animal. Ellis, Masson, Munro (1990) compared the protease enzyme profile of *L. salmonis* and *C. elongatus* (from whole animal homogenates) and found that both species possessed such enzymes although the number of different enzymes and their relative quantities were greater in *C. elongatus*. Ellis *et al.* (1990) proposed that the bulk of the protease activity detected in both species was involved in digestive processes and that the greater diversity and abundance of them in *C. elongatus* reflected its greater range of host species. Such a conclusion was also reached by Roper, Grayson, Jenkins, Hone, Wrathmell, Russell and Harris (1995) who identified three proteases and

one lipase in the epithelial cells of the midgut of *L. salmonis* and proposed that such a restricted complement of gut enzymes was a feature of a species so specifically adapted to one host fish species. Shinn (pers. comm) identified more than 30 different enzymes from *L. salmonis* including those identified by others as metabolic and digestive enzymes as well as catalase and GPX. Recent studies by Butler (pers. comm) have identified at least six enzymes in the culture media of copepodid stage larvae *L. salmonis* which are suspected of being involved with feeding although he considers the possibility that immunosuppression may also be a function of some secreted enzymes. The evidence of Chróst (1991), though. indicates that alkaline phosphatase and leucine aminopeptidase are produced and secreted in detectable quantities by bacteria as well as zooplankton and data pertaining to the detection of these enzymes must take into account production by such other means. Ross, Firth, Wang, Burka and Johnson (2000) demonstrated increased levels of hydrolytic enzymes in the epidermal mucus of Atlantic salmon infected with *L. salmonis* but failed to ascertain whether the detected enzymes were derived from exocrine gland systems of the parasite or were host-derived. The data obtained by Butler (pers. comm.) and that of Ross *et al.* (2000) suggest that such enzymes are derived from *L. salmonis*, either secondarily from the gut or directly from their exocrine glands.

5.1.2 Antioxidant enzymes

The principal reaction recognised for the DAB stain in the literature (Silveira and Hadler 1978, Angermüller and Fahimi 1981, Fredriksson *et al.* 1988, Cajaraville, Uranga and Angulo 1993, Gamble *et al.* 1995) is towards a wide range of peroxidase enzymes. TMB is also known to have specificity to a broad spectrum of peroxidase-type enzymes (Anne Brighty, pers. comm. Vector Laboratories).

Peroxidase enzymes are a class of mixed function oxidase enzymes ubiquitous in animal tissues. These enzymes are of fundamental importance to the general wellbeing of cells and are primarily involved in the protection of cells from destructive reactive oxygen species (ROS). ROS are a type of 'free radical', defined as a *'species capable of independent existence that contains one or more unpaired electrons'* (Halliwell and Gutteridge, 1985, page 11, para. 1). Such ROS arise in the tissues of aerobic

organisms as a consequence of the natural enzymatic degradation of oxygen (Wendel, 1980) in the course of normal metabolic processes. The unpaired electrons of the ROS cause them to be highly reactive and as a result they can have severe deleterious effects on the cells in which they occur. The consequences of intracellular exposure to ROS vary from protein degradation and DNA damage to the initiation of lipid peroxidation processes, all of which can result in tissue damage and cell death. The protective effects of peroxidase enzymes with respect to this lipid peroxidation has led to them frequently being described as 'anti-oxidant' enzymes (Pipe, Porte and Livingstone 1993, Bell and Smith 1994, Gamble *et al.* 1995, Hawkridge, Pipe and Brown 2000).

Many different enzymes with peroxidatic properties are found in animal tissues but two of those most commonly found are catalase (EC 1.11.1.6[1]) and selenium-dependent glutathione peroxidase (EC 1.11.1.9). Both these enzymes are responsible for the enzymatic reduction of intracellular hydrogen peroxide, albeit via slightly different chemical mechanisms. Intracellular hydrogen peroxide arises naturally in aerobic cells, as a result of organic molecules reacting with oxygen, and can damage enzymes directly via its own oxidising ability or it can react further to produce more damaging ROS species with more toxic effects.

Catalase and selenium-dependent glutathione peroxidase (Se-GPX) have been shown to be partitioned from one another within eukaryotic cells (Halliwell and Gutteridge, 1985). Catalase activity (demonstrated to be present in the cells of most aerobic organisms) is generally located in ovoid-shaped subcellular organelles (with diameters of 0.6 – 0.7μm) called peroxisomes (DeRobertis and DeRobertis, 1987). Peroxisomes are particularly prevalent in the livers and kidneys of vertebrates i.e. those tissues associated with detoxification. Cancio and Cajaraville (1997) and Pipe *et al.* (1993) have also demonstrated peroxisomes to be present in bivalve molluscs but they were not identified in the tissues of

[1] EC denotes Enzyme Commission number, an internationally recognised number appointed to an enzyme to serve as a specific identifier

four species of tunicate examined by Fredriksson *et al.* (1988) nor in two species of cnidarian examined by Hawkridge *et al.* (2000). In many tissues peroxisomes show a distinctive crystal-like core of urate oxidase that is apparent when viewed using TEM whilst other structures with similar activities, but lacking the crystalline core are also present in many tissues and are termed microperoxisomes. Catalase in peroxisomes is responsible for reducing intracellular hydrogen peroxide, produced as a consequence of oxidation of organic molecules, to water and oxygen. The established view is that in vertebrates peroxisomal catalase is synthesized in the cytosol on free ribosomes and from there is incorporated into peroxisomes (Wolfe, 1993). Between its synthesis and its translocation into peroxisomes catalase present in the cytosol takes the form of an apoenzyme i.e. an inactive precursor of the active form (holoenzyme) that only exists after its entry into the peroxisome. In vertebrates, catalase from peroxisomes is known also to be involved in the β-oxidation of fatty acids to produce acetyl-coenzymeA for anabolic reactions but no similar pathways have been demonstrated in invertebrates (DeRobertis and DeRobertis, 1987). Enzymes located in peroxisomes have also been shown to be important in the biosynthesis of phospholipids for incorporation into cellular membranes (DeRobertis and DeRobertis, 1987). As a consequence of its subcellular compartmentalisation free catalase in the cytosol of tissues has not been widely reported, although Bulitta, Ganea, Fahimi and Voelkl (1996) reported the presence of a cytoplasmic catalase, separate from the peroxisomal enzyme, in the liver of the guinea pig. There is a marked lack of information regarding the subcellular distribution of antioxidant enzymes of copepods, but their distribution has been described in other marine invertebrate taxa. Hawkridge *et al.* (2000) located subcellular sites of catalase and glutathione peroxidase in the tissues of the cnidarians *Anemonia viridis* (Forskal) and *Goniopora stokesi* (Edwards and Haime) and concluded that those sites did not conform to the recognised structure of peroxisomes described in vertebrates and plants. Bell and Smith (1994) identified catalase and glutathione peroxidase in the haemolymph and haemocytes of the green crab *Carcinus maenas* (L.).

The other common peroxidase is selenium-dependent glutathione peroxidase (Se-GPX) which reduces hydrogen peroxide to water or alcohols at the expense of the substrate glutathione, a compound

found in most tissues. Se-GPX is composed of four subunits that each contains one atom of selenium at its active site and many of the symptoms of selenium deficiency in vertebrates can be accounted for by a decreased Se-GPX activity (Taylor, 1997). The enzyme is primarily found free in the cell cytosol, in contrast to catalase, although some activity has also been demonstrated to occur in mitochondria (Halliwell and Gutteridge, 1985) and essentially none is found in the peroxisomes (Wendel, 1980). This enzyme is found in high levels in the livers of vertebrates because of its role in detoxification processes, (and has been demonstrated to be present in many other taxa such as plants and fungi) but it is also present at much lower levels in many other tissues. Levels of the enzyme, however, vary from tissue to tissue according to species and, in the few studies of marine invertebrates where Se-GPX activity has been measured, it has been detected at high levels in the digestive tissues (in contrast to respiratory or muscular tissues) where it is proposed to counteract the effects of ROS formed in the gut (Gamble *et al.* 1995, Cancio and Cajaraville 1997). Se-GPX utilises the thiol compound glutathione as its substrate as it catalyses the oxidation of the former to glutathione disulphide (Halliwell and Gutteridge, 1985). This is undertaken at the expense of hydrogen peroxide and releases water as its product, thereby removing the toxic hydrogen peroxide from the system.

Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids which, in the absence of suitable or sufficient enzymes to prevent peroxidation, can result in cell membranes becoming damaged (Wendel 1980, Mannervik 1985). The overall effects of peroxidation are decreased membrane fluidity and an increased 'leakiness' of membranes, which ultimately results in the loss of membrane integrity. Peroxidase enzymes serve to protect animal cells from lipid peroxidation processes by disposing of the free radicals (ROS) that initiate such processes.

A further type of glutathione peroxidase that does not depend on selenium for its activity has also shown to be present in animal tissues. The activity of this non-selenium-dependent form of the enzyme is frequently attributed to the actions of glutathione-S-transferases (EC 2.5.1.18) (Halliwell and Gutteridge 1985, Gamble *et al.* 1995). Studies have revealed that this non-selenium-dependent glutathione peroxidase is not active with hydrogen peroxide but is instead active with inorganic cumene

hydroperoxide. This activity with artificial substrates reflects the role of these enzymes in detoxifying 'foreign' matter by conjugating the foreign molecules to intracellular glutathione (Halliwell and Gutteridge, 1985). Glutathione-S-transferase (GST) is a widely abundant 'housekeeping' enzyme in animal tissues (Gamble *et al.* 1995, Cervi, Rossi and Masih 1999) where it is involved in the detoxification and excretion of physiological and xenobiotic substances. Like Se-GPX, GST activity is primarily located in the cytosol of cells with smaller amounts being present in mitochondria. GST exerts its cytoprotective effects by catalsying the addition of reduced glutathione to foreign, or damaging, organic compounds. The resultant conjugates are more readily soluble in water, a property that facilitates their elimination (Wilce and Parker, 1994).

To assess total GPX activity therefore, both organic and inorganic hydroperoxides must be used as substrates. Such distinction between the two forms of the enzyme is necessary, as the relative activities of both types of GPX are known to vary between tissues.

Information regarding the presence and function of antioxidant enzymes in invertebrates is relatively sparse and our understanding of these enzymes in these species is correspondingly deficient. Recent studies in this area have concentrated on the possible significance of parasite antioxidant enzymes in host immunosuppression. These studies, such as those by Cervi *et al.* (1999) on *Fasciola hepatica* and Vibanco-Perez, Jimenez, Merchant and Landa (1999) on *Taenia solium* (L.) quantified the activities of selected antioxidant enzymes and, although the use of specific antibodies in one study (Vibanco-Perez *et al.* 1999) showed the localisation of the enzymes within tissue sections, no evidence was provided to suggest that specific gland tissues were secreting the enzymes. Both of these studies concluded that antioxidant enzymes, in particular GST, were of significance in reducing host immune responses to parasitic infection by detoxifying the products of lipid peroxidation caused by free radicals produced as a result of immune response processes. Recent investigations by Sudararaju and Sundara Babu (1999) have demonstrated the presence of peroxidase enzymes (catalase and glutathione peroxidase) in the salivary glands of the neem mosquito bug *Helopeltis antonii* Signoret where they were suggested to function to prevent the formation of harmful ROS resulting from tissue damage as a consequence of their feeding

habits. Such a function is similar to that proposed by Felton and Duffey (1991) for the gut and salivary catalase of lepidopteran larvae feeding on plant tissues. According to their results the digestive fluids of the salivary glands and midgut of lepidopteran larvae contain substantial catalase activity that is vital in combating the hydrogen peroxide and other ROS released from damaged plant tissues, thereby preventing peroxidative damage to the lumen of the gut.

Most studies on antioxidant enzymes of marine invertebrates have focused on the role such enzymes play in digestive detoxification, as in studies of molluscs by Gamble *et al.* (1995) and Cancio and Cajaraville (1997) and cnidarians by Hawkridge *et al.* (2000). Bell and Smith (1994) considered the role such enzymes play in protecting host tissues of decapods from free radicals generated as a consequence of haemocytic phagocytosis of invasive organisms or particles. Another proposed function for peroxidase enzymes in invertebrates was suggested by Fredriksson *et al.* (1988) where they were reported to be present in the endostyle of marine tunicates and served to catalyse the binding of environmental iodine for intracellular use as an endocrine hormone. No previous studies have suggested that copepods possess a thyroid homologue that is involved in secreting hormones to mediate cellular processes.

5.1.3 Prostaglandins

The evidence found in the present study (Chapters 3 and 4), which indicated that both peroxidase enzymes and lipids were exocrine gland components of *L. salmonis*, indicate that the exocrine glands identified may be producing prostaglandins (which are a form of 'eicosanoid', or C20 fatty acid derivative (Gurr and Harwood, 1991)). The pathway of synthesis and physiological role of prostaglandins was discussed in Chapter 3 of this study and will only be briefly described here. An enzyme with peroxidatic activities (prostaglandin endoperoxide synthetase) is known to be essential in the oxygenation of polyunsaturated fatty acids (usually arachidonic acid) to form prostaglandins (Nutgeren and Hazelhof 1973, Gurr and Harwood 1991, Morita *et al.* 1995, Rowley *et al.* 1995, Bowman, Dillwith and Sauer 1996). Prostaglandins have been identified as components of salivary gland secretions in some parasitic arthropods where they are implicated in many roles including down-modulation of host immune response,

anti-coagulation of blood platelets and analgesia (Belley and Chadee 1995, Bowman *et al.* 1996) that all serve to facilitate feeding by the parasite. In a review of prostaglandins of invertebrates Stanley and Howard (1998, page 370, paragraph 5) proposed that such chemicals are likely to be present in '*most, if not all animals*'. Chemicals active in such a way would obviously be beneficial to parasites with a prolonged host association such as *L. salmonis*. In particular, the prostaglandin E_2 has been identified in the lone star tick, *Amblyomma americanum* (Ribeiro, Evans MacSwain and Sauer, 1992), the American deer tick, *Ixodes dammini* (Ribeiro, Makoul, Levine, Robinson and Spielman, 1985) and the cattle tick *Boophilus microplus* (Higgs, Vane, Hart, Potter and Wilson, 1976) as well as in many mammalian species and has been described as a 'key immune modulator' by Rowley *et al.* (1995) in their study of prostaglandins in fish immune modulation.

5.1.4 Lipid composition of *L. salmonis*

The histochemical analysis in Chapter 4 identified lipid material in the exocrine glands of *L. salmonis*. Such lipids may serve as the precursor for prostaglandin synthesis, may be part of the secretion proper, or alternatively may be present only in the membrane lipids that are likely to be highly abundant in such tissues with their proliferation of ER and Golgi complexes. Determination of the types of lipids present and their relative abundances can be used to infer functions for those lipids. Analysis of the lipid content of pelagic copepods has been extensively documented (Lee, Nevenzel and Paffenhöfer 1971, Kayama and Mankura 1980, Sargent and Henderson, 1986) whilst the lipid profile of *L. salmonis* has also been previously determined by Lee (1975). These studies of lipid content, though, concentrated solely on the total lipid composition of whole copepods and made no attempt to isolate different tissues for comparative analyses.

Lipids in pelagic marine copepods serve as energy reserves and provide buoyancy, whilst ultimately providing the major metabolic fuels to be utilised by the rest of the marine food chain (Lee *et al.* 1971). The total lipid content of high latitude copepods can vary widely with season reflecting the relative abundance of food Mauchline (1998) and Sargent and Henderson (1986) describe how the total

lipid content of calanoid copepods can range from 3 - 74% of the dry body weight, with wax esters accounting for between 9 – 92% and triacylglycerols 0 – 30% of the total. The sequestration of wax esters is assumed to be a feature of herbivorous species where they are generally believed to function as long-term reserves whilst triacylglycerols function as readily utilisable resources. Lee (1975) provides the only account of the lipid composition of *L. salmonis*, as well as three other species of parasitic copepod. The results of the research of Lee (1975) indicated that the storage lipids of parasitic copepods species differed from that of free-living species in that triacylglycerols predominated in the parasitic species whilst the stored energy of free-living species were mainly present as wax esters (Lee *et al.*, 1971). The percentage of lipid (as dry weight) was similar in all four species examined by that author, consisting of high levels of triacylglycerols and phospholipids with only traces of wax ester recorded. The pattern of lipid composition in the parasitic copepods strongly accorded with the lipid composition of the skin of the fish hosts indicating that dietary lipids were assimilated with little modification.

Waxes secreted onto the cuticles of terrestrial insects have been characterised for many species and their functional significance is widely recognised (Waku and Foldi, 1984). Such chemicals serve to protect insects from desiccation, prevent abrasive damage, provide protection from predators and parasites and may also allow the passage across the cuticle of essential lipid-soluble substances. Insect cuticular waxes are secreted both by general epidermal cells responsible for secreting the cuticle but also by specialised gland structures. The cuticular waxes of insects are often complex mixtures of true waxes and other substances such as lipids (Waku and Foldi, 1984). Secreted external lipid or wax layers in copepods could potentially have some effect on the hydrodynamic characteristics of the animal in question although their presence has never been demonstrated. Poorly defined external layers, frequently termed 'fuzzy layers' have been identified on the external surface of the cuticles of some species of copepod (Briggs 1978, Gotto and Threadgold 1980, Bresciani 1986, Hipeau-Jacquotte 1987, Bron, Shinn and Sommerville 2000) but their composition and functions are poorly understood. In the cases where histochemical tests have been performed, the fuzzy layer has been shown to consist of mucoid components (Briggs 1978, Hipeau-Jacquotte 1987), not waxes or lipids.

5.2 OBJECTIVES

The negative response of the exocrine gland tissues of *L. salmonis* to the Alcian Blue stain in this study suggests that peroxidase enzymes, of an unspecified nature, and not sulphated mucous substances are causing the positive reaction with the DAB and TMB chromogens. The work presented in this chapter therefore aimed to specifically identify the reactive components of those glandular tissues of *L. salmonis* that were giving the positive response to the DAB and TMB stains. Several different techniques to identify peroxidase class enzymes were utilised. These techniques varied in their sensitivity and applicability to different tissue preparations and were used on whole specimens, gland tissue homogenate and gland tissues at an ultrastructural level.

The possible presence of prostaglandin E_2, was investigated in the tissues and glands of *L. salmonis*. PGE_2 was chosen because it was the prostaglandin that appeared to be most prevalent of the prostaglandins described in haematophagous parasitic arthropods.

The lipid profile of gland and non-glandular tissues was also characterised and it was hoped that analysis of the results would reveal whether the lipid material identified in Chapter 4 was destined for secretion or was merely a component of the gland matrix.

The objectives of the following chapter were realised using the following techniques:

- Non-specific blocking trials utilising DAB
- Specific photometric enzyme assays
- Selective DAB staining utilising TEM
- Immunohistochemical detection of PGE_2
- High performance thin layer chromatography

5.3 MATERIALS AND METHODS

5.3.1 Non-specific enzyme blocking

An experiment was designed which involved blocking endogenous peroxidase enzymes of *L. salmonis* prior to staining with DAB in order to indicate whether peroxidase enzymes caused the positive reaction observed with this stain. Hydrogen peroxide was utilised as the substrate to block the activity of endogenous peroxidase enzymes. Only adult male *L. salmonis* were used in this study as the presumption was made that secretions of identified glands would have the same composition in both males and females.

Three different treatments were used to establish whether blocking the activity of endogenous peroxidase enzymes would result in a negative response with the previously demonstrated DAB staining technique as applied to whole, fresh specimens.

One group of 20 adult male *L. salmonis* was immersed in a 10% v/v solution of fresh hydrogen peroxide in 100% methanol for 10min before being stained with DAB according to the protocol given in Chapter 3. A second group was immersed in 100% methanol only for 10min before being stained with DAB whilst a third set of lice, serving as positive controls, were killed with a scalpel blade by severing them immediately posterior to the protocerebrum and main ganglion. These lice were immersed in seawater for 10min prior to staining with DAB. All three sets of lice were incubated in identically prepared staining media for approx. 12h. The stain incubation was terminated by the addition of 70% ethanol following removal of the staining solution.

5.3.2 Specific enzyme assays

Assays for catalase, Se-dependent and Se-independent glutathione peroxidase and glutathione S-transferase were performed on selected tissues of *L. salmonis*.

Urosomal gland regions (urosomes severed transversely immediately anterior to the urosomal glands), Anterior Gland Complexes and 'Gland-Free' postero-lateral cephalothorax margins (**Figure 5.1**)

were dissected from freshly acquired adult female *L. salmonis*. Lice had been maintained in clean seawater for approximately 24h prior to processing in order to reduce the risk of host contamination. Adult females were chosen for this experiment as their larger cephalothorax size relative to males meant that it was more feasible to remove regions of the cephalothorax which contained few glands. The smaller size of the male cephalothorax precluded this. The excised regions were dissected on ice into 1.5ml Eppendorf tubes (50 lice per sample) and buffered in a volume approximately five times that of tissue using 50mM phosphate buffer. Samples were stored at -70°C until required with the exception of those samples prepared for CAT analysis which were prepared immediately prior to use to minimise any denaturation of the enzymes. This precaution was taken as catalase is known to readily dissociate into its constituent subunits upon storage or freezing, resulting in a loss of enzymic activity (Halliwell and Gutteridge, 1985).

Samples were homogenised using sterile, disposable plastic homogenisers and centrifuged (IEC Micromax OM3590) at 4°C at 18,894RCF for 10min. The protein content of each sample was determined using the Folin protein assay (Lowry, 1951) (Bio-Rad Protein Assay, 500-0002) to enable the calculation of enzymatic activity relative to protein content.

Assays were carried out using a Novikon 940 dual beam spectrophotometer (specific details for each enzyme can be found in Appendix 1) and the resultant absorbance curve printed out. Each sample was read three times to obtain an average reading and to accommodate slight variances between readings. Enzymatic activity of each sample was calculated as μmoles min^{-1} mg^{-1} of protein (**Appendix 1**).

5.3.3 Selective DAB staining utilising TEM

Previous researchers (Angermüller and Fahimi 1981) utilised the peroxidatic staining qualities of DAB to selectively demonstrate the presence of different enzymes such as catalase and GPX in a variety of different tissues. Although the success of these techniques was subject to considerable variation, depending on the tissue under investigation, the fixation process and incubation time used, it was hoped that the approximate technique would give satisfactory results without having to be substantially altered.

The method of Angermüller and Fahimi (1981) was followed. The urosomes ten adult male *L. salmonis* were dissected out and fixed immediately for 5min in a modified Karnovsky's fixative (1% glutaraldehyde, 4% paraformaldehyde in 0.1M sodium cacodylate buffer (pH 7.2)). Following fixation, dissected regions were subjected to one of two different staining regimens. To detect catalase, samples were transferred, after fixation, to a Tris-HCl solution at pH10.4 containing 5mM DAB and H_2O_2 (0.15%). Ten urosomal regions were incubated in this solution at room temperature for 30min. Several samples were treated in the same manner but omitting the H_2O_2 substrate to serve as a negative control. To detect GPX a staining solution of Tris-HCl buffer at pH7.2 containing 2.5mM DAB and 0.02% H_2O_2 was used. Samples were incubated at room temperature for 10min and 20 min. A negative control, identical except for the omission of H_2O_2, was also run and sampled after 10min. Following incubation, the specimens were rinsed briefly in buffer before osmication in 1% OsO_4 in borax for 1.5h.

Following osmication, samples were processed for TEM (as described in Chapter 2) and embedded in Spurrs resin. Gold sections were counterstained for 1min in lead citrate only and examined at 80kV with a Philips 301 transmission electron microscope.

5.3.4 Prostaglandin E_2 immunohistochemistry

Anti-Prostaglandin E_2 polyclonal antibody (Sigma P5164) was used to identify sites in sea louse tissues where PGE_2 was present. Whole adult male *L. salmonis* were used as the test tissues due to their combined attributes of small size for ease of sectioning and because of the increased likelihood of encountering glandular tissue in any one section. Specimens were fixed in either Stefaninis (Stefanini, De Martino and Zamboni, 1967) or Bouins (Bancroft and Stevens, 1990) fixative and embedded in plastic embedding medium compatible with immunohistochemistry (Historesin Plus, Leica) prior to sectioning at 4μm on a rotary microtome. Historesin Plus was deemed suitable for this purpose as it was alleged to maintain antigen sites during processing thereby making it suitable for immunohistochemistry. A standard Peroxidase-Anti-Peroxidase immunohistochemistry protocol for antigen location was followed (**Appendix 2**). Dilutions of the Anti-PGE_2 antibody ranging from 1:50 - 1:1000 were tested against sea

louse tissues. A negative control was also run to provide an estimation of background staining. The negative control consisted of PBS without the antibody component at the appropriate incubation step.

5.3.5 HPTLC of glandular tissue

High performance thin-layer chromatography (HPTLC) was utilised to determine the full profile of the lipid material suspected of being a component of the glandular secretion (Section 4.4.1.4). Samples of AGC, non-glandular material and the urosomal gland region (as defined in Section 5.2.2), taken from thirty freshly acquired adult female *L. salmonis*, were stored in 50mM phosphate buffer and maintained at -70°C until required. This sampling process was repeated three times to provide replicate samples. Lipids were extracted using the method of Folch, Lees and Stanley (1957).

HPTLC analyses for both the polar and non-polar (neutral) fractions of the sample lipids were carried out using the method of Olsen and Henderson (1989). Polar lipids are those lipids with an amphipathic structure typical of lipids of biological membranes whilst non-polar (neutral) lipids are those that do not possess an amphipathic structure (possessing both hydrophobic and hydrophilic moieties), typified by triglycerides and waxes. Extracted lipids were weighed and redissolved in an appropriate volume of 2:1 solution of chloroform : methanol to give a final sample weight : volume ratio of approx. 25µg / 25µl. HPTLC plates (10 x 10cm) were predeveloped in chloroform to remove impurities and then air-dried. Samples for non-polar lipid analysis were each added (10µl per sample) to separate 5mm long origins at the base of each plate. The three replicates of each tissue type were run together on a single plate. A halibut liver lipid sample was run on each plate to serve as a standard. Non-polar lipids developed for 8cm using isohexane : diethyl ether : glacial acetic acid (85 : 15 : 1by volume). Plates for polar lipid analysis were prepared in the same manner but instead were developed for 8cm using methyl acetate : isopropanol : chloroform : methanol : 0.25% potassium chloride (25 : 25 : 25 : 10 : 9 by volume). Both polar and non-polar lipids were detected by spraying the plate with 3% cupric acetate in 8% phosphoric acid followed by charring at 160°C for 20min. Densitometry analysis of the resolved lipids

was undertaken using a Shimadzu Dual-Wavelength Flying-Spot Scanner CS-9000 at 370nm linked to a Shimadzu data recorder.

To detect wax esters a second set of non-polar lipid fractions were stained instead with 0.1% 2,7 dichlorofluorescein in methanol then charred as before and viewed under U.V light.

5.3.6 Statistical Analyses

The mean and standard error of the enzyme activities were calculated and, to compare if the mean enzyme activities differed between tissues, a single factor analysis of variance test (ANOVA) was used. ANOVA tests whether the difference between sample means is too great to be explained by the random error of the measurement. The ratio of the between group variance and the within group variation is given the symbol F, if the value of $F_{experimental}$ falls below the critical value for F (as indicated in standard statistical tables) then the data indicate that the difference between groups are too small to be recognised against variation of measurements within groups. If significant differences were detected using ANOVA Tukey-Kramer Multiple Comparison tests were employed to identify where the differences lay between the treatments.

ANOVA was also used to determine whether the differences between the mean values of identified lipid types were greater than would be expected by random errors. Significant differences were tested further using the Tukey-Kramer tests.

5.4 RESULTS

5.4.1 Non-specific enzyme blocking

A definite pattern of staining was consistent in all specimens in each of the three experimental treatments. These patterns of staining were markedly different between each of the three treatments. Lice immersed in hydrogen peroxide prior to DAB staining (Specimen C, **Figure 5.2**) showed almost none of the characteristic staining seen in the staining trial in Chapter 3 and in Specimen B of **Figure 5.2**. Some very slight positive responses to the stain were observed in Specimen C in locations that corresponded to some parts of the AGC and the FGC as well as in some of the DSG of the cephalothorax, genital segment and abdomen. This staining was markedly less intense than in those lice which had been stained following the normal protocol (Specimen B, **Figure 5.2**) following immersion in seawater and which displayed all the characteristic DAB staining demonstrated for this stage in Chapter 3.

Those lice that had been immersed in methanol only prior to staining (Specimen A, **Figure 5.2**) showed considerable staining, comparable to that of lice that had been stained in accordance with the normal protocol (Specimen B), but also showed some staining that was peculiar to them and that did not occur in the control lice. The most obvious staining difference between specimens A and B (**Figure 5.2**) was the strong DAB-positive staining of the midgut that is clearly apparent in methanol-only treated Specimen A as a dark streak, extending from the region of the oesophagus to the junction of the posterior mid-gut and the hind gut. Such staining was evident in all specimens treated with methanol only prior to DAB staining. The FGC, AGC, the urosomal glands and some of the DSG also stained positively in methanol-only treated lice but not as intensely as in those lice that were stained with DAB after immersion in seawater only (Specimen B, **Figure 5.2**). The stained glands of methanol-only treated lice however appeared as less distinct structures than those of the control specimens. The boundaries of the glands were fuzzy and the intensity of their staining was also generally less than that of the controls.

5.4.2 Enzyme assays

Catalase activity was detected in all three different tissue types examined and when these activities were calculated relative to the total amount of protein in the sample (activity min^{-1} mg^{-1}) clear differences between the tissues became apparent (**Table 5.1** and **Figure 5.3**). The tissue of the urosomal region had a significantly higher (more than double) catalase activity than the AGC (Tukey-Kramer, q-value = 14.6, $p < 0.001$) and was also significantly higher (more than triple) than the activity of the non-glandular tissue (Tukey-Kramer, q-value = 18.7, $p < 0.001$).

Table 5.1 Catalase activities of different *L. salmonis* tissues..

Tissue type	Uncorrected activity (μmoles min^{-1} μl^{-1})	Protein content (mg ml^{-1})	Activity (μmoles min^{-1} mg^{-1})
AGC	29	10	2.9 (±0.35)
Non-gland	28	15	1.9 (±0.23)
Urosomal region	45	7	6.4 (±0)

Values of activity are given as means of three replicates with standard errors in brackets.

The negative control 'non-gland' tissue also possessed a measurable catalase activity although no significant differences in catalase activity were detected between the AGC and the negative control tissue (Tukey-Kramer, q-value = 4.13, $p > 0.05$).

Glutathione peroxidase activity was also recorded in both glandular and 'non-glandular' tissues but at very much lower levels than the demonstrated catalase activity. This activity when expressed relative to protein content (**Table 5.2, Figure 5.4**) showed there to be less marked differences in activity between tissues with respect to Se-GPX activity than was apparent in the catalase activities of the three tissues. The AGC and the non-gland tissue displayed similarly low levels of Se-GPX activity, with no significant differences being apparent between them (Tukey-Kramer, q = 0.48, $p > 0.05$) whilst the tissue of the urosomal region displayed an activity almost twice as great as that of the other tissues. The Se-

GPX activity of the urosomal gland tissue was significantly different from both the AGC tissue and the non-glandular control ($q = 6.86$, $p < 0.01$; $q = 6.37$, $p < 0.01$ respectively).

Table 5.2 Selenium-dependent glutathione peroxidase activities of different *L. salmonis* tissues.

Tissue type	Uncorrected activity (μmoles min^{-1} μl^{-1})	Protein content (mg ml^{-1})	Activity (μmoles min^{-1} mg^{-1})
AGC	7.5	18	0.43 (±0.004)
Non-gland	7.8	18	0.45 (±0.03)
Urosomal region	9.6	14	0.71 (±0.05)

Values of activity are given as mean values of three replicates with standard errors in brackets.

Cumene hydroperoxide, used as a substrate to detect non-selenium-dependent glutathione peroxidase, failed to reveal any measurable activity in any of the three tissue types analysed and therefore a specific assay for the detection of GST was performed. This assay technique (**Appendix 1**) utilised the substrate 1-chloro-2,4-dinitrobenzene (CDNB) and was assessed to be a more sensitive technique than the use of cumene hydroperoxide for the detection of non-selenium-dependent glutathione peroxidase activity as typified by GST (Habig and Jakoby, 1981). The results of the assay are displayed in **Table 5.3** and represent the total sum of non-selenium-dependent glutathione peroxidase activities in the tissues assayed. The data displayed in **Table 5.3** is displayed graphically in **Figure 5.5**.

Table 5.3 Glutathione S-transferase activities of different *L. salmonis* tissues.

Tissue type	Uncorrected activity (nmoles min^{-1} μl^{-1})	Protein content (mg ml^{-1})	Activity (nmoles min^{-1} mg^{-1})
AGC	79	14	5.83 (±0.33)
Non-gland	109	22	4.93 (±0.15)
Urosomal region	81	15	5.44 (±1.3)

Values of activity are given as mean values of three replicates with standard errors in brackets.

Use of the CDNB substrate did reveal that a very small amount of GST activity was present, albeit at similar levels, in all three tissues analysed. ANOVA indicated that there were no significant differences ($F = 0.492$, $p = 0.644$) between the GST activities of any of the three tissues.

Activities of GST in all three tissue types were only measurable at the nanomolar level and as such were active at levels approximately 1% and 0.1% of Se-GPX and catalase respectively.

5.4.3 Selective DAB staining utilising TEM

Only gland D of the urosomal gland complex (as defined in Section 4.4.5.5) was examined using TEM. The staining technique successfully resolved the presence of catalase although, ultrastructural details were poor with respect to the standard of fixation. Sections of gland tissue incubated for 30min to demonstrate the presence of catalase (**Figures 5.6, 5.7**) showed that the flocculent material inside the secretory vesicles of gland D had stained very darkly with DAB whilst none of the cell organelles (mitochondria, ER, Golgi's) showed a positive reaction in any sections examined. No components of the cytosol showed any staining positive with DAB. Staining of vesicle contents was distinct from the background staining of tissues achieved using lead citrate as a background stain. Sections of control tissue incubated in the same medium did not show any comparable staining suggesting that the incubation was successful and that catalase was present within the secretory vesicle contents but nowhere else in the gland, at least at the limit of resolution of this technique.

Sections of gland D incubated to stain GPX (staining only highlights areas of Se-GPX activity on account of the use of H_2O_2) showed none of the staining of the vesicle contents evident in the catalase treatment, although some staining was apparent in some regions of the tissue as is evident in **Figure 5.8**. This staining was restricted to small granular inclusions, which were abundant in the cytosol of the gland tissue (**Figure 5.9**), and stood out above the background lead citrate staining. These stained granular inclusions varied very slightly in size but typically were approx. 20nm in diameter which places them in the correct size range of ribosomes. The granular inclusions stained very darkly, and selectively, indicating that it was a positive response to the DAB treatment being observed and not staining caused by

the lead citrate. Control tissues for both the catalase and glutathione peroxidase trials showed no staining detectable above the background lead citrate staining in any section examined.

5.4.4 Prostaglandin E_2 immunohistochemistry

The peroxidase-anti-peroxidase immunohistochemical protocol utilised in this trial indicated that PGE_2 is not present in any tissues of *L. salmonis*. No positive staining was observed in any section at any of the antibody concentrations tested. Some general background staining was observed on the slides, but none of this was specifically located in any of the tissues. This background staining was also evident in the negative control tissues and can therefore be deemed artifactual. The background staining appeared to be located in a film of material, which surrounded the entire tissue section, and probably represented serum residue that remained after washing with PBS.

5.4.5 HPTLC of glandular material

5.4.5.1 Analysis of non-polar lipids in sea louse tissues

HPTLC analysis revealed there to be no apparent differences in the lipid profiles of the material from the urosomal region, the AGC and the non-gland tissue control sample. Initial analysis of the densitometry data shown in **Figure 5.10** revealed that polar lipids constituted almost 60% of the total lipid content of these tissues. The relative proportions of the remaining non-polar lipid fractions of these tissues are summarised in **Table 5.4,** which also includes the whole polar fraction. All figures and tabulated data pertain to tissues of the AGC only as data from the non-gland and the urosomal gland tissues was indistinguishable from the lipid data of the AGC.

The non-polar fractions of each sample tissue were dominated by cholesterol, with consistently high levels of that lipid (approx. 20%) occurring in all three tissue types. The same was true for FFA levels, which were present in all tissues at a level of approximately 10%. A consistent level of unknown neutral (non-polar) lipid (approx. 6%) was also present within each tissue type. No triacylglycerols were detected in the non-polar fraction of the lipids of the samples examined. **Table 5.4** presents the mean

values derived from the three replicates of each of the three separate samples analysed. The lipid profiles obtained from replicates of the same sample were surprisingly consistent and one way ANOVA tests indicated that no significant differences were apparent between any of the tissues with respect to the levels of different lipids detected.

Table 5.4 Total lipid (including non-polar breakdown) profile of *L. salmonis* AGC tissues.

	AGC	Non-gland	Urosomal region
Total Polar (%)	64.60 (±0.29)	60.40 (±3.3)	56.30 (±2.0)
Unknown (%)	5.80 (±0.87)	7.80 (±1.17)	6.50 (±0.29)
Cholesterol (%)	21.90 (±0.58)	23.50 (±1.05)	23.20 (±0.14)
FFA (%)	7.80 (±0.53)	8.20 (±1.34)	12.30 (±1.44)

FFA free fatty acid. Data are mean values of three replicates (thirty lice per sample) and include standard errors in brackets. Data corrected to nearest tenth percentile.

5.4.5.2 Analysis of polar lipids present in sea louse tissues

Analysis of the polar lipids present in the samples (displayed in **Figure 5.11**) revealed there to be several lipid classes present in each type of tissue but none of the three tissues analysed showing a profile distinguishable from that of the other tissues analysed. This data is summarised in **Table 5.5**.

Table 5.5 displays mean data derived from the three replicates of each tissue sample. High levels of phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylethanolamine (PE) were present in all three tissues.

Phosphatidylcholine represented the greatest proportion of the polar lipids present in the tissues analysed, being present at an approximate 35% level in all cases. Phosphatidylserine (PS) and phosphatidylinositol were present as one peak on the densitometry data (**Figure 5.11**) averaging around 10% of the total polar lipid content of all three tissue types. Sphingolipid constituted the smallest fraction of the polar lipids of all tissues being consistently present at a level between 3 – 6%. In conclusion, no differences were detectable between the three tissue types in terms of either lipid class present or levels of lipids in each class. Results were markedly similar between replicates of each tissue with significant

differences being evident only between the percentage composition of the PI and PS component of the control, non-glandular tissue, and the urosomal tissue (Tukey-Kramer, $q = 4.603$, $p < 0.1$). No significant differences were detected between any other lipid values.

Table 5.5 Polar lipid profile of *L. salmonis* AGC tissues.

	AGC	Non-gland	Urosomal region
Glycolipids (%)	3.00	nd	1.20
SP (%)	3.60 (±0.37)	3.90 (±1.16)	6.30 (±1.05)
PC (%)	37.80 (±0.43)	35.60 (±1.22)	35.00 (±0.72)
PS + PI (%)	9.80 (±0.37)	7.50 (±0.88)	10.20 (±0.31)
PG + CL (%)	19.70 (±0.35)	20.70 (±1.74)	21.60 (±1.18)
PE (%)	26.00 (±1.2)	25.40 (±0.7)	24.00 (±0.32)

SP sphingolipid; PC phosphatidylcholine; PS phosphatidylserine; PI phosphatidylinositol; PG phosphatidylglycerol; CL cardiolipin; PE phosphatidylethanolamine. nd – none detected. Data are mean values of three replicates (thirty lice per sample) and include standard errors in brackets.

5.5 DISCUSSION

The data obtained in this chapter has revealed much useful information about the secretions of the DAB-staining exocrine glands of *L. salmonis*. The procedures utilised have provided the first evidence of the suspected components of these glands and also represent the first specific identification of an enzymatic component of copepod exocrine glands.

5.5.1 Enzyme characterisation

The results of the blocking trial (Section 5.4.1) support the initial hypothesis that the coloured reaction products found in the glands arises as a result of the reaction between DAB / TMB and peroxidase enzymes. The results demonstrated that immersion in a solution of hydrogen peroxide prior to DAB staining effectively prevented the characteristic gland staining evident in specimens stained without hydrogen peroxide pre-treatment. This failure of hydrogen peroxide treated specimens to stain fully is convincing evidence that endogenous peroxidase enzymes are active within the identified exocrine gland tissues of *L. salmonis*. Such a hypothesis was proposed earlier in this study when the failure of gland tissues to stain with Alcian Blue suggested that acidic mucus was not the reactive component causing the observed DAB-positive staining and that peroxidase enzymes were likely to be the cause of the observed staining. The evidence from this experiment clearly supports such a hypothesis. The slight positive staining observed in some regions of the hydrogen peroxide blocked lice might have occurred because the hydrogen peroxide had not fully penetrated the tissues to effect the blocking of all endogenous peroxidase enzymes. A longer period of incubation in the blocking solution may possibly have fully blocked the slight activity observed. The unusual pattern of DAB staining observed in methanol immersed specimens is likely to be explained in terms of the high volatility of the methanol that would allow it to penetrate the length of the gut via the mouth and anus and reveals DAB staining features that were not previously apparent. The reason for the observed staining of midgut cells can perhaps be accounted for if the cells of the midgut lumen contain peroxidase enzymes (as shown in some chalimus stages such as that shown in **Figure 3.13**). Such enzymes in the midgut may serve to protect the cells from the damaging ROS

released as a result of uncontrolled chemical interactions from disrupted host tissue, as has been demonstrated by Felton and Duffey (1991) to occur in the guts of some lepidopteran larvae. Perhaps only the ability of the methanol to penetrate and carry the DAB stain into the guts of lice has revealed this feature that may be present in all stages of *L. salmonis* but has never been observed due to the inability of the less volatile Tris-HCl of the standard DAB solution to penetrate the length of the gut. In this study, DAB staining of formalin-fixed wax sections of *L. salmonis* failed to reveal the staining observed in unfixed, whole specimens, probably as a result of either the fixation or the process of wax embedding. In light of this discovery it is advised that DAB staining of cryostat sections of sea louse gut tissues be undertaken to determine whether peroxidase enzymes are a feature of gut epithelium which have not been revealed by the other techniques utilised in this study. Exocrine glands that stained positively with DAB tend to be intimately associated with their cuticular pore and it is presumed that this allowed the stain to enter the gland tissue. The volatility of methanol may also have been the cause of the fuzziness observed in the stained glands in the methanol-immersed specimens, the methanol having leached the contents of the gland outwith the membranes of the syncytia. The hindgut did not stain at all, which suggests that it was the gut epithelium that gave rise to the positive staining as the hindgut is cuticularised and, in contrast to the midgut, contains no epithelium. Midgut tissues of *L. salmonis* have been shown to contain a range of enzymes presumed to be involved in digestion (Roper *et al.* 1995, Andrade-Salas 1997) although no previous study has indicated that peroxidase enzymes are a feature of the midgut epithelial cells. The majority of the gland regions identified in the control lice were stained successfully in the methanol treated specimens, suggesting that the negative response to the DAB stain of those lice treated with hydrogen peroxide in methanol arose as a consequence of the action of the result of the hydrogen peroxide and not the methanol component.

Hydrogen peroxide, however, is a particularly harsh oxidising agent and it may have impaired or destroyed other compounds or enzymes which may have been present, so although these results are compelling evidence for the presence of peroxidases, it cannot in itself, be said to be irrefutable proof of the presence of such enzymes at these sites.

The spectrophotometric enzyme assays performed in this study, however, substantially support the hypothesis that the DAB and TMB stains are reacting with peroxidase enzymes. The positive reactions of the gland tissues to those stains have been shown by the assays to be caused primarily by a catalase enzyme although a small Se-GPX component was also detected. The urosomal gland tissues contained a much greater (no statistical tests were utilised) catalase activity than the other two tissues and this increased activity was a constant feature in all samples tested, suggesting that some component of this region possesses a conspicuous catalase constituent which is very much less in both the AGC and the control tissues. It is suggested that this catalase component is principally a feature of the urosomal gland complex and not the surrounding tissues, including terminal setae, which were removed along with the gland complex (**Figure 5.1**) although low background activities of catalase in non-glandular tissues are not discounted. The values of urosomal gland catalase activity are comparable to those identified in other marine invertebrates by Gamble *et al.* (1995) and lie between the activity detected by those authors in *Mytilus edulis* (L.) digestive gland (5.87μmoles min^{-1} mg^{-1}) and the pyloric caeca of *Asterias rubens* (L.) (20μmoles min^{-1} mg^{-1}). The enzyme activities of these tissues of these species were proposed to counteract the production of ROS partly as a result of the uptake of xenobiotics but also, in part, were explained by the production of hydrogen peroxide as a result of protein catabolism in the carnivorous *A. rubens*. The value of catalase activity in the urosomal gland of *L. salmonis* is therefore equivalent to that found in tissues that generate large amounts of ROS as a by-product of their digestive activities.

Se-GPX was detected at low levels in the urosomal gland complex and at even lower levels in both the AGC and the control tissue which suggests either that that enzyme is absent as a product in the AGC and is present at low levels in all tissues, including non-glandular, or that the non-glandular tissues contained some dorsal surface glands whose contents possessed a Se-GPX component. The 'gland-free' posterolateral margins of the cephalothorax are not entirely gland-free as can be observed in **Figure 3.20**, but this region of the louse contained the fewest glands of any region of tissue that could have been excised in sufficient quantities to assay and was hoped to have significantly less activity than the glandular

tissues selected for analyses, which in fact is the case. Selenium-independent glutathione peroxidase activity was shown to be present in negligible amounts in both glandular and control tissues and, what little activity was detected, could be accounted for by a very low and, apparently generally distributed, glutathione-S-transferase activity. Glutathione S-transferases are recognised, in vertebrates, to be centrally involved in the metabolisation of 'foreign compounds' (Halliwell and Gutteridge 1985) and as such are valuable in protecting tissues from a wide range of damaging species. Such an action of GST may also occur in *L. salmonis*, given its apparent presence in all areas of the body.

The results indicate that catalase is present in the urosomal glands at levels three times higher than the control, non-glandular tissues and twice as high as the AGC whilst Se-GPX is also present at higher levels in the urosomal gland tissue than in the AGC. The reasons for these differences between gland systems with similar staining characteristics are unclear except that it is possible that different gland systems have different levels of activity depending on their role or upon the physiological or behavioural condition of the animal. What is clear however is that the two selected gland systems contain above background levels of both catalase and Se-GPX and a substantial fraction of that background activity may well be accounted for by components of the DSG which were removed along with the control tissue. This suggests that all of the DAB-stained glands in this species contain both catalase and Se-GPX. The physiological significance of the presence of these enzymes in gland tissues remains to be resolved.

The identification of catalase within the glands of *L. salmonis* probably accounts, at least in part, for the success of the commercial sea louse treatment for farmed salmonids, hydrogen peroxide. Hydrogen peroxide causes preadult and adult stage *L. salmonis* to become detached from the host fish, an effect apparently caused by the formation of emboli within the haemocoel of the parasite (Thomassen, 1993). McAndrew, Sommerville, Wootten and Bron (1998) demonstrated that although chalimus stages were not removed by hydrogen peroxide treatment, probably because they are attached by their filament, their subsequent development was impaired. The evidence from the present study that catalase is present in the exocrine glands of *L. salmonis*, and presumably also within the stained vesicles of the chalimus stages, suggests that the impaired development of treated chalimus may be due to the disruption of the

delicate gut epithelium likely to occur as an effect of the violent effervescent reaction between hydrogen peroxide and catalase. Such damage to the gut epithelium is likely to reduce the ability of those stages to digest and absorb nutrients.

The identification of Se-GPX in the exocrine glands of *L. salmonis*, however, provides an exciting link to the identified importance of selenium in crustacean cuticle dynamics. Selenium have been shown to be an important trace element in the culture of Cladocera and media deficient in selenium has been shown to result in a form of cuticular damage similar to that which occurs during senescence (Keating and Dagbusan 1984, Keating and Caffrey 1989). The biochemical and physiological processes underlying this phenomenon were not identified in those studies but the possibility that this dependence on selenium may be accounted for by its inclusion in Se-GPX should be considered. It is interesting to note that the regions in caligids where peroxidase-containing glands (as they can now be called) are most prolific, are those regions where large, and presumably essential, cuticular 'extensions' are located i.e. close to, and secreting onto, the terminal setae of the caudal rami, the natatory setae of the thoracic legs and the marginal membrane. These setae and the marginal membrane are essential to the survival of these animals. If the setae of the thoracic legs become damaged or fouled, their ability to function will presumably be severely impaired and, consequently, the ability of the louse to attach to the host (the thoracic legs being used to create negative pressure for attachment) will become lessened with the attendant threat of unintentional detachment. Similarly, the marginal membrane is essential in maintaining the seal necessary to maintain negative pressure beneath the cephalothorax to facilitate attachment (Kabata and Hewitt, 1971). The functions of the terminal setae of the caudal rami are more difficult to evaluate but are possibly involved in modifying the flow of passing water, assist in maintaining the louse on the fish or may also be used in steering (Kabata and Hewitt, 1971). Whether or not these proposed functions are indeed accurate, it can however be presumed that such elaborate structures must be important to the biology of these animals in some respect. How, if at all, Se-GPX is involved in maintaining the cuticle of *L. salmonis* in a functional condition will require further study. Se-GPX could

theoretically function in preventing damage to the cuticle by preventing its attack from ROS that may build up in the collections of still water favoured by cladocerans.

This study (Chapter 3) has demonstrated that *Daphnia magna*, does not possess peroxidase-containing exocrine glands, suggesting that although selenium may be an essential element in cuticle maintenance / formation, its activity is not mediated via the actions of Se-GPX, or at least is not secreted directly onto the cuticle surface from tegumental glands. Examination of the Cladoceran cuticle by Mauchline (1977) revealed great numbers of integumental pores to be present and the presence of such pores suggests that species of this genus do possess large numbers of integumental glands but, in *D. magna* at least, the contents of those glands do not stain for peroxidase enzymes.

It is interesting that peroxidase-containing glands are present, and possibly functional, in early larval stages of *L. salmonis*, where cuticular deterioration between moults is likely to be negligible although the DSR do not develop fully until the adult stage i.e. where there are no further moults. This may also explain the apparent regression of the activity of these glands in the chalimus stages i.e. where the marginal membrane is absent and the thoracic legs are not utilised as attachment organs, attachment being effected solely by the frontal filament. Kovac and Maschwitz (1991) demonstrated that larval stage corixid bugs possess functionally active gland systems similar to the adult stages but the secretions of the immature stages are not utilised and the setae supporting the hydrofuge were only groomed by stages of long duration i.e. the adults.

Further evidence that the secretions of these glands might be involved in some form of cuticle maintenance / repair is the appearance of positive DAB staining at sites of injury (tears, cuts etc.) in the cuticle as observed in this study. Whether this represents an actual response to these injuries or merely results from the exposure of internally expressed peroxidase components in the cuticle and epidermis or DSG still needs to be resolved.

The results of the TEM anlysis appear to confirm the results of the assays of this study by identifying at least two different forms of peroxidase enzyme within the glands as evidenced by the positive reactions for both catalase and glutathione peroxidase. The GPX test utilised here only detected

Se-GPX and not any selenium-independent activity. TEM evidence showed conclusively the sub-cellular localisation of two different enzymes within the glandular tissue. It appears that catalase is found only within the secretory vesicles of certain gland tissues (of the urosomal glands at least) and therefore appears to be a component of the secretion proper and is not likely to be involved in providing intracellular protection from reactive oxygen species, lipid peroxidation, or be involved in any other intracellular peroxidatic activity. How this enzyme is transported to, and enters the vesicles of the gland was unresolved in this study, as no staining was evident outwith the secretory vesicles. It is possible that the enzyme is not present in an active state until it is sequestered in the vesicles as has been shown to be the case in vertebrates where catalase only assumes its active form once within the peroxisome and not whilst it is being transported through the cytoplasm in its apoenzyme form (DeRobertis and DeRobertis, 1987). It is also possible that the region where the enzyme is produced was not encountered in the sections examined. However, it is most likely that the enzyme is very sparsely dispersed within the gland tissue generally and is only concentrated in detectable amounts within the secretory vesicles. It is unusual that the catalase is found solely in the vesicles, as this enzyme is normally specifically located within peroxisomes in the cell cytoplasm in contrast to glutathione peroxidase, which is generally found free in the cytosol and in the matrices of mitochondria (Halliwell and Gutteridge, 1985). The data here suggests that catalase is found only within the membrane-bound vesicles and Se-GPX only occurs in small granules within the cytoplasm. No structures comparable to typical peroxisomes were observed generally within the gland tissues or to have stained in either the catalase or the glutathione peroxidase test. The organelles seen to give a positive response in the glutathione peroxidase test appear, from their size, shape and general distribution, to be the free ribosomes observed in Chapter 4 of this study in gland D of the urosomal gland complex. This in itself is unusual as there are no existing accounts of GPX being present in ribosomes, or indeed any cellular organelle (with the doubtful exception of mitochondria) as it is always identified free within the cell cytosol (Halliwell and Gutteridge, 1985). This leads to the conclusion that, in the glands of *L. salmonis*, either Se-GPX activity is localised within the free ribosomes, or that some other ribosome-associated component gives a similar reaction. Certainly the subcellular localisation of

both catalase and Se-GPX is very different from the generally recorded sites for these enzymes but many other studies of marine invertebrates have also shown that the distribution of these enzymes does not conform to the typical vertebrate pattern (Fredriksson *et al.* 1988, Bell and Smith 1994, Hawkridge *et al.* 2000). The combination of TEM analysis with antibodies (or DNA probes) specific for catalase and Se-GPX would help resolve these apparently contradictory findings by highlighting the subcellular locations of these two enzymes, but in a less destructive manner which may preserve tissue morphology more satisfactorily.

Tissue fixation was found to be poor using this technique and the morphology of the sections was accordingly unsatisfactory but, in order to retain the enzymes in their active form, short fixation periods, are essential. Further refinement of this technique could be pursued to identify the optimal fixation and incubation periods but this was outside the scope of this study. It was sufficient here to be able to identify the presence of the enzymes in tissue sections and confirm its absence from control tissue. It was also likely that the violent reaction, which occurs upon immersion in hydrogen peroxide, would have had a serious deleterious effect upon tissue morphology. The evidence from the selective DAB staining TEM study supports the results of the enzyme assays – Section 5.4.2.

This is not the first record of peroxidase enzymes actively secreted by an exocrine gland, albeit as part of a mixture of components. Sundararaju and Babu (1999) have recently shown that the salivary gland secretions of the insect heteropteran species *Helopeltis antonii* possess both a catalase and a peroxidase fraction which were proposed to have dual roles: the protection of the insect from harmful ROS and to cause unspecified 'phytotoxaemia' presumably to facilitate feeding. The results of the present study do not necessarily indicate that the enzymatic fraction is the active component in terms of the primary role of the secreted material. The catalase may be responsible in some way for maintaining another, yet unidentified, component of the secretion – the secretion as a whole being effective. Too much emphasis should not be placed on the identification of this enzyme fraction until further evidence is uncovered especially as the catalase component of the secreted mucus is likely to be very dilute after hydration of the mucus and the accompanying increase in volume this is likely to entail (Cross and Mercer

1983, Verdugo 1991). Protection of the secretion from reactive oxygen species may also be a function of the catalase within the secretion, although no evidence has been found in other species to indicate that peroxidase enzymes have any role in protecting mucus compounds. It has been shown though that marine gastropod mucus (Davies, Hawkins and Jones, 1992) can persist for a long time in the environment and if a similar scenario occurred with the mucous of *L. salmonis* a protective enzyme may be of utility.

It is possible that glandular catalase is utilised to neutralise environmental hydrogen peroxide. Hydrogen peroxide can be produced in seawater as a consequence of UV light interacting with dissolved organic matter in sunlit surface waters (Hoigne, Faust, Hagg and Zepp 1989, Buchner, Abele-Oeschger and Theede 1996). Whether this environmental hydrogen peroxide can reach levels where it would exert toxic effects on copepods and would require specific neutralisation is undetermined. What is known, though, is that the polychaete *Arenicola marina* (L.) shows a marked increase in antioxidant enzyme activity during the summer months to cope with the increased levels of environmental hydrogen peroxide associated with the increased UV interaction of the summer period (Buchner *et al.* 1996). Copepodid stage larvae of *L. salmonis* have also been shown to exhibit a marked negative taxis in response to UV light entering a water column (Aarseth and Schram, 1999). Visible light with no UV did not produce this effect. The reasons for this response are unclear, and may be related to host seeking, but may possibly reflect an avoidance of UV irradiated surface waters with its increased hydrogen peroxide levels. Host-associated adult stages must endure the environmental conditions of their host and may have developed the ability to counteract the effect of environmental hydrogen peroxide and the damage it may cause. In contrast to the findings of Aarseth and Schram (1999) however, Bron, Sommerville and Rae (1993a) discovered a positive phototaxis of *L. salmonis* copepodid larvae although those authors did not selectively exclude UV from their light source.

Epidermal mucus of sea lice infested salmon has been shown to appear 'washed out' (Horne and Sims, 1998) but the cause of this change is unknown and may possibly be due to the effect of the glandular secretions of *L. salmonis*.

5.5.2 Prostaglandin immunohistochemistry

The immunohistochemical analysis conducted here suggests that PGE_2 does not form a component of the gland secretion. Of itself this cannot be taken as conclusive proof of the absence of PGE_2, or other prostaglandins, in the secretions of *L. salmonis* exocrine glands, although there is, as yet, no evidence to suggest that prostaglandins are present in sea louse tissues. False negative results could have occurred for a number of different reasons such as inappropriate fixation, which may have destroyed any antigen sites and the fact that prostaglandins are known to exist only transiently as they are metabolised very rapidly and are unlikely to be sequestered within the gland (Gurr and Harwood, 1991). Quantities of PGE_2 present at any given time are therefore likely to be extremely low although the immunohistochemical technique employed should be sensitive enough to detect even exceedingly small amounts of antigens within tissues. It would probably be more advisable to look for arachidonic acid, the fatty acid precursor of prostaglandins which is likely to be present at high levels in a gland system that is geared for PG production (Bowman *et al.*, 1996). Unfortunately no positive control could be obtained for the immunohistochemistry trial. Without further research we are left with the conclusion that prostaglandins do not appear to form a component of the secretion of the identified exocrine glands of *L. salmonis*. This then suggests that sea lice are not modifying the immune response of their hosts with these chemicals, although the possibility that some of the enzymes known to be secreted / excreted by these animals (Roper *et al.* 1995, Andrade-Salas 1997, Ross *et al.* 2000, Butler, pers. comm), as well as any currently undiscovered enzymes, function as immunomodulators should be considered.

This failure to identify PGE_2 in the tissues of *L. salmonis* may be attributable to any of several factors. The most likely cause though is that the DAB reaction of *L. salmonis* gland tissues has been shown in this study (Section 5.4.2) to be caused primarily by catalase and to a lesser extent by Se-GPX. Although no specific assay for prostaglandin endoperoxide synthetase was performed it seems probable (given the significant levels of CAT and Se-GPX identified) that they are solely responsible for the peroxidase activity of the identified exocrine glands of *L. salmonis*. This factor alone suggests that prostaglandin production is not a feature of these glands although it is possible that prostaglandins are

present at very low levels throughout the general body tissues. Prostaglandins have been demonstrated in a number of parasitic arthropod classes including protozoa, cestodes and nematodes (Belley and Chadee, 1995). Those parasites though are generally endoparasites of vertebrates and are therefore probably exposed to more developed and specific immune responses characteristic of higher animals. The identification therefore of catalase and Se-GPX supports the failure to identify PG's in the glands of *L. salmonis*.

5.5.3 Lipid analysis

Analysis of the lipid content of gland and non-gland material provided crucial data, not only regarding the composition of the gland secretions, but also the structure and the general biology of these animals. The combined results of the polar and non-polar lipid analysis indicate that there is not a specific lipid component to the secretions of the AGC and urosomal glands. The profiles of lipids from glandular tissue and control tissue were essentially similar and bore a marked similarity to the profiles obtained for general tissue membranes such as those of mammalian erythrocytes (Gurr and Harwood, 1991). This then suggests that the positive response of gland tissues to Sudan Black (Section 4.4.2.8) arose from the lipid constituents of membranes within the tissues of *L. salmonis*. The apparent positive response to Sudan Black observed with the secretory vesicles of the urosomal glands and AGC probably occurred due to lipids being present in the membrane that envelops each vesicle. The non-polar lipid profile identified in these samples is very similar to the profile obtained from analysis of typical structural membranes such as that of mammalian erythrocytes (Gurr and Harwood, 1991). No conspicuous differences in the levels of each lipid type were detectable between the AGC, the control tissue and that of the urosomal gland tissue suggesting that there is no specific lipid component to the secretions of either the AGC or the urosomal glands as their lipid profiles are essentially similar to that of control (non-glandular) tissue.

The high levels of phosphatidylglycerol and cardiolipin detected in all tissues of *L. salmonis* are more typically associated with mitochondrial membranes from animal cells whilst the high levels of phosphatidylethanolamine are typically associated with mitochondria. The high levels of these lipids

present in the tissues therefore may indicate the presence of large numbers of mitochondria. However, as similarly high levels of these lipids were detected in the control tissue as well as the glandular tissues, this profile cannot be explained in terms of some aspect of gland syncitium structure or secretory material. Phosphatidylcholine was the second most abundant polar lipid in *L. salmonis* tissues and is frequently reported to be the most abundant major lipid component of animal tissues and is the most abundant lipid in vertebrate lung surfactant secretions (Riley and Henderson, 1999). Detection of similar levels of phosphatidylcholine in both gland and control tissues of *L. salmonis* though suggests that phosphatidylcholine does not constitute a part of the gland secretory product.

In most organisms triacylglycerols serve as the main storage lipids to be utilised for energetic needs, although marine copepods are known to make use of wax esters as the predominant form of lipid for energy storage (Lee *et al.* 1971). No triacylglycerols were detected in the non-polar fraction of *L. salmonis* lipids, differing from the findings of Lee (1975) who found that triacylglycerols accounted for approximately 50% of the total lipid present in this species. Such an apparent discrepancy can be accounted for by the fact that copepod triacylglycerol levels show seasonal fluctuations and great variation may be expected in the levels of these lipids depending on the latitude of sampling and the time of year. Specimens of *L. salmonis* associated with farmed salmonids probably show less of a seasonal variation in lipid levels than their wild counterparts as the food supply of farmed fish is constant and is not constrained by seasonality of prey items. A further consideration is the sexual condition of the specimens tested. Lee (1975) does not state whether he tested males, or females, or a mixture of both in his analysis.

Wax esters were not detected in the tissues of *L. salmonis* in this study. Though for a marine copepod this is highly unusual, it is in accordance with the findings of Lee (1975) who demonstrated that wax esters constituted less than 0.5% of the total lipid content of *L. salmonis*. Free-living, especially herbivorous, marine copepods rely heavily on wax esters as an energy resource and probably to some extent to provide buoyancy (Kayama and Mankura 1980, Sargent and Henderson 1986). The apparent absence of such compounds in *L. salmonis* can be explained through an examination of their life history. Free-living species of copepod probably experience fluctuations in food availability either as a

consequence of seasonal variations or as a result of spatial patchiness of food resources. This instability of food availability means that storage of energy reserves is essential for long-term survival, in contrast to the permanently host-associated *L. salmonis*. Parasitic copepods with the host exclusivity of *L. salmonis* are unlikely to experience food shortages and therefore have no requirement to store reserves for use during times of hardship. It is also apparent that copepod lipid profiles reflect those of their food as the lipids of herbivorous calanoid copepods closely mirror those of their phytoplankton prey whilst those of *L. salmonis* mirror closely the lipid profile of their salmon host (Lee, 1975). As *L. salmonis* can exist off the fish host for in excess of 14 days (authors unpublished observations) it can be supposed that they are able to draw on some form of energy reserve. It appears from this study that this energy reserve is neither wax esters nor triacylglycerols. It is worth considering that this correlation between dietary and tissue lipid profiles of copepods may be of utility in inferring behaviour of specimens with no detailed life history.

As only samples of crude glandular material were used it is impossible to distinguish between those lipids, which are components of membranes and those which may form part of the secretion itself. Analysis of the culture water, or the cuticle surface, of *L. salmonis* would reveal conclusively whether any of these lipid components are destined for secretion. However, as there were no apparent differences in either lipid types present, or percentages of each lipid class, the conclusion here must be that the glands examined here are not secreting any form of lipid material. The quantities of lipids present in the two different glandular tissues are comparable to that found in other body tissues.

Insects commonly possess a secreted lipid layer on the external surface of their cuticles, which serve to protect them from desiccation, mechanical damage and attack from predators and parasites (Waku and Foldi, 1984). However, wax layers have never been identified in copepods and the present study seems to support this. The samples taken for lipid analysis contained substantial quantities of cuticle yet wax esters were not detected in any sample suggesting that *L. salmonis* does not possess an external lipid layer.

5.6 CONCLUSIONS

The experimental evidence obtained here demonstrates that the peroxidase enzyme catalase is present and is highly reactive within the exocrine glands of *L. salmonis*. There is compelling evidence (from its presence in vesicles) that this catalase is actively secreted by those glands although its precise role in that secretion remains unresolved. The evidence also suggests that Se-GPX, although present in the glands, is not a component of the secretion but exists only within the gland cytosol and is active at significantly lower levels than the catalase component. This is the first recorded identification of specific enzymes within the exocrine glands of any crustacean. The catalase and Se-GPX detected have characteristic subcellular localisations within the gland tissues that are not consistent with those recorded for vertebrate forms of those enzymes. The data also demonstrates that almost all the GPX activity can be attributed to the activity of Se-GPX alone as almost no selenium-independent GPX activity was recorded in any tissues.

High reactivity of enzymatic components in assays does not necessarily imply that large quantities of enzyme are present. Catalase, for example, is known to be a highly reactive enzyme that is difficult to saturate with hydrogen peroxide as its maximal velocity for breaking down that chemical is very large. This capability means that even small quantities of enzyme can give rise to high levels of substrate catalysis.

It is also concluded that *L. salmonis* does not secrete prostaglandins, for the purposes of modifying the host immune response, via its peroxidase-containing exocrine glands. This is in contrast to many haematophagous arthropod parasites of mammals where prostaglandins form a significant proportion of their salivary gland secretions. Such an absence of prostaglandins in *L. salmonis*, and presumably other caligids, may reflect the primitive immune system of their fish hosts (Woo 1991, Yano 1996) in contrast to the sophisticated immune systems of mammalian hosts.

The population of peroxidase-containing exocrine glands described here is apparently not involved in lipid production with the possible exception of the circum-oral glands. The lipid profile of

gland and non-gland tissues was almost identical and both correspond closely to the profiles described for this species by Lee (1975).

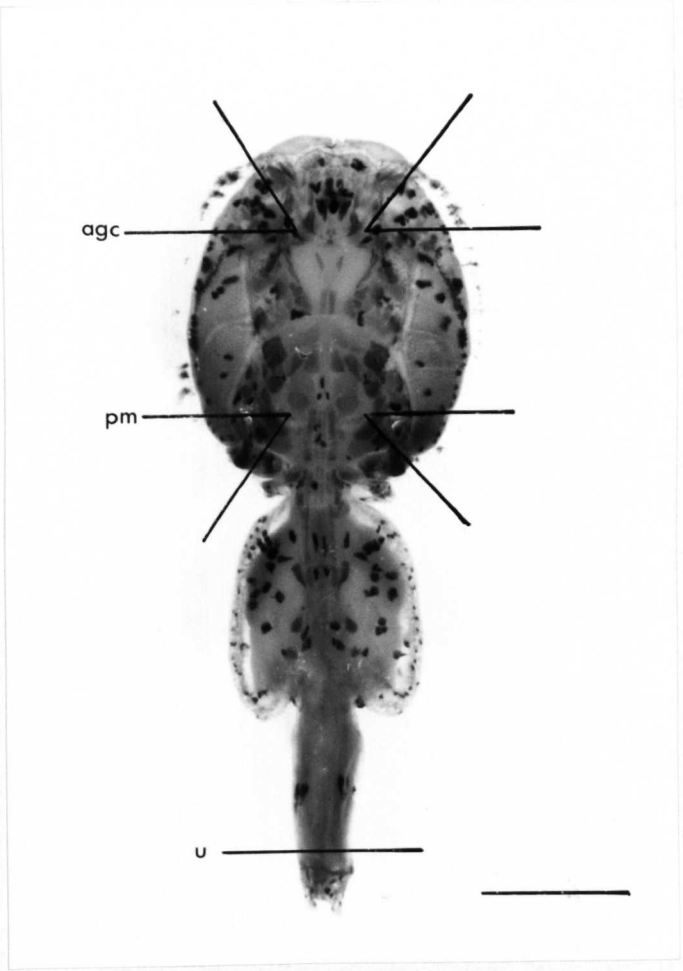


Figure 5.1 Diagram of an adult female *L. salmonis* showing the tissues removed for enzyme assays and HPTLC analysis. agc anterior gland complexes; pm posterolateral 'gland-free' margins; u urosomal region. Scale bar = 2mm.

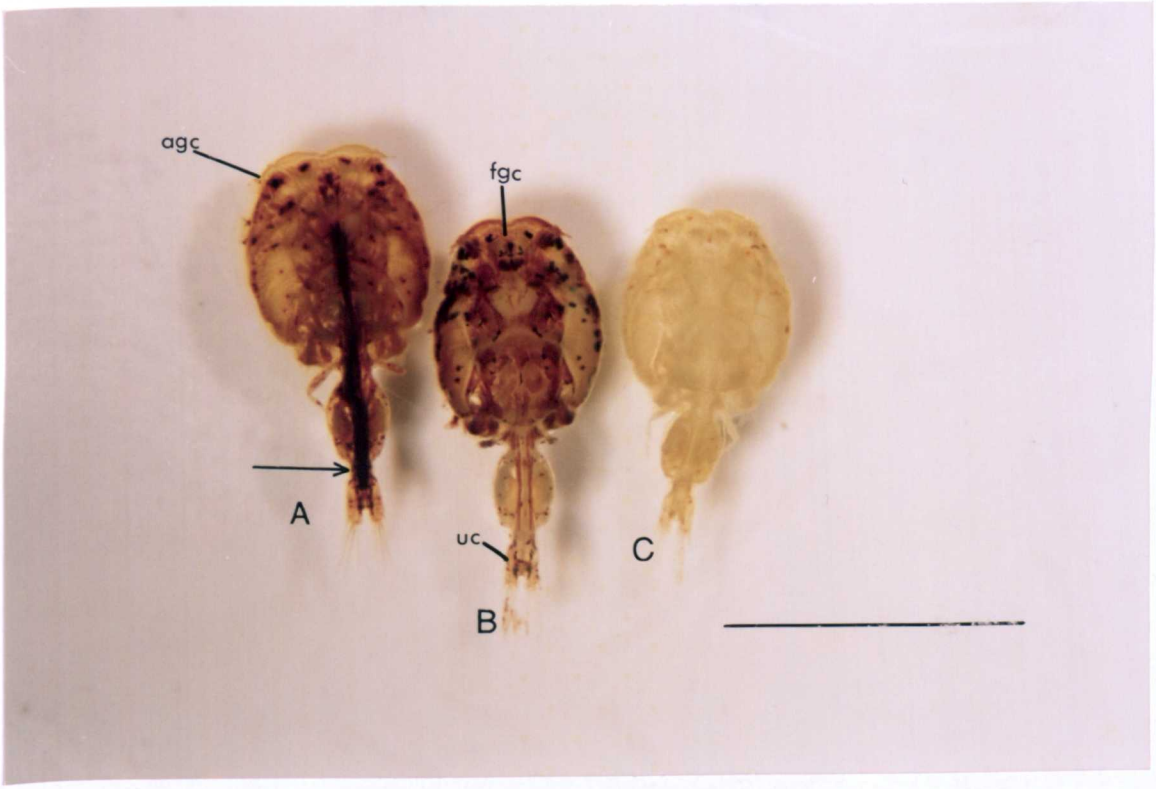


Figure 5.2 Three adult male *L. salmonis* each subjected to a different treatment regime prior to staining with DAB to demonstrate the blocking effect of hydrogen peroxide on the staining of exocrine glands. Specimen A - immersed in methanol only prior to DAB staining. Specimen B - stained with DAB after immersion in seawater. Specimen C - treated with hydrogen peroxide in methanol prior to DAB staining. fgc filament gland complex; agc anterior gland complex; uc urosomal gland. Arrow indicates stained gut of Specimen A. Scale bar = 5mm.

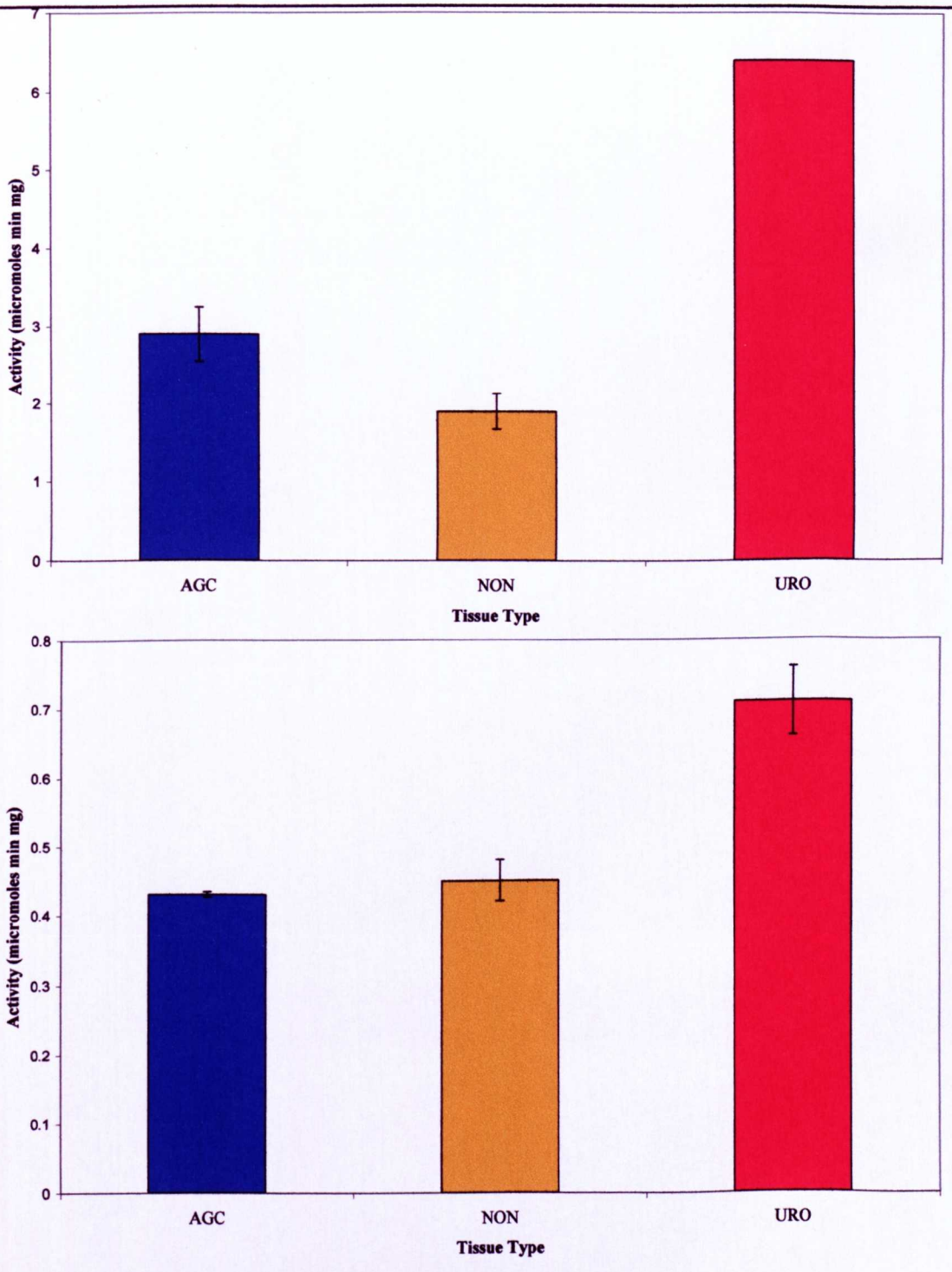


Figure 5.3 Graphical representation of the relative catalase activities of three different *L. salmonis* tissues as determined by spectrophotometric assays. AGC anterior gland complex; NON non-gland control; URO urosomal gland complex.

Figure 5.4 Graphical representation of the relative Se-GPX activities of three different *L. salmonis* tissues as determined by spectrophotometric assays. AGC anterior gland complex; NON non-gland control; URO urosomal gland complex.

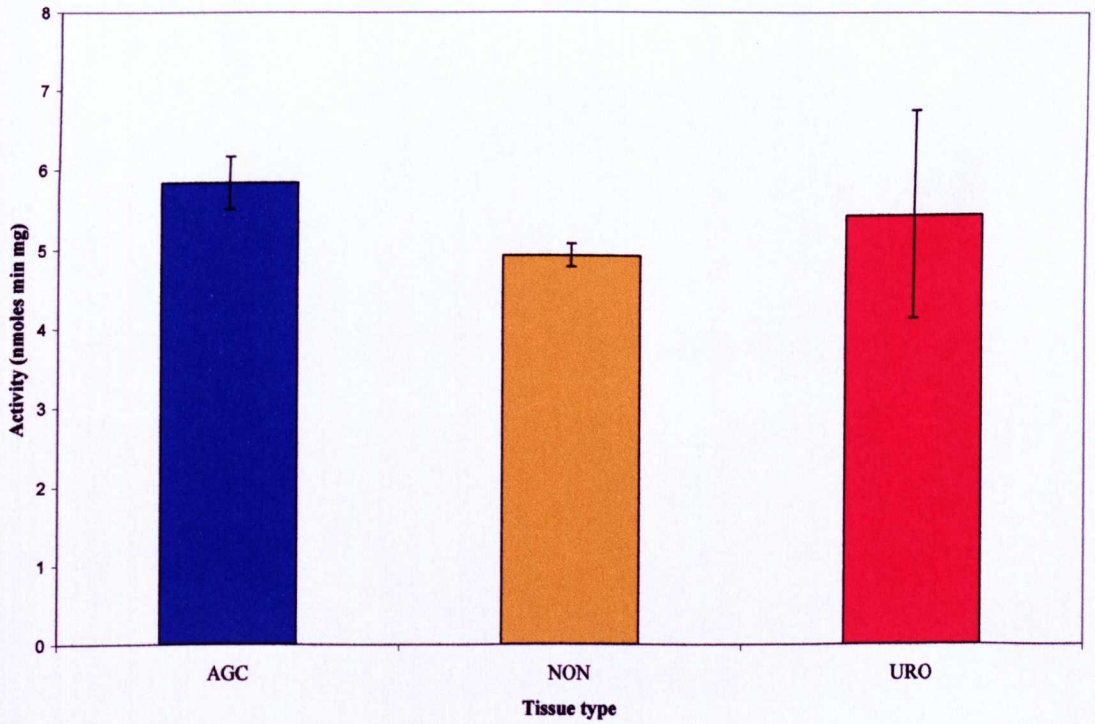


Figure 5.5 Graphical representation of the relative GST activities of three different *L. salmonis* tissues as determined by spectrophotometric assays. AGC anterior gland complex; NON non-gland control; URO urosomal gland complex.

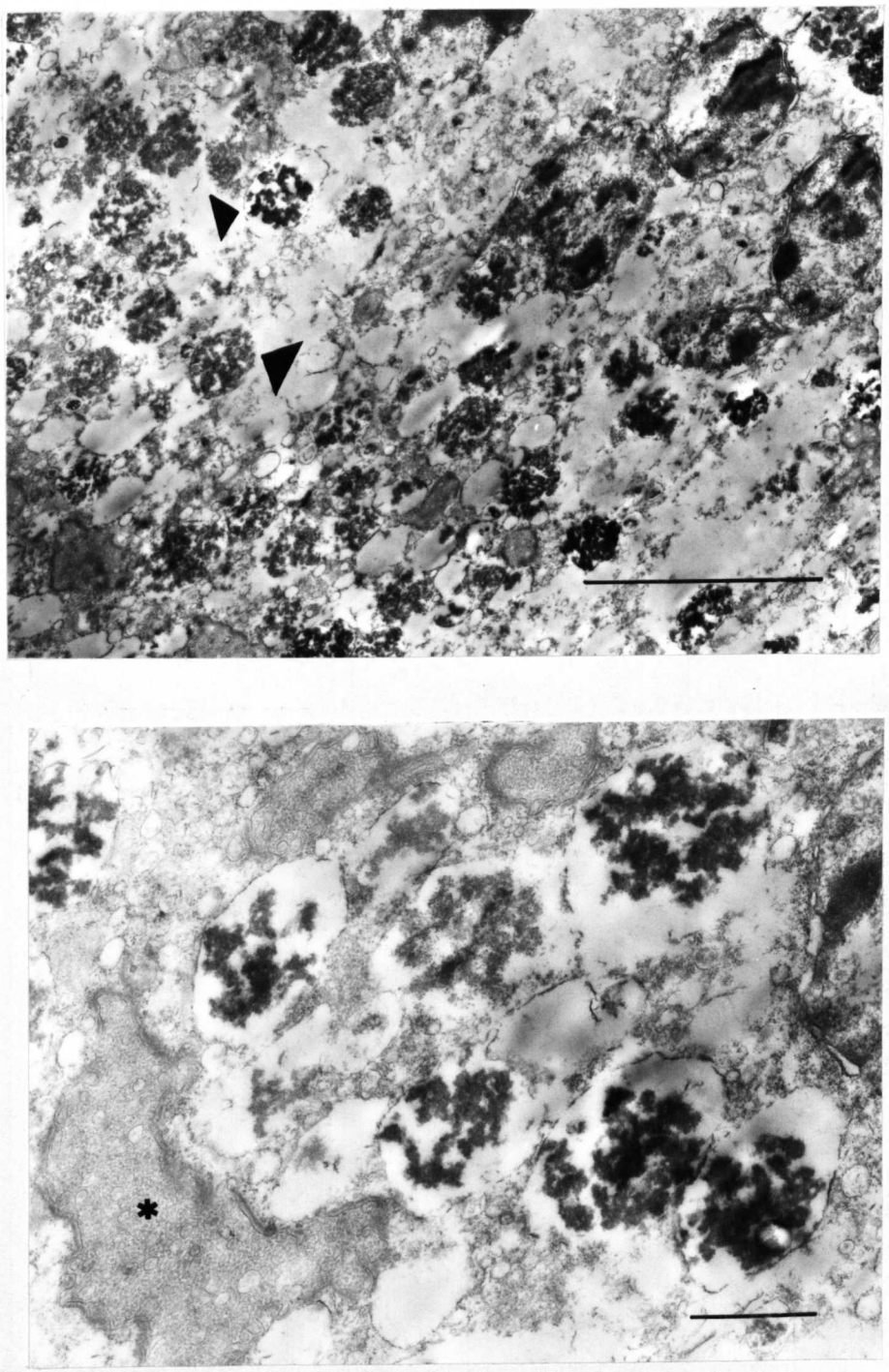
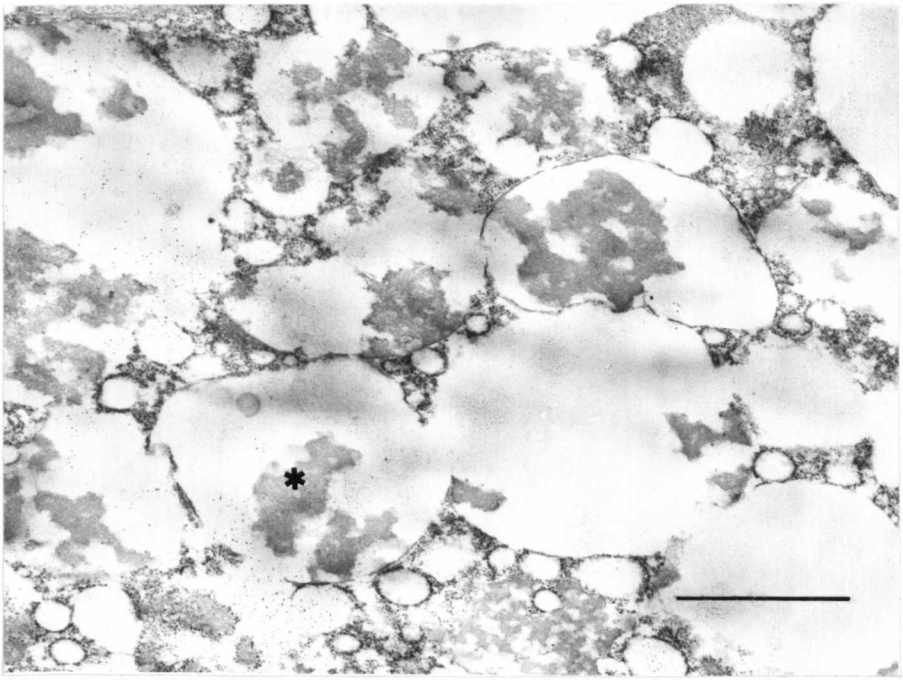


Figure 5.6 TEM micrograph of a typical section of urosomal gland D of an adult male *L. salmonis* after staining with DAB for 30min specifically to highlight areas of catalase activity. Regions of dark positive staining are apparent throughout the section (arrowheads) and correspond to the secretory vesicles of the tissue described in section 4.4.5.5. Scale bar = 4μm.

Figure 5.7 Higher magnification of gland D of the urosomal gland complex of *L. salmonis* stained for 30min with DAB to demonstrate areas of CAT activity. The strong positive staining of the vesicle contents is apparent as is the lack of positive staining of other components. A microvillous region is also evident (*) in the bottom left of the figure. Scale bar = 1μm.



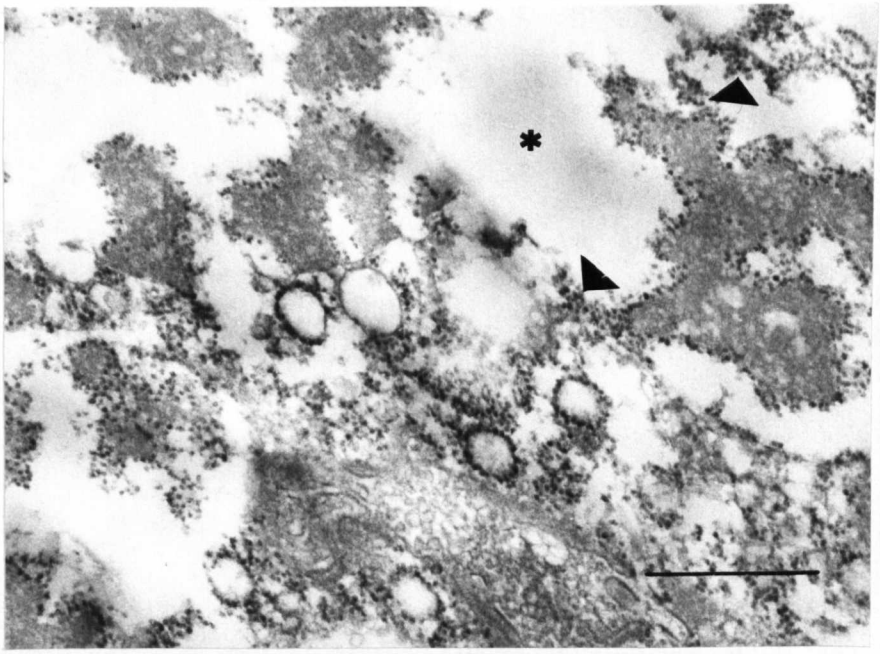


Figure 5.8 TEM micrograph of a typical section of urosomal gland D of an adult male *L. salmonis* after staining with DAB for 20min to specifically highlight areas of glutathione peroxidase activity. Note the absence of staining of the vesicles (*) and the staining of the small granular regions of the cytoplasm (arrow). Scale bar = 1μm.

Figure 5.9 Higher magnification of a region of Figure 5.8 demonstrating the staining of small granular components within the cytoplasm of gland D stained with DAB specifically to indicate the presence of GPX. The non-staining vesicles are clearly apparent (*) whilst the positive staining of the ribosomes is more evident (arrowhead) at this higher power. Scale bar = 0.5μm

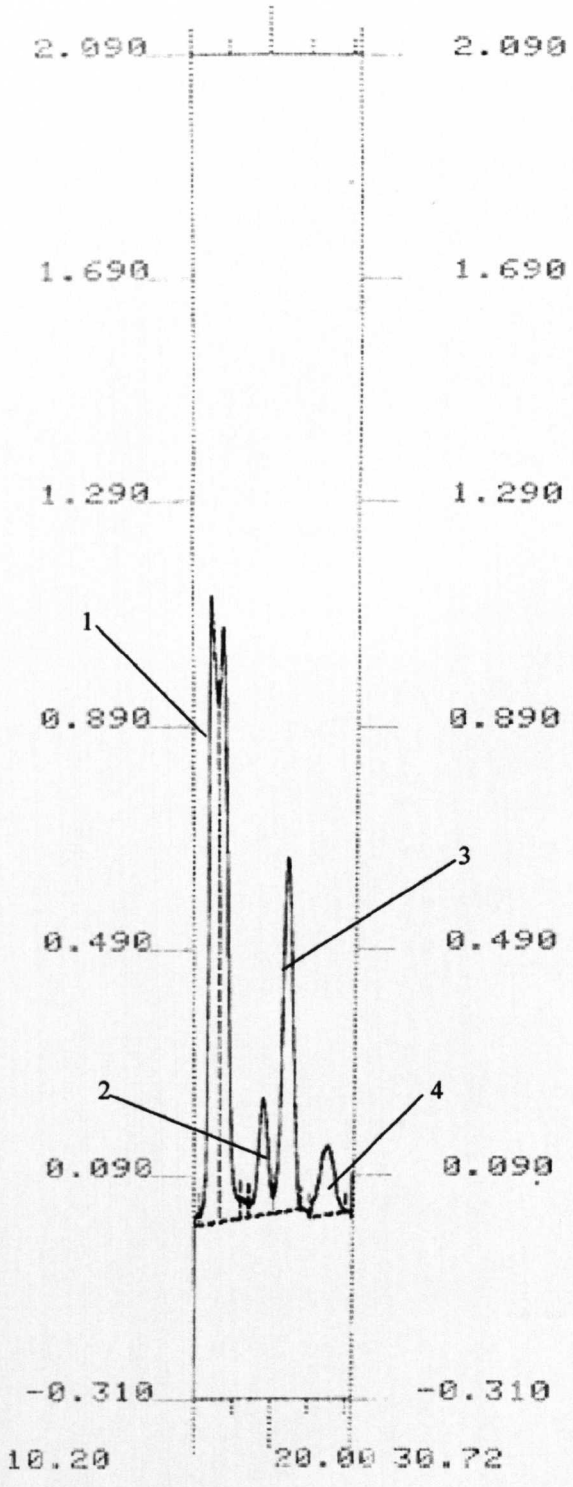


Figure 5.10 Neutral (non-polar) lipid profiles of *L. salmonis* AGC tissues as detected using densitometry. 1 total polar; 2 unknown; 3 cholesterol; 4 free fatty acid.

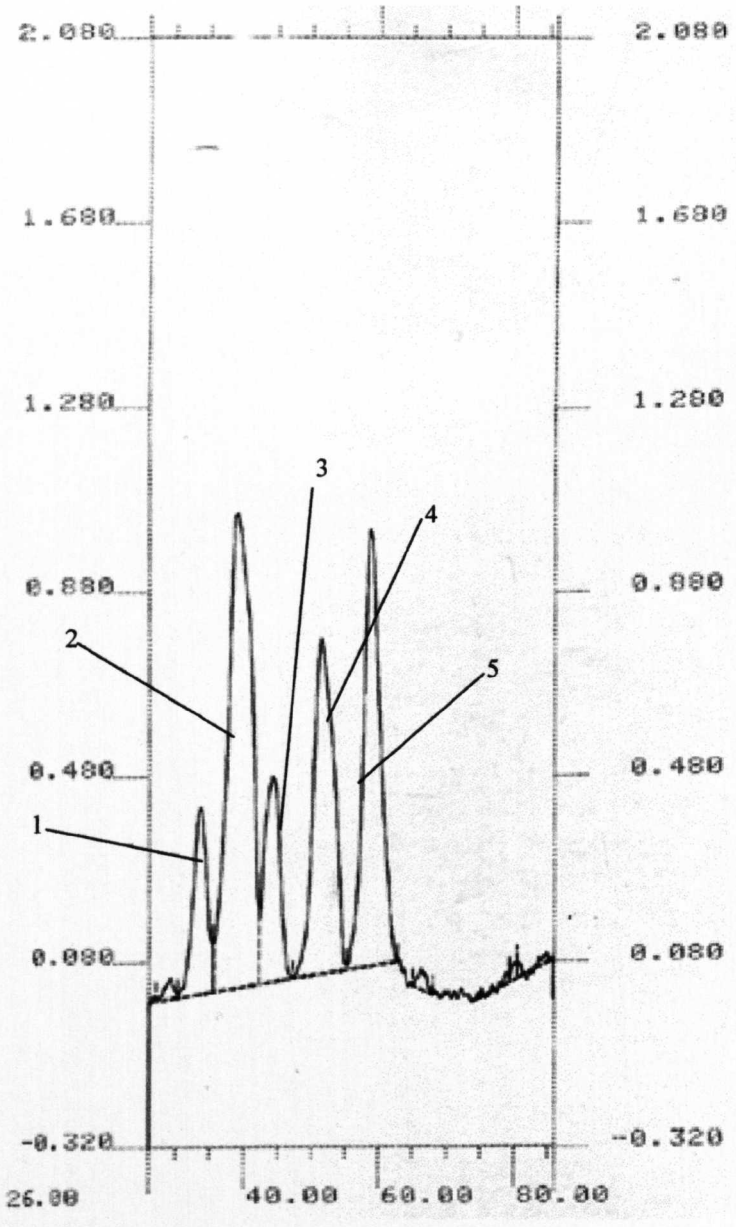


Figure 5.11 Polar lipid profiles of *L. salmonis* AGC tissues as detected using densitometry. 1 sphingolipid; 2 phosphatidylcholine; 3 phosphatidylserine and phosphatidylinositol; 4 phosphatidylglycerol and cardiolipin; 5 phosphatidylethanolamine.

6. ELECTROPHORETIC ANALYSIS OF GLAND SECRETIONS

6. ELECTROPHORETIC ANALYSIS OF GLAND SECRETIONS

6.1 INTRODUCTION

Having confirmed the presence of catalase within the glands of *L. salmonis* (Chapter 5) it was sought to characterise more precisely the quaternary structure of this protein by determining its molecular weight, and that of its constituent subunits. In order to do this, protein electrophoretic techniques were utilised to resolve the catalase component by separating all the proteins in a gland tissue sample on an electrophoretic gel. Theoretically, it would be possible to identify those components that occurred only in the gland tissues if workable quantities of gland and non-gland tissue could be collected. Non-gland tissue would serve as a negative control.

Polyacrylamide gel electrophoresis is a technique whereby complex protein mixtures can be separated and the constituent parts quantified and characterised and it is routinely used to determine the molecular weights of proteins to aid in their identification (Bollag, Rozycki and Edelstein, 1996). Resolved proteins can be identified either by comparing their molecular weight to that of a preparation of known protein standards or through the use of specific assays or staining procedures. The technique has many forms which are specific to the particular requirements of the type of proteins being studied. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used version of the technique where sample proteins are separated according to their molecular weights. The process first involves the denaturation of the proteins by heating them to 100°C in the presence of sodium dodecyl sulphate (SDS) while a thiol reagent (β-mercaptoethanol) is used to break disulphide bonds within the proteins (Bollag *et al.* 1996). SDS binds to the unfolded proteins at a specific rate per gram of polypeptide and forms rods roughly proportional to the molecular weight of the proteins, thereby allowing them to be separated based on their size alone. Although SDS-PAGE is the most commonly used method of gel electrophoresis for separating proteins, it is not suitable for all purposes. It is particularly unsuitable where intact protein complexes need to be retained and where the activity of an enzyme needs to be retained to aid in its identification. In these situations, non-denaturing (also termed 'native') gels, minus

SDS and thiol reagent, must be used. Electrophoresis under native conditions allows proteins to be separated based on both their size and charge whilst retaining their activity. The identification of an enzyme on a native gel can be achieved in many ways including the application of immunoblotting, autoradiography and fluorogenic and chromogenic reactions (Manchenko, 1994). Chromogenic reactions tend to be the easiest of these methods to implement, as they frequently require only one or two steps to the process and the enzymic reaction tends to be visible to the naked eye making detection of desired enzymes more rapid. Various chromogenic techniques have been described to allow the visualisation of catalases isolated on acrylamide gels (Manchenko 1994, Yamada and Wakabayashi 1998).

Many different animal catalases have been identified using acrylamide gel electrophoresis but they have all been shown to have similar quaternary structures. The quaternary structure of a protein refers to the specific composition of the whole protein molecule. Many proteins are not single chains of polypeptides (monomers) but are frequently found to be composed of several monomers in combination (an oligomer) the gross structure of which is referred to as the quaternary structure (Wynn, 1973). SDS-PAGE separates oligomeric proteins into their constituent monomers, each of which migrate separately within a gel. The molecular weights of described catalases vary from 124,000Da (Rocha and Smith, 1995) to 330,000Da (Wang, Tokusige, Shinoyama and Fujii, 1998) but most appear to have molecular weights in the low 200,000Da region (Kim, Lee, Hah and Roe, 1994, Kagawa, Murakoshi, Nishikawa, Matsumoto, Kurata, Mizobata, Kawata and Koyata-Minami, 1999). The quaternary structures of identified catalases indicates that they are usually composed of four identical protein subunits (Kim *et al.* 1994), although six subunits have been identified in some species (Kagawa *et al.* 1999). A subunit is the smallest covalent unit of the whole enzyme structure (Price and Stevens, 1982).

Glutathione peroxidases of varying molecular weights have also been identified in animal tissues using SDS-PAGE. Like catalase these enzymes are also known to consist of four protein subunits although the molecular weights of the complete molecule are lower than that of identified catalases. Identified glutathione peroxidases have molecular weights varying from 90,000Da to 105,000Da (Lyons, Wilhelmsen and Tappel 1981, Miwa, Adachi, Ito, Hirano and Sugiura 1983).

Although catalases and peroxidases have been identified in several marine invertebrates (Bell and Smith 1994, Gamble *et al.* 1995, Hawkridge *et al.* 2000) their structural characteristics have never been determined. The application of electrophoretic techniques in the determination of glandular secretions has been overlooked probably due to the difficulties associated with identifying an unknown protein component from a complex mixture of proteins as would occur if isolated secretory material was unavailable. Certainly, this difficulty occurs in copepods as their small size and aquatic nature make the isolation of secretory material almost impossible. Studies of other arthropod classes have achieved greater success in this area, such as that by Dezfuli, Capuano and Pironi (1999) where electrophoretic techniques were utilised to aid in the identification of an unknown protein component of the cement gland secretion of the acanthocephalan *Pomphoryhnchus laevis* (Müller, 1776). Studies such as those of insect spermatophores (Neuner, Peschke and Frohnmeyer, 1996), haematophagous insect saliva (Amino, Porto, Chammas, Egami and Schenkman, 1998) and stimulus-induced surface secretions Korchi, Farine and Brossut (1998) have described the isolation of gland secretions from terrestrial insects. The successful isolation of the secretion in these cases was achieved partly because of the relatively large size of the specimen and because of the mode of functioning of the gland i.e. the gland secretions isolated were those that were relatively straightforward to collect either due to their large size (in the case of spermatophores) or due to the fact that they are readily emitted by the animal upon stimulation (Korchi *et al.* 1998).

Electrophoretic techniques have never been utilised to analyse the components of the secretion of copepod exocrine glands. No evidence to show that either catalases or glutathione peroxidases have been isolated from copepods could be found in the literature. The detection of the catalase enzyme on an acrylamide gel would be the first step in the determination of its quaternary structure and would lay the foundations for the isolation and purification of the protein from whence sequencing or antibody production could be initiated. Following on from these steps, detection methods such as immunogold staining and *in-situ* hybridisation could be utilised to precisely determine the subcellular localisation of the enzyme whilst molecular techniques could also be applied to determine the evolutionary development of the enzyme system within different copepod orders.

Use of SDS-PAGE was unsuitable in retaining enzymes in their active form because it denatured protein complexes. The initial identification of the catalase component would require their enzymatic activity to be retained. The use of non-denaturing gels, in theory, would preserve any enzyme components in their native forms, thereby retaining their activities and allowing them to be assayed using zymographic techniques specific for each enzyme. SDS-PAGE could be used to resolve the structure of the identified enzyme component.

6.2 OBJECTIVES

The objective of the work presented in this chapter was to characterise, as far as possible, the structure of the catalase component of the exocrine glands of *L. salmonis* which had been identified by the techniques employed in Chapter 5. Acrylamide gel electrophoresis was utilised to assist in the isolation of proteins from whole gland homogenates as well as from the mixture of proteins secreted *in vitro* by *L. salmonis*. Techniques were employed to permit the identification of the enzyme amidst the numerous component proteins of the gland tissues. It was hoped to determine the molecular weight of the whole catalase molecule and its constituent subunits.

Attempts were also made to identifiy glutathione peroxidase in samples of *L. salmonis* gland tissues.

The program of electrophoretic techniques utilised is outlined below.

- Native polyacrylamide gel electrophoresis of *L. salmonis* gland tissues
- Zymography of native gels to detect enzymic components
- SDS-PAGE of *L. salmonis* gland tissues
- SDS-PAGE of *L. salmonis* secreted proteins

6.3 METHODS

6.3.1 Native gel electrophoresis of gland material

Specimens of *L. salmonis*, obtained as outlined before, were maintained in the laboratory for 24h prior to use and rinsed twice in clean seawater to minimise the risk of contamination by host proteins. Adult female lice were used for this trial because their greater size meant that it was easier to dissect glandular regions and regions of non-glandular material to serve as controls. The small size of male lice meant that it was difficult to obtain usable quantities of non-glandular tissue. Linear slab gels, where the acrylamide concentration of the gel is constant, and gradient gels, where the acrylamide concentration increases over the length of the gel were both utilised. Gradient gels were utilised because they allow a greater range of molecular masses to be fractionated on one gel (Shi and Jackowski, 1998).

For native gel analysis both AGC's and the urosome from fifty specimens were excised using a fine scalpel blade whilst trying to include as little extraneous tissue as possible. To serve as a non-glandular control, two regions from the posterolateral margins of the cephalothorax (**Figure 5.1**) were also removed from each specimen and stored in a separate sterile Eppendorf vial in 40µl of 5mM PMSF (phenylmethylsulphonylfluoride) protease inhibitor to minimise any degradation of the enzymatic fraction. It is generally regarded that catalase degrades when frozen but it was not feasible to obtain fresh samples for every trial. Samples therefore had to be collected and stored at -70°C. In some early analyses only the AGC and the non-glandular control tissues were removed and processed as described above, the urosomal region not being utilised.

It should be noted that those regions that were to serve as gland-free controls still contained a small number of DSG's as it was not possible to find a sufficiently large region which was completely gland-free to excise. Excised regions of tissue were snap frozen in liquid nitrogen whilst the dissections were carried out. Fifty lice were used for each sample giving 100 glandular / non-glandular regions per sample. Samples were stored at -70°C. Samples were analysed for protein content using the method of Bradford (1976).

Samples were homogenised for 5min in 140µl of homogenisation buffer (**Appendix 3**) and centrifuged in an IECM Micromax OM-3950 centrifuge at 13,000RCF at 4°C for 10min. To each of 4 wells, 5µl of sample were added and at least one high range (669,000 - 67,000Da) molecular weight standard (17-0445-01, Amersham Pharmacia Biotech Inc.) was also run in each gel.

Samples were electrophoresed for approximately 180min at 100 volts at 4°C using a minigel apparatus (Hoefer Mighty Small II) then stained in Coomassie Brilliant Blue overnight on an agitator. Gels were de-stained with three washes of de-stain solution (8:1:1 dH_2O : methanol : glacial acetic acid) and recorded using either standard photographic techniques or a digital camera.

Pre-cast mini polyacrylamide gradient (4-20%) gels (Sigma P5466) were initially used to allow a wide range of molecular weight components to be visualised on one gel. The information obtained from the gradient gels allowed proteins of interest to be targeted and run using more appropriate gel concentrations. Gels were run at 100volts at 4°C for a period between 180min and 240min and in all cases electrophoresis was terminated before the sample dye front reached the bottom of the gel. Photographs of the gels were taken after staining with Coomassie Brilliant Blue and destaining as detailed above.

The electrical charge of protein molecules in non-denatured samples meant that there would be a slight variability in migration distance of individual proteins depending upon the concentration of the gel. In order to accurately determine molecular weights from proteins in non-denaturing gels it is important to run several gels of different acrylamide concentrations. Further native gels of 8% and 12% acrylamide concentration, were also utilised to allow the accurate identification of protein molecular weights. All gels were run as described above for gradient gels. Each gel was run with two replicates of the three experimental tissues allowing different staining techniques to be applied and compared. Selected gels were stained with PAS (Periodic Acid-Schiff) to demonstrate neutral mucus groups whilst others were stained using one of the zymographic techniques outlined in **Appendix 4**. The molecular weights of the separated proteins were calculated from the molecular weight standard in each gel.

Ideally, the amount of protein present in each sample should be calculated in order to distinguish between genuine differences in abundance of proteins and the effects caused by unequal sample concentration. Due to the very small working quantities available, spectrophotometric protein estimation (method of Bradford (1976)), was performed only once in this study. The assumption was that all subsequent samples would contain approximately similar quantities of proteins.

6.3.2 Chromogenic detection

Chromogenic staining techniques were utilised to detect the position of separated enzymes present in the gel matrix. Techniques specific for the detection of catalase and glutathione peroxidase (Manchenko, 1994) were utilised in order to identify sites of enzyme activity. A description of each assay technique is included in **Appendix 4**. TMB (tetramethylbenzidine), as described in Chapter 3, was also used to stain native gels for sites of peroxidase enzyme activity.

6.3.3 SDS-PAGE of gland material

Samples of AGC, urosomal gland and non-glandular control material (as described in section 5.2.2 were subjected to SDS-PAGE to determine the effect of denaturation on the protein profile obtained. It was hoped that the denaturing effect of the SDS and mercaptoethanol would disrupt the carbohydrate material detected on native gels and allow proteins to be resolved fully.

Experiments utilised a discontinuous electrophoresis system employing both 8% and 12% SDS separating gels (buffers described in **Appendix 4**) overlaid with 5% stacking gels into which the samples are loaded. The loading of proteins into a stacking gel, from where they migrate into the separating gel, allows better resolution of sample proteins (Shi and Jackowski, 1998).

Ten samples (50 animals per sample) were processed as for native gel electrophoresis except for the fact that the centrifuged supernatants were diluted (1:4) in a sample buffer containing SDS and β-mercaptoethanol. The diluted sample was boiled for 5 minutes prior to 10μl of each being loaded onto the gel. A broad range molecular weight standard (161-0317, Bio-Rad) was used.

Electrophoresis was performed at 80 volts at 4°C for 180minutes. Gels were stained overnight in Coomassie Brilliant Blue, destained as described for native gel electrophoresis then photographed as described for native gels.

6.3.4 SDS-PAGE of *L. salmonis* culture water

It was assumed that the large and confusing protein profiles of samples of gland material arose as a result of all the constituent proteins of the various cell organelles being included in the sample. In order to successfully demonstrate that isolated enzymes actually originated from an exocrine secretion, it was necessary to identify them in a form where they were free from the contaminating effects of cellular material, in a form where only the secretion itself was analysed.

Attempts to stimulate individual specimens of *L. salmonis* to secrete the contents of their glands in response to a stimulus (freezing spray, solvents, and manual compression of gland tissue) met with little success. A more feasible method of secretion isolation was to extract the secretion from a volume of water in which the lice had been held. To achieve this it was proposed to concentrate the water from a high-density culture of *L. salmonis* and process it to a state where it could successfully be subjected to acrylamide gel electrophoresis. A comparison of the protein profile obtained from electrophoresis of these samples with those obtained from whole gland homogenates would demonstrate common proteins that could be presumed to be components of the secretion. Preliminary attempts to concentrate the culture water indicated that a starting volume of 300ml containing approx. 700 lice would be required in order to provide sufficient protein for electrophoresis. This was determined from a time series of lice in a specific volume of water followed by protein analysis of the culture water. From the results of the time series it was possible to calculate how many copepods were required, what volume of water and how long the lice had to be held to produce sufficient protein to concentrate into workable volumes.

Specimens used in this experiment had been removed from the fish host <24h previously and were rinsed briefly in sterile seawater to remove any contaminating host material. The copepods were maintained in 300ml of 10°C, sterilised seawater and gently aerated for 3h before the next step.

The histochemical analysis of Chapter 4 had indicated that the gland secretion consisted of a mixture of components, including mucus, so a detergent treatment was utilised in order to remove any mucus secretions that may have contained the active enzyme. Five minutes before the 3h had elapsed the non-ionic detergent Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma P1379) was added to the culture water (final concentration 0.05%) and slight agitation employed to assist in the removal of any adhering mucus secretion that may have been coating the lice. The culture water was immediately decanted after this step. Tween 20 at this concentration is known not to have an effect on either protein estimation or electrophoresis (Shi and Jackowski, 1998).

The culture water was then coarsely filtered through filter paper (Whatman, No. 1) to remove insoluble material and mucus and then vacuum filtered through a 0.45μm filter to remove any bacteria to prevent the solution spoiling as had been found to occur rapidly if filtering was not employed.

The filtered solution was then dialysed (Visking tubing, 12 – 14,000 molecular weight cut off) against a 20% solution of polyethyleneglycol 6000 (PEG) at 4°C for several hours until the volume of the sample had been reduced to approx. 80ml. This solution was further concentrated by spinning it through centrifuge tubes containing a 5,000MW filter (Vivascience, Lincoln, U.K). Each tube held 20ml that could be concentrated to 0.5ml by centrifuging at 3,000RCF for 240min. The final solution was divided into 200μl aliquots and stored at -70°C until required for use. The salt concentration, as measured using a salinometer, of the final sample was approx. 5%.

Prior to electrophoresis, samples were diluted (4:1 sample:5x SDS sample buffer) and boiled for 5min. Samples of 10μl were run on 12% linear SDS gels and 4-20% gradient SDS gels as described in section 6.3.3 for glandular tissue except for the fact that the molecular weight marker (Broad Range Protein Standards P7702S) was obtained from Biolabs, New England. Single samples of 50μl were run on gels of 8% SDS concentration.

6.4 RESULTS

6.4.1 Native gel electrophoresis of gland material

Initial trials using 4-20% gradient gels proved successful in allowing some separation of the proteins within the sample (**Figure 6.1**) although complete separation was not considered to be achieved using this technique, as only a small number of bands were observed. The range of protein masses resolved using gradient gels was from 559,800Da - 3,900Da. **Figure 6.2** provides a diagrammatic representation of **Figure 6.1** but displays only those protein bands considered to be of significance and mentioned in the text.

A conspicuous broad band of high molecular weight material (approx. 352,000Da - 270,00Da) was present in all three sample lanes. The thickness of this band, and the relatively few proteins clearly resolved in each sample suggested that there was a problem at this point of the gel that was causing some of the proteins to stop migrating properly. Smaller molecular weight proteins were apparent in all lanes, which indicates that some of the proteins were migrating successfully. This suggests that the thick band of proteins identified was not caused by either a fault in the gel or the buffer system. Only three other protein bands were identified that were present in all three tissue samples and weighed 119,000Da, 18,000Da and 7,400Da respectively.

Gradient gel electrophoresis did, however reveal several low molecular weight components unique to both the AGC and the urosomal tissue. Within the AGC tissue three such proteins were identified that were not apparent in either the non-glandular control or the urosomal gland tissue (**Figure 6.2**). The molecular weights of these components can only be roughly estimated, as they had migrated beyond the lowest molecular weight marker and so their size could not be stated with any degree of certainty. The approximate weights of these proteins were 9,900Da, 6,700Da, and 3,900Da. A small molecular weight component (approximately 5,200Da) was present in both the urosomal gland tissue (**Figure 6.2**) and the AGC which was absent from the control tissue. A single stained band of

approximately 50,800Da weight was evident in the control tissue sample that was not present in the glandular tissues (**Figures 6.1** and **6.2**).

Use of 8% and 12% linear native gels provided little more information than was derived from the gradient gels. A typical 8% linear native gel stained with Coomassie Blue is shown in **Figure 6.3**. In effect, the 8% gel displays the magnified top half of a gradient gel and does not display any proteins that were not identified on the gradient gels. The thick band of unresolved proteins is particularly clearly displayed, along with some streaked staining above the band. Use of the 8% gel to determine the molecular weight region of the thick band yielded a higher estimate of weights than obtained from the gradient gel. Data from the 8% gel suggested that the protein band occupies a range of molecular weights from 345,000Da – 385,000Da.

The Periodic acid-Schiff (PAS) stain was also used to stain native gels after electrophoresis. A strong reaction to the PAS stain indicates the presence of highly glycosylated proteins (Merril and Wishart, 1998). **Figure 6.4** shows a 12% native gel stained with PAS and clearly demonstrates the presence of a large band of highly glycosylated proteins (neutral mucus compounds) in both gland and non-gland sample lanes. This band of carbohydrate had migrated the same distance in each well and was present as a solid band in each lane that appeared to correspond to the broad band of dense staining observed in the 8% native gels between molecular weights 345,000Da and 385,000Da (**Figure 6.3**). The catalase from the molecular weight standard also stained positively suggesting that it also possessed a carbohydrate fraction. The sample wells in the stacking gel of all three samples also showed similar positive staining with PAS suggesting that a proportion of this mucus material had failed to enter the gel.

6.4.2 Chromogenic detection

The chromogenic detection techniques employed in this study proved difficult to utilise with any degree of success. The catalase detection technique was successful only in demonstrating the presence of the catalase molecular weight standard in 8 and 12% gels but did not identify any regions of catalase

activity within the sample lanes. The chromogenic method of detection of glutathione peroxidase did not reveal any areas of staining in any of the tissues tested.

TMB proved of the greatest utility in identifying regions of peroxidase activity in native gels. In 12% gels, regions of positive TMB staining were restricted to two bands in a region that extended both above and below the 232,000Da catalase standard (**Figure 6.5**) suggesting that the protein, or proteins, responsible had a molecular weight of at least 200,000Da but were possibly somewhat larger than this value. The molecular weights of the TMB stained components could only be crudely estimated from the position of the catalase marker as visualisation of the TMB stain meant that Coomassie Brilliant Blue staining had to be forsaken and so no relevant molecular weight standards could be visualised.

Of these two stained bands, the one that had a slightly larger molecular weight stained more intensely than the lower molecular weight band. The same two or three stained bands were also apparent in the non-gland control lane (**Figure 6.5**) although they stained much less intensely than those of the glandular tissues. The sample wells of the AGC and the urosomal gland sample both showed a positive response to the TMB stain indicating that the active compound / s had not entered the gel, but very little staining was evident in the well of the non-gland control sample. Native 8% gels stained with TMB showed a much less marked response with only one faint band appearing in the AGC lane. The reason for this difference in staining between gels of different concentrations is not clear.

6.4.3 SDS-PAGE of gland material

SDS-PAGE was effective at separating the components of the tissue samples revealing a range of proteins with molecular weights ranging from 6,000Da to at least 125,000Da (**Figure 6.6**). A thick band of unresolved proteins as shown in **Figure 6.6** was again present in all three tissue types. The approximate molecular weights of the proteins within the band were estimated to lie between 101,600Da and 109,900Da.

Only two of the resolved proteins were present in the gland lanes that were not present in the control lanes. A protein of approximately 41,700Da was identified exclusively in the AGC lane (**Figure**

6.6 and 6.7) whilst a smaller (approx. 6,000Da) (**Figure 6.6** and **6.7**) protein was detected exclusively in the urosomal gland lane. No other proteins were detected exclusively in the samples of urosomal gland tissue.

6.4.4 SDS-PAGE of *L. salmonis* culture water

SDS-PAGE of the concentrated culture water of *L. salmonis* proved to be of great utility in this study. The initial use of 12% gels revealed that only four closely spaced bands of protein were present in samples of the culture water. These bands had barely entered the separating gel and extended from above the second highest molecular weight marker (β galactosidase, 158,194Da) nearly to the highest molecular weight marker (myosin, 212,000Da) as shown in **Figure 6.8**. The gel was expected to identify the relatively low molecular weight subunits of the catalase molecule and therefore the result suggested that the proteins in the sample had incompletely denatured. To overcome this problem the samples were electrophoresed again using fresh buffer and using gels of different acrylamide concentrations: 8% and 4-20%. Use of 8% linear and 4-20% gradient gels allowed the protein subunits detected on the 12% linear gel to migrate more fully. The subunits formed two closely spaced bands in both concentrations of gel although the sharp delineation of the subunit bands in the initial 12% gel was not achieved. Due to the slight distortion of the bands that occurred during electrophoresis, accurate estimation of their molecular weights was difficult to achieve but they were calculated to lie approximately between 65,000Da and 70,000Da. The migration of these bands on an 8% SDS gel is shown in **Figure 6.9**.

6.5 DISCUSSION

Electrophoretic analysis of the glands and secretions of *L. salmonis* undertaken in this study has allowed the resolution of a catalase molecule from amongst the numerous proteins of the tissues studied. TMB staining of native gels and SDS-PAGE of the culture water of *L. salmonis* adults provided the greatest insights into both the activity and the quaternary structure of this enzyme.

SDS-PAGE of the culture water of *L. salmonis* provided the clearest evidence of a high molecular weight protein, which is considered to originate either within the gut or exocrine secretions of the animal. Proteins identified from the culture water should represent only those which are either secreted or excreted by the louse, although some host origin proteins may also be present. The fact that only four closely located protein bands (**Figure 6.8**) were detected accords with the theory that a single tetrameric catalase enzyme is present in the gland secretions of *L. salmonis*. The molecular weight of between 65,000Da and 70,000Da estimated from gels for these subunits suggests a whole enzyme weight of approximately 230,000Da. This figure accords with the range of values suggested for the complete enzyme by the TMB staining of native gels as well as catalase enzymes identified in other studies such as those by Kim *et al.* (1994) and Kurata *et al.* (1999) who identified catalases of 225kDa and 216kDa respectively. The stained subunits were less clearly defined in the gradient and 8% gels but protein bands are known to lose their clarity the further they migrate through a gel (K. Thompson, pers. comm). SDS-PAGE is a 'semi-empirical' method in that the values of molecular weights of unknown proteins are derived by comparison to standards, as opposed to empirical techniques where values are calculated from equations (Price and Stevens, 1982). Even under optimal conditions, the nature of this technique therefore precludes the precise determination of the molecular weights of proteins, although the determination of molecular weights by comparison with standards is asserted to be accurate within a 10% margin (Shi and Jackowski, 1998).

Native gel analysis was less accurate, in terms of molecular weight estimation, and suggested that the total weight of the protein lay between 200,000Da (from TMB staining of 12% gels) and 380,000Da (from Coomassie Blue stained 4-20% gradient and 8% linear gels).

The dense band of staining observed in all three samples in native gradient gels suggests that a substantial component of the protein profile was not fully resolved using this system. It is possible, but unlikely that all the proteins present within this band are of the same molecular weight. It is more likely that the large band of staining was artifactual and contained many unseparated proteins within it. Use of the PAS stain clearly showed that this band contained strongly glycosylated proteins or mucus compounds. The intensity of the PAS staining was similar in all three sample tissues despite the different tissue types presumed to be present in each. Molecular weight estimation of this band suggested that a range of proteins with weights ranging from 345,000Da to 385,000Da were present. Carbohydrates are known to migrate erroneously on electrophoresis gels (Price and Stevens 1982, Bollag *et al.* 1996) and their position in the gels may be looked upon as being artifactual. The TMB activity visualised so clearly in the samples, and shown in **Figure 6.5,** appeared to occur within this band of glycosylated proteins. Use of SDS-PAGE appeared to denature this glycoprotein fraction and allowed the protein profile to be more correctly visualised. The filtration step incorporated into the processing of the culture water for SDS-PAGE may have removed large mucus fragments and so may have removed entirely, or reduced the intensity of staining, of any components that may have been complexed to this material.

TMB stained gels frequently showed what appeared to be two, sometimes three, separate bands of activity as are evident in **Figure 6.5**. Gregory and Fridovich (1974) demonstrated, using electrophoresis, two different enzymes with catalase activity in the bacteria *Escherichia coli*, one with a molecular weight of 330,000Da and the other of 240,000Da. These two different forms of the enzyme had different specificitys, the lighter of the two acting solely against hydrogen peroxide and the heavier one having a broader range of activities. The presence of two separate staining proteins in the area of expected catalase activity enzymes in the gland tissues of *L. salmonis* suggest that more than one species of catalase is present. The presence of TMB positive staining in the AGC samples confirms the results of the enzyme

assays of this study where catalase activity was also detected in these tissues. The apparent difficulty of protein migration may have hindered the correct expression of this protein.

The almost identical pattern of staining observed with the PAS and TMB stains in native gels suggests that the catalase enzyme was contained entirely within the mucus fraction of the gland secretion. It is unclear whether this is merely a physical entrapment of the enzyme within its gland co-product or whether there is a chemical linkage between the two. Certainly, treatment with SDS successfully freed the catalase from its mucus associate.

The frequent identification of TMB-positive staining, and therefore peroxidase activity, in the sample wells of native gels suggests that the native gel electrophoresis system was not optimal for the separation of enzyme components of *L. salmonis* glandular proteins. The failure of proteins to migrate may have been attributable to any of several factors. Proteins may have failed to enter the gel if they were excessively large, if they carried the wrong charge or if they were contained within, or bound to, another compound which itself was unable to migrate under these conditions. It is unlikely that the activity detected in the sample wells was due to individual proteins that were too large to migrate, or enter the gel, as the highest molecular weight standard (thyroglobulin, 669,000Da) had successfully entered the gel and all characterised catalases and peroxidases have molecular weights considerably below this value (Lyons *et al.* 1981, Miwa *et al.* 1983, Kim *et al.* 1994, Rocha *et al.* 1995). It is also possible that enzyme-containing organelles such as peroxisomes were not disrupted by the homogenisation procedure utilised and these would be unlikely to be pelleted by the centrifugation used here. Whole peroxisomes in the sample would remain in the well and their peroxidatic enzyme activity would be detected there. TEM examination of DAB stained urosomal glands undertaken in Chapter 5 however indicated that peroxisomes were not a feature of these tissues and therefore the enzyme activity observed in sample wells is probably not attributable to them. It is most likely therefore that this failure to migrate arose as a result of the active component of the secretion being complexed to either a lipid or a glycoprotein, or a mixture of both. The small granular inclusions of the gland cytoplasm that stained positively for Se-GPX in Chapter 5 would also not have been pelleted by the centrifugation that was utilised in the sample

preparation. Positive TMB staining of the sample wells could have been caused by the presence of these organelles. The evidence from chapters 4 and 5 of this study suggests that only carbohydrates i.e. mucus, and not lipid compounds, are likely to be present in the gland tissues, probably as a component of the gland secretion. Lipids present in the tissue sample would be unlikely to be present in the processed sample loaded onto the gel, as they would be likely to aggregate at the surface of the homogenisation buffer due to their hydrophobic nature. Carbohydrates however are known to be problematic under native gel electrophoresis conditions and evidence suggests that they migrate erroneously during electrophoresis (Bollag *et al.* 1996). The evidence from the PAS staining of native gels indicated that mucus components were prominent in both the thick bands of unresolved proteins as well as in the sample wells.

Although peroxidase activity was identified in both the glandular and the non-glandular samples the activity was markedly more intense in the samples of gland material. Due to the difficulty of excising usable quantities of non-glandular material from *L. salmonis* some slight amount of enzymatic activity was to be expected in the non-gland samples as small numbers of dorsal surface glands were inadvertently and unavoidably included in those samples as was the case with the enzyme assays of Chapter 5. Without recourse to densitometry analyses, it is not possible to quantify relative activities / amounts of enzyme between the two samples but it would appear from **Figure 6.5** that peroxidase activities are more pronounced in those lanes containing glandular material, a phenomenon that was consistent throughout all samples analysed using this technique. As protein analysis indicated that similar quantities of proteins were present in both the glandular and non-glandular samples it is reasonable to assume that the greater reactivity of the samples in the gland lanes can be attributed to the activity of the enzymes and does not arise as a result of greater quantities of material in the sample. The equal protein content of the tissue samples may be supported by the appearance (i.e. density) of the non-specific bands on the gels. Bands of protein which were present in all three tissue samples displayed similar staining intensities, as would be expected for general tissue proteins.

The specific chromogenic detection techniques for catalase and glutathione peroxidase proved to be of limited utility in this study. The glutathione peroxidase assay did not indicate any regions of

observable activity and this may be explained by the generally low levels of this enzyme in all *L. salmonis* tissues as shown in chapter 5 of this study. There are several possible reasons why the chromogenic tests to identify catalase did not provide clear evidence of its presence but the most likely reason is that freezing is known to detrimentally affect the activity of catalase (Halliwell and Gutteridge, 1985) and all samples used in the electrophoretic analyses had been stored at -70°C. It is also possible that insufficient quantities of enzyme were present in the samples to give a positive response using the catalase and glutathione peroxidase assays, although the TMB stain was sufficiently sensitive to allow sites of peroxidase activity to be readily detected in native gels. No sites of enzyme activity that could be correlated to the activity of a protein of an appropriate molecular weight to suggest the presence of glutathione peroxidase were detected using either the TMB stain or the specific zymographic assay. The spectrophotometric assays employed in Chapter 5 indicated that Se-GPX was present only at very low levels in the gland tissues of *L. salmonis* and it may be that the tests employed here were not sufficiently sensitive to identify this enzyme at the levels at which it is present. From the results obtained in Chapter 5, indicating that the secretory vesicles of the urosomal gland stained positively for catalase and not GPX concurs with the data obtained from the electrophoresis of the culture water of *L. salmonis*.

SDS-PAGE of *L. salmonis* glandular tissue identified only one unique protein in both the AGC and urosomal gland tissues. Only the larger protein in the AGC lane could possibly correspond to the appropriate size for a catalase subunit, the protein weights suggesting a complete molecule weight of between 178,000Da and 266,000Da working on the presumption of a four subunit and a six subunit molecule respectively. Although SDS-PAGE of gland tissues of *L. salmonis* revealed a wide molecular weight range of proteins to be present in all three tissues other proteins are likely to have been present at levels beyond the limit of detection of the Coomassie Brilliant Blue staining technique.

SDS-PAGE of *L. salmonis* culture water indicated that the secretory catalase of *L. salmonis* exocrine glands is a tetrameric protein and that the molecular weight of the subunits lay between 65,000Da and 70,000Da. SDS-PAGE of the culture water of *L. salmonis* indicated very few proteins to be present at the limit of detection of Coomassie Brilliant Blue. No native gel analysis of culture water was

undertaken but it is presumed that the proteins present, and detected using SDS-PAGE, are those active components detected on native gels of whole gland homogenate. Problems of protein migration observed with this sample in some gels (such as that shown in **Figure 6.8**) may have been caused by the high salt content of the sample solution (approx. 5%). Use of three different gel concentrations (8%, 12% and 4-20% gradient gels) allowed more accurate determination of the structure of the secreted enzyme than a single gel concentration alone. No other protein components were detected in the culture water of *L. salmonis* in spite of the fact that other studies have shown that enzymes, presumed to be gut-derived, can be detected in sea louse conditioned media (Butler, pers. comm). This apparent absence of such proteins in the culture water may be attributable to the levels of enzyme present. Coomassie Brilliant Blue staining is sensitive to detect protein concentrations of 0.1μg, whereas a more sensitive method such as silver staining (sensitive to 2ng) may have revealed components that were present but not revealed by the Coomassie staining. It is unlikely that any other enzymes (especially proteases as might be found in the gut (Ellis *et al.* 1990, Andrade-Salas 1997, Perkins *et al.* 1997) were lost during sample preparation as the largest molecular weight limit of the processing schedule was 12,000Da - 14,000Da during dialysis. Most protease components are likely to be significantly larger than this (Karlstam and Ljunglof 1993, Bourtzis, Marmaras and Zacharopolous 1993, Kaga, Laurent, Doh, Luffau, Yvore and Pery 1998, Fetterer and Rhoads 2000). Research by Ross *et al.* (2000) has identified various proteases from the mucus of *L. salmonis* infected Atlantic salmon. The source of these enzymes was not identified in the study but may theoretically be from the louse itself, either as secreted or excreted compounds. Butler (pers. comm) identified several enzymes from the culture media of *L. salmonis* including leucine aminopeptidase, acid and alkaline phosphatase and β glucuronidase all of which have molecular weights within the size range that should have been retained in the processed culture water of this study (Karlstam and Ljunglof 1991, Bourtzis *et al.* 1993, Ribolla, Daffre and DeBianchi 1993, Polzer and Conradt 1994). The failure to identify these enzymatic components in the culture water of *L. salmonis* may be attributed to the fact that digestive proteases are likely to be present only at very low levels, as stated earlier, but may also be due to

a reduction in enzymatic activity as might be expected in specimens that had been starved for a minimum of 24h prior to sampling. The specimens from which Butler derived his secreted proteases were actively feeding and would be in the process of digesting and excreting ingested material and detection of enzymes under these conditions is to be expected. Butlers work did concentrate on host-associated copepodid stages that may be in the process of secreting compounds that may be released only whilst associated with the host e.g. feeding enzymes or 'immuno-active' substances. Chróst (1991) has demonstrated significant levels of bacterial ectoenzymes to be routinely present in seawater but as sterilised seawater was used in this study the possibility that the detected proteins were from a source other than *L. salmonis* can be discounted.

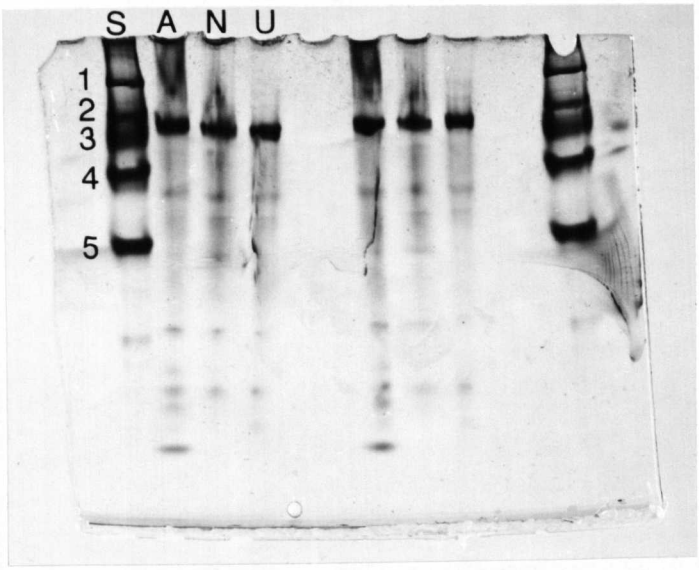
6.6 CONCLUSIONS

Application of electrophoretic techniques has allowed the confirmation of the presence of a catalase enzyme as suggested by the enzyme assays and TEM techniques utilised earlier in this study. No previous studies have utilised electrophoresis to identify the components of copepod exocrine gland secretions.

The results indicate that the secretory catalase of *L. salmonis* is a tetrameric molecule with a total molecular weight, derived from individual subunit weights, of 280,000Da. This is the first characterisation of the quaternary structure of an exocrine gland secretory enzyme of the exocrine glands of any crustacean. The techniques utilised did not however allow the identification of the glutathione peroxidase component identified through enzyme assays and TEM procedures as described in chapter 5.

Successful gel electrophoresis relies on the optimisation of many variables that can require considerable refinement of the technique to achieve. The results of the electrophoresis employed in this study were informative given the limitations due to unavoidable restrictions of time and material. The data presented here, though, requires further refinement of the protocols to provide more accurate estimates of the structure of the catalase molecule. The application of two-dimensional electrophoresis would be of utility in providing greater resolution of sample proteins and would allow the identification of

multiple protein components with identical molecular weights. Such analysis would help separate some of the extraneous proteins still contained within the sample. Now that the approximate molecular weight of the catalase molecule has been established, further purification steps could be incorporated prior to SDS-PAGE to increase the purity of the protein. Such steps could involve molecular sieving and affinity chromatography and other associated techniques.



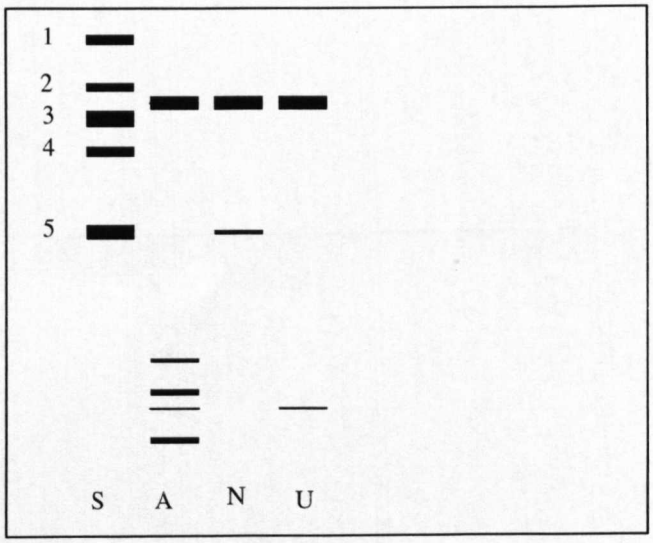


Figure 6.1 Native gradient (4-20%) polyacrylamide gel stained with Coomassie Brilliant Blue. S = High range MW standards, A = AGC tissue, N = control tissue, U = urosomal gland tissue. Standards, in order of descending MW: 1 669,000, 2 440,000, 3 232,000, 4 140,000, 5 67,000.

Figure 6.2 Diagrammatic representation of one half of a native 4-20% gel stained with Coomassie Brilliant Blue. S = High range MW standards, A = AGC tissue, N = control tissue, U = urosomal gland tissue. Standards as Figure 6.1.

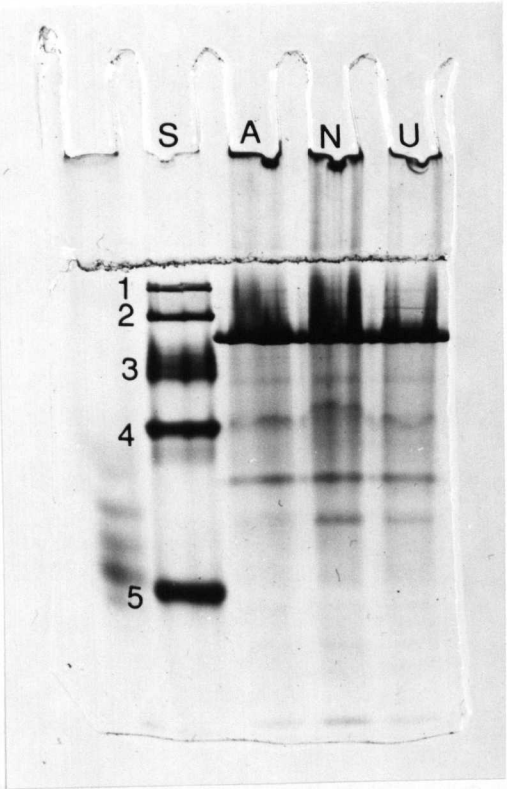
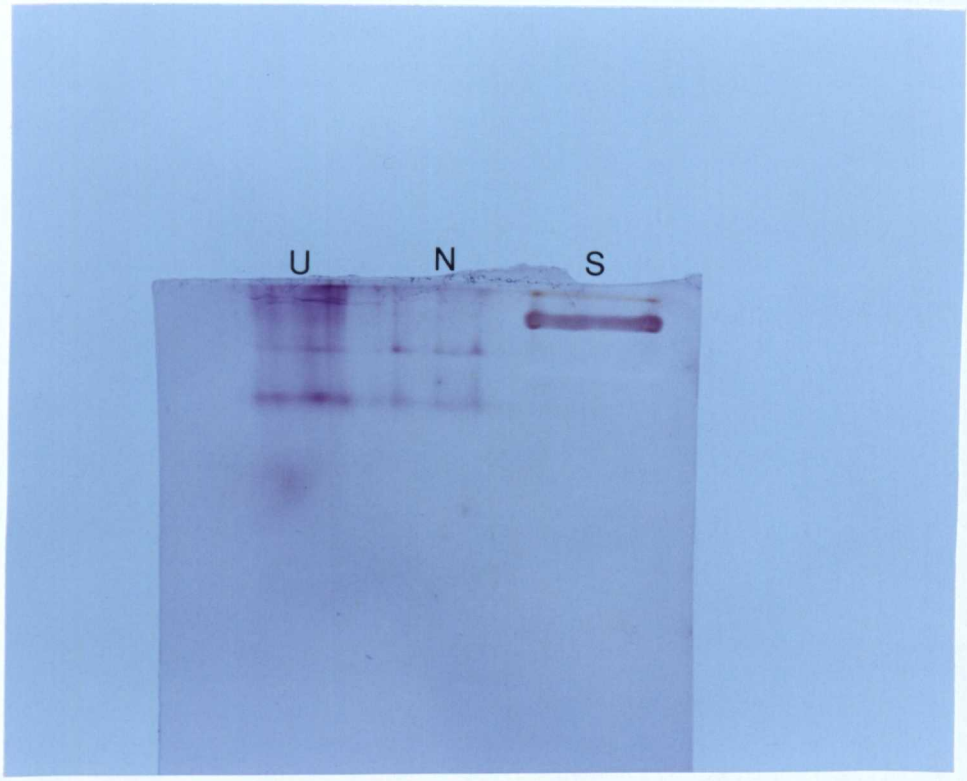


Figure 6.3 Native 8% linear gel of AGC, control and urosomal gland tissues stained with Coomassie Brilliant Blue. S = High range MW standards, A = AGC tissue, N = control tissue, U = urosomal gland tissue. Standards, in order of descending MW: 1 669,000; 2 440,000; 3 232,000; 4 140,000; 5 67,000.



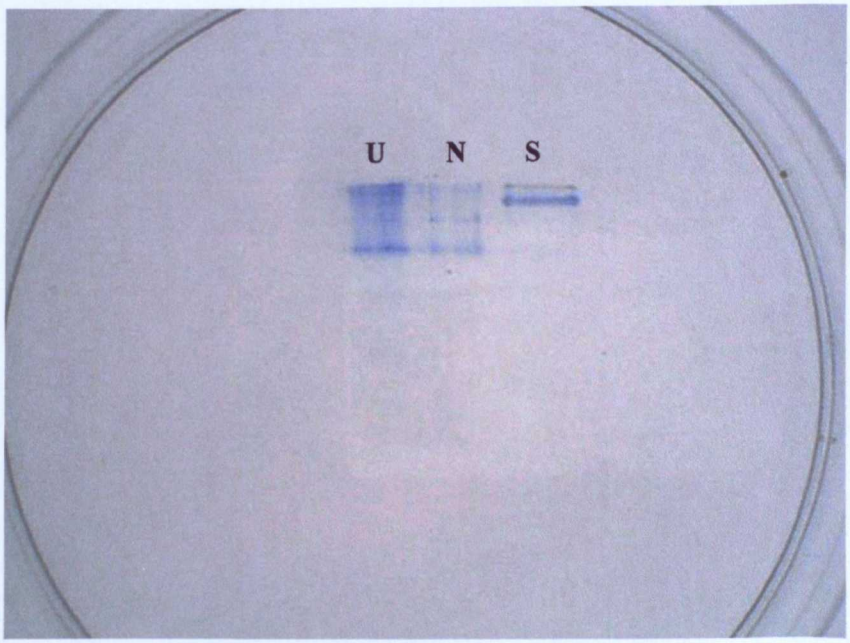
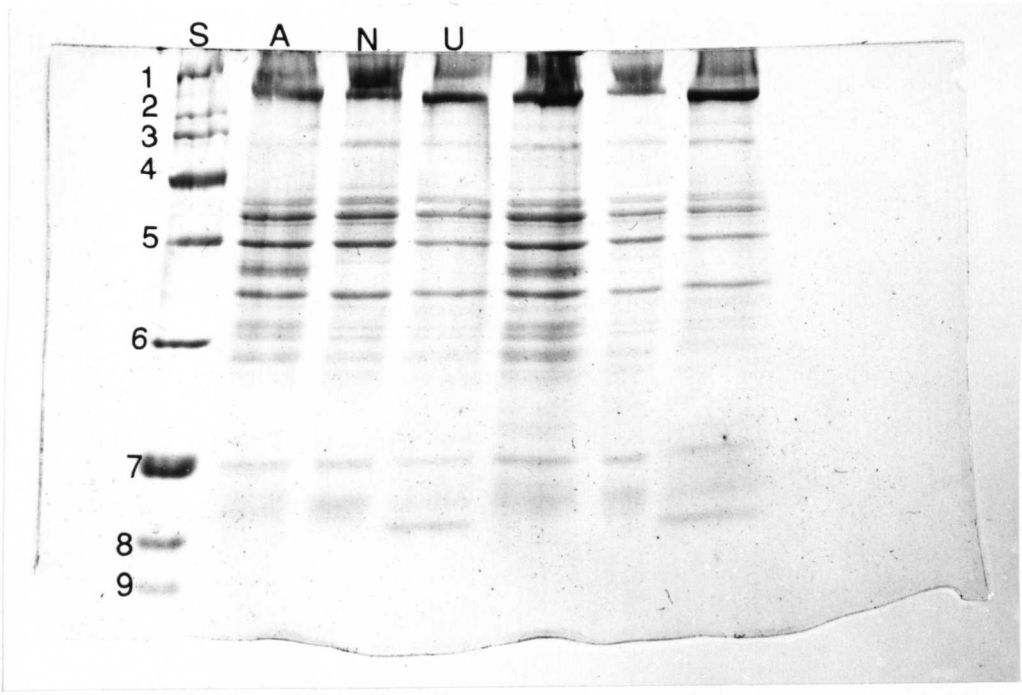


Figure 6.4 12% native gel stained with PAS to indicate sites of glycosylated proteins (mucus). U = urosomal, N = control tissue, S = molecular weight standard.

Figure 6.5 12% native gel stained with TMB to highlight areas of peroxidase enzyme activity. U = urosomal, N = control tissue, S = molecular weight standard.



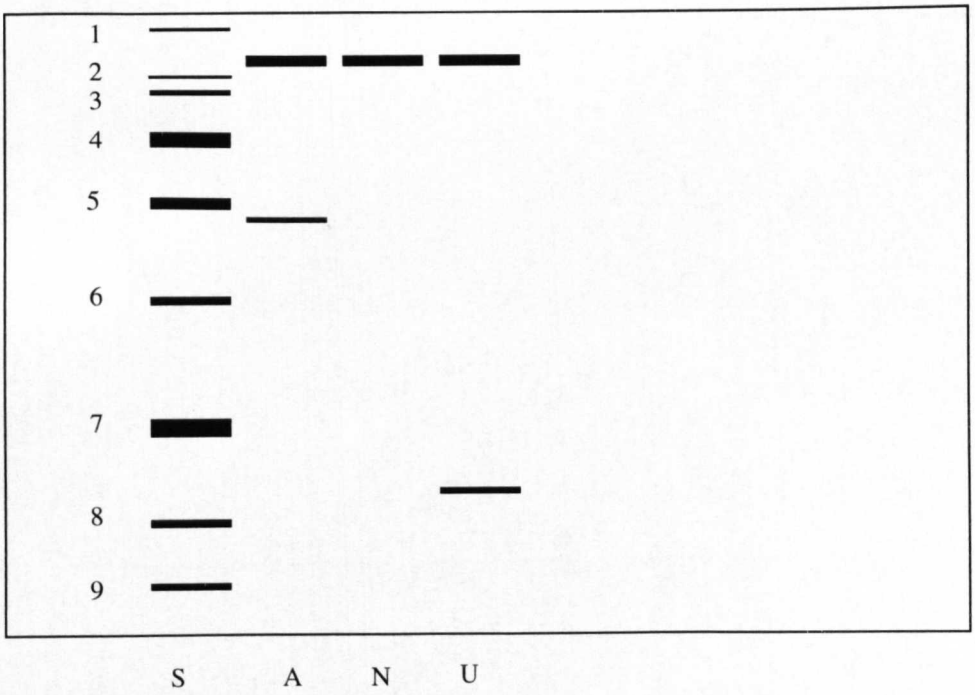
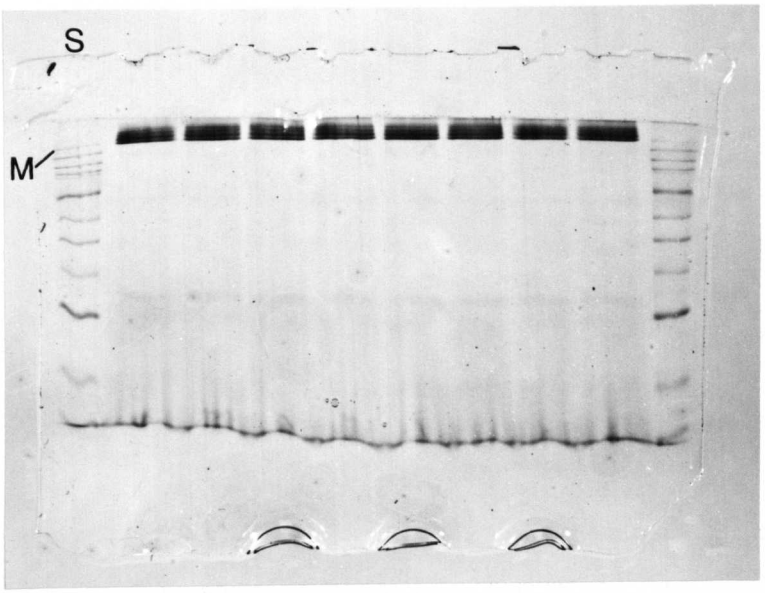


Figure 6.6 Coomassie Brilliant Blue stained 12% SDS gel displaying the protein profiles of the AGC, control and urosomal gland tissues. S = molecular weight standards; A = AGC tissue; N = control tissue; U = urosomal gland tissue. Molecular weight standards in descending weight order; 1 200,000; 2 116,500; 3 97,400; 4 66,200; 5 45,000; 6 31,000; 7 21,500; 8 14,400; 9 6,500.

Figure 6.7 Diagrammatic representation of the 12% SDS gel of Figure 6.6 highlighting bands of interest.



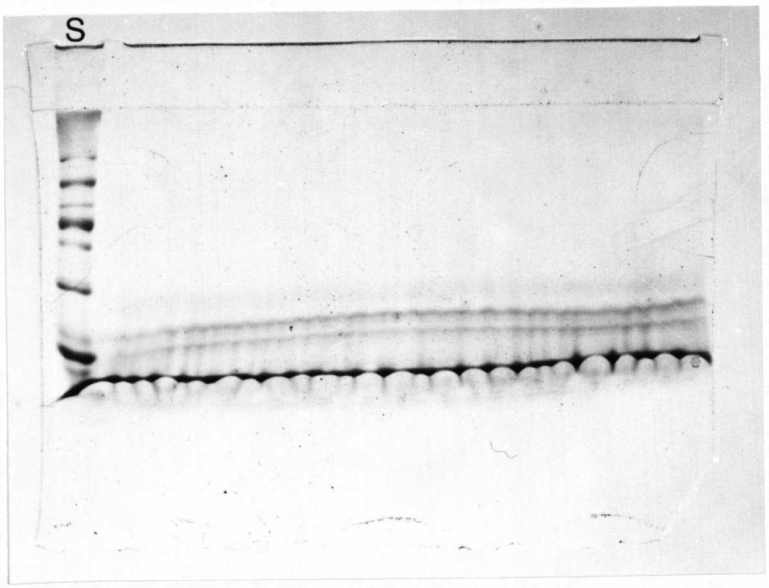


Figure 6.8 12% SDS gel demonstrating the proteins identified in the processed culture water of *L. salmonis*. S = molecular weight standards, M = myosin standard of 212,000Da. All other lanes are replicates of the same sample.

Figure 6.9 Protein profile of the culture water of *L. salmonis* as revealed by a single well (one 50µl sample) 8% SDS-PAGE. S = molecular weight standards.

7. FUNCTIONAL ATTRIBUTES

7. FUNCTIONAL ATTRIBUTES

7.1 INTRODUCTION

So far in this study we have established that caligid copepods possess a population of exocrine glands with unique staining characteristics and that these glands are largely absent in non-caligid species. The analyses of the present study have shown that the secretions of the described population of exocrine glands of the caligid copepod *L. salmonis* consist of at least a mucus component and a catalase enzyme. The work described in this chapter aims to investigate the significance of these glands, and their secretions, to the biology and life history of caligid species.

Secretions of exocrine glands of copepods have been suggested to serve a number of different purposes beside those directly associated with feeding such as, drag reduction (Von Vaupel Klein 1982, Brunet *et al.* 1991, Bannister 1993a), prevention of cuticle biofouling (Boxshall 1982, Bannister 1993a), predator deterrence (Pochon-Masson *et al.* 1975, Boxshall 1982), evasion of host defenses (Briggs, 1978) and pheromone production (Fleminger 1967, Gharagozlou-van-Ginneken 1979). Mucous secretions in non-copepod invertebrates have been widely researched and have been found to fulfill a wide variety of roles (Simkiss 1988, Denny 1989). These roles cover as diverse a range of functions as; locomotion, food capture (Beninger, St-Jean, Poussart, 1995), adhesion (Grenon and Walker, 1978), predator deterrence (Reel and Fuhrman 1981, Denny 1989), bactericide (McDade and Tripp 1967, Kubota, Watanabe, Otsuka, Tamiya, Tsuchiya and Matsumoto 1985) and physical protection from adhering epibionts (Thiesen 1972, Jones 1984). This list contains some of the more commonly occurring roles of secreted mucus, many other less common roles have also been identified. Surprisingly, despite the widespread occurrence and diversity of function of mucus compounds, the presence of mucous layers in copepods has been largely ignored; their presence generally accepted but with no real efforts to determine their significance having been undertaken.

Denny (1989) also alludes to the fact that mucus in marine species may function as a carrier for small molecular weight compounds that would be rapidly dispersed if freely released into the water.

Mucus will in these cases provide a stable layer on the animals surface which will retard the dispersion of secreted factors.

The evidence obtained so far in this study has discounted two possible functions of the secretions of the identified glands of *L. salmonis* i.e. cuticle tanning (as identified in amphipods by Stevenson (1961)) and prostaglandin production (Chapters 4 and 5). It is also unlikely that the glands will have an expressly pheromonal action, as the full complement of glands is not present until after mating has taken place. Other possible roles for these secretions must now be considered. These hypotheses are: firstly that the mucus secretion may either possess properties concerned with increasing the hydrodynamic efficiency of the copepod, or secondly, that they may provide some protection against organisms that foul the body cuticle. The work described in this chapter aims to test the feasibility of both of these hypotheses.

To understand how a mucus layer may be of hydrodynamic benefit to a copepod it is necessary to examine the swimming behaviour and morphological adaptations displayed by copepod species.

7.1.1 Copepod hydrodynamics

Studies of copepod hydrodynamic characteristics have tended to focus upon the propulsive mechanism of swimming (Morris, Gust and Torres, 1985) and the propagation of feeding currents (Andrews 1983, Emlet and Strathman 1985, Tiselius and Jonsson 1990, Yen and Strickler 1996) and have largely ignored the possibility of active secretions being involved in reducing turbulence and promoting hydrodynamically efficient swimming.

Morphology is likely to be closely linked to the hydrodynamic characteristics of copepods (Strickler, 1983) and therefore examination of selected morphological criteria may provide insights into the swimming mechanism of these animals.

7.1.2 Gland secretions and hydrodynamics

Hydrodynamically-active secretions have been suggested by some authors to be produced by exocrine glands of copepods. Von Vaupel-Klein (1982a, pg 382, para 6) proposed that slit-shaped pores in the integument of the calanoid copepod *Euchirella messinensis* (Claus, 1863) may '*be instrumental in*

extruding some fatty or oily secretion covering the copepod body with a hydrophobous layer' thereby explaining their ability to swim at speeds of up to 100cms^{-1}. That author also stated that, in copepods, there is a direct correlation between the number of integumental pores and swimming speed. This implies that the secretions passed from those pores have a quantifiable impact on water flow across the cuticle. It has been shown for several species of aquatic insects that gland secretions are utilised to propel them across the surface film of water (Vulinec 1987, Nachtigall 1974). The effect of surface active (surfactant) mucus secretions in some marine animals has also been investigated. Daniel (1981) showed that the epidermal mucus of the rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), reduced the friction drag of swimming by up to 50%. Bernadsky, Sar and Rosenberg (1993) also demonstrated clear drag-reducing effects in the epidermal mucus of several fish species as well as that of a benthic nudibranch although the degree of drag reduction varied greatly both between and within species and the complex interactions of these effects were never fully resolved. Such discoveries are recent additions to our understanding for, as recently as 1983, Alexander stated that there was *'no convincing zoological proof that secreted substances reduce local turbulence*'. To date no direct evidence of such a function has been demonstrated in copepods.

7.1.3 Sexual dimorphism and swimming behaviour in parasitic copepods

In recent years studies of the inter-host transfer behaviour of the copepod *L. salmonis* under experimental and culture conditions have been undertaken (Ritchie 1997, Hull, Pike, Mordue (Luntz), and Rae, 1998). These studies suggest that this species freely and frequently moves between hosts, demonstrating the capability of transfer at least at close quarters. Hull's study showed adult males to have significantly higher host transfer rates (3.5 times greater) over mated adult females, although this is in contrast to the results of Ritchie (1997) who found that male and female *L. salmonis* displayed similar transfer rates. Anstensrud (1990) demonstrated that male *Lepeophtheirus pectoralis* are capable of multiple fertilisations and display higher host transfer rates than females of the species. Whether this phenomenon of host transfer occurs in the wild with the lower host density conditions that pertain there

remains speculative. In contrast to females, males, after all suitable females on a host have been fertilised, have no vested interest to remain there. Males, with their capacity for multiple fertilisations, would presumably have greater incentives to transfer than females. Females usually mate once only and once mated can produce repeated batches of eggs from one pair of spermatophores. Salmon, in the wild, have lice densities which are sufficiently low (Todd, Walker, Hoyle, Northcott, Walker and Ritchie, 2000) that competition for food resources on any one fish would be unlikely to result in the decreased vitality of any one individual. Caligid copepods are relatively more adapted to parasitism than those of other genera such as the Ergasilidae where the male remains free-swimming throughout its life and such behavioural adaptations are likely to be reflected in morphological parameters.

Kabata (1979) gives an excellent account of the various pressures on parasitic copepods and the effect of these pressures on body morphology. In particular, he emphasizes the fact that males are often less modified than females, particularly regarding their locomotory ability. He also states (pg 4, para 4) that '*the foundations of sexual dimorphism are laid by the adoption of parasitism*', a statement which might be taken to suggest that more highly adapted parasitic species display greater dimorphism. Poulin (1996) suggests that copepods that parasitise fish display greater size sexual dimorphism over free-living species and those parasitic on sessile organisms. This is a feature he attributes to the decreased chance of the larvae of fish-associated species locating a suitable host having selected a greater capacity for reproduction in the females of such species. Poulin (1996) also states that males of such species tend to remain small and mobile to facilitate mate location but he does not explain how the small size of the male is of benefit in locating mates.

Hicks (1988) reinforced the conclusion of Kabata when he proposed that, in copepods, behavioural (including physiological) change precipitates morphological change. Swimming ability in copepods will in part depend on the mechanism of locomotion determined by morphological features such as position and length of natatory (and other) setae as well as the hydrodynamic efficiency of the organism which itself is contingent upon its morphology and possibly also its glandular secretions. These will in

turn depend on the specific life history attributes of the species in question such as host selection, reproductive strategy and the sex of the individual.

7.1.4 Reynolds number

To fully understand the significance of morphological adaptations of copepods to their hydrodynamic environment, knowledge of Reynolds numbers theory is required. The Reynolds number (Re) is a figure used to describe the relative importance of local viscosity to an animal and is essential when comparing the motion of differently sized and / or shaped objects through a fluid of constant viscosity or similarly sized and / or shaped objects through fluids of different viscosities. The Reynolds number is a dimensionless index that '*expresses the relative importance of pressure versus viscous drag*' (Alexander and Chen, 1990, page 409, para 2). As a general rule, as speed and size increase, so does the Re number.

At very low Re values (<1) viscous forces are dominant, a thick boundary layer coats the animal, the orientation of an animal has little effect on its drag profile and flow is essentially reversible. A boundary layer is the layer of water that separates an animal from the ambient water flow, the thickness of which depends on the viscosity of the fluid. The fluid against the solid surface in a boundary layer is stationary and its velocity increases moving away from the animal until the speed of flow reaches that of the ambient water flow. At very low Re's turbulence is negligible and friction drag ('*the force exerted on the sides of a body due to the viscosity of a fluid*', Alexander, 1983, pg 196, para 2) predominates, but as the size of an animal increases so does the Re, until inertial forces come to bear and turbulence and pressure drag effects come to the fore. In effect the animal experiences the surrounding water as a sticky medium, similar to our perception of honey. High Re environments ($>10^5$), on the other hand, are typified by thin boundary layers, pressure drag and moving bodies that leave disturbed, turbulent wakes. Pressure drag is the dominant drag force under these conditions and differs from viscous drag in that it is due to the difference in pressure of passing water between the front and rear of a body. Inertial conditions are required before pressure drag takes effect.

There is a wide, but loosely defined, range of Re conditions which lie between the two extreme conditions described above where both viscous and pressure drag will have an effect on a moving animal.

The term 'microenvironment' can be applied to the environment in the immediate vicinity of a small organism, and can be used to describe local effects that are not apparent above an organismal level. Copepods are generally considered to live at a low Reynolds number microenvironment (Svetlichnyy 1983, Strickler 1985, Mauchline 1998). In such a situation they exist in what is effectively a viscous medium, but during periods of increased swimming speed such as predator avoidance or host seeking, they may cross the transition between viscous and inertial flows (Mauchline, 1998). Herring *et al.* (1993) showed that the luminescent copepod *Oncaea conifera* Giesbrecht exists at a Re of between 10 and 48, where viscosity is so great that it would be impossible for it to escape from an ejected plume of luminescent material. Significantly that species is one of the few to retain its luminescent material within its glands, as it would be unable to swim away from the secreted material.

7.1.5 Setae, hydrodynamics and sinking rate

Many aquatic insects have elaborate caudal cerci, which are homologous with the terminal setae of the caudal rami of copepods. Whether these setae are involved in locomotion or have some other function such as predator deterrence, or spatial awareness (Kabata and Hewitt 1971, Gill 1990) is unclear. The aquatic nymph stages of the Ephemeropteran genus *Baetis* show marked differences in the morphology of their caudal cerci depending on the speed of flow in the streams in which they live (Vogel, 1994). Nymphs of species dwelling in higher speed flows have shorter caudal cerci than those species from streams with lower flow rates. This might suggest that long caudal cerci have some sort of detrimental effect at high flow speeds. These nymphs are dorsoventrally flattened and live on the surfaces of submerged rocks, a morphological condition that is similar to that of sea lice. We can reasonably surmise that longer setae cause problems of being swept off from their chosen substrate. Whether this is effected through increased drag or an increase in lift is unknown.

The setae of copepods (and small insects) may function in ways that are not consistent with our experience of similar structures in our own environment on account of the very low Re values experienced by very small organisms in fluids. It is often presumed that such small setae would function as paddles rather than as filters as we would expect in our inertia-dominated world (Strickler, 1983). The critical value that determines the function of such structures is the Reynolds number.

An increase in cephalisation, as displayed by caligid copepods, is generally supposed to be reflected in a decrease in strong swimming ability (Kabata, 1979). Hicks (1988), though, states that a calanoid body-form is not essential for efficient swimming, rather the use of setose cephalic appendages and anti-sinking devices are likely to permit effective swimming in species lacking the calanoid morphology. In his paper on the evolutionary implications of copepod swimming Hicks (1988) describes how meiobenthic harpacticoid copepods utilise beating of the swimming legs and flexion of the urosome to effect swimming movements and how apparently poorly adapted swimmers could display considerable swimming ability.

It has also been suggested that the terminal setae of *L. salmonis* serve to assist in spatial awareness. *In vitro* specimens sinking in a tail-down orientation were seen to initiate swimming movements in response to the terminal setae of the caudal rami touching the bottom of the experimental vessel, as recorded by Kabata and Hewitt (1971) and Wootten *et al.* (1982). This suggests that those setae may be innervated, as suggested earlier in Chapter 4. Such spatial awareness will undoubtedly be essential to the success of the animal and the maintenance of these setae to keep them in condition may also be a possible role of gland secretions.

Passive sinking rates of copepods may shed light on a number of aspects regarding their morphology and physiology. Sinking rates are likely to reflect physiological factors such as body composition as well as morphological characteristics such as cuticular ornamentation. Svetlichnyy (1983) studied the passive sinking rates of several species of copepod and found that an increase in surface area caused an increase in hydrodynamic resistance typified by low Re rather than particular body shape.

Interestingly, the copepods tested in that study had been fixed in formalin and the effects that immersion in this fixative might have on body morphology and any secretions is overlooked by the author.

Free-living copepods exhibit a range of feeding strategies that, in some species, includes passive sinking (Jonsson and Tiselius 1990, Tiselius and Jonsson 1990). This passive sinking is believed to make predatory copepods hydrodynamically inconspicuous and consequently more able to capture prey items before they can be startled by turbulent effects. Passive sinking may also allow raptorial feeders to detect moving food particles.

Some members of the Harpacticoida, in contrast to the majority of that Order, have become adapted to a planktonic existence. These species display adaptations proposed to slow their rate of sinking such as elongate caudal rami, elongate terminal setae or internal oil droplets (Huys and Boxshall, 1991). There appears to be some confusion regarding the utility of such morphological adaptations to retard sinking of planktonic species. The established view of early researchers (Lowndes, 1942 and references therein) was that setae and spines of planktonic species serve to slow the rate of passive sinking. Vega and Clausse (1998) demonstrated that the tail spine of *Daphnia middendorffiana* Fisher had no effect on the rate of sinking and instead suggested that such spines serve to protect smaller stages from predators. This is in contrast to the widely held belief that cuticular elaboration's in planktonic species serve to reduce sinking rates and help maintain the position of the animal in the surface waters. (Lowndes 1942, Hicks 1988, Mauchline 1998).

7.1.6 Method of propulsion

Kabata and Hewitt (1971) provide what is the only detailed description of the method of swimming propulsion of caligid copepods. According to Kabata and Hewitt (1971) caligids use the first two thoracic legs to provide propulsion and an alteration of the position of the genital segment and abdomen to effect directional changes – dorsiflexion causing movement in an upward direction and ventriflexion movement in a downward direction. This action is augmented by the caudal rami and associated setae. Kabata (1979) also suggests that the setae of the caudal rami of cyclopoids act as a steering oar or vane. The use

of the terminal setae to provide steering ability has also been described for the calanoid *Euchirella messinensis* (Claus, 1863) by Von Vaupel Klein (1982b) which may imply that calanoids as a family utilise their terminal setae in swimming movements.

It is evident therefore that a great combination of factors dictate swimming ability and hydrodynamic efficiency in copepods and all such factors must be considered when making predictions of these characteristics.

7.1.7 Copepod-associated bacteria

7.1.7.1 Bacterial colonisation of copepod cuticles

Anti-bacterial properties of copepod gland secretions have been suggested for several copepod species (Boxshall 1982, Bannister 1993) and evidence exists to suggest that secretions of selected glands of some terrestrial insect species are capable of reducing the numbers of bacteria colonising the cuticle e.g. Kovac and Maschwitz (1991). SEM examination of the cuticle of *L. salmonis* in this study (and in the study of Nese and Enger (1993)) has revealed that bacteria are commonly found adhering to the external surfaces of the cuticle, often in great numbers. The species present generally display two distinct morphologies being either short, stout and cylindrical or are longer and thinner and more thread like. The observed bacteria are more frequently found in protected regions of the body surface such as in the joints of limbs and on the setae of the caudal rami and thoracic legs. This appears to be a common feature of bacterial colonisations of copepod cuticles as Dumontet *et al.* (1996) describe the occurrence of bacteria on the cuticle of the calanoid *Acartia margalefi* Alcaraz, 1976 as being especially prominent in protected regions such as the swimming legs and in joints of the cuticle. Nagasawa, Simidu and Nemoto (1985) also provide evidence that bacteria colonising marine copepods appear to favour what are apparently sheltered areas of the body such as in the joints of legs and segments and in low-lying areas of cuticle.

Bacteria with chitinolytic properties are widespread in the marine environment and have been well documented (Simidu, Ashino and Kaneko 1971, Osawa and Koga 1995, Dumontet *et al.* 1996). Species

of the bacteria genus *Vibrio* have been suggested to play a significant role in the decomposition and recycling of chitin in the sea as well as in disease transmission to both humans (Dumontet *et al.* 1996) and fish (Wootten *et al.* 1982). Particularly, Simidu *et al.* (1971) suggest that *Vibrio* spp. and *Aeromonas* spp. are commonly associated with marine animals whilst Sochard, Wilson, Austin and Colwell (1979) state that *Vibrio* spp. are the predominant genus of bacteria colonising marine copepods. Nylund, Wallace and Hovland (1993) demonstrated the presence of more than ten different species of bacteria on the cuticle of *L. salmonis* whilst Nese and Enger (1993) identified *Aeromonas salmonicida* (the causative agent of furunculosis) as a species present on both the external surfaces and gut of *L. salmonis*. Those authors suggest that *L. salmonis* may serve as a vector of this disease as lice removed from furunculosis-infected fish harboured large numbers of these bacteria. However, lice from uninfected salmon were not examined and so it cannot be said whether the lice did indeed act as carriers of the disease or whether they had merely picked up the bacteria from the infected fish.

7.2 OBJECTIVES

This chapter reports the work carried out to determine, from an analysis of the sinking rates of anaesthetised copepods, whether the secretions from those glands exerted a measurable effect on the hydrodynamic characteristics of *L. salmonis*. Swimming speeds of male and female *L. salmonis* were determined to allow morphological characteristics to be assessed in relation to the Reynolds number environment of the animal. Analysis of the relative abundance of the identified glands between different caligid species was also undertaken, along with an analysis of the morphology of the terminal setae of those species, with the view to form a hypothesis regarding behaviour from such morphological characteristics.

Bacteriological studies were also conducted to determine the diversity of the external bacterial fauna of *L. salmonis*, the effect of exocrine gland secretions on that fauna and to determine whether any of the identified bacterial species possessed demonstrable chitinolytic properties that might pose a threat to the condition of the louse.

The objectives of this chapter were realised using the following procedures:

- Fuzzy layer examination
- Sinking rate analysis of *L. salmonis*
- Swimming speed determination
- Morphological comparison and sexual dimorphism analysis
- Bacteriological analysis

7.3 MATERIALS AND METHODS

7.3.1 Sinking rate analysis

An experiment was designed that would provide some direct evidence of the functional role of the gland secretions. It has been proposed that gland secretions may affect the hydrodynamic efficiency of copepods and therefore the hydrodynamic qualities of the secretion may help determine locomotory patterns in these organisms.

To determine whether the secretions of the identified exocrine glands of *L. salmonis* have a demonstrable effect on the hydrodynamic characteristics of that species, a straightforward sinking experiment was designed. The experiment had a simple premise whereby the passive sinking rate of an anaesthetised louse would initially be determined and then reassessed in the same specimen after the secreted fuzzy layer had been removed following chemical treatment. Adult males were chosen as the subject for this study because it was thought that males would be better adapted to swimming freely in search of hosts and might therefore have a greater requirement to assist the smooth flow of water over their cuticle. In addition to this, males are of a more uniform size than females as they do not vary in size according to the developmental state of the genital segment.

7.3.2 Examination of the Fuzzy Layer

Mucus compounds are known to be difficult to preserve during processing for TEM, due to their tendency to dissolve in aqueous fixatives, although recently, techniques have been developed which are suggested to preserve these layers, in fish at least, with greater integrity (Horne and Sims, 1999 and **Appendix 6**). This process involves the brief pre-fixation of mucus material, *in situ*, in a solution of 0.5% w/v osmium tetroxide in the perfluorocarbon solvent FC-72 (Acros Organics 12379-0100). Following this, the samples are processed for TEM as described in Chapter 2.

In order to assess the structure of the mucus layer on the cuticle *in vivo*, samples of *L. salmonis* cephalothorax cuticle were processed for TEM analysis using a conventional aqueous fixative (Karnovsky, 1965) and the novel technique of Horne and Sims (1998) which was designed to fix mucus layers *in situ*. The aim of this study was to determine whether the novel fixation process retained the fuzzy layer more completely than the standard process used (Section 2.2.2). Lice immersed for 5min in sterile seawater only, were used as a control and processed for TEM using both the novel and conventional fixatives to serve as negative controls.

7.3.3 Mucus Removal

As the sinking experiment relied upon the successful removal of the external mucus layer the mode of removal of this layer had to be carefully considered. To achieve the removal of this layer a brief trial was conducted that involved treating lice with different types and concentrations of detergent to determine the effect that these would have on the secreted fuzzy layer on the body cuticle. The intention of the detergent treatments was to remove this layer without damaging / removing any other layers of the cuticle. Freshly acquired *L. salmonis* were subjected individually to one of two different, nonionic detergent treatments designed to remove the external mucus layer. The two detergents were: 1% Triton X-100 (t-octylphenoxypolyethoxyethanol, Sigma T984) and 0.05% Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma P1379). Five specimens were gently agitated for 5min in one of these detergents made up in sterile seawater and this was followed by several changes of sterile seawater to remove all traces of detergent. Following the rinse washes, dissected regions of cephalothorax and urosome were processed for TEM using the specific mucus fixative of Horne and Sims (1999) described above.

The dorsal surface of the cephalothorax was preferentially examined for two reasons. Firstly, the dorsal surface bears the large DSG population (see Chapter 3), which is suggested to be responsible for secreting this fuzzy layer, but also because adult male lice were observed to sink with their dorsal surfaces facing downwards presenting the surface of the dorsal cuticle to the passing water.

7.3.4 Protocol for Sinking Experiment

To ascertain whether immersion in detergent had any other effects beyond the removal of the external mucus layer, a brief experiment was devised. Specimens were anaesthetised, weighed and had their sinking rate measured prior to the 5min treatment in the detergent solution. Following the detergent treatment the specimen was again weighed and its sinking rate determined and it was then subjected to a further 10min detergent treatment. Following weighing and sinking it was treated for a further 20min, weighed and it's sinking rate determined again. Following detergent treatment specimens were rinsed in clean seawater prior to weighing and sinking. Body weight was measured to the nearest 0.10mg.

After the optimum detergent treatment for mucus removal had been determined this was incorporated into the experimental setup, which was as follows. Untreated anaesthetised (CO_2 immersion) specimens were dropped through a 2L column (glass cylinder: 52cm height, 72mm diameter) of seawater. The seawater in the column was maintained at 10°C throughout the study by carrying out the whole experiment in a constant temperature room. The salinity, as determined using a salinometer was 35‰ and density, as determined by weighing a known volume, was 1.01295g ml^{-1}. All data was obtained in one session, using the same water to minimise any variations in salinity and density. Sinking rates were recorded as mm/s^{-1} using a hand operated digital timer. Anaesthetised copepods always sank with their dorsal surfaces facing down and with the anterior end markedly below the horizontal. This inclined body orientation caused sinking copepods to slowly move forward as they sank. To prevent specimens moving close to the wall of the cylinder, and therefore to combat any edge effects which might arise, specimens were released close to the back of the cylinder and their sinking monitored for a depth of 290mm. This distance was within the range where the sinking specimen had not moved forward to the front of the cylinder. Data from individuals that dropped close to the walls of the experimental vessel were disregarded. Fifteen specimens were each dropped through the column five times and a minimum of two minutes was allowed between drops to allow any turbulence caused by the retrieval of the animal to diminish. Following this step the same individual was subjected to the detergent treatment to remove its

adhering mucus layer, thoroughly in sterile seawater and again dropped through the water column five times and the sinking rate recorded as mms^{-1}.

The specific weight for each individual was calculated at the end of each experiment. Specific body weight was calculated by a combination of displacement volume analysis and actual weight measurement as follows: individual lice were placed inside a silanised 200µl graduated plastic disposable pipette tip. The tip was then replaced onto a Gilson 200µl pipette and seawater was pipetted up to the 200µl mark. The volume of water in the tip weighed to the nearest µg. This was repeated five times with each specimen, using a fresh tip for each measurement. The difference between the weighed value and the weight of 200µl of seawater was deemed to be the displacement volume of that specimen. An average value of the five measurements for each specimen was used. The specific weight of the animal could then be easily determined as a function of actual weight to volume ratio.

The total body length (excluding terminal setae), maximal body width across the cephalothorax and the surface area of the dorsal cuticle (cephalothorax, genital segment and abdomen) was also recorded for each specimen using a Zeiss KS 300 image analysis system.

Preliminary observations made of copepods sinking through the water column indicated that the sinking rate of individual specimens was not uniform over the course of large numbers of repeated observations. A trial was therefore run to determine whether there was a predictable pattern to this variation in sinking rate.

7.3.5 Swimming speed analysis of *L. salmonis*

Knowledge of the Reynolds number environment of *L. salmonis* can be used to imply how certain physical features of the animal are involved in its swimming behaviour and may also provide an indication of how secreted gland products behave.

Measurements of swimming speed were taken directly from adult lice maintained in the laboratory. Lice were used no sooner than 24h after capture but within 72h to minimise the risks associated with host mucus contamination and altered behaviour due to starvation effects. Both adult

males and adult females were used to determine whether there was a dimorphic component to swimming speed. Observations were taken directly from individuals swimming in a specially constructed glass aquarium (dimensions: length 32cm, height 25cm, width 6cm), shown in **Figure 7.1**, using a Sony VPC-G250 colour digital camera with consecutive images recorded every 100ms. To facilitate distance measurements a grid marked in 5mm squares was affixed to the outside of the rear wall of the aquaria. Illumination was provided by a combination of natural light and incident electric light. Measurements of swimming speed were made immediately the 10°C water was added to the aquaria, as it was necessary to perform measurements on the laboratory bench at ambient temperature. The temperature of the water in the aquaria was measured immediately after the film sequence had been shot to establish whether any increase in temperature had occurred. In no case was a detectable change in temperature evident by the end of the swimming experiment. Separate images were downloaded into a computer graphics program (Adobe Photoshop 5) and stacked into a single composite image containing the full number of images of each individual. From this composite image the swimming speed could easily be determined using the equation Speed = distance / time. To allow comparable measurements to be taken the lice were filmed during the first two seconds of a burst of swimming. The first second of swimming was ignored to minimise any acceleration effects. **Equation 7.1** (Alexander, 1983) was used to calculate the Re of *L. salmonis*.

Equation 7.1 $$\mathrm{Re} = \frac{UL}{v}$$

where U = velocity of the swimming specimen as ms^{-1}, L = length of specimen in metres and v = the dynamic viscosity of seawater. The dynamic viscosity of a fluid is obtained by dividing its viscosity (kg / m s^{-1}) by its density (kg m^3). Dynamic viscosity of seawater at 10°C = 1.35 x 10^{-6}. (Lide, 1999)

7.3.6 Dimorphism of terminal setae length

The terminal setae of the caudal rami of *L. salmonis*, and some related species, were investigated to identify any sexual dimorphisms that may have been present. Measurements of the lengths of the terminal setae were made for adult males and females of *L. salmonis*, *L. hippoglossi*, *L. pectoralis* and *C. elongatus*. Measurements were made from freshly killed specimens of these species. Data of terminal setae length were also taken from two other caligid species, *Caligus lacustris* Steenstrup and Lütken, 1861 and *Caligus rogercresseyi* (Boxshall and Bravo, 2000). Dr James Bron of the Institute of Aquaculture supplied specimens of these latter two species which were preserved in 10% formalin solution but were transferred to fresh seawater whilst measurements were taken.

Three seta from each caudal ramus were measured. These were, following the nomenclature of Huys (1988), the posterolateral setum, the outer terminal setum and the inner terminal setum. The positions of these setae on the caudal rami of an adult male *L. salmonis* are indicated in **Figure 7.2**. Setal length measurements were taken from specimens lying on their dorsal surface on a glass slide with the setae immersed in a small volume of seawater. The setae were not compressed under a coverslip. Point-to-point measurements of the setae were made using a Zeiss KS 300 image analysis system from images captured via a JVC KYF30-BE colour video camera mounted upon an Olympus BH-2 compound microscope. Measurements were made to the nearest 0.01mm.

Comparisons of setal length relative to total body length were made both between species and on a sexual basis within species.

7.3.7 Comparative morphology of *L. salmonis* setae

The shape, physical condition and orientation of both the natatory setae of the thoracic legs and the terminal setae of the caudal rami were recorded for both sexes. SEM was utilised to identify these structural characteristics. Specimens of adult male and female *L. salmonis* were processed for SEM examination as described earlier in Section 2.2.3.

7.3.8 Gland number : surface area ratio

Measurements were made of the surface area of the cephalothorax of adult male and female *L. salmonis*, *L. hippoglossi*, *L. pectoralis* and *C. elongatus*. Measurements were taken from specimens freshly fixed in 70% ethanol using an Olympus BH-2 dissection microscope linked directly to a Kontron 300 computer package via a JVC KYF30BE colour video camera. Measurements were made to the nearest 0.01mm.

Numbers of dorsal surface glands typical for both sexes for each species were determined using a combination of TMB staining (Chapter 3) and SEM observation.

7.3.9 Data analysis

The data obtained from the sinking experiment was analysed using two different statistical tests. The total sample data, untreated and treated specimens, was compared using the Mann-Whitney U-test for unmatched sample medians. The Wilcoxon Matched Pairs test was used to analyse the median value of each individual specimens treated and untreated sinking rate.

7.3.10 Bacteriological studies

7.3.10.1 Analysis of external bacterial fauna

In order to identify the species of bacteria colonising *L. salmonis*, the following experiment was utilised. Plates of marine agar (#2216, Difco Laboratories, UK) and tryptone soya agar (TSA) (Oxoid Cm131) were inoculated with bacteria from the cuticle of *L. salmonis* by wiping them with the dorsal surface of a freshly killed sea louse. The copepods were blotted on clean tissue paper prior to use to remove excess culture water. Three plates of each type of agar were incubated at either 10°C or 15°C for 4 days. After this time the plates were examined by eye to identify different bacterial colonies. Identified colonies were subcultured separately onto marine agar plates and incubated at 15°C for four days to obtain pure cultures from which identification could be made. Identification of bacterial species present was made from analysis of a variety of first and second stage characteristics as described by Krieg (1984) and

Frerichs and Millar (1993). These were Gram staining property, oxidase enzyme content, motility, antibiotic resistance (0/129 sensitivity) and respiratory attributes (O-F test). API test strips (API 20E, API Merieux, Cat. No. 20100), designed for enterobacteria identification, were also used to assist in the identification of isolated bacterial species.

7.3.10.2 Bactericidal properties

The urosomal gland complex was used exclusively in this experiment, as these glands appeared to possess greater catalase activity than other glands identified, their secretions often being seen to coat the long terminal setae of the caudal rami. To determine whether these secretions possessed any bactericidal properties the following experiment was designed. After maintaining the lice in clean seawater for 36h, to ensure gut evacuation, the posterior tips of the abdomen of 200 specimens were removed and snap frozen in liquid nitrogen. Immediately prior to use, they were homogenised in approx. 300µl of sterile seawater using a sterile, disposable plastic homogeniser, before being centrifuged for 10min at 6500rpm at 4°C. Adult male lice were again chosen for this experiment, partly for consistency and also because they frequently appeared to have larger, or more intensely staining, urosomal glands than females.

The supernatant was pipetted off and divided into two separate aliquots of approx. 150µl. One aliquot was added to a well in a plate of *E. coli* in the logarithmic phase and incubated at 22°C while the other was added to a well in a culture of *Vibrio* spp. and incubated at 6, 8 or 10°C. The plates were examined after 24h and 96h. Due to the difficulty in obtaining large quantities of gland product, only two species of bacteria were used in this experiment: *Escherichia coli* (environmental) and *Vibrio* (general marine bacteria). These bacteria were deemed suitable because as they are species that are frequently recorded in the marine environment (Krieg, 1984) and are presumably species that *L. salmonis* will be exposed to *in vivo*.

This experiment was repeated using fresh samples to eliminate any deleterious effects that freezing or storage may have had on any active enzyme component. Urosomal regions of 200 adult male *L. salmonis* were homogenised in 300µl of sterile seawater and centrifuged at 6,500rpm at 4°C for 10min.

In this trial, single plates of 96h cultures of *Vibrio anguillarum* and *Vibrio* spp. (species commonly found infecting marine salmonids) were each inoculated with 150µl of gland supernatant and incubated at 12°C for 2 days. Both bacterial cultures were in their log phase of growth at the 96h period.

7.3.10.3 Determination of chitinolytic properties of sea louse bacteria

An experiment was designed in order to determine whether those bacteria observed embedded in the 'fuzzy' layer of the cuticle of *L. salmonis* using TEM (**Figure 7.3**), displayed chitinolytic properties. Plates of marine agar had chitin incorporated into them at a 1% level, following the method of Danulat (1982) modified by the incorporation of the chitin suspension in marine agar (**Appendix 5**). The inclusion of chitin was intended to preferentially promote the growth of bacterial species dependent on this compound for their nutrition. Two methods of inoculation were followed. The first of these involved the direct inoculation of plates by rubbing the dorsal surface of a freshly killed adult female *L. salmonis* in one portion of the plate and then using a heat-sterilised loop to distribute the inoculate across the plate. The second method involved the inoculation of plates with a small quantity of one of the three species of bacteria that were successfully isolated from specimens of *L. salmonis* Section 7.3.6.1. For both methods control plates of marine agar without chitin were also inoculated with the same sample to assess any differential growth between the two media. Inoculated plates were incubated at 10°C and monitored daily for evidence of bacterial growth. The trial was terminated after day 28.

7.4 RESULTS

7.4.1 Sinking rate of *L. salmonis*

7.4.1.1 Mucus layer analysis

TEM examination of ultrathin sections of cuticle from the cephalothorax and urosome of detergent-treated and control lice showed that the novel fixative, incorporating Fluorocarbon-72, preserved the external fuzzy layer with significantly greater integrity than did the conventional fixation process. The observed fuzzy layer exhibited a marked homogeneity in terms of its electron density, appearing as a uniform layer of moderate density. The external surface of the layer was slightly dispersed where it interfaced with surrounding water. The fuzzy layer was substantially thicker in those lice fixed with the novel technique reaching a maximum of 0.54μm in **Figure 7.4** as compared to the 0.03μm in lice fixed using the standard procedure (**Figure 7.5**). The dorsal cuticle of the cephalothorax possessed a more consistent and uninterrupted fuzzy layer than was apparent on either the ventral surface of the cephalothorax or on the cuticle of the urosome. A fuzzy layer was present on the ventral cuticle of the cephalothorax but only at a barely detectable level (**Figure 7.6**) whilst a very thin layer was sparsely dispersed across the urosome surface.

7.4.1.2 Mucus layer removal

TEM examination (following FC-72 fixation) demonstrated that 1% Triton X-100 had an obvious effect on the integrity of the fuzzy layer of the cuticle (**Figure 7.7**), reducing its thickness by almost 50%. The application of 0.05% Tween 20, however, had the effect of almost entirely removing the adherent fuzzy layer (**Figure 7.8**). The Tween 20 treatment appeared to have no deleterious effects beyond that of removing the secreted layer on the cuticle surface as no discernible effects on the ultrastructure of the cuticle were detected. All four layers of the epicuticle as described for *L. salmonis* by Bron *et al.* (2000) (and for the harpacticoid copepod *Cletocamptus retrogressus* Schmankenwitsch by Gharagozlou-van-

Ginneken and Bouligand 1973 and other copepod species by Bresciani (1986) were all present and apparently unharmed both by the detergent washes and the fixation process.

7.4.1.3 Sinking trials

Preliminary observations indicated that there was a definite change in sinking rate of individual, untreated lice when they were repeatedly sunk through the water column in rapid succession. This change clearly occurred after the first two drops of a specimen through the water column, as is evident in **Figure 7.9.** To test this observation a trial was devised where CO_2 anaesthetised specimens were each dropped through the water column thirty times in succession and the sinking rate for each drop recorded. It was apparent that a clearly defined change in sinking rate occurred with each specimen over the course of their thirty drops through the water column. This change in sinking rate usually occurred soon after the second drop although the third, fourth and fifth observations of a specimen were generally of a slightly faster rate than the subsequent observations i.e. 6 - 30. After the sixth consecutive drop of a specimen however the sinking rate remained relatively constant. This pattern of sinking rate change, over the course of thirty observations can be seen in **Figure 7.9.** From this observation it was decided that, in the full experimental setup, all of the first five measurements of a specimen would be used for analysis. This decision meant that, despite a lower sinking rate being recorded than would be apparent if only the first two values were used, reasonable statistical analysis of the data could be performed.

The results of the experiment performed to determine the effects of repeated and progressively extended immersions in Tween 20 on the weight and sinking rate of *L. salmonis* clearly showed that immersion in Tween 20 had reached its maximal effect within 5min. Data of the wet weight of a typical specimen is presented in **Table 7.1** whilst the passive sinking rate of the same specimen is displayed in **Figure 7.10.**

The initial immersion in Tween 20 always served to reduce the body weight of the specimen by a small amount but further immersion had no detectable effect on either the body weight or sinking rate of

an individual. The mean percentage weight difference of a treated louse from an untreated one was a reduction of 4.79% wet weight, typically a reduction in the order of 70μg.

Table 7.1 Effect of repeated detergent treatments on the wet weight of a single specimen of *L. salmonis*.

	CO_2	5min Tween 20	10min Tween 20	20min Tween 20
Wet weight	4.8mg	4.6mg	4.6mg	4.6mg

This reduced weight could be correlated approximately to the removal of the theoretical weight of the attached mucus layer. A typical louse was calculated to have a dorsal surface mucus layer weighing 60μg. This approximation was determined from the measured surface area of a louse, the measured depth of the mucus layer (0.54μm), the weight difference in grams of a treated louse and an estimation that 90% of the mucus layer consists of water (seawater density of 1.01295gm ml^{-1} at 10°C). The approximate percentage water composition of marine invertebrate mucus was obtained from Davies and Hawkins (1998). This estimate of weight of the adherent mucus layer is somewhat less than the typical weight loss following detergent treatment but the calculation did not take into account the underestimate of surface area from a 2-D projection of the dorsal surface nor was the thinner mucus layer of the ventral surface taken into account.

The results of the full sinking experiment indicated that significant differences were apparent between the sinking rate of CO_2 anaesthetised specimens and the same specimen following the described detergent treatment. The Wilcoxon Matched Pairs Test indicated that the change in sinking rate after Tween 20 treatment was significant ($p<0.001$) whilst the Mann-Whitney U-Test suggested the difference between treated and untreated sinking rate was highly significant (<0.0000001). The data therefore suggest that some effect, possibly the removal of the mucus layer, caused the treated lice to sink at a significantly lower rate than prior to the detergent treatment. The observed change in sinking rate was apparent in all specimens examined. The data of sinking speed and the morphological measurements of each individual are given in **Appendix 7.**

The relationships of the measured morphological characters of each specimen were plotted in graph form to provide data relating to size, weight and volume of individuals. The data indicated that there were positive linear relationships between male copepod length and wet weight (**Figure 7.11**) and copepod surface area and wet weight (**Figure 7.12**). Surprisingly there was no relationship established between the measured copepod volume and wet weight (**Figure 7.13**). There was also no apparent correlation between weight or surface area and passive sinking rate. The heaviest louse (#4) had a sinking rate slower than specimens that weighed less (i.e. #10) whilst specimen #9 had the greatest surface area of all the specimens but had a passive sinking rate comparable, and less in some cases, to other specimens with smaller surface areas.

7.4.2 Swimming speed analysis of *L. salmonis*

Swimming speed estimates of both adult male and female *L. salmonis* were obtained from an analysis of the composite images obtained from series of individual images. Adult female lice were recorded at speeds of $25cms^{-1}$ during phases of their observed burst of swimming. Incorporating the data of specimen length, minus setae (**Table 7.2**), into Equation 7.1 we can determine that adult female *L. salmonis* possess a Re number in excess of 2000 whilst swimming at $25cms^{-1}$. Adult males were recorded swimming at speeds of $20cms^{-1}$ and therefore had a Re number of 859. These values of Re place both male and female *L. salmonis* within an inertial microenvironment.

This measurement is likely to be a substantial underestimate as the actual distance swum by a specimen in a 2-D image such as that shown in **Figure 7.14** is likely to be greater than it appears on a two dimensional image. Copepodid stage larvae of *L. salmonis* are known to, in response to stimuli, exhibit swimming behavior in a characteristic helical path that is likely to increase possible contact with passing hosts that elicited the swimming response (Bron *et al.* 1991, Bron *et al.* 1993). Such behaviour also appears from the present study, to be present in the adult stages. Adults of both sexes could be stimulated to initiate a burst of rapid swimming by passing a shadow across the experimental vessel. Typical bursts of swimming had a characteristic helical shape. Lice quickly became desensitised to this stimulus and

several minutes had to elapse before a similar burst of swimming could be elicited through a further shadow stimulus.

It is not possible to determine whether the swimming speeds measured here for males and females is the maximal swimming speed they can attain. It can reasonably be assumed that the location of a host fish is of ultimate importance to a detached louse and so swimming bursts associated with host location are likely to be close to maximal for both sexes.

7.4.3 Dimorphism of terminal setae length

A sexual dimorphism in the lengths of the terminal setae was strongly apparent in four of the six caligid species examined. The data in **Table 7.2** illustrates the extent of this dimorphism in both real and relative terms. This data is more clearly illustrated in **Figure 7.15**. With the exception of *C. lacustris* and *C. rogercresseyi* adult males had longer terminal setae than females both in real terms and relative to their overall body length. The terminal setae of adult male of *L. salmonis*, for example, account for more than 12% of the total body length, in contrast to the 3% that they contribute in the adult female. In five of the six species the female was the larger of the two sexes with regard to both total body length and body length minus the terminal setae, only in *C. rogercresseyi* was the adult male larger than the female in total length.

Only a single specimen of each sex of both *C. lacustris* and *C. rogercresseyi* were available for examination. From these specimens though it was apparent that in both C. *lacustris* and *C. rogercresseyi* the relative length of the terminal setae of the adult females exceeded that of the adult males.

There was considerable variation between species with regard to the relative lengths of the setae and also to the extent of the sexual dimorphism of the setae. In *L. salmonis* for example, the relative length of the male setae was more than four times greater than that of the adult females, whereas in *C. elongatus* the relative length of the male setae was only half again as great as that of the female. *C. elongatus* males had terminal setae that were almost twice the length, in relative terms of those of male *L. hippoglossi*.

Table 7.2 Terminal setae length relative to body proportions of examined caligid species.

	Body length (μm)	Length of setae (μm)	Relative length of setae (%)
L. salmonis Male (n=20)	6,575	749	12.9
L. salmonis Female (n=20)	10,427	321	3.2
C. elongatus Male (n=20)	4,829	743	18.2
C. elongatus Female (n=20)	6,158	618	11.2
L. pectoralis Male (n=9)	3,301	472	16.7
L. pectoralis Female (n=20)	5,779	328	6.0
L. hippoglossi Male (n=4)	5,751	489	9.3
L. hippoglossi Female (n=6)	10,086	522	5.5
C. lacustris Male (n=1)	5,260	585	12.5
C. lacustris Female (n=1)	5,850	821	16.3
C. rogercresseyi Male (n=1)	4,940	595	13.6
C. rogercresseyi Female (n=1)	4,840	797	19.7

Data of body length describes length from anterior cephalothorax to distal tip of terminal setae. Values given are mean values derived from specimen numbers given in brackets

7.4.4 Comparative morphology of *L. salmonis* setae

Conspicuous differences were apparent in the morphology of the terminal setae and the natatory setae of the thoracic legs both within and between sexes. The terminal setae of adult females were invariably in a very poor condition, as can be seen in **Figure 7.16**, frequently with many of their setules missing and often the setae themselves were broken off, with the tips missing. There was never any regular or definable overlap or interaction between the setules of neighbouring setae. The terminal setae of adult

males however, were always observed to be in good condition. The setae were long and robust and were rarely observed to be broken whilst the smaller setules that lined the lateral margins of the setae were seen to interlock with the setules of the neighbouring setae (**Figure 7.17**). The setules of the female often appeared to have a great deal of amorphous matter adhering to them whereas those of the adult males appeared to be clean and free from adhering material.

The natatory setae of the thoracic legs differed structurally from the terminal setae in that their setules were much longer, overlapping two, or even three of their neighbouring setae (**Figure 7.18**). The setules were compressed distally to form thin sheets that increased the surface of the setal area, almost forming a solid wall of cuticle (**Figure 7.19**). Compressed setules could measure as much as 0.5μm across.

7.4.5 Dorsal surface gland number : surface area ratio

Analysis of the data of surface area to DSG number revealed that within species there was, with one exception, no obvious sexual dimorphism in the number of glands per unit area of dorsal cuticle. Males and females of each species possessed similar densities of DSG on their cephalothoraxes. Due to the significant sexual dimorphism in size of the genital segment and abdomen the DSG's of these segments were not included in the density analysis. Due to the difficulty of obtaining sufficient numbers of specimens of some species, statistical analyses were not employed but the experimental data obtained is still useful in demonstrating general patterns. **Table 7.3** displays the data of DSG density for the four species of caligid copepod examined in this study.

There was a pronounced sexual dimorphism in the ratio of glands to surface area in *L. hippoglossi* where males had more than four times the number of glands per unit area than females. Due to the very large numbers of stained DSG present in this species, particularly in the males, there is likely to be a significant margin of error in the estimation of gland numbers, although this error was probably not sufficient to account for the measured difference. There were also very marked differences in gland densities between species. *L. salmonis* possess the lowest DSG density of all four species whilst *C. elongatus*, as a species, possessed the highest number. On account of the surface area measurements

being taken from two-dimensional projections of whole specimens the true surface area is likely to be somewhat greater and therefore the true gland density somewhat lower than calculated.

Table 7.3 Dorsal surface gland number to surface area ratio for four caligid copepod species.

	Surface area (mm^2)	Total gland number	Glands per mm^2
L. salmonis male	8.90	~60	~7
L. salmonis female	15.22	90 - 140	~6 - 9
C. elongatus male	3.35 (11)	~120	~36
C. elongatus female	5.49	~180	~34
L. hippoglossi male	8.71	>400	>46
L. hippoglossi female	23.50	>260	>11
L. pectoralis male	2.59	~58	~22
L. pectoralis female	5.01	~94	~19

Numbers of DSG from cephalothorax only. Numbers in parentheses indicate number of specimens examined where less than 20. Surface area measurements are from a 2-D projection of the dorsal surface.

7.4.6 Bacteriological studies

7.4.6.1 Isolation of bacterial fauna of L. salmonis

Following inoculation and incubation, mixed growth of bacteria was observed. Dominant growth of two species occurred whilst a third species was present only in a few colonies on each plate. All three species formed distinct colonies. It was apparent that marine agar was more suitable for the culturing of these species than was the TSA medium as evidenced by the more abundant growth of the bacterial colonies present on the former medium. Both media did however support the growth of the three species identified. Subculturing the bacteria provided pure cultures of each of the three species present. First stage identification tests and API strips were used in an attempt to identify the bacterial species isolated. The results of the identification tests are displayed in **Table 7. 4.**

The API test strips indicated that all three species were similarly unreactive, giving no positive response to any of the test reagents. Identification of the bacteria therefore had to be made solely from the data shown in **Table 7.4.** This data alone did not allow identification of these bacteria to species level.

All three species were Gram –ve rod-shaped bacteria whilst two others were oxidase-positive, features generally shown by members of the common marine bacteria genus *Vibrio*. The data displayed in **Table 7.4** was therefore compared against a checklist of diagnostic features of common marine *Vibrio* species. Marine *Vibrio* species are sensitive to O/129 (2,4-diamino-6,7-di-isopropyl pteridine phosphate). Sensitivity to O/129 is a feature that can be used to distinguish *Vibrio* species from *Aeromonas* species, as the latter are characteristically resistant to O/129. The sensitivity to O/129 displayed by the three species of bacteria isolated from *L. salmonis* indicates that they are not *Aeromonas* species.

Table 7.4 Characteristics of the three isolated bacterial species infesting the cuticle of *L. salmonis*.

Colony colour	**Cream**	**Pink**	**Yellow**
Gram	Negative	Negative	Negative
Shape	Rod	Rod	Rod
Motility	Negative	Positive	Negative
Oxidase	Positive	Positive	Negative
0 / 129 10μg	Resistant	Resistant	Resistant
0 / 129 150μg	Partially sensitive	Sensitive	Sensitive

The other data obtained from the first stage identification tests however provides information that is contradictory to any profiles of characterised *Vibrio* species and did not allow the identification of any of the species found.

7.4.6.2 Bactericidal properties of gland secretions

Using the methodology described here no evidence was found to suggest that the secretions of the urosomal glands of *L. salmonis* possessed any obvious bactericidal properties. In the first experiment, using samples that had been stored at -70°C the cultures of *E. coli* incubated at the lower temperature showed no sign of growth inhibition whilst the plate incubated at 22°C did show what appeared to be a very small ring of bacterial inhibition around the well.

The evidence from the second trial utilising fresh gland tissue directed against *V. anguillarum* and *Vibrio* spp. also gave no indication of bactericidal action after either 24h or 48h.

7.4.6.3 Chitinolytic properties of isolated bacteria

Analysis of the cuticle using TEM reveals that many of these bacteria appear to have digested, or eroded, small cup-shaped depressions in the cuticle of the louse (**Figure 7.3**). These cup-shaped depressions are of a size that matches the approximate dimensions of the end of the bacteria when orientated in a polar fashion on the cuticle. This suggests that at least some of the bacteria infesting *L. salmonis* have the capability to degrade the cuticle as the depressions presumably arose as a result of some activity of the bacteria themselves. No evidence was ever found to show that this degradation caused any substantial damage to the cuticle as the damage was never seen to extend deeper than the epicuticle (as classified for *L. salmonis* by Bron *et al.* (2000)).

After incubation at 15°C for 28 days there was no apparent utilisation of incorporated chitin by the yellow and pink coloured species of the three isolated species of bacteria. No rings of depleted chitin content were evident in the media of these cultures. The isolated bacteria did appear to grow equally well on the agar with incorporated chitin as they did on the marine agar without the chitin.

The cream coloured bacterial species did however show a marked zone of clearance of the chitin incorporated into the marine agar culture, as shown in **Figure 7.20**. This clearance only became apparent after day 20 of the incubation. Rings of clearance where chitin had been depleted from the media were apparent around all colonies of this species.

7.5 DISCUSSION

7.5.1 Sinking rate and implications of secretion interaction

Use of the FC-72 TEM fixative technique has allowed the visualisation of the full extent of the external layer that coats the cuticle of *L. salmonis*. This 'fuzzy' layer in other copepods is presumed to be derived from mucus producing tegumental glands (Briggs 1978, Bresciani 1986, Hipeau-Jacquotte 1987, Nishida 1989). It is here proposed that this fuzzy layer on the cuticle *of L. salmonis* is principally composed of the mucus secretions identified, in this study, in the exocrine glands of this species. This layer in *L. salmonis*, has previously been partially visualised (Bron *et al.* 2000) but never as clearly as in this study. The observation of the thick fuzzy layer of the dorsal cuticle in contrast to that of the ventral cuticle lends support to the hypothesis that the DSG are responsible for secreting this fuzzy layer, as very few glands are evident on the ventral surface of the cuticle. This fixative is therefore recommended in future studies where the dynamics of the cuticle are under investigation as the external layer should be looked upon as an integral part of the cuticular environment. The application of this fixation method to SEM samples was not explored in this study but again, it may be beneficial in revealing the condition of the mucus layer *in vivo*. The detergent treatment utilised in the sinking experiment was shown to successfully remove this mucus layer, with no observable alteration of the underlying cuticle. The fuzzy layer was substantially thicker on the dorsal surface of the cephalothorax than on the ventral surface, and was also thin on both surfaces of the urosome. This observation suggests that the secretion is not involved in protecting the cuticle either from epibionts or deleterious chemicals and may further indicate that the secretion has a beneficial effect on the hydrodynamic profile of the animal. Data pertaining to the depth of this fuzzy layer in copepods is scarce with only Hipeau-Jacquotte (1987) giving specific information relating to this feature. It would be interesting to compare the depth of this layer between species from different habitats and with different behaviours to determine whether any patterns are evident.

The data from the full sinking trial indicated that a definite, observable decrease in the sinking rate of treated *L. salmonis* over untreated specimens was apparent in all specimens. The difference in the

sinking rates of untreated and treated specimens were found to be significantly different and, as such, it is proposed that the results of the sinking trial do demonstrate a measurable and significant difference in the passive sinking rate between lice with their mucus layer intact and those with it removed. There is a point of contention however as to what actually causes this change in sinking rate. Immersion in Tween 20 was seen also to cause a reduction in the wet weight of specimens but further prolonged immersion in the treatment did not cause further reductions in weight (**Figure 7.10**), indicating that weight changes of Tween treated specimens have a maximal effect within the five minutes immersion utilised and are not likely to have been caused by osmolarity effects.

The decrease in sinking rate of treated specimens may not solely be attributable to the detergent treatment as it may be accounted for, either entirely or in part, by the reduced weight of the specimen observed after the detergent treatment. The pattern of sinking rate change observed in a single, repeatedly sunk, specimen even when untreated (**Figure 7.9**) also suggests that something occurred during the experimental process that altered the hydrodynamic characteristics of the animal. A distinct alteration of the sinking rate became apparent usually after the first two drops of a specimen but the first four or five drops through the column were obviously faster than subsequent observations. The apparent effect of removing the mucus layer would likely have been more pronounced if only the first two sinking measurements were taken, but for the sake of data analysis the first five were used. This change in sinking rate is possibly caused by the mechanical removal of the mucus layer on the cuticle of the untreated animal caused by a combination of sinking and the siphoning process used to recover individuals from the bottom of the experimental vessel. Unfortunately, no specimens were fixed for TEM using FC-72 after repeated sinking but without the detergent treatment so it cannot be stated with certainty whether the observed change in sinking rate was in fact caused by the mechanical removal of this layer. It appears though that the decrease in specimen weight had been achieved before the detergent treatment was applied. The alteration in sinking rate was likely to be accounted for by the removal of the mucus layer regardless of whether the change was effected by a reduced hydrodynamic ability or merely as a result of the decreased weight of the specimen. If, however, it could be determined that the increased sinking rate

of specimens with intact mucus layers was due solely to the weight of this layer then it would suggest that this layer provides no hydrodynamic benefit to the animal and the secretion must serve some other function.

The values obtained for the body density of individual *L. salmonis* in this study were not consistent with that which we would expect for a negatively buoyant copepod. The observed relationship of wet weight and specimen volume indicated that there were problems with the method of body density determination utilised in this study. All specimens in this study had calculated body densities lower than the stated value of seawater suggesting that they would be positively buoyant. This underestimate of body density is likely to stem from an overestimation of body displacement caused by water adhering to specimens during the displacement analysis. Svetlichny (1983) is responsible for almost the entire data that exists on individual copepod body densities derived from the degree of deformation of a specialised balance. Only Gross and Raymont (1942) and Lowndes (1942) have made attempts to determine the density of individual marine invertebrates but their methods were too impractical to be utilised in this study. The lack of data on body density in this study prevented the calculation of sinking speeds as a function of the specific weight of each individual and therefore prevents the determination of whether the observed decrease in sinking rate is caused by a decrease in weight or by a reduction in hydrodynamic quality. It was demonstrated that louse wet weight increased both with increasing surface area and length. Observations indicated that the heaviest louse did not have the fastest sinking rate, and the louse with the largest surface area did not display either a noticeably faster, or slower, sinking rate than other specimens. This suggests that a further factor, beyond the influence of morphological parameters, is involved in determining the sinking rate of *L. salmonis*. This observation may indicate that the surface mucus layer exerts an influence on the sinking rate of *L. salmonis*, as the extent and efficacy of such a layer is bound to vary both between copepods, and temporally within a specimen, which may be reflected in sinking rate variations between specimens. The decrease in body weight observed after the detergent treatment may not have a direct effect on the sinking rate of the copepod as weight seems, from the data of body weight and sinking rates, not to be a crucial factor in determining sinking rate. This though is likely to be the case

only within certain limits, obviously any dramatic increase in body weight is going to result in a faster sinking rate.

As a consequence of this inability to be certain as to whether the sinking rate is altered by a weight loss or the removal of a hydrodynamic secretion, or a combination of both, it cannot be stated conclusively that the observed mucus 'fuzzy' layer of *L. salmonis* confers upon them a beneficial hydrodynamic effect. However, analysis of further factors such as body weight and surface area is important when considering the hypothetical effect of the fuzzy layer on sinking rate.

Hutchinson (1967) observed a decrease in sinking rate in association with a mucus layer, suggesting that mucus serves to retard the sinking rate of marine organisms. To determine the effects of mucus secretions on hydrodynamics it would be preferable to test the mucus and its effect on water flow directly using a rheometer (Rosen and Cornford 1971, Bernadsky *et al.* 1993), but such an approach is not feasible with sea lice where such small quantities of mucus are available.

To ascertain therefore whether this adherent mucus layer could confer a hydrodynamic benefit to the animal it is necessary to consider the relative viscosity of the environment in which the lice exist. As was discussed earlier, the relative viscosity of an animals environment dictates (as described by the Re value) how morphological features interact with that environment. The calculated Re environment of *L. salmonis* may be used to imply whether a hydrodynamic secretion would be of utility to the animal as an aid to swimming. At Re environments <1 the animal lives in a viscosity dominated environment where the boundary layer is relatively thick, viscous drag will have a major effect and a hydrodynamic secretion is not likely to be of utility. Mauchline (1998) states that calanoid copepods live at an approximate Re of 1. The data obtained from Sullivan *et al.* (1983) for *Eurytemora affinis* yielded a Re number of 0.001 using the dynamic viscosity value of seawater at 10°C. This obviously is a far smaller value than that obtained for swimming *L. salmonis* and certainly places *E. affinis* within a viscous microenvironment. At a Re environment of this type a hydrodynamically active secretion is unlikely to be of any utility as the flow around the animal is likely to be slow and ponderous. Calanoid copepods however appear able to break out of their low Re environments as escape swimming speeds of copepods are significantly higher

than their routine maintenance swimming speeds (Mauchline, 1998). This higher swimming speed may propel them temporarily into an inertial environment where hydrodynamically active secretions may be of utility, but whether this requirement for higher speed swimming has precipitated the development of a mucus layer to facilitate this action is unknown although it was suggested by Von Vaupel Klein (1982a).

Re values calculated for *L. salmonis* in this study range from 859 for adult males to approximately 2000 for adult females. Due to difficulties in calculating the actual swimming speed of these copepods it is likely that their actual Re values were substantially higher than these given values. The value of Re for adult males calculated in this study was derived from the length of adult males excluding the terminal setae. The corrected Re, derived from the total length including the terminal setae yields a Re of 962, an increase of 12% suggesting that their setae may substantially increase their Re environment. Re environments below 1000 are difficult to describe, as both viscous and inertial forces will be involved depending on individual situations of animals. Adult male *L. salmonis* were close to the Re value of 1000 and may be expected to experience a largely inertial microenvironment. The evidence from this study therefore suggests that the Re of *L. salmonis* places them above the viscous flow situation (although individual limbs may act in a low Re microenvironment) and therefore a hydrodynamically active secretion may be of benefit to them, in contrast to smaller copepod species living under low Re conditions.

Sea lice are known to aggregate in particular regions and in a particular orientation (Wootten *et al.* 1982, Jaworski and Holm 1992, Jonsdottir *et al.* 1992, Ritchie *et al.* 1996b, Todd *et al.* 2000) on the salmonid host. Female *L. salmonis* seem to strongly prefer to attach in the regions immediately posterior to the adipose fin and the anal fin whereas males tend to be more widely dispersed across the body surfaces (Jonsdottir *et al.* 1992, Todd *et al.* 2000) with little site specificity being apparent. The reasons for this distribution pattern are unknown but it does appear that females are seeking out more sheltered areas whilst males appear to do so to a lesser degree although Todd *et al.* (2000) suggested that the larger females may competitively oust males from hydrodynamically favourable sites on the flat surfaces behind fins. The distribution pattern may also possibly occur as a result of specific behaviour of males designed to maximise encounters with sexually compatible female stages (Poulin, 1996) but possibly also because

their smaller size may make them less vulnerable to drag or water turbulence on the exposed surfaces of fish. Interestingly, females of *L. pectoralis* tend to congregate on the under surfaces of the pectoral fins of the fish species which they parasitise (Boxshall, 1974b, Kabata, 1979). The under surfaces of these fins are likely to be protected from both turbulence and abrasion from sediment particles in these bottom-dwelling fish. Males of this species again tend to show less regard towards the hazards presented by these environmental factors and have a wider distribution on the fish host, possibly again due to competition for favourable sites from adult females or to maximise their chance of encountering similarly sized, and presumably also ousted, sexually compatible preadult females. Parasitic branchiurans of the genus *Argulus* also display a very marked distribution upon the host fish with protected areas, such as the lee side of fins, being favoured over exposed body surfaces (Shimura, 1983a). This preferred distribution might then reflect a common behavioural adaptation to facilitate secure host attachment but might also reflect pressures from other factors. It should be considered that there may be a variation in the ease, or ability to feed, depending on the particular location of the parasite upon the host. The fins lack scales which cover the majority of other body surfaces, and so might facilitate easier feeding. Male *L. salmonis* appear to be preferentially distributed around the head region of their host fish, which shares the same epidermal characteristics as the fins although as Todd *et al.* (2000) pointed out, this distribution may be determined by factors not governed by choice on the part of the males. If males are displaced from preferred sites by females then maybe the corrugations and dimples frequently found on the salmonid head may provide shelter for the smaller males as opposed to the exposed flanks of the fish. The commonality of distribution between caligids and argulids might be proposed to support the theory that these parasites are actively seeking areas of reduced exposure to water currents and do not seek points of attachment solely on account of any aspect related to feeding. Shimura (1983a) also noted that argulid species had a more random distribution across the surface of inactive fish of slow moving waters, in contrast to those found on fast swimming fish and proposed that the distribution reflected requirements for shelter in those specimens on fast swimming fish. Such a proposal suggests that fish ectoparasites are subjected to drag forces as a result of the swimming activity of their host and therefore a secretion with hydrodynamic

properties on the exposed dorsal surface of these parasites may be of benefit to prevent them becoming dislodged. Ritchie *et al.* (1996b) have proposed that preadult females aggregate on the head region of fish to allow the 'chemical advertisement' (in other words, pheromonal attraction) of males situated more posteriorly on the animal. They further propose that this behaviour of the females facilitate the location of mates by adult males. Weissman, Lonsdale and Yen (1993) observed a reduced passive sinking rate of ciliate-infected *Acartia hudsonica* (Giesbrecht), a discovery that would seem to substantiate claims that projections and ornamentation's increase drag, unless of course epibionts have lower specific gravities than the copepod. This evidence may suggest that the secretions of *L. salmonis* are involved in preventing epibionts from attaching and affecting their drag profile.

Adult female lice on freshly killed salmon are generally orientated with their anterior end facing toward the anterior of the fish. Again, it seems reasonable that orientating themselves in this manner is likely to decrease the possibility of being detached from the a rapidly swimming host. Personal observations *in vitro* suggest that lice are more readily dislodged from surfaces by a strong jet of water directed at them from their posterior end than one directed at the front of the cephalothorax. Males do not generally exhibit such a marked uniformity of orientation, but are frequently found with some or all of their cephalothorax hidden underneath scales on the fish (Jonsdottir *et al.* 1992). This behaviour may be a deliberate attempt to 'hide' from the full force of the water flow, and reduce drag forces or to cover the 'liftable' front edge of the cephalothorax. Adult male *L. salmonis* and adult male and female *C. elongatus* are frequently observed (author's unpublished observations) to freely and rapidly move across the surface of the host fish after its removal from water. Female *L. salmonis* did not commonly exhibit such behaviour. The apparent positions of male *L. salmonis* and *C. elongatus* adults on captured fish however may not therefore represent their location *in vivo*.

The morphology of the sea louse cephalothorax is obviously well adapted for attaching to the host fish, and has a low profile that is likely to be beneficial hydrodynamically (i.e. low drag) whilst on the surface of the fish. Their shape though does not superficially appear to be hydrodynamically suited to free swimming in the water column. The ventral surface, being essentially concave with many protruding

appendages, is bound to counteract to a large extent any hydrodynamic benefit provided by the streamlined dorsal surface unless the secretion has evolved to facilitate the smooth passage of water over attached parasites to reduce the risk of their detachment from fast swimming hosts. An analysis of the swimming speeds of the hosts of different species of caligid copepod might reveal a relationship between parasite gland densities and host swimming speed, a topic that shall be addressed in the following section. This has not been investigated in the current study but should be examined in any future study of gland secretions of caligid copepods. An enhanced hydrodynamic profile on the dorsal surface however may serve to partially offset the irregularities of the ventral surface in a free-swimming specimen.

From observations *in vitro* and presumptions about the natural behaviour of sea lice both on and off the host it is possible to hypothesize that they will face two separate hydrodynamic difficulties. The first difficulty is that associated with being attached to a host which can swim at speeds of $2.25ms^{-1}$ (Økland, Finstad and McKinley, 1997). Lice attached to fish may be subjected to pressure and friction drag as well as the effects of lift caused by decreased pressure directly above them as a consequence of their streamlined shape. Both of these forces (drag and lift) will jeopardise the secure attachment of the louse on the host and may cause unintentional detachment from the fish unless countermeasures are employed.

Little research has been undertaken to determine the dynamics of fluid flow around free-swimming copepods (Andrews 1983, Emlet and Strathman 1985, Yen and Strickler 1996) and no studies have examined water flow around attached parasitic copepods. An assessment of the measurable effects that hydrophobic secretions present on the cuticle surface might have on the passage of water across the cuticle of copepods has never previously been made.

The data obtained in this study demonstrates that the mucus layer of *L. salmonis* does promote their faster passive sinking and that lice live in a microenvironment where a hydrodynamically active secretion would be of benefit to them. Whether this mucus layer does in fact provide such an advantage though has not been clearly resolved by the work undertaken so far.

7.5.2 Implications of setae structure, dimorphism and gland ratio analysis

The data on setal length indicate that the large terminal setae on the posterior margins of the caudal rami are much longer, both in real and relative terms, in adult male *L. salmonis* than in their female counterparts, a feature that was observed also in *L. pectoralis*, *L. hippoglossi* and *C. elongatus*. The difference in the relative lengths of the terminal setae between sexes is very pronounced and is such a conspicuous feature that it seems likely that there is a behavioral factor underlying it, rather than just being a random genetic event. Both *C. rogercresseyi* and *C. lacustris* displayed dimorphisms in the opposite direction from the other caligids examined with the females having longer terminal setae (in both real and relative terms) than the males of the species. *C. rogercresseyi* also showed a much less pronounced sexual dimorphism in body size than was typical for *L. salmonis*, *L. pectoralis*, *L. hippoglossi* and *C. elongatus*, whilst in *C. lacustris* the typical sexual dimorphism of body size was reversed with the male being larger than the female. The morphological characters of these latter two species suggest that the females have not evolved the same level of host association as *L. salmonis*, *L. pectoralis*, *L. hippoglossi* and *C. elongatus* if a dimorphic increase in female body size is a characteristic of a greater level of host association (Poulin, 1996). The natural behavior of *C. lacustris* and *C. rogercresseyi* are not well known and so it is not possible to correlate these findings with direct evidence of their lifestyle.

The reasons for the dimorphism of setae length are unknown but are possibly related to the mode of life of the male. It is unclear quite how males differ in their behaviour but it is likely that they are more active and mobile than females on the host surface as they will be seeking out preadult females for mating purposes (Poulin, 1996). A recent study by Hull *et al.* (1998) suggested that male *L. salmonis* may even swim freely in the water column to seek out new hosts, presumably in order to maximise reproductive output. A similar strategy was proposed for males of the related species *L. pectoralis* by Anstensrud (1990). It is possible that the terminal setae of the males serve to increase surface area of posterior part of body to enhance swimming ability, as suggested for several microcrustaceans by Lochhead (1974). Female caligid copepods have a larger posterior region than their male counterparts and may not need

setae to enhance this ability, coupled with the fact that they may not routinely swim freely off the host once the adult stage has been reached.

The unsubstantiated observation of a reduced urosomal gland activity of females (Chapter 3), if it is correct, may be of significance in respect to the differences in the condition of these setae between the sexes. SEM examination of the terminal setae of adult female *L. salmonis* revealed that they were of a significantly lower condition than those of the males were. The SEM images (**Figures 7.16** and **7.17**) of the terminal setae of the two sexes are particularly striking as they clearly demonstrate this marked difference in setae condition between the sexes. The SEM evidence is not artifactual as light microscope observations (incidental to obtaining measurements of terminal setae length) revealed that, invariably, the setae of the females would be torn, fractured or in many cases missing entirely. No TEM analysis of the setae was undertaken in this study but FC-72 fixed setae would provide interesting data pertaining to mucus layers on the terminal setae and may indicate whether the apparently more active urosomal glands of males provide a more substantial layer than the females. Adult female *L. salmonis* are still competent swimmers, a fact that may in part be explained by their larger genital segment and abdomen which may provide the thrust and steering capabilities provided in the males by their terminal setae. Von-Vaupel-Klein (1982b) has stated that terminal setae are important in the swimming mechanism of calanoid copepods whilst Kabata and Hewitt (1971) have suggested the same for *L. salmonis* in particular. Such observations are further indications that the dimorphism in terminal setae size, condition and associated gland activity of *L. salmonis* is reflected by differences in swimming requirements.

The known host-transfer activity of males might suggest that the gland secretions are either directly involved in improving the swimming ability of these mobile stages or is involved in some way to maintain the setae in a functional state. Such a function would be pertinent particularly if the longer terminal setae of the males were an adaptation for swimming / surface mobility on the host. The data from the swimming speed analysis undertaken in this study has shown that specimens of *L. salmonis* have Reynolds numbers, approximately in the region of 2000, although this is liable to be a substantial underestimate. At this sort of environment the terminal setae are likely to be useful as a 'vane' for

steering as has been suggested by Kabata and Hewitt (1971) and may even serve to increase the surface area of the posterior of the animal which may be utilised to provide propulsive flexing movements. Sierszen, Maki, Remsen and Brooks (1982) describe how the uropodal setae of the mysid *Mysis relicta* Loven are interlocked by their setules and describe the uropod as an appendage that is integral in the swimming behaviour of the animal whilst Mauchline (1998) states that the urosome of some calanoid copepods is utilised in escape reaction swimming. Kutash and Craig (1998) describe how, in aquatic nymphs, propulsive methods of swimming such as rowing are effective at low Re but that abdominal dorso-ventral oscillations are more effective at higher Re environments where inertial forces dominate. Lochhead (1974) suggested that terminal setae of aquatic arthropods might have some role in reducing the turbulence of water flowing across the animal. The larger size of the male terminal setae may also explain the apparently greater activity of male urosomal glands over female (Section 3.3.5.6) i.e. larger setae will require more secretion if the secretion has a direct effect on the functioning of those setae. Analysis of the free-swimming behaviour of *L. salmonis* would provide evidence relating to the urosome and its involvement in swimming.

The longer terminal setae of the caudal rami of male *L. salmonis* are likely to be a relict feature as males are unlikely to have evolved longer setae, rather females are likely to have lost them possibly as a result of their reduced swimming requirement. The males can therefore be looked upon as being less adapted to parasitism than females and consequently better adapted to a free-living mode of existence. If this does represent an evolutionary dimorphism it is at a very poorly developed stage. Huys (1988) describes a sexual dimorphism in the terminal setae of a group of harpacticoid copepods within the family Paramesochridae though he provides no hypothesis to explain this observation. Both sexes of *L. salmonis* retain essentially similar body morphologies in terms of their overall shape and locomotory appendages suggesting that the ability to swim is still a requirement. However, as both sexes are entirely dependent on host fish as a food source they can be deemed to have wholly committed themselves to a parasitic lifestyle. The retained swimming ability in the host-associated female stages can be assumed either to be in the process of being lost or is still a fundamental requirement. Dimorphisms of the terminal setae of

free-living copepods was not investigated in this study but again should be included in any future work undertaken in this area.

Differences were observed between the morphologies of the terminal setae and the natatory setae of the thoracic legs. The setules of the terminal setae (**Figure 7.16**) are fine and appear cylindrical in shape whilst those of the thoracic legs are flattened to form blades that presumably increase the surface area for the propulsive swimming stroke (**Figures 7.18** and **7.19**). These morphological differences suggest different functions for these setae. It suggests that the terminal setae are not used for propulsion, or at least are less effective at providing propulsion, but may instead be used for directional control or possibly to reduce turbulence at the trailing edge of the parasite.

A reduction in swimming ability, as a parasite becomes more adapted to its host, should be accompanied by a concomitant development of attachment, or holdfast organs as Kabata (1979) has proposed. There appears to be no sexual dimorphism of the attachment organs of *L. salmonis*. We can presume that female *L. salmonis* still have a requirement to swim after the settling stage as evidenced by the retention, and even development, of the swimming legs in the preadult and adult stages. It is possible that they are in the process of evolving away from this free-swimming state but there is no evidence to support such a hypothesis. It should be borne in mind though that the ability to move over the surface of a host fish is largely dependent on the action of the thoracic legs so retention of these structures does not necessarily imply a free-swimming habit in adult females. The copepods need to move across the surface of the fish host to facilitate their mode feeding (Andrade-Salas, 1997). During this feeding action the copepods are unlikely to attach to the host via their maxillipeds and antennae but are likely to rely solely on the negative pressure created under the cephalothorax by the action of the thoracic legs, as described by Kabata and Hewitt (1971). During this phase the copepods are less securely attached to the fish than they would be whilst using their prehensile thoracic appendages, and it may be possible that a hydrodynamically active gland secretion may be of utility during these periods. A drag reducing secretion may facilitate the passage of water over the cuticle, making the copepod less hydrodynamically 'conspicuous' and lessening the threat of the copepod being swept from the host by passing water flow.

So can morphology, and implied or observed behaviour of a parasitic copepod be used to provide an indication as to the host of that parasite? The data obtained in this study suggests that it may at least be possible to identify some aspects of the behaviour of species from the structure, condition and relative size of their terminal setae but this approach is unlikely to be of great utility for the following reasons. One difficulty of trying to correlate morphological differences in caligid copepods to differences in life-style and swimming behaviour is that the caligid copepods are all very similarly adapted and display few pronounced morphological differences between species. As such it is difficult to identify clear trends in morphological parameters, especially with the few species available in this study. Comparison with other parasitic species of copepod, both more and less adapted to their hosts may provide further evidence regarding the development of glands and the structure of the terminal setae in relation to life-style.

One aspect of louse biology that we have not considered, and may have relevance to the secretion of these glands and their proximity to the setae of the louse, is the fact that lice living within the host epidermal mucus layer may be prone to becoming 'clogged' with host mucus. Host mucus functions as a physical barrier as well as a chemically protective one (Whitear 1970, Shephard 1994). Presuming that the setae are essential in swimming then the consequences for a louse with setae smothered with host mucus are that their ability to move either on or off the host is likely to be severely impaired. The secretions of the exocrine glands may well serve to prevent host mucus from sticking to the cuticle of the louse. Such an anti-adherent effect may be effected through two paths, a chemical incompatibility between mucus types preventing adhesion, or a chemical 'digestion' of the host mucus where it contacts the louse. No identified component of the louse secretion could accomplish such a 'digestion' but the mucus types themselves may be chemically different and may remain discrete from one another, thereby preventing adhesion. Several reports have shown that the epidermal mucus of louse infested salmon is thinner and more watery than the mucus of uninfected fish (Horne and Sims, 1999). This altered state has always been attributed to the stress response of the host and an active involvement of louse-secreted factors in altering the state of the host mucus has never been considered.

The data in **Table 7.3** illustrates that in all four species of caligid copepod studied, males and females of the same species possessed comparable numbers of dorsal surface glands, as a ratio of glands to dorsal cephalothorax surface area. It is possible that differences in gland density may reflect differences in behaviour of copepods as the extent of the adherent mucus layer is likely to be dependent on the ratio of glands to surface area. Between the species examined in this study though there are clear differences in the numbers of glands per unit area. *Lepeophtheirus salmonis* have a considerably lower density of glands on their dorsal surface than do the other three species of caligid examined here. The reasons for this disparity are unclear but may reflect some behavioural differences in which the secretions of the glands are involved and these differences may be revealed if we roughly separate the four copepod species into three different behavioural categories according to their host species. *C. elongatus* is widely distributed with numerous different host species (Kabata, 1979) whilst *L. salmonis* is restricted to one species of migratory marine fish and *L. pectoralis* and *L. hippoglossi* are associated with a restricted number of benthic fish species. These parasites are likely to exhibit behaviour that reflects these differences in their hosts' distribution. This behaviour is likely to contrast that of *C. elongatus* with its high number of potential host fish.

It is reasonable to assume that, as behaviour precipitates morphological change, parasitic copepods with a specific host, with *its own* specific and characteristic behaviour and habitat, will show modifications acquired as a result of being associated exclusively with that host. For example, species such as *L. hippoglossi* associated with non-shoaling benthic hosts, are likely to be subjected to different pressures regarding host and mate location than species such as *C. elongatus* associated with multiple species of abundant pelagic fish. Determination of the passive sinking rates of the other three species of caligid copepod examined in this study would be of great utility. Particularly it would be of great significance if *C. elongatus* showed a markedly greater reduction in relative sinking rate after removal of its cuticular mucus layer than observed in *L. salmonis* as it would suggest that increased gland densities result in a faster passive sinking rate which would suggest that the secretions from those glands had a direct effect on the hydrodynamics of the animal.

Gland densities of caligid copepods appears to be greater than those described for cuticular pores of other species e.g. *Hemidiaptomous ingens* has been shown by Brunet *et al.* (1991) to have only 19 pores on the dorsal surface of the body cuticle but less than those described for other arthropod groups i.e. 1,400 glands/mm^2 in the Pycnogonida (Fahrenbach, 1994). Von Vaupel Klein (1982b) describes the calanoid copepod *Euchirella messinensis* to be capable of swimming at 100cms^{-1} yet the number of gland pores on its body cuticle is much less than those of the caligids described here (approximately 40 in adult females) which suggests either that gland secretions are not related to swimming ability or that variations in the efficacy of those secretions exists between species. Data concerning gland numbers per unit area in caligids may not be of much utility as glands may originally have evolved for another purpose so may not give any indication as to present swimming ability. It is likely that gland density does not reflect swimming behaviour in all species of copepod but is rather likely to be an indicator of specific requirements of the individual species habitat and behaviour.

Glands in the abdominal segment, termed 'telson glands' by Elofsson and Hessler (1998), have been identified in the cephalocarid species *Hutchinsoniella macracantha*. These glands are very large and fill the last two abdominal segments and exit on the tip of the caudal ramus and would appear to be homologous with the urosomal glands described in this study for caligid copepods, by Boxshall (1982) for misophrioid copepods by Fahrenbach (1962) and Williams-Howze (1996) for harpacticoid copepods and by Chapman (1981) for calanoid copepods. Cephalocarids although not necessarily interstitial live on soft depositional bottoms: muds and oozes. How a proposed hydrodynamic secretion would benefit such species is less clear although it is possible that small interstitial species can move freely in water between the particles. The fact that such glands are widespread throughout the Copepoda and other crustacean orders though might suggest that the glands are adapted to serve different functions according to habitat and are unlikely to fill the same role in all species that they occur in.

However, it is important not to ascribe all morphological differences to a specific evolutionary adaptation. Caligid copepods are unlikely to have evolved an entirely novel set of exocrine glands to suit their adopted environment. It is more probable that the secretions of the extant gland system of their free-

living ancestors adapted, over time, to suit their particular needs. Therefore the distribution of the glands is still likely to reflect that of free-living species (as discussed in Chapter 3), although the function of their secretions may have become adapted to suit particular needs.

7.5.3 Bacteriological studies

The isolation of three different species of bacteria from the cuticle of *L. salmonis* was successfully achieved in this study. The three species of bacteria isolated were all identified as belonging to the genus *Vibrio* but the characteristics of each species did not allow them to be identified to genus level. The *Vibrio* group is well represented in the marine environment and indeed Simidu *et al.* (1971) suggested that this genus accounted for more than 70% of the bacterial flora of plankton whilst Sochard *et al.* (1979) also demonstrated that *Vibrio* species were the predominant species of bacteria colonising marine copepods. It is likely that more than three bacterial species colonise the cuticle of *L. salmonis*, but variations in the extent of colonisation are likely to occur due to seasonal, anthropogenic, and other factors.

The evidence from the bacteriology experiments described in this study suggests that the secretions of the urosomal glands of *L. salmonis* have no directly bactericidal properties. Consequently, there is no evidence that the stained secretions observed on the caudal rami and natatory setae are functioning to directly control, or modify, numbers of bacteria infesting sea lice. In the one instance when a small ring of apparent growth inhibition was observed it was so small as to be inconclusive in terms of a bactericidal action. The mild inhibition observed was probably artifactual and could have been caused by a number of factors. For bactericidal properties to be apparent it might be expected that the secretion would act rather more swiftly and dramatically, although at ambient sea temperatures the growth of bacteria is probably very slow so even an apparently slow or moderate reaction may be sufficient for the purposes of the lice. The fact that substantial numbers of bacteria are frequently observed on the cuticle and setae of *L. salmonis* does not necessarily preclude the possibility of a bactericidal secretion being involved. The numbers present may represent a reduced burden over what would have occurred without the protection of an active compound.

In an apparent corroboration of the evidence from TEM, a species of bacteria with chitinolytic properties was isolated from the cuticle of *L. salmonis*. This bacterial species was distinguishable macroscopically from the other two isolated species by its cream colouration. The depletion of the chitin incorporated into the marine agar by the cream coloured species of bacteria suggests that they rely on chitin, at least in part, for their nutrition. As the cream coloured bacteria apparently grew well on marine agar alone, it would appear that this species is not wholly dependent on chitin for its nutrition or that the apparent removal of the chitin is caused as a secondary effect of the growth of the bacteria, possibly as a result of some excreted product. *Vibrio harveyii* has been shown to possess chitin-degrading enzymes (Svitil, Ni-Chadhain, Moore and Kirchman, 1997), as has *V. cholerae* (Dumontet *et al.* 1996) whilst Krieg (1984) states that chitinases are a common feature of *Vibrio* species, and so the identification of a chitinoclastic *Vibrio* species in this study is not without precedent.

Osawa and Koga (1995) identified 48 different species of bacteria capable of utilising chitin as their sole source of nutrients within one sea area of Japan alone suggesting that bacteria with chitinolytic properties are very common in the aquatic environment. Dead copepods have been shown to become rapidly and extensively colonised by bacteria that are suggested to degrade the cuticle resulting in the recycling of nutrients held in copepod carcasses (Kirchner, 1995). The fact that healthy copepods can be assumed ordinarily to have a relatively low bacterial burden suggests that some aspect of either the behaviour or physiology of healthy animals prevent them from being overrun by these bacteria. As secretions of the identified exocrine glands of *L. salmonis* have been shown in this study to have little or no antibacterial effects yet specimens had relatively few bacteria adhering to their cuticles, may suggest that the secreted fuzzy layer prohibits the establishment of such bacteria. This prohibition may be effected in a purely physical way by preventing the bacteria from reaching the cuticle (see Parsons and Mulholland, 1978), or by entangling them and reducing their mobility. The mucus layer may also provide a temporary or unstable surface for attachment. Such an action would explain the inability of *L. salmonis* gland homogenate to inhibit the growth of general marine bacteria although their overall effect could still be termed 'anti-bacterial'. The role of the catalase enzyme component within this context of physical

barrier protection remains enigmatic. It may have a specific role in affecting the mobility or virulence of bacterial species but such an action was not identified in this study.

All of these hypotheses leave one question unanswered. How does the ventral cuticle cope with the assault of bacteria with chitinolytic properties? The ventral cuticle of *L. salmonis* is thinner than that of the dorsal surface, and is likely to be exposed to similar levels of environmental bacteria as the dorsal cuticle, yet it has no glands, other than the thoracic leg glands to provide it with a protective coating of mucus, although the epidermal mucus of the host fish may in part perform this job for the parasite. This paucity of glands is reflected by the very sparse fuzzy layer observed on the ventral cuticles of *L. salmonis*. No detailed analysis of the ventral cuticle of *L. salmonis* was undertaken in this study so we cannot say whether it suffers greater damage from such bacteria, but the very poor condition of the terminal setae of adult females may be reflected in the apparent reduced activity of their urosomal glands, in contrast to the adult male of the species. This study has only considered the effects of the urosomal gland secretion on bacterial species. The possibility remains that the secretions of other gland groups of *L. salmonis* may exhibit anti-bacterial or anti-foulant properties.

7.6 CONCLUSIONS

From the experimental procedures utilised in this chapter it is possible to conclude that adult stage *L. salmonis* (although the same may also hold for other stages) possess a conspicuous mucus layer that coats their body cuticle and is especially pronounced on the dorsal surface, and that this layer appears to have a demonstrable effect on the passive sinking rate of this species. It is still undecided whether the decreased sinking rate of specimens with their mucus layer removed is due to a reduction in weight that appears to result from the removal of the mucus layer or whether this weight loss has a negligible effect on sinking rate. It was not wholly conclusive from the evidence collected in this study whether the apparent hydrodynamic property of the mucus layer confers a significant hydrodynamic benefit to the louse but the evidence suggests that this might be the case.

The clearly demonstrable decrease in the sinking rate of untreated specimens that were repeatedly sunk in rapid succession indicates that some removable component with a positive effect on the hydrodynamics of the louse is naturally present on the cuticle. The decline in sinking rate to a specific, and steady, rate suggests that this component is mechanically washed off the cuticle during the sinking procedure. If the mucus layer is the feature which is causing this effect then a constant supply must be required to maintain a constant layer on the cuticle and may explain the full reservoir of secretory vesicles identified in all glands examined.

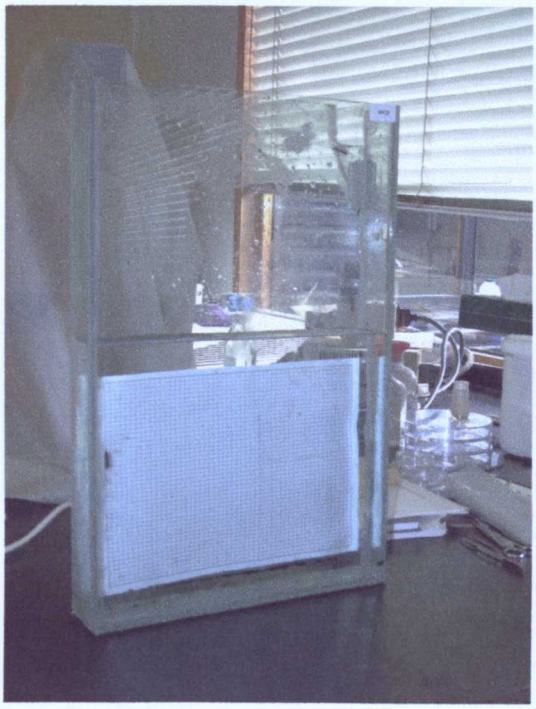
The morphological data presented here reveals many interesting features which may be related to the swimming ability of the copepod. Clear differences are apparent between numbers of glands per unit area between species and in the relative lengths of the terminal setae between the different sexes of the same species. Further research involving other species, both free-living and parasitic, is required to provide a greater understanding of these morphological differences and to clarify how they relate to the biology and behaviour of these animals.

Sexual dimorphism was noted in the lengths of the terminal setae of the caudal rami of all caligid species examined. In five out of six species the males had relatively longer setae than the females despite their smaller total body size. The implications of this dimorphism to swimming behaviour and life history were considered and are suggested to reflect, not necessarily a greater swimming ability of males but a feature that enables males to swim more effectively on account of their generally smaller body size. There was however no apparent sexual dimorphism in the numbers of glands per unit area of the cephalothorax in the four species of caligid copepod examined. Such a discovery correlates well with the belief that new gland systems are unlikely to be developed in a species regardless of sexually divergent behaviour and that gland numbers are therefore likely to be similar in both sexes. The functional significance if there is one, regarding the differences in gland density between species has been explored and may reflect differences in swimming speeds of host fish.

Three different bacterial species were isolated from the cuticle of *L. salmonis*, although more are likely to be present depending on the host habitat and the temperature of the water. None of the isolated

species could be identified beyond genus level. The secretions of the urosomal gland complex did not display any bactericidal properties against general marine bacterial species, but the secretions of other gland groups may have such activities.

The role of the identified catalase component of the secretion has not been revealed in the experiments performed in this study. It may serve some role in the dynamics of the mucus secretion or it may have a specific role of its own that remains to be elucidated.



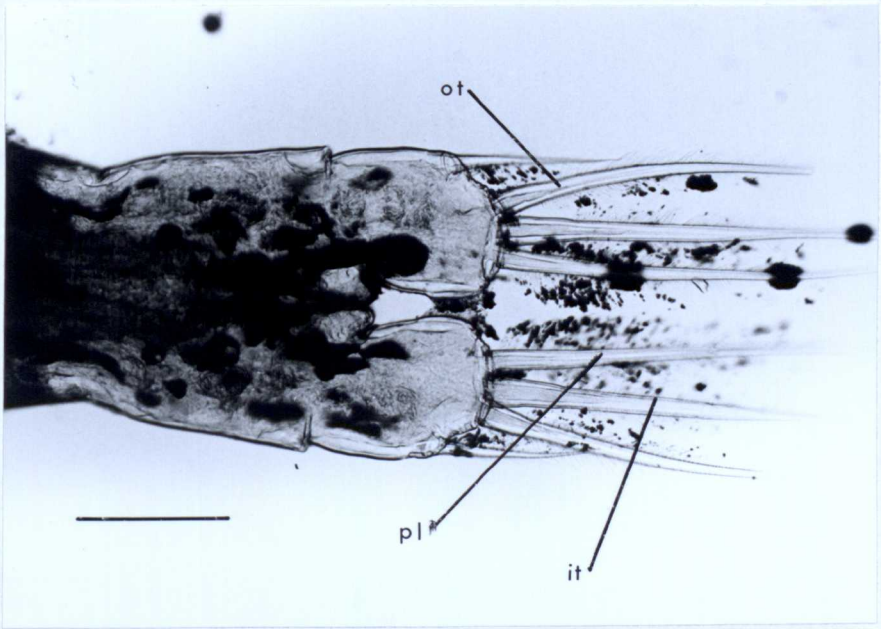


Figure 7.1 Glass aquarium used for swimming speed analysis. Grid is marked in 5mm divisions.

Figure 7.2 Dorsal view of the urosome of an adult male *L. salmonis* showing the positions of the terminal setae measured for analysis. Nomenclature of setae follows Huys (1988): ot outer terminal setae; pl posterolateral setae; it inner terminal setae. Scale bar = 200μm

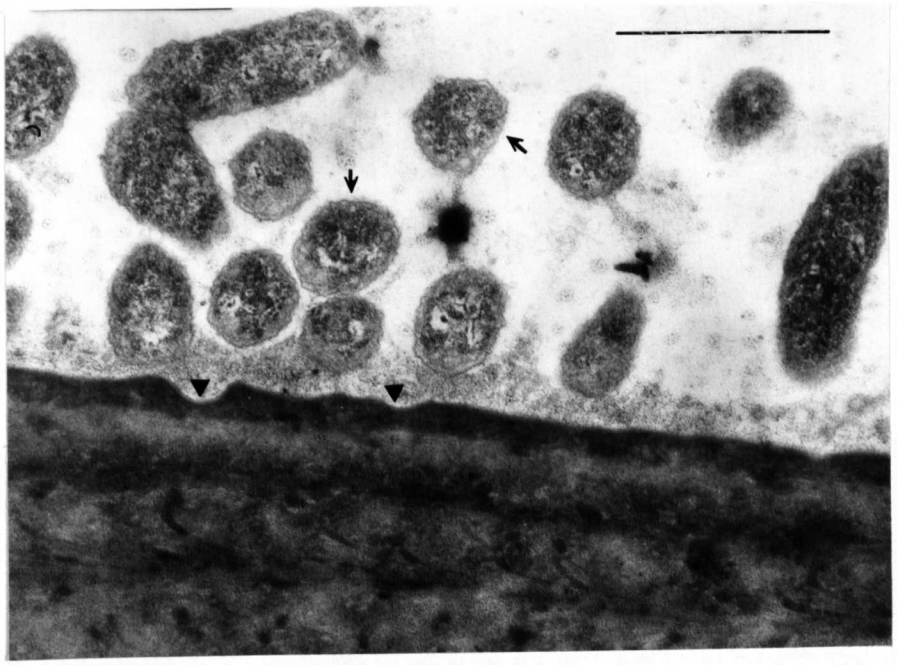
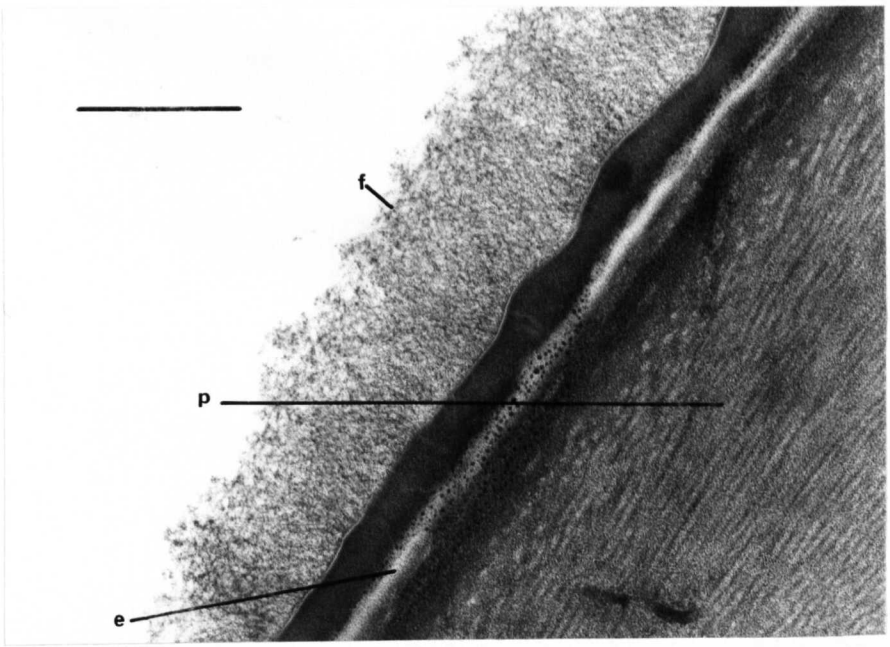


Figure 7.3 TEM micrograph of the cuticle of the urosome of *L. salmonis* showing associated bacteria (arrowed) and pits eroded in the cuticle (arrowheads). Scale bar = 1μm.



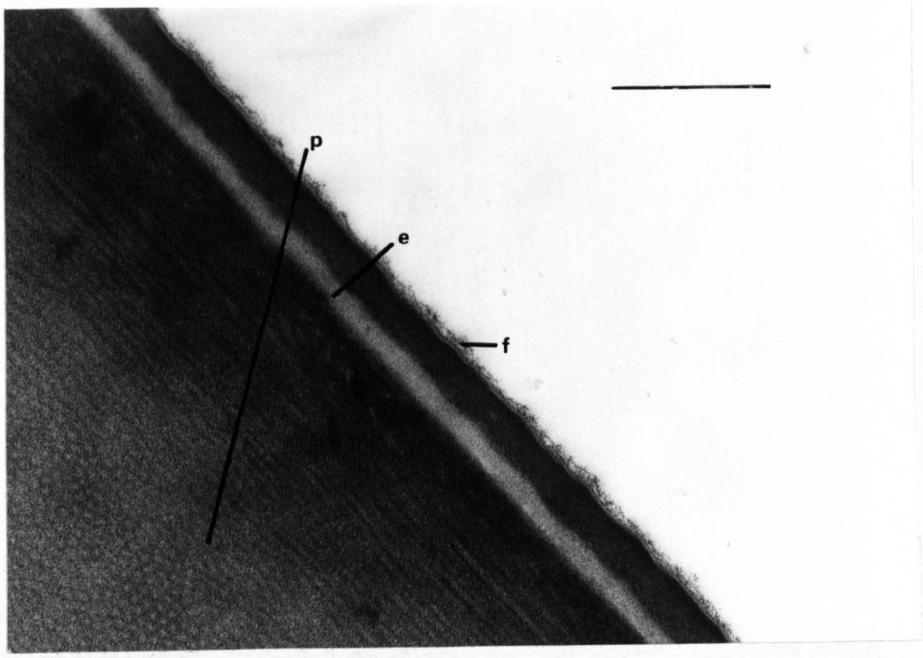


Figure 7.4 Fuzzy layer adhering to the dorsal cuticle of the cephalothorax of *L. salmonis* following fixation with FC-72. e epicuticle; p procuticle; f fuzzy layer. Scale bar = 0.5µm.

Figure 7.5 Fuzzy layer adhering to the dorsal cuticle of the cephalothorax of *L. salmonis* following fixation with Karnovskys fixative. e epicuticle; p procuticle; f fuzzy layer. Scale bar = 0.5µm.

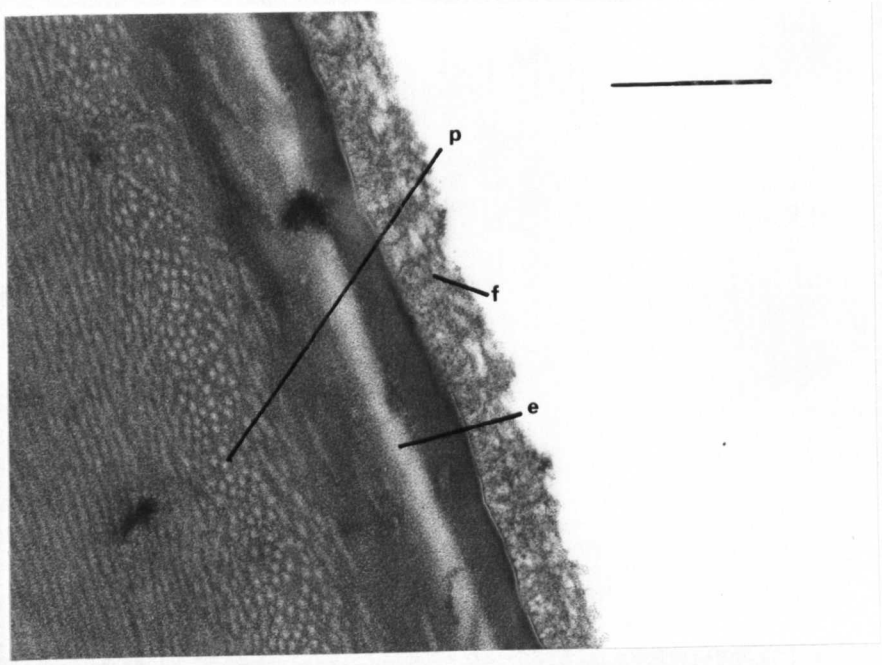


Figure 7.6 Cuticle of the ventral surface of the cephalothorax of *L. salmonis*, fixed using FC-72, demonstrating the extent of the associated fuzzy layer (arrowed). Scale bar = 3μm.

Figure 7.7 Fuzzy layer adhering to the dorsal cuticle of the cephalothorax of *L. salmonis* following rinsing with 1% Triton X-100 detergent and subsequent fixation with FC-72. e epicuticle; p procuticle; f fuzzy layer. Scale bar = 0.5μm.

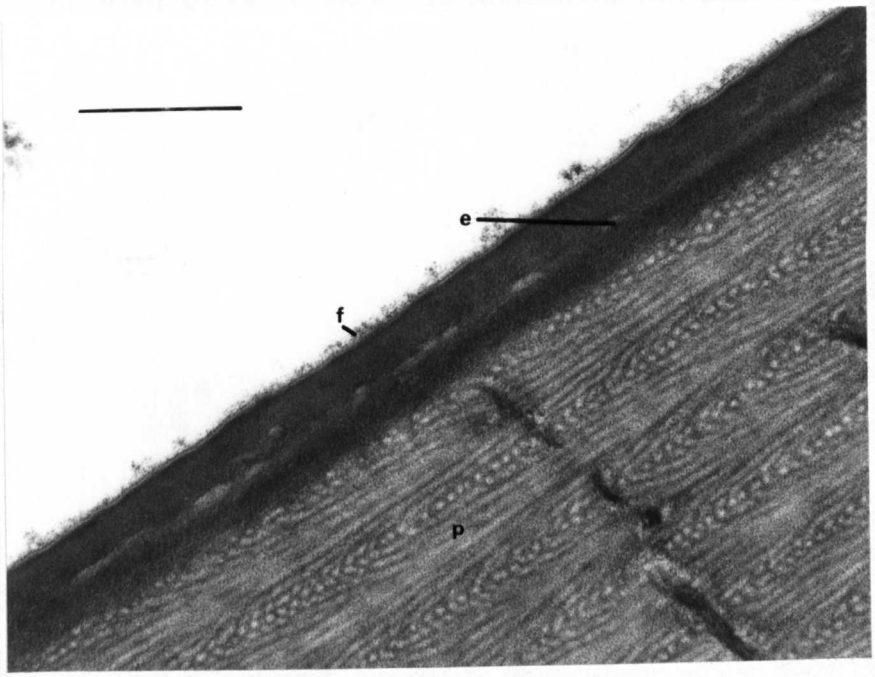
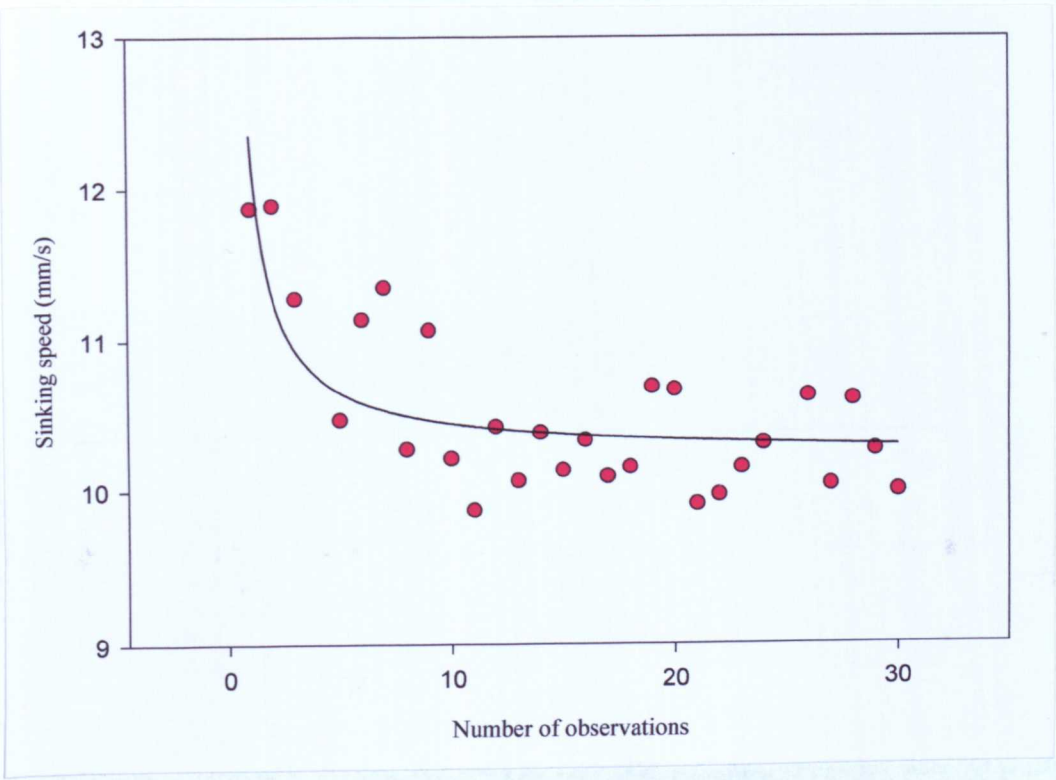


Figure 7.8 Fuzzy layer adhering to the dorsal cuticle of the cephalothorax of *L. salmonis* following rinsing with 0.05% Tween 20 detergent and subsequent fixation with FC-72. e epicuticle; p procuticle; f fuzzy layer. Scale bar = 0.5μm.



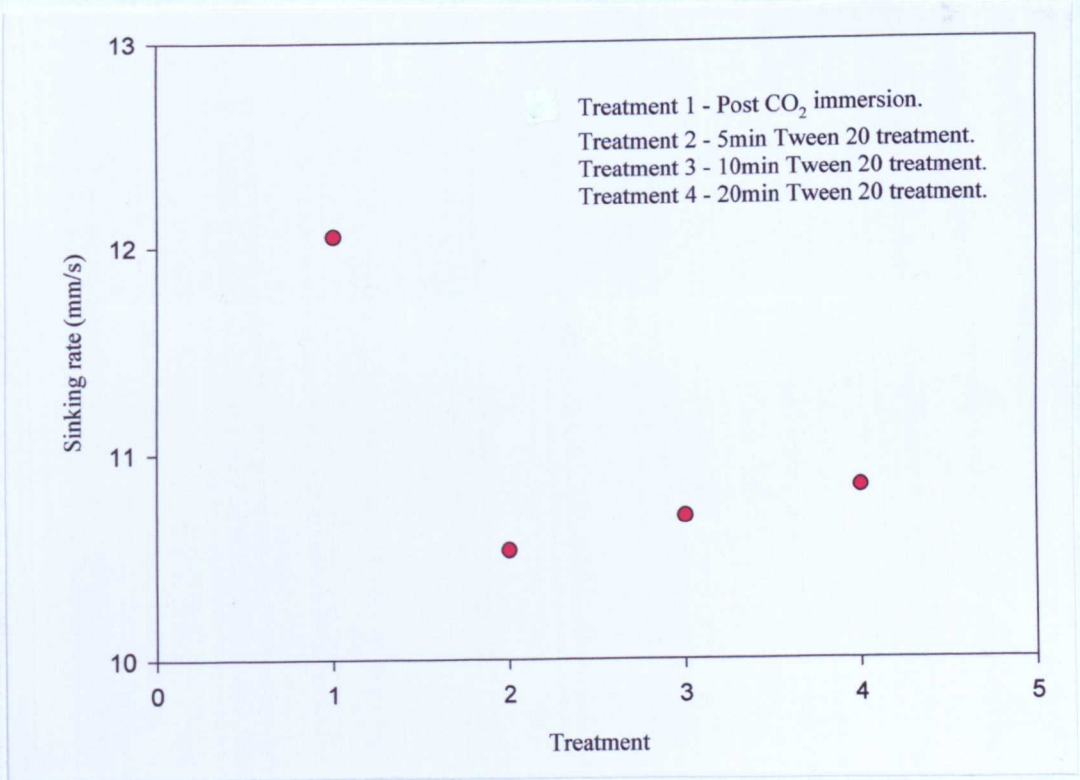


Figure 7.9 Change in sinking rate over time (number of drops) for a single CO_2 anaesthetised specimen of *L. salmonis*.

Figure 7.10 Effect of repeated, and extended, rinses of Tween 20 on the passive sinking rate of an individual specimen of *L. salmonis*.

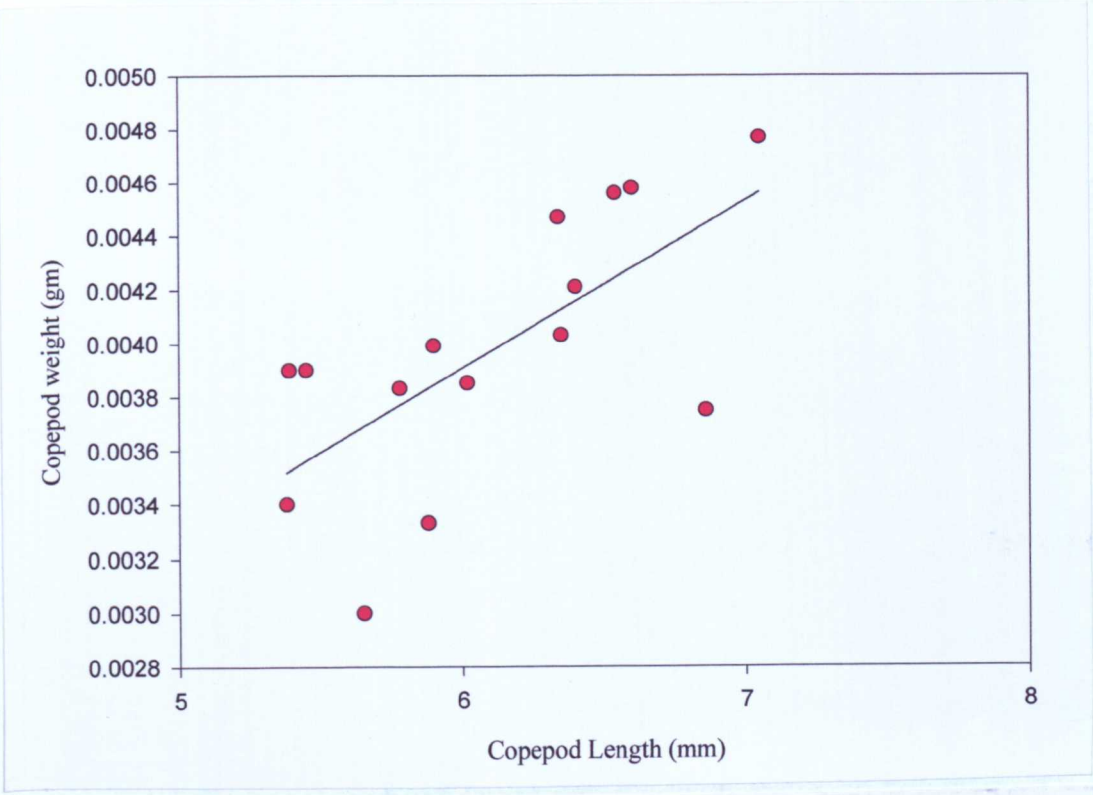


Figure 7.11 Relationship of *L. salmonis* wet weight (gm) and length (mm).

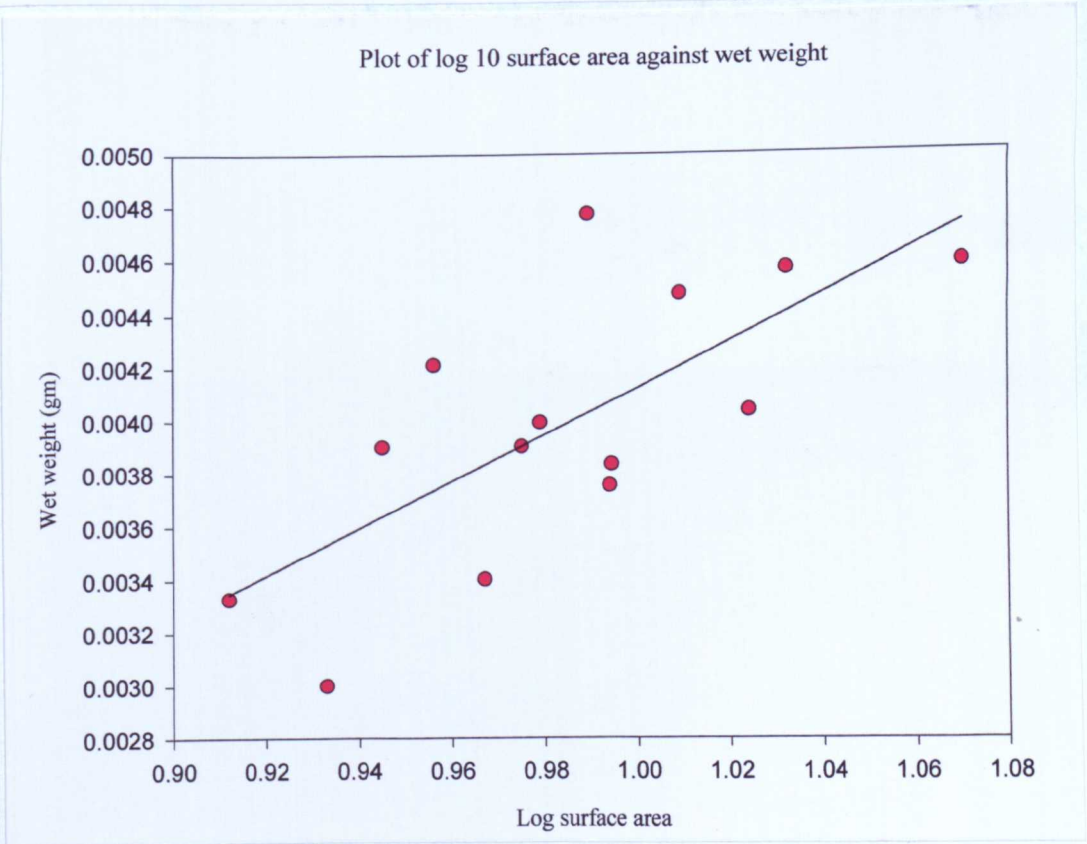


Figure 7.12 Relationship of *L. salmonis* wet weight (gm) and log 10 surface area (mm^2).

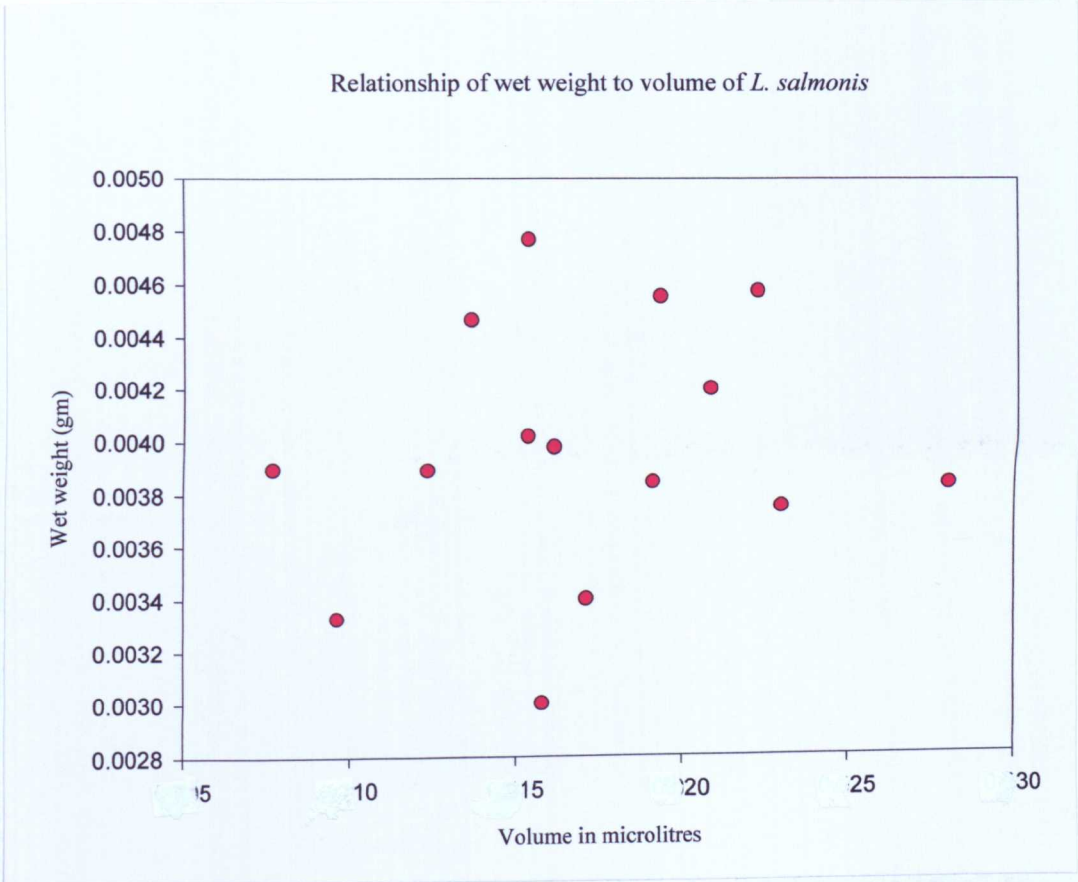


Figure 7.13 Relationship of *L. salmonis* wet weight (gm) and copepod volume (µl).

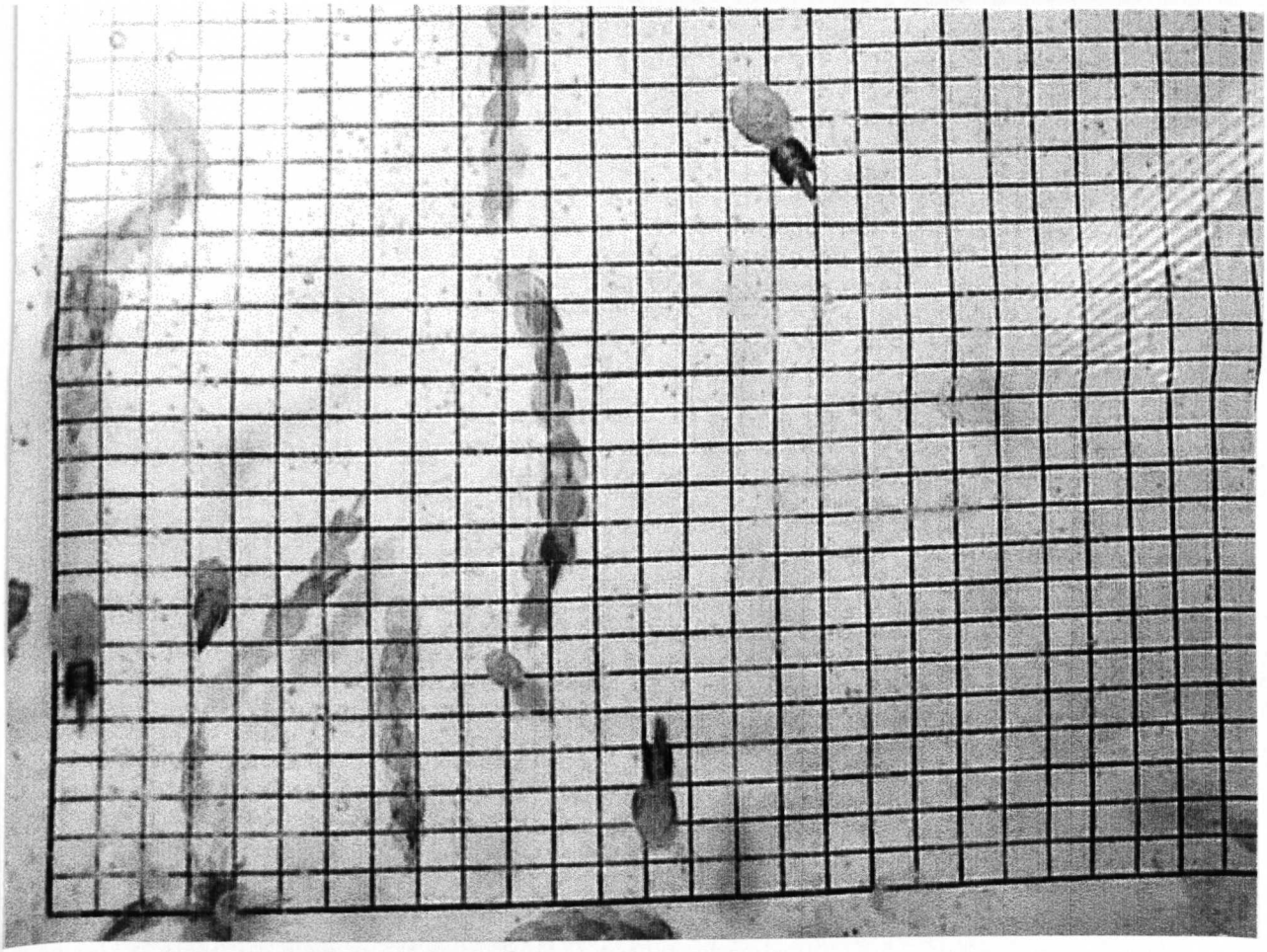


Figure 7.14 A typical composite image of the swimming path of *L. salmonis* as described in the text. Each square is 5mm by 5mm.

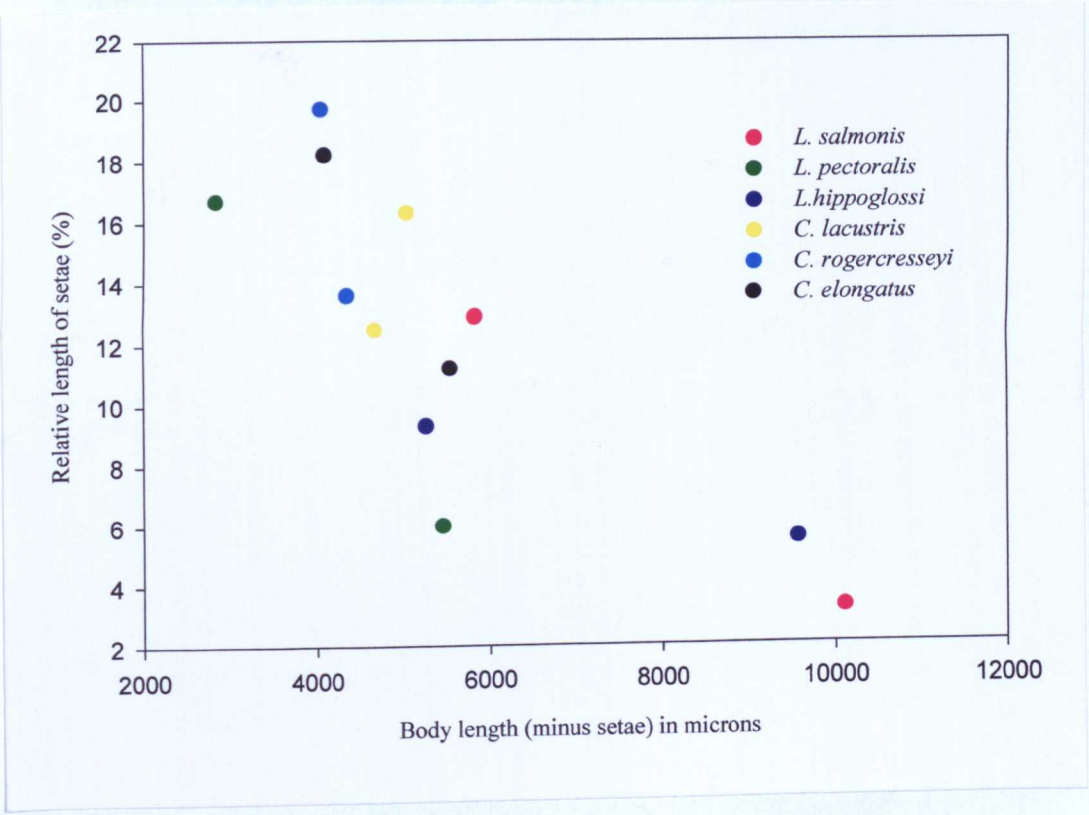


Figure 7.15 The relationship of caligid copepod terminal setae length as a percentage of total body length. In all cases except *C. lacustris* and *C. rogercresseyi* the male of the species is the highest of the two values on the y axis.

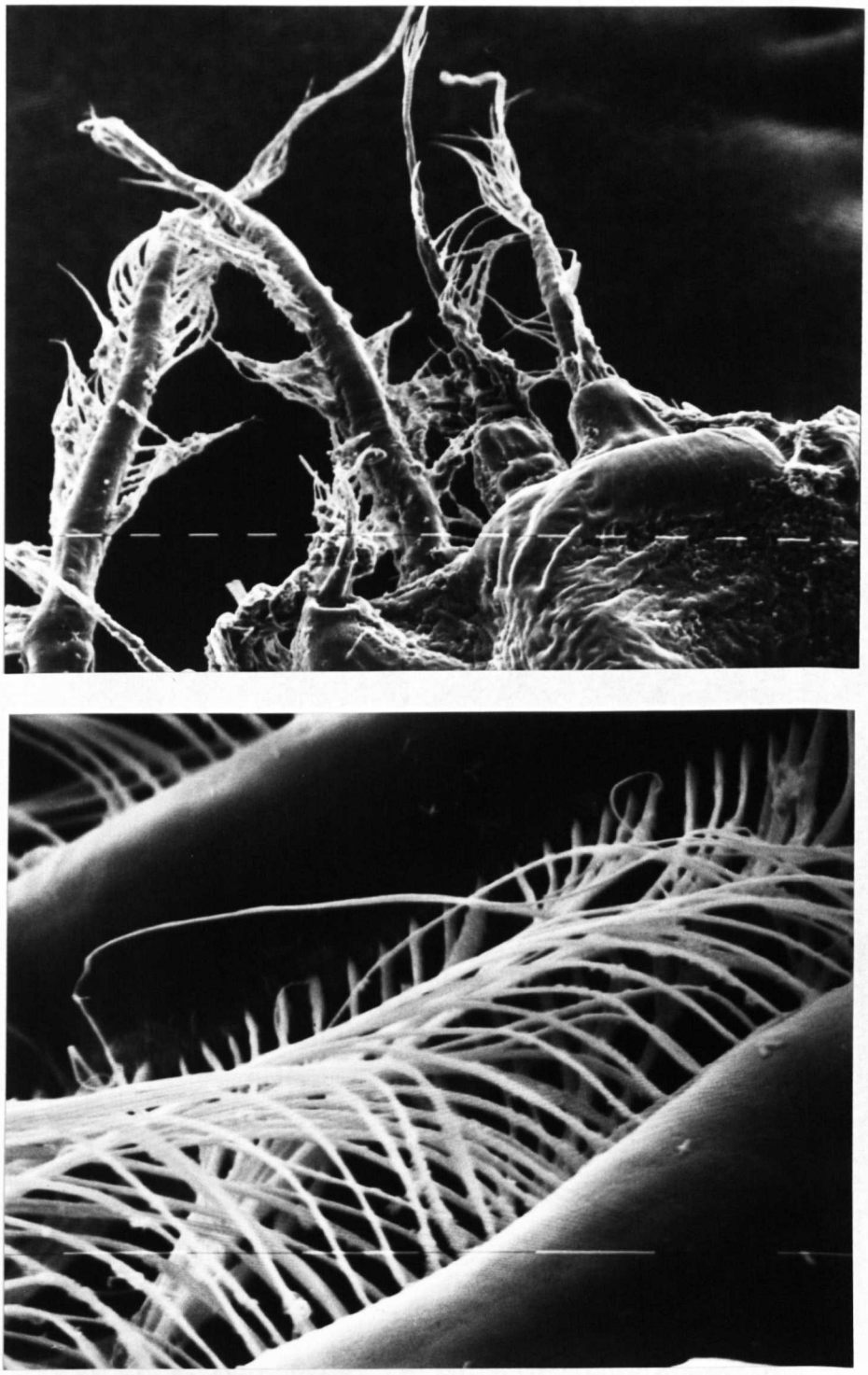


Figure 7.16 SEM micrograph of the terminal setae of an adult female *L. salmonis*. Note the lack of setules on the lateral margins of the setae and the general poor condition of the setae and setules. Scale bar marked in 1μm intervals.

Figure 7.17 SEM micrograph of the terminal setae of an adult male *L. salmonis*. The setules between setae IV and V are shown. Note the lack of damage to the setules and how the interdigitate with the setules of neighbouring setae. Scale bar marked in 1μm intervals.

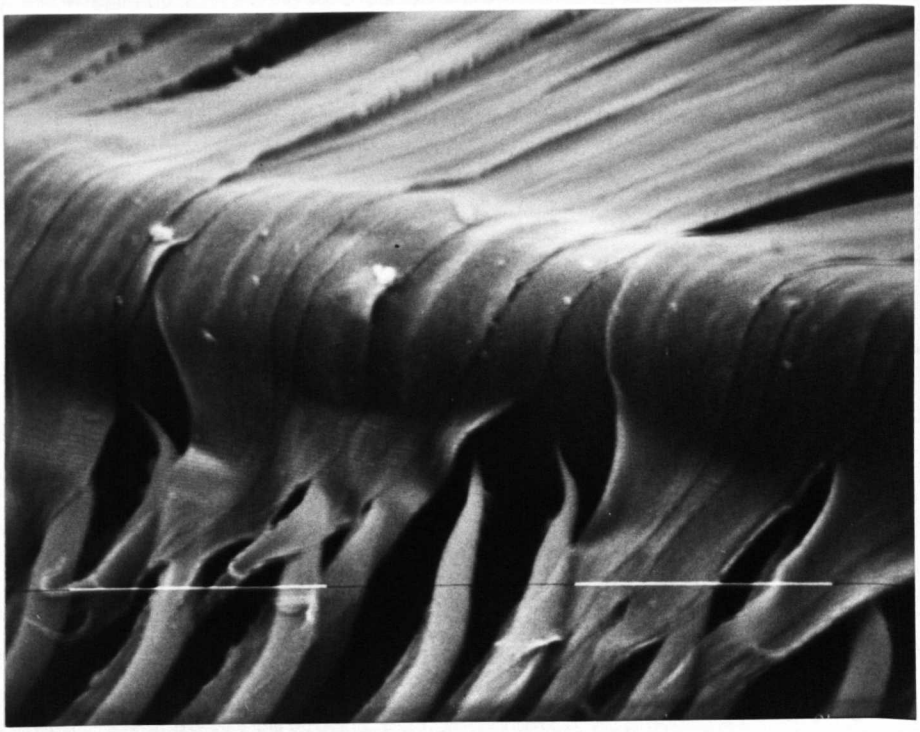


Figure 7.18 SEM micrograph of the setae and setules of the sympod of the th_{II} of an adult male *L. salmonis*. Scale bar marked in 1μm intervals.

Figure 7.19 Higher magnification of the sympod shown in Figure 7.12 clearly displaying the flattened nature of the setules and the extent of their overlap across other setae of the leg. Scale bar marked in 1μm intervals.

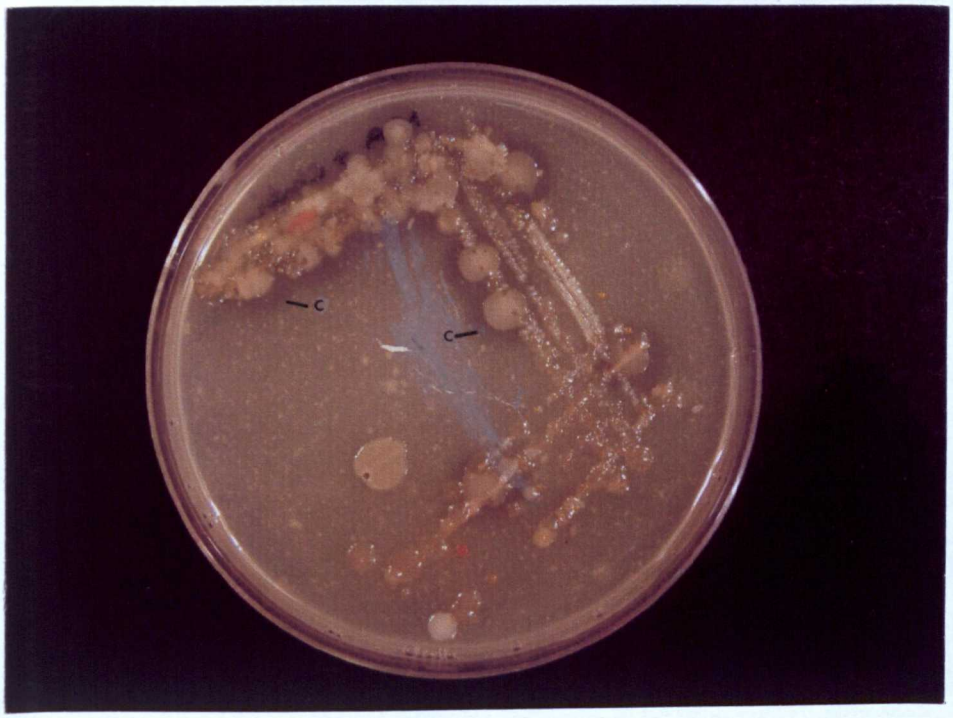


Figure 7.20 Zone of clearance (c) of incorporated chitin in a marine agar plate inoculated with the cream coloured bacterial species isolated from the cuticle of *L. salmonis*

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8. CONCLUSIONS

8. CONCLUSIONS

This study provides the first systematic description of a previously undescribed population of exocrine glands within 4 species of two genera of the copepod family Caligidae. Such glands were present in four representatives of the family suggesting that such glands are a common feature of the caligid copepods and it is likely that such glands are present in other members of the same family. Examination of several harpacticoid copepods demonstrated that glands with similar staining characteristics are also a feature of these species and the presence of a reduced number of similarly staining glands has been shown in members of the Calanoida.

Glands were present from the first larval stage (and even in unhatched nauplii) and the progressive development of some groups of larval glands could be followed to the adult stage, whilst others developed only in the later life-stages. The possibility that other glands, which do not stain positively with DAB, are present in caligid copepods but no evidence was found in this study to support the belief that other, unstaining glands, were present. Glands were often located close to regions of large expanses of cuticle, such as the terminal setae of the urosome, the natatory setae of the thoracic legs and the marginal membrane. The significance of this distribution is not obvious and may indicate that they are regions that require a beneficial drag-reducing secretion or they may be regions of exposed cuticle that require protection from attaching epibionts or reactive oxygen species.

No pore signature pattern, that may be useful as a species-specific identifier, was ever determined in any of the caligid species examined although the presence of such a pattern, as shown for other species of copepod, is not precluded.

Light and electron microscope analysis clearly demonstrated that the identified glands were exocrine in nature and possessed regions of secretory vesicle accumulation. Histochemical analyses suggested that the identified exocrine glands were rich in neutral mucus compounds as well as proteins. Histochemistry also suggested that the glands contained a demonstrable lipid component. Use of the

chromogens DAB and TMB initially suggested that the identified glands contained either a peroxidase enzyme or a form of sulphated mucus but the histochemistry data demonstrated that the gland tissues and secretions contained no sulphated mucus component (as demonstrated by a negative response to the Alcian blue stain) and suggested therefore that the positive response to the DAB and TMB stains was caused by some peroxidatic enzyme component. TEM examination demonstrated that at least the urosomal, labral and anterior gland complexes were exocrine glands with large zones of accumulated secretory vesicles. Slight differences were apparent between the morphologies of the vesicles of the different glands but the significance of these differences is unclear. Both the labral gland and the main secretory gland of the urosomal gland showed ultrastructural characteristics typical of mucus production i.e. significant amounts of RER, active Golgi complexes and plentiful secretory vesicles. The AGC showed a somewhat different ultrastructural morphology from those of the labral and urosomal glands. The secretory vesicles of the AGC were very different in their morphology and the relative proportions of RER and Golgi complexes differed from those of the labral and urosomal glands. The reason for these differences was not determined in this study and is in contradiction to the results of the histochemical analyses that suggested that all of the identified glands had similar properties in terms of their chemical constituents.

The application of specific enzyme assays indicated that the gland tissues of *L. salmonis* possessed substantial catalase enzyme activity that was significantly higher than the activity detected in the general body tissues. Catalase activity varied between gland types but was present in both gland types at levels significantly higher than background. Variation was observed in the catalase activities of the different tissues tested, with activities three and two times greater than control tissues for the urosomal gland complex and the AGC tissues respectively. The detection of catalase in body tissues other than the glands was probably accounted for by small portions of individual glands being inadvertently removed along with the negative control tissue and the actual level of catalase activity in the general body tissues of *L. salmonis* is likely to be only a fraction of that detected in the exocrine glands described. The detection of this catalase enzyme in the gland tissues proves conclusively that the observed DAB and TMB staining

of glands is caused principally by the presence of this enzyme and not by any acidic mucus components. Selenium-dependent glutathione peroxidase and glutathione S-transferase were also detected in the tissues of *L. salmonis*. Se-GPX activity was significantly greater in the urosomal gland complex than both the AGC and non-glandular control, which had similar levels of Se-GPX activity. The measured GST activity was similarly low in both glandular and non-glandular tissues. Specific staining techniques also demonstrated that the catalase was present in the secretory vesicles of at least one component of the urosomal glands whilst Se-GPX was present only in the cytosol of the gland tissues, in structures closely resembling ribosomes. The increased level of Se-GPX activity observed in the urosomal gland complex therefore probably occurred due to the large number of ribosomes present in this tissue.

Prostaglandin E_2 was not identified in the exocrine glands of *L. salmonis* which, along with the specific identification of catalase in the secretions, suggests that these glands are not involved in secreting these compounds, under the conditions tested. It is possible though, that such compounds may only be produced as a result of some host stimulus which was absent in the experimental system. HPTLC demonstrated that the identified glands of *L. salmonis* do not secrete a lipoidal material, as lipid profiles were essentially identical between the glandular and non-glandular samples. This suggests that the positive lipid staining of the glands observed with histochemistry was due to the positive reaction of the membranes of the subcellular organelles and the membranes of the secretory vesicles.

SDS-PAGE identified a protein of approximately 230kDa (consisting of 4 subunits of 65-70kDa) in the culture water of *L. salmonis*. This protein corresponded to the region of TMB staining of native gels indicating that it was the enzymic fraction of the gland secretions. The calculated molecular weight of this component is very similar to that of other identified catalases.

The fact that all the gland groups identified in *L. salmonis* displayed similar histochemical staining characteristics does not necessarily suggest that those glands serve the same role in the biology of the animal, a fact also suggested by the different levels of enzyme activities identified in different gland groups. Glands that are known to serve radically different functions such as the filament glands and the dorsal surface glands all stained with DAB and TMB, suggesting that there is a point of commonality in at

least a part of their secretion. The ultracellular characteristics of the AGC and urosomal glands, and even the different components of the urosomal glands, displayed considerable variation in their morphology and the relative proportions of organelles suggesting that the secretions of those glands differed in their constituents and possibly therefore, their roles.

Both calanoid species examined in this study were observed to have some DAB staining glands in their mouth areas and this may suggest that the function of such glands is to combat the effects of free radicals produced as a result of host / prey damage. Parasitic species will have an increased likelihood of encountering free radicals and such contact may exert effects on their physiology beyond the mouth and gut alone. Such a hypothesis would indicate that the extant gland system of calanoid copepods became more extensive in species with greater exposure to free radicals, or immune-related chemicals. This would explain the progressive development of these glands through commensal species to the obligate parasitic species. The development of the system in those species that have evolved to become even more closely associated with their hosts than *L. salmonis* should be explored.

From the roles filled by secreted catalases in other arthropods it is possible to formulate hypotheses concerning the role such secretions may play in the Copepoda. The most plausible hypothesis is that it serves to counteract the harmful effects of some of the chemicals associated with the host epidermis and mucus layer, although environmental ROS may also be deleterious to the parasite. Such chemicals may either be normally present in these tissues, or may be released specifically in response to parasitic infection, or may arise as a consequence of tissue damage caused by the feeding of these species. Such hypotheses serve also to explain the presence of similarly staining glands in phytal-associated harpacticoid species and their much reduced presence in the representatives of the calanoid species examined in this study. Although calanoid copepods did show some staining in the mouth region and the harpacticoids also showed strong DAB staining in their labral glands. Therefore if the glands of caligids evolved from the extant gland system of the calanoids then there may be similarities in terms of function. Calanoid copepods are known to possess many exocrine glands yet only their labral glands stained with DAB in this study, (possibly indicating the presence of peroxidase enzymes, although acidic mucus

compounds may also have caused the positive reaction) suggesting that their other glands fill different roles from those of the labral glands. In caligids and harpacticoids peroxidase enzyme secretion has been adopted by a significant proportion of their exocrine gland system. The increased development of these glands may therefore indicate the commensal and parasitic species experience more extreme conditions of the same kind that the free-living species experience in the region of their mouthparts. We therefore need to look at areas of commonality between the three groups. The potential presence of catalase in the labral gland secretions suggests that free-radical (ROS) neutralisation is one of their roles. Exposure to ROS may increase in severity from free-living species through the algal-associated species to the obligate parasitic species. There are three possible sources of these ROS in parasitic and commensal species: environmental hydrogen peroxide in shallow sunlit waters, host-derived ROS either in the host mucus or as a result of tissue damage as a consequence of feeding, or from the degradation of their own secreted mucus layer. An interesting extension of this work would be to determine the level of ROS production of the epidermis of intact and damaged salmon skin to determine empirically whether damage does cause the release of such chemicals. Glands with similar staining characteristics were shown not to be a feature of the freshwater fish louse *Argulus,* nor were they present in a number of other non-copepod marine crustaceans.

The different ultrastructural characteristics of the exocrine glands and the different morphologies of the secretory vesicles of the glands of *L. salmonis* may indicate that the secretions of different glands serve different functions but that all have become adapted to secreting catalase in addition to these particular functions.

The TEM fixation method designed specifically to retain mucus compounds revealed, on the external cuticle of *L. salmonis*, the presence of a conspicuous layer that had only been partially visualised by previous researchers. Bresciani (1986), in his extensive review of copepod cuticles, mentions only briefly the external fuzzy layer on the cuticle described by other authors (although he does mention glandular secretions) despite the fact that such layers may be an integral part of the cuticle. The

visualisation of the true extent of this mucus layer, that is presumed to be secreted by the exocrine glands identified, has been achieved in this study.

Experimental evidence indicates that specimens of *L. salmonis* display a significantly slower passive sinking rate following the removal of the cuticular mucus layer. The data obtained did not allow the distinction between a change in sinking rate as a consequence of altered hydrodynamic efficiency or as a consequence of a reduced weight following removal of the adherent layer. Analysis of morphological parameters though, suggested that weight was not the primary factor determining sinking rate. This may indicate that the decreased weight of specimens with their mucus layer removed was not the reason for their decreased sinking rate. This may provide support for the hypothesis that properties of the external mucus layer modify the sinking rate of *L. salmonis*.

Any theories regarding a specific drag-reducing property of the secretions of the glands described in this study have to be countered against the presence of similarly-staining glands in those copepods which are associated with algae (Chapter 3). If we make the presumption that these glands have the same function in all species they have been shown to be present in then we must consider the similarities and differences in the life history and behaviour of those species to find common elements. It is probable that copepods associated with algae are wholly dependent upon that algae as a source of food (either directly or indirectly) and / or as a source of protection against predation. In that case it can be readily argued that a drag-reducing secretion may serve to assist them in not being dislodged from their host during times of hydrodynamic duress i.e. wave action, tidal currents etc. The second point of commonality between caligid copepods and algal commensals / parasites is the possibility of both being subjected to chemical attacks from their chosen hosts. These attacks may be in the form of either immunological factors known to be present in fish mucus, or toxic chemicals released (i.e. free radicals) as a consequence of the tissue damage both groups cause either as a direct result of their feeding in caligids or by both feeding and burrowing activities in phytal-associated species. These factors, taken together, indicate that the described glands function to promote successful attachment to the host substrate either by facilitating adhesion to the host by reducing problematic drag elements or by directly protecting the animal from its local

environment. This protection could extend as far as the specific protection of the cuticle by means of secreted factors.

The catalase component of the exocrine glands of *L. salmonis* may explain the efficacy of hydrogen peroxide as a treatment against sea lice as routinely used in commercial salmon farming operations (McAndrew *et al.* 1998). Hydrogen peroxide has been proven to be effective at removing sea lice from salmon (Thomassen 1993, McAndrew *et al.* 1998) in commercial farm operations and its effect is believed to occur as a result of the formation of bubbles in the haemolymph of the sea louse (Thomassen, 1993). Such bubble formation could easily occur as a consequence of the action of endogenous catalase on the hydrogen peroxide.

The data collected in this study provide a starting point for further studies of copepod gland secretions and in particular, those of parasitic and commensal species. Experiments could be designed to test the validity of the hypotheses derived from the data collected so far. These experiments could incorporate radio-labeling techniques to identify the source of the mucus compounds that form the base of the secretion of the exocrine glands of *L. salmonis*, to determine if the carbohydrate fraction of the secretion is derived directly from host tissues or whether it is assimilated from DOM in the surrounding water. Studies could be used to determine whether sea lice, isolated from the host fish display greater survival in water enriched with glucose, or other DOM.

Further work should aim to extend the scope of the present work by screening other species of copepod for the presence of such glands including species from further, unexamined copepod families to determine the extent of such glands within the Copepoda. Knowledge of distribution and evolutionary path may suggest functions.

In retrospect several other avenues of research could be explored more fully to provide a greater understanding of the secretions of *L. salmonis* and the role they fill in the biology of this species and others of the same family. In particular a repetition of a section of the bacteriological study i.e. the gland secretions of *L. salmonis* should be tested specifically against the chitinolytic bacteria isolated from the cuticle of *L. salmonis*. A more detailed examination of the effects of removing the external mucus layer

on passive sinking rates may provide data that would allow the conclusive distinction between hydrodynamics and ROS neutralisation as a function for these glands.

An important consideration, which must be borne in mind, is the artificiality of the experimental situation. It is not possible to replicate *in vitro*, the specific conditions that lice will be subjected to *in vivo*. Sea lice in the wild would rarely be subject to starvation such as lice removed from the host for experimental purposes are. The effects of this starvation on any of the physiological processes of the lice are entirely unknown but it should be presumed that pronounced effects must occur. Life processes which are not fundamentally essential to the louse may be suspended or cease altogether under such conditions of stress and food deprivation. Further, the effects of keeping large numbers of lice in relatively small volumes of water are also unknown. These unknown factors although difficult to quantify may possibly produce what are effectively artifactual results. These effects though are more likely to be pronounced in behavioural studies whilst the histochemistry and ultrastructure of tissues is likely to be largely unaffected by such factors in the short times between removing the lice from the host and processing. The construction of many of the experimental procedures utilised in this study precluded the possibility of using lice straight from the fish.

9. REFERENCES

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10. APPENDICES

APPENDIX 1

Spectrophotometric enzyme assay techniques:

I. Catalase

II. Glutathione peroxidase (both Se-dependent and independent forms)

III. Glutathione S-transferase

I. Catalase

Assay solutions:

50mM phosphate buffer, pH 6.5

30% H_2O_2 solution

Supernatant of homogenised and centrifuged tissue samples

Assay procedure

The reaction was initiated by adding 20µl of sample to a 1cm quartz cuvette containing 455µl buffer and 5µl of the hydrogen peroxide substrate. A cuvette containing buffer and substrate alone served as a blank. Blank and sample-containing cuvettes were read simultaneously in a Uvikon 940 dual beam spectrophotometer at 240nm with the blank values automatically subtracted from the sample readings. Absorbance readings were taken every 10s for 180s. Three assays of each sample were taken to accommodate slight variability in reactivity. A freshly prepared blank was used for each assay. A sample of blue mussel (*Mytilus edulis*) digestive gland was used as a positive control to demonstrate that the procedure was working properly. The protein content of each of the tissue samples was calculated following the method of Lowry (1951). Enzyme activity (as µmoles min^{-1} mg^{-1}) was calculated from the absorbance data using the following calculation: E240nm / 0.044 x 1000 / vol fraction (µl) x dilution = µmoles ml^{-1} min^{-1}. This calculated value and the data from the protein analysis were used to calculate the rate of activity relative to the protein present in each sample.

II. Glutathione peroxidase (both Se-dependent and independent forms)

Assay solutions:

0.1M phosphate buffer (pH 7.5), including 4mM sodium azide and 2mM EDTA

20mM reduced glutathione (GSH) in buffer

10mM NADPH in distilled water

1:4 dilution of glutathione reductase (Sigma G-4759)

5mM H_2O_2 (substrate for the Se-dependent form of GPX)

40mM cumene hydroperoxide in acetone (substrate for the Se-independent form of GPX)

Supernatant of homogenised and centrifuged tissue samples

Assay procedure

The reaction mixture contained 400µl buffer, 50µl GSH, 10µl NADPH, 5µl of the enzyme preparation, 2.5µl glutathione reductase and 25µl of either H_2O_2 for Se-dependent GPX detection or cumene hydroperoxide for Se-independent GPX detection. The decrease in absorbance at 340nm was recorded every second for 5min using a Uvikon 940 dual beam spectrophotometer. Blank and sample-containing cuvettes were read simoultaneously and the blank values automatically subtracted from the sample readings. A sample of blue mussel (*Mytilus edulis*) digestive gland was used as a positive control to demonstrate that the procedure was working properly.

The protein content of each of the tissue samples was calculated following the method of Lowry (1951). Enzyme activity (as µmoles min^{-1} mg^{-1}) was calculated from the absorbance data using the following calculation: E240nm / 6.22 x 1000 / vol fraction (µl) x dilution = µmoles ml^{-1} min^{-1}. This calculated value and the data from the protein analysis were used to calculate the rate of activity relative to the protein present in each sample.

III. Glutathione S-transferase

Assay Solutions:

0.1M phosphate buffer (ph 6.5) including 1.5mM EDTA (ethylendiaminetatraceticacid) and 0.3% Triton X-100

50mM CDNB (1-chloro2,4-dinitrobenzene) in ethanol

20mM GSH in buffer

Supernatant of homogenised and centrifuged tissue samples

Assay procedure

The reaction mixture was combined in a 1cm plastic semimicrocuvettes and consisted of 870µl buffer, 100µl GSH, 25µl of the enzyme preparation. The reaction was initiated by the addition of 25µl of the CDNB substrate to the cuvette and the increase in absorbance at 340nm recorded every second for 2min using a Uvikon 940 dual beam spectrophotometer. A sample of blue mussel (*Mytilus edulis*) digestive gland was used as a positive control to demonstrate that the procedure was working properly. Negative controls consisting of the reaction mixture with 25µl buffer substituted for the CDNB were run concurrently with each sample and automatically subtracted from the sample values accommodate background absorbance. The protein content of each of the tissue samples was calculated following the method of Lowry (1951).

The activity of each sample (µmoles/min/mg) was obtained from the calculation: E340nm / 9.6 x 1000 / volume fraction (µl) x dilution. From this value and the data of protein content the specific activity in each tissue type could easily be determined.

APPENDIX 2

Peroxidase-anti-peroxidase immunohistochemistry method (Rainbow, 1996)

Peroxidase-anti-peroxidase immunohistochemistry method

Procedure

Follow steps 1 through 13

1.	Dewax sections in xylene	5min
2.	Rehydrate in alcohol series	
3.	Incubate with 10% H_2O_2 in methanol	10min
4.	Wash in TBS	
5.	Block slides in 10% serum	10min
6.	Tap slides dry	
7.	Incubate at 37°C in PGE_2 antibody dilutions in PBS (1-1:100,000)	1h
8.	Wash in TBS	
9.	Incubate with anti-mouse HRP	30min
10.	Wash in TBS	
11.	Apply diluted DAB solution until desired staining intensity is obtained	
12.	Wash in tap water	
13.	Counterstain, dehydrate and mount.	

Solution

0.05M Tris-buffered saline (TBS) (pH 7.4)

4.84g Tris base

58.48g NaCl

Dissolve Tris base and NaCl in 2L distilled water and adjust pH using HCl

APPENDIX 3

Electrophoresis buffers:

I. Native gel electrophoresis buffers

II. Denaturing gel electrophoresis buffers

I. Native gel electrophoresis buffers

4x Separating Gel Buffer (1.5M Tris-HCl (pH 8.8))

18.2g Tris in 40ml H_2O

pH to 8.8 and add H_2O to 100ml

4x Stacking Gel Buffer (0.5M Tris (pH 6.8))

6.0g Tris-HCl in 40ml H_2O

pH to 6.8 and add H_2O to 100ml

Electrophoresis Buffer

6g Tris

28.8g glycine

H_2O to 1L (pH 8.3)

Homogenisation Buffer

15.5ml 1M Tris-HCl

7ml H_2O

5x Sample Buffer

15.5ml 1M Tris-HCl (pH 6.8)

25ml glycerol

2.5ml 1% w/v bromophenol blue

7ml H_2O

II. Denaturing gel electrophoresis buffers

4x Separating Gel Buffer

75ml 2M Tris-HCl (pH 6.8)
4ml 10% SDS
21ml H_2O

4x Stacking Gel Buffer

50ml 1M Tris-HCl (pH 8.8)
4ml 10% SDS
46ml H_2O

5x Electrophoresis Buffer

3g Tris
14.4g glycine
1g SDS
H_2O to 1L (pH 8.3)

5x Sample Buffer

0.6 ml 1M Tris-HCl (pH 6.8)
5ml 50% glycerol
2ml 10% SDS
0.5ml β-mercaptoethanol
1ml 1% bromophenol blue
0.9ml H_2O

APPENDIX 4

Chromogenic assay techniques (Manchenko, 1984)

I. Catalase

II. Glutathione peroxidase

I. Catalase

Staining solutions:

A. 3% Hydrogen peroxide

B. 2% Potassium ferricyanide

C. 2% Ferric chloride

Procedure

The gel is incubated in solution A for 15min. Pour off solution A, rinse gel with water and immerse it in a 1:1 mixture of solutions B and C. Gently agitate the tray containing the gel for a few minutes. Yellow bands of catalase activity appear on a blue-green background. Wash stained gel in water. Stained gels were photographed with a digital camera.

N.B. Solutions A, B and C should be prepared immediately prior to use.

II. Glutathione peroxidase

Staining solution:

0.1M Potassium phosphate buffer, pH 7.0	10ml
Reduced glutathione (GSH)	30mg
Glutathione reductase (GR)	12units
5.4mM EDTA (pH 7.0)	2ml
NADPH	15mg
t-Butyl-hydroperoxide (add just before use)	50µl

Procedure

The staining solution is applied to the gel surface on a filter-paper overlay. Incubate the gel at 37°C for 10-30min. Remove filter paper and view gel under long-wave UV light. Dark (non-fluorescent) bands of GPX are visible on the light (fluorescent) background of the gel. Photograph zymogram using a yellow filter.

APPENDIX 5

Mucus fixation protocol for TEM (Horne and Sims, 1999)

Mucus fixation for TEM (method of Horne and Sims (1998))

The fixative consists of a solution of the fluorocarbon solvent FC-72 (Acros Organics 12379-0100) with 0.5% w/v dissolved osmium tetroxide. Trimmed tissue samples were immersed in this fixative for 20min, then dehydrated through an acetone series before being embedded in Spurrs resin as described for routine TEM analysis in Chapter 2.

APPENDIX 6

Incorporation of chitin into agar plates (Danulat, 1984)

Incorporation of chitin into agar plates

The method for incorporating chitin into marine agar plates was as follows: 7.5g of chitin (C7170, Sigma) were added to 75ml ice-cold H_2SO_4 (1:1 diluted with distilled water) and kept in the refrigerator overnight. The following day the chitin was precipitated in 2.5L distilled water and stirred with a magnetic stirrer for 1h. Following this, mixing was stopped, the chitin allowed to settle and the water poured off. This was repeated twice, after the second period of settling, 1L of water including the settled chitin, were centrifuged (5,525RCF for 5min), the water decanted and the chitin resuspended. This process was carried out three times after which the chitin was resuspended in 250ml of sterile seawater.

Twenty five millilitres of the chitin suspension was then added aseptically to 500mls of sterilised marine agar (Difco 0797-17-8) pH 7.6. The agar was then poured into 90mm triple vent petri dishes and allowed to set. The final percentage of incorporated chitin was therefore 4.76%.

APPENDIX 7

Data of passive sinking experiment

Data of passive sinking experiment

Specimen Number	Untreated Sink rate	Treated sink rate	Weight (mg)	Length (mm)	Width (mm)	Displacement (cm^3)	Surface Area (mm^2)
1	11.36 (0.39)	10.69 (0.37)	4.21	6.4	3.02	0.02077	9.039
2	12.68 (0.41)	11.35 (0.60)	4.56	6.54	3.09	0.01928	10.789
3	11.64 (0.68)	9.99 (0.41)	3.75	6.86	2.92	0.02297	9.880
4	11.74 (0.49)	10.40 (0.35)	4.77	7.05	2.76	0.01533	9.756
5	11.23 (0.61)	10.23 (0.21)	3.00	5.65	2.65	0.01579	8.587
6	11.74 (0.49)	10.41 (0.35)	3.85	6.02	2.74	0.01907	9.25
7	11.47 (0.42)	10.65 (0.23)	3.33	5.88	2.50	0.00960	8.180
8	12.10 (0.82)	10.09 (0.45)	3.83	5.78	2.98	0.02800	9.880
9	11.83 (0.70)	11.21 (0.63)	4.58	6.60	3.19	0.02220	11.76
10	11.27 (0.72)	10.87 (0.46)	3.90	5.39	2.69	0.00766	8.83
11	11.39 (0.76)	10.74 (0.48)	3.99	5.90	2.88	0.01608	9.54
12	12.32 (0.63)	10.36 (0.21)	3.40	5.38	2.81	0.01710	9.27
13	11.53 (0.35)	10.07 (0.41)	3.90	5.45	2.83	0.01230	9.45
14	12.20 (0.45)	11.01 (0.30)	4.03	6.35	2.94	0.01530	10.58
15	12.33 (0.44)	10.97 (0.29)	4.47	6.34	3.00	0.01360	10.22

Values of sinking rate given are mean values of 5 observations. Figures in brackets represent standard deviations of those mean values. Untreated = CO_2 anaesthetised specimens. Treated = detergent-rinsed specimens.