# ASPECTS OF THE BIOLOGY OF SOME MARINE ASCARIDOID NEMATODES

A thesis presented for the degree of

Doctor of Philosophy to the University of Stirling

by

ANGELA M. JONES

Parasitology Laboratory
Institute of Aquaculture
University of Stirling
Stirling FK9 4LA

October 1994

# BRITISH THESIS SERVICE

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## DECLARATION

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

**Angela M. Jones**

For Blakey,

My little lady.

Who sat with me every night during

the writing of this thesis, and

who gave me so much love.

*Where do nematodes live? Almost anywhere. Nematodes antedated man in their conquest of the whole earth..... As Cobb (1915) so aptly put it, "They occur in arid deserts and at the bottom of lakes and rivers, in the waters of hot springs and in the polar seas where the temperature is constantly below the freezing point of freshwater. They were thawed out alive from Antarctic ice in the Far South by members of the Shakleton Expedition. They occur at enormous depths in Alpine lakes and in the ocean. As parasites of fishes they traverse the seas; as parasites of birds they float across continents and over high mountain ranges." Man, without wings, flies in aeroplanes. Nematodes, without wings, fly in birds, bats, bees, flies, or fleas, or just catch on as these go by and sail with them. ....they need not exert themselves in walking for representatives of the whole animal kingdom act as their common carriers, and even winds may on occasion stoop to lift them and take them to their destination.*

Chitwood and Chitwood (1950)

**ACKNOWLEDGEMENTS**

Firstly, I would like to extend my appreciation to my supervisors, Dr. Rod Wootten, at the Institute of Aquaculture, Stirling, and Dr. John Smith, at S.O.A.F.D. Marine Laboratory, Aberdeen. Rod deserves special mention for his (mostly!) infinite patience and good humour, especially over the last year when ploughing his way through the vast drafts of thesis which I gave him to read. John must also be acknowledged particularly for his organisation of my bits and pieces of work in Aberdeen, and for being a mine of information with regard to the literature on marine ascaridoids.

All my love, appreciation and undying thanks to all the parasites at the Institute of Aquaculture. I could not have done this Ph.D. without them - they have been there through all the laughter and tears, and in addition to help and support, they have also managed to put up with me and my numerous crises over the last four years! There are several people whom I must thank profusely - James, for his advice on statistics and all things computer-related, and for his constant enthusiasm when I thought the writing of this thesis would never end; Andy Shinn - for being the kindest, smiliest, most helpful man in the whole world, for cheering me up whenever I saw him, and for managing to iron out all the problems which inevitably occured whenever I touched a computer at the Institute; Jim - for just about everything over the years, from driving me miles around the west coast of Scotland with samples, to driving me mad with frustration at how easily his electron microscopy material sectioned and mine didn't; David - for help with photographs, driving to collect samples, and numerous other bits and pieces; and Will, and Andy and Alison Bell (the latter an honourary parasite) for always being ready and willing to discuss things over lots of alcohol, in addition to being three of the most caring people I know. Thank you also Helen, Eva, Vino, and Obdulio! I would also like to take this opportunity to apologise to everyone for putting up with the smell I created when digesting my fish!

There are two very special ladies at the Institute of Aquaculture who deserve well-earned thanks. Maureen Menzies - for always being there when I needed her, either for practical help or a shoulder to cry on - and Marguerite Kobbs, for her advice, expertise, patience (and sympathy!) in relation to all the electron microscopy work.

Still others at the Institute must be acknowledged - Tom Turnbull, for helping me to discover the mysterious world of histopathology, and also for helping with photographs; Mairi, Debbie, Laura and Jane Brown for always making me laugh as well as advising me on many different matters, and Betty, Sheila and Beatrice, for making my life that little bit easier with regard to all the administration etc. behind the research.

My fondest thanks to Ken MacKenzie (and that infamous Welshman - Dr. Williams!) for making my first research cruise absolutely unforgettable; additional thanks to Ken for collecting samples at sea when I was unable to attend the cruise myself.

I am indebted to both Dr. David Gibson and Eileen at the Natural History Museum, London, in particular for allowing me to come and "steal" some specimens of adult ascaridoids from the parasitic worms collection. Also, I must extend my special "angelic" thanks and affection to David Gibson for always being ready to respond, in such an expert and entertaining way, to my many enquiries and communications.

Finally, but without a doubt most importantly, I would like to thank my husband Mark for putting up with me over the last year, and ask his forgiveness for having to endure the first year of marriage with his wife constantly in the "cupboard under the stairs", and staying up till the early hours nearly every night in order to finish this thesis. In addition to this, in practical terms, I could not have finished this thesis without his help, particularly with computer figures, and in finally putting the last few bits of the thesis together. I can't begin to express how grateful I am to have had his love and support over the last 12 months.

## ABSTRACT

Larval **Anisakis simplex**, **Pseudoterranova decipiens**, **Contracaecum osculatum** and larvae and adults of **Hysterothylacium aduncum** were recovered from specimens of cod, haddock, blue whiting and bull rout; however, only **A.simplex** were retrieved from long rough dab. The epidemiology of infection by these four nematode species was examined both in whole fish, and in individual host tissues and organs. Frequency distributions of nematodes were found to be generally overdispersed in fish. Preliminary investigations revealed no strong evidence to suggest that competitive interactions occurred between ascaridoid nematodes within fish.

Stomach lesions in gadoids were associated with single (partially penetrated) and multiple (throughout stomach wall) worm infections of larval **A.simplex**; such lesions were discrete and raised in appearance. Lesions associated with 1-3 larval **P.decipiens** in an open cavity within the stomach of angler fish were diffuse and not significantly raised. Histological examination of each form of ulcer revealed general similarities in pathology, with infiltration of inflammatory cells being the initial response to the nematode\s.

Changes in the cephalic morphology of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** were examined at different life cycle stages under scanning electron microscopy. Due to their small size, newly hatched third stage larvae of **P.decipiens** were cultured in a bacterial mat prior to fixation for S.E.M., and the external ultrastructure of these larvae is described. The most prominent external feature at this stage is the cephalic boring tooth.

Aspects of the internal ultrastructure of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** were examined using transmission electron microscopy. Newly hatched third stage larvae of **P.decipiens** show little differentiation of internal organs. The ultrastructure of sensory amphids in **H.aduncum** and **A.simplex** is consistent with that of a chemoreceptor,

that of the single papilla in **P.decipiens** - a mechanoreceptor. The ultrastructure of the digestive tract, excretory gland and body wall of marine ascaridoids were also examined.

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## CHAPTER ONE : GENERAL INTRODUCTION

Seventeen families of nematodes occur in fish, and at least 650 species of adult nematodes have been recorded, generally occurring in the intestine. Additional species occur at the larval stage in fish (Möller and Anders 1986). Ascaridoid nematodes parasitise all major vertebrate groups, although they are scarce in amphibians (Gibson 1983). The superfamily Ascaridoidea is large, and is divided into several families and subfamilies, including the Anisakidae, which are mainly found as adults in piscivorous vertebrates, such as mammals, birds and fish (Myers 1970) and the Raphidascaridae. Nematodes parasitic in marine fish are extremely common, and although the number of species reported is not large, the number of specimens -especially of larval nematodes - found in individual fish may be very large (Berland 1961). The larval stages of certain ascaridoid genera occur commonly in the flesh and viscera of marine fish (eg. Myers 1970) and have assumed economic and medical significance. Four species of ascaridoid nematodes commonly infect marine teleosts in British waters -**Anisakis simplex**, (Rudolphi, 1809) Dujardin, 1845; **Pseudoterranova decipiens** (Krabbe, 1878); **Contracaecum osculatum** (Rudolphi, 1802), all anisakines, found as third stage larvae, and **Hysterothylacium aduncum** (Rudolphi, 1802) Deardorff and Overstreet, 1981 - a raphidascarine, found as third stage larvae in the viscera, and as adults in the intestinal tract. **Anisakis** sp. is one of the most widespread parasites of marine teleost fish (MacKenzie 1979), being found in a variety of species (eg. Smith and Wootten 1984b). **P.decipiens** have also been reported from a variety of marine teleosts (eg. Smith and Wootten 1984a), and heavy infections of **P.decipiens** have been reported particularly from the fillets of cod and other fish species (eg. McClelland **et al.** 1983a,b), with Young (1972) stating that cod are the major fish host for **P.decipiens** in the Atlantic. **Contracaecum** sp. and **H.aduncum** also occur in a number of marine fish species (eg. Smith and Wootten 1984c;

Berland 1961, 1991). All of these parasites are found in a wide range of fish species and thus do not appear to be host specific. Lick (1991) found 78% of 41 fish species from the outer Elbe estuary to be infected with nematodes of a number of species including **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum**.

## 1.1 Classification and Taxonomy

The taxonomic histories of **Anisakis**, **Pseudoterranova**, **Contracaecum** and **Hysterothylacium** are confusing, and ascaridoid nematodes in general appear to be inadequately characterised with regard to nomenclature. The morphology of the excretory system in nematodes is considered to be of taxonomic significance (Gibson 1983, Sprent 1983), and Hartwich (1974) formulated a classification for nematodes of the superfamily Ascaridoidea, based on features of the excretory system. Hartwich recognised five families - one of which was the Anisakidae - which contains most of the ascaridoids parasitic in fish. Hartwich distinguished the subfamily Raphidascaridinae (containing **Hysterothylacium**) from Anisakinae (containing **Anisakis**, **Pseudoterranova** and **Contracaecum**) by virtue of the non ribbon-like excretory system of the former, with an excretory pore near the nerve ring. The excretory system of Anisakinae is ribbon-like, and the excretory pore is found at the level of the lips. Sprent (1983) considered that there may be no justification for the family Anisakidae, as Hartwichs' system of classification contained defects with regard to the oesophago-intestinal junction, and Sprent suggested that the family Ascarididae comprised five sub-families, including Anisakinae (containing the genera **Anisakis** and **Pseudoterranova**) and Contracaecinae (containing **Contracaecum**). However, Gibson (1983), in the same treatise, also reviewed the systematics of ascaridoid nematodes, and retained the family Anisakidae containing only the subfamilies Anisakinae and Raphidascaridinae.

However, the currently accepted classification for the four species of ascaridoid nematodes examined during the present study is as follows (D.I.Gibson pers.comm.):-

Class Nematoda
Subclass Secernentea
Order Ascaridida
Superfamily Ascaridoidea
Family Anisakidae

| | | |
|---|---|---|
| Subfamily | Anisakinae | Raphidascaridinae |
| Genus | **Anisakis** | **Hysterothylacium** |
| | **Pseudoterranova** | |
| | **Contracaecum** | |

Difficulties may arise when identifying or assigning the larval stages to genera, and much of the literature is in a state of confusion with regard to larvae in fish (eg. Templeman **et al.** 1957; Berland 1961; Myers 1975). Identification is often based on tenuous morphological grounds, and this, along with a lack of knowledge on the life histories, is further complicated by the fact that few of the characteristics used to identify adults are present in the larval stages (Hurst 1984a).

Nematodes of the genus **Anisakis** have been described under a vast number of names - many of which have now been shown to be incorrect (see Davey 1971, Smith and Wootten 1978a, and references therein). Grainger (1959) had reared larvae to the pre-adult stage and positively identified them as belonging to the genus **Anisakis**. Van Theil **et al.** (1960) identified **Anisakis** as **Eustoma rotundatum**, but subsequently (Van Theil 1966) corrected this to **A.marina**, and proposed that all **Anisakis** species belonged to this taxon. However, several authors including Khalil (1969) and Davey (1971) rejected this proposal. Davey (1971) examined taxonomic characters of various members of the genus **Anisakis** and concluded that there are only three valid distinguishable

species, of which **A.simplex** is one (with several synonyms, including **A.marina**), the others being **A.typica** and **A.physeteris**, and four **species inquirendae**. **A.simplex** is the most common species of this genus (Munger 1983). Berland (1961) identified two types of **Anisakis** larvae which he designated types I and II - probably belonging to separate species. Positive specific identification of **Anisakis** larvae from fish was made by, amongst others, Pippy and Van Banning (1975), Grabda (1976b), Hurst (1984a) and Carvajal **et al.** (1981) who reared third stage larvae type I, from the North Sea, Baltic Sea, New Zealand waters and Chilean waters respectively, into adults of **A.simplex**.

The confusion with regard to **Pseudoterranova decipiens** occurs at the generic level. The scientific name has undergone a number of changes over the last 50 years. Originally called **Ascaris**, then **Porrocaecum** and **Terranova** (see Myers 1959, Gibson 1983 and references therein), Myers (1959) created the genus **Phocanema** to include **Porrocaecum decipiens** and **Terranova decipiens**, with **Phocanema decipiens** being the only species. The genus **Pseudoterranova** was initially erected by Mozgovoi (in Skrjabin **et al.** 1951, see Gibson 1983), however Hartwich (1974) did not include **Pseudoterranova** in his key as he considered it to be incompletely described. Gibson (1983) reviewed the taxonomic confusion surrounding the genus **Phocanema**, and stated that current criteria for distinguishing **Phocanema** and **Pseudoterranova**, as distinct from **Terranova**, were weak or defunct. Authorities on nematode systematics agree that the generic name **Porrocaecum** should no longer be used, but opinions vary as to the use of **Terranova** or **Phocanema** (Margolis 1977). Gibson (1983) concluded that **Pseudoterranova** must be recognised as the oldest name with **Phocanema** as its' synonym. **Terranova** larvae type A, from Japanese waters are synonymous with **P.decipiens** (Ishikura 1990).

Baylis (1937) listed synonyms of **C.osculatum**. Berland (1963) subsequently suggested that **Contracaecum** sp. be termed

**Phocascaris**, but Smith and Wootten (1984c) believed that they are in fact synonymous. However, interlabia are absent in the genus **Phocascaris**, and present in **Contracaecum** spp. (Hartwich 1974). Various Japanese authors have classified **Contracaecum** larvae into types A and B (see, for example, Ishikura 1990). Arthur **et al.** (1982) found **Contracaecum** type B larvae from walleye pollock to be morphologically similar to third stage **C.osculatum**. Confusion may also occur with regard to **C.aduncum** - which has at least two synonyms - **Thynnascaris adunca** and **Hysterothylacium aduncum** (Soleim 1976a). **Thynnascaris** was formerly considered as a synonym of **Contracaecum** (Soleim and Berland 1981) but these genera are now regarded as belonging to separate subfamilies within the family Anisakidae (Hartwich 1974). Deardorff and Overstreet (1980) resurrected the genus **Hysterothylacium** to include those species previously considered as **Thynnascaris** and others described in the genus **Contracaecum** that mature in fish. These authors (1980, 1981a) and Berland (1961) reviewed the previous taxonomy and classification of this genus. Among others, both **Contracaecum clavatum** (eg. Kahl 1936) and **C.gadi** are regarded as synonyms of **H.aduncum** (D.I. Gibson, pers. comm.). Again, Japanese authors have classified larvae of **Thynnascaris** (= **Hysterothylacium**) into types e.g. A and B (eg. Fukuda **et al.** 1988).

Recently, methods have been developed which further contribute to the identification of these nematodes. A number of studies, principally by Italian workers, have investigated the gene-enzyme systems of **A.simplex**, **P.decipiens** and **C.osculatum**, and reproductively isolated sibling species have been recognised ie. nematodes which are similar morphologically, and grouped together as one species, have been found to belong to different species. Multi-locus electrophoretic studies on larval **A.simplex** collected from fish hosts in the Mediterranean and North-East Atlantic, confirmed that two separate species exist (Mattiucci **et al.** 1983, 1989; Nascetti **et al.** 1983,1986; Orecchia **et al.** 1986). Specimens were assigned as either **A.simplex** A or **A.simplex**

B, with most type A specimens recovered from the Mediterranean, and type B specimens from the North-East Atlantic.

Further genetic studies by Paggi **et al.** (1991) revealed genetic evidence for three sibling species within **P.decipiens** larvae and adults, from fish and seals in the North Atlantic, Norwegian and Barents Seas. These reproductively isolated species were provisionally designated **P.decipiens** A,B and C. In addition to genetic variability, differences between these three types were also found with respect to morphology of caudal papillae in adult males, geographical distribution and final hosts. **P.decipiens** B and C were widespread in the areas studied, whereas **P.decipiens** A was found only in the North-East Atlantic. Although both **P.decipiens** A and B were found in grey and common seals, including individual seals with mixed infections, in eastern Atlantic waters, **P.decipiens** A was found to be most common in the grey seal (**Halichoerus grypus**), with common seals (**Phoca vitulina**) acting as the main hosts for **P.decipiens** B. In Canadian waters, only **P.decipiens** B and C were found, with **P.decipiens** B infecting both grey and common seals, and occasionally hooded seals (**Cystophora cristata**). **P.decipiens** C were only recovered from the bearded seal (**Erignathus barbatus**). Subsequently, Bristow and Berland (1992) found **P.decipiens** C to occur primarily in long rough dab (**Hippoglossoides platessoides**) in northern Norwegian waters; being rare or absent from other fish species. In addition, these authors found this parasite to be absent in fish outwith the normal range of bearded seals. Similarly, Appleton and Burt (1991) used isoelectric focusing of soluble proteins to find two "variants" of **P.decipiens** in fish from Canadian Atlantic waters, with a geographical difference in occurrence, although both types were found in all sites sampled. There was also some evidence to suggest that different variants matured and reproduced preferentially in different final hosts. Burt **et al.** (1990b) found differences in the ability of larval **P.decipiens** from grey and harbour seal origins to infect crustaceans, with only

larvae of grey seal origin infecting **Gammarus oceanicus** directly.

Nascetti **et al.** (1993) also identified three sibling species within **C.osculatum** from the Atlantic Arctic-Boreal region, and designated these A,B and C.

## 1.2 General Appearance and Identification of Marine Fish Ascaridoids

The identification of larval ascaridoids can be complicated as they do not possess adult features. With regard to **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum**, the main features for species identification of third stage larvae, are the structure of the cephalic and caudal regions, the position of the excretory pore, and the structure of the transitional region between the oesophagus and the intestine (Myers 1975, Möller and Anders 1986).

Many authors have examined the morphology of the above species in fish; Table 1 illustrates the major identifying characteristics of third stage larvae of **Anisakis** sp., **P.decipiens** and **Contracaecum** sp., and third stage larvae and adults of **H.aduncum**, as reported by Soleim (1976a), Smith and Wootten (1983a,b,c), Möller and Anders (1986) and Berland (1991). Figure 1 illustrates these features. All four species, possess an oesophagus (split into an anterior muscular preventriculus and smaller posterior glandular ventriculus), intestine, rectum and anus. A nerve ring is situated around the preventriculus immediately posterior to the head, and an excretory canal is present to a point immediately above the ventriculus, where it forms the excretory duct, leading to the excretory pore.

TABLE 1: IDENTIFYING CHARACTERISTICS OF ASCARIDOID NEMATODES IN FISH

| | Length (mm) | Boring tooth | Position of excretory pore | Structures between preventriculus and intestine | | | Tail structure |
|---|---|---|---|---|---|---|---|
| | | | | Ventriculus | Ventricular appendix | Intestinal caecum | |
| THIRD STAGE LARVAE | | | | | | | |
| **Anisakis sp.** | 9-36 | YES | Level of mouth | YES | NO | NO | Blunt; with mucron |
| **P.decipiens** | 9-58 | YES | Level of mouth | YES | NO | YES | Blunt; with mucron |
| **Contracaecum sp.** | 7-35 | YES | Level of mouth | YES | YES | YES | Narrows to sharp point; no mucron |
| **H.aduncum** | 30(max) | YES | Level of nerve ring | YES | YES | YES | Tapering; no mucron |
| FOURTH STAGE LARVAE & ADULTS | | | | | | | |
| **H.aduncum** | 9-47m,12-90f | NO | Level of nerve ring | YES | YES | YES | Tapering tail with spines |

(m=male, f=female)

Compiled from Soleim (1976a), Smith & Wootten (1983a,b,c), Moller & Anders (1986), & Berland (1991)

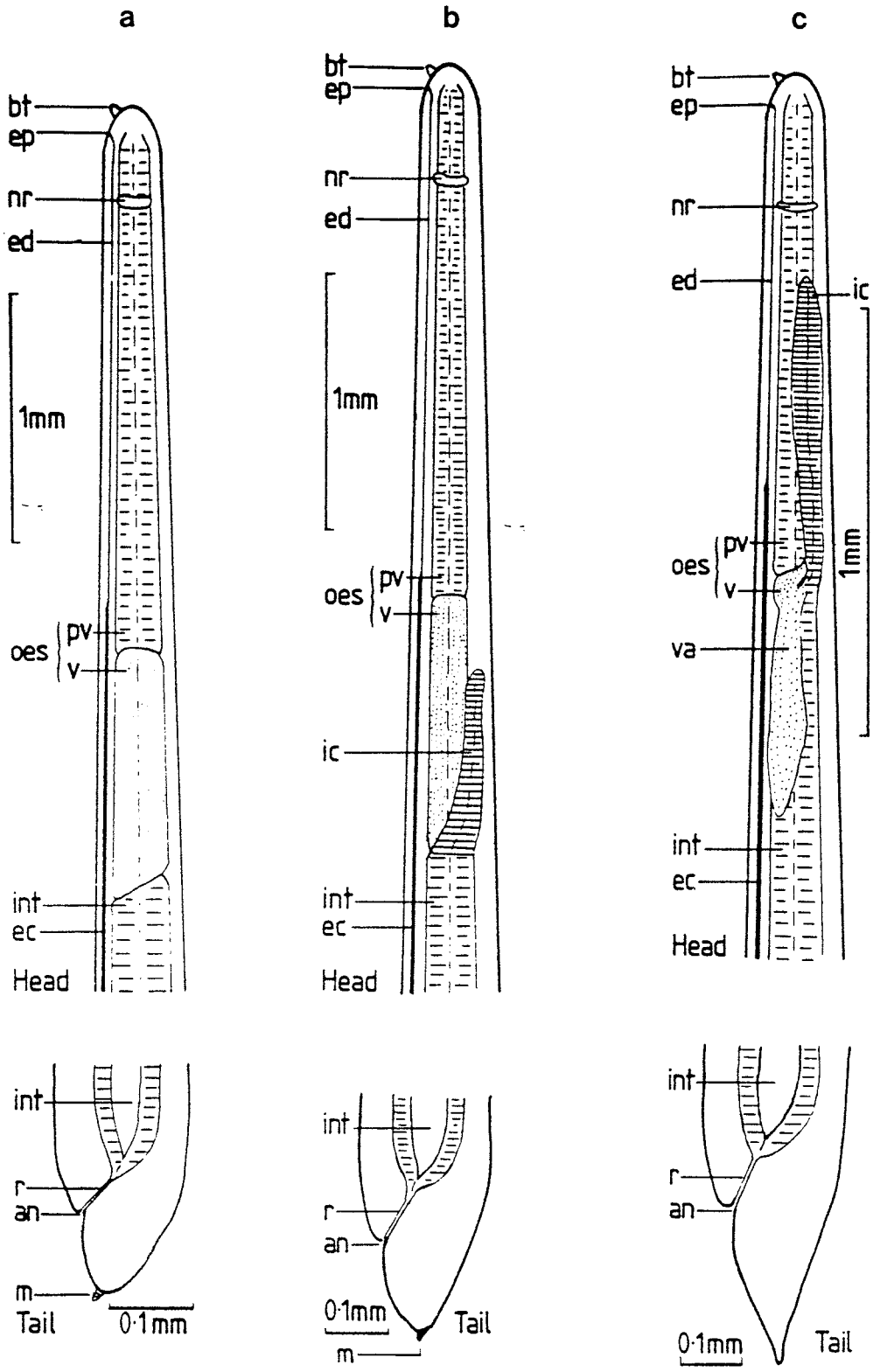



FIGURE 1: Identifying Characteristics of Marine Ascaridoids
(a) Third Stage Larva of A.simplex
(b) Third Stage Larva of P.decipiens
(c) Third Stage Larva of Contracaecum sp.
(Figures a-c reproduced with permission from Smith and Wootten 1984a,b,c; see overleaf for key)

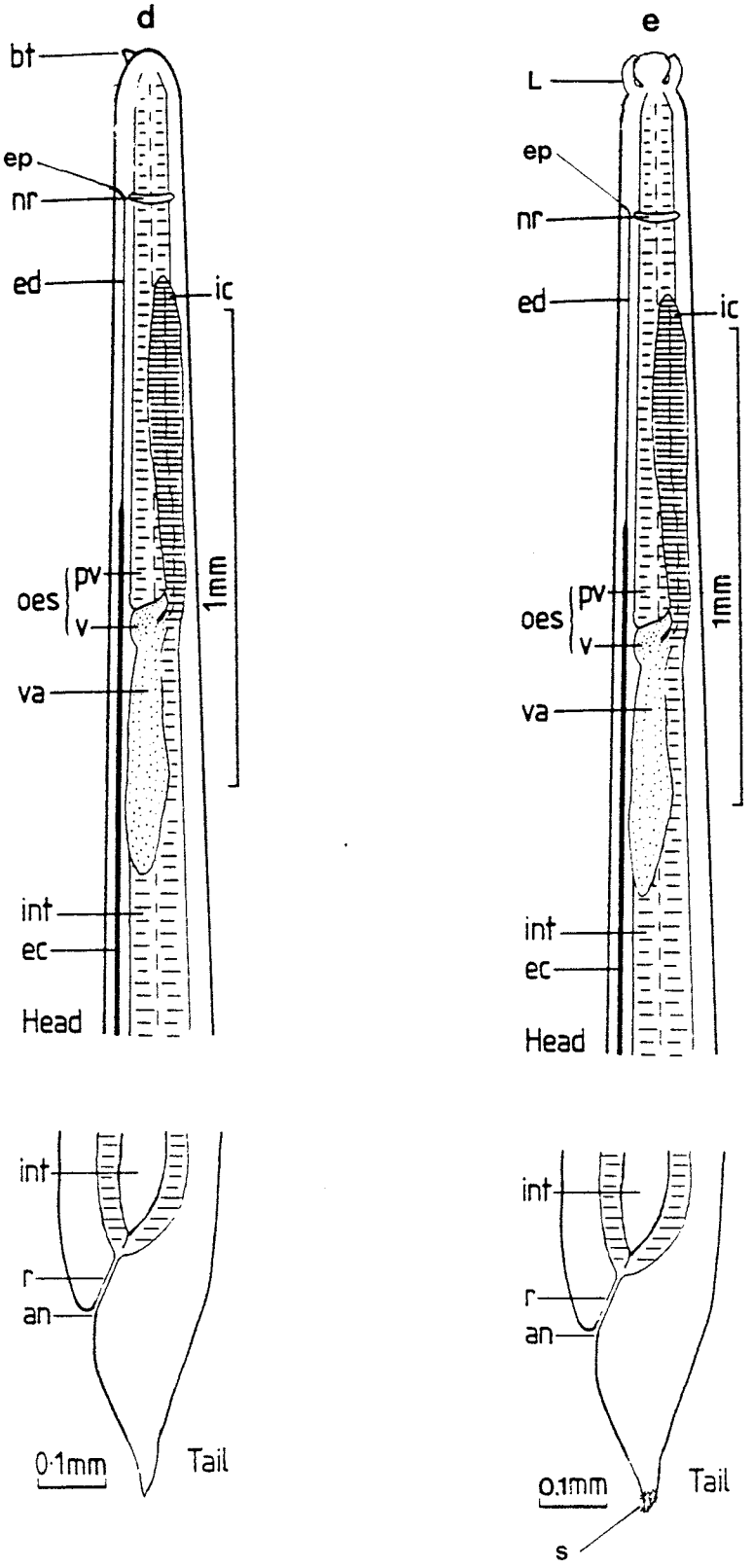



FIGURE 1 contd.
(d) Third Stage Larva of H.aduncum
(e) Adult H.aduncum
(KEY: an = anus, bt = boring tooth, ec = excretory canal, ed = excretory duct, ep = excretory pore, ic = intestinal caecum, int = intestine, L = lips, m = mucron, nr = nerve ring, oes = oesophagus, pv = preventriculus, r = rectum, s = spines, v = ventriculus, va = ventricular appendix)

## 1.3 Life Cycles

The general life cycle of marine ascaridoid nematodes involves both free-living and parasitic stages (Cheng 1976; Smith and Wootten 1978a), and comprises four larval and one adult stage (Möller and Anders 1986). The life cycles of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** are indirect; using intermediate host\s in their transmission.

Cheng (1976) reviewed the life cycle patterns of anisakid nematodes from marine fish, and discussed the physical factors known to influence development. More specifically, Smith and Wootten (1978a) reviewed the life cycle of **Anisakis**, Myers (1960) and McClelland **et al.** (1983a) discussed the life cycle of **P.decipiens**, and Berland (1961) reviewed the life cycle and development of **C.aduncum** (= **H.aduncum**).

### 1.3.1 Eggs, Hatching and Free Living stage

Eggs develop in the uterus of mature female worms in the digestive tracts of the final host and are released with the faeces (eg. Scott 1955; Young 1972; McClelland 1980b; Smith 1983a,b; Möller and Anders 1986; Berland 1961,1991). Cheng (1976) stated that it would appear that eggs of marine anisakids all undergo an incubation period in sea water. The first stage larvae develop within the egg, and several authors have considered that the first moult also occurs in the egg, with the second stage larvae hatching as the free living stage - retaining the cuticle of the first larval stage as a sheath eg. **Anisakis** (Smith 1971, 1983a; Grabda 1976b), **P.decipiens** (Scott 1955, Myers 1960, McClelland and Ronald 1974a), **C.osculatum** (Davey 1969) and **H.aduncum** (Berland 1961, 1991; Norris and Overstreet 1976; Yoshinaga **et al.** 1987). However, Koie and Berland (in press) observed two moults to occur in the egg of **A.simplex**, with the third

stage larvae emerging, ensheathed in the second stage cuticle, and McClelland (1982) also suggested that **P.decipiens** larvae were at the third larval stage on hatching. It would seem likely that third stage larvae also hatch from the eggs of **C.osculatum**, this being a closely related species to **A.simplex** and **P.decipiens**. It is of relevance to note that Soleim and Berland (1981) found second stage larvae of **Thynnascaris adunca** (= **H.aduncum**) in the eggs within the uterus of female worms, and, among others, Svendsen (1990) considered that it was this second stage larvae which hatched from the eggs. In addition, Yoshinaga **et al.** (1987) observed second stage larvae of **H.aduncum** to hatch from eggs of specimens recovered from freshwater fish.

Eggs of **Anisakis** sink in seawater (Sluiters 1974, in Smith 1983a). Grabda (1976b) observed larvae within eggs of **A.simplex** by 2-3 days. Smith (1983a) also obtained ensheathed hatched larvae of **A.simplex** from eggs after incubation in seawater at 15°C, and calculated the possible depth of hatching of **A.simplex** eggs under natural conditions in the North Sea, adding that free-living larvae which have hatched mid-water will continue to sink.

Eggs of **P.decipiens** sink to the bottom of the sea and hatched larvae attach to the substrate until they are eaten by a suitable host (Scott 1954, Myers 1960, McClelland and Ronald 1974a, McClelland 1982). A number of papers have investigated the hatching of **P. decipiens** eggs and survival of newly hatched larvae (Scott 1955, Myers 1960, McClelland and Ronald 1974a, McClelland 1982, Likely and Burt 1989, Burt **et al.** 1990c). Scott investigated hatching in sea water, and found that development was very slow at 2-4°C, with no hatching observed, but was more rapid at higher temperatures, hatching being observed after 13-14 days at 13-14°C, and with the most rapid development occurring at 17-25°C - hatching occurring after 8-9 days. Similarly, McClelland (1982) found hatching time to decrease with increasing temperature, although temperatures over 24°C were found to be lethal (Myers 1960). Brattey (1990) also examined the effect of

temperature on egg hatching in **P.decipiens** and discussed possible times of hatching and development for this species in their natural environment. Free living **P.decipiens** larvae are thought to survive for 2-3 weeks, survival decreasing with increasing temperatures on the sea bed (McClelland and Ronald 1974a, McClelland 1982).

Eggs of **C.osculatum** also sink in sea water (Davey 1969). Davey found the optimum temperature for hatching of **C.osculatum** eggs to be 16°C; this temperature giving hatching by day 13. Development was slow below 10°C. McClelland and Ronald (1974b), and Brattey (1990) additionally examined hatching in this species.

Berland (1961) stated that eggs of **C.aduncum** (= **H.aduncum**) sink slowly to the sea bed. Normal development of **H.aduncum** larvae was observed at 5-20°C, although it was noted that survival in the egg was reduced in freshwater; however, first stage larvae of this species did develop in salinities ranging from 0 - 32‰ (Möller 1978). Yoshinaga **et al.** (1987), observed development of **H.aduncum** in eggs recovered from specimens in freshwater fish, and observed hatching by day 10, in a variety of media at 15°C.

If it is the third stage larvae of **A.simplex**, **P.decipiens** and **C.osculatum** which hatches from the egg, the subsequent invertebrate and fish hosts, which harbour larval stages, must be regarded as transport (= paratenic) hosts, whereby no further moults take place until the parasite enters the final host. However, further growth of these larvae are reported as occurring within the invertebrate and fish hosts (eg. McClelland 1982). In the case of **Hysterothylacium**, where second stage larvae hatch, and the moult to the third stage is thought to occur in the first host (eg. Svendsen 1990), the first host must be regarded as being a true intermediate host, with the subsequent hosts to the larvae acting as transport hosts, and this was similarly suggested by Berland (1961).

### 1.3.2. First Invertebrate Hosts

Free living larvae of all four nematode species are thought to be ingested by invertebrate hosts. Davey (1969) considered the passage through the first host important, as the larvae are released (exsheath) from the cuticles of the previous stage. Although Davey (1969) studied the early development of **C.osculatum**, other authors eg. Scott (1955) and Smith (1983b) were of the opinion that **A.simplex** and **P.decipiens** also exsheathed on ingestion by the first host. In invertebrate hosts, ascaridoid larvae penetrate the gut wall, and are found free in the haemocoel.

The first major intermediate hosts of **Anisakis** appear to be planktonic crustaceans (Platt 1975), with euphausiids being particularly important (Smith 1971, Smith 1983a,b,). Smith (1971; 1983a,b) reported larvae of **A.simplex** from the euphausiids **Thysanoessa longicaudata**, **T.inermis** and **Nyctiphanes couchii** in the north east Atlantic and northern North Sea. The overall prevalence rate of **A.simplex** in euphausiids from individual localities was low (Smith 1971,1983b), and although reaching 4%, was often less (Smith 1971). In addition, only one larvae was found in each euphausiid (Smith 1971). Hurst (1984b) had also found the euphausiid **Nyctiphanes australis** to harbour **A.simplex** in New Zealand waters. Makings (1981) recovered a larval **Anisakis** sp. from a specimen of **Mesopodopsis slabberi** (Mysidacea), collected from the west of Scotland, and it was stated that this was probably **A.simplex**. Smith (1983a,b), and Smith and Wootten (1978a) discussed reports of **Anisakis** sp. in invertebrates in greater detail, noting that records of **Anisakis** larvae in invertebrates are all from the subclass Malacostraca and mainly the Euphausiidae - indicating that **Anisakis** appears to be "loosely" host specific at this stage (Smith and Wootten 1978a).

It is thought that the first intermediate host of **Pseudoterranova** is a benthic crustacean, and various species of benthic, epibenthic and natant copepods may become

infected, as McClelland (1982) showed experimentally. Burt **et al.** (1990b,c) also experimentally infected copepods, and **Gammarus oceanicus** with **P.decipiens** larvae. Natural invertebrate intermediate hosts of **P.decipiens** larvae are largely unknown, and few have been reported, although larvae have been recovered from, among others, isopods of the genera **Idothea** (Smith and Wootten 1984a). McClelland **et al.** (1983a) discussed the possibility of **P.decipiens** larvae being transferred from copepod hosts to benthic macroinvertebrates as second "intermediate" hosts.

Davey (1969) experimentally infected crustaceans with newly hatched larvae of **C.osculatum** and observed only one case of a **Gammarus** sp. (amphipod) infection, but two species of harpacticoid copepods did successfully become infected. Huizinga (1966) had previously stated that copepods were suitable hosts for larval **Contracaecum** and this author discussed previous reports of **Contracaecum** sp. in invertebrate hosts.

The first intermediate hosts of **H.aduncum** are several species of invertebrates (Hamerlynck **et al.** 1989). Larvae of **H.aduncum** have been reported from both planktonic and benthic marine invertebrates (Möller 1978, Svendsen 1990), mainly polychaetes, crustaceans and molluscs (Norris and Overstreet 1976). In Norwegian waters, Svendsen (1990) recovered larvae of **Hysterothylacium** sp. from seven planktonic species of invertebrates, including **Sagitta elegans** (a chaetognath), **Thysanoessa rashii**, two species of copepods, and a single species of amphipod, ctenophore and hydrozoan,; with **S.elegans** being the most commonly infected host. Øresland (1986) also recovered larvae of **H.aduncum** from **Sagitta setosa** in the English Channel, and indeed it was the commonest parasite recovered, accounting for 56% of all parasites found. Øresland stated that it seemed likely that **S.setosa** harboured **H.aduncum** throughout most of its' distributional area. Among other organisms, Hurst (1984b) found **Sagitta** sp. to be a host of **T.adunca** (= **H.aduncum**) in New Zealand waters. Smith (1971) had previously referred to single worm

infections of larval **Contracaecum** in the euphausiids **T.rashii** and **N.couchii**, and in the chaetognath **Sagitta elegans** from Scottish waters, but subsequent examination indicated that they were actually larvae of **Hysterothylacium** sp.. Subsequently, Smith (1983a) recovered third stage larvae of **Hysterothylacium** sp. from **Thysanoessa** spp. and **N.couchii**, from two inshore localities to the east and west of Scotland, although their presence was rare and Smith stated that euphausiids may not be important hosts for these parasites. As with **A.simplex**, no single euphausiid held more than one larval nematode (Smith 1971,1983a). Svendsen (1990) noted that most of the invertebrate hosts for **Hysterothylacium** were predators, and were thus likely to have become infected by ingesting smaller infected plankton. Berland (1961) had also suggested this previously, generally assuming that eggs and larvae were eaten by small benthic invertebrates, which may serve as first intermediate hosts. Freshwater mysids were observed to become infected with **H.aduncum** on exposure to both eggs and hatched larvae (Yoshinaga **et al.** 1987).

### 1.3.3 Fish Transport Hosts

The second transport hosts of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** are teleost fish, which become infected by eating infected invertebrate hosts. Parasites are released from infected prey during digestion, penetrate the digestive tract and migrate to various organs within the body cavity of the fish - or to the musculature - where they are generally encapsulated (eg. Berland 1961, 1991; Platt 1975; Smith and Wootten 1984a,b,c). Parasites of marine fish in northern waters show a low degree of host specificity (Appy and Burt 1992) and indeed the life cycles of the above parasites are characterised by a general lack of host specificity within fish and are thus capable of infecting a large variety of unrelated fish species at the third larval stage, and, in the case of **H.aduncum**, also at the fourth

larval and adult stage eg. Smith and Wootten 1984a,b,c; Hamerlynck **et al.** 1989). Larvae of **A.simplex** occur commonly in pelagic species of fish, presumably as a result of feeding on pelagic invertebrates (Platt 1975). Prevalence and intensity of infection in teleosts is also higher in offshore than inshore waters (Wootten and Waddell 1977), and this fits the pattern of infection in euphausiids, which are also commoner in offshore than inshore waters (Smith 1983a,b), and the distribution of final cetacean hosts. **P.decipiens** are thought to predominate in demersal fish species, which feed on infected benthic invertebrate hosts (eg. Platt 1975); and infections of **P.decipiens** and **C.osculatum** are common in fish from inshore waters eg. Templeman **et al.** (1957), McClelland **et al.** (1983a,b); which these authors, among others, related to the distribution of the seal final hosts.

Berland (1961) suggested that fish may become infected with **C.aduncum** (= **H.aduncum**) by eating eggs or larvae whilst free in the water; in which case these fish will act as first transport hosts. Fish feeding on infected plankton will act as second transport hosts. It is also of relevance to note that Smith **et al.** (1990) experimentally infected rainbow trout with freshly hatched larvae of **P.decipiens** and **C.osculatum**, and found that **C.osculatum** were able to develop in the body cavity without prior passage through an invertebrate host.

Scott (1954) and Smith (1974) demonstrated transfer of third stage larvae of **P.decipiens** and **Anisakis** sp. respectively from fish to fish; whilst Burt **et al.** (1990a), observed sequential invasion by the same **P.decipiens** larvae through a maximum of two fish. Thus, larvae of **A.simplex**, **P.decipiens** and **C.osculatum** may be transported through several fish, via the food chain ie. larger piscivorous fish feeding on smaller infected species; larvae are re-encapsulated in new fish hosts, until an infected fish is eaten by a suitable final host (eg. Platt 1975).

Larvae may remain within fish for a long period of time eg. Van Banning and Becker (1978), Smith (1984c), Des Clers

and Wootten (1990), Hemmingsen **et al.** (1991). The occurrence of third stage ascaridoid larvae in fish hosts is discussed in further detail in Chapter Two.

In addition to fish, Smith (1984d) recorded third stage larvae of **A.simplex** from four species of squid from waters around Scotland, and in the northern North Sea, and found a larval contracaecinean in one species, which he stated was possibly **C.osculatum**. Berland (1961) and Threlfall (1982) also reported **A.simplex** third stage larvae from squid.

### 1.3.4 Final Hosts

Third stage larvae ingested in prey by suitable final hosts, are released during digestion, remain in the alimentary tract and moult twice to the fourth stage (= pre-adult), and then the adult stage.

**Anisakis simplex** develops to the adult stage in the stomachs and intestines of a wide variety of marine mammals, predominantly cetaceans of various species (eg. Davey 1971, Young 1972, Myers 1979, Smith 1983a, Smith and Wootten 1978a, 1984b). Van Theil (1966) stated that **A.marina** (= **A.simplex**) was very common in cetaceans from the North Sea, with some cases of thousands of worms per host eg. the total worm burden of a white whale (beluga) was estimated at 22,000. Among other cetaceans, **A.simplex** have been recovered from porpoises **Phocaena phocaena** (eg. Young 1972), with Lick (1991) stating that **P.phocaena** appeared to be a major host for adult **A.simplex** in the eastern North Sea and western Baltic; and Young (1972) also reported **A.simplex** as being the most common nematode species recovered from minke, fin, sperm and pilot whales. Davey (1971) reported **A.simplex** from the blue whale **Balaenoptera musculus**, a typical plankton feeder, and this and other evidence suggests that third stage larvae in euphausiids, in addition to fish, are directly infective to final hosts (Smith 1983b). Seals appear to be

insignificant as final hosts of **Anisakis** (Smith and Wootten 1984b). Young (1972) recovered no **A.simplex** from the stomachs of common seals, and only small numbers from grey seals, whilst **A.simplex** was found rarely, and only at the juvenile stage, in the stomachs of harbour (= common) seals from the Wadden Sea (Lick 1991).

At least 18 species of seal have been recorded as final hosts for **P.decipiens** (eg. Myers 1959). Grey seals (**Halichoerus grypus**) are important final hosts in the eastern Atlantic (Young 1972), whilst Lick (1991) found harbour seals (**Phoca vitulina**) to be the main host for adult **P.decipiens** in the Wadden Sea (south-eastern North Sea). Grey and harbour seals have variously been reported as the major final hosts of **P.decipiens** in Canadian Atlantic waters (eg. Scott and Fisher 1958b, McClelland 1980c). Among others, **Porracaecum\ Phocanema\Pseudoterranova decipiens** have also been recorded from harp seals **Pagophilus\Phoca gröenlandicus**, bearded seals **Erignathus barbatus**, ringed seals **Phoca hispida**, and hooded seals **Cystophora cristata** (Myers 1957, Scott and Fisher 1958b, Bishop 1979, Paggi **et al.** 1991). McClelland (1980a) observed moulting of **P.decipiens** in seals; the fourth larval stage occurring 2-5 days post-infection, the adult 5-15 days p.i., maturity after 15-25 days and eggs detected in the faeces by day 16. Scott (1953) made similar observations. Growth, reproduction and survival of **P.decipiens** in seals were examined in detail by McClelland (1980b). Young (1972) reported **P.decipiens** from cetaceans, as did Scott and Fisher (1958a) and Lick (1991); however the latter two authors found this nematode to be generally immature, often with a low prevalence, and Young (1972) considered pinnipeds as being more important hosts for **P.decipiens** than cetaceans.

A wide range of seals also act as final hosts to **C.osculatum**, with Baylis (1937) stating that almost every seal species in the northern hemisphere has been recorded as a host for this species, in addition to it being reported several times from seals in the southern hemisphere. **C.osculatum** has been reported mainly from phocids, but has

also been found in otariids (Fagerholm and Gibson 1987), with Johnston and Mawson (1941b) recovering **C.osculatum** from the Australian sea lion and leopard seals. Beverley-Burton (1971) recovered **C.osculatum** from the stomachs and intestines of Weddell seals, **Leptonychotes weddelli**, from the Antarctic. In northern waters, **C.osculatum** have been recovered from the stomachs of, among others, grey, harbour, harp and ringed seals (eg. Myers 1957, Young 1972, Bishop 1979, Lick 1991). In British waters, Young (1972) found the majority of nematodes in the alimentary tracts of grey seals to be **C.osculatum**; Valtonen **et al.** (1988) reported heavy infections of **C.osculatum** in grey seals from the north-eastern Baltic, but this species was only found occasionally in resident ringed seals, **Phoca hispida botnica**, from this area. Scott and Fisher (1958a) also recovered larvae and adults of **Contracaecum** from porpoises.

Fourth stage larvae and adults of **Hysterothylacium** (= **Thynnascaris**) are found free in the intestinal tracts of many piscivorous species of marine teleost fish (eg. Norris and Overstreet 1976; Möller 1978; Hamerlynck **et al.** 1989; Svendsen 1990; Berland 1961,1991).

**Anisakis** sp. third stage larvae were observed, **in vitro**, to moult to the fourth stage after 3-7 days (Grainger 1959, Khalil 1969, Grabda 1976b, Hurst 1984a); to the adult 12-14 days after the previous moult, and eggs were produced 4-9 days after the moult to the adult (Grabda 1976b). In rats, pre-adults were observed 3-4 days after infection (Gibson 1970, Weerasooriya **et al.** 1986).

**In vitro** culture of third stage larvae of **P.decipiens** to pre-adult and adult stages has been undertaken by several authors eg. Grainger 1959, Townsley **et al.** 1963, Carvajal **et al.** 1981, Weerasooriya **et al.** 1986, Likely and Burt 1989. McClelland and Ronald (1974a,b) cultivated **P.decipiens** and **C.osculatum in vitro** and examined development from eggs to adults, although obtaining no adults of **P.decipiens**.

Sommerville and Buzzell (1974) concluded that the

stimulus for **in vitro** development of **Anisakis** larvae from fish was a combination of increased temperature and relatively high concentrations of carbon dioxide, and it is reasonable to suppose that similar conditions to stimulate development would be found in the stomachs of the mammalian final hosts. Grainger (1959) had also considered that temperature was an important factor in determining the moult of larvae to the fourth stage.

## 1.4 Geographical Distribution

**A. simplex** is generally distributed in temperate waters, particularly in colder temperate and polar waters. Davey (1971) considered the distribution of this species to be worldwide, except for the region between 40°N and 36°S (warmer temperate and tropical waters) where **A.typica** occurs. However, Smith (1983b) commented on the apparent absence of infection by **A.simplex** in Antarctic euphausiids and baleen whales, and suggested that this parasite may not circulate there.

**P.decipiens** has been reported worldwide from mammalian hosts (Davey 1971), with Margolis (1977) stating that **P.decipiens** occurs in both hemispheres, particularly in temperate and polar climatic zones. Similarly, **C.osculatum** is known to have a wide geographic distribution in seals, being found throughout the northern hemisphere, and also in seals from the southern hemisphere including the Antarctic eg. Baylis 1937, Johnston and Mawson 1941b, Beverley-Burton 1971).

Berland (1961) considered the geographic range of **H.aduncum** to be extensive as both adults and larvae have been reported from many species of fish. Subsequently, Berland (1991) stated that **H.aduncum** has a circumpolar distribution in the northern hemisphere, infecting marine teleosts in temperate and cold waters.

Many marine parasites extend throughout the range of

their hosts (Sprent 1982), and Smith and Wootten (1984a,b,c) considered larvae of **Anisakis** sp., **P.decipiens** and **Contracaecum** sp. to be most prevalent where the greatest number of hosts occurred ie. inshore waters in the case of **P.decipiens** and **Contracaecum** sp., and offshore waters in the case of **Anisakis** sp.

Davey (1971) noted that environmental temperature appeared to influence geographic distribution of **A.simplex**, and McClelland (1982) also stated that the distribution of **P.decipiens** in the north Atlantic appeared to be related partly to oceanic temperatures; **P.decipiens** in fish being virtually absent from regions where the sea bed temperatures were less than 2°C, with moderate-heavy infections between 4-17°C. McClelland found the latter temperature range to be optimal for the early development of this species.

## 1.5 Relevance to Man

The presence of larval nematodes in fish fillets and viscera constitutes not only a potential human health hazard, as some species are pathogenic to man (Williams and Jones 1976), but also lowers the market value of fish for human consumption (Myers 1970, Chandra and Khan 1988).

The nematodes, particularly **P.decipiens**, being large and brown\red in colour, are macroscopically visible leading to infected fish being rendered unaesthetic in appearance, and are thus a source of concern to the fishing industry (Templeman **et al.** 1957). Platt (1975) suggested that a practical solution would be to candle the fillets and remove the nematodes before sale of the fish. McClelland **et al.** (1983a) discussed control of the **P.decipiens** problem in fish. Adult **H.aduncum** may also cause hygienic problems when whole fish are presented at market, as they may migrate out of the mouth or anus (Möller 1978).

Ingestion of live **Anisakis** and **Pseudoterranova** larvae by humans, as a result of consuming insufficiently cooked or

raw fish containing the larvae, may produce a condition known as anisakiasis, or phocanemiasis (eg. Smith and Wootten 1984a,b). Infections in humans are well documented in the literature. The first documented case of anisakiasis, caused by **Anisakis** sp. larvae, was recorded in the Netherlands (Van Thiel **et al.** 1960). Numerous cases have now been reported worldwide (eg. Jackson 1975, Margolis 1977), particularly from Japan where raw fish and lightly cooked fish are commonly eaten eg. Asami **et al.** (1965), Yokogawa and Yoshimura 1967, and in certain areas of Europe where lightly salted or pickled fish are consumed eg. Van Theil and Van Houten (1967), Van Theil (1976). Among others, a large number of cases have been reported from the United States eg. Kliks 1983. Most North American cases have involved **P.decipiens** (Fredericksen and Specian 1981, Kliks 1983).

Man is an "accidental" host to these nematodes and the larvae survive for various periods of time, but may not develop to the adult stage (Van Thiel **et al.** 1960, Van Theil and Van Houten 1967, Myers 1970). In such cases, ingested larvae may attempt to penetrate the gastro-intestinal wall, and cause acute abdominal symptoms (Van Thiel **et al.** 1960, Van Theil and Van Houten 1967) and elicit a series of host reactions, including nausea (Kliks 1983), fever (Van Theil **et al.** 1960), abdominal pain (Van Theil **et al.** 1960, Asami **et al.** 1965, Kliks 1983, Takahashi **et al.** 1986), gastrointestinal disorders and lesions in the stomach and intestine, which can be fatal (Sommerville and Buzzell 1974).

**Contracaecum** sp. have also been implicated in human infection eg. Jackson (1975), Williams and Jones (1976), Smith and Wootten (1984c). However, Smith and Wootten considered this species of less importance than **Anisakis** or **P.decipiens** as **Contracaecum** rarely occur in the flesh. Several authors have reviewed aspects of human infection by marine ascaridoids eg. Jackson (1975), Van Theil (1976), Williams and Jones (1976), Margolis (1977) and Smith and Wootten (1978a). Norris and Overstreet (1976) reviewed the public health importance of larval **Thynnascaris** (=

**Hysterothylacium**) from shellfish, reporting that larvae of **Thynnascaris aduncum** have been implicated, but not confirmed, as agents of human gastrointestinal eosinophilic granulomata in France (Petter 1969). Norris and Overstreet (1976) also demonstrated that a type of larval **Thynnascaris** from the cutlass fish, **Trichiuris lepturus**, infected and produced gastrointestinal lesions in the mouse.

Adequate cooking or freezing kills nematode larvae in fish flesh (Davey 1972; Smith and Wootten 1984a,b,c; Berland 1991). Experimental studies reviewed by Bier (1976) showed that fish must be cooked at 60°C for 1 minute, or frozen to -20°C for 52hours to kill anisakid larvae. Norris and Overstreet (1976) stated that larval **Thynnascaris**, were killed by the usual methods of food processing and preparation.

Van Theil **et al.** (1960) observed that salt had a paralysing effect on **Anisakis** larvae, although fairly high concentrations were needed to kill the worms; **Anisakis** larvae were also highly resistant to acids. Smoking fish does not necessarily kill parasites eg. Gardiner (1990) found that samples of smoked salmon examined 27 days after storage in a refrigerator at 4°C yielded live **Anisakis**, and Khalil (1969) observed the salt concentration and heat during the smoking process of herring to be insufficient to kill **Anisakis** larvae, suggesting that deep freezing before or after smoking should eliminate the danger to humans. Grabda and Bier (1988) used **in vitro** cultivation to recover motile (viable) larvae of **A.simplex** from salted and spiced herring, and found that larval mortality in fish increased as brining increased from 1-4 weeks.

The Dutch have significantly reduced the incidence of human anisakiasis in Holland by gutting fish at sea, thus preventing migration of larvae from the viscera into the fillet (Roderick and Cheng 1989).

## 1.5 Aspects of the Present Study

Four aspects of the biology of marine ascaridoid nematodes were investigated during the present study :-

(a) Occurrence and Distribution of Ascaridoids in Marine Teleosts -encompassing epidemiological studies of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** in five fish species; distribution of these nematodes in different tissues and organs of the fish and a preliminary analysis of interactions between species within fish.

(b) Pathology of Stomach Lesions Associated with **A.simplex** and **P.decipiens** Larvae in Marine Teleosts - describing gross and histological observations of stomach ulcers in gadoids and angler fish (**Lophius piscatorius**).

(c) External Ultrastructure of the Cephalic End of Marine Ascaridoids - general examination and comparison of the developmental morphology of the cephalic region of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** through different life cycle stages.

(d) Internal Ultrastructure of Marine Ascaridoids - an investigation into aspects of the fine morphology of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum**, at different life cycle stages.

# CHAPTER TWO : OCCURRENCE AND DISTRIBUTION OF MARINE ASCARIDOIDS IN FIVE SPECIES OF MARINE TELEOSTS

## 2.1 INTRODUCTION

### 2.1.1 Previous Epidemiological Studies

There have been numerous studies on larval ascaridoid nematode infestation in a wide variety of marine fish species from a large number of geographic locations. The majority of such studies have been carried out in the northern hemisphere, particularly in Atlantic waters.

Some authors have examined widely varied aspects of ascaridoid infections. Some have carried out general examinations of single species of fish, encompassing a wide range of parasite species, including nematodes, in single host species eg. haddock (**Melanogrammus aegelfinus**) (Scott 1981), Pacific herring (**Clupea harengus pallasi**) (Arthur and Aria 1980a,b), walleye pollock (**Theraga chalcogramma**) (Arthur **et al.** 1982) and blue whiting (**Micromesistius poutassou**) (Grabda 1978, MacKenzie 1979). Others have examined infections by **Anisakis** sp. and\or **Pseudoterranova decipiens** larvae in single host species, mostly concentrating on infections in commercially important fish species eg. **H.platessoides** (American\Canadian plaice \ Long Rough Dab) (McClelland **et al.** 1987); herring (Bishop and Margolis 1955, Khalil 1969, Davey 1972, Van Banning and Becker 1978, McGladdery 1986); cod (**Gadus morhua**) (Scott and Martin 1959; Wiles 1968; Young 1972; Platt 1975; Chandra and Khan 1988) and blue whiting (Smith and Wootten 1978b). Still others have studied a wide range of fish species, often examining only a few specimens, for nematode infections eg. Jackson **et al.** (1978) examined over 1000 fish of 23 different species from waters around Washington D.C. in the United States; Berland (1961) examined 260 fish from 64 different species in Norwegian waters. Authors such as Kahl (1939), Templeman **et**

**al.** (1957), Berland (1961), Wootten and Waddell (1977), Wootten (1978), Smith and Wootten (1979), McClelland **et al.** (1983a,b), Munger (1983), Smith (1984e), Jensen and Andersen (1991), Myjak **et al.** (1991) and Strømnes and Andersen (1991) reported on the occurrence of one or more ascaridoid species in several species of fish.

Most research on the occurrence of larval nematodes in fish has concentrated on **Anisakis** sp. and **P.decipiens**, and their prevalence and intensity in various localities. Such studies are often only concerned with infection in the musculature eg Templeman **et al.** (1957), Scott and Martin (1959), Wiles (1968), Wootten and Waddell (1974, 1977), Platt (1975), Smith and Wootten (1979), Haukkson (1984, 1992), Chandra and Khan (1988) and records of numbers of these worms in the fillets alone do not provide an accurate indication of true abundance of the parasites. In addition, such examinations do not reveal infection levels of **Contracaecum** sp. or **Hysterothylacium aduncum**, as these nematodes rarely occur in the musculature. However, even those reports which have examined infection by larval nematodes in the viscera and body cavity, have rarely included studies of **Contracaecum** sp. or **H.aduncum**. Among the exceptions to this were Grabda (1976a), who studied infections by third stage larvae of **C.aduncum** (= **H.aduncum**) in Baltic cod, finding only this and **Anisakis simplex** sp. in the specimens examined, and McClelland **et al.** (1983a), who included **Contracaecum** sp. in their studies on cod and flatfish from eastern Canadian waters. Palsson **et al.** (1985) appear to be the only authors who have studied infection levels of **A.simplex**, **P.decipiens**, **Contracaecum** sp. and **H.aduncum** in fish, albeit from a single species (cod), and this study only included third stage larvae of **H.aduncum** - fourth stage larvae and adults in the alimentary tract were not examined.

Whilst third stage larvae of **A.simplex** and **P.decipiens** commonly occur in the viscera and musculature of many marine fish species. **Contracaecum osculatum** and **H.aduncum** third stage larvae generally only occur in the viscera, and

**H.aduncum** fourth stage larvae and adults in the alimentary tract. Few studies have paid detailed attention to the numbers of these four nematode species within separate organs or tissues of individual fish. Where studies have been conducted on the infection levels of nematodes within the entire fish, many authors have simply reported their presence, and have not counted the nematodes, or stated where the nematodes were found. Others have simply split the areas into two broad microhabitats - the viscera and the flesh. Several authors have examined the distribution of larvae in the musculature of fish eg. Templeman **et al.** (1957) examined the distribution of larval nematodes in the fillets of various fish species, including cod, haddock, **H.platessoides** and longhorn sculpin; Wootten and Waddell (1977) examined the location of larval nematodes in the musculature of cod and whiting. Other authors, including Berland (1961), Khalil (1969) and Strømnes and Andersen (1991) have commented on the variable distribution of ascaridoid nematodes in fish, although did not provide detailed results. Among others, McClelland **et al.** examined the distribution of larval **Anisakis**, **P.decipiens** (1983a,b) and **Contracaecum** sp. (1983a) in various host tissues of cod and flatfish, Kahl (1939) examined the distribution of **Anisakis** sp. and **P.decipiens** within cod and Grabda analysed the occurrence of **A.simplex** and **H.aduncum** in different organs and tissues of Baltic cod (1976a) and blue whiting (1978), while Kusz and Treder (1980) also examined the distribution of the latter two parasites in blue whiting. The most comprehensive study was carried out by Palsson **et al.** (1985) who examined the distribution of **A.simplex**, **P.decipiens**, **Contracaecum** sp. and **H.aduncum** larvae in various regions of cod from Icelandic waters.

### 2.1.2 Geographical and Temporal Variations in Infection Levels

#### 2.1.2.1 Geographical Variations

In Scottish waters, Wootten and Waddell (1974, 1977) noted variations in infection levels of larval nematodes in the same fish species from different areas. Differences were also observed in other British waters (Khalil 1969, Davey 1972, Young 1972, Van Banning and Becker 1978), Canadian Atlantic waters (Templeman **et al.** 1957; Scott and Martin 1959; McClelland 1983a,b; McGladdery and Burt 1985), west coast waters of Canada (Bishop and Margolis 1955, Arai 1969), North Atlantic and Arctic waters (Platt 1975), Norwegian waters (Hemmingsen **et al.** 1991) and the Baltic sea (Grabda 1976a). Scott (1981) also noted differences in the infection levels of **H.aduncum** in the intestine of haddock sampled from different areas.

Several factors appear likely to govern the geographical distribution and abundance of nematodes in fish. The most significant of these is the life cycle of the parasites themselves, in relation to the hosts they utilise. Young (1972) for example, regarded the presence or absence of suitable final hosts as the factor most responsible for the geographical distribution of larvae in fish hosts, and stated that the absence of suitable invertebrate hosts was also a limiting factor. Young thus related differences in the distribution of **Anisakis** and **P.decipiens** from different areas to the distribution of the mammalian final hosts, suggesting that numbers of nematodes in fish will be at a maximum in those areas containing most final hosts, and both Platt (1975) and Wootten and Waddell (1977) considered variations in fish infections in relation to the geographical distribution of the invertebrate and mammalian hosts of the parasites.

McClelland (1983a,b) discussed **P.decipiens** abundance in fish populations from different regions in relation to the

distribution of grey seals, finding the intensity of **P.decipiens** larvae in cod and flatfish to increase with proximity to grey seal colonies. Several other authors have associated the presence of seals with areas of heavy **P.decipiens** infection in fish eg. Kahl (1939), Templeman **et al.** (1957), Scott and Martin (1959), Myers (1960), Platt (1975).

Templeman **et al.** (1957), Young (1972), Wootten and Waddell (1977), Wootten (1978) and McClelland **et al.** (1983b) found larval **Anisakis** sp. to be more common in fish from offshore areas. Infection of fish with **Anisakis** larvae must be considered in relation to the distribution of, and infection levels in, the cetacean final hosts, and also to the distribution of invertebrate hosts (Platt 1975). Thus, Platt (1975), Myers (1979) and Hemmingsen **et al.** (1991) considered that heavy infection by **Anisakis** larvae in fish may be due to the presence of large numbers of infected cetaceans, which are more common in offshore areas (Hemmingsen **et al.** 1991). High prevalences and intensities of **A.simplex** in fish occurred in the central region of the northern North Sea (Wootten and Waddell 1977) and this may be related to the higher prevalence of **A.simplex** infections in euphausiids in offshore waters in the northern North Sea (Smith 1971, 1983a,b).

Wootten (1978) found larval **Thynnascaris** (= **Hysterothylacium**) **aduncum** to be abundant in small gadoids from both inshore and offshore waters, but larval **Contracaecum** were only found in fish from the Moray Firth, which he suggested may reflect the coastal distribution of seal final hosts, and he added that the intermediate hosts of this parasite may also be restricted to coastal areas. High levels of **Contracaecum** larvae in fish were related to the occurrence of harp seal populations by McClelland **et al.** (1983a).

### 2.1.2.2 Temporal Variations

Comparisons of previous surveys in fish from Scottish waters (Wootten and Waddell 1974, 1977; Smith and Wootten 1979) revealed significant fluctuations in levels of larval nematode infection in fish over different years. These authors discussed the possible causes of these fluctuations. A number of authors have related increases in infection of fish to increases in the population of mammalian final hosts eg. McClelland **et al.** 1983a,b; Kliks 1983; Hauksson 1984; Chandra and Khan 1988, Des Clers 1989.
Davey (1972) stated that the level of infection to which herring are exposed every year may fluctuate due to differences in the distribution and infection of invertebrate hosts or with the extent to which fish are feeding on this host.

Grabda (1974, in Williams and Jones 1976) found that Baltic herring could harbour low or heavy infections with larvae of **A.simplex** depending on the season, and suggested that infected populations left the area and were replaced by non-infected ones. Hamerlynck **et al.** (1989) found larval **H.aduncum** in the body cavity of sand gobies throughout the year, although highest infection levels were recorded from mature fish in spring. Möller and Anders (1986) noted that the intensity of infection of **H.aduncum** decreased significantly after summer. Scott (1981) noted seasonal differences in the prevalence of **H.aduncum** in the intestine of haddock from eastern Canadian waters, with prevalence of infection decreasing by approximately 60% from summer to winter; this was thought to be due to a decreased intake of intermediate hosts. Berland (1961) and Grabda (1976a) suggested that annual and seasonal increases in infection by anisakine larvae in fish are related to periodic migrations of the marine mammal hosts. Hamerlynck **et al.** (1989) also suggested that seasonal changes in parasitic infection of fish may be a result of the seasonal occurrence of intermediate hosts. In Norwegian waters, Strømnes and

Andersen (1991) found no distinct seasonal fluctuations in infection of saithe, cod and redfish with **A.simplex** larvae, and Valtonen **et al.** (1988) observed no seasonal variation in infection levels of **C.osculatum** in fish from the north-eastern Baltic.

### 2.1.3 Length \ Age Relationship to Infection Levels

It is well documented that the infection by larval nematodes generally increases with fish length\age eg **Anisakis** in cod (Kahl 1939, Scott and Martin 1959, Grabda 1976a, Wootten and Waddell 1977, Smith and Wootten 1979, McClelland **et al.** 1983b, Myjak **et al.** 1991), herring (Bishop and Margolis 1955, Khalil 1969, Van Banning and Becker 1978, McGladdery and Burt 1985, McGladdery 1986, Myjak **et al.** 1991) and blue whiting (Smith and Wootten 1978b); **P.decipiens** in cod (Kahl 1939; Scott and Martin 1959; Young 1972; Wootten and Waddell 1977; Smith and Wootten 1979; McClelland **et al.** 1983a,b) and redfish (Kahl 1939); **Contracaecum** sp. in cod (Myjak **et al.** 1991) and herring (Myjak **et al.** 1991); **H.aduncum** in cod (Grabda 1976a, Myjak **et al.** 1991) and herring (Myjak **et al.** 1991).

Two main factors account for the increase in infection with age of the fish - longevity of third stage larvae within fish and increases in the amount of food eaten by fish they grow older. Van Banning and Becker (1978) stated that **Anisakis** larvae can remain in fish hosts for years. Smith (1984c) studied larvae of **A.simplex** of known minimum age from herring and found that the oldest third stage larvae examined may have spent at least three years in herring. Such longevity is also likely in other species of larval nematodes. Larval nematodes in fish are released only on death or ingestion by a subsequent host (McClelland **et al.** 1983a), and they therefore remain in the fish throughout its life span, becoming increasingly numerous in older fish through cumulative infections. Accumulation of nematodes with

size can also be related to the food consumption of the host fish species, which increases with size, therefore large and older fish (which consume more) will be exposed to more parasites than small and younger fish (Khalil 1969, Wootten and Waddell 1977, Wootten 1978, Des Clers 1989). Des Clers (1989) found that increases in average **P.decipiens** burden in individual fish agreed well with her predicted increase in food consumption as a result of fish growth.

McClelland **et al.** (1983a) stated that the rate at which fish accumulate nematodes will depend not only on the quantity of prey consumed but also on the types of food eaten. This can be extended to include dietary differences over the life span of the fish eg. as herring increase in size, they prey on larger organisms which may act as intermediate hosts for **Anisakis** (Khalil 1969), and indeed McGladdery and Burt (1985) found **A.simplex** infection in herring to increase between the ages of two and three, which corresponds to the age at which the diet changes from consisting mainly of copepods to one predominantly of euphausiids; Burt **et al.** (1990a) stated that small cod which feed mainly on crustaceans harboured relatively few third stage larvae, but as they grow and become largely piscivorous the number of larvae increases, presumably acquired from prey fish. Smith and Wootten (1978b) found most blue whiting to become infected with **Anisakis** within the first two years of life, and the subsequent increase in infection probably reflected continual heavy feeding on euphausiids. McGladdery and Burt (1985) stated that although adult herring can still acquire **H.aduncum**, infection levels do not increase as it is the younger herring which feed more extensively on the intermediate hosts. Wootten (1978) found a general increase in infection level of **Anisakis** sp, **Contracaecum** sp. and **Thynnascaris** (= **Hysterothylacium**) **aduncum** larvae with increasing age and length of small gadoids, however, a decrease was noted in the infection of **T.aduncum** with increasing length of haddock. Khalil (1969) and Scott (1981) also found the infection of **H.aduncum** to decrease with

increased size in herring and haddock respectively. Decreases in **Anisakis** infection with age were also noted in herring (Davey 1972) and cod from certain locations in Scottish waters (Wootten and Waddell 1977). Young (1972), found infections with **Anisakis** to increase in cod up to 72cm in length, and then to decrease, and Templeman **et al.** (1957) found that in areas of low infection, nematodes did not become more abundant in larger cod.

The biology of the fish host must also be considered in relation to variations in infection level with length eg. larger cod usually live in deeper water than smaller cod, and are thus generally further away from the inshore site of much of the infection of fish with **P.decipiens** (Templeman **et al.** 1957).

Samples of cod, haddock, blue whiting, long rough dab and bull rout (**Myoxocephalus scorpius**) from different localities in Scottish waters were examined for ascaridoid nematode infections. These species of fish were particularly chosen as they exhibit a range of life histories and feeding habits, rather than being, with the exception of cod and haddock, of commercial importance. Many studies have concentrated on infection by larval nematodes in species of commercially important fish, and have not compared infection in fish from different ecological types. In particular, infections by ascaridoid nematodes in bull rout have rarely been examined. Determinations were made of the abundance of **A.simplex**, **P.decipiens**, **C.osculatum** larvae and **H.aduncum** in nine organs or tissues of the fish examined. Nematodes in the alimentary tract were also recovered, thus including fourth stage larvae and adults of **H.aduncum** in the studies as well as third stage larvae of this species from the viscera and body cavity. Examination of the four parasite species from five different fish species also enabled comparisons to be made between the parasite populations of each fish species, in relation to the feeding behaviour of fish, and to the life

histories of both the host and individual parasites. Such aspects have rarely been discussed in detail, although notable exceptions to this are Wootten (1978), Scott (1981) and Smith (1984e).

A preliminary investigation into possible interactions between third stage ascaridoid larvae in fish was also carried out.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Collection of Fish

Specimens of cod, haddock, blue whiting and long rough dab were collected from several localities in northern Scottish waters in January 1991 (Figure 2). Table 2 shows the sampling locations, dates of sampling, along with the numbers of fish species collected and range in length. Specimens were collected by Aberdeen bottom trawl, each haul being one hour in duration, on the research vessel "Scotia". A minimum of ten specimens of each fish species were collected where possible (some hauls yielding less than ten specimens of each fish species), from each locality. Samples comprised of ten or more fish were chosen randomly from individual catches. Specimens of cod from the St. Abbs and Bell Rock area in southern Scottish waters were similarly collected in June 1991 (Figure 2, Table 2).

Some specimens were examined for nematodes on board ship, immediately after capture at sea, however, the majority were deep frozen and stored for later examination in the laboratory. All fish were examined fresh or deep frozen within one hour of capture, thus removing any possibility of migration of nematodes from the viscera after death of the host (see Smith and Wootten 1975, Smith 1984e).

Specimens of bull rout were collected by Mr A. MacDonald (SOAFD) from coastal waters near Stonehaven (Figure 2, Table 2), using a bottom trawl, in February 1991 and June 1991.

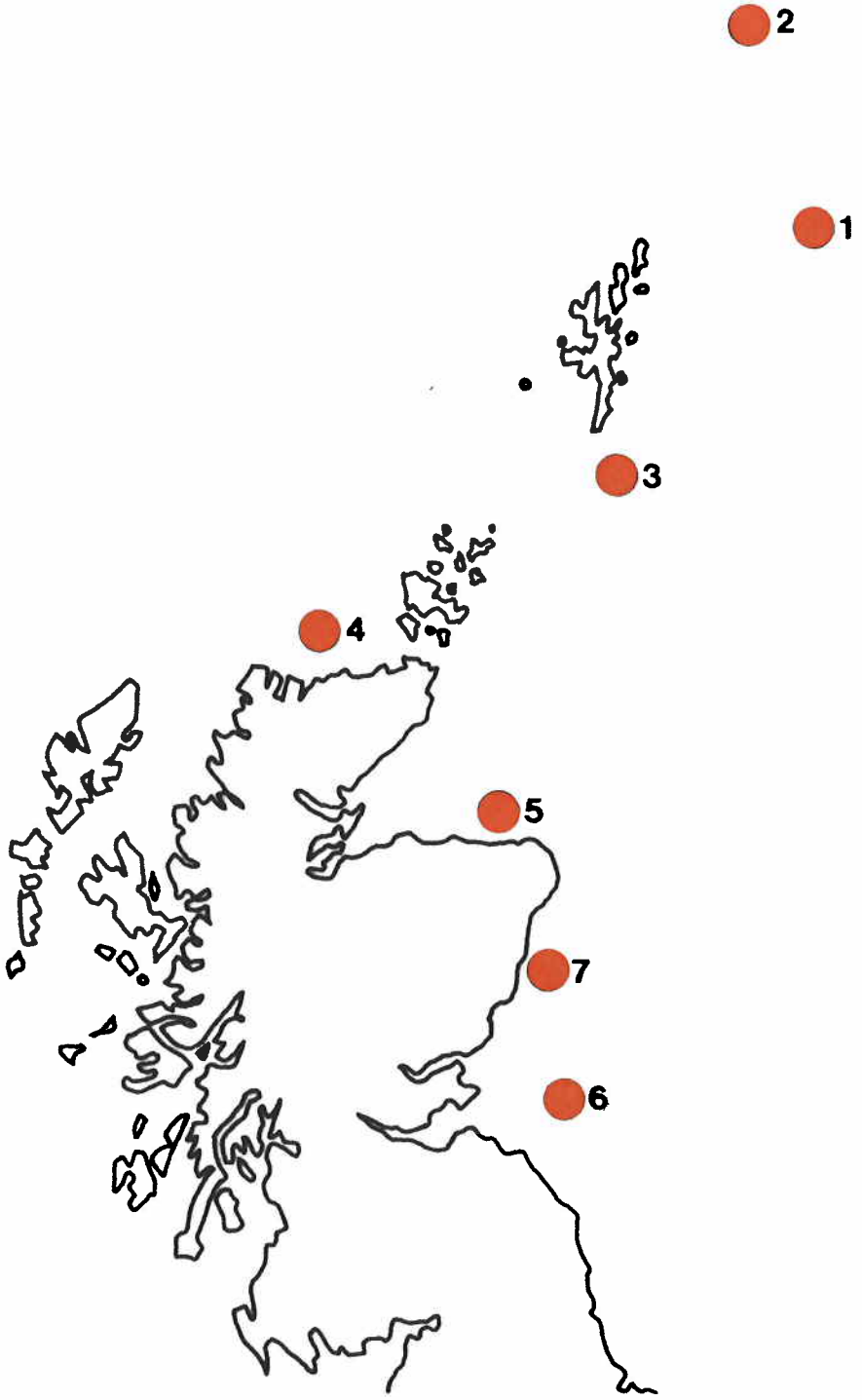



FIGURE 2: Sampling Locations
1 = East Shetland Basin, 2 = North-East Shetland,
3 = Fair Isle, 4 = North Coast, 5 = Moray Firth,
6 = St. Abbs\Bell Rock, 7 = Stonehaven

TABLE 2: SAMPLE DETAILS

| Fish | Location | Date | No.of fish | Length range (cm) |
|---|---|---|---|---|
| Cod | E.Shetland basin | 9/1/91 | 11 | 40-66.5 |
| **(G.morhua)** | N.E.Shetland | 10/1/91 | 8 | 47-66.5 |
| | Fair Isle | 13/1/91 | 6 | 34-95 |
| | Moray Firth | 16/1/91 | 7 | 49-60 |
| | St Abbs/Bell Rock | 7-8/6/91 | 10 | 37-60 |
| Haddock | E.Shetland basin | 10/1/91 | 10 | 27-47 |
| **(M.aeglefinus)** | N.coast | 14/1/91 | 17 | 30-37 |
| Blue Whiting | N.E.Shetland | 10/1/91 | 22 | 21.5-32 |
| **(M.poutassou)** | | | | |
| Bull Rout | Stonehaven | 21/2/91 | 16 | 25-33.5 |
| **(M.scorpius)** | | 2/5/91 | 12 | 24-32 |
| Long Rough Dab | E.Shetland basin | 9/1/91 | 10 | 14.5-21 |
| **(H.platessoides)** | N.coast | 14/1/91 | 4 | 12.5-21 |

These fish were transported alive to the Marine Laboratory in Aberdeen, where they were retained in a large seawater tank for one - two months, after which they were killed and examined. Bull rout were fed on fish which had previously been frozen, thus eliminating the possibility of obtaining further infections from fresh fish. Specimens taken originally in February were examined immediately on death, specimens taken in June were deep frozen and examined subsequently.

### 2.2.2 Examination of fish

Fish examined were weighed (ungutted) and the fork length measured to the nearest 0.5cm. The sex of the fish was noted, if reproductive organs were distinct.

The viscera and musculature of the fish were examined as described below. The viscera were dissected under tapwater in large Petri dishes. Nematodes were removed initially using the naked eye, then the organs were re-examined using an Olympus dissecting microscope.

(1) Stomach - The stomach was dissected out, cutting at the oesophagus and above the pyloric caeca. The stomach was then dissected open, washed repeatedly to remove the contents, and both the contents of the stomach and the stomach wall itself were then examined for nematodes. Any nematodes in the lumen were retrieved and identified, as were any which were present in the lumen but were in the process of penetrating the stomach wall. No distinction was made between the cardiac or pyloric stomach. The exterior of the stomach was also examined and any nematodes present in the connective tissue were removed and included under "mesenteries".
(2) Pyloric Caeca - The pyloric caeca were dissected out and any nematodes present on the caeca were removed. Fine seekers were also used to tease the individual caeca apart and a close examination was made for nematodes between the caeca.

(3) Intestine - The intestine, including the rectum, was removed, cut open from the posterior forward, the contents washed out and both the contents and the intestinal lumen itself examined for nematodes. The exterior of the intestine was also examined and any nematodes found amongst the connective tissue were included under "mesenteries".
(4) Mesenteries - All mesenteries associated with the viscera were examined for nematodes. The mesenteries closely associated with the exterior of the stomach and intestine were also included in this category. No distinction was made between mesenteries from different areas of the body cavity.
(5) Liver - The surface of the liver was examined for nematodes, before cutting the liver into small pieces and teasing each section apart, closely examining the parenchyma for worms.
(6) Kidney - The surface of the kidney was examined for nematodes.
(7) Gonads - Where gonads were present, the surface was examined for nematodes.

Fillets (epaxial musculature) and flaps (hypaxial musculature - surrounding the body cavity) were removed and examined together as:-
(8) Left Fillet and flaps, and
(9) Right Fillet and flaps

A number of methods were used to retrieve nematodes from fillets and flaps :-

(a) Removal of nematodes with the naked eye.

With a little practise, nematodes present on the surface of the fillets or flaps could be detected relatively easy with the naked eye, and were removed. **P.decipiens** lying beneath the fillet or flap surface could often be detected due to their large size and prominent colouring, and could also be easily retrieved. However, **A.simplex**, being relatively small in size, and of a similar colour to that of

the muscle tissue, are often difficult to observe in the muscle underlying the surface of the flesh; in addition, in large fish specimens where the muscle is thick, even **P.decipiens** cannot be observed if buried deeply within the tissue. Therefore, once the nematodes on the surface of the musculature were removed, the fillets and flaps were further examined using one of the following methods.

(b) Candling and Slicing.

Such examinations were carried out on freshly killed fish only. Individual fillets and flaps were placed over a Kodak Coldlight Illuminator Model 3 (candling table containing 2 x 15 watt fluorescent tubes) to allow nematodes to be detected within the tissue. Any nematodes present were then removed. Fillets and flaps were then systematically sliced transversely into thin sections along the length of the fillet using a scalpel, over the light box, into thin strips, approximately 7-10mm. in thickness, which were then teased apart and carefully examined for nematodes. This method allowed detection of any nematodes buried deep within the tissue, which might otherwise have gone undetected. Power (1961) reported that longitudinal slicing (parallel to the skin surface) of cod fillets, into layers approximately 13mm. thick, could increase the efficiency of candling fillets for **Porrocaecum** (= **Pseudoterranova**) **decipiens** to over 95%.

(c) Slicing.

Fillets and flaps from fish which had been deep frozen for storage purposes were removed, once the fish had defrosted, and examined for nematodes by slicing (as described above). The musculature in this case was not examined over a light box, slicing alone being considered as allowing the majority of nematodes present in the flesh to be detected.

When the above methods (a-c) were used to recover nematodes, the location (fillet or flaps) and the nematode

species found, were noted for each individual infected fish examined.

(d) Pepsin - Hydrochloric Acid Digest (after Smith and Wootten 1975)

This method was used to release larvae from the fillets and flaps of small fish. The musculature of all long rough dab collected and approximately 50% of blue whiting were examined using this method. Digest solution was prepared by mixing 10g of pepsin powder in 1 litre of 1% HCl. Individual left and right fillets and flaps from fish were removed and placed in large test tubes containing digest solution. The tubes were then placed in a water bath at 52°C until the tissue had been digested (approximately 2-3 hours), leaving the worms intact. The resulting liquid was then examined in a large petri dish, and any worms present were retrieved. These were subsequently identified and counted. This method could not be used to give data on the location of the worms in the tissue.

### 2.2.3 Identification of nematodes

Only **A.simplex**, **P.decipiens**, **H.aduncum** and **C.osculatum** were recovered - no other parasite species were examined. Cut or broken nematodes were not collected. Nematodes were removed from their capsules (if present), fixed in hot 70% alcohol and cleared in beechwood creosote. Nematodes were examined at high power under a light microscope and were initially identified by looking at the position of the excretory pore, the structure of the digestive system and the structure of the tail (Table 1, in Introduction, gives general identifying characteristics for third stage larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and third stage larvae and adults of **H.aduncum**. Figure 1 shows the general distinguishing features of these nematodes). Additional references for identification included Baylis 1920, 1937;

Scott 1954; Myers 1959,1960, 1975; Grainger 1959; Berland 1961; Van Theil and Van Houten 1967; Davey 1971; Hartwich 1974; McClelland and Ronald 1974a,b; Pippy and Van Banning 1975; Beverley-Burton **et al.** 1977; Smith and Wootten 1978a; Deardorff and Overstreet 1981a; Smith 1983a,b. No attempt was made to differentiate possible sibling species. With practice, specimens could be identified by eye, thus removing the need for fixation and clearing of specimens. **A.simplex** larvae in fish are often found in the form of a flat coil, and can be identified by this shape alone (Berland 1982), thus **A.simplex** could often be identified with the naked eye due to their coiled nature and distinct opaque white ventriculus. **P.decipiens** were also generally easy to identify due to their large size and colour (red/brown - yellow), particularly when present within white fish fillets. Larvae of **C.osculatum** and **H.aduncum** larvae were always examined under a dissecting microscope. **C.osculatum** larvae were often tinged with colours, whereas **H.aduncum** were always white. In some cases, where differentiation could not be made using a dissecting microscope, specimens of **C.osculatum** and **H.aduncum** were mounted, in seawater, on a slide and examined under a light microscope for identification purposes. **H.aduncum** adults, removed from the digestive tract of fish were usually easily identifiable due to their size, and the presence of either spicules or a uterus.

For **Hysterothylacium aduncum**, no distinction was made between third or fourth stage larvae and adults in the digestive tract. Only third stage larvae of this species are found in the body cavity of fish.

### 2.2.4 Analysis of Data

Results from the same fish species, recovered from different areas were grouped together for simplicity in analysing the data.

After each fish had been examined, and the nematodes

recovered from each area had been counted and identified, numbers of each nematode species recovered from the different regions were marked on an individual sheet, corresponding to a single fish, along with the data on the fish itself, date of capture and locality of capture.

The abundance of larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and larvae and adults of **H.aduncum** was determined in the viscera and flesh of the fish species collected, allowing prevalences and intensities of infection of each parasite to be calculated for each fish species, and also the proportions of the four nematode species present in the fish species, and within individual organs or tissues of the fish. Where possible, figures were converted into percentages to allow comparisons to be made between fish species, thus avoiding variability in the numbers of nematodes recovered from different fish species.

The relationship between numbers of **A.simplex** and **P.decipiens** in the body cavity and musculature of the fish was assessed. Frequency distributions of the nematodes in each species of fish were calculated, and a preliminary investigation of possible interactions between the four nematode species was made.

Correlation co-efficients were calculated using a statistical computer package (Lotus 1-2-3, Release 4 for Windows), and probabilities determined from a statistical table given in Parker (1986).

### 2.2.5 Terminology

The terminology used to describe nematode infections in this study is that of Margolis **et al.** (1982). Thus:

Prevalence (%) - Number of fish infected with particular parasite\s divided by number of fish examined (= % of fish infected with particular parasite\s).

Mean Intensity - Total number of parasites in sample of fish examined divided by number of infected fish within the sample (= mean number of parasites per infected fish in a sample).

## 2.3 RESULTS

### 2.3.1 Epidemiological Data

The prevalences, mean intensities and range of infection of individual and total nematode species recovered from the five fish species examined are shown in Table 3. All cod, haddock , blue whiting and bull rout examined were infected with nematodes, however, only 64.3% of long rough dab harboured nematode infections. In addition, all four species of nematodes were recovered from cod, haddock, blue whiting and bull rout, however, **A.simplex** was the only nematode species to be recovered from long rough dab.

All individuals of cod and blue whiting were infected with **A.simplex**, with blue whiting having the highest mean intensity (27.3). Prevalence and mean intensity of **A.simplex** was lowest in long rough dab. The highest infections of **P.decipiens** and **C.osculatum** were observed in cod and bull rout. Haddock was the least infected species, with respect to infections of **P.decipiens** and **C.osculatum**. 100% of cod and 95.4% of blue whiting were infected with **H.aduncum** (mean intensities = 14.8 and 22.7 respectively). In terms of infection by all four species of nematode, blue whiting had the highest mean intensity, followed by cod, bull rout, haddock and long rough dab.

Table 4 shows the infection levels by **A.simplex** and **P.decipiens** infections in the body cavity and musculature (nematodes recovered from the alimentary tract were not included in this analysis), and infection levels of **H.aduncum** third stage larvae, in the body cavity, and fourth stage larvae and adults in the digestive tract. While some of the worms of this latter species recovered from the alimentary

TABLE 3: INFECTION LEVELS OF NEMATODES IN WHOLE FISH

| | | A.simplex | P.decipiens | C.osculatum | H.aduncum | Totals |
|---|---|---|---|---|---|---|
| Cod | Prevalence(%) | 100 | 85.7 | 90.5 | 100 | 100 |
| | Intensity | 15.9 | 5 | 7.2 | 14.8 | 41.6 |
| | Range | 3-67 | 0-23 | 0-47 | 1-74 | 6-136 |
| Haddock | Prevalence(%) | 77.8 | 33.3 | 81.5 | 77.8 | 100 |
| | Intensity | 4.9 | 1.2 | 2.4 | 6.4 | 10.9 |
| | Range | 0-18 | 0-3 | 0-6 | 0-52 | 2-55 |
| Blue Whiting | Prevalence(%) | 100 | 54.5 | 86.4 | 95.4 | 100 |
| | Intensity | 27.3 | 2 | 6.2 | 22.7 | 55.4 |
| | Range | 1-75 | 0-5 | 0-18 | 0-89 | 3-105 |
| Bull rout | Prevalence(%) | 96.4 | 78.6 | 96.4 | 78.6 | 100 |
| | Intensity | 11.9 | 9 | 12.1 | 10.6 | 39.2 |
| | Range | 0-52 | 0-25 | 0-49 | 0-57 | 2-143 |
| Long Rough Dab | Prevalence(%) | 64.3 | - | - | - | 64.3 |
| | Intensity | 3.8 | - | - | - | 3.8 |
| | Range | 0-14 | - | - | - | 0-14 |

TABLE 4: INFECTION LEVELS OF **A.simplex, P.decipiens, C.osculatum & H.aduncum** IN DIFFERENT REGIONS OF THE FISH

| | | **A.simplex** Body Cavity | **A.simplex** Muscle | **P.decipiens** Body Cavity | **P.decipiens** Muscle | **H.aduncum (L3)** Body Cavity | **H.adumcum (L4)** Alim. tract |
|---|---|---|---|---|---|---|---|
| Cod | Prevalence(%) | 100 | 40.5 | 57.1 | 71.4 | 78.6 | 85.7 |
| | Intensity | 12.3 | 3 | 2.9 | 3.3 | 6.6 | 11.1 |
| | Range | 2-63 | 0-8 | 0-12 | 0-22 | 0-38 | 0-43 |
| Haddock | Prevalence(%) | 77.8 | 25.9 | 25.9 | 7.4 | 63 | 48 |
| | Intensity | 4.1 | 1.6 | 1.1 | 1 | 1.7 | 8 |
| | Range | 0-17 | 0-3 | 0-2 | 0-1 | 0-4 | 0-52 |
| Blue Whiting | Prevalence(%) | 100 | 72.7 | 55.6 | 4.5 | 95.4 | 45.4 |
| | Intensity | 23 | 4.4 | 1.8 | 1 | 21.3 | 2.9 |
| | Range | 1-68 | 0-13 | 0-4 | 0-1 | 0-89 | 0-9 |
| Bull rout | Prevalence(%) | 96.4 | 46.4 | 57.1 | 78.6 | 67.9 | 64 |
| | Intensity | 8.5 | 3.5 | 3.7 | 6.6 | 7.7 | 4.9 |
| | Range | 0-43 | 0-7 | 0-11 | 0-19 | 0-57 | 0-20 |
| Long Rough Dab | Prevalence(%) | 64.3 | - | - | - | - | - |
| | Intensity | 3.8 | - | - | - | - | - |
| | Range | 0-14 | - | - | - | - | - |

tract may be recently acquired third stage larvae recovered prior to penetration of the alimentary tract, initial examinations showed that these were very few and the great majority are fourth stage larvae and adults. No distinction was therefore made between different stages of this species.

All species of fish examined harboured infections of both **A.simplex** and **P.decipiens** in both the body cavity and musculature, with the exception of long rough dab, where infections of **A.simplex** only occurred in the body cavity. All individuals of blue whiting and cod contained **A.simplex** in the body cavity. Prevalence of this parasite in the body cavity was lowest in long rough dab. Highest intensities were observed in the body cavity of cod and the lowest in long rough dab. The musculature of blue whiting was by far the most frequently infected with **A.simplex**, followed by cod and bull rout. **A.simplex** infection was lowest in the musculature of haddock. Infections of **P.decipiens** were highest in the body cavity of cod and bull rout, and were very similar - the prevalence in both being 57.1% with mean intensities of 2.9 (cod) and 3.7 (bull rout). Over 50% of blue whiting were also infected with **P.decipiens** in the musculature. Infections were again lowest in haddock. Of **P.decipiens** in the musculature, prevalences were again highest in cod and bull rout, however, the mean intensity of infection in the musculature of bull rout was twice that observed in cod. Low infections of this species in the musculature were observed in haddock and blue whiting.

Infection with third stage **H.aduncum** was most common in blue whiting, followed by cod and bull rout. Infections were lowest in haddock. Fourth stage larvae and adult **H.aduncum** were most frequently found in cod and bull rout. Less than 50% of blue whiting and haddock harboured infections in the alimentary tract, however, the mean intensity of infection in haddock was 8.

Figures 3-7 show the percentages of worms recovered from both whole fish and individual regions within the fish (Figures 3-6). In terms of the whole fish, in cod and haddock

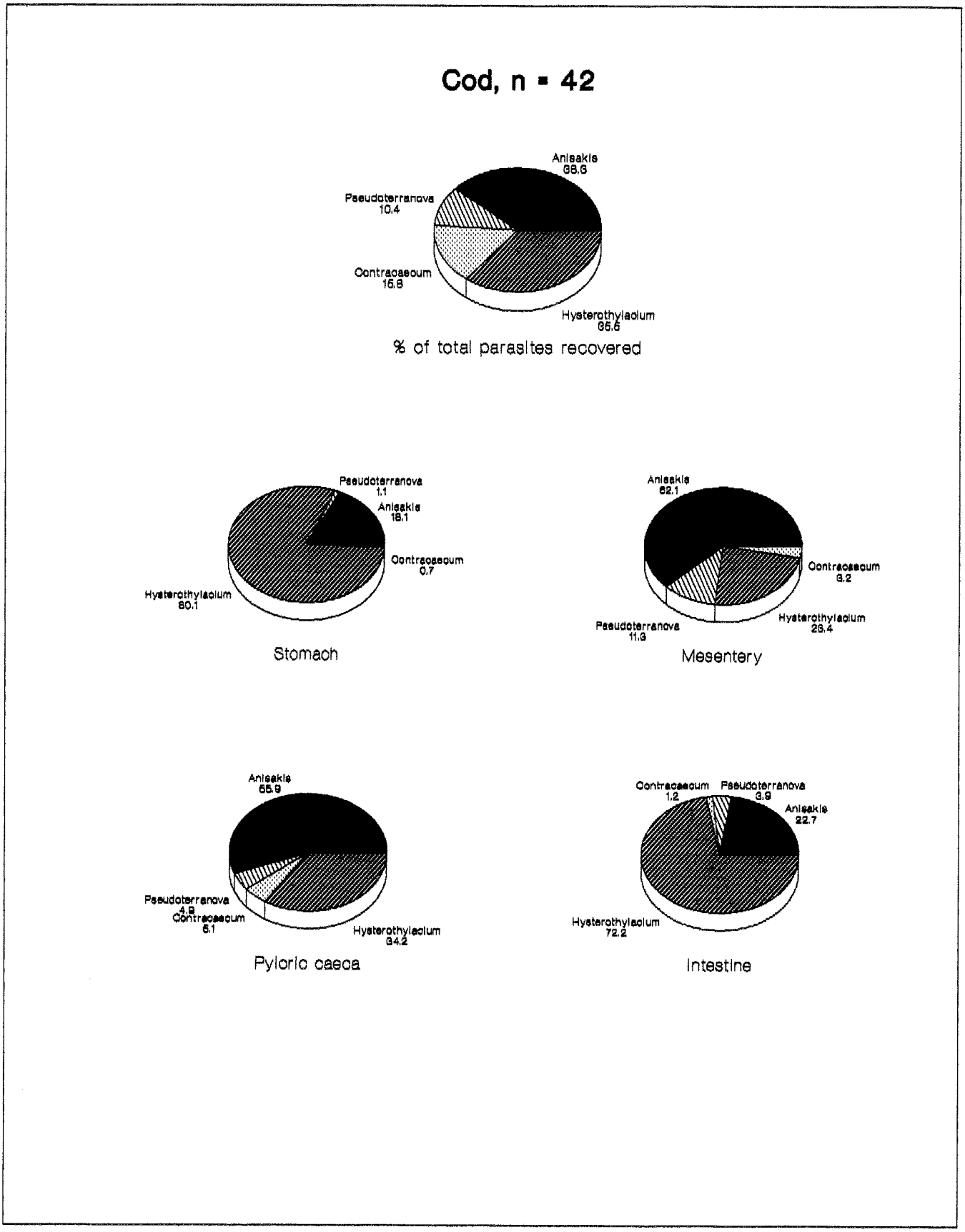
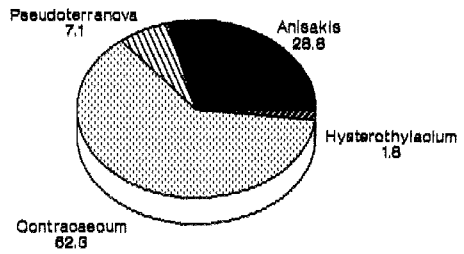
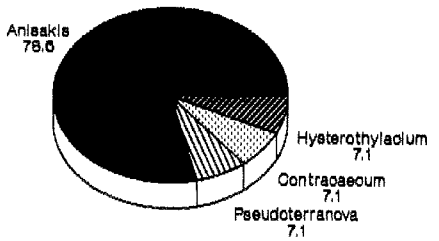



FIGURE 3: Percentage of nematodes recovered from whole, and individual organs and tissues of, cod




Liver

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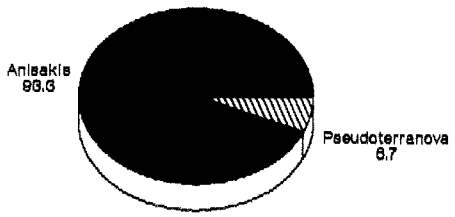


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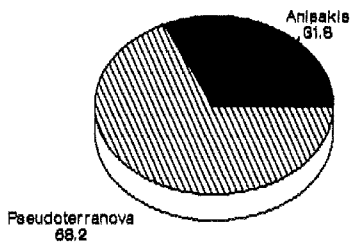


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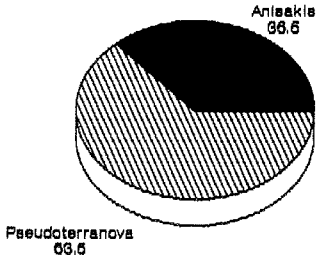


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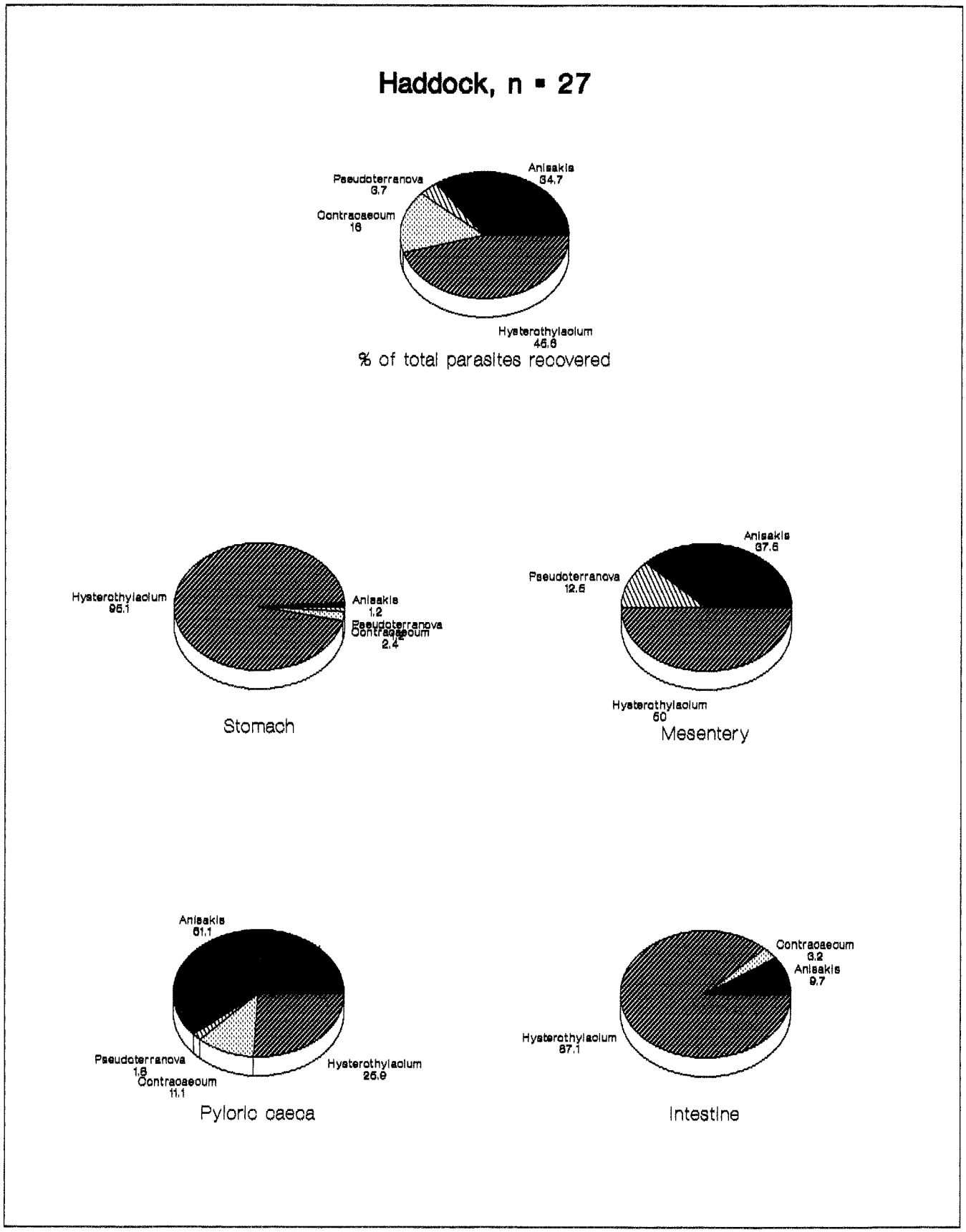
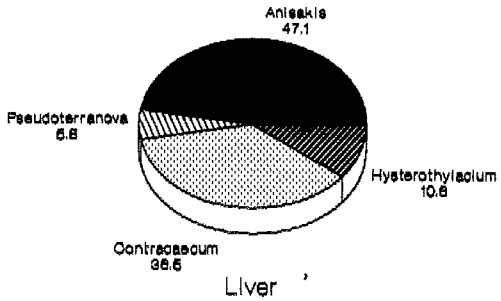
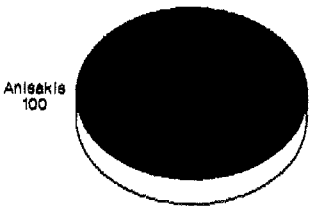



**FIGURE 4: Percentage of nematodes recovered from whole, and individual organs and tissues of, haddock**




Liver

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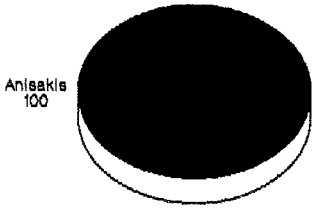


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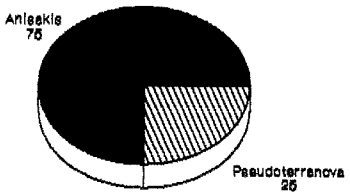


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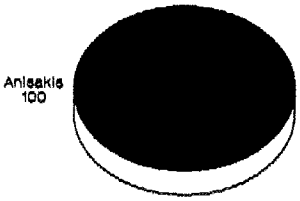


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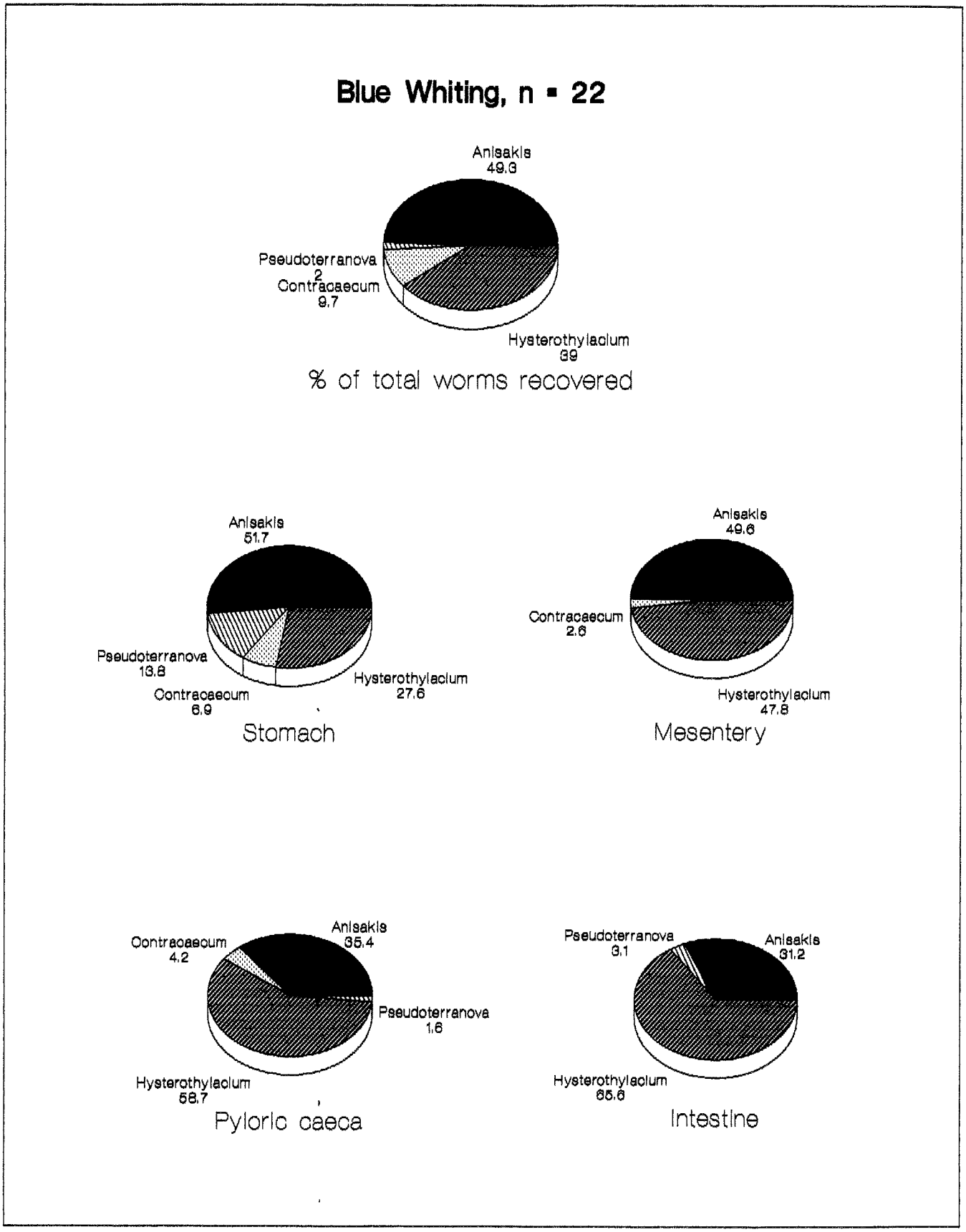
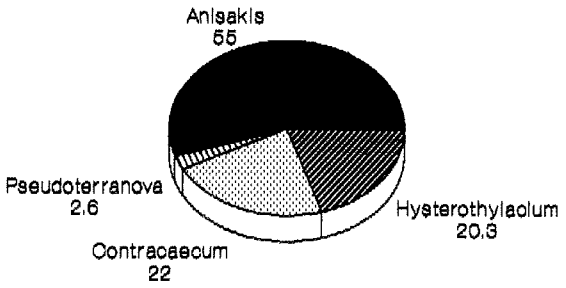



**FIGURE 5: Percentage of nematodes recovered from whole, and individual organs and tissues of, blue whiting**

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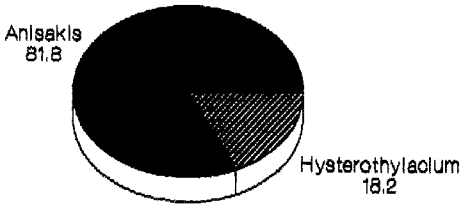


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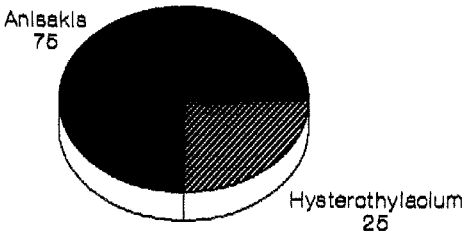


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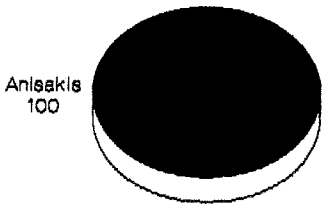


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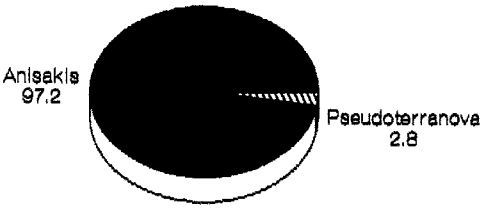


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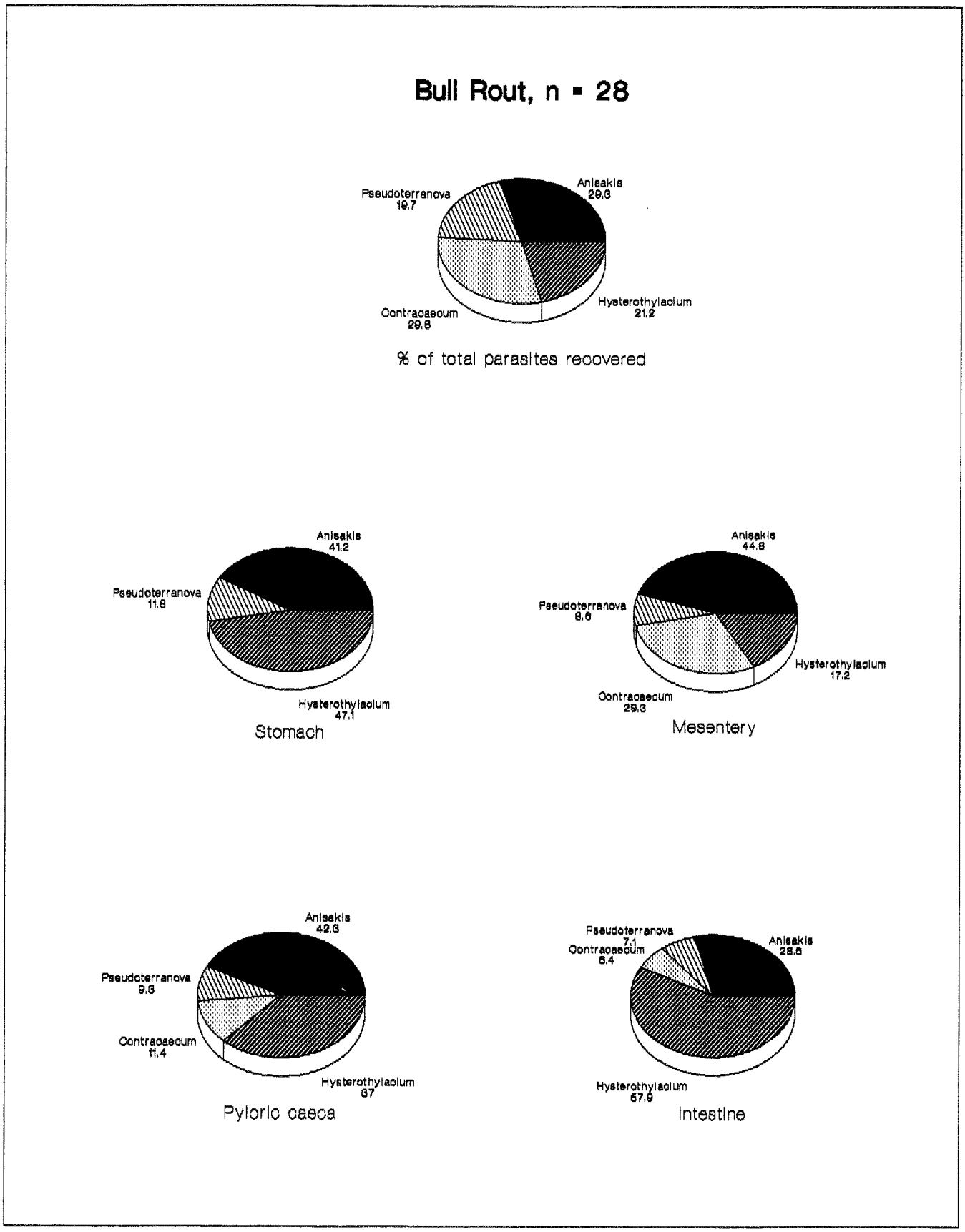
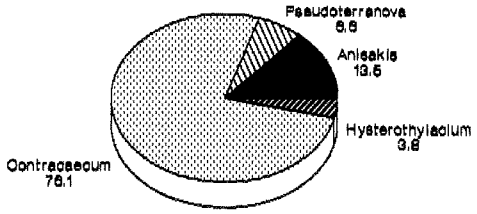
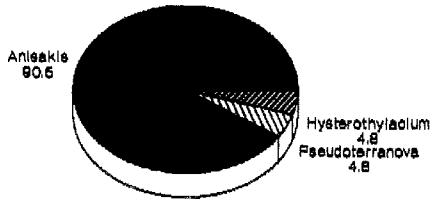


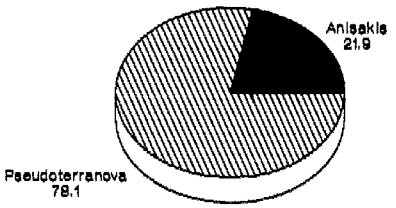

**FIGURE 6: Percentage of nematodes recovered from whole, and individual organs and tissues of, bull rout**




Liver




Gonads

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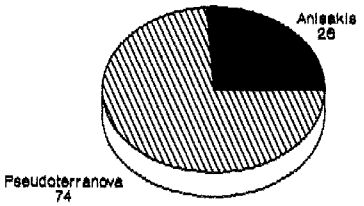


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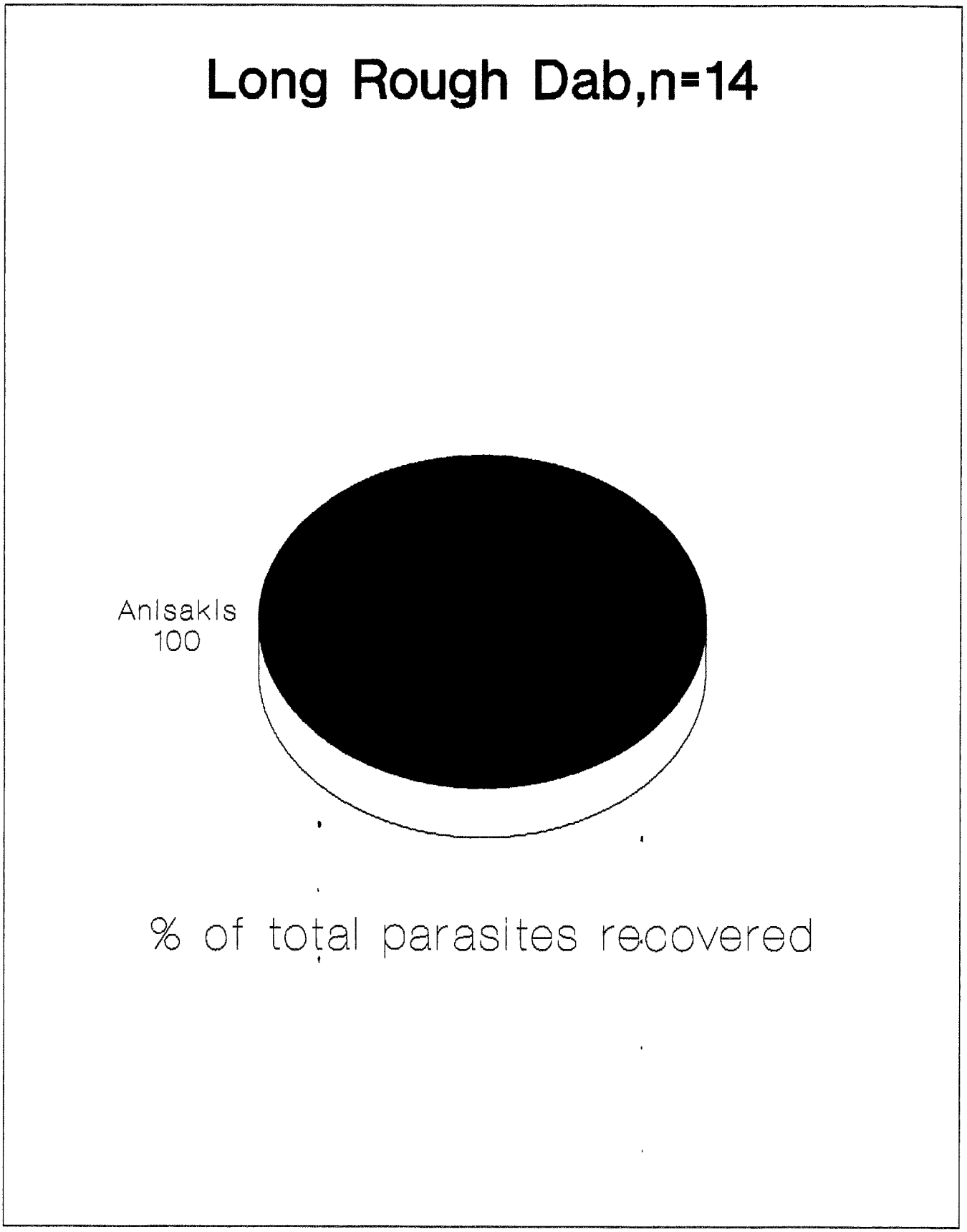


**FIGURE 7: Percentage of nematodes recovered from whole long rough dab**

(Figures 3 and 4), the proportions of worms recovered were similar. Almost 50% of the nematodes recovered from blue whiting (Figure 5) were **A.simplex**, with **P.decipiens** and **C.osculatum** accounting for approximately 12%. Proportions of the four species in bull rout (Figure 6) were fairly similar, with **P.decipiens** and **C.osculatum** accounting for almost half of the worms recovered. All of the nematodes recovered from long rough dab (Figure 7) were **A.simplex**. However, if the percentages of third stage larvae only are calculated (ie. **H.aduncum** from the digestive tract excluded), these proportions alter (Table 5); **A.simplex** in cod and haddock now accounting for half of the nematodes recovered, **P.decipiens** and **C.osculatum** for over 30%. the proportion of **H.aduncum** in bull rout is also decreased from 21% to 14%. Figures in blue whiting remain similar, indicating that most of the **H.aduncum** in this species are third stage larvae.

The relative proportions of **H.aduncum** third stage larvae, and fourth stage larvae and adults in fish are shown in Table 6. The majority of **H.aduncum** in haddock (78.4%) were fourth stage larvae and adults, while cod also harboured higher numbers of fourth stage larvae and adults than third stage specimens. In blue whiting, almost 94% of the **H.aduncum** recovered were third stage larvae, while bull rout were also infected with a greater proportion of third stage larvae than fourth stage and adults.

### 2.3.2 Frequency Distributions

Figures 8-12 show the frequency distribution of the total numbers of nematodes, and numbers of individual nematode species recovered from cod (Figure 8), haddock (Figure 9), blue whiting (Figure 10), bull rout (Figure 11) and long rough dab (Figure 12). It should be noted that the categories used in the figures were selected for the purpose of illustration only. The choice of categories did not influence the dispersal pattern.

TABLE 5: PERCENTAGES OF THIRD STAGE ASCARIDOID LARVAE RECOVERED FROM FISH

| | A.simplex | P.decipiens | C.osculatum | H.aduncum | Total |
|---|---|---|---|---|---|
| Cod | 50 | 13.5 | 20.5 | 16 | 100 |
| Haddock | 54 | 6 | 25 | 15 | 100 |
| Blue Whiting | 50.5 | 2 | 10 | 37.5 | 100 |
| Bull Rout | 32 | 21.5 | 32 | 14.5 | 100 |
| Long Rough Dab | 100 | 0 | 0 | 0 | 100 |

TABLE 6: PERCENTAGE OF H.aduncum THIRD STAGE LARVAE, AND FOURTH STAGE LARVAE & ADULTS

| | % L4 & adults | % L3 |
|---|---|---|
| Cod | 64.7 | 35.3 |
| Haddock | 78.4 | 21.6 |
| Blue Whiting | 6.1 | 93.9 |
| Bull Rout | 38.2 | 61.8 |
| Long Rough Dab | - | - |

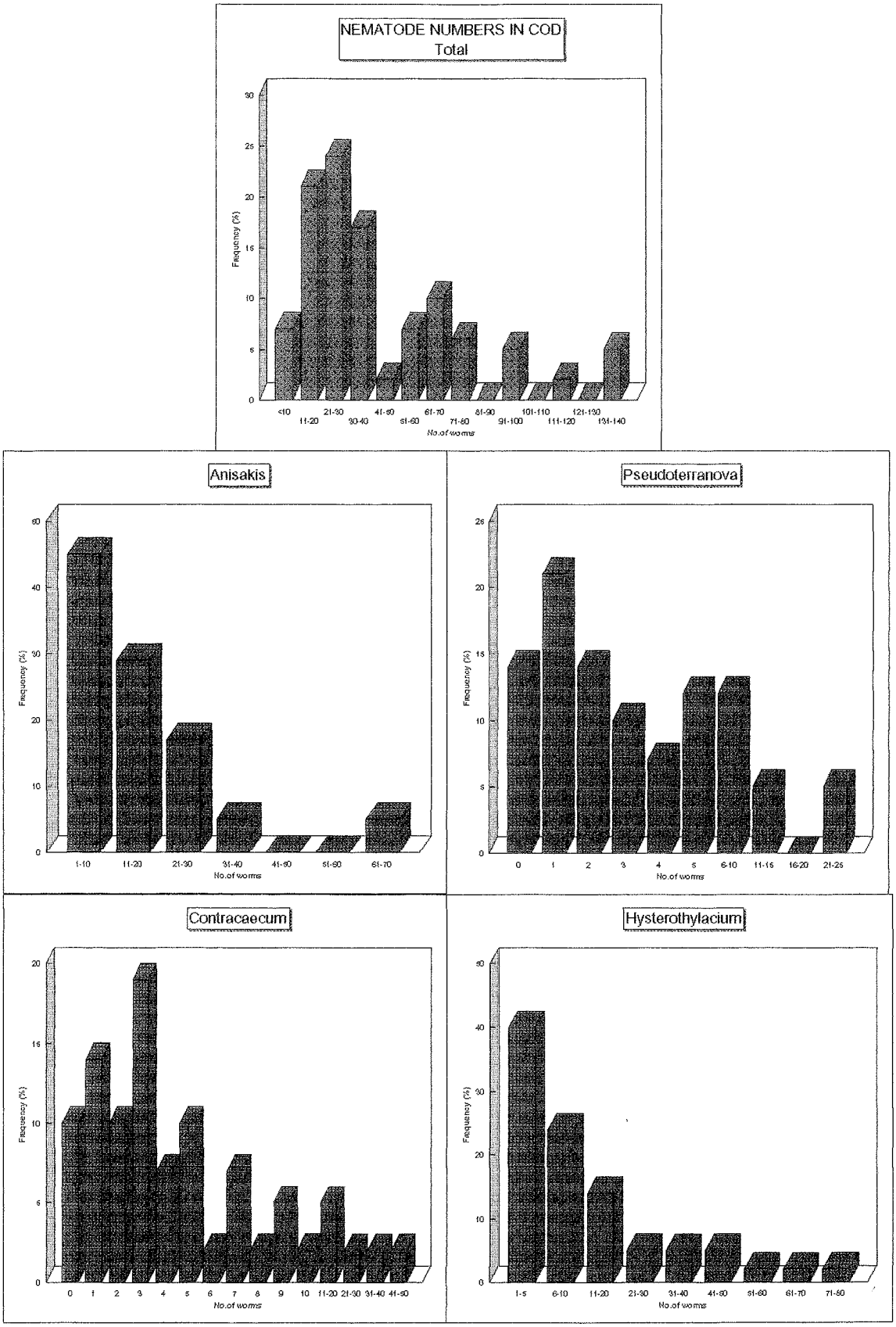


**FIGURE 8: Frequency distributions of nematodes in cod**

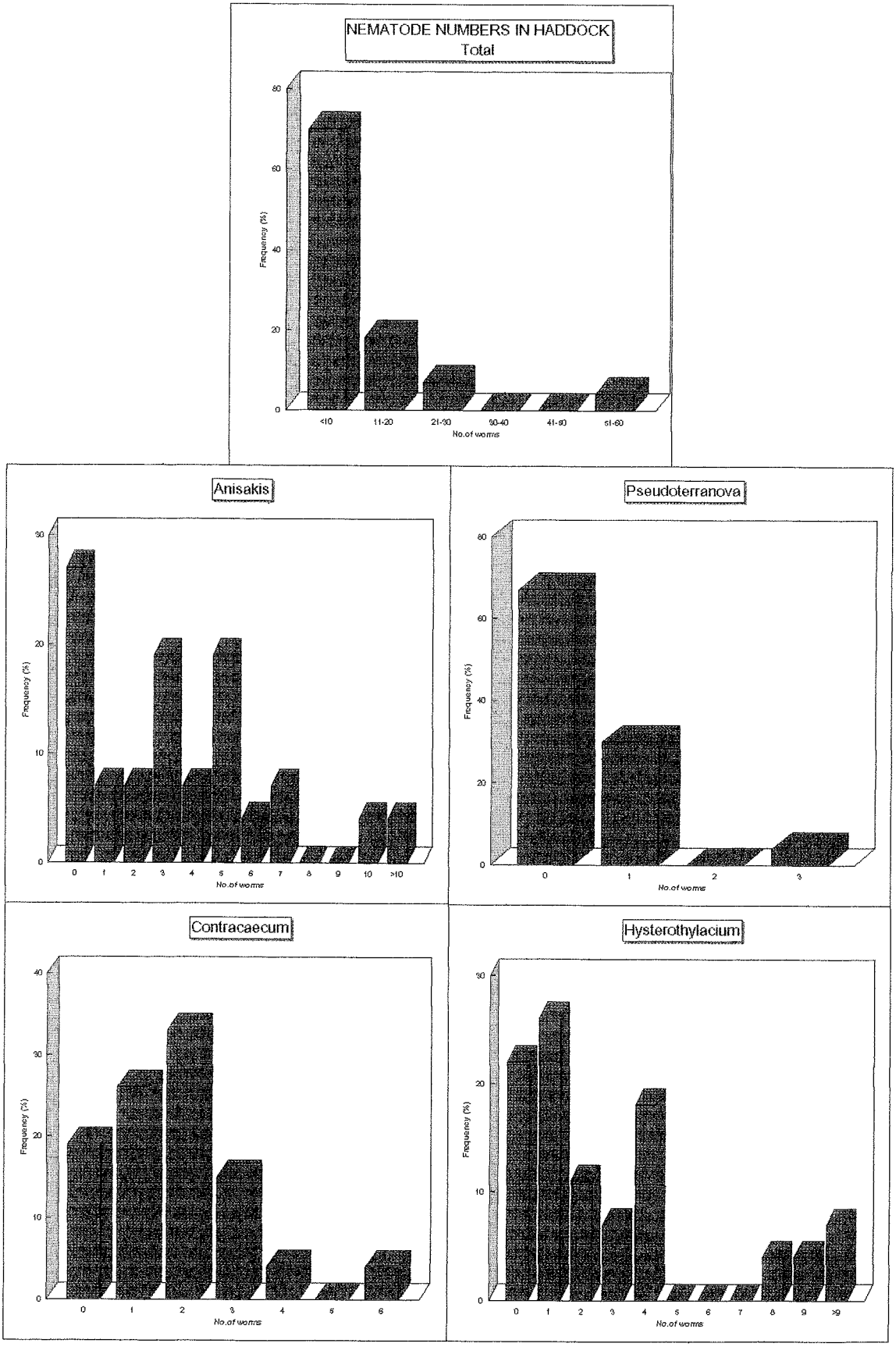


**FIGURE 9: Frequency distributions of nematodes in haddock**

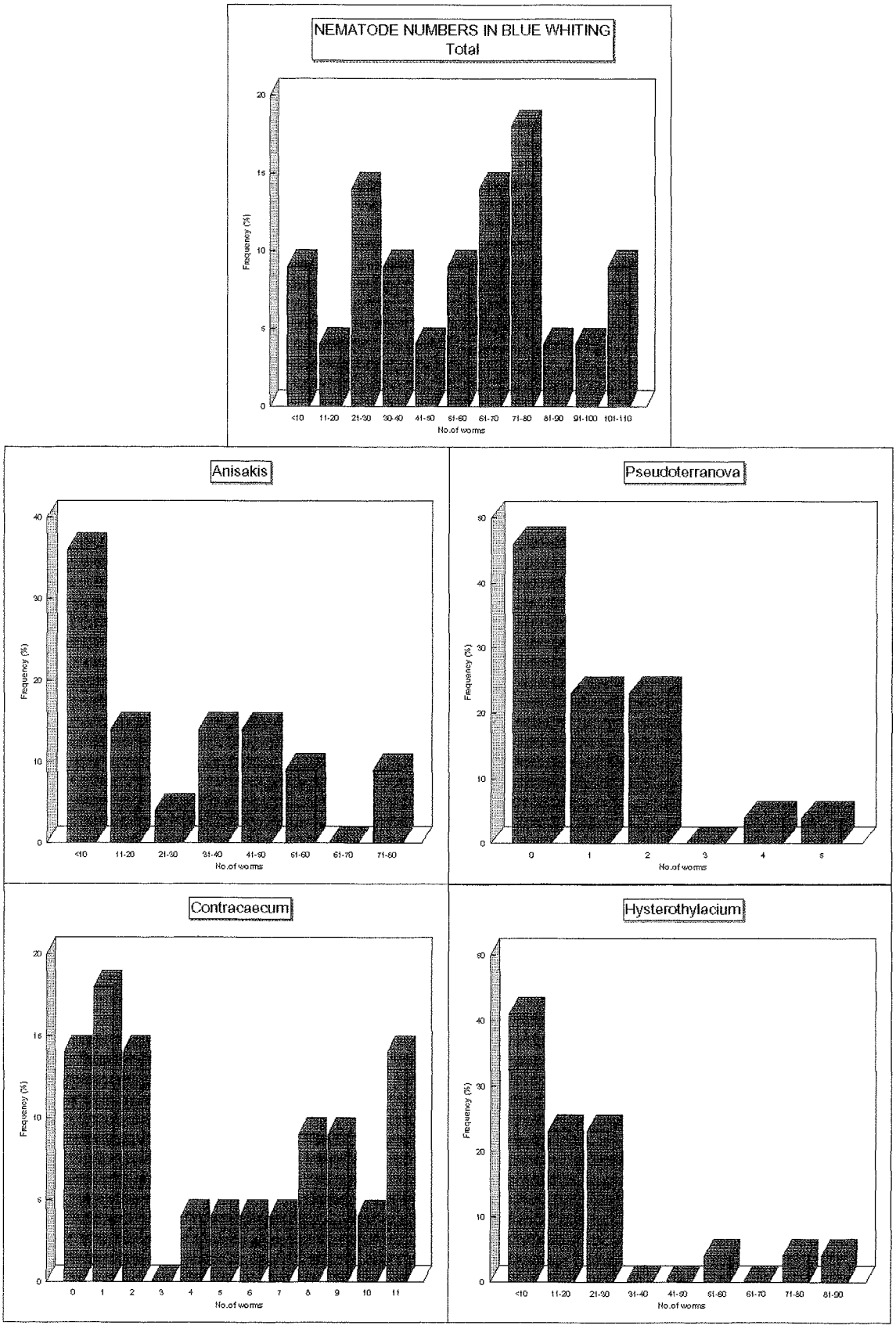


**FIGURE 10: Frequency distributions of nematodes in blue whiting**

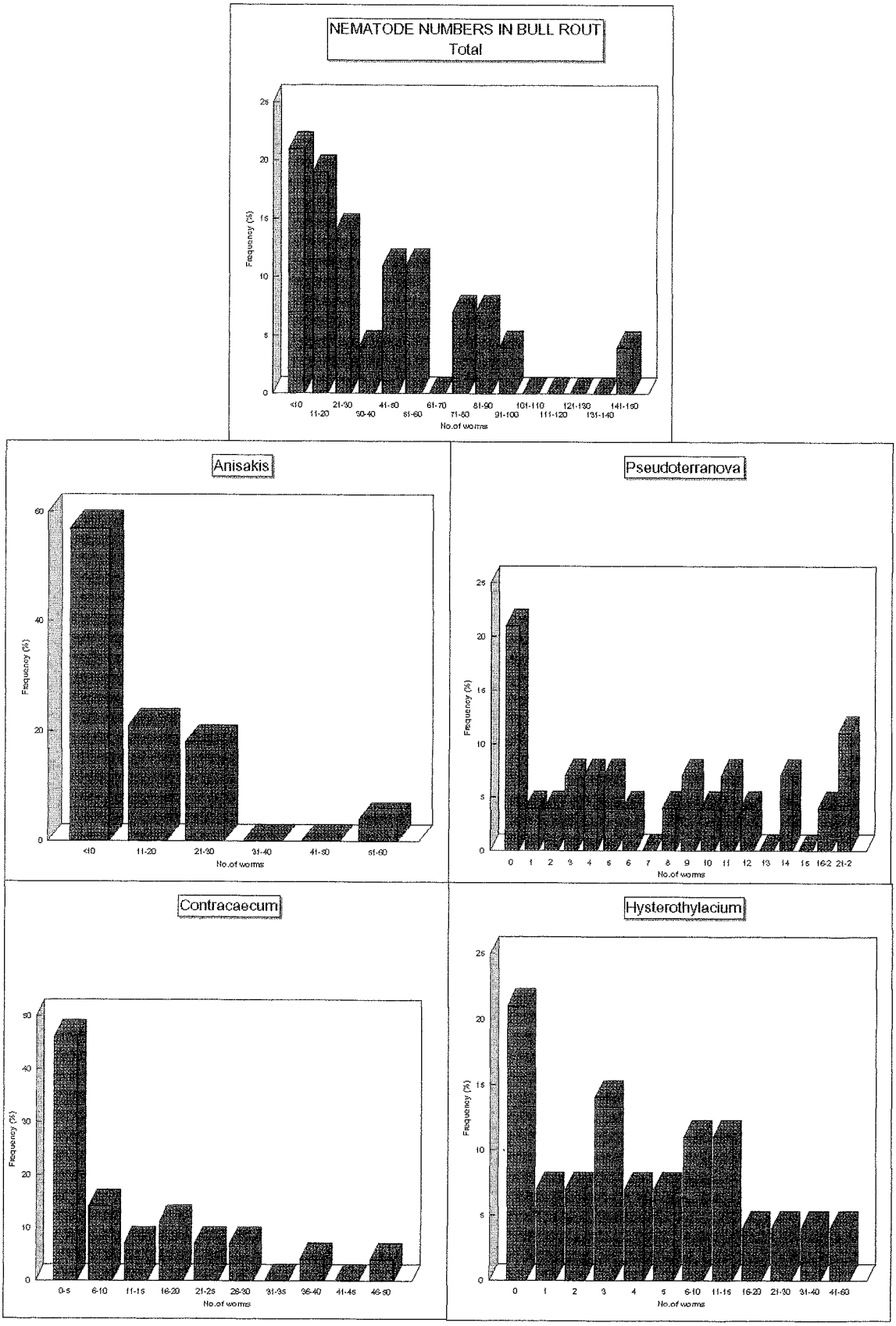


**FIGURE 11: Frequency distributions of nematodes in bull rout**

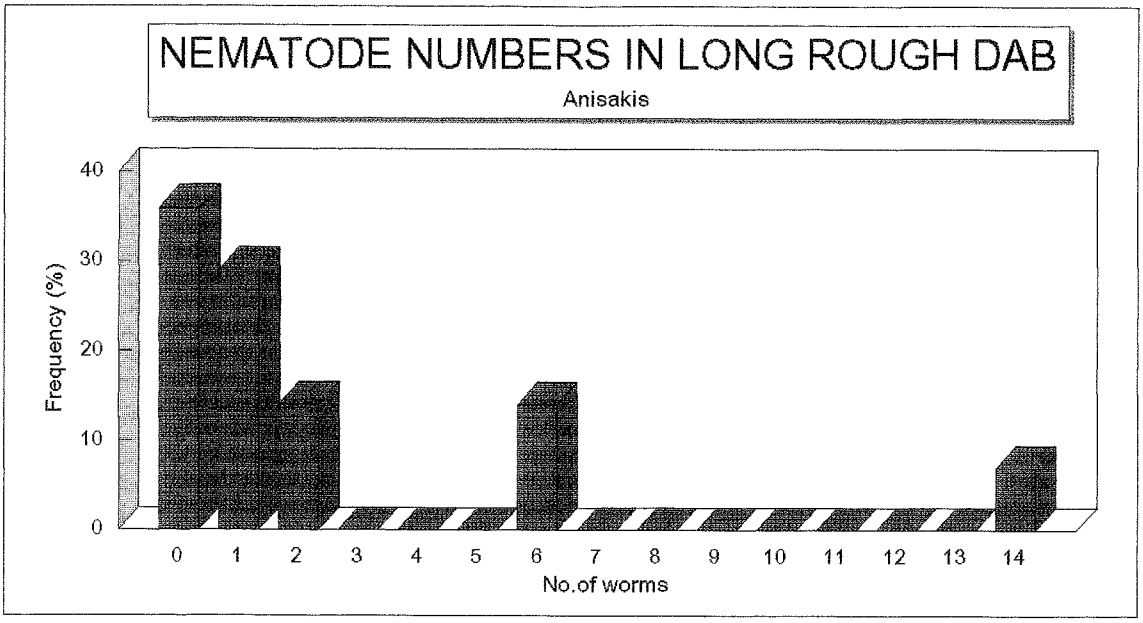


FIGURE 12: Frequency distribution of A.simplex in long rough dab

In the majority of cases, for both total numbers of nematodes, and numbers of individual parasite species, the frequency distribution was skewed, whereby most fish were infected with a low number of worms; the frequency showed a general decrease with increasing numbers of worms so that only a few fish were infected with large numbers of worms. This trend was also seen in long rough dab, even though only 14 fish had been examined for nematodes. The only exceptions to this distribution were the total numbers of nematodes, and numbers of **C.osculatum** in blue whiting (Figure 10), where the frequency of total nematodes in fish was irregularly distributed eg only 9% of fish examined were infected with less than ten worms, but 18% with 71-80 worms. Although most blue whiting were infected with either 0,1 or 2 **C.osculatum**, infections with 8,9 or 11 parasites of this species were also common.

### 2.3.3 Distribution Of Nematodes Within Fish

Figures 3-6 show the percentages of worms recovered from the nine individual regions examined within cod (Figure 3), haddock (Figure 4), blue whiting (Figure 5) and bull rout (Figure 6). **H.aduncum** was observed to be the most common nematode recovered from the stomach of cod, haddock and bull rout, although 41% of the nematodes recovered from the stomachs of bull rout were **A.simplex**. Over 50% of the nematodes in the stomachs of blue whiting were **A.simplex**, with **H.aduncum** accounting for almost 28%. The majority of the nematodes recovered from the mesenteries of all four fish species were **H.aduncum** and **A.simplex**, although almost 28% of nematodes in the mesentery of bull rout were **P.decipiens** and **C.osculatum**. The majority of nematodes in the pyloric caeca of all four fish species were **A.simplex** and **H.aduncum**. **A.simplex** was the most common nematode in the pyloric caeca of cod, haddock and bull rout, followed by **H.aduncum**. The

proportions of nematodes in the pyloric caeca of cod and haddock were similar; **A.simplex** accounted for 56% (cod) and 61% (haddock) of the nematodes recovered, **P.decipiens** and **C.osculatum** accounting for 10% (cod) and 13% (haddock). **A.simplex** and **H.aduncum** comprised 42% and 37% respectively of the nematodes in the pyloric caeca of bull rout. In blue whiting, almost 60% of the nematodes in the pyloric caeca were **H.aduncum**, followed by **A.simplex** (35%). **A.simplex** and **H.aduncum** were also the most common species recovered from the intestine, in all fish species examined. **H.aduncum** was the most common, varying from 58% of the total nematodes in bull rout intestine to 87% in haddock. The percentages of **P.decipiens** and **C.osculatum** recovered from the intestine were generally low, accounting for 3% of the nematodes recovered in blue whiting (**P.decipiens** only) and haddock (**C.osculatum** only) to 5% in cod. However 13.5% of the nematodes observed in the intestine of bull rout were **P.decipiens** and **C.osculatum**.

The proportions of nematodes recovered from the liver of cod and bull rout were similar. **C.osculatum** comprised 62% and 76% of the nematodes recovered from the livers of cod and bull rout respectively. **P.decipiens** and **H.aduncum** accounted for only 9% and 10% of the nematodes recovered from the liver in cod and bull rout respectively. Similarly, the percentages of nematodes recovered from the liver of blue whiting and haddock were similar, comprising mainly **A.simplex** (55% in blue whiting, 47% in haddock), followed by **C.osculatum** (22% in blue whiting, 36.5% in haddock) and **H.aduncum** (20% in blue whiting, 11% in haddock) - the remainder being **P.decipiens**. The majority of nematodes found on the gonads were **A.simplex** varying from 79% in cod to 100% in haddock. A similar distribution was observed in the kidney where **A.simplex** varied from comprising 75% of the nematodes found in blue whiting kidney to 100%, again in haddock. No nematodes were observed on the kidney of bull rout.

**A.simplex** and **P.decipiens** were the only nematodes to be recovered from the musculature. The proportions of **A.simplex**

and **P.decipiens** in the left and right fillets varied between species. **A.simplex** was found to be the most common nematode in both left and right fillets of blue whiting and haddock, and were the only nematodes recovered from the left fillet of blue whiting, and the right fillet of haddock. **P.decipiens** comprised 25% of the nematodes in the left fillet of haddock, and only 3% of the nematodes in the right fillet of blue whiting. In cod and bull rout, **P.decipiens** were the most commonly recovered nematodes from the fillets, percentages of **A.simplex** varying from comprising 22% of the nematodes in the left fillet of bull rout to 36.5% of the nematodes in the right fillet of cod.

Table 7 shows the relative proportions of **A.simplex** and **P.decipiens** in the musculature (left and right fillets combined) of the fish examined. Both blue whiting and haddock harbour a significantly larger proportion of **A.simplex** in the musculature than **P.decipiens**. 66.2% of the nematodes in cod musculature were **A.simplex** and the muscles of bull rout were most commonly infected with **P.decipiens**.

Figures 13-17 show the percentage distribution of individual nematode species throughout the nine regions examined, in each fish species. In cod, haddock and blue whiting (Figures 13-15), **A.simplex** were recovered from all nine regions examined. **A.simplex** were absent from the kidney of bull rout (Figure 16), and were found only in the stomach, mesentery, intestine and liver of long rough dab (Figure 17). With the exception of long rough dab, most **A.simplex** larvae in the fish examined were found in the pyloric caeca or liver. In cod and bull rout, **A.simplex** occurred most commonly in the pyloric caeca (44.6% and 42.6% respectively), followed by the liver (16.5% and 14.6% respectively). However, if the stomach and intestine are combined, 16% and 14.6% of **A.simplex** are seen to occur in the alimentary tract of cod and bull rout respectively. In haddock and blue whiting, **A.simplex** were most commonly recovered from the liver (48% and 38.3% respectively), with 32.4% (haddock) and 25.1% (blue whiting) being found on the pyloric caeca. 7.6% and 10.8% of

TABLE 7: RELATIVE PROPORTIONS OF A.simplex & P.decipiens IN THE MUSCULATURE OF FISH (%)

| | A.simplex | P.decipiens |
|---|---|---|
| Cod | 66.2 | 33.8 |
| Haddock | 84.6 | 15.4 |
| Blue Whiting | 98.6 | 1.4 |
| Bull Rout | 24 | 76 |
| Long Rough Dab | 0 | 0 |

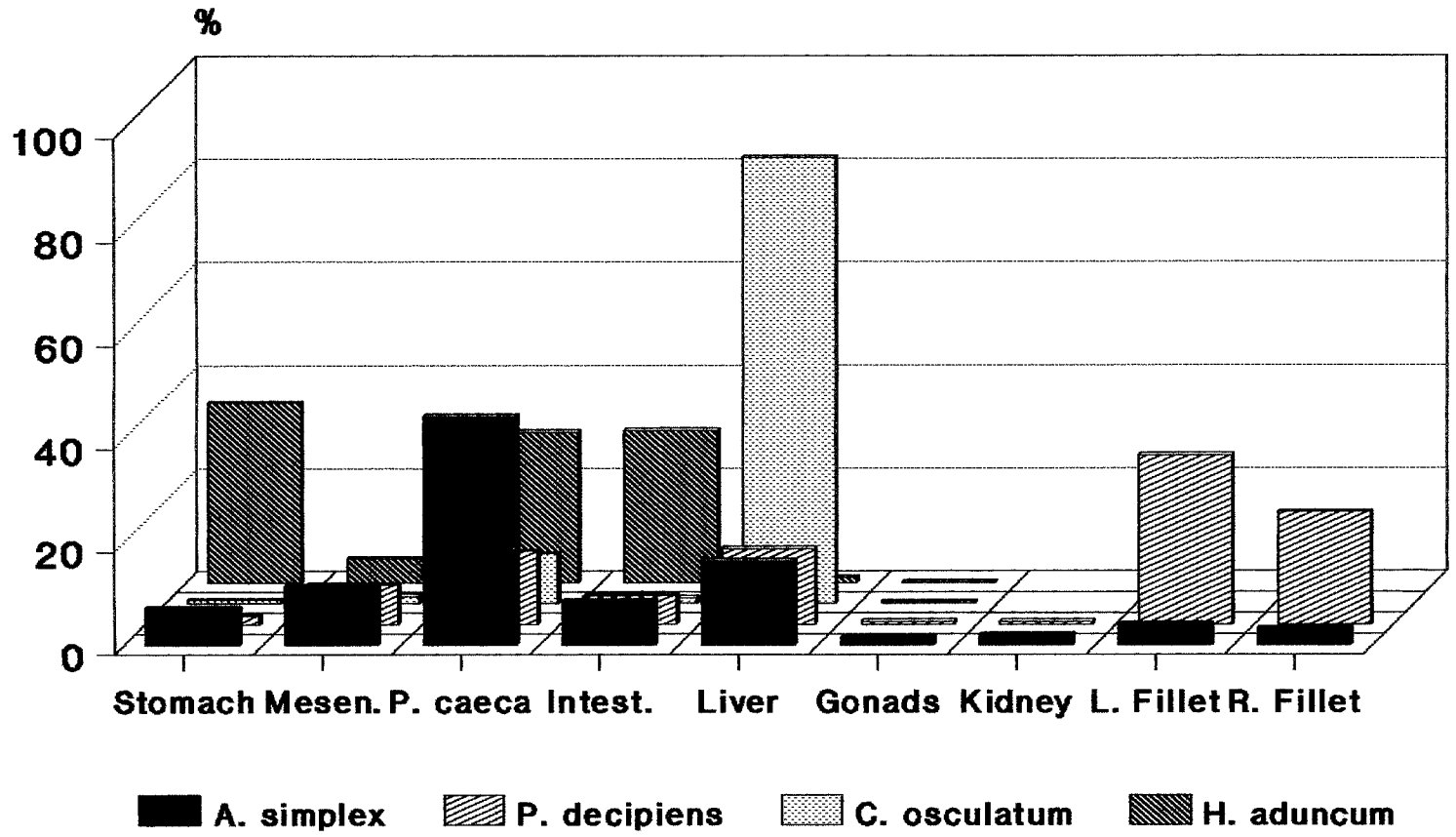


**FIGURE 13: Percentage distribution of individual nematode species in nine organs and tissues of cod**

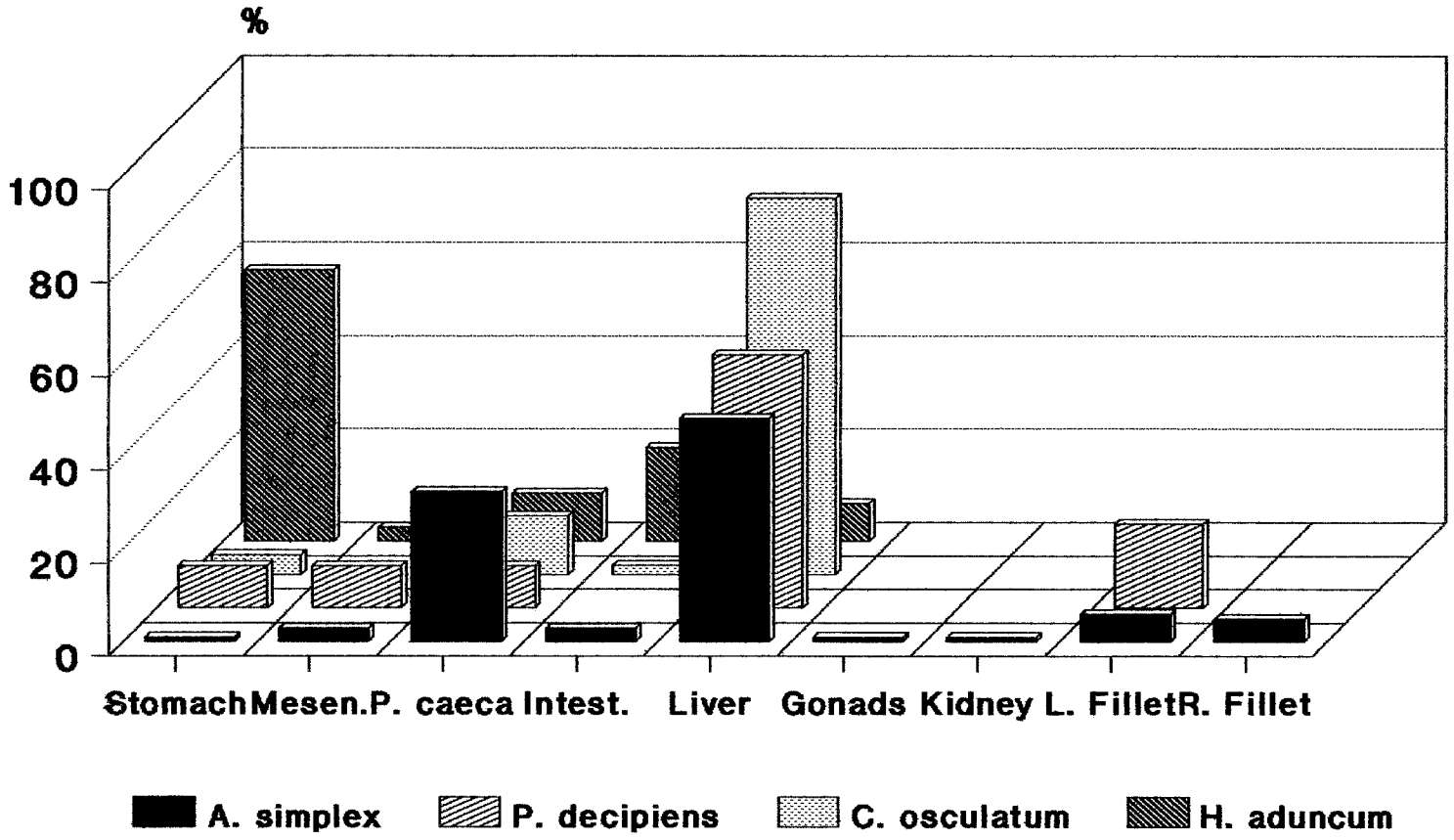


**FIGURE 14: Percentage distribution of individual nematode species in nine organs and tissues of haddock**

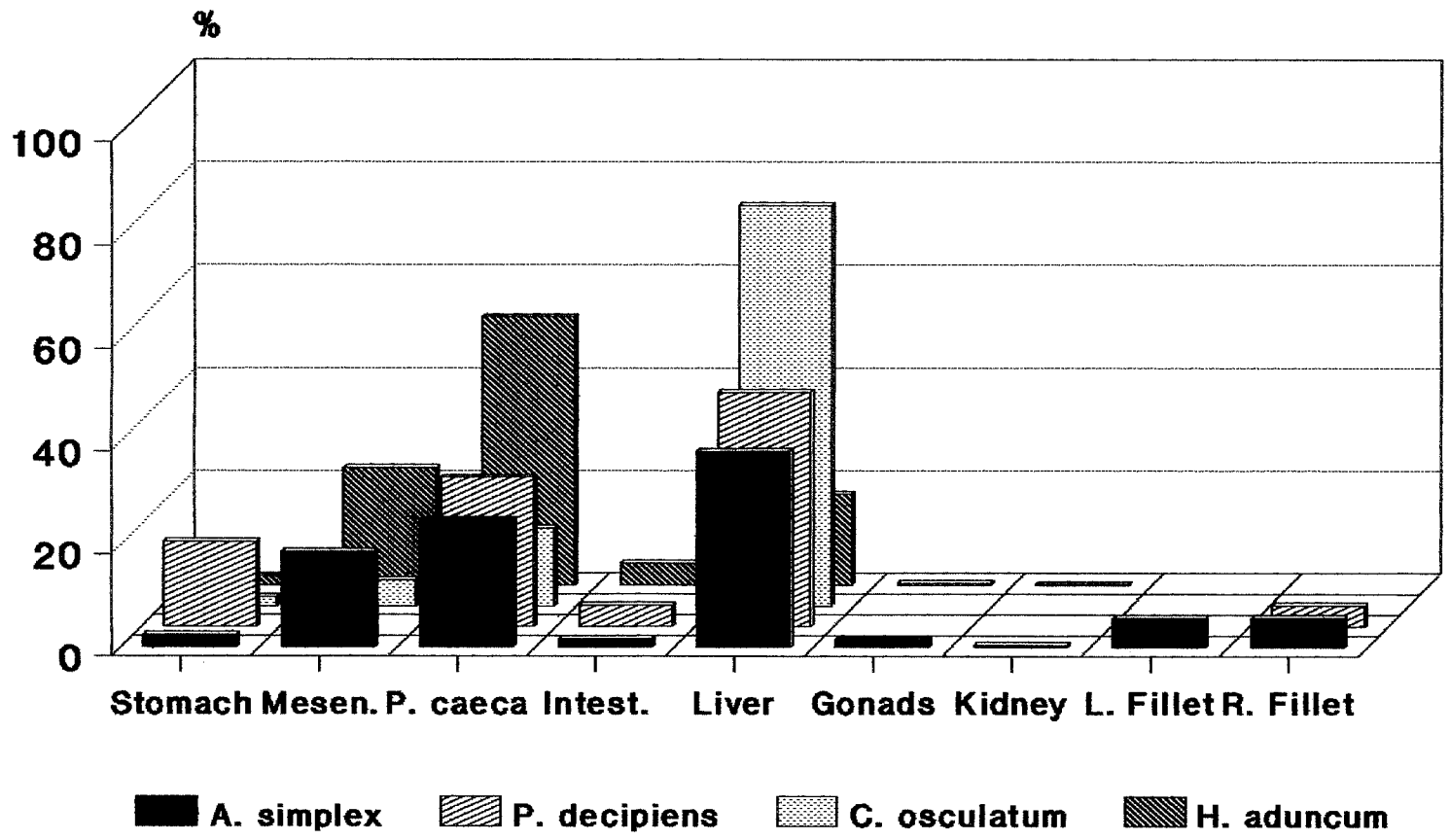


**FIGURE 15: Percentage distribution of individual nematode species in nine organs and tissues of blue whiting**

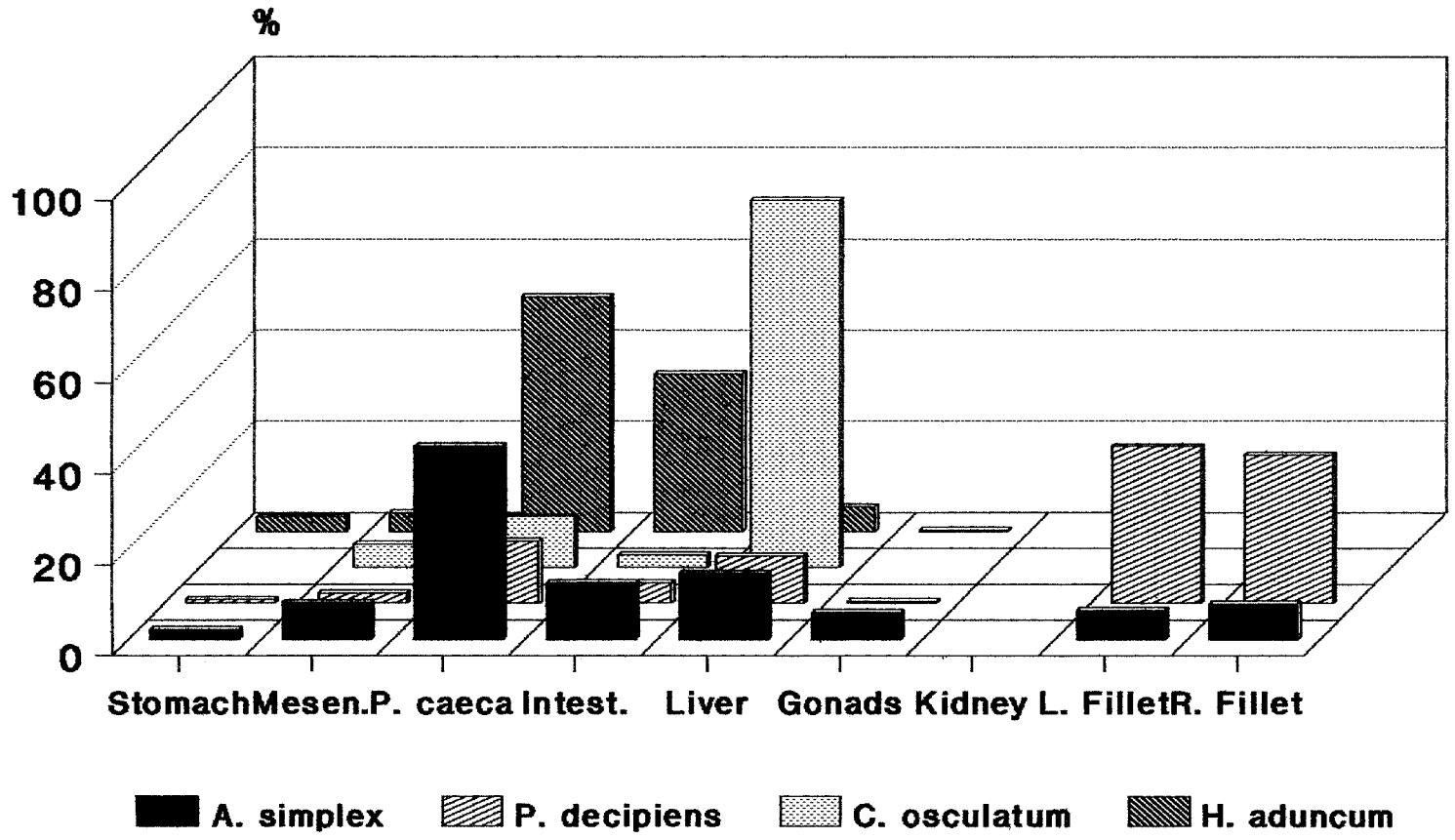


**FIGURE 16: Percentage distribution of individual nematode species in nine organs and tissues of bull rout**

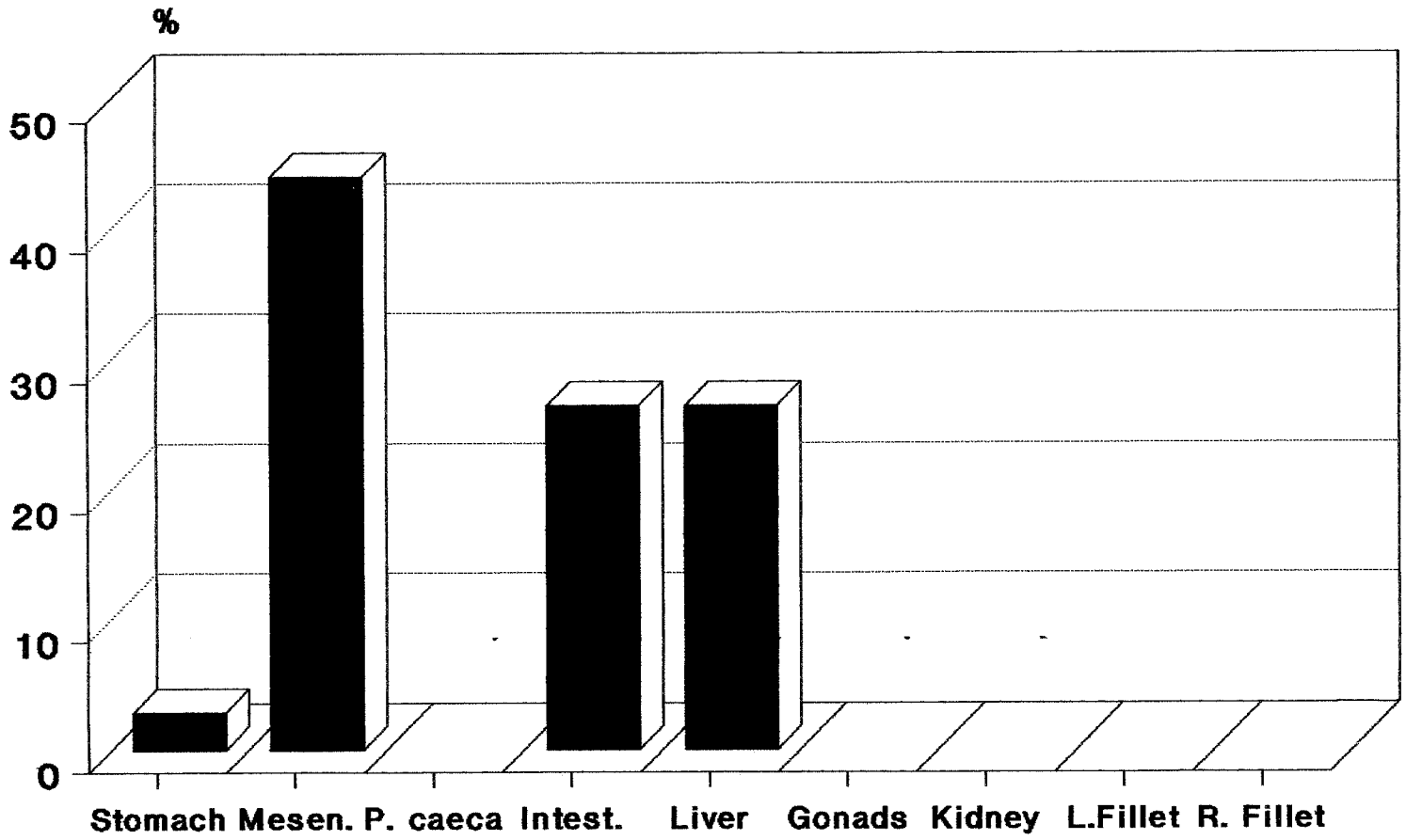


**FIGURE 17: Percentage distribution of A.SIMPLEX in nine organs and tissues of long rough dab**

**A.simplex** were recovered from the musculature of cod and haddock respectively, in both cases with slightly more being found in the left fillet than the right (4.2% left, 3.4% right in cod; 5.9% left, 4.9% right in haddock). 12% of **A.simplex** occurred in the musculature of blue whiting - with 6% in both the left and right fillets. In bull rout, 14.3% of **A.simplex** were retrieved from the musculature, with slightly more in the right fillet (7.8% as compared to 6.5% in the left). 44% of **A.simplex** were recovered from the mesentery of long rough dab, followed by the intestine and liver (both 26.5%), then the stomach.

Only in cod were all nine regions of the fish infected with **P.decipiens** (Figure 13), although in bull rout, this species was only absent from the kidney (Figure 16). Both cod and bull rout harboured the majority of their **P.decipiens** infections in the musculature, with 55% of **P.decipiens** in cod, and 67.3% in bull rout occurring in this region. Infections were slightly heavier in the left fillet (33% left, 22% right in cod; 34.6% left, 32.7% right in bull rout). After the musculature, **P.decipiens** was next commonly found in the liver (14.8%), then mesenteries (7.7%) of cod, and in the pyloric caeca (13.8%) and liver (10.6%) of bull rout. In haddock and blue whiting (Figures 14 and 15), the liver was the organ most commonly infected with **P.decipiens**. Over 50% of **P.decipiens** occurring in the liver of haddock, and 45.8% in the liver of blue whiting. 18.2% of **P.decipiens** were observed in the left fillet of haddock; this species was never recovered from the right fillet of any of the haddock examined. The stomach, mesentery and pyloric caeca of haddock all contained 9.1% of the **P.decipiens** recovered. After the liver, the pyloric caeca were the next most commonly infected organ in blue whiting (29.2%), then stomach (16.7%). Only 4.2% of **P.decipiens** occurred in the musculature of blue whiting, and only the right fillet was infected.

In the viscera and body cavity of cod, **C.osculatum** were only absent from the kidney (Figure 13), and indeed this species was never recovered from the kidneys, or gonads, of

haddock, blue whiting and bull rout (Figures 14-16). The distribution of **C.osculatum** was similar in all four fish species, with the great majority occurring in the liver (varying from 78% in blue whiting to 86.5% in cod), followed by the pyloric caeca (varying from 9.8% in cod to 15.2% in blue whiting).

**H.aduncum** were recovered from the stomach, intestine, mesentery, pyloric caeca and liver of cod, haddock, blue whiting and bull rout (Figures 13-16). In cod and haddock, the majority of **H.aduncum** occurred in the stomach and intestine (64.7% in cod; 78.4% in haddock), followed by the pyloric caeca (29.4% in cod; 10.4% in haddock). In bull rout and blue whiting, **H.aduncum** occurred most commonly on the pyloric caeca (51.5% and 52.5% respectively), followed by the stomach and intestine in bull rout (38.2%), and liver in blue whiting (17.9%).

The percentages of **H.aduncum** recovered from the alimentary tract, split into stomach and intestine, are shown in Table 8. Both cod and haddock harboured more **H.aduncum** in the stomach; in blue whiting and bull rout, **H.aduncum** was recovered more commonly in the intestine.

Table 9 shows the proportion of nematodes recovered from the fillets only, split into left and right fillets. Only two and one **P.decipiens** larvae were found in haddock and blue whiting respectively. Apart from this, figures were approximately equal for the percentages of nematodes in left and right fillets, although 60% of **P.decipiens** in cod musculature were found in the left fillet, and 62% of total nematodes from the flesh in the left fillet of blue whiting.

Table 10 shows the percentage distribution of **A.simplex** and **P.decipiens** larvae in the body cavity and musculature of each fish species (nematodes in the alimentary tract were not included in this analysis). All five species of fish harboured a significantly greater proportion of their **A.simplex** burden in the body cavity, with percentages varying from 83.3% in bull rout to 100% in long rough dab. The musculature in both cod and bull rout contained the majority

TABLE 8: PERCENTAGE OF H.ADUNCUM RECOVERED FROM STOMACH & INTESTINE OF FISH

| | Stomach | Intestine |
|---|---|---|
| Cod | 54 | 46 |
| Haddock | 74 | 26 |
| Blue whiting | 9 | 91 |
| Bull rout | 28 | 72 |

TABLE 9: PERCENTAGE OF NEMATODES RECOVERED FROM LEFT AND RIGHT FILLETS OF FISH

| | **A.simplex** | | **P.decipiens** | | Total | |
|---|---|---|---|---|---|---|
| | Left | Right | Left | Right | Left | Right |
| Cod | 55 | 45 | 60 | 40 | 58 | 42 |
| Haddock | 55 | 45 | 100 | 0 | 62 | 38 |
| Blue whiting | 50 | 50 | 0 | 100 | 49 | 51 |
| Bull rout | 46 | 54 | 51 | 49 | 50 | 50 |

TABLE 10: PERCENTAGE DISTRIBUTION OF A.simplex & P.decipiens IN BODY CAVITY AND MUSCULATURE OF FISH

| | | Body cavity | Muscle |
|---|---|---|---|
| Cod | **A.simplex** | 91 | 9 |
| | **P.decipiens** | 40.8 | 59.2 |
| Haddock | **A.simplex** | 88.8 | 11.2 |
| | **P.decipiens** | 80 | 20 |
| Blue Whiting | **A.simplex** | 87.8 | 12.2 |
| | **P.decipiens** | 94.7 | 5.3 |
| Bull rout | **A.simplex** | 83.3 | 16.7 |
| | **P.decipiens** | 28.8 | 71.2 |
| Long Rough Dab | **A.simplex** | 100 | 0 |
| | **P.decipiens** | 0 | 0 |

of the **P.decipiens** infection - 59.2% and 71.2% respectively, while only 20% and 5.3% of **P.decipiens** occurred in the musculature of haddock and blue whiting respectively.

Correlations between the numbers of **A.simplex** and **P.decipiens** in the body cavity and musculature are shown in Table 11. Although all correlations were positive, they were generally weak, with a particularly low relationship between the numbers of **A.simplex** in the body cavity and musculature of haddock (0.02), and **P.decipiens** and **A.simplex** in cod (0.09 and 0.1 respectively). The highest correlation observed was between the numbers of **A.simplex** in the body cavity and musculature of blue whiting (0.63).

Figure 18 shows the percentage distributions of **A.simplex** and **P.decipiens** in the fillets and flaps (left and right combined) of cod, bull rout and specimens of blue whiting which had been examined by candling and\or slicing. Haddock and long rough dab were not examined in such a manner as infection of nematodes in the musculature of the former species was low, and the musculature of the latter species had been digested to release the nematodes (nematodes were absent from the fillets of the long rough dab examined in any case). The majority of **A.simplex** in the musculature of blue whiting and bull rout occurred in the fillets. **A.simplex** were recovered in equal numbers from the fillets and flaps of cod. In bull rout, **P.decipiens** in the flesh were recovered primarily from the fillets, and in cod, mainly from the flaps. The figure for blue whiting (100% of **P.decipiens** in the fillets) is misleading as it represents a single worm only.

### 2.3.4 Preliminary Analysis for Competitive Interactions

Preliminary investigation for possible competitive interactions between the four nematode species was examined by calculating the proportions of fish infected with single species infections only, and mixed infections of two or more

TABLE 11: CORRELATION COEFFICIENTS BETWEEN THE NUMBERS OF A.simplex and P.decipiens IN THE BODY CAVITY AND MUSCULATURE OF FISH

| | FISH SPECIES | | | |
|---|---|---|---|---|
| | Bull Rout | Haddock | B Whiting | Cod |
| CORRELATION | | | | |
| Anisakis body cavity vs Anisakis muscle | 0.37* | 0.02 | 0.63** | 0.1 |
| Pseudoterranova body cavity vs Pseudoterranova muscle | 0.45** | | | 0.09 |

*P<0.1

**P<0.05

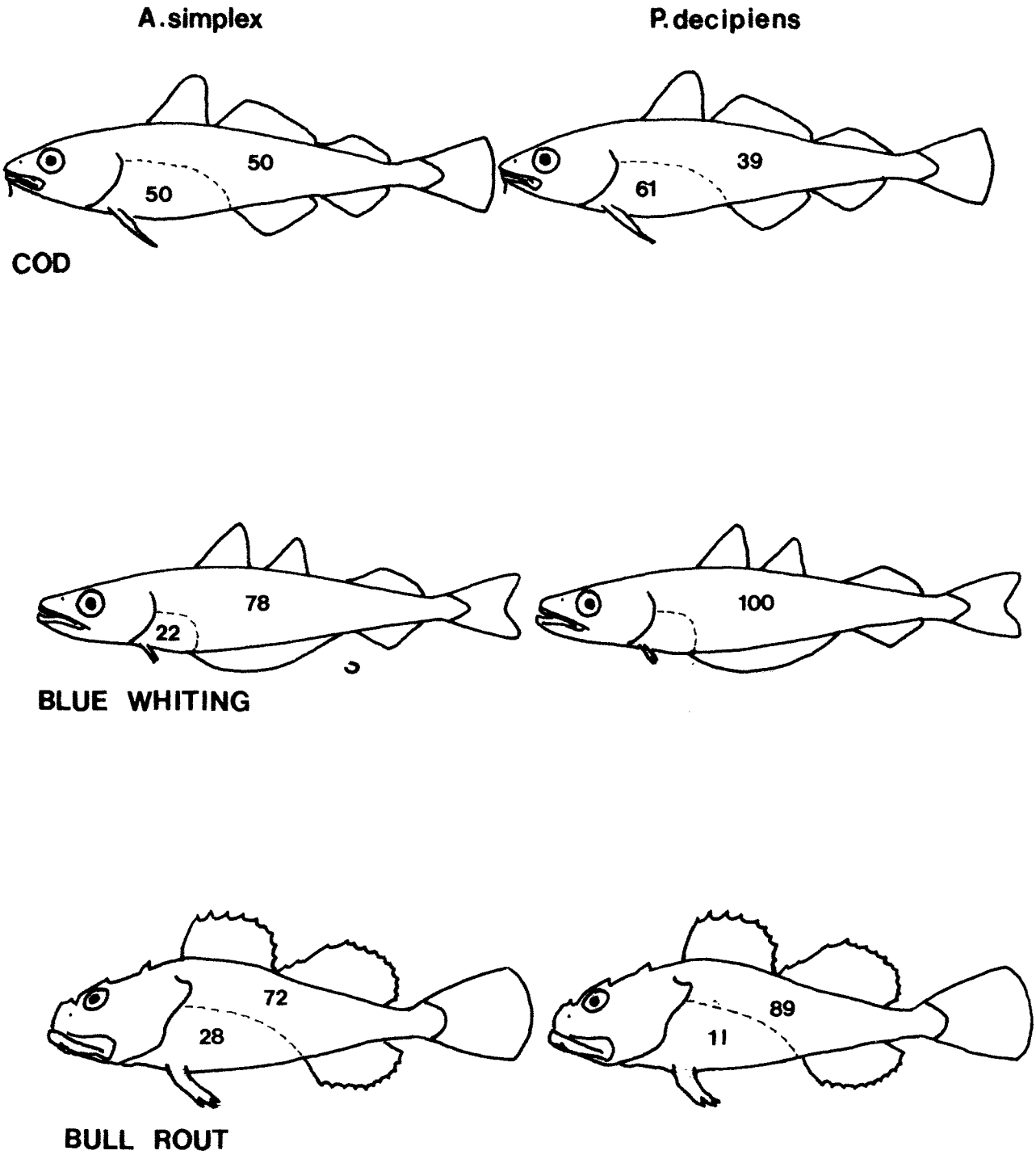


FIGURE 18 : Percentage distribution of larval A.simplex and P.decipiens between the fillets and flaps in cod, bull rout and blue whiting.

of the parasite species, both in the body cavity and in the musculature. **H.aduncum** fourth stage larvae and adults in the intestine were excluded from the analysis, so that effects between third stage larvae of each species only were assessed. In addition, correlations between individual parasite species were calculated -these being between both the total numbers of individual parasite species present in each fish and the numbers of individual parasite species in the body cavity only. The correlation between the numbers of **A.simplex** and **P.decipiens** in the musculature of each fish species was also calculated (see previous section).

Table 12 shows the proportion of single species and mixed species infections observed for each species of fish (long rough dab excepted as only **A.simplex** were recovered from this species).

Mixed infections of the four nematode species were found in all four fish species - and indeed, in cod, blue whiting and bull rout, the majority of infections found were mixed infections of all four species. 22.2% of haddock were infected with all four species, and the same proportion with infections of **A.simplex** + **C.osculatum** only. Infections of two to three parasite species varied between species, both in terms of the percentage of fish in which particular mixed infections occurred, and in terms of the presence or absence of certain mixed infections. However, all four fish species examined also had mixed infections of **A.simplex**, **C.osculatum** + **H.aduncum**, in addition to single species infections with **A.simplex**. No species of fish harboured infections of **P.decipiens** only, or of **P.decipiens**, **C.osculatum** + **H.aduncum**.

The percentages of single and mixed infections of **A.simplex** and **P.decipiens** in the musculature are shown in Table 13. In both haddock and blue whiting most of the muscle infections consisted of a single species infection by **A.simplex**. No infections of **P.decipiens** only were observed. In cod and bull rout, a small percentage of muscle infections were of **A.simplex** only, the remainder being split fairly

TABLE 12: PERCENTAGE OF SINGLE AND MIXED SPECIES INFECTIONS OF NEMATODES IN WHOLE FISH
(excluding digestive tract)

| | A | P | C | H | A+P | A+C | A+H | P+C | P+H | C+H | A+P+C | A+P+H | A+C+H | P+C+H | A+P+C+H |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cod | 2.4 | | | | 2.4 | 4.8 | 2.4 | | | | 11.9 | 2.4 | 7.1 | | 66.7 |
| Haddock | 7.4 | | 3.7 | 11.1 | | 22.2 | 3.7 | | 3.7 | 3.7 | 3.7 | | 18.5 | | 22.2 |
| Blue whiting | 9.1 | | | | | | 4.5 | | | | | | 36.4 | | 50 |
| Bull rout | 3.6 | | | | | 7.1 | | 3.6 | | | 17.9 | | 10.7 | | 57.1 |

TABLE 13: PERCENTAGE OF SINGLE AND MIXED SPECIES
OF NEMATODES IN THE MUSCULATURE OF FISH

| | A | P | A+P |
|---|---|---|---|
| Cod | 9.1 | 48.5 | 42.4 |
| Haddock | 71.4 | 0 | 28.6 |
| Blue whiting | 93.8 | 0 | 6.2 |
| Bull rout | 4.4 | 43.5 | 52.2 |

Key:

A = **A.simplex**

P = **P.decipiens**

C = **C.osculatum**

H = **H.aduncum**

evenly between single species infections of **P.decipiens** and mixed **A.simplex** + **P.decipiens** infections.

Table 14 shows the correlation coefficients for relationships between individual parasites. Fifteen negative relationships were found, seven in blue whiting, and four in cod and haddock. Eleven involved **H.aduncum**, and nine **A.simplex**. Although the negative correlations were generally weak (-0.33 being the strongest observed - between **C.osculatum** + **H.aduncum** in blue whiting), negative correlations occurred between the total numbers of **A.simplex** + **H.aduncum**, numbers of **A.simplex** in the body cavity + **H.aduncum**, and **C.osculatum** + **H.aduncum** in both haddock and blue whiting.

Despite these findings, the strongest positive correlation (0.8) was found between the total numbers of **A.simplex** + **H.aduncum** in bull rout. Correlations between **A.simplex** + **P.decipiens** in the musculature were all positive, although fairly weak. Five of the twelve correlations between **A.simplex** in the musculature of fish + **P.decipiens**, **C.osculatum** and **H.aduncum** in the body cavity were weakly negative, with all correlations between these being negative in cod, and both cod and blue whiting having negative correlations between **A.simplex** in the musculature and **H.aduncum** in the body cavity.

## 2.4 DISCUSSION

### 2.4.1 Comments on General Analysis of Results

Although the sex of the fish was noted where possible, no attempt was made to analyze nematode infections with respect to host sex. Van Banning and Becker (1978) stated that the means of infection and the feeding habits of the herring offers no reason to suspect differences in **Anisakis** infection between sexes, and this applies equally well to the

TABLE 14: Correlation coefficients between individual parasites within fish

| | FISH SPECIES | | | |
|---|---|---|---|---|
| CORRELATION | Bull Rout | Haddock | B Whiting | Cod |
| Total Anisakis vs total Pseudoterranova | 0.58** | 0.27 | 0.59** | 0.31** |
| Total Anisakis vs total Contracaecum | 0.34* | 0.18 | 0.66** | 0.15 |
| Total Anisakis vs total Hysterothylacium | 0.8** | -0.28 | -0.26 | 0.49** |
| Total Pseudoterranova vs total Contracaecum | 0.46** | 0.24 | 0.66** | 0.19 |
| Total Pseudoterranova vs total Hysterothylacium | 0.57** | 0.45** | -0.41* | 0.34** |
| Total Contracaecum vs total Hysterothylacium | 0.1 | -0.06 | -0.33 | 0.41** |
| | | | | |
| Anisakis body cavity vs total Contracaecum | 0.02 | 0.24 | 0.21 | 0.07 |
| Anisakis body cavity vs total Hysterothylacium | 0.56** | -0.18 | -0.17 | 0.45** |
| Anisakis body cavity vs Pseudoterranova muscle | 0.24 | 0.06 | 0.02 | 0.26* |
| Pseudoterranova body cavity vs total Contracaecum | 0.23 | 0.11 | 0.45** | 0.23 |
| Pseudoterranova body cavity vs total Hysterothylacium | 0.43** | 0.27 | -0.33 | 0.26* |
| Pseudoterranova body cavity vs Anisakis body cavity | 0.3 | 0.29 | 0.51** | 0.08 |
| | | | | |
| Anisakis muscle vs Pseudoterranova muscle | 0.44** | | | 0.29* |
| Anisakis muscle vs Pseudoterranova body cavity | 0.48** | 0.33* | 0.6** | -0.13 |
| Anisakis muscle vs total Contracaecum | 0.09 | -0.01 | 0.36* | -0.11 |
| Anisakis muscle vs total Hysterothylacium | 0.23 | 0.13 | -0.12 | -0.12 |
| | | | | |
| Pseudoterranova muscle vs total Contracaecum | 0.16 | | | -0.03 |
| Pseudoterranova muscle vs total Hysterothylacium | 0.45** | 0.23 | -0.12 | 0.15 |

* P<0.1

**P<0.05

fish species examined during this study, and to the other nematode species. Other authors found no significant differences between sexes eg. for **Anisakis** larvae in herring (Bishop and Margolis 1955, Davey 1972, Van Banning and Becker 1978), and blue whiting (Smith and Wootten 1978b); **H.aduncum** in the alimentary tract of haddock (Scott 1981). Templeman **et al.** (1957) also found no differences in infection levels between **P.decipiens** and **Anisakis** in the musculature of male and female cod, and McClelland **et al.** (1983a,b) noted that abundances of larval anisakines in cod and flatfish did not differ significantly with host sex.

During the present study, specimens from the same species of fish, but collected from different geographical locations, were pooled together both for simplicity in analysing the data on the location of nematodes within the fish, and also because some sample sizes were too small to compare infections between localities. Geographical differences in infection levels are thus not discussed here. Although significant variations in ascaridoid infection are known to occur between different areas of Scottish waters (see Section 2.1.2.1) and the epidemiological data presented must be regarded as indicating only the general level of infection of nematodes in fish from waters to the north and east of Scotland. Problems therefore arise in comparing these results with those of other authors. In addition to observed variations in infection levels of nematodes within fish - be they seasonal, annual or geographical (see Section 2.1.2) - the method of retrieval and analysis and the length\age of the fish sample examined may also differ eg. Platt (1975) stated that if only fillets have been examined for nematodes, and not flaps, and also if the fillets have not been sliced during candling, large numbers of nematodes may potentially have not been found; the mean number of parasites per fish, in all fish examined, may have been calculated rather than mean intensity, which is calculated from infected fish only; the prevalence and intensity of infection in the same species of fish examined by different

authors will vary depending on the size range of the fish examined (see Section 2.1.3.).

### 2.4.2 Infection Levels in Fish

#### 2.4.2.1 Infections in cod (Gadus morhua)

Cod are generally found from the shore line to 600m and deeper, and form shoals which tend to remain between 30 and 80m from the sea bed, although they are known to forage both on the sea bed and near the surface. In addition to feeding migrations of the adults, mature cod also migrate to within the 200m line to spawn. Young cod feed on copepods whilst young, and become demersal at a length of approximately 2cm (Wheeler 1969). On the change to a demersal mode of life, the feeding habits also alter whereby the main food items eaten are crustaceans, primarily euphausiids (**Thysanoessa**), amphipods, mysids, decapods and some copepods (Wheeler 1969). As cod mature and increase in size, they become largely piscivorous (Bishop and Odense 1966), consuming herring, sand eels, capelin, haddock and codlings in particular, although adult cod will feed on almost any suitable fish present in sufficient numbers (Wheeler 1969). Adult cod are non-selective feeders, however, and if fish are not available they will feed on invertebrates (Myers 1960), particularly benthic organisms (Bishop and Odense 1966).

All specimens of cod examined were infected with both **A.simplex** and **H.aduncum**, which accounted for almost 75% of the total nematodes recovered, being retrieved in approximately equal numbers. **P.decipiens** and **C.osculatum** comprised 10.4% and 15.8% respectively of the nematodes recovered. Palsson **et al.** (1985) examined the infection by all four nematode species in the body cavity and musculature of cod from Icelandic waters. The proportions of **A.simplex** and **P.decipiens** found by these authors are comparable to those recorded here. Excluding those **H.aduncum** in the

alimentary tract, 50% of the nematodes recovered from cod during the present study were **A.simplex**, and 13.5% were **P.decipiens**. Palsson **et al.** (1985) found 40.4% and 12.1% of the nematodes recovered from Icelandic cod to be **A.simplex** and **P.decipiens** respectively. However, the percentages of **C.osculatum** and **H.aduncum** recovered differed, being 20.5% and 16% respectively during the present investigation, while Palsson **et al.** found these to be 6.3% and 41.2% respectively. Such differences in the proportions of the latter two species may be explained by feeding differences and\or differences in the level of infection of these species in invertebrate and final hosts from Scottish and Icelandic waters.

Cod are common hosts of **P.decipiens** in Scottish waters (Wootten and Waddell 1977), and of the five fish species examined during this study, the highest prevalence of infection with **P.decipiens** was found in cod (85.7%).

The musculature of cod is well documented as being infected with **A.simplex** and **P.decipiens** larvae in all British coastal waters (Young 1972), including waters around Scotland (Wootten and Waddell 1974, 1977; Wootten 1979; Smith and Wootten 1979). In the central North Sea, Young found the mean number : range per fish of **Anisakis** and **T.decipiens** (= **P.decipiens**) in cod musculature to be approximately 3.1 : 0-31 for **Anisakis**, and 0.2 : 0-57 for **T.decipiens**.

Wootten and Waddell (1977) found **P.decipiens** and **A.simplex** to be common in the musculature of cod from Scottish waters, although infection differed in different areas with 0-81.2% of cod infected with **Anisakis** sp., and <0.1-95.2% with **Phocanema** (= **Pseudoterranova**). Of those areas sampled by Wootten and Waddell for cod in Scottish waters, which were also sampled during the present study (north coast of Scotland and Moray Firth), Wootten and Waddell found the prevalence : mean number per fish : range of **Phocanema** (= **P.decipiens**) and **Anisakis** in the musculature to vary (depending on the age of the cod), being 3.1-58.3% : <0.1-1.8 : 0-29 for **Phocanema**, and 5.4-29.4% : 0.1-0.7 : 0-14 for **Anisakis**. In cod musculature only, **A.simplex** infection

during this study, expressed in terms of prevalence : mean intensity : range of infection was 40.5% : 3 : 0-8, that of **P.decipiens** in the musculature 71.4% : 3.3 : 0-22.

In other waters, **Anisakis** larvae in cod musculature was found to have a prevalence : range of 95% : 0-334 in Arcto-Norwegian cod (Platt 1975); 80% : 0-74 in cod from the Faroes (Platt 1975) and 75% prevalence in Icelandic cod (Hauksson 1992). The prevalence of infection of **A.simplex** in the musculature was considerably lower in the present study than that found by these authors. However, McClelland **et al.** (1983a) found **Anisakis** sp. prevalences of generally <2% in fillets of cod from eastern Canadian waters. For **P.decipiens** in the musculature, Platt (1975) found prevalences and ranges of infection of 31% : 0-11 and 7% : 0-27 in fillets of cod from the Faroes and Arcto-Norwegian waters respectively. 0-80% and 80% prevalence of **P.decipiens** was reported in the fillets of cod from eastern Canadian waters (Templeman **et al.** 1957) and Icelandic waters (Hauksson 1992) respectively.

Total **A.simplex** infection in cod examined during this study was 100% : 15.9 (prevalence : mean intensity). Wootten (1978) found 88.9% prevalence of **Anisakis** in small cod (28-29cm) from the North Sea, with a mean of 5.3 per fish. In other waters, infection levels of **Anisakis** were significantly lower in terms of prevalence in Baltic cod (Grabda 1976a), whilst the maximum prevalences of this parasite in cod from eastern Canadian waters (McClelland **et al.** 1983a) and Norwegian waters (Berland 1961) were 97% and 83% respectively. The prevalence of total **P.decipiens** infection found in the present study was 85.7%. In cod from Canadian Atlantic waters, prevalence of infection with **P.decipiens** varied from 8-100% (Appy and Burt 1982, McClelland **et al.** 1983a). In the musculature of cod, Young (1972) found larval **Anisakis** to be more abundant than **P.decipiens** in cod from the central North sea. Kahl (1939) also found higher infections of **Anisakis** in the musculature compared to **P.decipiens**. Wootten and Waddell (1977) found infection levels of **Anisakis** and **Phocanema** (= **P.decipiens**) in the flesh of cod to differ

between areas, prevalences and mean number of larvae per fish of **P.decipiens** being generally higher than **Anisakis** in all areas with the exception of the Ling Bank. In the present study, infection of **P.decipiens** in the musculature was greater than that of **A.simplex**. The reverse was true for larvae of these species in the body cavity, with the level of infection with **A.simplex** being significantly higher than **P.decipiens** (100% prevalence, 12.3 intensity for **A.simplex**; 57.1% prevalence, 2.9 intensity for **P.decipiens**). Kahl (1939) found a significantly greater prevalence of **Anisakis** in the body cavity of cod from the North Sea, as compared to the flesh. This agrees with the findings of the present study.

Infection of **C.osculatum** in cod examined during this study was 90.5% :7.2 : 0-47. In the North Sea, Wootten (1978) found only 1.6% of small cod (<30cm) infected with larval **Contracaecum**. McClelland **et al.** (1983a) found the prevalence of **Contracaecum** in cod from eastern Canadian waters to vary from 0-70%. Valtonen **et al.** (1988) found a 15% prevalence of infection with **C.osculatum** in cod from the north-eastern Baltic, with an intensity of infection of 1.5 (Range = 1-2), however, **C.osculatum** was found in higher prevalences and intensities in cod 500km south of this area (Fagerholm 1982b, in Valtonen **et al.** 1988).

In the North Sea, Wootten (1978) found small cod (<30cm in length) to be heavily infected with larval **Thynnascaris aduncum** (= **H.aduncum**), and to a lesser extent with **Anisakis.** Grabda (1976a) also observed that **C.aduncum** larvae occurred more frequently in Baltic cod than **A.simplex** larvae. The cod examined during the present study were larger than 30cm, and harboured higher infections of **A.simplex** than **H.aduncum** larvae, both in terms of prevalence and mean intensity. In the North Sea, Wootten (1978) found higher infection levels of larval **Thynnascaris aduncum** (= **H.aduncum**) in cod 28-29cm in length, than were observed in cod during the present study, whereas the prevalence of **C.aduncum** (= **H.aduncum**) in Baltic cod was lower than reported here (Grabda 1976a),

although the maximum figures for mean intensity and range were higher than in the present study. The prevalence and mean intensity of fourth stage and adult **H.aduncum** in cod was similar to that found in cod from Canadian Atlantic waters (Appy and Burt 1982). Berland (1961) did not distinguish between larval and adult stages of **C.aduncum**, and found the prevalence of this species in cod to vary from approximately 62-100% in Norwegian waters. The prevalence of total **H.aduncum** in cod examined during this study was 100%.

The wide variety of food types consumed by cod during their life cycle, renders them susceptible to infection by all four species of nematodes found here. Scott and Martin (1959) showed that cod may be infected with larval nematodes by the time they are one year old and have reached a length of 13cm. The large range of invertebrates eaten whilst young may include the hosts of all four nematode species. Small cod feeding on euphausiids probably become initially infected with **A.simplex** at this stage, and Wootten and Waddell (1977) also stated that infection with **A.simplex** larvae does not occur until the fish start feeding on larger plankton such as euphausiids. The wide range of invertebrates (particularly copepods) taken whilst young may also lead to primary infection by third stage larvae of **H.aduncum**. In Scottish waters, young cod become infected with **T.aduncum** (= **H.aduncum**) larvae earlier than they do with **A.simplex** larvae (Wootten and Waddell 1977). Wootten (1978) additionally noted heavier infections of **T.aduncum** than **Anisakis** in young cod, and McClelland **et al.** (1983a) also mentioned that **Anisakis** infection was low in small cod. This supports the suggestion that infection with third stage **H.aduncum** occurs whilst the cod are young, and is obtained from small invertebrates. **P.decipiens** and **C.osculatum** are also likely to be acquired via infected benthic invertebrates. McClelland **et al.** (1983a) suggested that juvenile cod feeding primarily on invertebrates, probably became infected with **P.decipiens** via amphipods or other hosts. The spawning migration of cod will lead to further acquisitions of **P.decipiens** and **C.osculatum**,

in the shallower coastal waters.

However, the piscivorous feeding habits of adult cod, on a wide range of fish species, is likely to be the major source of infection of all four nematode species. Scott and Fisher (1958b) suggested that larger fish probably much of their **Porrocaecum** (= **P.decipiens**) infection by eating infected smaller fish, and Smith (1984e) stated that most **A.simplex** harboured by cod would appear to have been acquired from prey fish.

**P.decipiens** have been recovered from a number of fish species, some of which are known to form part of the diet of cod in Scottish waters (Rae 1967). Templeman **et al.** (1957) stated that larger cod are usually found in deeper water than smaller cod and are thus usually further away from inshore sources of **P.decipiens** infection, and Kahl (1939) presumed that the possibilities for infection of cod with **P.decipiens** were slight during time spent offshore, but fish entering shallower waters, where seals are present, will be exposed to a higher degree of infection by this parasite. Indeed, Young (1972) found larval **Anisakis** to be more abundant than **P.decipiens** in cod from the North Sea, particularly in offshore areas. Wootten and Waddell (1977) also observed **P.decipiens** to be rare in fish from the central North Sea. Similarly, **A.simplex** larvae are also found in numerous fish species, so cod may acquire this parasite from many different prey fish. In particular, cod are known to consume herring (Templeman **et al.** 1957, Wheeler 1969), which harbour large numbers of **Anisakis** in British waters (Khalil 1969, Davey 1972). In the Baltic, euphausiids are absent, and Grabda (1976a) stated that herring were the source of **Anisakis** infection for cod. Again, fourth stage larvae and adults of **H.aduncum** may be acquired from numerous fish species eg. sand eels (Wheeler 1969), which often harbour large numbers of **H.aduncum** (personal observation).

Wootten (1978) considered that small gadoids were a significant reservoir of nematode infection for larger fish, given the levels of infection he found in such fish, and the

fact that small gadoids are an important food source for many economically important fish species in the north-east Atlantic.

Wootten (1978) did not recover **P.decipiens** from cod less than 30cm in Scottish waters, and stated that absence of this species from small cod, may reflect the offshore locations where most fish were collected and also, small cod may not feed on the intermediate hosts of **P.decipiens**, or other infected fish which may be an important source of infection for older, larger cod (Wootten and Waddell 1977). Such considerations may apply equally to the low numbers of **Contracaecum** larvae recovered in small cod by Wootten (1978).

### 2.4.2.2 Infection of haddock (Melanogrammus aeglefinus)

Haddock generally occur near the sea bed, however, both eggs and larvae are pelagic - young haddock becoming demersal at approximately seven months old, and at a length of approximately 5cm (Wheeler 1969), although Scott (1981) noted the change from planktonic to benthic feeding at a length of 18-30cm. Planktonic feeding is thus indicated in young haddock, whereas adult haddock feed primarily on benthic fauna, and their diet consists mainly of echinoderms, annelids, molluscs and a wide variety of crustaceans, including euphausiids and amphipods (Jones 1954). Jones found fish to increase in the diet as the haddock aged, but fish were intermittently important as a food item. The main fish species eaten in Scottish waters by small haddock (<26cm in length) was **Ammodytes** (sandeels), but in larger haddock, herring were often found, and occasionally long rough dab. Almost half of the worms recovered from haddock in the present study were **H.aduncum**, followed by **A.simplex**, **C.osculatum** and **P.decipiens**, in that order. However, fourth stage larvae and adults of **H.aduncum** in the digestive tract account for a large proportion of the total burden of this

species (78.4%), and only 15% of worms in the body cavity are **H.aduncum**. Thus, excluding these, the proportions of the other species are increased, with **A.simplex** accounting for over 50% of the worms recovered.

Wootten (1978) also found larval **Anisakis** to be abundant in small haddock (<30cm in length) from the Moray Firth and northern North Sea, with prevalences similar to those recorded in the present study. Haddock are likely to have become infected with **Anisakis** through eating euphausiids, and ingestion of other fish, such as herring (Jones 1954).

Scott (1981) found that **H.aduncum** was the most abundant intestinal parasite in haddock from the Scotian shelf, with prevalences of 82.3% (summer) and 29% (winter) in fish 30-75cm in length. The prevalence of **H.aduncum** in the alimentary tract (stomach and intestine) of haddock examined during this study, and collected in January, was 48%, although the mean intensity of **H.aduncum** recorded by Scott was lower than that found here. Larval **T.aduncum** (= **H.aduncum**) were found to be abundant in small haddock from the Moray Firth and Scottish northern North Sea waters (Wootten 1978), with similar prevalences and mean number of larvae per fish to those recorded in the present study.

**H.aduncum** utilizes planktonic intermediate hosts eg. copepods and **Sagitta** spp. (see, for example, Berland 1961), and Scott (1981) stated that the parasites of young haddock are consistent with a planktonic mode of feeding. Wootten (1978) considered that haddock probably acquire larval **T.aduncum** at a young stage mainly by feeding on pelagic copepods which form an important part of their diets; as fish grow older, and the diet becomes more diverse, other hosts may become more important in the transmission of this parasite. Fourth stage larvae and adults of **H.aduncum** are likely to be acquired from predation on fish infected with third stage larvae; in particular, sandeels have been noted as one of the main fish species eaten by haddock (Jones 1954), and these are often heavily infected with **H.aduncum** (personal observation). Ingestion of such fish may explain

the high proportion of fourth stage larvae and adults in this species - which account for 78.4% of the total **H.aduncum** recovered.

Wootten and Waddell (1977) recorded a 6.3% prevalence of **P.decipiens** in the musculature of small haddock (15-25cm) from the west coast of Scotland. No **P.decipiens** were recovered from haddock taken from the Moray Firth or the north coast of Scotland. The prevalence of **P.decipiens** in the musculature of haddock examined during this study was 7.4%. 25.9% of the fillets were also infected with **A.simplex**. Templeman **et al.** (1957) found nematode infection in the fillets to be low in haddock (prevalence varying from 0-2%), even in areas where cod were highly infected. In general, the musculature of haddock was found to be lightly infected by nematodes, with mean intensities of 1.6 and 1 for **A.simplex** and **P.decipiens** respectively.

In small haddock (<30cm), Wootten (1978) only found larval **Contracaecum** sp. in fish from the Moray Firth; the prevalence and mean number of larvae per fish, in the largest fish examined, was 27% and 0.4. During the present study, prevalence and mean intensity of **C.osculatum** in haddock was 81.5% and 2.4. Smith (1974) found a mean intensity (in ten haddock examined) of 2.1 for **Contracaecum**.

Parasites which use benthic intermediate hosts were absent in young haddock and the change from planktonic to benthic feeding was indicated at lengths of 18-30cm (Scott 1981). Wootten (1978) found **Contracaecum** sp. restricted to larger haddock, which suggested that the intermediate host may be a relatively large benthic organism, possibly a crustacean. As both **C.osculatum** and **P.decipiens** utilise benthic invertebrate hosts, haddock are thus likely to become infected with these species after the transition to a benthic mode of life. In particular, Jones (1954) reported amphipods in the diet of haddock, and Myers (1960) reported these as intermediate hosts of **P.decipiens**. **P.decipiens** and **C.osculatum** larvae may also be acquired through predation on infected fish, although fish are eaten only occasionally.

### 2.4.2.3 Infection of Blue Whiting (Micromesistius poutassou)

Blue whiting are pelagic, oceanic fish, although young fish are found in shallower water in summer, and may also be found inshore. The young blue whiting feed on copepod crustaceans, small amphipods and mysids, while the principal food of the adult fish is crustaceans, primarily euphausiids, mysids, the amphipod **Themisto** and the shrimp **Pandalus**. They are rarely piscivorous (Wheeler 1969).

Kusz and Treder (1980) found **A.simplex** to be the most abundant parasite of blue whiting, and the results of the present study are in agreement with this. **A.simplex** accounted for approximately 50% of the total worm burden in the blue whiting examined, and all specimens were infected with nematodes of this species. MacKenzie (1979) also found 100% prevalence of **Anisakis** sp. larvae in blue whiting from the north of Scotland (the nearest locality sampled in comparison to the area examined during this study). Heavy infections of **Anisakis** in blue whiting from Scottish waters have also been reported by Smith and Wootten (1978b), with individual worm burdens ranging from 0-583. Comparisons of data from the north of Shetland (the area sampled here being North East Shetland) revealed a prevalence of 94.5%, and a mean worm burden of 28.9 (Smith and Wootten 1978b), which is similar to the present study. Smith (1984e) found means to range from 21.4-67.4 **Anisakis** larvae per fish in the body cavity of blue whiting from Scottish waters, and 5.2-17.1 in the flesh. In the present study, the mean intensities of **A.simplex** in the body cavity and musculature of blue whiting were 23 and 4.4 respectively.

Levels of **Anisakis** infection were also found to be high in blue whiting from other European waters (Grabda 1978, Kusz and Treder 1980). Muscle infection by **A.simplex** in the blue whiting examined during the present study is similar to that observed by both of these authors.

The high levels of **A.simplex** observed during this study in blue whiting can be attributed to heavy feeding on

euphausiids, as Smith (1984e) had previously suggested. Although Smith (1983b) found the prevalence of larval **Anisakis** in euphausiids to be low, the abundance of these invertebrates, the quantities in which they are eaten, and the apparent longevity of third stage larvae in fish hosts, lead to the high worm burdens seen in fish (see Smith 1983b).

**H.aduncum** levels were also high in the specimens of blue whiting examined. 93.9% of **H.aduncum** were third stage larvae in the body cavity (prevalence 95.4%), and the remainder were found as fourth stage larvae and adults in the alimentary tract (prevalence 45.4%). Berland (1961), MacKenzie (1979) and Kusz and Treder (1980) also observed high **H.aduncum** levels in blue whiting from waters around Norway, the north of Scotland and the Faroes.

The high infections of third stage larvae of this species are likely to have been acquired at a young stage, when feeding on copepods. The lower infections of fourth stage larvae and adult **H.aduncum** may be acquired from occasional feeding on small fish infected with third stage larvae.

That blue whiting are pelagic and oceanic fish is likely to account for the small proportions of **P.decipiens** and **C.osculatum** present, which may be acquired from invertebrates whilst young, in inshore waters. Small numbers of **P.decipiens** and **C.osculatum** may also be acquired via occasional predation on smaller infected fish. Although the proportions of these two nematode species are low in terms of the total nematodes recovered, and these worms occurred in relatively small numbers in individual fish, **P.decipiens** occurred in over 50% of the fish examined, **C.osculatum** in over 80%. MacKenzie (1979) recorded **C.osculatum** larvae in blue whiting from Scottish waters, and found the prevalence in fish from the north of Scotland (the nearest location to the area sampled during this study) to be 10%.

### 2.4.2.4 Infection of Long Rough Dab (Hippoglossoides platessoides)

The diet of long rough dab consists chiefly of echinoderms and crustaceans, including shrimps and hermit and spider crabs (Wheeler 1969), but fish are also consumed occasionally (Templeman **et al.** 1957). The smallest length classes (varied from $\leq$ 25-35cm) of **H.platessoides** examined from different areas in Canadian Atlantic waters by McClelland **et al.** (1983a) had a mean prevalence of **P.decipiens** of 33%. In addition, Wootten and Waddell (1977) stated that although this parasite was found in long rough dab from all the areas they examined in Scottish waters, the prevalence of infection was low, varying from 2.3% in the Clyde to 9.1% in the southern Minch. **H.platessoides** in Canadian Atlantic waters are often heavily infected with **P.decipiens** (Templeman **et al.** 1957, McClelland **et al.** 1983a,b) and Bristow and Berland (1992) stated that **H.platessoides** was the major intermediate host for **P.decipiens** C in northern Norwegian waters. The absence of **P.decipiens** and **C.osculatum** in the specimens of long rough dab examined, is surprising, considering that these fish are benthic feeders and have previously been reported as a host for **P.decipiens** in Scottish waters (Wootten and Waddell 1977), although these authors did not examine specimens from areas sampled during this study. Templeman **et al.** (1957) found **H.platessoides** in Canadian Atlantic waters to be heavily infected with **P.decipiens** at a size of 11-20cm. The absence of **P.decipiens** from long rough dab in this study may simply be a reflection of the relatively small number of fish examined and the low prevalence of this parasite in this species. Other species of fish from the same areas were infected with this parasite.

Templeman **et al.** (1957) noted prevalences of nematodes up to 21% in the fillets of **H.platessoides** in different areas, however, only 2% of the nematodes in the fillets were **Anisakis** sp., the rest being **P.decipiens**. In addition to

**P.decipiens**, McClelland **et al.** (1983a) had also reported **Anisakis** and **Contracaecum** sp. from **H.platessoides** in Canadian Atlantic waters. All the nematodes recovered from long rough dab during this study were **A.simplex**, and none were recovered from the fillets, although 64.3% of the fish examined did harbour infections of **A.simplex** in the body cavity. Of the **H.platessoides** examined by McClelland **et al.** (1983a), and which were $\leq$ 25cm in length, only one **Anisakis** was recovered from 93 fish; the mean prevalence of **Anisakis** in the Canadian waters examined by these authors only increased to 1.2% when length classes up to and including 35cm were considered. The fish examined during this study were generally lightly infected with **A.simplex**, each infected fish harbouring a mean of 3.8 parasites, although 14 were retrieved from one individual. Berland (1961) found a prevalence of 40% for **Anisakis** in long rough dab from Norwegian waters.

The presence of larval **A.simplex** is difficult to reconcile with the feeding habits of this fish species, however, the infections were low and may have been acquired through predation on smaller fish or occasional ingestion of infected invertebrates.

The small size of the fish examined during this study may account for the absence of **C.osculatum**. McClelland **et al.** (1983a) found no **Contracaecum** in any of the smallest length classes (varied from $\leq$ 25-35cm) of **H.platessoides** examined from different areas. Long rough dab may not feed on the invertebrate intermediate hosts of this species until they have reached a larger size than those examined here. **H.aduncum** were also absent from the long rough dab examined. Presumably this was because long rough dab, being benthic fish, were not feeding on the planktonic invertebrate hosts of this parasite or indeed on fish infected with this species of worm.

Levels of nematode infection in species of pleuronectids generally appear to be low. Myers (1979) examined eleven species of Pleuronectidae from the Pacific coast of the United states, for anisakine nematodes and found no

**P.decipiens** in the flesh of any of them, although **Anisakis** were present in the flesh of individuals from five of the species; prevalences of nematodes were generally low, as were the mean intensities which mostly ranged from 0 to 5. Myjak **et al.** (1991) found only a 0.9% prevalence of Anisakidae larvae in 649 flounders from the southern Baltic, although it was not stated which species of nematodes were found. Oikawa and Ikeda (1990) found a mean intensity of 1.8 for infection with Anisakinae larvae (again the species were not given) in **Hippoglossoides dubius** from Japanese waters, with a prevalence of 40%.

### 2.4.2.5 Infection in Bull Rout (Myoxocephalus scorpius)

Bull rout are coastal, littoral species in northern waters (Wheeler 1969). The diet is varied and is composed mainly of fish and crustaceans, with small bull rout consuming gammarids, **Idothea** and young brown shrimps, and larger fish eating crabs, prawns and pink and brown shrimps. Various fish are eaten, including young herring, whiting, sand eels, gobies and dragonets. The coastal habitation of bull rout leads to high levels of infection by **P.decipiens** and **C.osculatum**, these two species accounting for approximately 50% of the total parasite population observed - significantly higher than the levels observed in cod, haddock and blue whiting. Kerstan (1991) considered bull rout to be important in transmitting infections to seal populations. The mean intensities of **P.decipiens** and **C.osculatum** found here were 9 and 12.1 respectively, and were the highest observed in any of the fish species examined. Presumably **P.decipiens** and **C.osculatum** larvae are acquired both from invertebrate intermediate hosts throughout the life span of the fish, and via smaller infected fish, eaten as the bull rout grow. Infections by larval **A.simplex** and larvae and adults of **H.aduncum** are likely to have derived from feeding on smaller fish infected with these species.

In terms of prevalences and mean intensities of individual nematodes, 78.6% of fish were infected with **P.decipiens**, and 96.4% with **C.osculatum** during this study. Jensen and Andersen (1991) found that 95% of sculpin from southern Norwegian waters were infected with **P.decipiens**, with some individuals harbouring up to 300 larvae in the flesh. During the present study, the maximum number of **P.decipiens** observed in an individual fish was 25 (19 in the musculature). Valtonen **et al.** (1988) found a 20% prevalence of **C.osculatum** in bull rout from the north-eastern Baltic, with an intensity of infection of 1.8. The maximum number observed in a single fish by these authors was 4, as compared to 49 during the present study.

Both Berland (1961) and Kerstan (1991) reported **H.aduncum** from **M.scorpius**.

Infections of nematodes in **Myoxocephalus** spp. in the western Atlantic and North Pacific oceans appear to be generally low eg. Templeman **et al.** (1957) recovered no nematodes from the fillets of longhorn sculpin 21-30cm. in length, and 10.5% prevalence in fish 31-40cm; Arai (1969) examined 18 species of sculpin from Canadian Pacific waters and found 12 species to harbour infections of **Anisakis**, **Contracaecum** and **Porrocaecum** (= **Pseudoterranova**), but few individuals appeared to be infected, and when they were, infections were generally low and often only one species of parasite was found within any one species of fish.

### 2.4.2.6 General Infection In Fish

The nematodes examined during this study are not host specific in fish, and are found in a wide variety of marine teleosts. Third stage larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** in the body cavity, and larval and adult **H.aduncum** from the alimentary tract were recovered from all species of fish examined with the exception of Long Rough Dab in which only **A.simplex** were found. Parasites of marine fish in northern waters generally show a wide range of host

specificity, and this low degree of host specificity can lead to a rich parasite fauna in individual fish species (Appy and Burt 1982). Mixed infections of two or more ascaridoid species were common, with the majority of individual cod, blue whiting and bull rout harbouring all four nematode species. The frequency of one or more nematodes within individual fish is probably related to diet, rather than to possible competitive effects, whereby some individuals may simply not consume prey infected with certain species of nematodes, and others consume a range of food items infected with all four parasite species.

The infection levels of nematodes in different fish species can be attributed to the life cycle of the parasite, the habitats (eg. pelagic or demersal, oceanic or coastal), diet and migrations of each fish species. Demersal species of fish are more likely to acquire infections of **P.decipiens** and **C.osculatum**, as the second stage larvae are eaten by benthic invertebrates, which in turn are eaten by demersal fish. Similarly, pelagic species of fish are more likely to become infected with **Anisakis** as these parasitise planktonic crustaceans. In addition, **P.decipiens** and **C.osculatum** tend to be more common inshore, in coastal regions, because seals are their final hosts, whereas **Anisakis** are more common offshore, where cetaceans are most abundant. **Hysterothylacium aduncum** are common both in and off shore, with several species of benthic and planktonic invertebrates acting as first intermediate hosts. The wide host range of this parasite may account for the presence of this parasite in all fish examined, as piscivorous and non-piscivorous species of fish feeding at all depths, over a wide area can potentially become infected with different stages of this species. Adult **H.aduncum** occurs in the alimentary tracts of many species of teleosts, especially cod and other gadoids (Soleim and Berland 1981), and certainly, during the present study, cod were found to harbour the highest infections of **H.aduncum** in their alimentary tracts. Möller and Anders (1986) also identified adult **H.aduncum** from the intestines of nearly all

fish species in the western Baltic sea. The high levels of third stage **H.aduncum** larvae found in eg. cod, haddock and blue whiting indicate that these fish may themselves be important intermediate hosts for this parasite.

**P.decipiens** and **C.osculatum** were found to have the highest prevalences and intensities in cod (a demersal species, found in shallow waters when young, and during spawning migrations) and bull rout (a coastal species). Jensen and Andersen (1991) also found **M.scorpius** and cod to be the most heavily infected fish species, out of twenty examined, with **P.decipiens**. **P.decipiens** has previously been recorded as being common, and having high infections, in cod eg. Young (1972), McClelland **et al.** (1983a,b), Wootten and Waddell (1977), Hauksson (1992). Des Clers and Wootten (1990) stated that cod were the most commonly infected fish host of **P.decipiens**, and the findings of this study are in agreement with this (85.7% prevalence), although the mean intensity of this species was higher in bull rout (9 as compared to 5 for cod).

The highest infection levels of **A.simplex** were observed again in cod (found in deeper waters when older), and also in blue whiting (a pelagic, oceanic species). This corresponds with the general offshore life history of this parasite, utilising cetaceans and planktonic crustaceans as final and invertebrate hosts respectively.

Piscivorous fish have been reported as harbouring higher infections of nematodes than planktivorous fish, or fish feeding on benthos (Myers 1979, Smith 1984e). In the present study, the observed mean intensities of total nematodes in haddock and long rough dab (which feed primarily on benthos) were significantly lower than those observed in other fish species (10.9 and 3.8 respectively). The mean intensities of total nematodes in cod and bull rout, which are piscivorous were 41.6 and 39.2 respectively. The high infection levels of total nematodes seen in piscivorous fish are probably due primarily to the likelihood of acquiring large numbers of these parasites from infected prey fish, whereas the

prevalence of infection in invertebrates generally appears to be low. However, the highest mean intensity (55.4) was observed in blue whiting (a planktonic feeder). This species is known to feed heavily on euphausiids as an adult, and copepods when young, and approximately 90% of the total nematodes recovered from these fish were **A.simplex** and **H.aduncum**, which utilise euphausiids and copepods respectively as invertebrate hosts. Berland (1961) commented on a low incidence of **C.aduncum** (= **H.aduncum**) in planktivorous fish. This was not observed in the blue whiting examined during this study, where 95.4% of fish were infected with nematodes of this species, and the mean intensity was 22.7. However, only 6.1% of the **H.aduncum** recovered were fourth stage larvae and adults (prevalence : mean intensity = 45.4% : 2.9). In haddock, bull rout and cod, a significantly higher proportion of **H.aduncum** were fourth stage larvae and adults, and this may be explained by the higher levels of fish eaten by these species, whereby third stage larvae in infected prey fish mature in the alimentary tract of the predator.

### 2.4.3 Frequency Distribution

The nematode species examined here generally showed an overdispersed distribution within the host fish, with most fish harbouring small numbers of parasites, and a few having high numbers of nematodes. The only exceptions to this general trend were the total numbers of all four species of nematodes in blue whiting, and the numbers of **C.osculatum** in blue whiting.

Numerous authors have reported an overdispersed distribution of larval nematodes in fish eg. **P.decipiens** in **H.platessoides** (McClelland **et al.** 1987), redfish, smelt (Kahl 1939) and long rough dab (Bristow and Berland 1992); **P.decipiens** and **Anisakis** sp. in cod (Young 1972, Platt 1975); **Anisakis** sp. in blue whiting (Smith and Wootten 1978b) and

herring (Bishop and Margolis 1955, Davey 1972 and Van Banning and Becker 1978).

Overdispersion of a parasite in a host population is typical for the majority of parasite frequency distributions studied from natural populations (Anderson 1978), with a large proportion of the parasite population occurring in a few hosts. A large number of processes are known to produce overdispersion in a population. However, Smith and Wootten (1978b) considered that it was not yet possible to deduce which processes were involved in the **Anisakis**\blue whiting relationship.

Van Banning and Becker (1978) stated that the possibility of some herring feeding on heavily infected individual euphausiids, or in areas where euphausiids were heavily infected, leading to high numbers of **Anisakis**, while other fish had low numbers of nematodes can be ruled out. Their evidence for this was based on the observations of Smith (1971), who observed multiple infections in euphausiids to be rare and found that prevalences of infected euphausiids in the northern North Sea, although reaching 4% in individual areas, was often lower. Differences in individual food selectivity, whereby some fish eat many invertebrate hosts, and others eat none, was also considered an unlikely explanation for the observed frequency distribution. Van Banning and Becker (1978) suggested that "mixing" of specimens from a stock of heavily infected herring with a stock in which infection was lower, may explain the skewed distribution.

### 2.4.4 Distribution of Nematodes within Fish

#### 2.4.4.1 Stomach and Intestine

**H.aduncum** was found to be the most common nematode species recovered from the stomachs of cod, haddock and bull

rout, and from the intestines of these species and blue whiting. Fish act as final hosts to **H.aduncum** and adult nematodes in fish are generally found in the stomach and intestine (Berland 1961), therefore the large proportions of **H.aduncum** found in this region are likely to be fourth stage larvae and adults. Appy and Burt (1982) also recovered most adult **H.aduncum** from the intestine of cod, followed by the stomach. Haddock were also found to have a greater percentage of **H.aduncum** in the stomach, whereas blue whiting and bull rout harboured the majority of their **H.aduncum** alimentary tract infections in the intestine. Kusz and Treder (1980) found 2.7% of **T.adunca** (= **H.aduncum**) in the alimentary tract of blue whiting, with the majority of these in the stomach. In the present study, approximately 2% of total **H.aduncum** were also found in the stomach, however approximately 6% were found in total in the digestive tract.

Berland (1980, in Berland 1991) stated that adult **H.aduncum** is thought to penetrate into food ingested by the fish host, and to break it up, speeding digestion. Many of the fish stomachs containing **H.aduncum**, examined in this study, harboured food items with which the **H.aduncum** present were associated, and indeed often retrieved from. MacKenzie and Gibson (1970) found adult **C.aduncum** (= **H.aduncum**) to migrate in the flounder, where **C.aduncum** was observed to enter the stomach regularly when food was in the gut; Soleim (1976a) also observed that the stomachs of fish only contained **H.aduncum** when food was present.

In addition to being observed in the alimentary tract during the present study, **Anisakis**, **P.decipiens** and **Contracaecum** larvae have previously been reported from the stomach and intestine of fish (Scott 1954, Aria 1969). The presence of these species in the stomach and intestine is likely to be transient; such worms are probably relatively recent arrivals, and have been recovered from these regions prior to penetration into the body cavity. A similar explanation may account for the high proportion of **A.simplex** recovered from the stomach of blue whiting, as these feed

primarily on euphausiids - a source of **A.simplex** infection. In addition, only 6% of the **H.aduncum** recovered were found in the alimentary tract of this species.

### 2.4.4.2 Body Cavity and Viscera

#### 2.4.4.2.1 A.simplex

In the body cavities of cod and bull rout, **A.simplex** were most commonly recovered from the pyloric caeca, followed by the liver and mesenteries. In haddock and blue whiting, the liver was the site from which most **A.simplex** were retrieved, followed by the pyloric caeca and mesenteries. **A.simplex** in the body cavity of long rough dab occurred most frequently in the mesentery followed by the liver. In cod, haddock and blue whiting, **A.simplex** occurred in every organ; in bull rout, only the kidney was found to be free of infection by this species. The gonads, and kidney of each fish species, were however, only lightly infected with **A.simplex**.

Cannon (1977) and Berland (1961) found **Anisakis** larvae in fish to be particularly common in the mesenteries, on the outside of the alimentary tract and in the pyloric caeca. **A.simplex** larvae in herring were recovered mainly from the mesentery and pyloric caeca (Myjak **et al.** 1991).

Unlike the results obtained in the present study, **Anisakis** in cod examined by other authors appears to have been recovered most commonly from the liver eg. McClelland **et al.** (1983a,b), Myjak **et al.** (1991); followed by the pyloric caeca (McClelland **et al.** 1983a,b). The opposite was observed for **A.simplex** in cod examined during the present study. However, in cod $\geq$ 71cm in length, McClelland **et al.** (1983a) did find **Anisakis** larvae to occur most frequently on the pyloric caeca followed by the liver. Wootten (1978) recovered the majority of **Anisakis** larvae from the

musculature of small cod, however, in the body cavity **Anisakis** were found most frequently in the liver. Appy and Burt (1982) recovered **A.simplex** larvae most frequently from the mesentery of cod. The mesentery was the third most common site from which **A.simplex** were retrieved from during the present study.

In agreement with the present study, Grabda (1978), Smith and Wootten (1978b) and Kusz and Treder (1980) found **A.simplex** to occur most commonly on the liver of blue whiting; whilst Smith (1974) also found **Anisakis**, in 10 haddock examined, to occur most frequently in the liver, followed by the pyloric caeca. Kusz and Treder (1980), however, found that the mesentery was the next most commonly infected organ in blue whiting, followed by the pyloric caeca; the opposite was true in the present study. These authors also stated that virtually every organ in blue whiting was infected with **A.simplex**.

McClelland **et al.** (1983a) found **A.simplex** in long rough dab to occur most commonly in the liver, followed by the mesentery; the opposite was observed in long rough dab examined during the present study.

#### 2.4.4.2.2 P.decipiens

The majority of **P.decipiens** were recovered from the flesh of cod and bull rout. However, in the body cavity and viscera, larvae were found to occur most frequently on the liver and pyloric caeca of cod and in the pyloric caeca and liver of bull rout. In both haddock and blue whiting, **P.decipiens** occurred most commonly on the liver, followed by pyloric caeca, with haddock also harbouring an equal number of **P.decipiens** in the mesenteries and the liver. No infections by this species were observed in the mesenteries of blue whiting. **P.decipiens** were absent from the kidneys of all fish examined, with the exception of cod; and occurred only in the gonads of cod and bull rout. Infections in the

kidney and gonads were low, consisting of one larvae from one fish, for all species examined.

Cannon (1977) found **Terranova** (= **Pseudoterranova**) larvae in fish within the connective tissue and mesenteries of the viscera. McClelland **et al.** (1983a,b) recovered **P.decipiens** primarily from the musculature of cod from Canadian Atlantic waters. Similarly, Appy and Burt (1982) found **P.decipiens** in cod to occur most commonly in the flesh, followed by the mesentery.

#### 2.4.4.2.3 C.osculatum

In all fish species examined, 78% (blue whiting) to 86% (cod) of **C.osculatum** occurred in the liver. Larvae were also found in the pyloric caeca of all fish examined, and in the mesenteries of all except haddock. None were recovered from the kidneys of any fish examined, and only one cod out of 42 examined harboured a single larva on the gonad.

Smith and Wootten (1984c) and Möller and Anders (1986) stated that **Contracaecum** larvae were found particularly in the liver of fish and the findings of the present study agree with this. MacKenzie (1979) also found **C.osculatum** larvae on the liver surface of blue whiting, and Smith (1974) and Myjak **et al.** (1991) observed **Contracaecum** larvae mainly in the liver of haddock and cod respectively. However, in cod from Canadian Atlantic waters, Appy and Burt (1982) recovered **Contracaecum** most frequently from the mesenteries, and McClelland **et al.** (1983a) retrieved 96% of this species from the pyloric caeca, with only 0.5% from the liver.

#### 2.4.4.2.4 H.aduncum

During the present study, most larval **H.aduncum** recovered from all fish species were found on the pyloric caeca. Larvae were also retrieved from the mesentery and

liver in all fish species. This species was rarely recovered from the gonads and kidney of fish, and was completely absent from the gonads of bull rout and haddock, and the kidneys of all but blue whiting.

**H.aduncum** were recovered most frequently from the pyloric caeca of cod, followed by the mesentery and liver. In Baltic cod, **C.aduncum** (= **H.aduncum**) larvae were most often found in the liver, followed by pyloric caeca then mesentery; larvae were also observed in the gonads (Grabda 1976a), as they were in cod during this study. Myjak **et al.** (1991) found **Hysterothylacium** larvae mainly in liver of cod, and Appy and Burt (1982) reported larval **Hysterothylacium** from the mesentery of cod in Canadian waters. In the blue whiting examined most **H.aduncum** were found on the pyloric caeca, mesentery and liver. Kusz and Treder (1980) found a similar distribution in blue whiting. Grabda (1978) also found most **T.adunca** larvae on the pyloric caeca of blue whiting, with larvae also observed in the liver and between the viscera.

### 2.4.4.3 Distribution of A.simplex and P.decipiens between Viscera and Musculature

The percentage distribution of larval **Anisakis** and **P.decipiens** in the musculature and viscera differ greatly between fish species. In whiting, Wootten and Waddell (1977) found over half the total **Anisakis** burden in the musculature, rather than in the viscera; in cod, less than 12% of the total **Anisakis** larvae present occurred in the musculature of fish >30cm in length. However, differences were related to the size\age of the host, as in young cod <30cm in length, more than 40% of the **Anisakis** burden occurred in the flesh (Wootten 1978). In Canadian Atlantic waters, McClelland **et al.** (1983a,b) found approximately 2-3% of **Anisakis** in the musculature of cod and Palsson **et al.** (1985) found 25% in the musculature of Icelandic cod. During this study, 91% of **A.simplex** larvae were recovered from the viscera of cod, and

9% from the musculature. Approximately 12% of **Anisakis** were found in the musculature of blue whiting; this compares to approximately 20% found in the flesh of this species by Smith (1984e), and 4.6% found in the in muscle by Kusz and Treder (1980). Grabda (1978) also found the majority of **A.simplex** to occur in the body cavity of blue whiting. Wootten (1978) found approximately 6% of **Anisakis** larvae to occur in the musculature of haddock, 11% were found in this region in haddock examined during the present study. All **A.simplex** were recovered from the body cavity and viscera of long rough dab. McClelland **et al.** (1983a) also found virtually all **Anisakis** larvae in **H.platessoides** to occur in the body cavity of fish. Bull rout examined in this study also harboured the majority of their **A.simplex** burden in the body cavity.

In fish, third stage larvae of **P.decipiens** normally migrate to the musculature and are found less frequently in the body cavity (Kahl 1939, Margolis 1977). McClelland **et al.** (1983a,b) found approximately 97% of **P.decipiens** to occur in the musculature of cod from Canadian Atlantic waters and Palsson **et al.** (1985) found approximately 94% in the musculature in Icelandic cod. In both cod and bull rout examined during the present study, 59% and 71% respectively of the **P.decipiens** burden (excluding those in the digestive tract) occurred in the musculature. In haddock and blue whiting, the great majority of **P.decipiens** occurred in the viscera.

## Association between Infections in the Viscera and Musculature

Wootten and Waddell (1977) found no evidence of association between the levels of **Anisakis** infection in the viscera and musculature of cod, but a positive correlation was found between the numbers of **Anisakis** in the musculature and viscera of whiting. Positive associations between infections of **Anisakis** larvae in the viscera and in the flesh were also observed in blue whiting (Grabda 1978) and in

herring (Smith and Wootten 1975, McGladdery 1986). Kahl (1939) noted that in some fish, such as mackerel and herring, **Anisakis** larvae occurred only in the muscles when there was a high rate of infection.

McGladdery (1986) suggested that the number of **Anisakis** in the viscera may effect the establishment of infections in the muscle, however, Boczon **et al.** (1989) found no correlation between the intensity of muscle and visceral infection of **A.simplex** in Baltic herring, finding larvae in the muscles even when the intensity of visceral infection was low. Correlations of **A.simplex** and **P.decipiens** between the viscera and musculature of the fish examined during the present study were generally weak and did not indicate a strong relationship between numbers of these larvae in the viscera and in the musculature. In herring, Davey (1972) found a small but constant proportion of **Anisakis** larvae in the musculature of all length groups examined, and considered that the tendency for each **Anisakis** larva to penetrate the musculature was constant and independent of other factors such as length and age of host, or numbers of other larvae present in the same host. This may also be the case for **A.simplex** and **P.decipiens** in the fish examined during the present study.

#### 2.4.4.4 Musculature

During the present study, only **A.simplex** and **P.decipiens** were found to occur in the musculature of the fish examined; these were recovered from the musculature of all fish species with the exception of long rough dab. Small numbers of **H.aduncum** have been recorded previously from the musculature of cod (McClelland **et al.** 1983a, Palsson **et al.** (1985), and bull rout (Kerstan 1991); McClelland **et al.** (1983a) also recovered 0.1% of the total **Contracaecum** sp. larvae from the flesh of cod in Canadian Atlantic waters.

#### 2.4.4.4.1 Relative Proportions of A.simplex and P.decipiens in the Musculature

During the present study, the majority of nematodes found in the musculature of cod, haddock and blue whiting were **A.simplex**. 66.2% of nematodes in the musculature of cod examined during the present study were **A.simplex**. In British waters, 39.8% of all larvae recovered from cod flesh by Young (1972) were **Anisakis** sp.. Wootten and Waddell (1974, 1977) found infection by **P.decipiens** and **A.simplex** in the musculature of cod from Scottish waters to vary in different areas. In the Wadden Sea, Kerstan (1991) found **A.simplex** to comprise 79% of the nematodes present in commercial cod fillets, although **P.decipiens** was the most dominant nematode in the fillets of juvenile cod.

Grainger (1959) and Palsson **et al.** (1985) found **P.decipiens** and **A.simplex** to be present in approximately equal numbers in fillets of cod from Icelandic waters. In Canadian Atlantic waters, **P.decipiens** appears to greatly outnumber **Anisakis** in cod fillets eg Templeman **et al.** (1957), Scott and Martin (1959), McClelland **et al.** (1983a), although there were regions where **Anisakis** was most abundant.

Most of the nematodes found in haddock fillets during the present study were **A.simplex**, the remainder **P.decipiens.** Templeman **et al.** (1957) found 75% of the nematodes in the fillets of haddock from Canadian Atlantic waters to be **P.decipiens.**

Kerstan (1991) found **P.decipiens** to be the dominant nematode in the fillets of bull rout, as found in this study.

#### 2.4.4.4.2 Distribution between Fillets and Flaps

**A.simplex**

Young (1972), Platt (1975), Smith and Wootten (1975), Wootten and Waddell (1977), Wootten (1978) and Arthur **et al.** (1982) found the majority of **Anisakis** larvae in the

musculature of cod, whiting, herring, blue whiting and walleye pollock to be situated in the flaps (hypaxial musculature surrounding the body cavity). However, during the present study, **A.simplex** were observed to be distributed equally between the flaps and the fillets of cod.

**P.decipiens**

In cod (Young 1972, Platt 1975, Wootten and Waddell 1977, McClelland **et al.** 1983a,b) and walleye pollock (Arthur **et al.** 1982) found larval **P.decipiens** in the musculature to occur in both the fillets and flaps; Young (1972) found numbers in each region to be approximately equal, but Platt (1975), Wootten and Waddell (1977) and McClelland **et al.** (1983a,b) found a larger proportion in the fillets. However, Young (1972), Platt (1975), and Wootten and Waddell (1977) found infections of **P.decipiens** in larger cod to be higher in the flaps. In the present study, 89% of **P.decipiens** in the musculature of bull rout were retrieved from the fillets, however, 61% of **P.decipiens** in the flesh were of cod were recovered from the flaps.

### 2.4.4.4.3 Distribution between Left and Right Fillets

Unpublished work by J.W.Smith has suggested that there is a clear difference in the numbers of nematodes retrieved from left and right fillets. Cutting and Burgess (1960) also stated that there appeared to be more worms in the left fillet of fish than in the right fillet. Kerstan (1991) observed that, in addition to cod, saithe and redfish harboured approximately twice as many nematodes in their left fillets than in their right. However, Arthur **et al.** (1982) found no significant differences in infection of either **Anisakis** or **P.decipiens** between the left and right musculature of walleye pollock. In the present study, of those nematodes in the musculature, the percentages of **A.simplex**, **P.decipiens** and total nematodes (musculature only)

in left and right fillets were generally approximately equal. However, cod harboured 20% more **P.decipiens** in the left than right fillet, and blue whiting 24% more of the total nematodes in the flesh in the left fillet.

### 2.4.4.5 Comparisons of site distribution of nematodes in cod with the results of Palsson et al. (1985).

Apart from the area examined, the results of Palsson **et al.** (1985), on cod in Icelandic waters are directly comparable with the results obtained during this study for the distribution of the four species of larval nematodes in different regions within the body of the host. In addition, the average length of cod examined by Palsson **et al.** was 52.5cm; that of cod examined during the present study, 50.6cm. Palsson **et al.** examined two samples of cod, which have been pooled here for simplicity of comparison.

The data obtained during the present study was grouped to be comparable to that of Palsson **et al.**: nematodes from the alimentary tract were not included in the analysis; numbers of larvae in the mesentery, gonads and kidney were pooled together under the "Other Organs \ Abdominal Cavity" category of Palsson **et al.**; and the results from left and right musculature were combined. Table 15 shows the comparative data for the percentage distribution of each nematode species within different regions of the cod, and the percentage distribution of different nematode species in individual regions of the host.

Palsson **et al.** found **A.simplex** to be generally distributed evenly between the musculature, liver and pyloric caeca. During the present study, over 50% of **A.simplex** were recovered from the musculature of cod, with even distributions being seen between the liver and the rest of the body cavity. Over 90% of **P.decipiens** occurred in the musculature of Icelandic cod, with few (0.8) in the liver; in Scottish waters, approximately 59% occurred in the

TABLE 15: COMPARISON OF DISTRIBUTION OF ASCARIDOID NEMATODES IN COD, WITH THE RESULTS OF PALSSON ET AL. (1985)

1] % DISTRIBUTION OF INDIVIDUAL NEMATODE SPECIES

| | Anisakis | Pseudoterranova | Contracaecum | Hysterothylacium |
|---|---|---|---|---|
| Muscle | 9.1 (24.4) | 59.2 (93.3) | - | 0 (0.2) |
| Liver | 19.6 (30.2) | 16 (0.8) | 88.2 (3.7) | 3.2 (3.7) |
| Pyloric caeca | 53.1 (30.6) | 15.4 (4.1) | 10 (76.8) | 83.1 (75.2) |
| Body cavity | 18.2 (14.7) | 9.5 (1.8) | 1.8 (19.6) | 13.7 (21) |
| (excl. alimentary tract) | | | | |
| TOTAL | 100 (100) | 100 (100) | 100 (100) | 100 (100) |

2] % DISTRIBUTION OF NEMATODES IN DIFFERENT HOST REGIONS

| | Anisakis | Pseudoterranova | Contracaecum | Hysterothylacium | Total |
|---|---|---|---|---|---|
| Muscle | 66.2 (46.8) | 33.8 (52.8) | - | 0 (0.4) | 100 (100) |
| Liver | 28.8 (86.8) | 7.1 (0.6) | 62.3 (2) | 1.8 (10.6) | 100 (100) |
| Pyloric cecae | 55.9 (25.3) | 4.9 (0.9) | 5.1 (10) | 34.2 (63.8) | 100 (100) |
| Body cavity | 66.7 (36.4) | 10.5 (1.3) | 3.3 (7.5) | 19.6 (54.8) | 100 (100) |
| (excl. alimentary tract) | | | | | |

(Palsson et al. (1985) in brackets)

musculature with 16% in the liver. Palsson **et al.** recovered only 3.7% of **Contracaecum** from the liver of cod, with approximately 77% in the pyloric caeca; during this study 88% of **Contracaecum** occurred in the liver, and 10% from the pyloric caeca. The distribution of **Hysterothylacium** was similar in both Icelandic and Scottish cod, with the exception being that Palsson **et al.** did recover a small number of **Hysterothylacium** from the musculature.

Palsson **et al.** observed **P.decipiens** to be the most common nematode in the musculature, whereas in Scottish cod, **A.simplex** was more common. In the liver, 87% of the nematodes recovered from Icelandic cod were **A.simplex**, with **Contracaecum** accounting for only 2%; during the present study, the majority of nematodes within the liver were **Contracaecum** (62%), with **A.simplex** comprising 29%. Similarly, in Icelandic waters, **Hysterothylacium**, followed by **A.simplex**, were the most common nematodes in the pyloric caeca and remainder of body cavity; in Scottish waters the opposite was observed.

#### 2.4.4.6 Reasons for Distribution of Nematodes within Fish

The differing sites of infection of third stage larval nematodes in fish result from migrations carried out by the larvae after penetration through the gut.

Smith (1990) related the topography of the visceral organs of cod (in particular the stomach, pyloric caeca and liver) to the locations occupied, and distributions in the flesh, by larval ascaridoids. Cutting and Burgess (1960) also suggested that the larger numbers of parasites in the left filet of fish was due to the asymmetry of the digestive tract. Scott (1954) considered the spatial arrangements of the fillets and flaps in relation to the body cavity of cod, and stated that most **P.decipiens** larvae must enter the flaps before the fillets can be reached. This applies equally well to **A.simplex** larvae. Larvae in the flaps will thus generally

be of a more recent arrival than those in the fillets (Scott 1954). Several authors have attempted to explain the distribution pattern of larval **Anisakis** and **P.decipiens** in the musculature and viscera of fish. Young (1972) suggested that the distribution of these species may be explained if larvae are assumed to have an optimum distance which they will travel through tissues of the host. This author thus assumed **Anisakis** sp. to have a shorter penetration range than **P.decipiens**. This would certainly explain the observation of the present study, that **A.simplex** larvae were more common in the body cavity and viscera of fish than in the musculature. However, the fish examined did not have higher infections of **A.simplex** in the flaps, as compared to the fillets. If penetration into the musculature is indeed unrelated to the number of larvae in the body cavity, then infection of the fillet may occur at an early stage, when the fish is smaller, and the distance between the alimentary tract and fillet is less than when the fish is larger. It may be significant that Wootten (1978) found 40% of the total **Anisakis** burden in small cod in the musculature. As the fish increases in size, **A.simplex** may only migrate as far as the flap, and this may account for the equal numbers of **A.simplex** observed in the fillets and flaps of cod. It is also relevant to note that Smith and Wootten (1975) and Wootten and Waddell (1977) found a distinctly greater proportion of larval **Anisakis** to occur in the fillets of whiting and herring respectively as compared to cod (Wootten and Waddell 1977). Wootten and Waddell (1977) presumed that larval **Anisakis** could penetrate relatively further into the musculature of smaller fish such as whiting and herring compared to larger cod. This seems likely to be the case in the cod, blue whiting and bull rout examined during the present study - the blue whiting and bull rout examined being an average of 27 and 28cm in length respectively, that of cod approximately 51cm. With the exception of long rough dab, cod harboured the lowest proportion of **A.simplex** in the musculature, and this too may be a result of its larger size, whereby fewer **A.simplex**

larvae can reach the muscle.

Young (1972) suggested that a larger penetration range of **T.decipiens** (= **P.decipiens**) larvae will allow more larvae to reach the fillets (as observed in cod during the present study), but as the fish increase in size, infection increases in the flaps as larger numbers of larvae fail to reach the fillets. Again this would explain the higher proportions of **P.decipiens** observed in the musculature, as compared to the viscera, of cod and bull rout; and also explains the higher proportion of **P.decipiens** found in the flaps of cod, as compared to the higher proportions found in the fillets of bull rout and blue whiting. McClelland **et al.** (1983b) also found that infections in the flaps and body cavity of cod were most common in large fish. 41% of **P.decipiens** were recovered from the viscera of cod, as compared to 29% in bull rout - again this may be due to the smaller size of bull rout, allowing a larger proportion of larvae to reach the musculature.

Third stage larvae of **C.osculatum** and **H.aduncum** may have a shorter optimum distance for migration than **A.simplex** or **P.decipiens**, which would explain their rare occurrence, and in the present study, complete absence, from the flesh of fish - being restricted to the viscera and body cavity. Alternatively, fish musculature may be an unsuitable physiological habitat for **C.osculatum** and **H.aduncum**.

Smith (1984e) stated that differences in the locations of third stage larvae within fish might be explained by examining the relationship between the sites of penetration of larvae from the alimentary canal into the body cavity, and the spatial arrangement and contiguity of organs in the various fish species. Wootten and Smith (1975) experimentally infected trout with **Anisakis** larvae and observed penetration to occur in the stomach, pyloric caeca and anterior intestine sequentially over time, as worms passed down the gut; larvae were never observed penetrating the posterior part of the intestine. These authors stated that the distribution of larvae in trout reflected the site of penetration through the

anterior regions of the gut, as most larvae were found in the body cavity along the length of the alimentary tract. Smith (1974) observed **Anisakis** larvae penetrating the stomach wall and pyloric caeca of haddock and whiting, and Scott (1954) stated that larvae of **P.decipiens** penetrated the stomach wall of cod. All four species of nematode examined during the present study were recovered from both the stomach and intestine of fish. The four nematode species were recovered from the mesentery, pyloric caeca and liver of cod, haddock, blue whiting and bull rout, and **A.simplex** from the liver and mesenteries of long rough dab. In all fish species examined, most **A.simplex**, **P.decipiens** and **H.aduncum** larvae were generally recovered from either the pyloric caeca or liver. This is likely to reflect the areas through which these nematodes have penetrated - many of those which have penetrated through the stomach may be the specimens which have been recovered from the liver; which is located immediately beneath the stomach and is the first organ parasites reach after penetration through the stomach. The nematodes found on the pyloric caeca may have penetrated through this region. In all fish examined, third stage larvae of **H.aduncum** were recovered most frequently from the pyloric caeca, and this may be the preferred site of penetration for this species.

In the case of **C.osculatum**, over 78% were found in the liver of all fish species examined. Although the data do not indicate that more **C.osculatum** occur in the stomach than in the intestine, it may be that larvae of this species mostly penetrate through the stomach, and this, along with a potentially short migration distance of this species, leads to accumulations of this parasite in the liver. Alternatively, it possible that this species has some physiological preference for this organ. It is relevant to note that larvae of this species in the liver were generally recovered from within the liver parenchyma, whereas larvae of the other nematode species were rarely found in this area of the liver, being most commonly observed on the surface of

this organ, under the liver capsule. Infections of nematodes in the gonads and kidney of all fish species were generally low. This may reflect the position of these organs in the fish or their suitability as a site of encystment.

Thus the distribution of third stage larvae within fish may depend both on the location of penetration through the alimentary tract, and on the optimum distance which larvae can migrate, as well as particular site preferences.

### 2.4.5 Competitive Effects

Analysis of the percentages of single species infections and mixed infections of two or more species, revealed that in cod, blue whiting and bull rout, the majority of infections observed were of third stage larvae of all four parasite species. In haddock, both infections of all four species, and infections of **A.simplex** + **C.osculatum** only were the commonest type seen. Long rough dab harboured infections of **A.simplex** only. In areas where both **Anisakis** and **Phocanema** (= **P.decipiens**) occurred in cod, Wootten and Waddell (1974, 1977) found **Anisakis** and **Phocanema** to rarely occur together in the musculature of the same individual. During the present study, 42.4% of cod did harbour infections of both **A.simplex** and **P.decipiens** together in the musculature, with 48.5% having **P.decipiens** only. Over 50% of bull rout also had mixed infections of these species in the musculature, with 43.5% with **P.decipiens** only.

Eight negative correlations between different species of third stage larvae (totals and body cavity only) were found, and these were generally weak. The remaining positive correlations varied from 0.02 to 0.8 (the latter for total **A.simplex** + **H.aduncum** in bull rout). Correlations between **A.simplex** and **P.decipiens** in the musculature were all positive, but again generally weak. Wootten and Waddell (1977) found no general evidence of either positive or negative associations between **Phocanema** and **Anisakis** in cod

musculature. Although all eight negative correlations (total and body cavity only) observed in the present study involved **H.aduncum**, and three of these occurred in both blue whiting and haddock, this trend was not regular over all fish species examined eg. the correlation between total **A.simplex** + **H.aduncum** in cod was fairly strong, yet it was negative in both blue whiting and haddock; two of the negative correlations were only observed in blue whiting.

McClelland **et al.** (1985) speculated that **P.decipiens** infection in fish may be mitigated by the influence of **C.osculatum**, which appears to compete with and displace other anisakine sp. in the alimentary tract of seals (Berland 1963), adding that grey seals in the Gulf of St. Lawrence which are heavily infected with **C.osculatum** had significantly fewer **P.decipiens** than seals from the Scotian shelf where **C.osculatum** is uncommon. Correlations calculated during the present study revealed no strong associations between these two species in any of the fish examined.

The method of acquisition, and the location and behaviour of these third stage larvae in fish is not consistent with the theory of competitive inhibition. Larvae enter the fish within food items, and penetrate through to the body cavity. Once through, premunition (the ability of an existing infection to prevent reinfection) cannot take place, as the larvae are already within the body cavity and cannot leave until death of the host. Unless ascaridoids, especially **H.aduncum**, can actively "eliminate" other parasite species (eg. by secreting antigens which adversely effect the other species present, as observed by Durborow **et al.** 1988 in tapeworm\acanthocephalan infections in the intestines of largemouth bass), then premunition seems unlikely to occur in this system. However, it is possible that competitive displacement occurs, whereby the presence of **H.aduncum**, and potentially other species, in one region of the fish may effect the establishment, and cause a reduction in the numbers, of other species in that region. Most studies documenting competitive displacement of parasites have

concerned helminths in the alimentary tract eg. Chappell (1969) observed partial spatial separation of the acanthocephalan **Neoechinorhynchus rutili** and the cestode **Proteocephalus filicollis** in concurrent infections in stickleback, with the intensity of infection of each species remaining unaltered. Such an effect may influence the site distribution of parasites within the fish examined during the present study, whereby the presence of one species of ascaridoid causes displacement of other species to other organs.

Holmes (1961) stated that the intraspecific competition resulting in a limitation of the distribution of **Hymenolepis diminuta** (Cestoda) and **Moniliformis dubius** (Acanthocephala), in the intestine of rats, was for food. Third stage larvae in fish are not thought to feed, and are therefore unlikely to compete for this resource. However, overcrowding, leading to competition for space, may occur. McGladdery (1986) previously suggested that the absence of muscle infections in herring with less than 15 **A.simplex** in the body cavity may be due to intraspecific crowding in the body cavity, however, over 50% of herring infected with more than 15 **A.simplex** in the body cavity had no muscle infections. Certainly, the correlations for the numbers of **A.simplex** and **P.decipiens** in the musculature against the numbers of both their own species and **C.osculatum** and **H.aduncum** in the body cavity, observed during the present study, do not show any strong positive correlations to indicate that as numbers of parasites in the body cavity increase, nematodes are displaced to the musculature. Competition for space in the body cavity of fish seems unlikely, given that all regions of the body cavity can potentially be occupied, although it is possible that such competition would occur under conditions of extremely heavy infestation.

Initial analysis suggests that no competitive effects are occurring between different parasite species, although correlations were not calculated for the four nematode species in individual organs of the body cavity. Thomas

(1964) found little evidence of interspecific competition between helminths in the alimentary tract, gills and urinary bladder of brown trout. Thomas explained the positive correlations between some, by stating that infective stages have overlapping distributional areas. This is also the case for third stage ascaridoid larvae observed in fish during the present study.

The occurrence of two or more congeneric species of helminths in the same host population and individual is fairly common in nematodes, digeneans and cestodes (Holmes 1973, Dobson 1985), and this is certainly true for marine ascaridoids in fish. Kennedy and Moriarty (1987) also demonstrated that congeneric acanthocephalans can co-exist in fish without obvious interactions. However, it is relevant to note that Dobson (1985) stated competing species may co-exist if each is overdispersed and the probability of an individual host harbouring large numbers of each is very low. In the case of the fish examined during the present study, the populations of nematodes were generally overdispersed, and in cod and haddock over 50% of third stage larvae recovered were **A.simplex**. In blue whiting, large numbers of both **A.simplex** and **H.aduncum** third stage larvae were recovered, and all correlations involving **H.aduncum** in this species were negative.

It would generally appear that the correlations observed are a reflection of the numbers of parasites, of different species, which accumulate in the fish over time rather than a result of competitive interactions, eg. where one parasite generally increases in number and the other occurs in irregular numbers in fish. This in itself can be related to the feeding habits of the fish. For example, blue whiting will accumulate **A.simplex** through heavy feeding on euphausiids but may have variable numbers of other parasites depending on the other food items which individual fish have eaten; the correlations observed in cod and bull rout were all positive, although generally weakly so, and can be attributed to their broad diet over their life cycle, whereby

parasites of all species are acquired but some will occur more commonly in their diet than others. Similarly, the low percentage of certain mixed infections of parasites in fish, and differences in the proportion of individual parasites recovered from different fish species can be explained by diet rather than competition. Wootten and Waddell (1977) found it difficult to explain why larval **Phocanema** and **Anisakis** rarely occurred together in the musculature of the same cod and suggested that differences in feeding habits of individual cod may provide an explanation.

## 2.5 CONCLUSIONS

**A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** were found to occur in cod, haddock, blue whiting and bull rout. Only **A.simplex** were observed in long rough dab. All fish examined were infected with these ascaridoids with the exception of long rough dab; 100% prevalence of **A.simplex** and **H.aduncum** occurred in cod, with blue whiting also having 100% prevalence of **A.simplex**. Numerically, in cod, haddock and blue whiting, **A.simplex** and **H.aduncum** were found to be the most common species; in bull rout, numbers of the four nematode species were approximately equal. Differences in the infection levels of the four parasite species were observed between individual fish species, and these can be attributed to differences in the diet and habitat of each fish species, in addition to the life cycles of the individual parasite species.

The frequency distributions of individual parasite species, and the total numbers of all worms recovered were generally overdispersed in fish.

The proportion of each parasite species in individual organs, and the percentage distribution of individual nematode species in different regions of the fish, differed between fish species. Third stage larvae of **A.simplex**, **P.decipiens** and **H.aduncum** in the body cavity of fish were

most frequently found in the liver or pyloric caeca in all species of fish; over 78% of **C.osculatum** were retrieved from the liver in all fish species examined. Only **A.simplex** and **P.decipiens** were recovered from the musculature. A higher proportion of **A.simplex** occurred in the viscera and body cavity of each fish species compared to the musculature. **P.decipiens** was found to be more frequent in the musculature, rather than the viscera and body cavity, of cod and bull rout. There was no strong evidence to suggest an association between the numbers of **A.simplex** and **P.decipiens** in the body cavity and in the musculature. Variations were also observed in the distribution of **A.simplex** and **P.decipiens** between the fillets and the flaps, with the former parasite recovered more frequently from the fillets of blue whiting and bull rout, with an equal distribution between the fillets and flaps in cod. **P.decipiens** were found more commonly in the fillets of bull rout, and in the flaps of cod. Variations in the site distribution of third stage larvae in each fish species are likely to be a result of differences in the site of penetration through the alimentary tract, and the optimum distance over which each species of larvae can migrate. **C.osculatum** and **H.aduncum** are presumed to have a lower optimum distance of penetration than **A.simplex** and **P.decipiens**, allowing the latter two species to migrate as far as the musculature, but not the former two. It is possible that **C.osculatum** also show a predilection for the liver.

A preliminary study for competitive interactions between nematode species revealed no strong evidence to suggest that interactions were occurring between different species, with mixed infections of all four nematode species being the most frequent type of infection observed in all fish species, with the exception of long rough dab, and correlations between nematode species in fish generally showing no strong negative associations. The correlations are thought to be a reflection of the feeding habits, and thus numbers of individual parasite species acquired, by the fish.

# CHAPTER THREE : THE PATHOLOGY OF FISH STOMACH LESIONS ASSOCIATED WITH LARVAL A.SIMPLEX AND P.DECIPIENS

## 3.1 INTRODUCTION

### 3.1.1 General Pathology of Marine Ascaridoids in Fish

The pathology of marine ascaridoids in teleost fish has previously been described in a number of fish species. Kahl (1938a) observed inflammation of the musculature of fish caused by the movements of nematodes, and discussed tissue changes in fish infected with **P.decipiens**. Ramakrishna and Burt (1991) examined the tissue response to invasion by **P.decipiens** larvae in rainbow trout, noting an inflammatory reaction and capsule formation around the larvae. The majority of larvae were recovered in the musculature, where tissue destruction, damage to blood vessels, muscle necrosis with haemorrhages and infiltration of inflammatory cells was seen. Smith and Wootten (1984a,b,c) stated that **P.decipiens**, **Anisakis** and **Contracaecum** sp. were of little significance as pathogens of fish. However, muscle infection with **P.decipiens** larvae has been found to significantly reduce swimming speed in smelt (**Osmerus eperlanus**) by up to 32.2% (Sprengel and Lüchtenberg 1991), which may make infected fish more susceptible to predation, capture by fishing methods or adversely effected in terms of feeding behaviour.

A number of authors have described lesions in various species of fish infected with **Anisakis** larvae eg. Kahl (1938b) and Margolis (1970). Boczon **et al.** (1989) observed that **A.simplex** in fish muscle was associated with degenerated muscle cells. Hauck and May (1977) described histopathologic alterations associated with **Anisakis** larvae in various organs of Pacific herring, observing mechanical compression of the pancreas and liver, inflammation of the liver, and mechanical injury to the exterior of the pyloric caeca. These authors did not, however, observe gross lesions.

The tissue response to liver infection by marine ascaridoid larvae has been described histologically by several authors eg. Kahl (1938b), Petrushevsky and Shulman (1955), Ramadan **et al.** (1981), Elarifi (1982). Poole and Dick (1984) examined the liver pathology of larval **Raphidascaris acus** in yellow perch, and observed encapsulation of the parasite by host tissue, leading to eventual death of the worm. Sindermann (1966, 1970) stated that larval nematodes which invaded the liver of gadoids, disrupted normal function and often caused extensive atrophy. A number of Russian authors (summarised in Sindermann 1966, 1970) noted that heavy infection of cod liver by larval **C.aduncum** caused loss of body weight and liver mass, with liver fat and oil content also significantly reduced.

Rosenthal (1967) reported mortalities in larval herring likely to have been caused primarily by infections of larval **Contracaecum** sp.. Möller and Anders (1986) found a distinct decrease in the condition factor of smelt, older than two years, infected with **P.decipiens**. Palsson **et al.** (1985) however, observed a positive relationship between condition factor of cod, and infection by **P.decipiens**.

Sindermann (1970) stated that adult nematodes in fish appear to do little damage to hosts than larval worms. **H.aduncum** is, apparently, not important as a pathogen of fish (Berland 1991), and infection of wild fish is of little significance, with only one possible report of mortality in salmonids being known (Berland 1991).

Margolis (1970), Cheng (1976), and Smith and Wootten (1978a) reviewed aspects of the pathology of nematodes within fish.

### 3.1.2 Stomach Lesions Associated with Marine Nematodes

#### 3.1.2.1. Birds and Mammals

Stomach lesions, caused by penetration of marine anisakids into the stomach wall, appear to be relatively common in mammalian hosts eg. Vik (1964), Migaki **et al.** (1982), Lambertsen and Kohn (1987) and Smith (1989) reported infections with **Anisakis** spp. in the digestive tracts of cetaceans; Wilson and Stockdale (1970), Liu and Edward (1971), Bishop (1979) described lesions associated with **Contracaecum** sp. in pinnipeds. Lesions associated with **P.decipiens**, **Anisakis** and **Contracaecum** were described from both experimentally and naturally infected harbour and grey seals by McClelland (1980c). King (1983) also stated that anisakines in the stomachs of seals attach to the walls and cause inflamed and ulcerous lesions. Young and Lowe (1969) demonstrated an association between stomach lesions and anisakine nematodes in both grey seals and harbour porpoises. Reactions caused by marine ascaridoids have also been described from the digestive systems of free living birds (Riley 1972, Liu and Edward 1971) and experimentally infected laboratory mammals, including rats (Young and Lowe 1969, Gibson 1970, Deardorff **et al.** 1983), mice (Deardorff **et al.** 1991) and rhesus monkeys (Overstreet and Meyer 1981). Gastric and intestinal lesions in humans, associated with infection by anisakids, are well documented in the literature eg. Van Thiel **et al.** (1960), Asami **et al.** (1965), Yokogawa and Yoshimura (1965), Van Theil and Van Houten (1967), Aji **et al.** (1982), Fujino **et al.** (1984), Kikuchi **et al.** (1990).

#### 3.1.2.2 Fish

Stomach lesions associated with nematodes have been reported previously from fish. Arai (1969) reported that nematodes of the genus **Anisakis** were found in three ulcerous

cavities, almost three cm in diameter, in the stomach wall of a single large lingcod, **Ophiodon elongatus**, although an illustration was not given, and the ulcers were not examined histologically. Menezes and Lima (1980) reported heavy infections of larval **Anisakis** sp. in the stomach submucosa of large hake **Merluccius polylepis** from waters off Chile, and Berland (1981, in both Möller and Anders 1986 and Smith 1984e) observed distinct ulcers associated with clusters of **Anisakis** larvae on the mucosal surface of the stomach in some large cod. Lick (1991) found stomach granulomas in twelve species of fish examined from the outer Elbe estuary, with which three species of nematodes were associated - **Cosmocephalus obvelatus**, **Paracuaria tridentata** and **Hysterothylacium** sp. cf. **cornutum**. Obiekezie **et al.** (1992) subsequently examined these stomach wall granulomas in detail, in smelt (**Osmerus eperlanus**). Miyazaki **et al.** (1988) observed ulcers in the gastric mucosa of paddlefish, **Polydon spathula**, caused by larval **H.dollfusi**, and Jilek and Crites (1982b) described the intestinal histopathology of common bluegill, **Lepomis macrochirus**, infected with **Spinitectus carolini**.

Stomach lesions characterised by an infection of anisakid nematodes, were observed in specimens of gadoids and angler fish (**Lophius piscatorius**) caught in Scottish waters. These lesions were found only in fish over 60cm in length, and varied in size from small single worm infections, to large ulcers containing hundreds of nematodes. Such lesions, being distinct and associated with anisakid larvae, have rarely been documented from fish, and therefore a gross and histopathological examination of the ulcers was made. In addition, stomach lesions in angler fish have not previously been noted.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Collection of Ulcers and Normal Stomach Tissue from Fish

#### 3.2.1.1 Gadoids

Stomachs with ulcerous lesions, associated with single and multiple worm infections, were collected from cod (**Gadus morhua**) and saithe (**Pollachius virens**) by Dr. R. Wootten during research vessel cruises in the northern North Sea in 1983 and 1984. Fish were caught by bottom trawl and were over 60cm in fork length. Ulcers were preserved in 10% formalin and some smaller examples were also fixed in Bouins fluid. These ulcers were kept in storage until 1991, when they were re-examined as part of this project.

The stomach of a freshly caught cod, captured during a research cruise in June 1992, was dissected out and examined grossly. No evidence of lesions on the mucosa was observed, and sections of tissue were excised for histological examination.

#### 3.2.1.2 <u>Lophius piscatorius</u>

During a research vessel cruise in June 1992, three specimens of angler fish (**Lophius piscatorius**), caught by bottom trawl from the northern North Sea, were examined routinely for larval ascaridoids. On dissection of the stomachs, small crater-like lesions were observed on the mucosa, each of which were seen to contain at least one large nematode. More than one lesion was found in each individual stomach. These lesions were dissected out, along with surrounding normal stomach tissue and preserved in McKnights fixative (see Appendix 1a), and taken back to the laboratory for further gross and histological examination.

### 3.2.2 Gross Morphology

The gross morphology of normal stomach mucosa from cod and **Lophius piscatorius** was briefly examined, in order to compare the normal appearance of the stomach to ulcerated tissue.

A full description of the gross appearance of the stomach ulcers was made, from gadoids and **Lophius piscatorius**, along with measurements of their fixed size (diameter and height above normal mucosal surface) and, in the case of the large gadoid ulcers, the thickness of the stomach wall. The height was measured from the base of the ulcer to the apex; the base of the ulcer being the point at which it was dissected from the mucosa. In multiple worm lesions from gadoids, basic estimations of the cross sectional area and volume of the ulcers were made, to examine the relationship between size variables. Cross sectional area was calculated by multiplying the length and width of the ulcers. Although the ulcers were roughly circular in shape, and this method of estimating cross sectional area will not give a true indication of actual size, it was useful for comparative purposes. Volume of the ulcers was calculated by multiplying cross-sectional area by the height of the ulcer, again not giving a true indication of actual volume but useful for comparisons. The nematodes present in each ulcer were identified, and their positions within each lesion noted.

### 3.2.3 Assessment of the number of nematodes recovered from large ulcers in gadoids

An attempt was made to assess the number of nematodes present in the large stomach ulcers found in cod and saithe. Nematodes not totally embedded in the stomach wall could be counted quite easily, by their removal from the lesion using a fine forceps. The number of nematodes totally embedded

within the stomach wall itself could not be assessed. Distinction was made between nematodes removed from different areas of the lesion, and also between nematodes which had been recovered alive and dead on fixation of the ulcer. Distinction of "dead" from "live" larvae was determined by the former appearing opaque and flaccid.

### 3.2.4 Histopathological Examination

#### 3.2.4.1 Stomach Ulcers from Gadoids

Large ulcers from gadoid stomachs were cut into longitudinal sections, approximately 5-7mm thick, and processed for histology. All samples were automatically processed in a Shandon Citadel 2000 tissue processor. Single worm lesions were similarly processed. Sections of tissue were embedded in paraffin wax. Due to the tough nature of the ulcerous tissue in large lesions, and the presence of large numbers of nematodes within the stomach wall, blocks were cut to expose the tissue and decalcified in acid decalcifier for up to an hour before section cutting. Longitudinal ulcer sections were cut at 5µm, using a Leitz Wetzlar microtome. Sections were then placed on slides and dried overnight before being stained with both haematoxylin and eosin, and with Van Gieson stain to show collagen (Drury and Wallington 1980).

#### 3.2.4.2 Stomach Ulcers from <u>Lophius piscatorius</u>

Due to their smaller size, whole angler fish lesions were processed for histology as described above. Sections were again cut at 5µm and stained with both haematoxylin and eosin, and Van Gieson stains.

#### 3.2.4.3 Normal Stomach Tissue

Sections of normal stomach tissue, from both cod and angler fish, were similarly processed for histology. Such samples were cut longitudinally, to show the different regions which compose the stomach wall. Sections were cut at 5µm, and stained with haematoxylin and eosin, and Van Gieson stains.

All slides were examined under an Olympus CH2 light microscope, and photographs were taken using a Rioch XR-X SLR camera.

Abbreviations to the Plates are given facing Plates 3.1a and b.

## 3.3 RESULTS

### 3.3.1 Gross Morphology

#### 3.3.1.1 Gadoids

**Normal Stomach Mucosa**

The normal stomach mucosa of cod, and other gadoids, including saithe, has large longitudinal folds running along its' length when the stomach is empty. These folds allow the stomach to expand in size when food is ingested, and the folds disappear when the stomach is full. The stomach surface itself, including the folds, is smooth in appearance.

**Stomach Ulcers**

The lesions found in gadoid stomachs consisted of raised ulcers which protruded above the normal level of the stomach mucosa. These ulcers were divided into two types :-
(a) Single worm infections which consisted of a single

nematode within a small, raised lesion (Figure 19). In this case, more than one lesion was typically observed within individual fish. One particular individual harboured four single worm lesions, and a lesion bearing no worm but similar to those lesions containing worms, and with a small hole in the centre, indicating that a worm had been present at one stage. In this stomach, each lesion was separated by a mean distance of 16mm.

(b) Multiple worm infections which contained numerous nematodes within a large, raised tumour-like lesion. Most fish observed harboured one large ulcer. Such ulcers varied enormously in size between individual lesions but were morphologically similar in appearance. (Plates 3.1a,b).

In single worm infections, the anterior end of the nematode was observed to have penetrated through to the submucosa of the stomach, with the posterior end of the worm remaining free in the stomach lumen. In these lesions, the worm was seen to have penetrated at an oblique angle to the stomach surface, causing a displacement of the gastric mucosa to build up around one side of the worm thus causing the stomach tissue in this area to become slightly raised above the normal mucosal surface. On examination of the serosa on the body cavity side of the stomach, no evidence of the ulcer on the inner surface of the stomach was observed. Nematodes retrieved from single worm infections appeared to have been alive on fixation and were identified as third stage larvae of **A.simplex**. Figure 20 diagrammatically illustrates a longitudinal section through a single worm stomach lesion.

In multiple worm infections the ulcers were roughly circular in shape (Plate 3.1a), and raised significantly above the normal mucosal surface. They appeared to consist of hard fibrous tissue, being solid to the touch, as compared to the softer, more pliable nature of normal fixed stomach tissue. The mucosa of the stomach covers the exterior of the ulcer, as it lies **in situ** within the stomach, and the folded

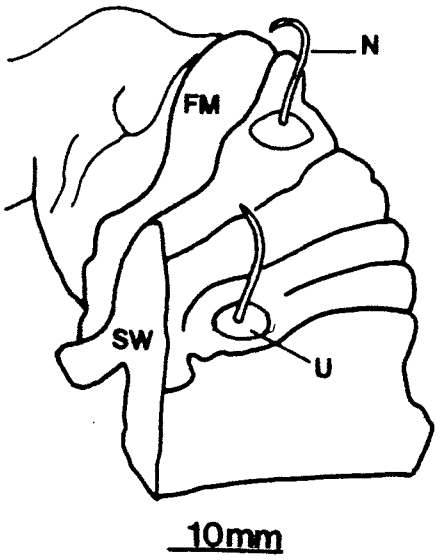


FIGURE 19 : Portion of gadoid stomach containing two single worm lesions (FM = folded mucosa, N = nematode, SW = stomach wall, U = ulcer).

ABBREVIATIONS TO PLATES 3.1 - 3.7

Ca = Cavity
CF = Collagen Fibres
CM = Circular Muscle
DN = Dead Nematode
E = Epithelium
IC = Inflammatory Cells
LM = Longitudinal Muscle
N = Nematode\s
NM = Necrotic Material
Sm = Submucosa

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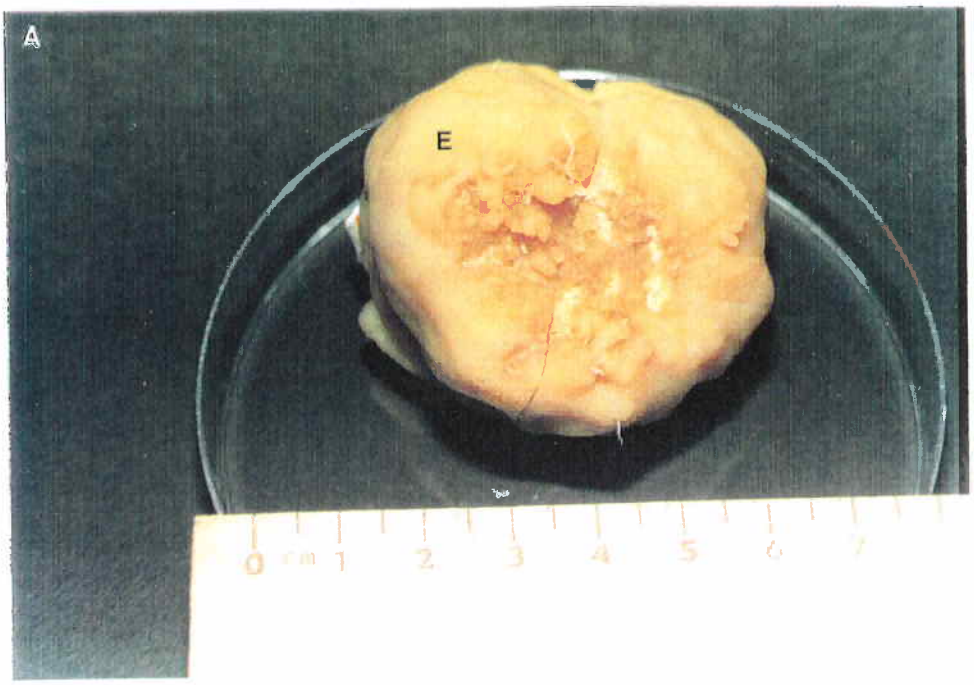
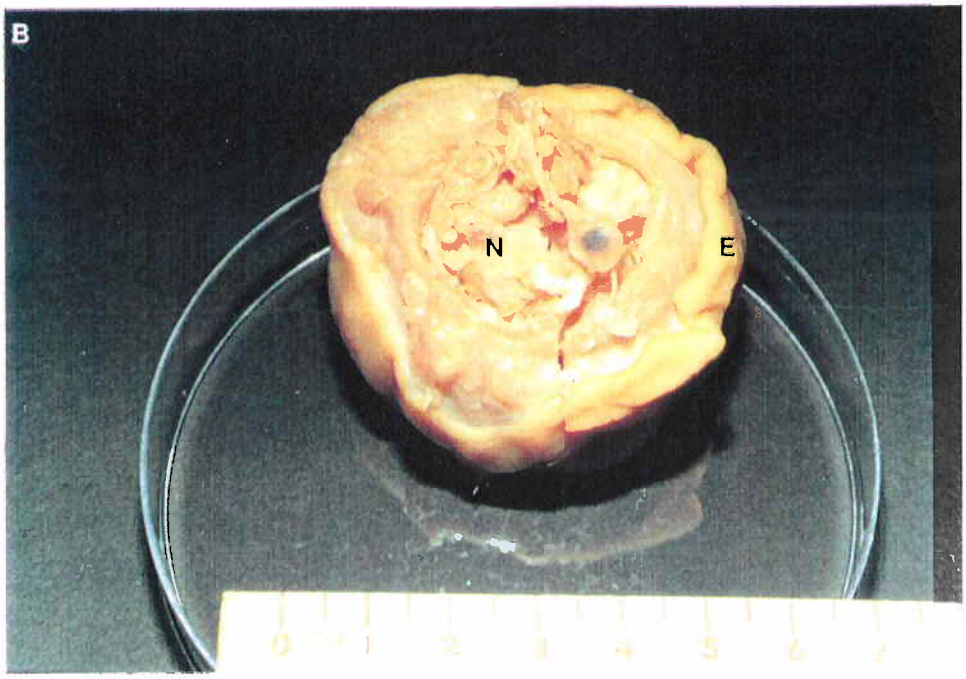



**PLATES 3.1(a,b): Multiple worm stomach lesion from a gadoid**
(a) <u>En face</u> (mucosal) view of isolated stomach lesion
(b) View of stomach lesion from body cavity side

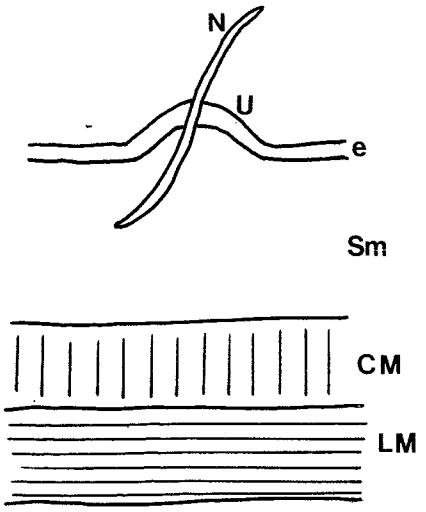



FIGURE 20: Diagrammatic representation of a longitudinal section through a single worm stomach lesion from a gadoid (CM = circular muscle, e = epithelium, LM = longitudinal muscle, N = nematode, Sm = submucosa, U = ulcer). Not to scale

nature of the normal stomach mucosa continues onto the lesion itself. The top of the ulcers were flattened in appearance, often with a slight circular depression in the centre of the lesion. The lesions involved the entire stomach wall. On the serosal side of the ulcer, numerous worms were observed coiled and encapsulated within the connective tissue (Plate 3.1b). If ulcers were cut longitudinally, it was observed that the interior tissue was solid and appeared to be homogeneous. The stomach wall is also significantly thicker within the lesion than in normal stomach tissue. Figure 21 shows a diagrammatic representation of a longitudinal section through a multiple worm stomach ulcer.

Nematodes in multiple worm infections were found in three differing regions of the ulcer:-

(1) Mucosa - many nematodes were found attached to the mucosa at the top of the ulcer. The nematodes were concentrated in the centre of this area, and in some lesions, a few individuals were seen attached to the mucosa outwith this central area. The nematodes were attached in the same manner as described for single worm infections, but at different stages of penetration. Generally, the anterior ends, or upper halves, were buried, with the rest of the body projecting freely into the lumen of the stomach. However, in some cases, only the most posterior parts were observed, the rest of the body having penetrated into the stomach wall. Where only the anterior ends had penetrated, only the upper mucosal layer had been breached. In other cases, the penetrated portions of the nematodes had entered the submucosa. Each nematode had penetrated individually, with the mucosa between the buried larvae remaining intact and appearing undamaged. The nematodes in this area proved fairly difficult to remove and most could not be recovered whole as the anterior ends of the nematodes were buried too firmly in the lesion, and attempts to free them resulted in breakage of the parasite at the point where the worm entered the stomach tissue. Upon examination of those specimens which had been recovered whole, it was noted that the anterior regions of these

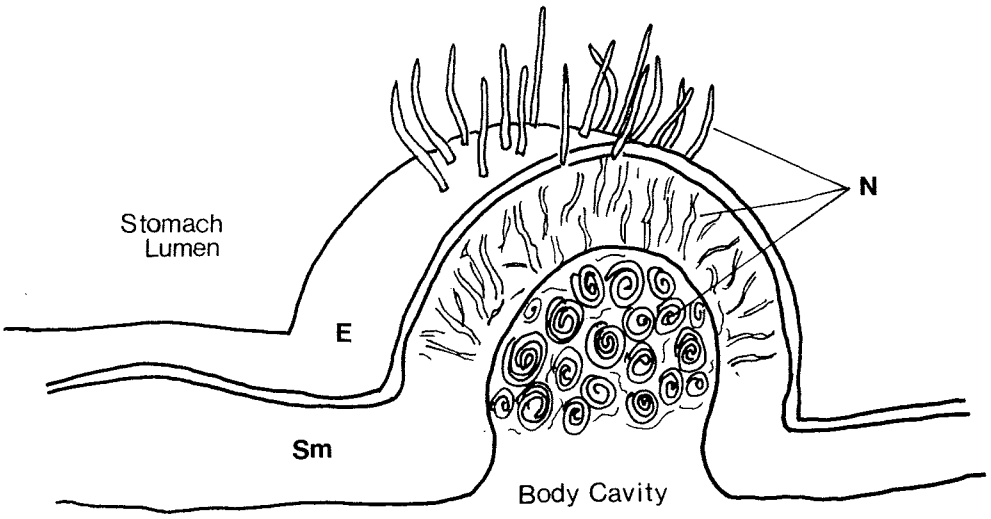



FIGURE 21: Diagrammatic representation of a longitudinal section through a multiple worm stomach lesion from a gadoid (E = epithelium, N = nematodes, Sm = submucosa). Not to scale

specimens, which had penetrated into the mucosa, were compressed and twisted in shape. The majority of the nematodes in this region appeared to have been alive on fixation of the lesion, however a proportion (mean = 19%) of dead worms were also present. Those nematodes which had been alive upon fixation were identified as third stage larvae of **A.simplex**.

(2) Submucosa - on sectioning of the ulcers, it was found that the submucosa of the stomach walls was packed with nematodes which were completely enclosed by the host tissue. These nematodes proved impossible to retrieve, and their numbers could not be estimated, and many of the specimens appeared to have been already dead on fixation.

(3) External to serosa - underneath the ulcer, on the serosal side of the stomach (body cavity side), numerous nematodes were found, firmly entwined in the connective tissue covering the serosa of the stomach. These lay completely outwith the stomach wall, and all were found to be coiled and encapsulated in clusters within the connective tissue. These nematodes were removed individually, and identified as third stage larvae of **A.simplex**. Again, the majority of these specimens appeared to have been alive on fixation of the ulcer, but some dead nematodes were also retrieved from this area (mean = 5% of total).

Given that the nematodes from the mucosa and serosal area of the ulcer were identified as third stage larvae of **A.simplex**, it seems reasonable to assume that the nematodes within the submucosa were also **A.simplex**. In terms of numbers of nematodes in each of these three areas, more worms were generally observed in Region (3) than in Region (1). Numbers of worms in Region (2) could not be estimated, although it appeared that a large number of individuals were present in this area.

**Size of ulcers**

Table 16 shows the mean and range of fixed sizes of gadoid stomach ulcers containing single and multiple worm infections.

As can be seen from the table, the mean fixed diameters and height above normal mucosal surface of multiple worm ulcers were significantly larger than that of the single worm ulcers.

The thickness of the stomach wall in multiple worm infections ranged from 7 - 18mm, with a mean of 12mm..

A positive relationship (correlation co-efficient = 0.9, $p = <0.001$) was observed between the area of the ulcer and volume of the ulcer, indicating that the larger the total size of the ulcer, the larger was the area of the ulcer.

**Assessment of the number of worms recovered from large lesions in gadoids**

The total numbers of nematodes recoverable from ten ulcers, encompassing a range of sizes, were assessed, and separated into worms recovered from the mucosa, and worms recovered from the serosal area. These were further separated into nematodes which appeared to be alive and dead on fixation of the lesion. The average number of nematodes recovered from both areas in each ulcer was 84, with a range of 31-106. The mean of total worms recovered from the mucosa of each ulcer was 42, with a range of 17-90. Of these, a mean of 34 (81%) were alive on fixation (range = 13-77), and a mean of 8 (19%) were dead on fixation (range = 3-13). The mean number of total nematodes recovered from the serosal area of each lesion was also 42, with a range of 5-116. Of these, a mean of 40 (95%) were alive on fixation (range = 4-115), and a mean of 2 (5%) were dead on fixation (range = 0-6).

TABLE 16: SIZE OF STOMACH LESIONS OBSERVED IN GADOIDS

| | Diameter of fixed ulcer | | Height of fixed ulcer above normal mucosal surface | |
|---|---|---|---|---|
| | Mean (mm) | Range (mm) | Mean (mm) | Range (mm) |
| Single worm infections (n=7) | 3 x 2.4 | 1-5 x 1-3.5 | 0.8 | 0.5-1.75 |
| Multiple worm infections (n=20) | 25.6 x 21.8 | 13-49 x 12-39 | 14.4 | 9-25 |

### 3.3.1.2 Lophius piscatorius

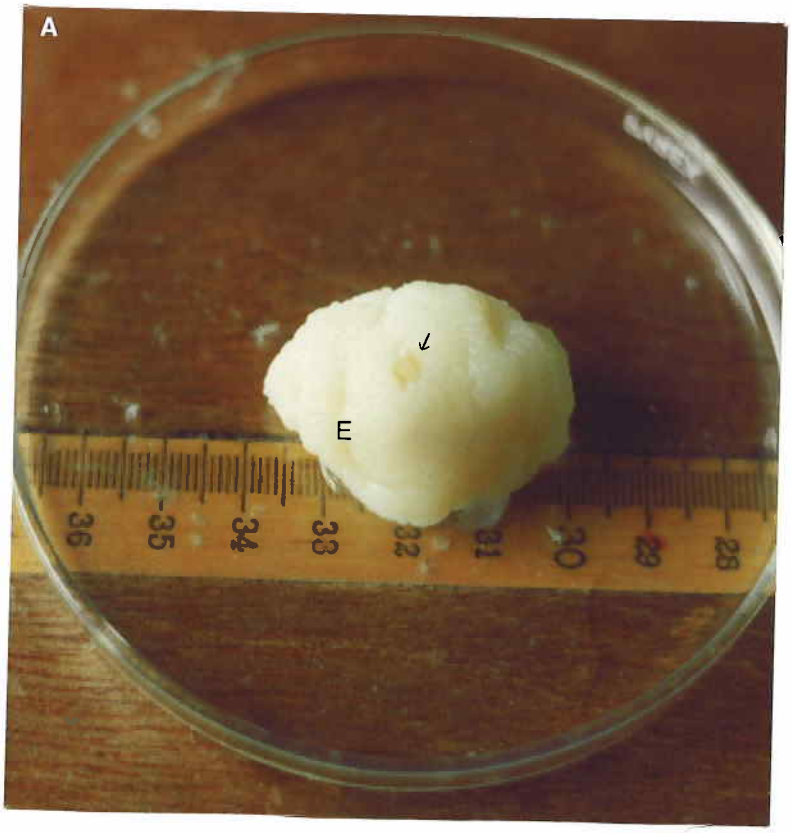
**Normal Stomach Mucosa**

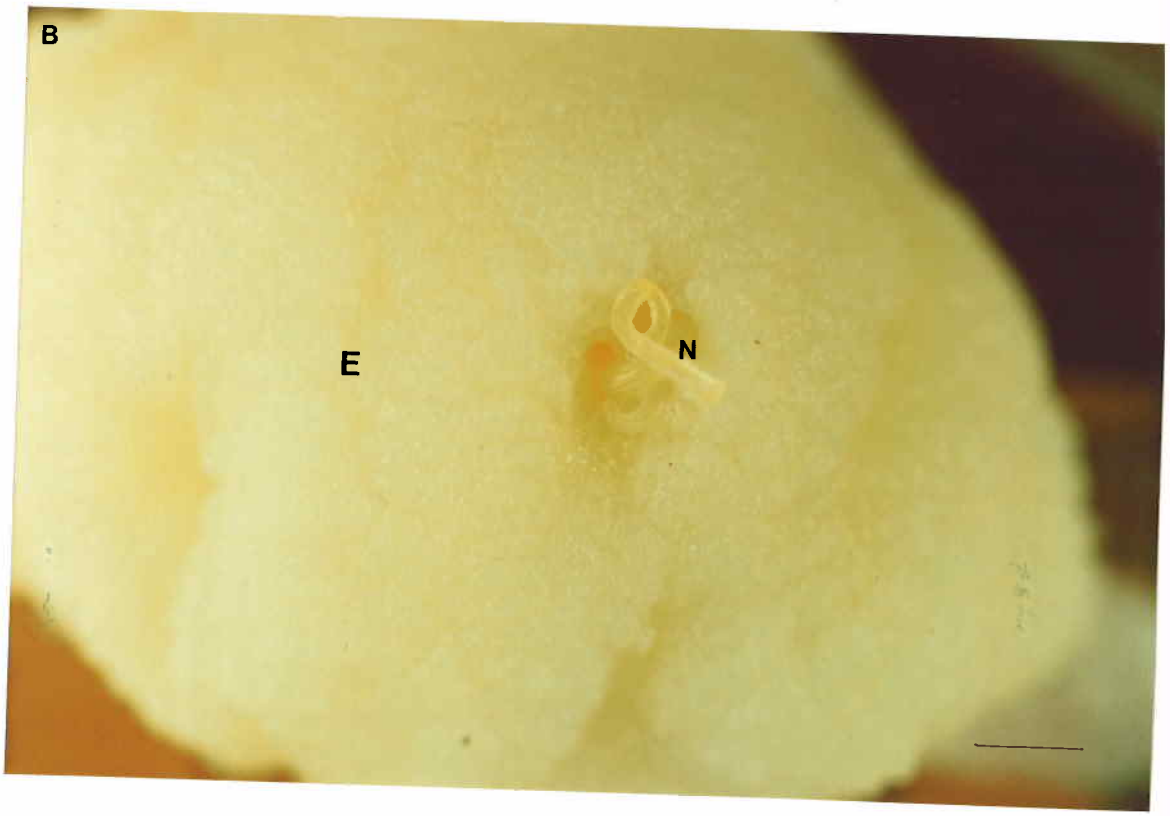
The first angler fish stomach in which lesions were detected contained a large gadoid, and the stomach was thus fully expanded; the surface of the mucosa appearing smooth. This stretching of the stomach revealed the discrete ulcers on the mucosa, which might otherwise have gone undetected if the stomach had been empty, and the mucosal folds present. In empty stomachs, the mucosa in angler fish was also folded, but these folds appeared more numerous and fine than in cod. The surface structure was generally smooth in appearance, although it appeared more "spongy" in nature than that seen in cod.

**Stomach Ulcers**

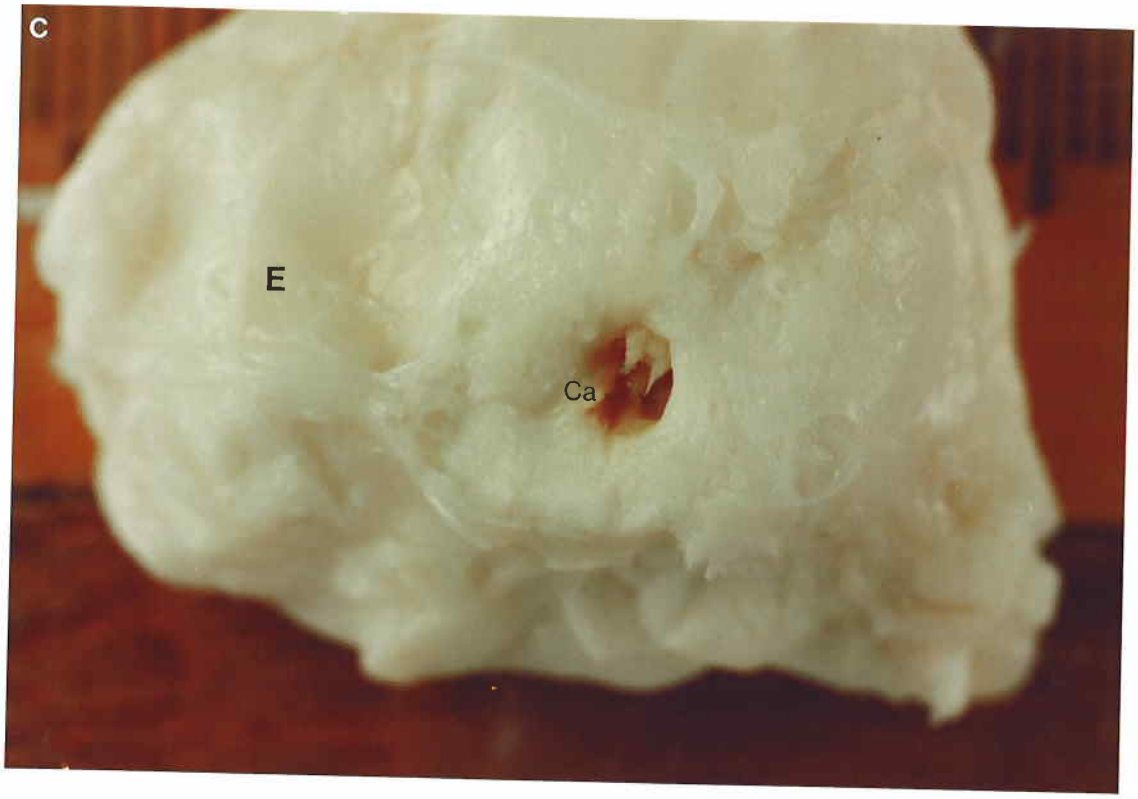
The ulcers retrieved from **L.piscatorius** were flatter and more diffuse in nature than those from gadoids, having a less discernable boundary with the rest of the stomach tissue (Plates 3.2a-c). The ulcers were not significantly raised above the stomach surface, but tended to be formed within the upper layers of the stomach wall. The roughly circular ulcers had a central -relatively large - crater which was ovoid in shape, in the mucosa and open to the stomach lumen. These craters were lined by the submucosa, and appeared to extend down to the outer muscular layer of the stomach. Within these craters lay one to three large nematodes, which were identified as third stage larvae of **P.decipiens**. The nematodes were found to be slightly coiled, but were free in the cavity, and were not encapsulated or attached by any connective tissue. No evidence of the lesion was apparent on the serosal side of the stomach. Two stomach ulcers were found in each of the three angler fish in which lesions were observed.

Figure 22 diagrammatically illustrates a longitudinal section through an angler fish stomach lesion.




**PLATES 3.2(a-c): Stomach lesions from <u>L.piscatorius</u>**
(a) Lesion within a section of stomach (arrow = crater)
(b) High power of 3.2(a) showing cavity containing <u>P.decipiens</u> third stage larva (scale bar = 3.5mm)
(c) Lesion and cavity within a section of stomach (OVERLEAF)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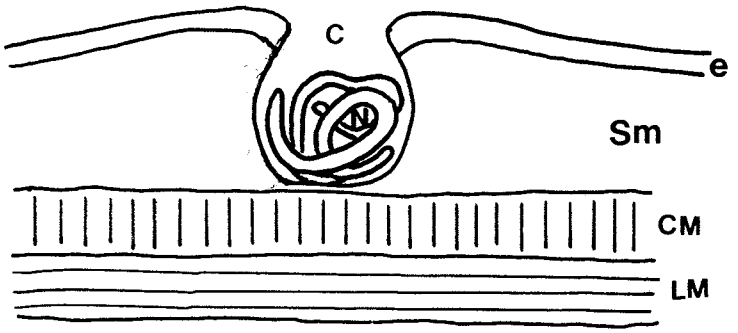



FIGURE 22: Diagrammatic representation of a longitudinal section through a stomach lesion in L.piscatorius (C = cavity, CM = circular muscle, e = epithelium, LM = longitudinal muscle, N = nematodes, Sm = submucosa). Not to scale

**Size of Ulcer**

Measurements of the ulcers are approximate as the boundary with the rest of the stomach tissue was generally not distinct. The mean fixed diameter of the ulcers was 20 x 18mm (Range = 10-30 x 10-23mm). The ulcers were not significantly raised above the mucosa.

The mean diameter of the crater in the centre of the lesion was 3.7 x 2.3mm. (Range = 1-5 x 1-3mm.). The size of the crater did not appear to be related to the size of the ulcer.

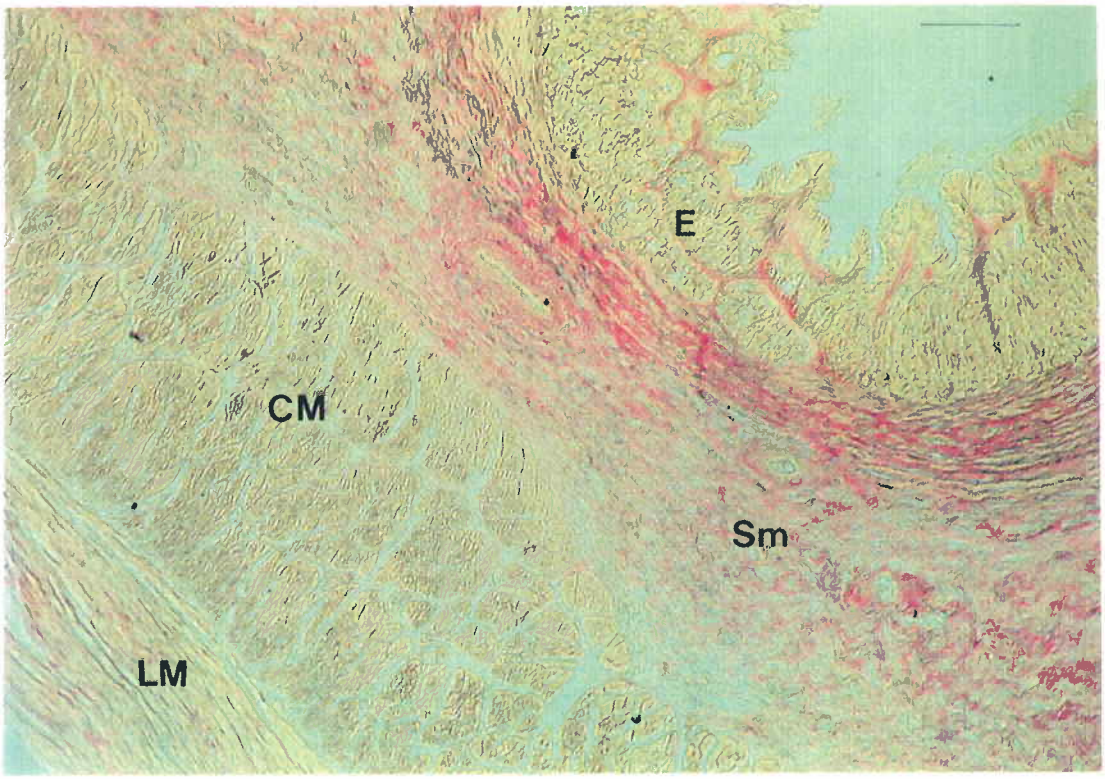
The stomach wall did not appear to be significantly thickened in the area of the lesion.

### 3.3.2. Histopathological Examination

#### 3.3.2.1 Gadoids

**Normal Stomach Tissue**

In the sections taken from normal cod stomach, four distinct areas can be seen, corresponding to the four main layers of the stomach wall of fish (Plate 3.3). The mucosa consists of the surface epithelium (columnar cells), the lamina propria (connective tissue), the stratum granulosum (granular cell layer), the stratum compactum (collagenous cells) and the muscularis mucosa (a layer of smooth muscle fibres). The columnar epithelium is glandular, and is composed of long cells with basal nuclei. The epithelium can clearly be seen as a folded layer, approximately 700µm thick (maximum). The submucosa in fish is generally a thin layer of loose connective tissue, which also contains blood vessels and some muscle fibres. In fish, all the connective tissue under the epithelial lining, is generally termed the "submucosa". The true submucosa, as compared to mammalian stomach histology, is often a very shallow area. In this case, the submucosa is approximately 900µm thick and contains dense, loosely-orientated red-staining (with Van Gieson) collagen fibres, which are denser immediately under the



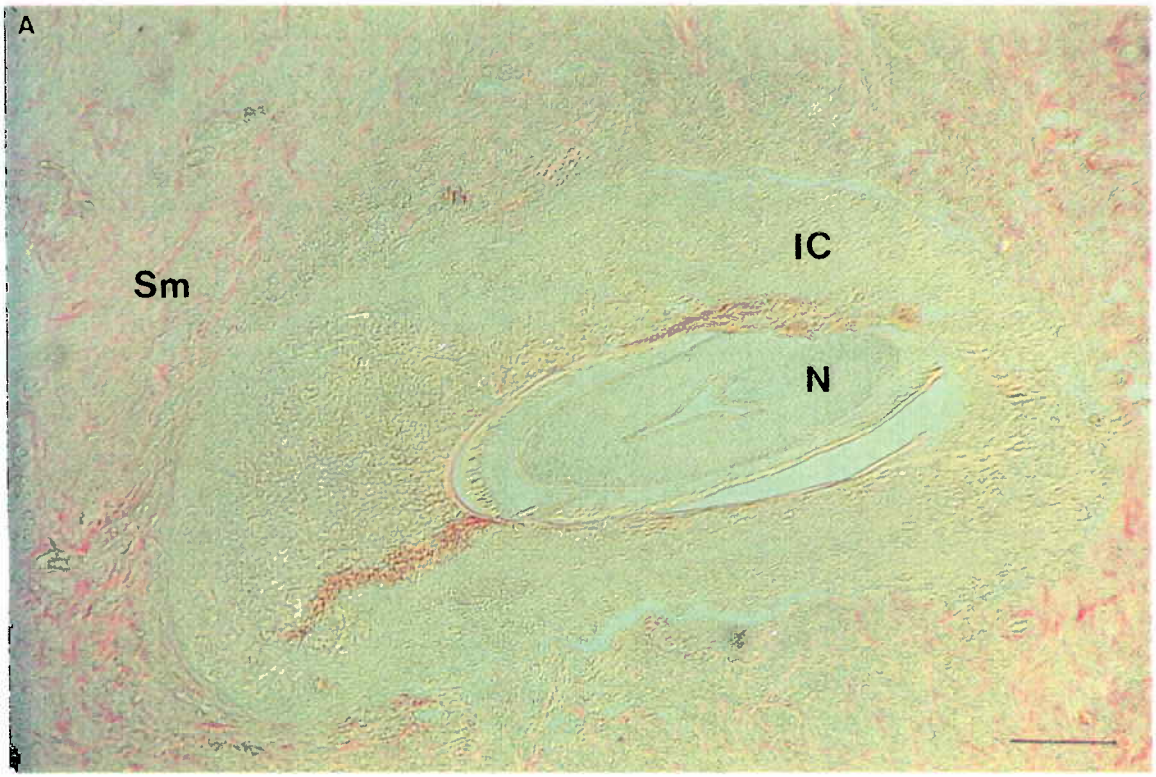

PLATES 3.3: Longitudinal section through normal cod stomach (Van Gieson) (scale bar = 250 µm)

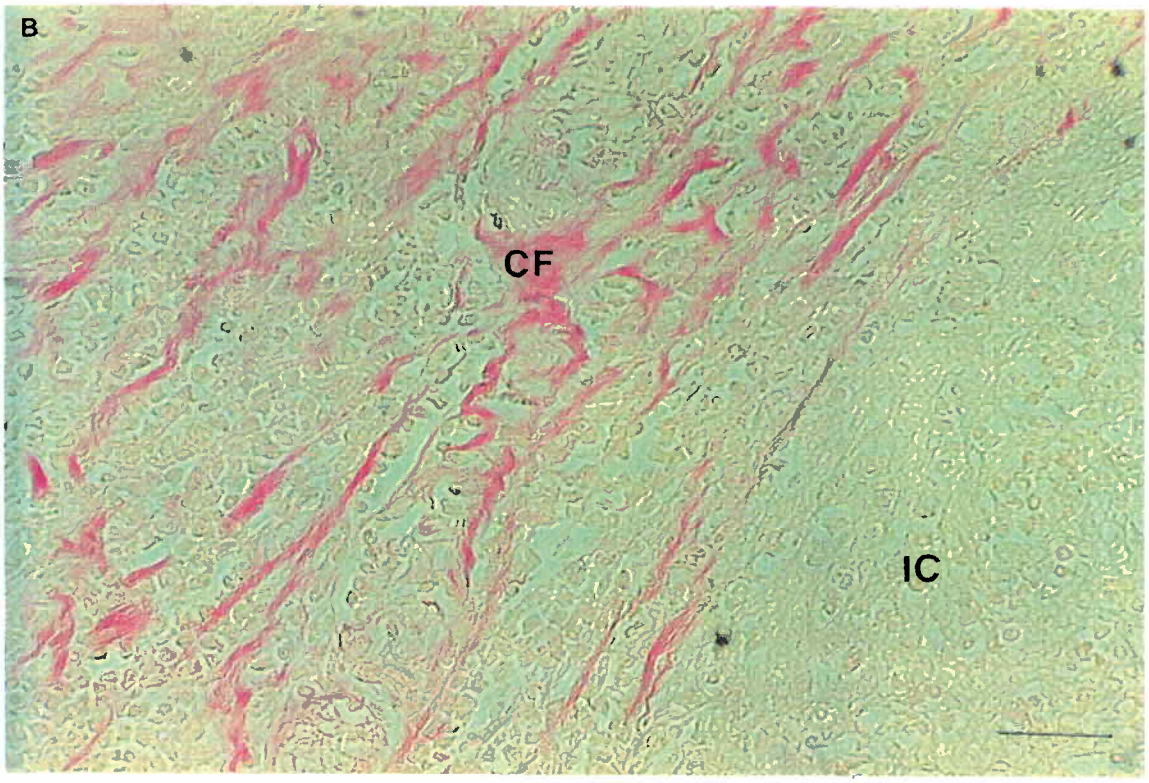
epithelium, and yellow-staining connective tissue. The muscular coat of the stomach is comprised of a layer of circular muscle (muscularis circularis) and a layer of longitudinal muscle (muscularis longitudinalis), and these two smooth muscle layers can also be clearly seen, approximately 1.5mm thick. The serosa consists of a single layer of simple squamous epithelium and a small amount of underlying connective tissue. In this case, the serosal layer was composed of a very thin layer of single cells and connective tissue. The entire stomach wall is approximately 3.5mm in thickness. Bishop and Odense (1966) and Morrison (1987) give fuller descriptions of the histology of the stomach of cod.

**Stomach Ulcers**

**Single Worm Infections** (Plates 3.4a,b)

Sections from single worm infections reveal the anterior end of the nematode in the submucosa. A distinct circular layer of inflammatory cells approximately 330μm thick immediately and completely surrounds the parasite. Plate 3.4b shows part of the inflammatory cell layer around the parasite and the surrounding submucosa containing randomly orientated red-staining collagen fibres, similar to those observed in normal stomach submucosa. Cells close to the parasite are indistinct and appear to have lost their cellular outline. From H&E staining it was observed that these cells contain small, dark, condensed pyknotic nuclei. Some red blood cells are also present in the submucosa, indicating leakage from the capillaries. The two muscle layers in the stomach wall remained normal with no evidence of pathology, and the stomach tissue outwith the region of inflammation also appeared to be normal. The structure of the nematodes showed no degenerative changes, and they appeared to have been alive on fixation.




**PLATES 3.4(a,b): Single worm stomach lesion from a gadoid (Van Gieson)**
(a) Nematode within submucosa (scale bar = 250µm)
(b) Inflammatory cells and collagen fibres surrounding worm (scale bar = 50µm)

**Multiple Worm Infections** (Plates 3.5a,b,c)

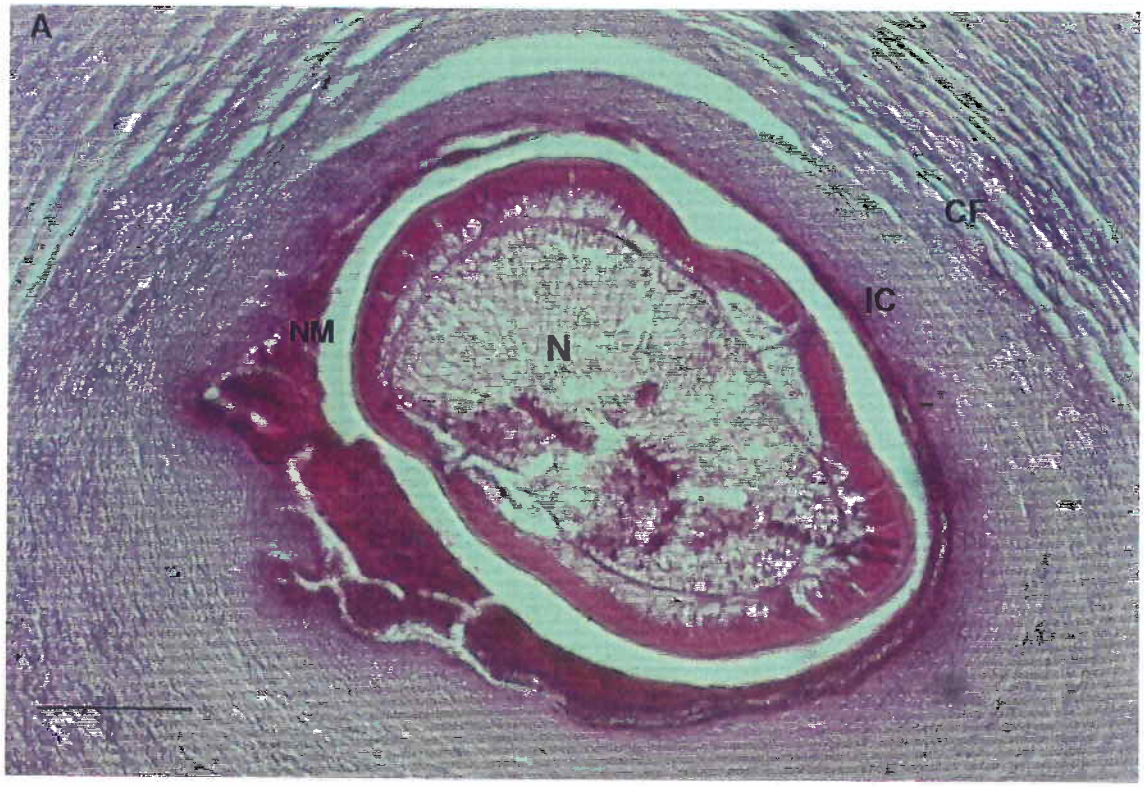
The submucosa in multiple worm infections is congested with parasites, many of which appear to have been dead prior to fixation (Plate 3.5b). Parasites were observed in various positions, with both longitudinal and transverse sections of worms being observed, and many appeared to lie coiled within the submucosa. The nematodes are immediately surrounded by a coagulative layer of eosinophilic necrotic cell debris (Plates 3.5a,c), approximately 50-80µm thick. A layer of inflammatory cells surround the necrotic material (Plates 3.5a,c), this layer being on average approximately 50µm in thickness. Fine details of the cells were not evident owing to the quality of the fixation and\or the age of the samples. Exterior to the inflammatory cell layer lie layers of closely packed collagen fibres in the submucosa (Plates 3.5a,c), gathered and orientated concentrically around the inflammatory cells and which become loose and less organised further away from the area of response. This is clearly illustrated using the Van Gieson stain in Plate 3.5b. This region, rich in collagen fibres, forms a thick layer around the parasite and inflammatory cells.

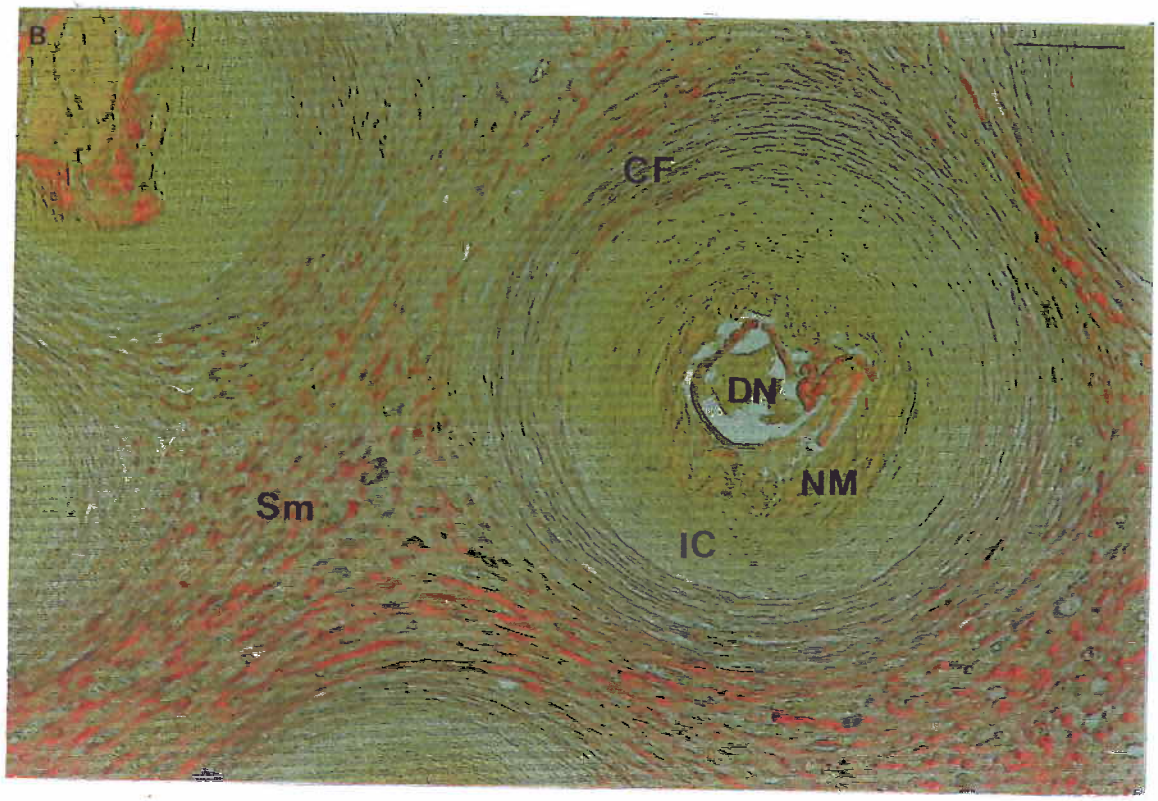
The muscle layers of the hosts stomach are generally absent from the area of the lesion, indicating that they have been broken down, although some muscle fibres are present.

### 3.3.2.2 Lophius piscatorius

**Normal Stomach Tissue**

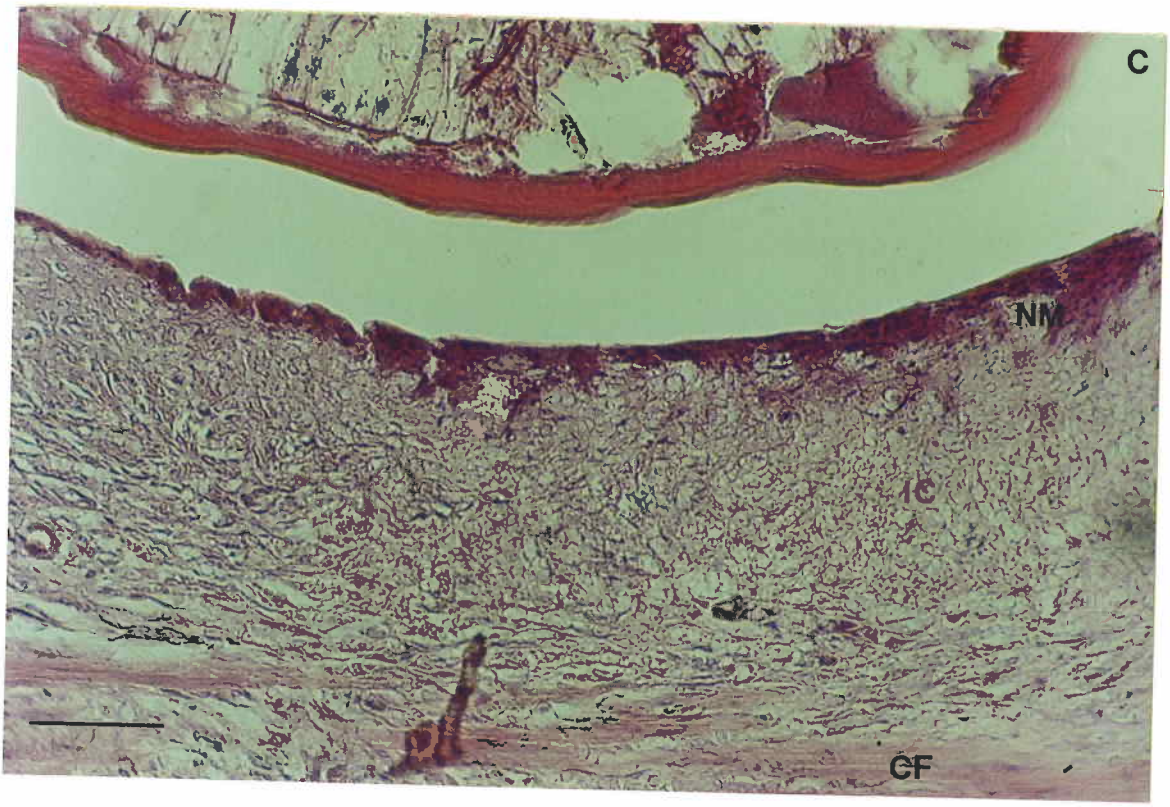
Plate 3.6 shows a longitudinal section through the stomach wall of **L.piscatorius**. The general appearance of the stomach is similar to that of other teleosts (see above). However, the stomach wall, and its constituent layers appear thicker than is normally observed in teleosts, being approximately 5mm thick in its entirety. The folded epithelium is approximately 1mm thick, and the glandular nature of these cells is distinct. The lamina propria is




**PLATES 3.5(a-c): Multiple worm stomach lesions from Gadoid**

(a) Nematode within submucosa (scale bar = 50µm) H&E
(b) Degenerated nematode and associated histopathology (scale bar = 125µm) Van Gieson
(c) Necrotic material, inflammatory cells, and collagen fibres surrounding worm (scale bar = 25µm) H&E (OVERLEAF)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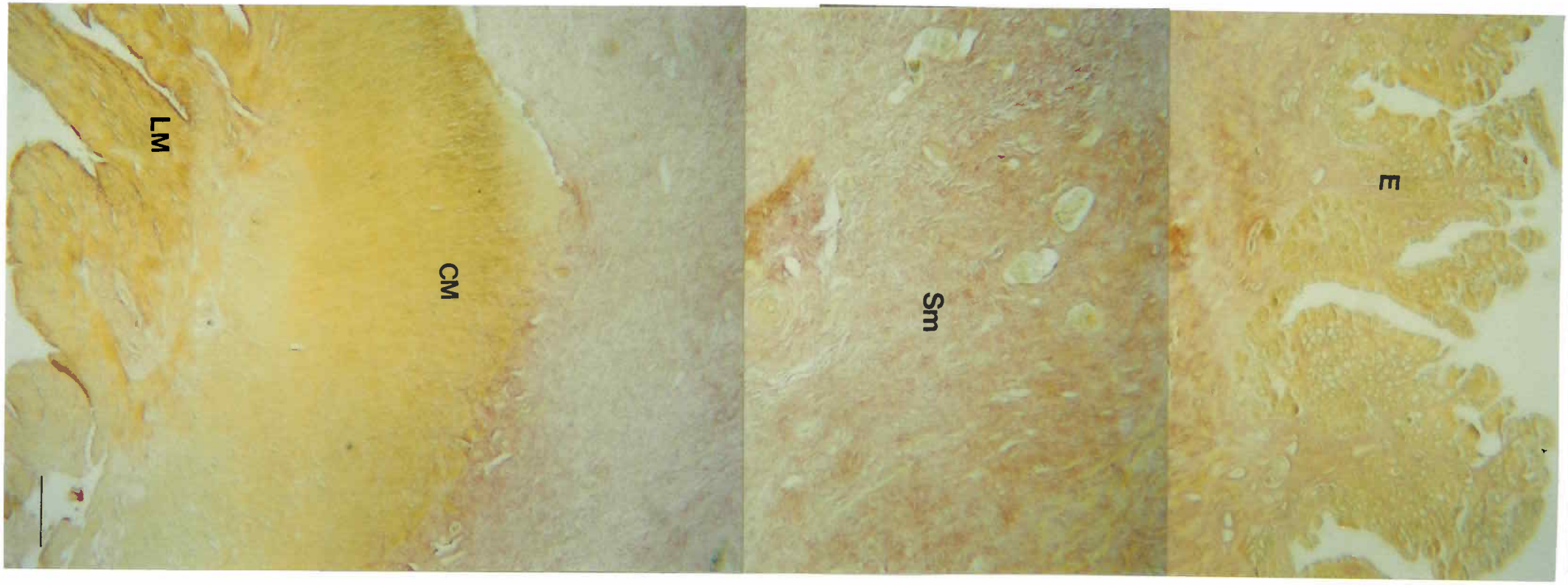


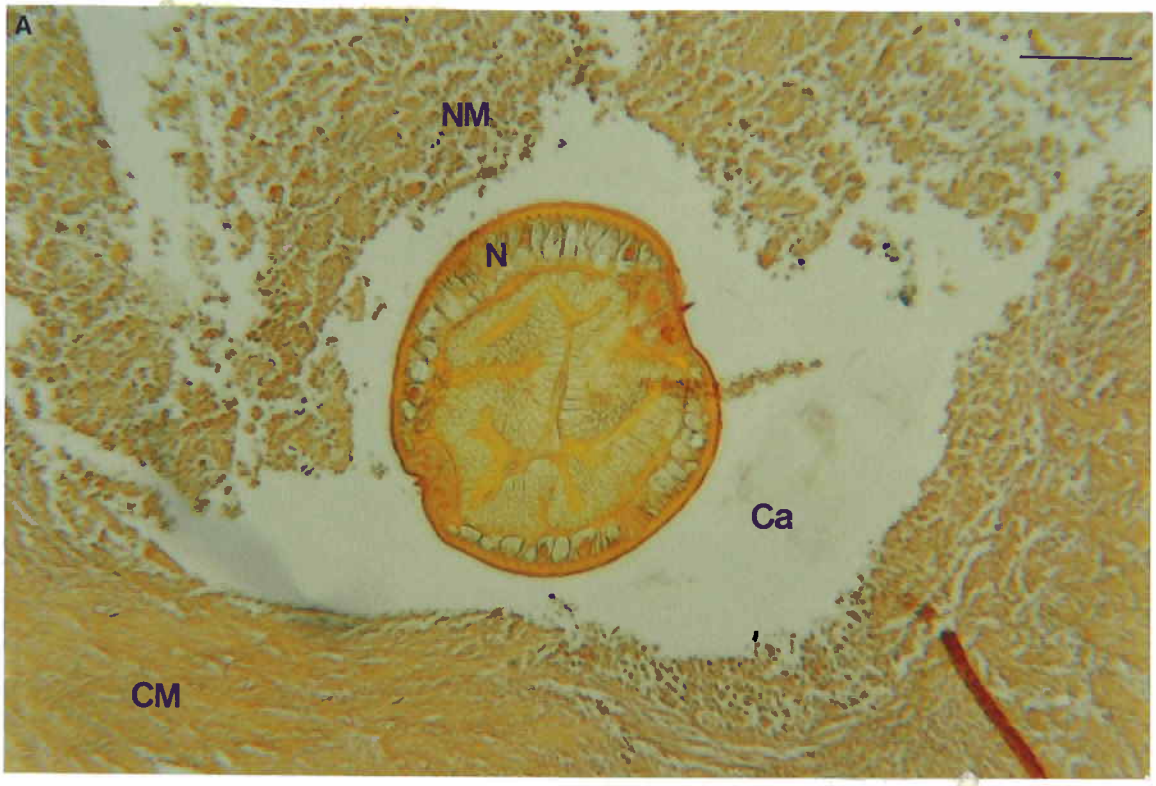

**PLATE 3.6:** Longitudinal section through normal L.piscatorius stomach (Van Gieson) (scale bar = 250μm)

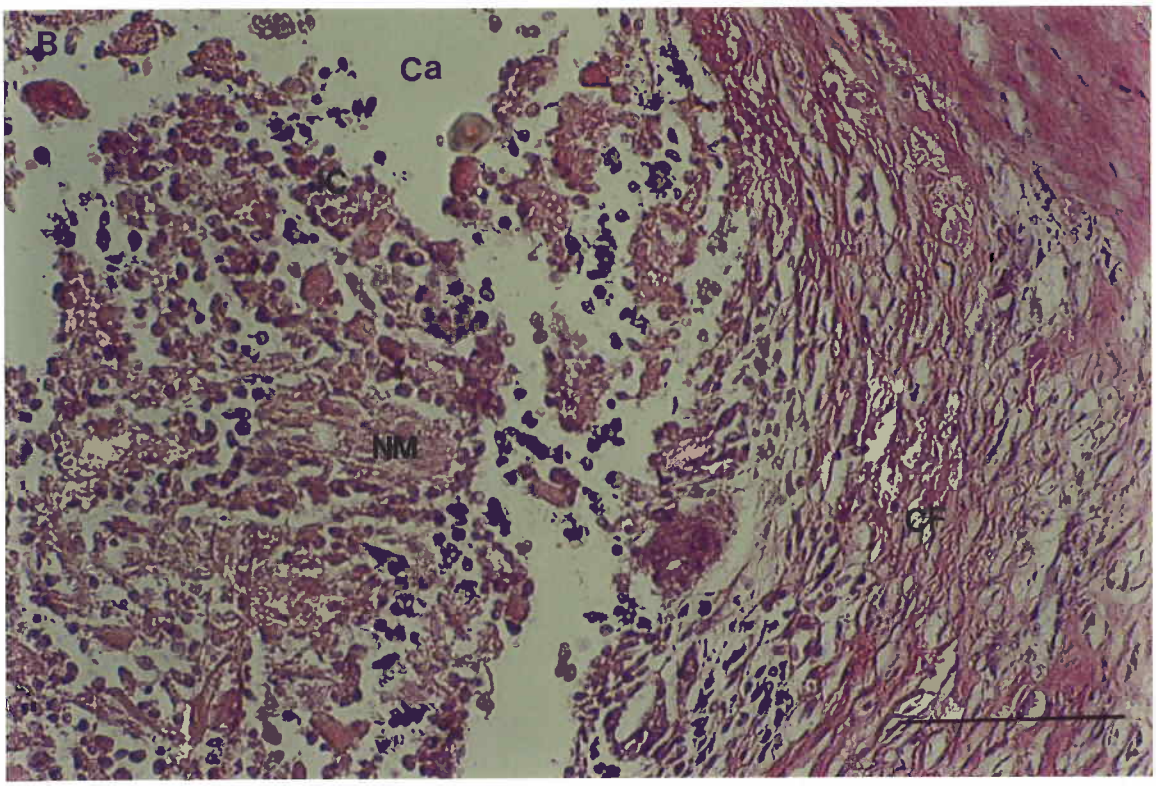
composed of strands of muscle tissue, with the muscularis mucosa consisting of a more distinct band of muscle. The submucosa is approximately 2mm thick and contains numerous collagenous and elastic fibres, which presumably aid in extension of the stomach during feeding. Beneath the submucosa, the muscle layers can be seen, approximately 1.8mm in thickness.

**Stomach Ulcers**

Plates 3.7a shows the parasite lying within the single cavity, in the submucosa. The cavity itself is approximately 1.5mm in diameter. Necrotic cellular debris can also be observed within the cavity (Plates 3.7a and c), with some large cells, appearing to be macrophages, gathered around this debris (Plate 3.7b). Proliferation of inflammatory cells was observed around the lesion, and some necrotic cellular debris was also seen in this area, around the edges of the cavity. The inflammatory cells gathered around the cavity showed evidence of being concentrically arranged around the lesion. The inflammatory cells themselves appeared to be mixed, with some large cells being vacuolated, indicating that they were either undergoing degenerative changes, or were phagocytotic. Some cells were observed to be ingesting material, and the majority of the cells around the lesion appeared to be monocytes\macrophages. Other cells had a granular appearance and were probably granular leucocytes. All cells appeared to have single, discrete nuclei, with no cells appearing polymorphic.

There was some evidence of fibrous tissue (red-staining) concentrating around the lesion (Plate 3.7c). The concentric nature of the cells around the cavity is suggestive of early attempts to enclose the lesion, and may represent early fibrosis, or alternatively, it may have been due to mechanical pressure on either side of the lesion. In either case, the tissue on either side of the cavity, outwith this area, appeared to be normal. Bacteria were observed within the area of the lesion.



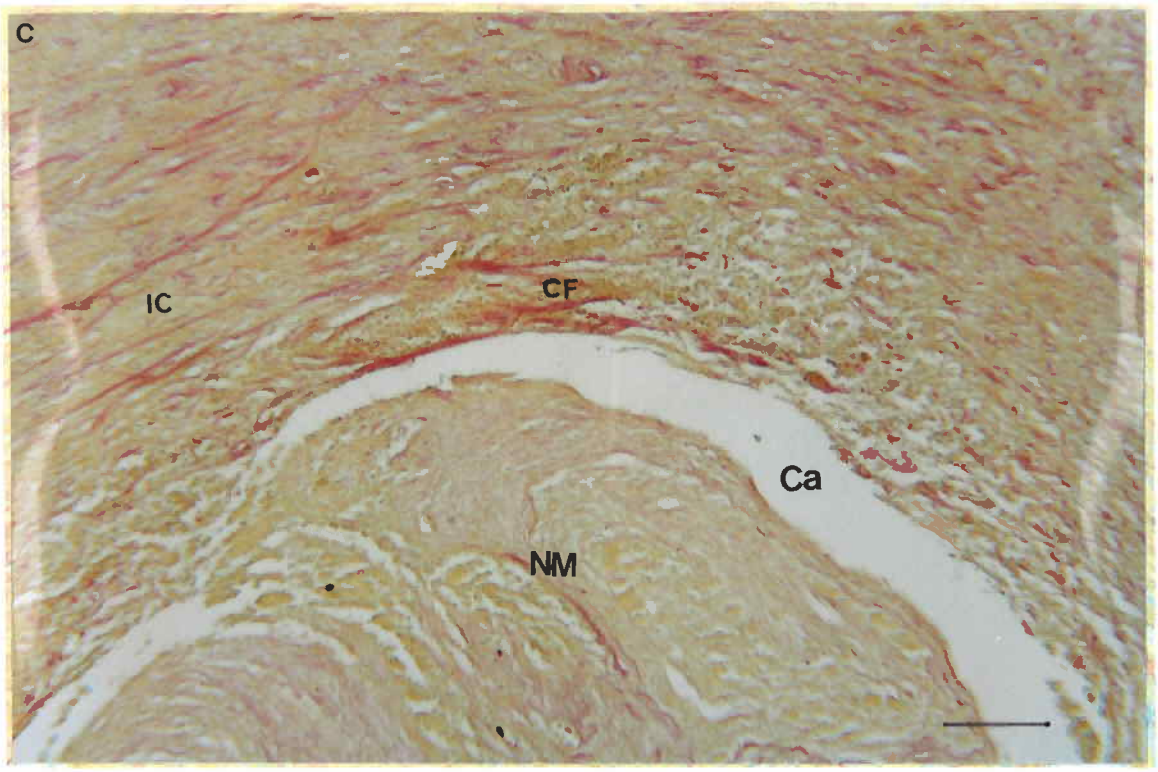




**PLATES 3.7(a-c): Stomach lesion from *L.piscatorius***

(a) Nematode within cavity above muscle layer (scale bar = 125µm) Van Gieson

(b) Nematode within cavity in submucosa (scale bar = 25µm) H&E

(c) Edge of cavity showing inflammatory cells and collagen fibres (scale bar = 125µm) Van Gieson (OVERLEAF)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The outer muscle layers of the stomach wall were not affected by the lesion, and the cavity can be observed in the submucosa, immediately above the layer of muscle (Plate 3.7a). The entire stomach wall in this area is approximately 7mm thick.

## 3.4 DISCUSSION

### 3.4.1 Normal Stomach Tissue

The normal gross appearance, and histology, of the cod stomach examined in this study agrees with that of Bishop and Odense (1966) and Morrison (1987). The structure of the stomach of **L.piscatorius** has not been studied previously, however, it is similar to that of other teleosts, although the stomach wall and constituent layers appeared much thicker than is normally seen in teleosts. This may be related to the size of the stomach, which occupies a large area within the body cavity of the fish, even when this organ is devoid of food (personal observation); and the size of the stomach itself is presumably related to the carnivorous diet.

### 3.4.2 Gross Morphology of Stomach Lesions

In single worm ulcers from gadoids, the nematode was observed to have penetrated into the mucosa by its anterior end only, and a small raised ulcer was formed around the larvae. Riley (1972) had noted that penetration in the stomach of fulmars by single **Anisakis** larvae, as well as clusters, caused inflamed lesions, and McClelland (1980c) observed that **P.decipiens** which occurred singly in the stomach wall of seals were associated with raised inflammatory areas 10-20mm in diameter.

By contrast, in multiple worm lesions from gadoids,

numerous nematodes were seen in three distinct regions of the stomach - penetrating the mucosa, completely within the submucosa and attached to the serosal surface, and such infections were associated with a grossly raised lesion. The nematodes found in the serosal region were coiled and encapsulated, and had successfully penetrated through the stomach wall. Such coiling and encapsulation is normal for **A.simplex** infection in the body cavity of fish. The presence of apparently dead nematodes in the submucosa indicates that not all the nematodes can successfully penetrate the entire stomach wall. The nematodes attached to the mucosa had penetrated anterior end first, and this would be normal behaviour for initial migration through the stomach wall. Those within the submucosa were presumably also attempting to migrate as normal through the stomach wall.

Liu and Edward (1971) found the lesions associated with **C.osculatum** in sea lions to encompass the entire stomach wall, and they were swollen and firm. This description is similar to the multiple worm lesions observed in gadoids. Gastric nodules in bowhead whales associated with **Anisakis** type fourth stage larvae were also firm in nature (Migaki **et al.** 1982).

The nematodes attached to the mucosa in multiple worms lesions from gadoids were firmly attached and often proved difficult to remove. Several authors have commented on the firm attachment of anisakids to gastro-intestinal lesions in the natural mammalian final hosts eg. Montreuil and Ronald 1957, Young and Lowe 1969, Wilson and Stockdale 1970, Smith 1989), and in birds (Liu and Edward 1971, Riley 1972). The anterior regions of **A.simplex** which had penetrated into the stomach mucosa in multiple worm infections in gadoids were observed to be compressed and twisted, and such a feature is likely to be a result of mechanical pressure on the anterior end of the larvae as it attempts to migrate through the solid and thickened stomach wall. From the distended nature of the anterior ends of the larvae, it is assumed that they are embedded so firmly as a result of the host reaction within

the underlying host tissue, and around the larvae themselves, as Smith (1989) suggested for **A.simplex** in porpoise ulcers.

The size of multiple worm lesions in gadoids appears to be related to the number of nematodes present, and therefore large lesions are not necessarily older.

Stomach lesions caused by nematodes do not appear to have been reported previously from **L.piscatorius**, and indeed such ulcers associated with **P.decipiens** in fish have not been documented. It is possible that stomach ulcers in this species may be common, but have not been observed previously as they are often quite diffuse, and not as gross and apparent as those in gadoids. The flatter, and more diffuse appearance of the lesion, with no significant thickening of the stomach wall may be related to the presence of only 1-3 nematodes in the submucosa.

Although **A.simplex** were also observed in the submucosa of multiple worm infections from gadoids, the gross appearance of this area differs from that in **L.piscatorius** lesions. In gadoids, the submucosa contains many **A.simplex** throughout, which are firmly enclosed within the tissue. In **L.piscatorius**, **P.decipiens** were only found within in a single large cavity which had formed within the submucosa, where they were observed to lie freely. The stomach tissue did not appear to differ grossly from normal. From the general appearance of the lesions in angler fish, it appears that **P.decipiens** larvae had penetrated through to the submucosa, then ceased penetrating and remained in this region, moving freely within the tissue, and forming the cavity within. In gadoid ulcers, **A.simplex** were observed to penetrate the mucosa individually and were tightly surrounded by host tissue, whereas in **L.piscatorius** lesions, a single open crater was observed in the mucosa, through which all larvae had presumably entered. Stomach ulcers with a large central crater, were reported in association with **P.decipiens** in seals by McClelland (1980c), and **Anisakis** in porpoises (Smith 1989) and fulmars (Riley 1972). Vik (1964) also found larvae of **Anisakis** in single crater-like depressions in the centre

of ulcers in porpoises, and attributed the open form of the ulcers to penetration of the mucosa of the stomach wall, and the movement of the larvae.

Arai (1969) reported ulcerous lesions containing **Anisakis** larvae, from the stomach wall of lingcod, also a gadoid. The gross description given of these lesions is similar to that described in gadoids here. Aria found the ulcers to be approximately three centimetres in length, which falls within the size range of the large gadoid lesions observed during this study. Menezes and Lima (1980) reported intense infections with larval **Anisakis** sp. in the stomach submucosa of **Merluccius polylepis** from Chilean waters, and recovered several hundred parasites from this region in individual fish. In addition, Berland (1981, in both Möller and Anders 1986 and Smith 1984e) observed stomach ulcers associated with clusters of **Anisakis** in large cod.

Lick (1991) found stomach lesions associated with nematodes in species of marine fish from the outer Elbe estuary. Subsequently, Obiekezie **et al.** (1992) examined this phenomena further and identified the larval nematodes **Hysterothylacium cornutum**, **Cosmocephalus obvelatus** and **Paracuaria tridentata** from stomach wall lesions in smelt. In contrast to the stomach lesions observed during this study, which were found in large gadoids and angler fish, the lesions observed by Obiekezie **et al.** were found in smelt 15-20cm in length, and appeared to be relatively common, being found in 72% of all stomachs examined. Both live and dead nematodes were observed in the lesions. Miyazaki **et al.** (1988) noted that invasions of **H.dollfusi** larvae in the stomach of paddlefish caused small ulcerous lesions containing the worms.

#### 3.4.2.1 Gastro-Intestinal Lesions in Non-Fish Hosts

Anisakid nematodes are well documented as causing ulcers and lesions in the gastrointestinal tracts of their natural

final hosts eg. **P.decipiens** in seals (Young and Lowe 1969, Bishop 1979, McClelland 1980c); **Contracaecum** sp. in seals (Young and Lowe 1969, Wilson and Stockdale 1970) and sea lions (Liu and Edward 1971); **Anisakis** sp. in seals (Young and Lowe 1969) and cetaceans (Vik 1964, Young and Lowe 1969, Migaki **et al.** 1982, Lambertsen and Kohn 1987, Smith 1989).

Gastrointestinal lesions caused by marine ascaridoids have also been widely reported from turtles, birds, experimental mammals and humans eg. **Sulcascaris sulcata** in turtles (Lester **et al.** 1980, Berry and Cannon 1981); **Contracaecum** sp. (Liu and Edward 1971) and **Anisakis** sp. (Riley 1972) in birds; **Terranova** sp. in rats (Young and Lowe 1969, Deardorff **et al.** 1983), **Anisakis** sp. in rats and mice (Young and Lowe 1969, Deardorff **et al.** 1991) and **Thynnascaris\Hysterothylacium** sp. in mice and rhesus monkeys (Norris and Overstreet 1981, Overstreet and Meyer 1981); **Anisakis** sp. in humans (Van Theil **et al.** 1960, Asami **et al.** 1965, Yokogawa and Yoshimura 1965, and Van Theil and Van Houten 1967, Fujino **et al.** 1984, Aji **et al.** 1982 and Kikuchi **et al.** 1990).

### 3.4.2.2 Comparisons of fish lesions with the gross morphology of gastro-intestinal lesions reported from other hosts.

**Natural Final Hosts**

The general morphology of multiple worm ulcers in gadoids is strikingly similar to many of the ulcers reported from the mammalian final hosts of **Anisakis** sp., **P.decipiens** and **Contracaecum** sp. Such ulcers are raised above the normal mucosal surface and are associated with a large cluster of nematodes embedded by their anterior ends in the centre of the lesion. McClelland (1980c) observed clusters of **P.decipiens** in lesions from grey seals to have penetrated individually, and the mucosa between adjacent nematodes in clusters was intact. This was also observed for the larvae

of **A.simplex** attached to the mucosa in multiple worm lesions in gadoids.

A range of sizes has been reported for stomach ulcers in final hosts varying from 1-2mm in diameter (Bishop 1979, Smith 1989) up to 60mm in diameter (Young and Lowe 1969, Smith 1989); although Lambertsen and Kohn (1987) did observe a lesion of over 20cm in diameter caused by **Anisakis physeteris** in a sperm whale. The height of lesions in marine mammals is noted as ranging from 6-50mm (Young and Lowe 1969, Liu and Edward 1971, Migaki **et al.** 1982, Smith 1989), with numbers of nematodes recovered varying from 4-400+ (Vik 1964, Young and Lowe 1969, Liu and Edward 1971, McClelland **et al.** 1980c, Smith 1989).

McClelland (1980c) observed lesions in grey seals to generally range from 10-20mm in diameter, and harbour 3-60 worms, but the lesion associated with a cluster of 60 adult **P.decipiens** was 40-50mm in diameter; this supports the suggestion that in multiple worm lesions in gadoids the numbers of worms present in the ulcer may be related to the size of the lesion. Penetration by single nematodes in seal stomachs were not usually associated with gross lesions, gross lesions normally being associated with clusters of partially embedded nematodes, as observed during this study for **A.simplex** in gadoids.

**Humans**

Gastro-intestinal lesions in humans ranged in size from 13-65mm (Asami **et al.** 1965, Yokogawa and Yoshimura 1965), although Van Theil and Van Houten (1967) did observe a 15cm lesion. The former sizes of lesion generally fall within the range observed in multiple worm infections from gadoids, although in humans, lesions were only associated with a single worm. It seems likely that the host reaction is more severe in man.

**Birds**

In birds, stomach ulcers ranged from approximately 5-11mm, with 2-15 worms attached to each ulcer (Liu and Edward 1971, Riley 1972). The largest lesions in fulmars were 8mm in height (Riley 1972). Riley also observed that in fulmar stomachs infected with less than ten nematodes, **Anisakis** sp. larvae either lay free in the lumen, or had penetrated individually, with a small inflammatory reaction occurring around the anterior end of the worm.

### 3.4.3 Histopathology of Stomach Lesions

The histopathology of the lesions correlates with their gross appearance, eg. in multiple worm infections from gadoids, there are severe histological changes and **A.simplex** can be seen throughout the submucosa, firmly enclosed and surrounded by tissue; however, in single worm lesions in gadoids there is little disruption to the normal stomach tissue, other than immediately around the larvae; in **L.piscatorius**, the cavity containing **P.decipiens** can be seen histologically and macroscopically to be held immediately above the outer muscle layers.

From the histological observations, it can be seen that the larvae appear to induce a distinct inflammatory response within the host submucosa. The host response differs between single and multiple worm lesions in gadoids, and those in **L.piscatorius.** All lesions show an inflammatory cell response around the larvae, however, the inflammatory cells can clearly be seen within a distinct layer around the larvae in gadoid ulcers. Young and Lowe (1969) had also observed a thick layer of infiltrated cells to form around **Anisakis** and **Terranova** larvae in the submucosa of rats. In **L.piscatorius**, the response is more diffuse, and is marked by a proliferation of inflammatory cells within the tissue around the area of the cavity. Asami **et al.** (1965) observed **Anisakis** larvae to induce a diffuse inflammatory reaction in the

submucosa in a human infection, and Jilek and Crites (1982b) also found the lesions caused by **Spinitectus carolini** in the intestinal wall of bluegill to generally be diffuse.

In both single worm ulcers from gadoids and ulcers in **L.piscatorius**, the remainder of the stomach tissue within the lesion appears normal, whereas the entire submucosa in multiple worm infections from gadoids is full of parasites, with associated host reaction surrounding each parasite. Young and Lowe (1969) described complete destruction of the submucosa in the area of the lesion caused by **Anisakis** sp. in porpoises.

Only in multiple worm lesions from gadoids were distinct concentric aggregations of collagenous fibres observed surrounding the inflammatory cell layer. Richards **et al.** (1978) described inflammation associated with fungal infection in internal organs of salmon, with fibrin deposition seen in association with extensive fungal growth but rarely present in association with small quantities of fungus. Fibrin deposition also appeared to occur in association with rapid fungal growth, and cellular destruction. It is probable that the fibrous response in the multiple worm lesions from gadoids is also related to the large number of parasites present in the submucosa and the associated extensive tissue damage. Extensive formation of fibrous tissue has also been observed in the body musculature of herring infected with fungus (Sindermann 1970). Fibrous tissue around the inflammatory cell layer in single worm infections from gadoids was similar to that observed in normal cod submucosa. However, the fibrous tissue in lesions from **L.piscatorius** did show a slight concentration around the lesion, although this was nowhere near as distinct as in multiple worm infections from gadoids. Necrotic tissue was seen immediately surrounding the parasites in multiple worm infections from gadoids, and the lesion cavity in the case of **L.piscatorius** lesions, but was not observed in single worm infections from gadoids. In multiple worm lesions in gadoids, this tissue formed a clear dark-yellow staining layer. This

mass of necrotic material derives from the inflammatory cell layer and represents gathered and degenerated inflammatory cells. The pyknotic nuclei of the cells close to the parasite in single worm infections in gadoids, and their indistinct cell outline suggest that these cells are also dying. This may be the onset of necrosis, as observed around larvae in multiple worm gadoid lesions. Single nematodes embedded in seal stomachs were surrounded by both necrosis and inflammatory infiltration (McClelland 1980c).

Encapsulated larvae were also observed in stomach lesions associated with nematodes in smelt, with fibrotic tissue and varying degrees of cellular infiltration surrounding the larvae (Obiekezie **et al.** 1992). Gastro-intestinal ulcers associated with **H.dollfusi** in paddlefish histologically revealed damaged tissue with cellular infiltration; deeper penetration of larvae caused capsule formation, with thickly layered circular collagen fibres surrounding the worms and a necrotic inner layer (Miyazaki **et al.** 1988). It is possible that the capsules which have formed around the larvae in the submucosa of multiple worm lesions in gadoids are also a result of deep penetration, where the entire larvae were found within the submucosa. Capsules may not have formed around the single worm in the smaller gadoid lesions, as only the anterior ends of the larvae had penetrated. In **L.piscatorius**, the cavity opening to the stomach lumen and containing the larvae, along with probable movement of larvae, may have prevented capsule formation, as although the larvae are situated within the submucosa, they are not entirely surrounded by host tissue.

The outer muscle layers of the stomach wall were present in single worm infection in gadoids, and in **L.piscatorius** lesions, but were absent from multiple worm lesions in gadoids. Miyazaki **et al.** (1988) noted that worms in the muscle layers of paddlefish were also walled off, and the muscle fibres around the area were compressed and atrophied. The host response to nematodes in stomach lesions of smelt was also observed to encompass a large region of the

musculature (Obiekezie **et al.** 1992). Liu and Edward (1971) observed that the fibrous tissue in ulcers associated with **C.osculatum** in the stomachs of sea lions extended down between the muscles of the stomach wall, and Young and Lowe (1969) had noted that a few nematodes associated with stomach lesions in seals had penetrated to the muscle, causing a response. Van Theil **et al.** (1960) and Kikuchi **et al.** (1990) also noted cellular infiltration in the muscle layers of human intestines infected with **Anisakis** larvae.

Miyazaki **et al.** (1988) noted cellular components and debris in the spaces surrounding **H.dollfusi** in the stomach wall, as was seen within the cavity containing **P.decipiens** in angler fish lesions.

Overstreet and Meyer (1981) suggested that the number of worms may have an influence on the intensity of the inflammatory response. The extent and severity of the host reaction in the submucosa, and the absence of the outer muscle layer in multiple worm infections from gadoids is presumably related to the number of parasites present within the stomach wall, but an age related effect is also likely with regard to the host response in the submucosa. The age of these lesions is, unfortunately, not known, but, from the histopathology, and observations by other authors (Figure 23), it is possible to speculate on their possible formation over time.

The presence and location of inflammatory cells around single larvae in gadoids suggests that initially a rapid cellular infiltration occurs around the parasites. The cellular response seen surrounding the parasites probably consists of macrophage-like cells attracted to the area as part of the immune mediated response. Evidence from multiple worm infections in gadoids suggests that these inflammatory cells cannot break the parasite down and therefore a fibrous response occurs, with collagen fibres being laid down to encapsulate the parasite within the host tissue. This is confirmed by the location of these collagen fibres in multiple worm infections in gadoids, and from the slight

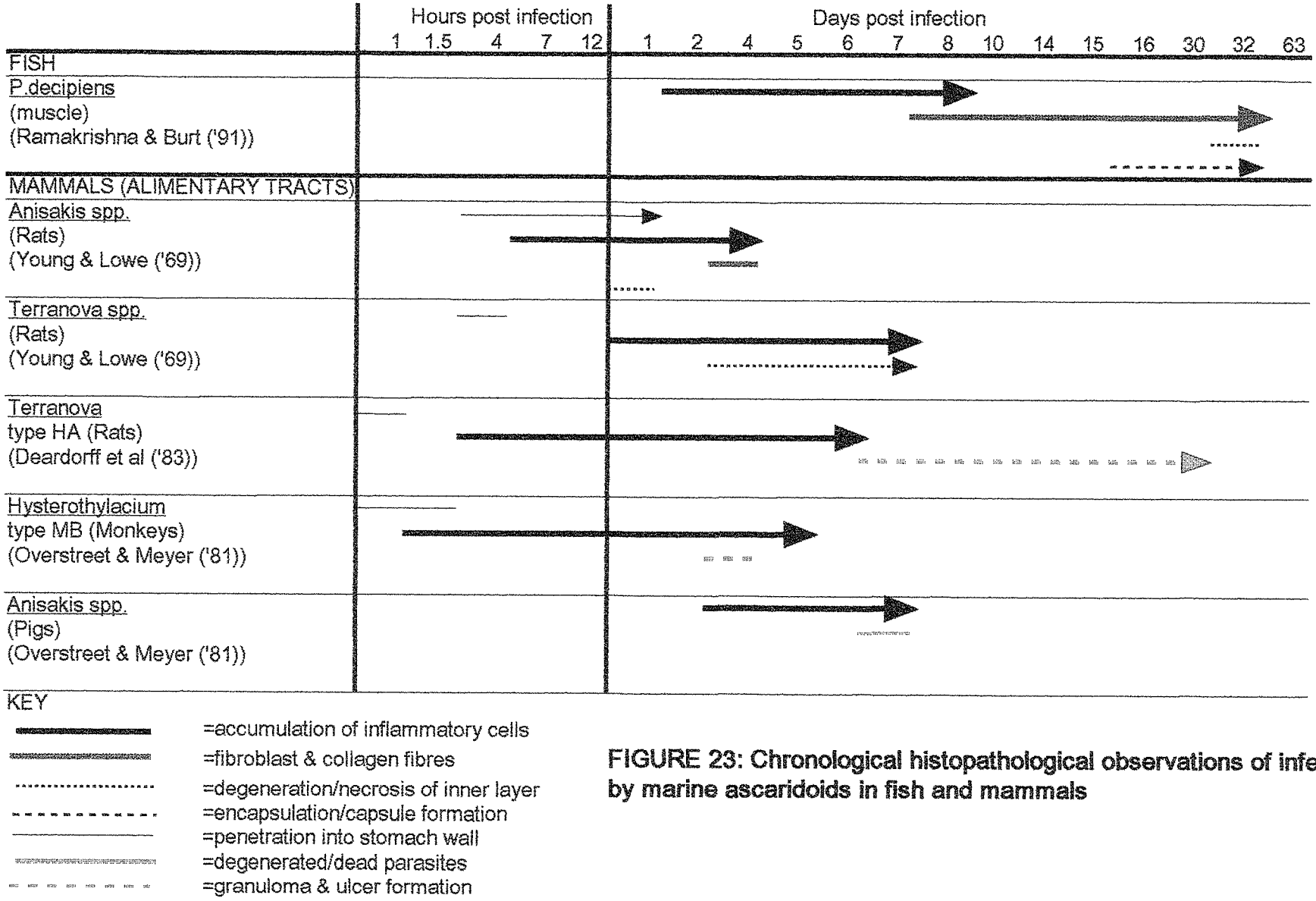



**FIGURE 23: Chronological histopathological observations of infection by marine ascaridoids in fish and mammals**

concentration of fibrous tissue in **L.piscatorius** lesions, which are not directly around the worm, but surround the inflammatory cell layer. The final stage of the host response appears to be complete encapsulation of the parasites by both the inflammatory cells and the dense accumulation of collagenous fibres leading ultimately to death of the parasite, as observed in multiple worm infections from gadoids. These collagen fibres are closely packed and form a concentric pattern encircling and enclosing the nematodes in the submucosa. In addition, the strands of collagen laid down become less organised further out from the worm (that the collagen fibres are not directly around the worm and are not observed in single worm infections indicates that the fibrous reaction is a secondary response). The fibrous response is accompanied by necrosis immediately surrounding the parasites. Necrosis is normally caused by factors such as the physical pressure of the parasite within the tissue, or a lack of oxygen. In single worm infections from gadoids, only an inflammatory layer is observed around the parasite - with the collagen fibres showing no apparent deviation from normal tissue in terms of the number and orientation of these fibres - indicating that a fibrous response has not yet occurred. A full response is only observed in the large ulcers due to the larger numbers of worms and perhaps because these lesions may have been produced over a long period of time. It is not clear if the single worm lesions represent the initial stage in the formation of large lesions and whether the former lesions resolve on death or successful penetration of the larva. Local pathology associated with heavy infections of **Anisakis** larvae in the stomach submucosa of **Merluccius polylepis** consisted of inflammation, and the host response eventually led to the formation of a capsule isolating the parasites within the host stomach (Menezes and Lima 1980).

The histopathology of third stage larvae of **P.decipiens** in experimentally infected rainbow trout muscle was similar to that in naturally infected cod (Ramakrishna and Burt

1991), and to the **A.simplex** infections observed in the stomach of multiple worm lesions in gadoids during this study. Ramakrishna and Burt found that early infections in naturally infected cod had few inflammatory cells and in older infections most larvae were encapsulated in a two layered capsule, with an inner layer of inflammatory cells showing gradual degeneration of inflammatory cells, which formed a dense substance with embedded nuclear debris, and an outer layer of fibroblasts and concentric collagen fibres. The sequential formation of these infections in trout (see Figure 23) is similar to that described above for stomach lesions in fish.

Ramakrishna and Burt (1991) considered that the tissue response of rainbow trout to **P.decipiens** was a chronic inflammatory reaction, and this is also the case at least for the multiple worm lesions in the stomach of gadoids. Similar chronic inflammatory reactions have been reported in fish to anisakid larvae (Hauck and May 1977, Elarifi 1982), and to digeneans (Sommerville 1981) and heat-killed bacteria (Finn and Nielson 1971). It is suggested that in fish, nonspecific inflammatory reactions are important in defence against parasitic invasions and ultimately result in encapsulation of the parasite. Young and Lowe (1969) also stated that gastric lesions associated with anisakids in marine mammals appeared typical of chronic nematode infection, and Deardorff **et al.** (1991) had also observed chronic lesions around third stage larvae of **A.simplex** in mice. Kikuchi **et al.** (1990) classified four types of "chronic" infections in man, including a form where degenerating larvae were surrounded by a layer of necrotic tissue. This is similar to the multiple worm lesions observed in gadoids.

The histological similarity between lesions in mammals eg. marine final hosts (Vik 1964, Young and Lowe 1969, Wilson and Stockdale 1970, Liu and Edward 1971, McClelland 1980c, Migaki **et al.** 1982), experimentally infected laboratory mammals (Young and Lowe 1969, Overstreet and Meyer 1981, Deardorff **et al.** 1983), humans (Van Theil **et al.** 1960, Asami

**et al.** 1965, Yokogawa and Yoshimura 1965, Van Theil and Van Houten 1967, Aji **et al.** 1982, Kikuchi **et al.** 1990), birds (Riley 1972, Liu and Edward 1971), and lesions in fish suggests that the types of inflammation caused by penetration of nematodes do not appear to be host-specific, species-specific or indeed organ-specific, the latter as indicated from studies of anisakid nematodes in fish liver (Hauck and May 1977, Elarifi 1982), and muscle (Ramakrishna and Burt 1991).

The host response to invasion by nematodes observed here is likely to have been invoked by mechanical injury to the tissue (Myers 1963, Poole and Dick 1984), and\or excretory\secretory products of the worms (Liu and Edward 1971). Boczon **et al.** (1989) suggested that a combination of these two factors contributed to the pathology observed in muscle infections of **A.simplex** in fish. In either case, the host response to larvae in fish appears to be elicited as a result of active penetration of the nematodes into the stomach, as suggested by eg. Young and Lowe (1969), McClelland (1980c). In a normal situation, larvae presumably migrate through the wall before the host response is fully activated, but in some large fish the larvae appear to have become entrapped in the stomach wall by the host reaction. Anisakid larvae in fish normally migrate rapidly through the stomach wall into the body cavity, however, the larvae observed in the stomach lesions of fish must move slowly enough for a host response to occur around them. Gibson (1970) found **Anisakis** to have little difficulty in penetrating the gut walls of rats, and suggested that differences in the thickness of the gut were related to ease of penetration of larvae, in particular stating that the human gut wall, being thick, would be more difficult to migrate through, and there would be more time for host reactions to occur. This may be true for large fish also, which would have a thicker stomach wall than smaller ones, however, if this were the case, then all large fish would potentially harbour ulcers. However, the increased thickness

of the wall due to parasites already encapsulated within it, in multiple worm lesions in gadoids, is likely to make penetration more difficult for further larvae. In addition, it is possible the nematodes associated with ulcers in these large fish, may have previously penetrated through many fish and may have used up their energy reserves in penetrating through a number of previous hosts. Burt **et al.** (1990a) considered migration of larval anisakids through fish to only occur a limited number of times, and discussed this in relation to energy stores within larvae.

### 3.4.4 Possible Causes of Stomach Ulcers

Menezes and Lima (1980) stated that the degree of infection of **Anisakis** sp. larvae in the stomach submucosa of **Merluccius polylepis** was apparently related to the advanced age of the population (the youngest infected fish being estimated at approximately 16 years old), and with continued accumulation of parasites. The large length of lingcod (over three feet) associated with ulcers harbouring **Anisakis** larvae (Aria 1969), and the fact that ulcers were recovered only from large gadoids and angler fish during this study, also seems to suggest that the degree of infection in these fish is related to the advanced age of the population and continued accumulation of parasites from the food chain.

#### 3.4.4.1 Resistance and Immunity

Ramakrishna **et al.** (1993) described the responses of experimentally infected rainbow trout to **P.decipiens** larvae after sensitization with live **P.decipiens**, extract of **P.decipiens** and non-homologous extract. Fish immunised with **P.decipiens** extract showed a delayed type hypersensitivity reaction to live **P.decipiens**. These fish manifested a significantly stronger secondary response to live sealworm

than was observed in live worm challenges after control immunizations or with live sealworm, and it was concluded that **P.decipiens** could elicit a cell mediated immune response in fish immunised with antigen in form of **P.decipiens** extract. Fish immunised with non-homologous extract or live **P.decipiens** and later challenged with live **P.decipiens** showed a poor secondary host response similar to that of naive fish. Ramakrishna **et al.** considered this unsurprising as multistage infections are common in naturally infected cod. The host response to secondary infections of live worms in naturally and experimentally infected fish suggested that **P.decipiens** released antigens slowly, delaying the response, or that they are "hidden" from the specific immune reaction, and Ramakrishna **et al.** considered that the long survival of **P.decipiens** in natural fish hosts, shows its ability to avoid lethal consequences of immune sensitization. Such an effect is also likely to be true for third stage larvae of **A.simplex** in fish, however, the morphology and pathology of the stomach lesions observed during this study indicates that **A.simplex** and **P.decipiens** in some large fish are not managing to avoid the cellular host response.

The pathology observed in stomach lesions in fish, particularly in the multiple worm lesions in gadoids, appears to indicate a breakdown in the mutual tolerance of the host and parasite, as suggested for the ulcers reported from seals by McClelland (1980c). From the fish examined during this study, it seems that some large fish have also become less tolerant to infections by certain species of nematodes.

Sindermann (1966,1970) stated that resistance to disease in fish involves a number of interacting factors including individual variability, species characteristics, seasonal influences and nutritional effects. Environmental factors may also be important in response to disease eg. temperature or diet (Sindermann 1966). Khalil (1969) suggested that fish may develop a resistance with ageing, as he observed infection with larval **Contracaecum** (= **Hysterothylacium**) **aduncum** to decrease with an increase in length\age in herring, and Smith

(1983b) suggested that resistance of certain species, sizes and ages of teleost hosts to penetration of the alimentary tract by ascaridoid larvae may possibly occur. This is apparently occurring in the specimens of large (and therefore older) gadoids and angler fish for infection by **A.simplex** and **P.decipiens** respectively.

There may be differences in the responses of different host fish species to anisakid larvae eg. Burt **et al.** (1990a) stated that in their experiments on fish - fish transmission of larval **P.decipiens**, larvae harvested from sea raven may have suffered a more severe host response than those harvested from cod. McClelland (1980c) noted that **P.decipiens** appeared to be more pathogenic in harbour than grey seals, with inflammatory reactions being less severe in the latter. Whilst it is possible that **P.decipiens** is more pathogenic in angler fish than in gadoids, and **A.simplex** more pathogenic in gadoids than in angler fish, additional factors must be involved in the response to these parasites, as both species of nematode are likely to be present in the body cavity of the fish examined, having penetrated successfully previously. However, it is of relevance to note that **P.decipiens** may be the most common nematode found in angler fish - these being benthic, piscivorous fish, generally found in shallower waters (Wheeler 1969) whilst cod (as shown in the present study) harbour significantly larger numbers of larval **A.simplex** than **P.decipiens**.

It is also possible that some large cod and angler fish have become hypersensitive to infections by **A.simplex** and **P.decipiens** respectively. Resistance or hypersensitivity may have resulted in the development of a host response around larvae attempting to migrate through the stomach (the first organ to be breached), and would also account for the presence of single worm lesions in gadoids, where several individual ulcers were observed within the same stomach; the host may elicit an inflammatory reaction (to each of these worms) if they were all in the process of penetration at the same time, or within a relatively short space of time.

Presumably, resistance and hypersensitivity would be specific to the parasite species involved.

### 3.4.4.2 Mass Attack, Attraction and Clustering Behaviour

Sindermann (1970) stated that in parasitic diseases acquired immunity takes the form of heightened resistance to superinvasion. This may be the case in multiple worm lesions from gadoids, where a large number of **A.simplex** may have derived from a single invasion.

In the present study, **A.simplex** was observed to exhibit clustering behaviour at the third larval stage in fish. Clustering behaviour has been suggested for adult **A.simplex** in natural final hosts (Smith 1989) and third stage larvae in fish appear to be exhibiting such behaviour in association with multiple worm lesions from gadoids. Such attraction of **A.simplex**, and the resulting ulcer formation, would also account for the fact that no **P.decipiens**, **C.osculatum** or **H.aduncum** were observed in the stomach wall, as these would not be attracted to those areas and would penetrate in other, non-ulcerous, regions of the stomach. Young and Lowe (1969) had found mixed infections of **Contracaecum** and **Anisakis** in lesions in seal stomachs but the two species did not occur together in the same lesion. It may be that third stage larvae of **P.decipiens** do not exhibit attraction and aggregation behaviour in fish, as evidenced by the small numbers of larvae found lesions from angler fish. Lesions in **L.piscatorius** are formed by one to three **P.decipiens**, and those containing more than one **P.decipiens**, may have resulted from nematodes in close proximity to each other in a prey fish, being released together during digestion, and penetrating in the same area.

The age of the lesions is not known, and the stage at which larvae entered the ulcer cannot be estimated, however, the presence of both live and dead larvae at all levels of the lesion in multiple worm gadoid infections suggests that

larvae are being attracted to the lesion in successive infections over several periods of time and the lesion was not a result of a single mass infection. Smith (1989) suggested that **A.simplex** in porpoise lesions represented successive waves of infection, and added that further nematodes entering the porpoise may be attracted to the ulcer and attach.

Ingested **A.simplex** from further food eaten may be attracted to these areas where **A.simplex** are already present and the presence of additional nematodes attempting to penetrate through the same region of the stomach may result in the massive host response observed in multiple worm ulcers, whereby the host is attempting to isolate the large number of parasites. Such a response can obviously be effective - leading to the immobilisation and eventual death of the parasite within the stomach wall. The worms observed on the serosal side of these ulcers may have been the first to penetrate and have penetrated successfully before the host response was fully activated, but may have been caught up in a milder host reaction leading to normal encapsulation, as seen in the body cavity, but concentrated in the area of the ulcer. If attraction does occur, any further nematodes will presumably experience difficulties in attempted penetration due to the host response in the submucosa, as evidenced from the morphology of the anterior ends of **A.simplex** larvae recovered from the mucosa.

In addition to Smith (1989), other authors have reported gastro-intestinal lesions associated with large numbers of anisakids which have penetrated in a cluster and have formed a raised ulcer eg. **Anisakis** sp. (Vik 1964, Young and Lowe 1969), **Contracaecum** sp. (Young and Lowe 1969, Wilson and Stockdale 1970, McClelland 1980c, Valtonen **et al.**1988) and **P.decipiens** (McClelland 1980c). McClelland (1980c) stated that clustering of nematodes in the gut of marine mammals may be detrimental to the host by causing or aggravating lesions.

Aggregation of nematodes at one site, if no encapsulation occurred, may be potentially advantageous with regard to

penetration (Lee 1970), and subsequent larvae entering the stomach may be attracted to this location. Aggregation or clustering of nematodes in one area has previously been described. Riley (1972) suggested that the clustering behaviour of **Anisakis** larvae in the stomachs of fulmars, was probably related to feeding activity. A feeding related function for clustering is unlikely to be the case for anisakid larvae in fish, as third stage larvae are not thought to feed. McClelland (1980c) postulated that clustering of **P.decipiens** in seals may increase the opportunities for copulation. Again, a reproductive role for aggregation can be discounted for infections in fish, as the larvae are at the third stage and have thus not yet matured.

Smith (1989) stated that penetrating the defensive barriers of the stomach in a previously uninfected host may be difficult for single third stage larvae in porpoises, and suggested that a mass attack may be more successful, and collective excretions\secretions from excretory and\or oesophageal glands may be involved. The large fish examined during this study are likely to already harbour nematode infections in their body cavity and musculature, and therefore difficulty in penetrating the stomach of an uninfected host can be discounted.

Clustering in other species of nematodes has previously been reported eg. **Nippostrongylus brasiliensis** (Lee 1970). Chemical communication, in the form of sex and aggregation attraction has been demonstrated in both free living and zooparasitic nematodes eg. **Panagrolaimus rigidus** (Greet 1964), **Panagrellus silusiae** (Cheng and Samoiloff 1971), **Nippostrongylus brasiliensis** (Bone **et al.** 1977) and **Heligmosomoides polygrus** (Riga and MacKinnon 1987,1988). As mentioned, sexual attraction can be discounted in third stage larvae, but aggregation attraction, may well occur, and indeed Smith (1989) suggested that **A.simplex** in porpoise lesions may stay together due to aggregation attractants. Aggregation attraction functions in bringing together individuals of the same species (Riga and MacKinnon 1987).

Riga and MacKinnon showed that in control experiments, with no attractant target present, responding worms travelled randomly. This may well be the normal situation for larvae in fish, the nematodes released by digestion of infected fish penetrating individually at separate sites. However, if larvae are present within the stomach wall, larvae may be attracted to these sites. Riga and MacKinnon observed increases in attraction to groups of worms rather than to single nematodes, and Bone **et al.** (1977) also found that the number of target worms was important for chemical communication in **Nippostrongylus brasiliensis**. It may well be that attraction in third stage larvae in fish is also based on the number of worms present in a target area ie. ulcer.

Smith (1989) also suggested that as third stage larvae in porpoises were freed from the fish host, gravity may take them to one site where they concentrate and form an ulcer. This may also be possible for the heavy infection of **A.simplex** observed in the multiple worm lesions from gadoids.

### 3.4.4.3 Re-infection

Gibson (1970) stated that the main reason for ease of penetration of **Anisakis** larvae through rats rather than humans was likely to be that in humans multiple infections had previously occurred. This may be true for fish as there seems no doubt that large fish will harbour previously acquired anisakid infections in the body cavity, but this would still not explain why not all large gadoids are affected by ulcers.

Sindermann (1970) stated that the severe localised reaction of fish intestinal wall tissue against penetration of larvae appeared to be confined to areas sensitised by previous penetration of other larvae. Young and Lowe (1969) observed that a second exposure of **Anisakis** sp. into rats

which already harboured **Anisakis** sp. infections in their stomachs, induced a similar host response to that of the first infection, irrespective of the number of days after the first infection when the second infection was given, but the response was more rapid and infiltration was significantly more extensive, and they concluded that gastric lesions in mammals were caused by repeated penetration of large numbers of anisakine larvae in one area. In addition, Oyangi (1967, in Gibson 1970) noted that re-infection of rabbits previously sensitised by infection of larval **Anisakis** sp. normally resulted in a large lesion. In humans, Van Theil and Van Houten (1967) suggested that gastro-intestinal lesions developed only after two **Anisakis** larvae, infecting at different times, penetrated into the intestinal wall at the same area; and Van Theil **et al.** (1960) also considered that the cellular response in man was caused by repeated infection with **Anisakis** larvae.

However, McClelland (1980a,c) stated that neither grey seals or cod demonstrated a significant level of resistance to re-infection by **P.decipiens**. However, numerous previous larval penetrations, perhaps in the same area and within a short time period - may lead to an increased response within the stomach. Once such a reaction has been invoked further penetrating larvae will be adversely affected by the host response.

Van Theil **et al.** (1960) stated that it was not known if the host response in humans started at the moment the worm penetrated the mucosa, or if the host response becomes strong enough to cause a reaction only after penetration by subsequent worms in the same area. McClelland (1980c) noted that there appeared to be an immediate inflammatory reaction to larval **P.decipiens** from challenge exposures in seals. It seems likely that in the fish examined during this study, the host response was invoked on penetration, as an inflammatory reaction was observed around even the single worm infections in gadoids. Although the severe host response seen in multiple worm infections in gadoids may have been due, in

part, to the penetration of further worms into the area.

### 3.4.5 Fate of Nematode Larvae in Stomach Lesions

The presence of dead nematodes in the mucosa shows that a proportion of nematodes die in the process of attempting to penetrate the stomach wall.

It seems reasonable to assume that, in fish, it is encapsulation, associated with deposition of concentrically arranged collagen fibres, as seen in multiple worm infections from gadoids, which eventually leads to the destruction and death of the parasites within the stomach wall, as dead parasites were not observed in either single worm infections in gadoids or in lesions from **L.piscatorius**, which were not associated with a distinct fibrous response. Death of **Raphidascaris acus** in the liver of yellow perch was also observed after the formation of a thick-walled collagenous nodule around it (Poole and Dick 1984), and Johnston and Mawson (1951) found degenerating larvae of **Goezia fluviatilis** within cysts of fibrous tissue in fish. Necrotic **Terranova** type HA were also surrounded by dense concentrically arranged tissue fibres in the submucosa of rats (Deardorff **et al.** 1983). Death of larvae within fibrous capsules may be associated with a lack of oxygen and\or mechanical pressure on the body. Deardorff **et al.** (1991) observed destruction of third stage larvae of **A.simplex** within lesions from mice. They suggested that macrophages, attached to and acting on the worm cuticle, may be involved in the killing of larvae, and that larvae of **A.simplex** in human or mice infections perhaps do not survive due to an inability to slough the cuticle and avoid the immune response of the host and cytotoxic effects associated with the adherence of macrophages.

Van Theil and Van Houten (1967) suggested that larvae which penetrated the gastro-intestinal walls of humans remained there until they were killed by defence mechanisms

of the host. This also appears to be the case for larvae in the submucosa of the multiple worm lesions in gadoids. Larvae of **Anisakis** were observed to die by 7 and 14 days in experimental infections in the alimentary tracts of pigs (Overstreet and Meyer 1981) and rats (Deardorff **et al.** 1991) respectively. It is not known when the onset of larval destruction and death begins in stomach lesions in fish, but it does appear to occur after encapsulation in collagenous tissue. Anisakid larvae probably take longer to die in fish as the immune response is not as well developed as in mammals. Encapsulation of **P.decipiens** in **L.piscatorius** ulcers, leading to death of the larvae, may only occur after the cavity itself has been completely enclosed, including the crater open to the stomach lumen.

### 3.4.6 Possible Adverse Effects of Stomach Ulcers in Fish

It is not known what effect, if any, such stomach ulcers may have on fish. Hauck and May (1977) considered that organ function in Pacific herring was unlikely to be seriously affected by **Anisakis** infection, and the chronic pathology observed would only be serious in older and more heavily infected fish. Arai (1969) found that large lingcod which harboured ulcerous lesions associated with **Anisakis** larvae appeared to be emaciated, in comparison with other specimens, and that they weighed approximately half that of a healthy fish of comparable length. Despite the presence of these stomach lesions, the fish examined during this study appeared to be unaffected in terms of general appearance, and thus it would seem that the stomach lesions in fish are not affecting normal functioning of the stomach. Obiekezie **et al.** (1992) also stated that the condition factor of smelt could not be related to the number of stomach wall lesions. Riley (1972) noted that stomach lesions, caused by **Anisakis** larvae, did not appear to adversely effect the health of the fulmars. The large size of the proventriculus in this species meant that

only a small area of the stomach was made inoperative, even under heavy infections and it seemed unlikely that infections would significantly effect the functioning of the stomach. This may also be the case in large gadoids and angler fish.

The nature of the parasitism, however, may allow infection of secondary pathogens which could lead to death of the fish. Margolis (1970) stated that it has been speculated that parasites which penetrate the gut wall may harbour, or provide a means of entry for, pathogenic micro-organisms and may lead to secondary infections of the digestive tract and body cavity. The cavity caused by **P.decipiens** in angler fish opens to the stomach lumen via a large crater, and evidence of bacteria within the lesions in **L.piscatorius** was observed. However, these cells were not extensive, and unless proliferation of bacteria may subsequently take place, it is unlikely that these would cause severe infection.

### 3.4.7 CONCLUSIONS

The major differences between single and multiple worm stomach lesions in gadoids and **L.piscatorius** lesions, is the differing morphology, both grossly and histologically, and difference in the species and number of parasites present. However, the host response may not yet have been fully developed in single worm lesions from gadoids and lesions in **L.piscatorius**. Potential differences in the pathogenicity of **A.simplex** and **P.decipiens**, differences in the host response of gadoids and **L.piscatorius**, and the numbers of worms present must also be considered in relation to the morphology of the lesions. Ulcers in gadoids were discrete, raised and associated with infections of **A.simplex** larvae, and both single and multiple worm infections were observed. Single worm infections consisted of partially penetrated larvae, with an inflammatory response surrounding the anterior ends of the larvae within the stomach tissue. Multiple worm

infections contained partially penetrated larvae, innumerable larvae within the submucosa and aggregations of larvae associated with the serosal side of the lesion. Larvae within the submucosa were surrounded by a layer of necrotic cell debris, followed by a layer of inflammatory cells, and were encapsulated by fibrous tissue. Lesions in angler fish were associated with 1-3 **P.decipiens** larvae, situated within an open cavity in the submucosa. The ulcers were diffuse in gross appearance, and were not significantly raised. Histologically, these lesions consisted of a diffuse inflammatory reaction, with associated cell necrosis, with slight evidence of a fibrous response occurring.

Infections by penetrating nematodes in the digestive tracts of fish, bird or mammalian hosts, including man, are similar in terms of histopathology and gross morphology - generally involving raised lesions, nematodes often in clusters, and inflammatory, and sometimes fibrous, responses in the alimentary tract wall. The histopathology of lesions caused by nematodes in other organs of fish is also similar to that observed during the present study, as is the reaction caused in response to other parasites eg. digenea, fungal and bacterial infections. Therefore, it would seem that the response observed in the stomach lesions during the present study, is normal for any fish, or indeed vertebrate, host on encountering a pathogen.

It seems likely that a combination of factors are involved in the manifestation of stomach lesions in fish, including potential development of resistance to the nematode species involved, and, in the case of multiple worm lesions from gadoids, possible initiation of ulcer formation by a mass attack of worms, followed by attraction of further worms to the area.

# CHAPTER FOUR : SCANNING ELECTRON MICROSCOPY OBSERVATIONS OF THE CEPHALIC REGIONS OF MARINE ASCARIDOIDS

## 4.1 INTRODUCTION

Scanning electron microscopy (SEM) has proved useful in the study of the external morphology of nematodes, supplementing information gained from conventional light microscopy, and offering an improved method for verification of morphology above the light microscope level. The high resolution, depth of focus and magnifications of SEM enable morphological characteristics to be studied in greater detail, often revealing structures which cannot be observed, or are difficult to discern, by light microscopy, and leading to an increased knowledge of the structures present. For example, the comparative ultrastructural morphology of four North American species of **Spinitectus**, a member of the Spirurida, parasitic in the intestinal tracts of fish, was examined by Jilek and Crites (1982a), who revealed the presence of deirids, amphids and papillae which were previously incorrectly described. SEM has already proved useful in the study of nematode identification, taxonomy and systematics. For example, Gibson (1973) compared cephalic morphology of **Pseudanisakis** spp. using light and scanning electron microscopy and redefined the genus; Fagerholm and Gibson (1987) redescribed the species **Contracaecum ogmorhini** using SEM; Sprent (1977a,b;1978a,b;1980) used SEM to supplement light microscopy in several papers reviewing ascaridoid nematodes of amphibians and reptiles, and also found (1977b) that fourth stage larvae of **Sulcascaris sulcata** from turtles were identical to fourth stage ascaridoid larvae from scallops. SEM has also been used in demonstrating differences between species of the same genera, thus Ansel and Thibaut (1973) described distinct differences in the morphology of the cephalic lips and denticles of **Ascaris lumbricoides** and **A.suum**, when examined by SEM; Madden **et al.** (1970), Maung (1973) and Weise (1973) also found different

arrangements of lip denticles in **A.suum** and **A.lumbricoides**; Jilek and Crites (1982a) observed distinct morphological differences between species of **Spinitectus** recovered from freshwater hosts, as compared to **S. beaveri**, from a marine host; Fagerholm (1988b) observed differences in the pattern of caudal papillae in males of **Contracaecum** spp. : and within the same species (eg. Fagerholm 1988b found differences in the patterns of caudal papillae from cultured specimens of **C.osculatum**, and preliminary examination indicated differences in the numbers of cloacal papillae in **C.osculatum** from different geographical regions).

The morphology and ultrastructure of the superfamily Ascaridoidea have been extensively studied and the use of SEM has supplemented the systematics of this Superfamily. SEM has been used to investigate the ultrastructure of the cephalic region of many genera of Ascaridoidea, a region which bears important criteria for generic and specific identification, eg. the structure of the cephalic regions, along with the excretory organ and definitive hosts, are regarded as being systematically important for **Thynnascaris** (= **Hysterothylacium**) (Soleim 1976b).

Species of the genus **Ascaris** have been examined with SEM by several authors, including Ansel and Thibaut (1973), Maung (1973), Weise (1973), Madden **et al.** (1970), Madden and Tromba (1976) and Lysek (1980). Sprent (eg. 1977a,b; 1978a,b;1980) used SEM to examine several species in various genera of ascaridoid nematodes, including **Dujardinascaris, Sulcascaris, Goezia, Gedoelstascaris, Ortleppascaris, Angusticaecum** and **Krefftascaris**. Barus **et al.** (1983) examined the external morphology of the cephalic end of **Porrocaecum ensicaudatum** using SEM. **Toxocara** sp. were examined by Prociv (1989) and Sanmartín **et al.** (1992). Kazacos and Turek (1982) and Snyder (1989) examined the labia and caudal region respectively of adult **Baylisascaris procyonis**. De and Dey (1992) examined the external ultrastructure of adult **Raillietascaris varani** from the digestive tract of an Indian monitor lizard.

### 4.1.1 Scanning Electron Microscopy of Marine Ascaridoids

A number of authors have used Scanning electron microscopy to describe the ultrastructure of marine ascaridoid nematodes. Several authors have described ultrastructural characteristics from third and fourth stage larvae of **Anisakis** and **Pseudoterranova decipiens** using SEM. Aji **et al.** (1982), Smith (1983b) and Fujino **et al.** (1984) examined third stage larvae of **Anisakis** only. Valter **et al.** (1982) described third stage larvae of **Anisakis**, **Phocanema** (= **Pseudoterranova decipiens**), **Contracaecum** and **Thynnascaris** (= **Hysterothylacium**), whilst Tongu **et al.** (1990) described the surface ultrastructure of third stage larvae of **Anisakis** types I and II, **Terranova** (=**P.decipiens**) type A, **Raphidascaris**, **Contracaecum** type A and **Thynnascaris** type B. Fukuda **et al.** (1988) examined the surface ultrastructure of third stage larval **Anisakis** types I and II, **Raphidascaris**, **Contracaecum** type A and third and fourth stage larvae of **Thynnascaris** (=**Hysterothylacium**) types A and B but only noted the presence of cephalic structures, and neither described them in any great detail nor described the general form of the lip masses. Weerasooriya **et al.** (1986) also examined the surface structures of third stage larvae of **A.simplex**, **P.decipiens**, **Contracaecum** type B and **Hysterothylacium**, and compared third and fourth stage larvae of **Anisakis** type I and **P.decipiens** from different sources. Carvajal **et al.** (1981) briefly examined the ultrastructure of the cephalic region of cultured adults of **A.simplex** and **P.decipiens**. Adults of **Contracaecum aduncum\Thynnascaris adunca** (= **H.aduncum**) were examined under SEM by Soleim (1974, 1976b) and Soleim and Berland (1981), whilst Yoshinaga **et al.** (1987) illustrated the ultrastructure of the cephalic and caudal regions of second, third and fourth stage larvae, and adults of **H.aduncum**. Fagerholm (1988a) used SEM to supplement light microscopy in the examination of fourth stage larvae and adults of **C.osculatum**. Fagerholm (1988b, 1989) also used SEM to examine the pattern of caudal papillae in **C.osculatum** and

other species of this genus from different regions of the world and to examine intra-specific variability of morphology in a single population of **C.osculatum**. In addition to differences in the cephalic structures of these species, significant differences in the distances between the transverse striations of the cuticle of larval Anisakidae, and the diameter of the worm trunk were also observed by some authors (eg. Fukuda **et al.** 1988, Tongu **et al.** 1990), allowing larvae to be identified, even if available only as a fragment. Aji **et al.** (1982) also used SEM to identify an **Anisakis** larva recovered from the intestine of a human patient.

### 4.1.2 General Cephalic Morphology

A diagnostic character of ascaridoids is the presence of three lips - one dorsal and two subventral, or ventrolateral (to avoid confusion the term subventral will be used for the latter) at the cephalic end, which generally become distinct at the fourth larval stage. In the centre of the three lips, a mouth opening is present leading to the oesophagus. The morphology of the cephalic region is important in taxonomic terms, for identifying nematodes at various levels of classification (eg. Davey 1971, De and Dey 1992). The lip structure of ascaridoid nematodes is also important in evolutionary terms. Sprent (1983) suggested that ascaridoids have adapted their labial and associated structures to their hosts in accordance with methods of feeding and anchorage, and this has led therefore to a wide variety of modifications of the anterior ends of these nematodes.

The cephalic structure of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** has been documented by numerous authors throughout their life cycle stages, using light microscopy, eg. Baylis (1937), Grainger (1959), Myers (1960), Berland (1961), Van Theil and Van Houten (1967), Davey

(1969,1971), Koyama **et al.** (1969), Gibson (1970), McClelland and Ronald (1974a,b), Pappy and Van Banning (1975), Grabda (1976b), Beverley-Burton **et al.** (1977), Cannon (1977) and Hurst (1984a). Descriptions of the early larval stages are generally lacking, although McClelland and Ronald (1974a,b) and Grabda (1976b) did describe the morphology of newly hatched **P.decipiens**, **C.osculatum** and **A.simplex** respectively. Third stage larvae of Anisakidae usually possess a boring tooth, and indeed, third stage larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** all have a single boring tooth at their anterior end (eg. Grainger 1959, Van Theil and Van Houten 1967, Davey 1971, Grabda 1976b, Gibson 1983, Hurst 1984a, Smith and Wootten 1984a,b,c, Berland 1991). This boring tooth is situated between the mouth opening and the excretory pore in **A.simplex**, **P.decipiens** and **C.osculatum** (Smith and Wootten 1984a,b,c), and in the same position relative to the mouth in **H.aduncum**, but in this species, the excretory pore is situated at the level of the nerve ring. The lips of these species are usually poorly developed at this stage. Townsley **et al.** (1963) stated that the preadult (= fourth larval) stage is characterised by loss of the boring tooth and the presence of three well developed lips.

These lips can be observed distinctly in these species at the fourth larval stage. Each lip of fourth stage larvae and adults of **A.simplex** and **P.decipiens** carries a bilobed anterior projection and a dentigerous ridge (eg. Grabda 1976b, Myers 1960). Adult **C.osculatum** and **H.aduncum** do not possess a bilobed anterior projection or denticles, but do have interlabia between each of the lips, termed semi-interlabia in **H.aduncum** - see Berland (1961) for definition - (Baylis 1937, McClelland and Ronald 1974b, Berland 1991).

### 4.1.3 Cephalic Sense Organs

The presence of cephalic labial sensory organs in nematodes has long been established. These sensory organs (papillae and amphids) are modifications of the cuticle, and are associated with underlying nerve processes (Wright 1980). Ascaridoids have both an external and internal ring of papillae. The external papillae are comprised of four large double papillae (two on the dorsal lip and one on each subventral lip) and a smaller single lateral papilla on each of the subventral lips. Two inner papillae are situated on the anterior margins of each lip (however, inner labial papillae were not examined during the present study). Each subventral lip also possesses a single amphidial pore in the lip cuticle which is the external indicator of the amphid. The amphids are situated in close proximity to the single papillae. The sense organs of nematodes are generally small in size (Wright 1980) and this prevented detailed studies regarding their fine structure and functioning until electron microscopy studies were carried out.

It was decided to examine the external ultrastructural morphology of the mouthparts and cephalic sense organs in the four nematode species **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum**, as far as possible throughout their life cycle stages, in an attempt to ascertain external morphological changes in the structure of the mouthparts and sense organs during development and to compare the ultrastructure between these and other nematode species. In general, the sense organs of these species have not been previously described in great detail, generally only the positions of the sense organs having been noted, with little data on the morphology or relative size of the organs themselves. In addition, few comparisons have been made of different species at different life cycle stages, and indeed, only Yoshinaga **et al.** (1987) appear to have previously documented the ultrastructural

morphology of newly hatched larvae - in this case of **H.aduncum** second and third stage larvae recovered from experimentally infected mysids; although the structure of the newly hatched larvae of **P.decipiens** and **C.osculatum** has been described using light microscopy by McClelland and Ronald (1974a,b) and that of **A.simplex** by Grabda (1976b). With the exception of the brief study by Carvajal **et al.** (1981), adults of **A.simplex** and **P.decipiens** do not appear to have previously been examined using SEM. The cephalic ultrastructure of fourth stage larvae of **H.aduncum** also appears to have been insufficiently described.

SEM of the species studied in the present work has not been examined previously from British waters, with the exception of Smith (1983b), who examined third stage larvae of **A.simplex** from euphausiids and fish. Hurst (1984a) suggested that large variations in length of third stage larvae of **A.simplex** from different areas may be indicative of geographic variation and the results of Fagerholm (1988b) indicated differences in the number of cloacal papillae in **C.osculatum** from different geographical areas; therefore, slight variations in cephalic ultrastructural morphology which cannot be observed in specimens by light microscopy may be observed by SEM in specimens from different areas. The known presence of sibling species amongst these nematodes must also be considered when making morphological comparisons between nematodes of these species from different areas. Burt **et al.** (1990b), in an abstract, noted morphological variations in specimens of **P.decipiens**, including the mouth structures of adults, and stated that scanning (and light) microscopy were used to examine these differences with regard to host species and geographical distribution, in view of the likely presence of sibling species of this parasite. Sprent (1952) stated that the specific distinction between pig and human strains of **Ascaris** had been a matter of uncertainty, with epidemiological and experimental observations indicating that there was a difference between the two species, although no morphological difference had been found to distinguish

between them. Examination of the denticles of these species by Sprent and others, eg. Ansel and Thibaut (1973), Madden **et al.** (1970), Maung (1973) and Weise (1973), did reveal differences and Sprent suggested that examination of denticles may be of taxonomic value. Ansel and Thibaut (1973) also observed differences in cephalic lip morphology of **A.suum** and **A.lumbricoides**. Anderson and Townshend (1980) found slight variations in the size, shape and position of the amphids in specimens of **Pratylenchus penetrans** from different host plants. Therefore, there may be differences in ultrastructure of sibling species of the nematodes examined during this study, when compared to nematodes of these species from other areas, in addition to possible geographic variation.

In general, SEM studies of the external morphology of nematodes have tended to concentrate solely on descriptions of the surface structure, and few authors have attempted to compare their results with those for other nematode species, to describe development of cephalic structures through the different life cycle stages, to discuss background information on the structures reported, or to relate their findings to the behaviour or habitats of the species examined. Those worthy of note, however, are: Soleim and Berland (1981) who discussed the functions of the auricles in lips of **H.aduncum**, but did not discuss possible functions of the sense organs or compare their findings with the cephalic morphology of other nematode species, with the exception of the interlabia in **C.osculatum**; Smith (1983b) who discussed the morphology of the lips and sense organs of **A.simplex** in relation to a typical adult ascaridoid; De and Dey (1992) who briefly compared their ultrastructural findings on **Raillietascaris varani** with other species of ascaridoid nematodes, and discussed additional information which they felt may be useful in understanding the relationship of **R.varani** with other ascaridoids and in general comparative studies of ascaridoids; Kazacos and Turek (1982) who compared their SEM findings on the labia of

**Baylisascaris procyonis** with other members of this genus and with the findings of other authors for other nematode species examined by SEM.

Scanning electron microscopy was also used in this study to examine the internal ultrastructure of the anterior end of some of these nematodes. Internal ultrastructure, using SEM, has rarely been examined previously in nematodes. Gibbons (1984) used SEM to examine the internal structure of the buccal cavity in four **Strongylus** species, and Soleim and Berland (1981) examined the ultrastructural morphology of the female reproductive tract and body wall musculature in adults of **Thynnascaris adunca** (= **H.aduncum**). However, no descriptions of digestive tract ultrastructure in nematodes, using SEM, appear to have been made.

## 4.2 MATERIALS AND METHODS

Several preparation methods for SEM of nematodes have been documented in the literature and Eisenback (1986) suggested that each genus of nematodes may require a different procedure for preservation, and indeed different methods of preparation were used for initial fixation of specimens in the present study, some of which were a result of "trial and error", after various attempts to ascertain the procedure which gave the best results (see parasite stages for details).

### 4.2.1 Conventional SEM Processing

Some modifications to the conventional method of SEM processing were made during this study, as specimens of some larvae were found to be insufficiently fixed after the normal initial fixation time (see parasite stages for details).

Conventional preparation for SEM specimens was as follows:
(1) Fixation in 1% glutaraldehyde in sea water - 1 hour. This stage was generally omitted as it was found that the increased fixation time of stage (2) gave equivalent results. Third and fourth stage and adult nematodes were, however, killed in hot (50-60°C) 70% ethanol (to allow extension\straightening of the nematodes for examination) and immediately transferred to glutaraldehyde as in stage (2).
(2) Fixation in 3% glutaraldehyde in sea water (see below for individual stage details). Short periods of glutaraldehyde fixation are not suitable as they do not render tissues resistant to osmotic changes, and in any case short periods of fixation did not appear to kill the parasites from the third larval stage onwards.
(3) Rinse in sea water - minimum of four hours, although specimens were usually left overnight.

Sea water was used as the buffer for SEM processing, as this was the medium in which these parasites were retained prior to fixation.

Stages (2) and (3) were carried out in a refrigerator at 8°C.

(4) Post-fixation in 2% osmium tetroxide in seawater buffer - 6 hours.
(5) Dehydration through acetone series.
70% Acetone - overnight
90% Acetone - 2 hours
100% Acetone - 2 hours
100% Acetone - overnight
(6) Fresh change of 100% acetone and critical point drying in a Biorad Critical Point Drier.
(7) Mounting of specimens on aluminium stubs.
Two methods were used :
(a) Silver Paint
A paintbrush was used to place a small amount of silver

paint in the centre of the stub. Specimens were then mounted carefully on the paint, using fine forceps to position them as required for examination. Specimens were held in this position with the forceps until the paint began to dry. This method was used for third and fourth stage larvae and adults.

(b) Araldite Resin

10ml of araldite resin (CY212) was mixed with 11ml of hardener (DDSA) and 1.1ml of accelerator (BDMA). A thin layer of this resin mixture was spread over the stub using a toothpick, and specimens were then dropped onto the stub and left until the resin mixture set. This method was used for early third stage larvae, owing to their small size and the likelihood of silver paint partially obscuring some of the larvae at the base of the stub.

(8) Sputter coat stubs with gold for two minutes, in an Edwards S150B Sputter Coater, giving a gold layer 15nm in thickness over the stubs.

### 4.2.2 Culturing of Early Third Stage Larvae of Pseudoterranova decipiens

Koie and Berland (in press) found that it was the third stage larva of **A.simplex** which hatched from the egg, and not the second stage, as was previously thought. McClelland (1982) suggested that this was also the case for the closely related **P.decipiens**, and the results of McClelland and Ronald (1974a) -who found that newly hatched larvae of **P.decipiens**, cultured **in vitro**, grew to the infective stage without further moults, were within the length range of larvae recovered from fish and were morphologically similar to larvae from fish, support this hypothesis. Therefore the newly hatched larvae in this study are regarded as early third stage larvae of **P.decipiens**.

Mature female adult worms of **P. decipiens** were obtained from the excised stomachs of grey seals, **Halichoerus grypus,** from the West Coast of Scotland. Several of these worms were dissected in a petri dish full of sea water to release the eggs. A longitudinal incision was made in the body of the worm, through which the uterus was teased out. The uterus was then cut several times and the eggs allowed to flow out into the petri dish. The eggs of **P. decipiens** sank to the bottom of the petri dish where they adhered, making it relatively easy to change the seawater in the dish without losing many of the eggs. The dish was kept in a refrigerator at 8°C, and approximately 90% of the seawater in the dish was changed daily, after examination of the eggs for development. Spontaneous hatching was observed after 15 days, and the highly active larvae remained attached to their egg cases, which were still adhering to the bottom of the dish, by their posterior ends. These hatched larvae were regarded as third stage, but they retained the cuticle of the second stage larvae as a sheath. Hatched larvae were artificially exsheathed, allowing the third stage larvae to be released for examination. Artificial exsheathment followed the method of Davey (1969), using a 0.05% sodium hypochlorite solution. The sea water in the dish was carefully pipetted out and the sodium hypochlorite solution poured in and left for 15 minutes at room temperature. However, after this period, the larvae appeared to remain ensheathed. The larvae were left in the solution for a further 15 minutes, by which time the majority of the larvae appeared to be exsheathed (free) and the sodium hypochlorite solution was pipetted out and fresh sea water poured in. Some specimens did not artificially exsheath.

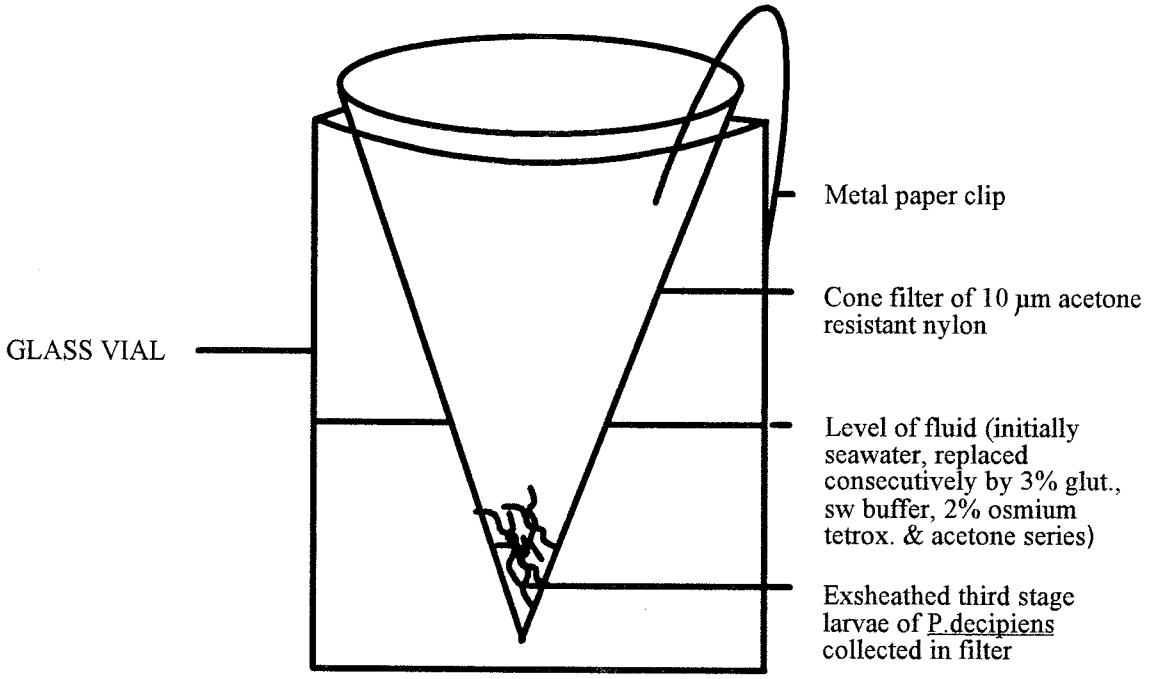
### 4.2.3 Fixation of Early Third Stage Larvae of Pseudoterranova decipiens

The small size of the third stage larvae (approximately 200µm in length by 15µm wide) made conventional fixation for scanning electron microscopy impossible. Two alternative fixation methods were tested :-

(1) FINE MESH FILTER

Exsheathed third stage larvae and seawater were collected in a pipette and released into a cone shaped filter, made from a 10µm mesh of acetone resistant nylon. This mesh was held in a cone shape by a metal paper clip, which also allowed the cone to be supported against the top of a small glass vial (Figure 24). When third stage larvae and sea water were released into this cone, the larvae were trapped in the filter, and the seawater passed through into the base of the vial. This process was repeated several times to ensure that a large number of larvae became trapped in the filter. The level of seawater in the base of the vial was allowed to cover the base of the cone, to ensure that the larvae trapped within did not dehydrate. Excess seawater was pipetted out from the side of the vial if the level reached more than halfway up the vial. After a large number of larvae had been collected, all the seawater surrounding the cone was quickly pipetted out and 3% glutaraldehyde in seawater was pipetted into the vial, via the filter, until the level of glutaraldehyde covered the base of the filter. The vial was then placed carefully in a larger pot, sealed with a lid, and left in the refrigerator at 8°C for one day. The glutaraldehyde was then pipetted out from the side of the vial, and seawater buffer pipetted in via the filter, until the level of the buffer covered the base of the filter. The vial was then placed in the refrigerator overnight. The following day, the seawater was replaced with 2% osmium tetroxide in seawater buffer for 4 hours, and this in turn

(1) FINE MESH FILTER




(2) BACTERIAL MAT CULTIVATION

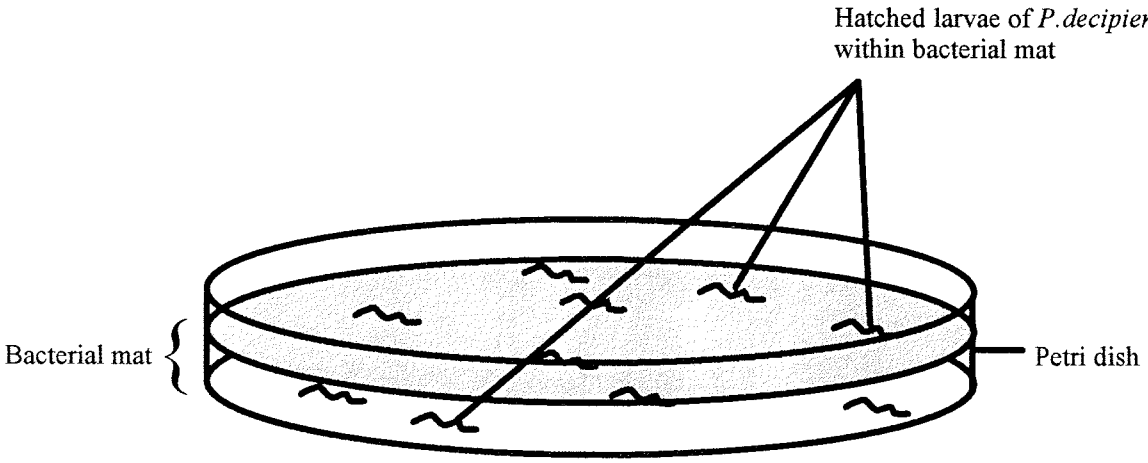



FIGURE 24 : Methods of S.E.M. fixation for early third stage larvae of P.decipiens

(1) Fine Mesh Filter (glut. = glutaraldehyde, sw = seawater, tetrox. = tetroxide).

(2) Bacterial Mat Cultivation

was replaced by 70% acetone and dehydration through the acetone series (as in Step 5 above) was carried out, with the filter remaining in the vial.

After the final acetone change, the filter was removed carefully from the vial, and the top folded over securely to prevent the specimens from falling out during critical point drying. Specimens were critical point dried, then carefully brushed out of the filter onto a piece of folded filter paper, using a fine paint brush, where the now black larvae could be seen under a dissecting microscope against the white paper. The specimens were brushed into the fold of the paper and were transferred onto a stub smeared with a thin layer of wet araldite, by brushing them down the fold onto the stub beneath. The araldite was allowed to dry before sputter coating the stubs.

This method gave limited success, with many larvae being lost both at the critical point drying stage and whilst transferring them to the filter paper and the stub - the small size of the larvae rendered them liable to be lost from the final stages of preparation with the slightest movement. Method (2) was therefore developed to avoid this problem.

(2) BACTERIAL MAT CULTIVATION

A mat of bacteria was grown on the bottom of the petri dish, around the larvae, after hatching had occurred (Figure 24). This mat was cultivated by changing the water in the dish weekly, instead of daily, and over a period of a few weeks the entire base of the petri dish was covered by bacteria. The larvae remained alive throughout this time. Artificial exsheathment was carried out, then pieces of this mat, containing larvae, were broken off and fixed conventionally for SEM; initial fixation in 3% glutaraldehyde in seawater being for one day. After critical point drying, the pieces of mat were directly placed on stubs spread with a thin layer of araldite, using fine forceps, and sputter coated.

This method gave good results, with many larvae being

trapped in the bacterial mat and different regions and angles of the larvae being available for examination, although it was generally found that sections of each larva were partially obscured by the bacterial mat.

The size of the boring tooth was seen to vary in each specimen, and measurements of the length of the tooth were taken, and related to the width of the head.

### 4.2.4 Collection and Fixation of Third Stage Larvae from Fish

Third stage larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** were obtained from the viscera of freshly dissected cod from waters to the north of Scotland, placed in vials of seawater and kept in the refrigerator prior to processing. Several changes of seawater were made before fixation of the specimens, and specimens were vigorously shaken in the seawater in an attempt to dislodge any mucus or debris adhering to the body surface. Specimens were initially processed whole, using the conventional method for SEM fixation. Specimens were kept in 3% glutaraldehyde in seawater for at least one week to allow full penetration through the cuticle. Following fixation with glutaraldehyde, the cephalic ends of these nematodes were cut off with a razor blade approximately 3 to 4mm down the body, before being placed in the osmium tetroxide and processed conventionally. Specimens were mounted on the stubs, using silver paint, with the cephalic end upwards, and sputter coated.

It was noted that some specimens fixed using this method remained alive in the glutaraldehyde for up to one week after initial fixation. It was therefore questionable whether these specimens were fully fixed. It was thought that the cuticle acted as a virtually impermeable barrier to the penetration

of glutaraldehyde into these worms. The body wall of many species of nematodes is known to be highly resistant to adverse chemical environments (Smith 1970). Following the processing of these first samples, further live specimens were cut into sections (cephalic end and sections of the body below this area - approximately 4mm in length) before being placed in the glutaraldehyde, in the hope that the glutaraldehyde could then penetrate directly into the body tissues. Observations made three days after initial fixation revealed that the individual sections were still actively moving; however, after one week in glutaraldehyde this movement had ceased. Specimens were then processed conventionally and mounted, sputter coated and examined as above. Sections of the body itself were mounted vertically, to enable the internal cross-section of the body to be examined.

### 4.2.5 Collection and Fixation of Fourth Stage Larvae

Attempts were made to culture fourth stage larvae of **A.simplex**, **P.decipiens** and **C.osculatum** from third stage larvae collected from fish. The initial culture attempt using Eagle's minimum essential medium + 50µg\ml Glutamine + 50µg\ml Penicillin\Streptomycin + 50µg\ml Kanamycin at 37°C, resulted in the death of many of the larvae, in which cases the cuticle was observed to have loosened around the worm, though this may have been a result of the death of the nematode. Of those which did not die, only one **P.decipiens** and one **C.osculatum** appeared to have moulted to the fourth larval stage; the remaining specimens did not appear to have moulted, even after 14 days. No further attempts were made to culture third stage larvae to the fourth stage, owing to pressure of time, and attempts by other authors, eg. Fagerholm (1988a), to culture larvae of **C.osculatum** have met with limited success. The fourth stage larva of **P.decipiens** appeared to be damaged at the cephalic end, but the fourth

stage larva of **C.osculatum** was processed conventionally, cutting the cephalic end off prior to fixation.

Fourth stage larvae of **H.aduncum** were easily obtained from the intestines of fish, and processed as above.

Specimens were mounted with the cephalic ends upwards.

### 4.2.6 Collection and Fixation of Adults

Owing to ethical difficulties in obtaining adult specimens of **A.simplex** from their cetacean final hosts, formalin fixed specimens of this species were obtained from the collection at the Natural History Museum, London. Adult specimens of **C.osculatum** were found within the stomachs of grey seals examined from the West Coast of Scotland, but as they appeared damaged, formalin fixed specimens of these were also obtained from the Natural History Museum. A number of studies have been carried out concerning the best fixation methods of nematodes for SEM, and formalin was found to give good results (eg. De and Dey 1992), particularly if the nematodes are fixed for a long period of time. In this case, the specimens of **A.simplex** had been collected and fixed in 1991, and those of **C.osculatum** were collected and fixed in 1968. The specimens of **A.simplex** were obtained from a dolphin (**Delphinus delphinus**) in Cornwall, and those of **C.osculatum** from a grey seal in Orkney. The cephalic ends were cut off and processed conventionally from Step (3) onwards.

Adult specimens of **P.decipiens** were obtained from the grey seal stomachs collected (although such specimens were observed to be in a generally poor condition) and adult specimens of **H.aduncum** were obtained directly from the intestines of freshly dissected fish examined for third stage larvae. These specimens were fixed and processed conventionally, again cutting the cephalic ends off prior to fixation.

All adult specimens were mounted with the cephalic end upwards.

### 4.2.7 Examination of Specimens

All stubs were examined under a Philips 500 Scanning Electron Microscope, operating at 12kV.

Images were recorded using photographic equipment attached to the microscope.

Abbreviations to the plates, are given facing Plates 4.1a-d.

## 4.3 RESULTS

Some of the specimens were observed to be in a poor condition and adhering debris still remained, despite washing, a common problem in SEM studies, eg. Fagerholm (1988a).

Shrinkage and distortion of specimens also appears to be a common problem with nematodes examined under SEM, (eg. Green 1967, Allison **et al.** 1972, Weise 1973, Ansel **et al.** 1974, Maung 1975). Anderson and Townshend (1980) stated that most of their specimens of **Pratylenchus penetrans** examined showed some distortion; however, these authors considered that the value of such specimens for morphological examination was not greatly affected, when compared to similar specimens which were free of distortion. Morphological details can often become obscured as a result of poor preservation and by artifacts produced by processing techniques. Eisenback (1986) compared techniques used for preparing nematodes for scanning electron microscopy and found that if fixation was prolonged, shrinkage occurred and surface precipitates formed. In many of the specimens examined here, there was no choice but to fix for longer periods than normal, as the nematodes, and even sections of nematodes, remained alive after a number of days in the fixative.

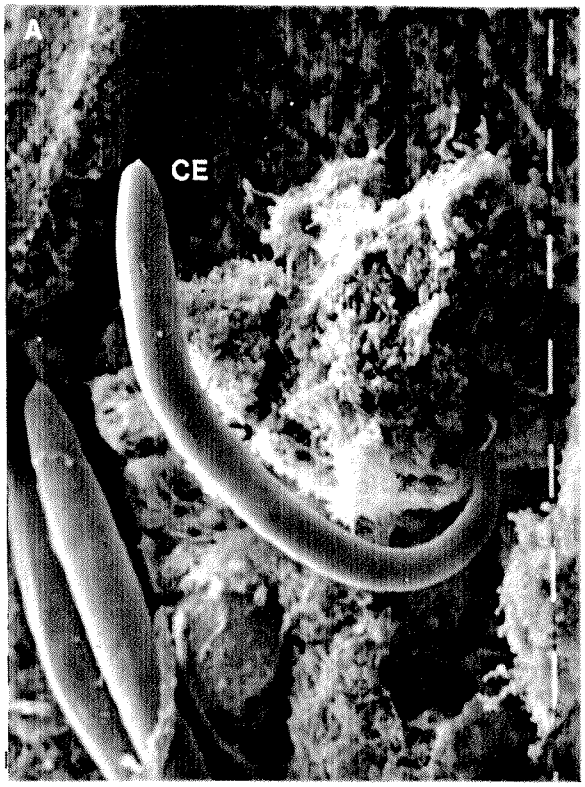
### 4.3.1 Early Third Stage Larvae of Pseudoterranova decipiens

Plate 4.1a shows an exsheathed early third stage larva of **P.decipiens** protruding from the bacterial mat. The visible part of the worm is approximately 120µm in length. The width of the body (at the widest point) is approximately 12.5µm. The club-shaped anterior end shows little external cephalic structure, apart from the prominent boring tooth at the anterior end of the larva. The larvae are widest in the anterior third of the body and gradually taper to a long, pointed tail at the caudal end, which cannot be seen in this specimen. The larvae appear to have a longitudinal ridge running down the body (Plate 4.1b), which is perpendicular to the transverse striations of the cuticle. Plate 4.1c shows the boring tooth at a higher power. The boring tooth is pointed, conical in shape and is directed anteriorly outwards from a medial position on the head; examination of other specimens revealed this tooth to be situated medially, but displaced to one side of the larvae. The boring tooth is not separated from the body but rather seems to derive from the cephalic cuticle itself, and is large relative to the size of the head. The tooth varies in height between approximately 1.0µm and 1.9µm. The height of the tooth correlates positively with the width of the head taken immediately behind the tooth (correlation co-efficient 0.86, $p = <0.01$). No mouth opening appears to be present, although there was a small depression in the centre of the head behind the boring tooth which presumably corresponds to the position of the mouth. No indications of the excretory pore or possible sensory structures were observed.

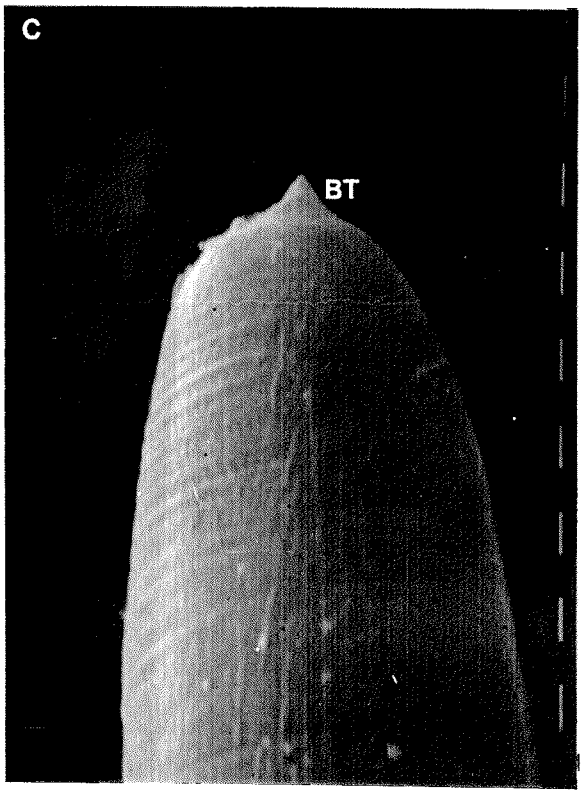
Plate 4.1d shows a coiled ensheathed third stage larva lying on the bacterial mat. The ends of the larva are rounded and a narrow channel of cuticle can be seen to project out from either end. The larva is again club-shaped, and from this it is possible to distinguish the anterior region. The striated nature of the second stage cuticle can be seen, particularly where the larva coils, and the cuticle appears

ABBREVIATIONS TO PLATES 4.1 - 4.9

A = amphid
aa = anterior auricles
ak = antero-lateral knob
AL = anterior lobe
AP = anterior projection
BT\bt = boring tooth
bwm = body wall musculature
CE = cephalic end
cm = cuticular margin
CS = cuticular striations
D = denticles
DL = dorsal lip
DP\dp = double papilla
ec = extended cuticle
EC = external cuticle
EG = excretory gland
EO = exterior of oesophagus
ep = excretory pore
g = groove
I = interlabia
ic = intestinal caecum
IL = intestinal lumen
IN = intestine
lc = lateral chord
lf = lateral flange
LG = longitudinal groove
LR = longitudinal ridge
m = mouth
mf = muscle fibres
O = oesophagus
ol = oesophageal lumen
pg = papillar groove
pr = processes
PS = papillar stalk
RA = raised area
RP = raised protrusions
SI = semi-interlabia
SL = subventral lip
SP = single papilla









**PLATES 4.1(a-d): Early third stage larvae of <u>P.decipiens</u>**
(a) Exsheathed larva (scale bars = 10μm)
(b) Mid-body of exsheathed larva (scale bars = 10μm)
(c) Cephalic region of exsheathed larva (scale bars = 1μm)
(d) Ensheathed larva (scale bars = 10μm)

loose in this area. A longitudinal cuticular indentation can be seen running down most of the body of the larva. No head structure can be seen in this specimen owing to the presence of the loose and extended cuticle at the anterior end of the larva.

### 4.3.2 Cephalic Regions of Third Stage Larvae from Fish

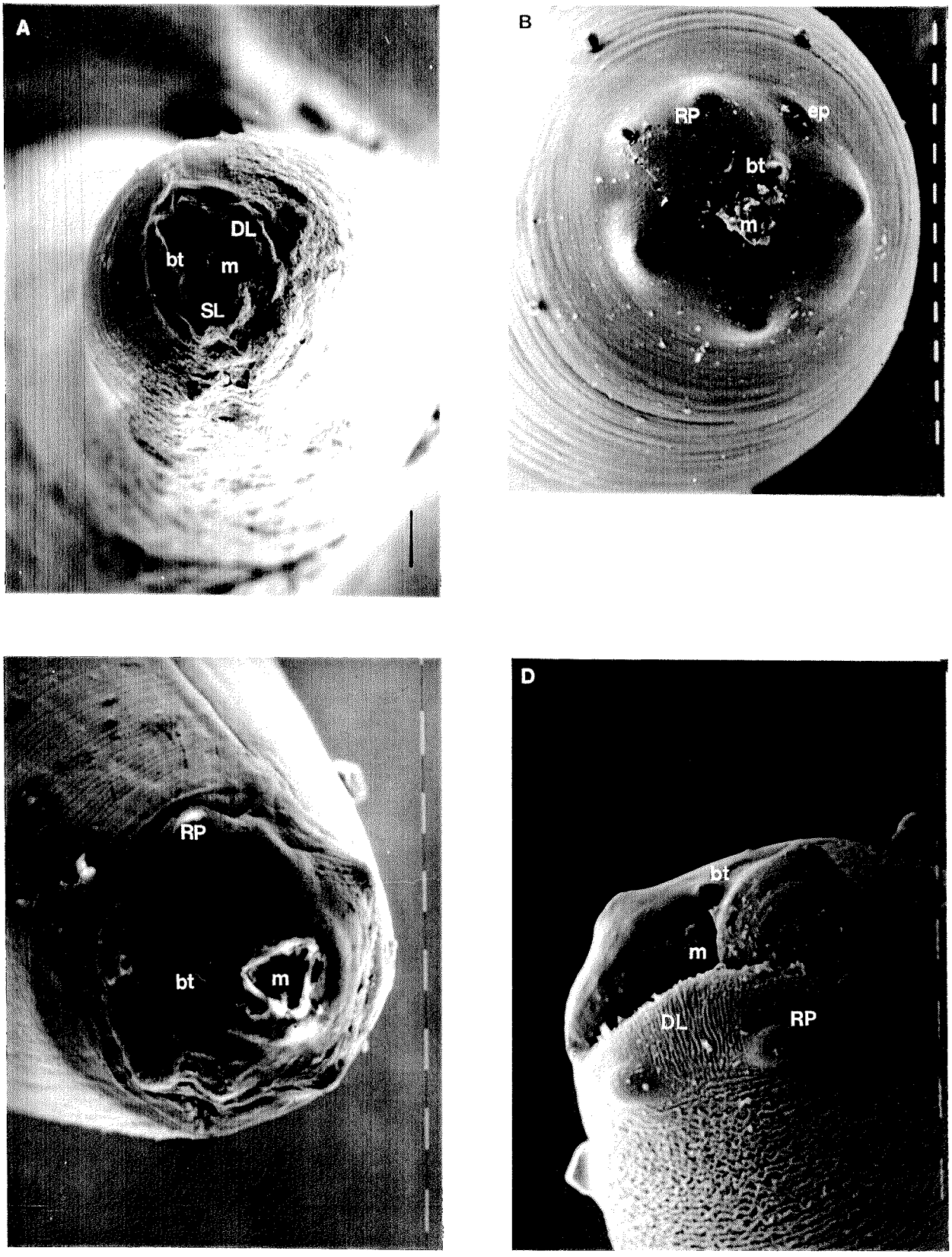
Third stage larvae in fish of all species in general show poorly developed lip masses.

#### 4.3.2.1 **Pseudoterranova decipiens** (Plate 4.2a)

Late third stage larvae of **P.decipiens** from fish are significantly larger and thicker than the newly hatched early third stage. The width of the head is approximately 200µm in this specimen. The triangular mouth opening can be seen (approximately 20µm wide), leading to the tri-radiate oesophagus, with the edges of the triangles parallel to the three lips. Three flat rudimentary lips of equal sizes can be seen, separated by triangular indentations, although they are attached to each other at their bases, and to the posterior tissue. It is possible to distinguish the dorsal lip from the subventral lips by the position of the boring tooth and excretory pore. The subventral lips appear to be bilobed at their outer margins, with one projection at either side. Unfortunately, the specimens were too poor to discern whether any sensory structures were present at this late third stage.

#### 4.3.2.2 **Anisakis simplex**

Two different forms of the anterior end of **A.simplex** third stage larvae from fish were observed.




**PLATES 4.2(a-d): Cephalic regions of third stage larvae**
(a) P.decipiens (scale bar = 100μm)
(b) A.simplex form 1 (scale bars = 10μm)
(c) A.simplex form 2 (scale bars = 10μm)
(d) C.osculatum (scale bars = 10μm)

Form 1 (Plate 4.2b) - A roughly quadrangular lip mass can be seen in the centre of the anterior end - this is not separated from the remainder of the body. This mass is approximately 150µm in width. The triangular mouth opening, full of debris, can be seen in the centre of this mass, and is approximately 25µm wide. The cuticular boring tooth projects slightly outwards and upwards from the lip mass, is roughly triangular in shape, with a rounded end, and approximately 8µm in height. The excretory pore can be seen, approximately 12µm wide. The lip mass itself is generally smooth in appearance, and bears four raised, rounded protrusions, each approximately 22µm wide, at each corner, which are continuous with the cuticle. From the position of the boring tooth, excretory pore and the edges of the mouth opening (parallel to the lips), it is possible to ascertain the positions of the future dorsal and subventral lips, and it can be seen that the dorsal lip bears two of these protrusions, while each subventral lip bears one. These protrusions appear to be situated in the same positions as the double papillae found on adult specimens, and it was concluded that these are incompletely developed double papillae. Two minute slightly raised circular areas - one on each of the subventral lips, situated approximately 20µm to the dorsal side of the rounded protrusions - are likely to be either the single papillae or the amphids, but were impossible to distinguish at this stage.

Form 2 (Plate 4.2c) - This specimen was of a poorer quality than that of the specimen described above. The quadrangular lip mass is approximately 70µm in width, encompasses the entire anterior end, is generally smooth and flat in appearance and appears to be slightly separated from the remainder of the body. A large triangular mouth opening is present in the centre of the lip mass. This is approximately 18µm wide. The triangular cuticular boring tooth (approximately 7µm long) can be seen to project outwards, parallel to the cephalic surface, and is located at the edge

of a raised circular area which surrounds the mouth opening. The boring tooth in this specimen is more sharply pointed than in the above form. As above, from the position of the mouth and boring tooth, it is possible to deduce the position of the future dorsal and subventral lips. There are four raised, rounded protrusions (approximately 27µm in width) at each corner of the quadrangular lip mass, again continuous with the cuticle, although owing to the quality of the specimen these are not very clearly demarcated. Again, these are likely to be sensory papillae which are not yet fully developed, as the position of these protrusions appears to relate to the position of the double papillae which occur in later stages - two on the dorsal lip and one on each of the subventral lips. No evidence of single papillae or amphidial structures was observed, but no firm conclusions can be made about the presence of these owing to the poor quality of the specimen. The excretory pore was also obscured.

#### 4.3.2.3 Contracaecum osculatum (Plate 4.2d)

Third stage larvae of **C.osculatum** have three clear lip bulges - a large dorsal one and two smaller subventrals. The width of the dorsal lip is approximately 70µm. The lips are generally smooth in appearance, and are not fully separated one from another, or from the remainder of the body, and encompass the entire anterior end. The mouth opening lies within the transverse "slit" formed between the dorsal lips and the subventral lips, and extends across the entire lip mass. The cuticular boring tooth can be seen between the subventral lips, and this projects slightly inwards, towards the centre of the head. It is approximately 7µm in height and roughly triangular in shape, with a rounded end. The excretory pore cannot be seen from this angle. As in **A.simplex**, four oval, raised elevations are present on the lip bulges - two on the dorsal lip and one on each subventral lip, and these elevations are continuous with the cuticle of

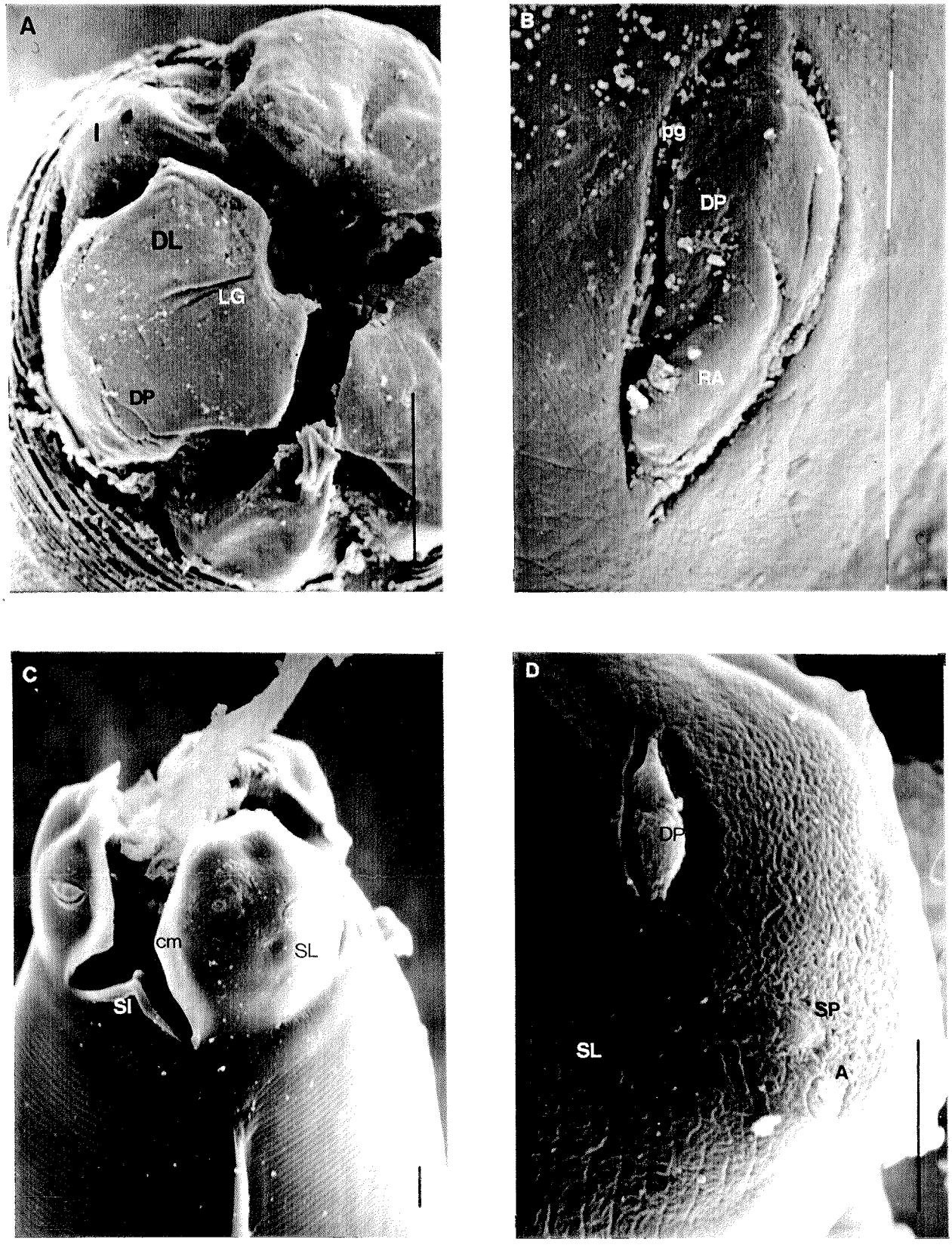
the lips. The elevations measure approximately 15µm across, and 5µm in height, and their position and general shape appear to relate to the position and shape of the double papillae in the fourth stage larvae and adults of this species. No amphids or possible single papillae were observed; however, this plate does show an example of the shrinkage problem that occurred in some of the specimens.

Third stage larvae of **H.aduncum** were observed to be damaged at the cephalic ends, and examination of this stage was not carried out for this species.

### 4.3.3 Cephalic Regions of Fourth Stage Larvae

#### 4.3.3.1 Contracaecum osculatum (Plate 4.3a)

Three fully separated lips of equal size can be seen at this stage (each approximately 150µm wide, at the widest point). The anterior margin of each lip is shallowly indented medially, dividing the margins into two regions. The lip surface generally appears smooth, although there is a shallow longitudinal groove running down the centre of each lip at the anterior region. Partially developed interlabia can be seen between the lips. The interlabia appear to be triangular, tapering from a broad base to a round point, and do not appear to be completely separated from the rest of the lip mass. The mouth opening was obscured in this specimen. The dorsal lip bears two double papillae - one at either side, approximately halfway down the lip. Each subventral lip bears one double papilla, situated near the ventral lateral edges of these lips, and a single papilla and amphid (see adult description for details) near the dorsal lateral edges. The double papillae are seen externally as single structures. Plate 4.3b shows a double papilla, approximately 30µm in length, from a fourth stage larva of **C.osculatum**. The double papilla is leaf-shaped, slightly raised, and is




**PLATES 4.3(a-d): Cephalic regions of fourth stage larvae of C.osculatum & H.aduncum**

(a) Dorsal lip and adjacent interlabia of C.osculatum (scale bar = 100µm)
(b) Double papilla of C.osculatum (scale bars = 10µm)
(c) Subventral lip and adjacent semi-interlabia of H.aduncum (scale bar = 10µm)
(d) Sense organs on right subventral lip of H.aduncum (scale bar = 10µm)

attached to the rest of the lip cuticle at two points on either side of the structure; thus it can be seen that the cuticle of the lip passes directly onto the papillae. A groove around the papilla separates the structure from the rest of the lip, apart from at the two aforementioned points. An additional raised area is present within the papilla itself. This area is situated to one side of the papilla, encompasses just under half of the papillar area, and appears smoother in structure than the rest of the papilla.

#### 4.3.3.2 **Hysterothylacium aduncum** (Plate 4.3c)

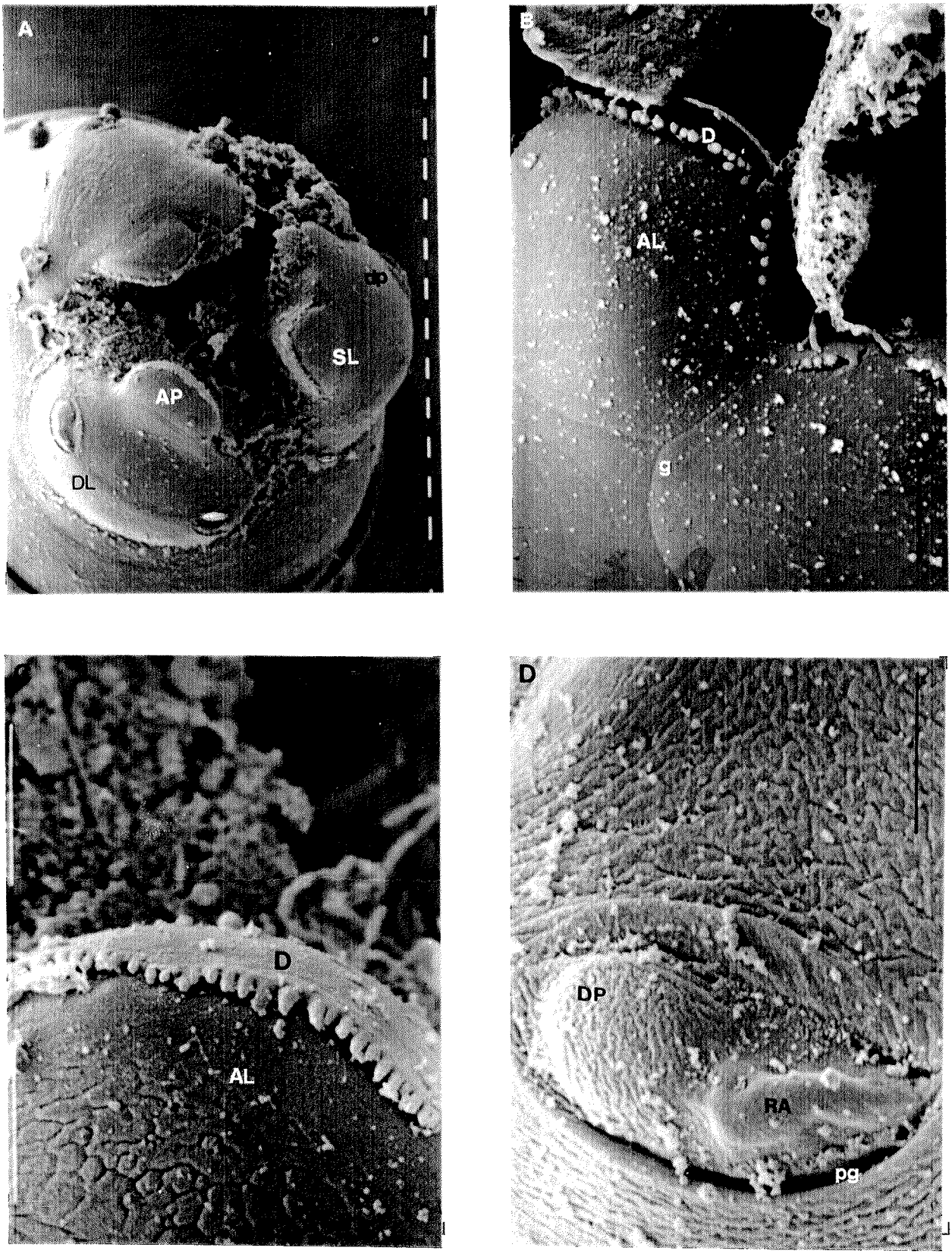
Fourth stage larvae of **H.aduncum** have three fully separated roughly hexagonal lips (approximately 53µm in width), of approximately equal size, the free sides of which have straight edges with a thin cuticular margin. The cuticle of the lips is continuous with the cuticle of the body. The surface of the lips appears to be relatively smooth. A triangular semi-interlabium and associated grooves lies between each lip. The semi-interlabium has a broad base, and is approximately 8µm in height. The grooves on either side of the interlabia separate the lips. The width of the base of each lip, from groove to groove, is approximately 42µm, and the bases of the lips are broad in relation to their size. The mouth opening is obscured in this specimen, owing to the presence of material within the cavity formed by the lips. Such material was observed in all specimens of **H.aduncum**, including adults. Two double papillae are situated approximately halfway down the dorsal lip, one on either side of the lip. One double papilla, a single papilla and an amphid are situated on each subventral lip, in the same positions as described above for **C.osculatum**. Plate 4.3d shows the sensory structures on the right subventral lip of a fourth stage larva of **H.aduncum**, and shows the positions of the single papilla and amphid in relation to the double papilla and to the lip itself. The double papilla is

approximately 10µm. in length. The papilla is surrounded by a deep groove, and is attached to the rest of the lip surface most notably by a stalk-like structure at the side of the papilla nearest to the edge of the lip. The surface of the papilla appears to be smooth. These double papillae do not appear to be as fully developed as those observed in the adults. The single papilla and amphid are found on the opposite side of the lip to the double papilla. The amphid is situated between the single papilla and the lip margin, is approximately 2.7µm. wide, circular and appears to be separated from the rest of the lip. The single papilla is a smooth, raised circular structure, and is approximately the same size as the amphid, but does not appear to be separated from the rest of the lip cuticle. Although the surface of the lip appears to be slightly striated and ridged at this magnification, it also appears that a region of small pitting occurs immediately around the single papilla at this stage. The thin cuticular margin and straight anterior edge of the lip can also be seen in this plate.

### 4.3.4 Cephalic Region of Adults

#### 4.3.4.1 <u>Pseudoterranova decipiens</u> (Plate 4.4a)

Whilst the adults of **P.decipiens** examined were of poor quality, the general cephalic structure can be seen. The lips encompass the entire anterior end, are fully separated, rounded and approximately equal in size (135µm in width), each with a rounded bilobed anterior projection situated centrally on the lip margin. The projections are approximately 25µm in height, and 65µm in width. These lobes are separated from each other and from the rest of the lip tissue by shallow grooves (Plate 4.4b). A row of denticles is present on the anterior margin of each lobe, separating the inner and outer surfaces of the lips. The teeth on the subventral lips of this specimen appear to be severely worn,




**PLATES 4.4(a-e): Cephalic regions of adult P.decipiens**
(a) En face (scale bars = 10μm)
(b) Anterior lobes of a subventral lip (scale bar = 10μm)
(c) Denticles on dorsal lip (scale bars = 10μm)
(d) Double papilla on dorsal lip (scale bar = 10μm)
(e) Sense organs on subventral lip (scale bars = 10μm) (OVERLEAF)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being absent in areas, and where they are present they are short and rounded and of uneven size. Plate 4.4c shows the denticles on the dorsal lip of this specimen. They can be seen to be formed from the inner surface of the lip, and they overlap the anterior margin of the outer lip surface. The denticles are observed as single structures, thin and stubby and of varying size (up to approximately 2µm in height). These denticles also appear to be worn, although not to such an extent as those on the subventral lip. The mouth opening and excretory pore were obscured in this specimen.

The dorsal lip bears two double papillae, one situated near each of the lateral margins, and each subventral lip bears one double papilla situated near the ventral lateral margins. These papillae are seen externally as raised, single structures (Plate 4.4d), and are oval in shape and taper to a point at one side. They are approximately 27µm in length, and are separated from the rest of the lip cuticle by a groove. Within these raised structures is a further raised area at the tapering side of the papilla. This area is thin and irregular in shape, appears smoother than the rest of the papilla, and extends over approximately half of the total papillar length. Each subventral lip also bears a single papilla and an amphid (Plate 4.4e). The single papillae are situated adjacent to the double papillae, towards the dorsal side. These structures are flat, and are separated from the rest of the lip cuticle by a groove. They are roughly heart-shaped, with the base surrounding the edge of the double papillae. The single papillae are approximately 16µm in diameter, and their surface structure does not appear to differ from that of the rest of the lip surface. The amphid is situated approximately 10µm from the single papilla, towards the dorsal side. It is a circular structure, approximately 4.5µm in diameter, and appears to be separated from the rest of the lip cuticle by a groove.

Figure 25 shows comparative **en face** diagrams of a third stage larva and adult of **P.decipiens**.

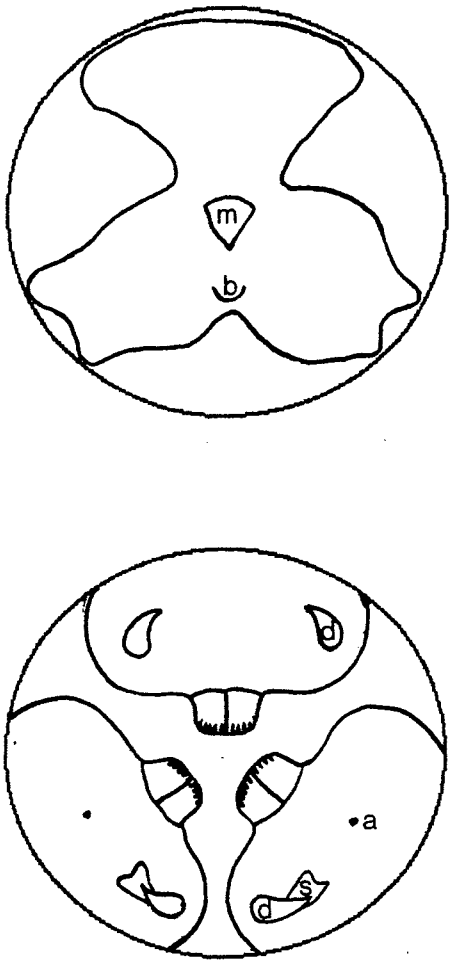


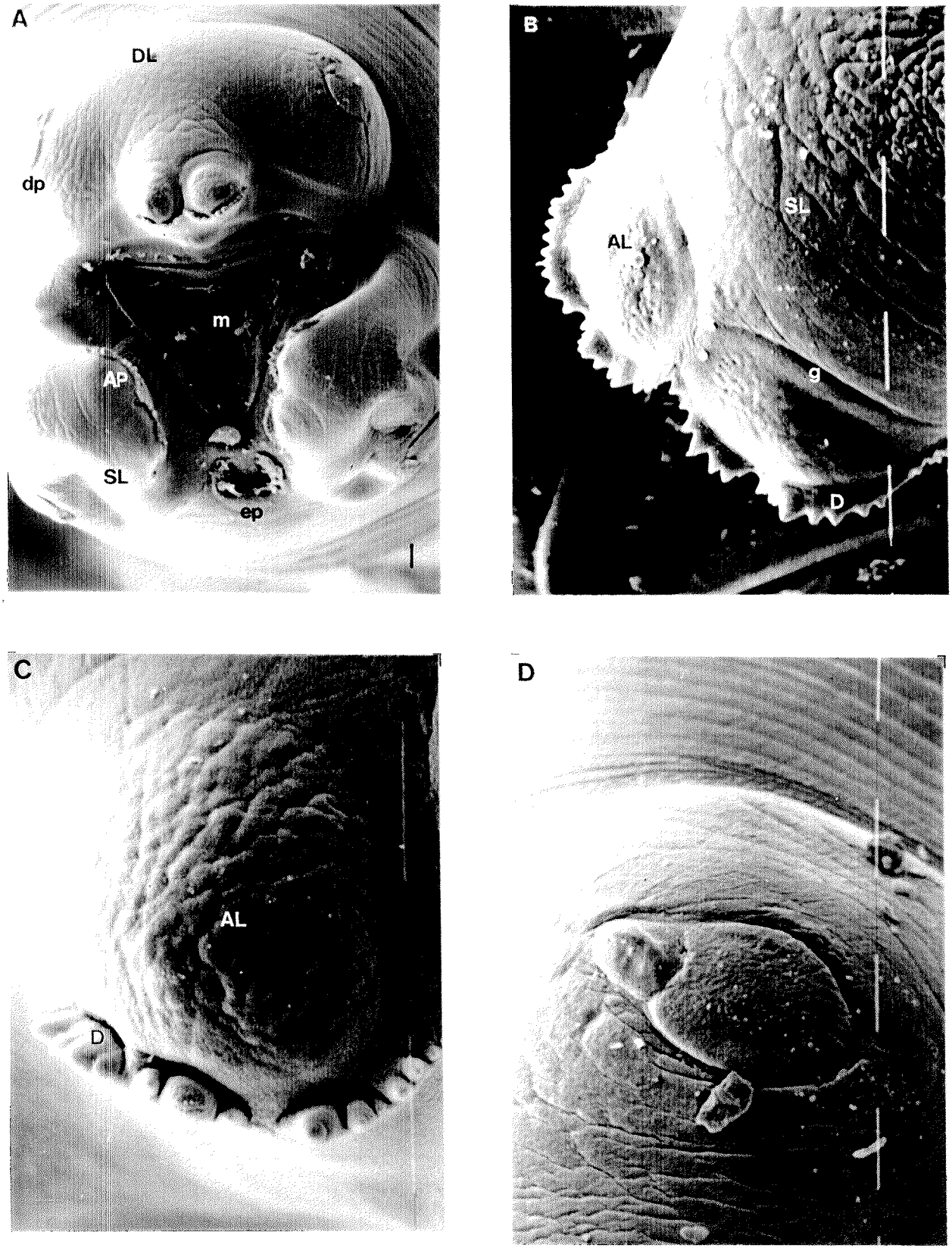

FIGURE 25 : En face diagrams of P.decipiens
(a) Third Stage Larva
(b) Adult
(Not to scale; dorsal lips uppermost)

a = amphid, b = boring tooth, d = double papilla,
m = mouth, s = single papilla

#### 4.3.4.2 **Anisakis simplex** (Plate 4.5a)

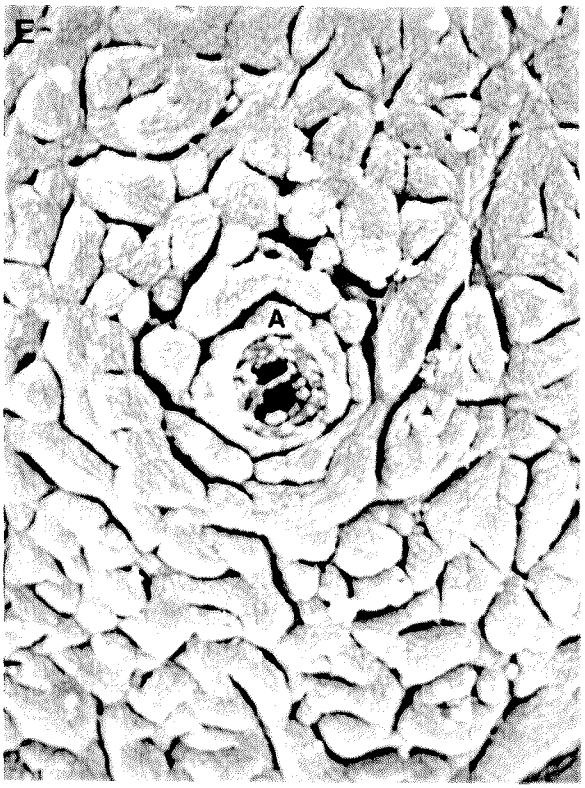
Three fully separated, rounded lips can be seen in adult specimens of **A.simplex**, encompassing the entire anterior end. The cuticle of the body appears continuous with that of the lips. The dorsal lip is much larger than the subventral lips, being approximately 180µm in diameter, whilst the subventrals are approximately 110µm in diameter. The mouth is well demarcated, approximately 88µm wide, and although still triangular, has a triradiate opening. The excretory pore can clearly be seen positioned between the subventral lips. The anterior central regions of the lips bear bilobed projections, separated one from another and from the rest of the lip by shallow grooves (Plate 4.5b). The anterior projections are approximately 60µm wide, with two single rows of dentigerous ridges, separated by a gap, running along the anterior edge of each lobe, separating the inner and outer surfaces of the lips. The ridges on the outer edges of the lips are larger, extending down the edges of the bilobed projection. The denticles are manifested on molar-like ridges approximately 5µm in height, and vary morphologically within the same specimen. The denticles differ in size (although they appear to be smaller on the outer edges of the lobes) and gaps of varying size also occur between them. In general, they are triangular in shape, when viewed from the side, but appear thick and blunt when viewed **en face** (Plate 4.5c). The number of denticles on the two ridges of each lip of three specimens was counted (twice - from different angles) and was found to range from 37 to 47 on the subventral lips and from 45 to 52 on the larger dorsal lip. In addition, the outer ridge of each lobe contained on average twice as many teeth than the inner ridge of the lobe.

Two double papillae are situated laterally on the dorsal lip -one on either side - with one on the lateral ventral sides of each of the subventral lips. The double papillae in adult **A.simplex** (Plate 4.5d) appear as double structures and are approximately 35µm wide. They are ovoid in shape and




**PLATES 4.5(a-e): Cephalic regions of adult <u>A.simplex</u>**

(a) <u>En face</u> view of cephalic region (scale bar = 10μm)
(b) Anterior lobes of a subventral lip (scale bars = 10μm)
(c) <u>En face</u> view of denticles on dorsal lip (scale bars = 10μm)
(d) Double papilla (scale bars = 10μm)
(e) Amphid and surrounding cuticular ornamentation (scale bars = 10μm) (OVERLEAF)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consist of a large and a small lobe - the smaller of which is smoother in appearance than the larger. On the dorsal lip, the smaller lobe is situated on the side of the papilla facing towards the centre of the lips, whereas on the subventral lips the smaller lobe is situated on the side of the papilla facing towards the ventral margins of the lips. The double papillae appear to lie flat against the lip surface - although the smaller lobe appears to be slightly elevated - and these papillae appear to be separated from the surrounding lip cuticle. The amphids in **A.simplex** (Plate 4.5e) are circular and are approximately 2µm wide with a large central pore surrounded by a narrow rim. One amphid is situated on the dorsal side of each subventral lip. The amphids are surrounded by deeply ridged cuticular ornamentation, and this may be the external manifestation of the single papillae.

Figure 26 shows comparative **en face** diagrams of the two **A.simplex** third stage larval forms observed and the adult stage of this species.

#### 4.3.4.3 Contracaecum osculatum (Plate 4.6a)

The lips of adult **C.osculatum** are fully separated and are of equal size (approximately 120µm wide, at the widest point of their posterior halves), and are roughly square in shape. The edges of each lip are concave in the anterior portion (Plate 4.6b), leading to antero-lateral knobs, which are manifested as triangular projections, approximately 10µm in length, one on either side of the anterior lip margin. There is a small but distinct depression in the centre of each lip, approximately 28µm below the anterior lip edge, and a longitudinal groove leads from the anterior of this depression to the anterior margin of the lip. The anterior margins of each lip are shallowly indented at the centre of the margin. Full interlabia are present between the lips, and these are large and hooked, with a rounded tip at their

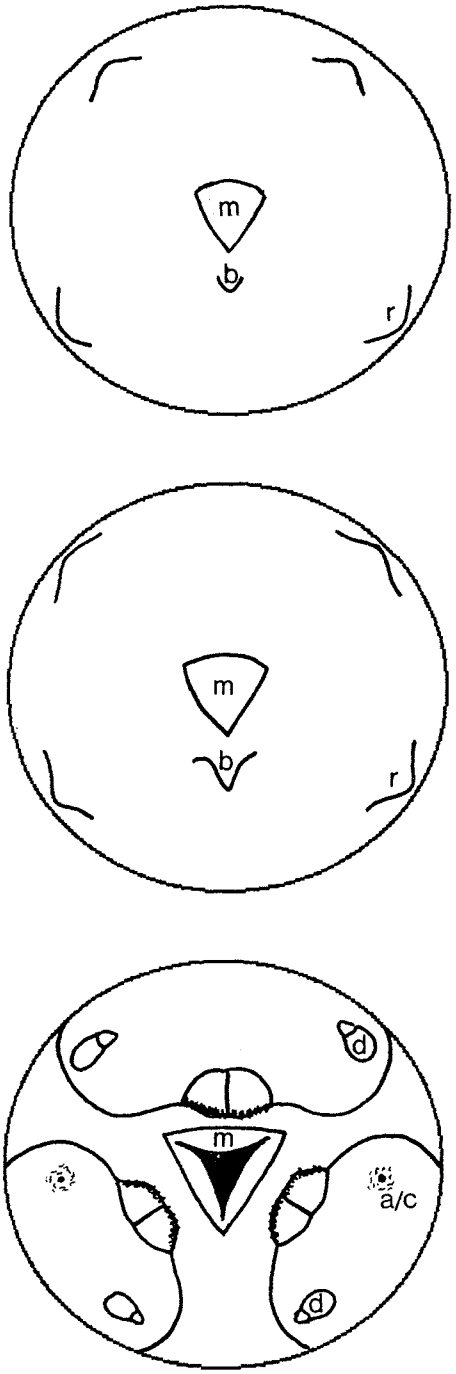
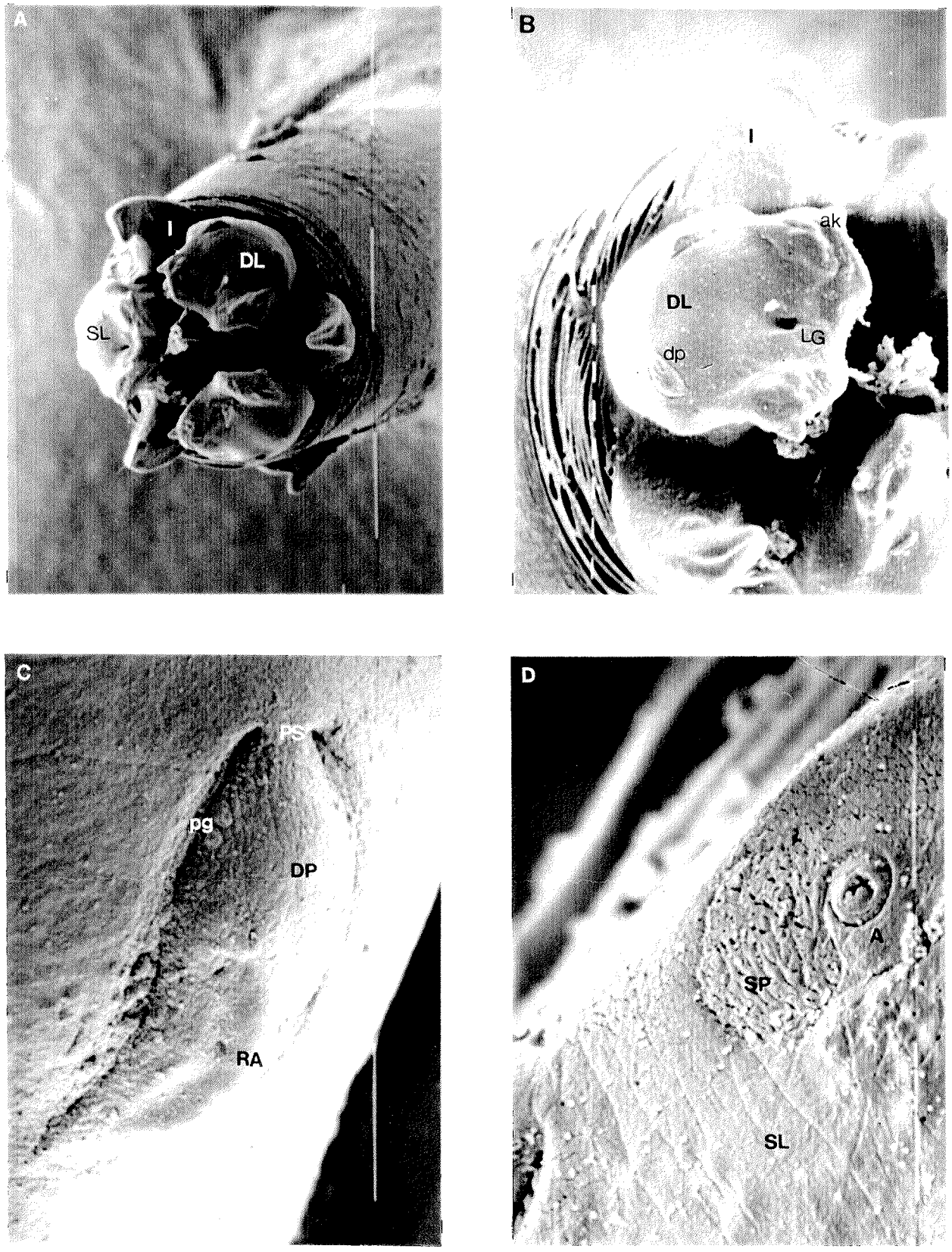



FIGURE 26 : En face diagrams of A.simplex
(a) Third Stage Larva, Form 1
(b) Third Stage Larva, Form 2
(c) Adult
(Not to scale; dorsal lips uppermost)

a/c = amphid surrounded by cuticular ornamentation,
b = boring tooth, d = double papilla, m = mouth,
r = rounded protrusions




**PLATES 4.6(a-d): Cephalic region of adult C.osculatum**
(a) En face (scale bars = 100μm)
(b) Dorsal lip and adjacent interlabia (scale bars = 10μm)
(c) Double papilla (scale bars = 10μm)
(d) Single papilla and amphid (scale bars = 10μm)

anterior ends. The interlabia taper from a broad, wide base to their anterior ends, and they are triangular in shape, when viewed both from the side and **en face** (if the hook is removed). The lips and interlabia encompass the entire anterior end of the specimen, and the interlabia protrude over the edge of the anterior end. The excretory pore and mouth opening are obscured in this specimen.

Two double papillae are present on the dorsal lip (one on each side of the posterior half of the lip), and each subventral lip also has one double papilla, situated ventrally on the posterior half of the lips. The double papillae in adult **C.osculatum** (Plate 4.6c) are raised single structures, approximately 32µm in length, and have a papillar stalk connecting them to the rest of the lip, so that the cuticle of the papillae is a continuation of the lip cuticle. With the exception of this stalk, a papillar groove surrounds the structure. The papillae appear to be leaf-shaped and taper to a point at the end opposite to the stalk. These structures also have an additional elevation within them, similar to that seen in the double papillae of **P.decipiens**, which appears smoother than the rest of the papilla and which extends over approximately half the total length of the papilla. This elevation is positioned on different sides of the papilla, depending upon which lip it is on. The double papillae on the dorsal lip have this elevation at the side nearest the papillar stalk, whilst the double papillae on the subventral lips have this elevation on the side furthest from the papillar stalks.

Each subventral lip bears a single papilla and an amphid (Plate 4.6d). The single papilla and amphid are situated towards the dorsal side of each subventral lip. The single papilla is marked by a roughly circular flat area of cuticular pitting, approximately 12µm in diameter, which is separated from the rest of the lip cuticle by a shallow groove. The amphid is circular, approximately 4.5µm in diameter, appears separated from the rest of the lip cuticle and has a transverse slit. The amphid is adjacent to the

single papilla.

Figure 27 shows comparative **en face** diagrams of a third and fourth stage larva and adult of **C.osculatum**.

#### 4.3.4.4 **Hysterothylacium aduncum** (Plate 4.7a)

The head of **H.aduncum** is smaller in width than the body immediately behind it. The lips are fully separated and are approximately equal in size, being approximately 80µm in width and 60µm in length. Each lip has a pair of rounded anterior auricles of the same size, at each side of the anterior lip margins, which can interlock. The anterior lip margins are a shallow V-shape. Each lip also has lateral flanges on its edge, which are extensions of the cuticle. These flanges occur over the lower three quarters of the lips, are widest at their anterior ends, and taper slightly towards the base of the lips. Triangular semi-interlabia (with a broad base), approximately 20µm high, are present between each of the lips, and the associated grooves appear to extend further than those observed in the fourth stage larvae. The width of the bases of the lips (from groove to groove) is approximately 30µm, and the basal attachment is narrow compared to the size of the lips. The cuticle of the body is continuous with that of the lips. The mouth opening is obscured in this specimen.

Two double papillae can be seen on the dorsal lip, one at each side near the ends of the flanges. Double papillae are present in the same position, relative to the flanges, on the subventral lips, but these lips bear only one papilla, situated on the ventro-lateral edges. The double papillae are manifested as single structures. Plate 4.7b is a high power photograph of a double papilla in **H.aduncum**. The double papillae are pear shaped and approximately 15µm in length. They protrude out from the surface of the lips. A papillar stalk at the narrow end connects the structure to the lip itself, so that the cuticle of the structure is an extension

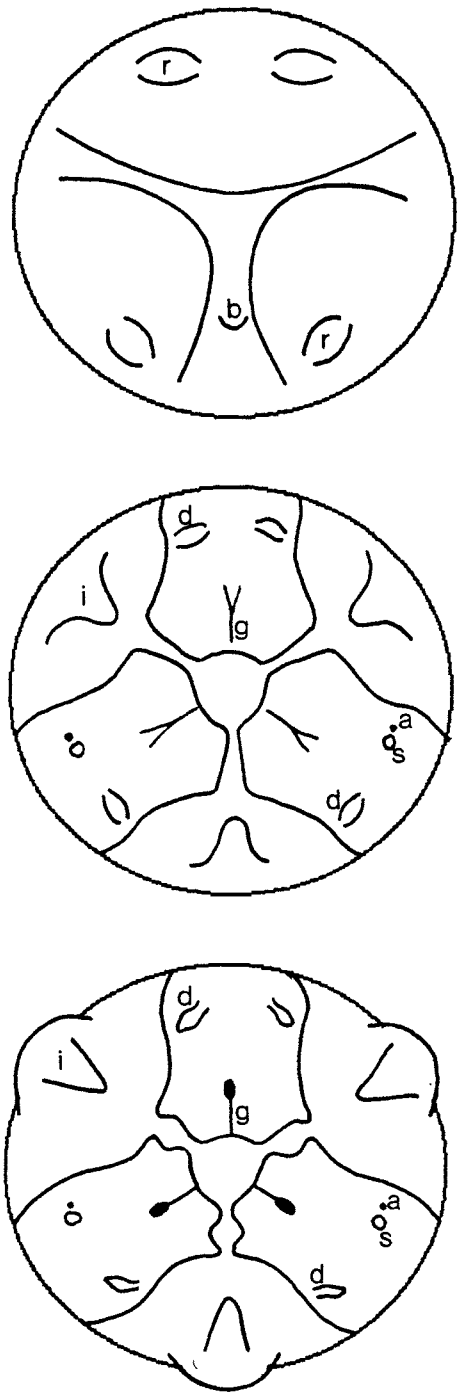
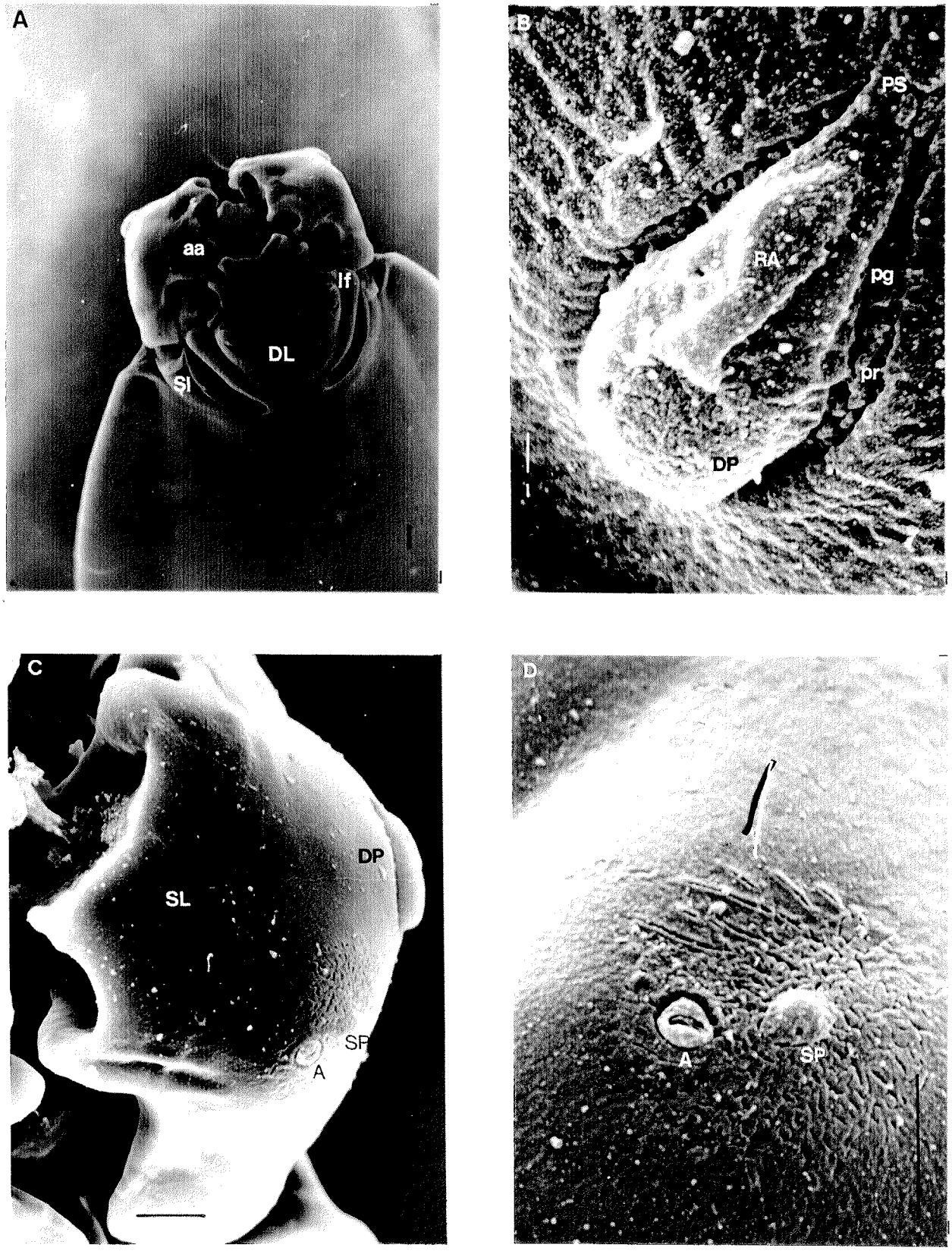



FIGURE 27 : En face diagrams of C.osculatum
(a) Third Stage Larva
(b) Fourth Stage Larva
(c) Adult
(Not to scale; dorsal lips uppermost)

a = amphid, b = boring tooth, d = double papilla,
g = groove, i = interlabium, r = rounded protrusions,
s = single papilla




**PLATES 4.7(a-d): Cephalic region of adult <u>H.aduncum</u>**
(a) Lateral view of cephalic region (scale bar = 10μm)
(b) Double papilla (scale bar = 1μm)
(c) <u>En face</u> view of subventral lip (scale bar = 10μm)
(d) Single papilla and amphid (scale bar = 10μm)

of the lip cuticle, manifested as a "flap". The papillar stalks are situated towards the centre of the dorsal lip, and towards the margins on the subventral lips. With the exception of the stalk, a wide papillar groove surrounds these structures and these grooves contain what appear to be numerous small processes. A thin, raised elevation similar to that observed in **P.decipiens** and **C.osculatum** is also present within the double papillae of this species. The elevation is smooth in appearance and extends across approximately half of the total papillar length. Each subventral lip also possesses one amphid and a single papilla positioned close together. Plate 4.7c shows the position of the single papilla and amphid, in relation one to another, and to the double papillae, on the right subventral lip. Plate 4.7d is a higher power photograph of the amphid and single papilla on the subventral lip of **H.aduncum**. The amphid is a circular structure approximately 4µm wide, separated from the rest of the lip cuticle by a slit-like opening. The single papilla is a smooth, round structure similar in diameter to the amphid, and approximately 2.5µm high. The cuticle surrounding these structures is irregularly pitted and ridged, but does not appear to be separated from the rest of the lip cuticle.

Figure 28 shows comparative **en face** diagrams of a fourth stage larva and adult of **H.aduncum**.

### 4.3.5 Internal Structure

Plate 4.8a is a low power view of a section through the oesophagus and body wall of a third stage larva of **A.simplex**. The oesophagus can be seen to project slightly out from the body wall, and is approximately 420µm in diameter. The body musculature can be seen as radial strands of fibres immediately under the cuticle. This muscle layer is approximately 75µm thick. Plate 4.8b is a higher power photograph of the oesophagus in this specimen. The tri-

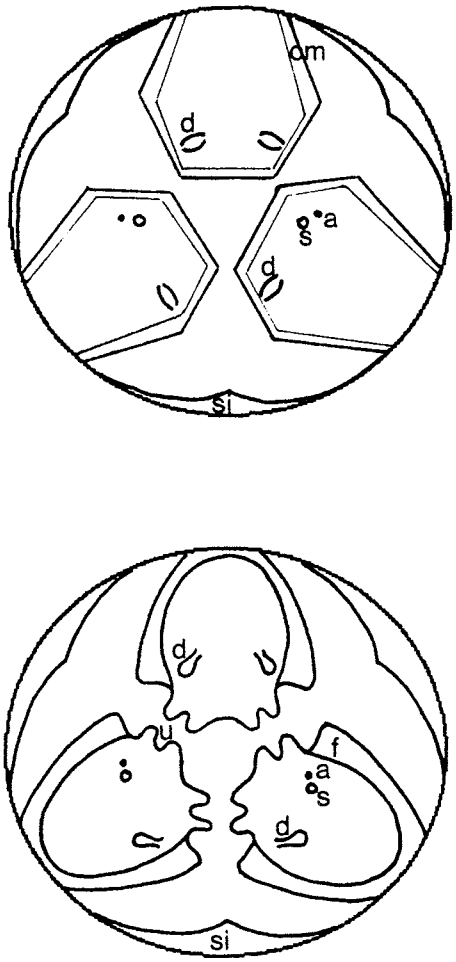
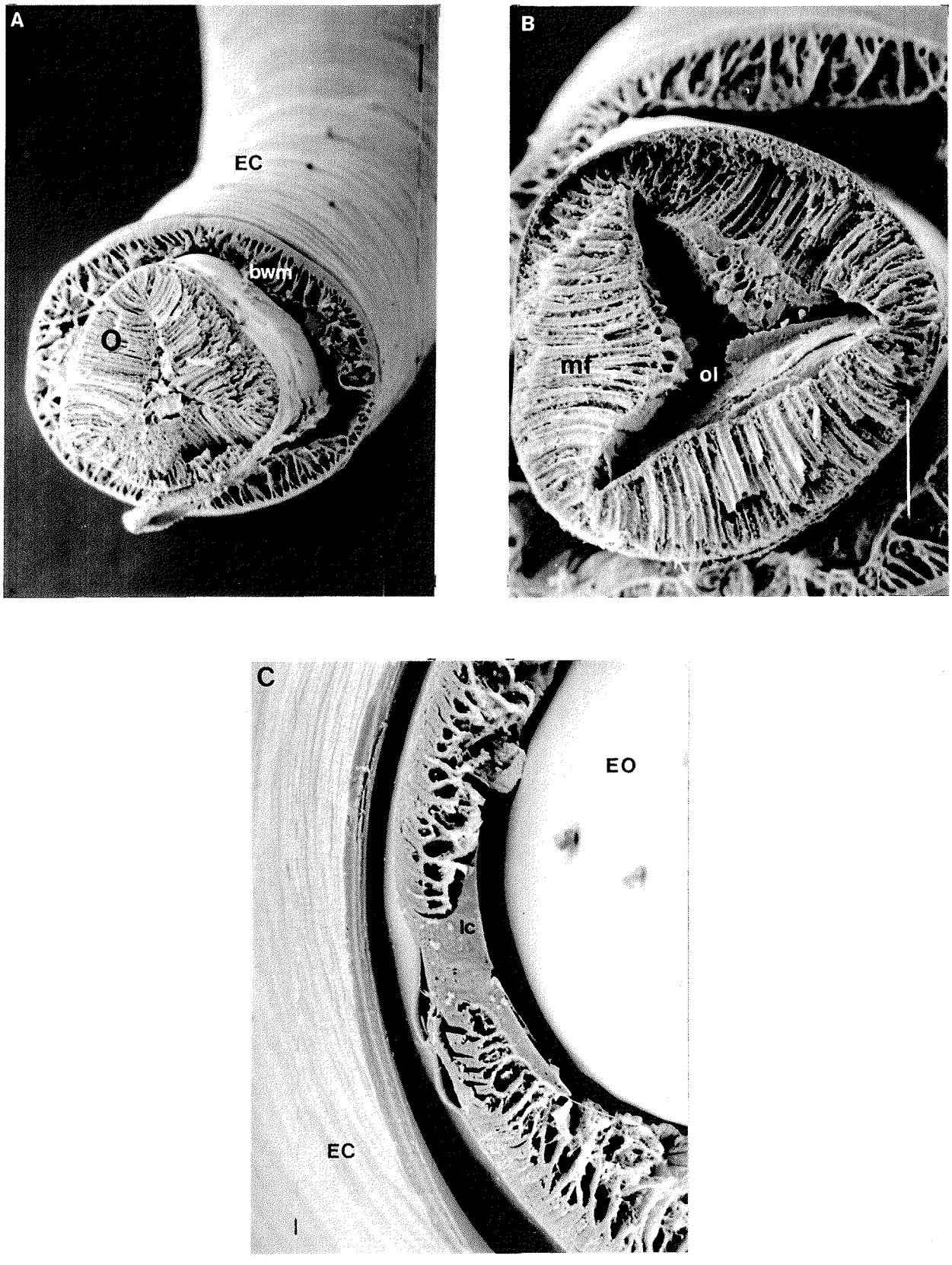



FIGURE 28 : En face diagrams of H.aduncum
(a) Fourth Stage Larva
(b) Adult
(Not to scale; dorsal lips uppermost)

a = amphid, cm = cuticular margin, d = double papilla, f = flange, s = single papilla, si = semi-interlabium, u = auricles

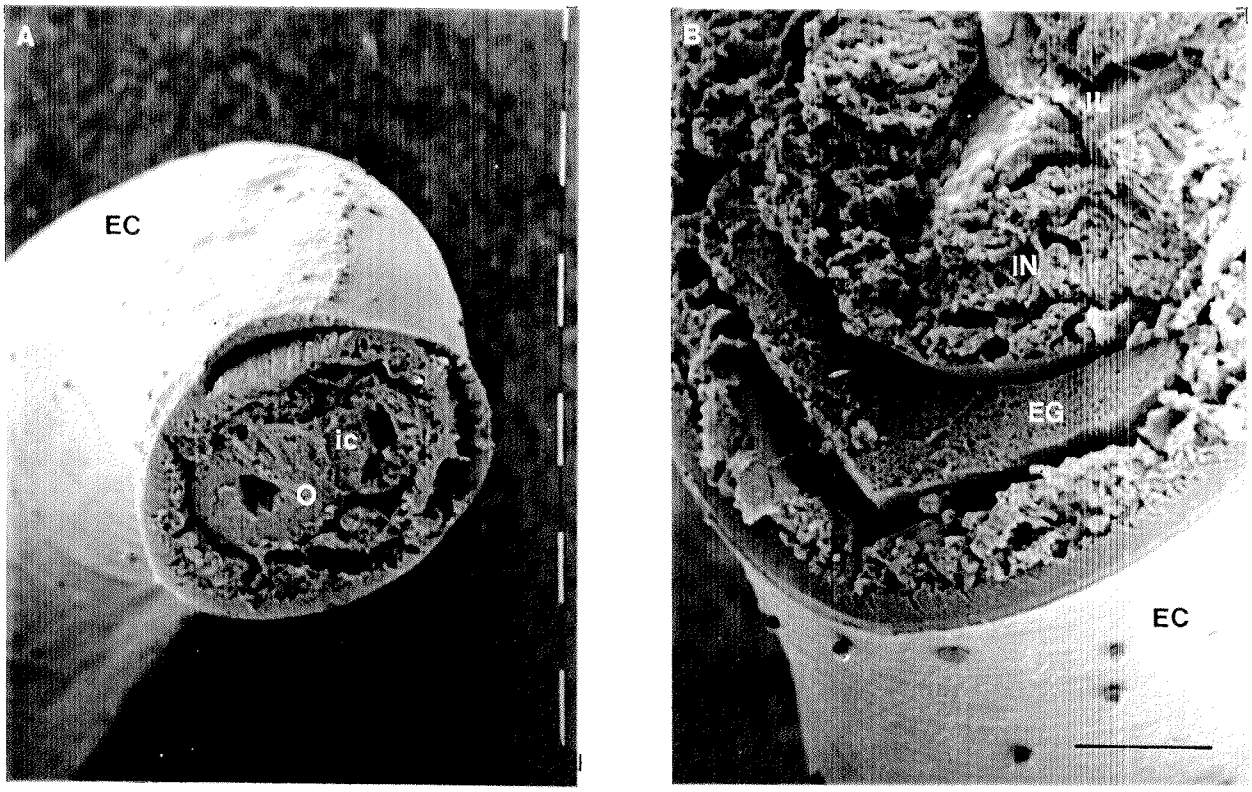



**PLATES 4.8(a-c): Internal structure of third stage larvae of <u>A.simplex</u>**

(a) Cross section through the oesophageal region (scale bars = 100µm)
(b) Cross section through the oesophagus (scale bar = 100µm)
(c) Cross section through a lateral chord and body wall (scale bar = 10µm)

radiate lumen can be seen to be slightly open, and appears to contain globular particles of food. Radial muscle fibres can be seen clearly spanning the entire oesophageal wall. Plate 4.8c is a high power view of a lateral chord, situated within the body wall and spanning the musculature, extending outwards in either direction over the inner surface of the musculature, giving it an "anvil" shape. The chord is smooth in appearance, and also appears to be homogeneous in nature. The smooth exterior of the oesophagus can also be seen in this photograph. The musculature of the body wall can be seen to be composed of two distinct layers. The first, outer layer, closest to the cuticle, appears to be composed of tightly packed muscle fibres, giving a solid appearance. The second, inner layer is thicker and consists of loose muscle fibres, which are extensions of the fibres from the outer layer.

Sections through the body of third stage **P.decipiens** larvae were of a poorer quality; however, the general form of the internal structures can be seen. Plate 4.9a is a cross-section through the oesophagus and intestinal caecum of a third stage larva of **P.decipiens**. The intestinal caecum can be seen above the oesophagus in this photograph, and is only slightly smaller in diameter (180µm) than the oesophagus (200µm). The intestinal caecum can be seen to have an almost semi-circular lumen, as compared to the triangular lumen of the oesophagus. Plate 4.9b shows a cross-section through the intestinal region of a third stage larva of **P.decipiens**. The multi-radiate lumen of the intestine can be seen, and adjacent to the intestine lies the excretory gland. This is roughly triangular in shape in this section, and partially surrounds the intestine. The "pebbled" nature of the gland can be seen, and the tissue itself appears to be homogeneous. The diameter of the intestine is approximately 340µm, and that of the excretory gland, at the widest point, is approximately 92µm.




**PLATES 4.9(a,b): Internal structure of third stage larvae of <u>P.decipiens</u>**

(a) Cross section through the oesophagus and intestinal caecum (scale bars = 100μm)

(b) Cross section through the intestine and excretory gland (scale bar = 100μm)

## 4.4 DISCUSSION

The appearance of the mouthparts differs greatly both between genera at each individual life cycle stage and between different life cycle stages of the same genus of nematodes.

### 4.4.1 Early third stage larvae of Pseudoterranova decipiens

The culturing of specimens in a bacterial mat for subsequent examination under the scanning electron microscope appears to be a novel technique. This technique gave good results and may be potentially useful for studies of other marine or freshwater nematodes which have a small free living stage.

McClelland and Ronald (1974a) did not find sodium hypochlorite suitable for exsheathing newly hatched larvae of **P.decipiens** using the method of Davey (1969) for **C.osculatum**. Davey's method involved incubation of larvae in this medium for 15 minutes. During the present study, exsheathed larvae of **P.decipiens** were not observed after this time, but a further 15 minutes incubation did stimulate the majority of larvae to exsheath. No apparent deleterious effects on the larvae were observed as a result of the increased incubation time.

#### 4.4.1.1 Body, Cuticular and Caudal Structure

The early third stage larvae of **P.decipiens** examined with the scanning electron microscope are slightly smaller than live larvae, owing to the effects of fixation.

Grabda (1976b) noted that the larval bodies of newly hatched free living larvae of **A.simplex** were thickest at the anterior region and gradually tapered posteriorly, forming a long, pointed tail. Sprent (1959) stated that second stage

larvae of **Toxascaris leonina** were widest in the anterior third and gradually tapered to the tail end. These descriptions agree with the findings of this study for early third stage larvae of **P.decipiens**, both ensheathed and exsheathed. A long tapering pointed tail appears to be a common feature of early nematode stages, being observed also in newly hatched larvae of **C.osculatum** (Davey 1969) and first stage larvae of **Philometra** sp. (Crites 1980, in Kelly and Crites 1993; Moravec 1980).

The longitudinal indentation observed down the second stage cuticle which ensheaths the third stage larvae may be the area along which the cuticle splits during the moult to release the third stage larvae. However, other authors have observed exsheathment of larval stages via separations at the anterior end. McClelland and Ronald (1970, 1974a) observed that hatched larvae of **P.decipiens** exsheath from their cuticles via an opening of the cuticle at the anterior end. Davey (1969) observed second stage larvae of **C.osculatum** to bend the head region during artificial exsheathment, as if to slit the cuticle with their boring tooth. Huizinga (1967) noted that second stage larvae of **C.multipapillatum** exsheathed from the first stage cuticle via a separation at the anterior end. Therefore, it appears that the cuticle of these worms splits at the anterior end rather than longitudinally down the body. Grainger (1959) stated that the presence of cuticular striations in third stage larvae of **P.decipiens** from fish appeared to depend on the tension on, and degree of extension of, the cuticle - where the worm was bent, the cuticle was stretched and no striations were seen. The presence of distinct striations on the second stage larval cuticle examined here, particularly where the worm is bent, suggests that the cuticle is loose. Scott (1955) and Myers (1960) noted that newly hatched larvae of **P.decipiens** were encased in loose cuticular sheaths. Gibson (1970) observed detachment of the fourth stage cuticle of **Anisakis** along part of its length, especially at the anterior end, which suggested that a moult was about to occur, and Moravec

(1980) noted that first and second stage larvae of **Philometra ovata** which were about to moult had loosened cuticle along the whole length of the body. The apparent loose nature of the second stage cuticle observed in this study may also indicate that the parasite is about to exsheath, and this may be further substantiated by the fact that these parasites had previously been placed in artificial exsheathing medium, in which the majority of specimens had artificially exsheathed, but some specimens had appeared to remain ensheathed. It seems likely that the latter specimens would have been on the point of exsheathing, but were removed from the medium before actual exsheathment took place.

It is difficult to suggest a function for the longitudinal ridge seen running down the body in the exsheathed larvae; however, Lian-Yin **et al.** (1984) also described a short longitudinal ridge on the body surface cuticle of third stage larvae of **Angiostrongylus cantonensis.**

#### 4.4.1.2 Boring Tooth and Cephalic Structure

Light microscopy studies have previously revealed the presence of a boring tooth in newly hatched larvae of **P.decipiens** (Scott 1955, Myers 1960, McClelland and Ronald 1974a, McClelland 1982) and **C.osculatum** (Davey 1969, McClelland and Ronald 1974b). McClelland and Ronald observed a blunt boring tooth in the latter species. The boring tooth observed here in **P.decipiens** was pointed. Grabda (1976b) also noted an anterior boring tooth in newly hatched larvae of **Anisakis simplex**, which was slightly asymmetrically placed, like those of newly hatched **P.decipiens** observed here. The presence of a boring tooth also appears to be a common feature of the early larval stages of various nematode species, eg. in **Hysterothylacium** (Deardorff and Overstreet 1981a), **Raphidascaris acus** (Smith 1984b), **Contracaecum spiculigerum** (Huizinga 1966) and **C.multipapillatum** (Huizinga 1967), **Sulcascaris sulcata** (Berry and Cannon 1981),

**Philometra** sp. (Kelly and Crites 1993) and nematodes of the superfamily Dracunculoidea (Chitwood and Chitwood 1950).

Boring teeth are common on many nematode larvae that use copepod intermediate hosts (Kelly and Crites 1993), as indeed has been shown experimentally for **Pseudoterranova decipiens** (McClelland 1982), and it has been suggested that the boring tooth may aid in the penetration of the digestive systems of hosts, eg. Kelly and Crites (1993), Huizinga (1966, 1967). Davey (1969) suggested that larvae of **C.osculatum** appeared to slit the cuticular sheath using their boring tooth, and this may also be true for **P.decipiens**. Valter **et al.** (1982) found the height of the boring tooth in third stage larvae of **Thynnascaris** (= **Hysterothylacium**) varied with the age\size of the larvae. The boring tooth in early third stage larvae of **P.decipiens** also varies in size (depending on the size of the head) from approximately 1 to 2µm high, and is large in relation to head size. It appears that in the early stages, the tooth grows along with the head; however, the boring tooth in third stage larvae from fish is approximately 7µm high, and is small in relation to head size, which suggests that growth of this structure has ceased or slowed down at some point. Valter **et al.** (1982) found no correlation between the height of the boring tooth in third stage larvae of **P.decipiens** and the size of the larvae, which may suggest that the tooth does stop growing at some point during the development of this stage. It is possible that the tooth of the third stage larva is only used for slitting the second stage cuticle and\or penetration through invertebrate hosts, and is non-functional in the fish host, but is retained as there are no further moults until the final host is reached. That the tooth of early third stage larvae of **P.decipiens** is large relative to the head size, whereas the tooth in the later third stage from fish is significantly smaller in relation to head size, may indicate that the tooth has a reduced function in the fish host.

The presence of a triangular mouth opening, sensory structures and an excretory pore have been reported

previously from newly exsheathed larvae of **P.decipiens** (McClelland and Ronald 1974a, McClelland 1982) and **C.osculatum** (McClelland and Ronald 1974b) using light microscopy. These authors also observed two papillae on the dorsal lip and one papilla and an amphid on each of the subventral lips of these species, and their illustrations do indeed show these apparently in the same positions as they occupy in later stages. No mention was made of the presence or absence of the two single papillae present on the subventral lips of later stages of these species. No mouth opening, sensory structures or excretory pore were observed in the specimens of newly hatched **P.decipiens** examined during this study and Myers (1960) also stated that the cephalic structures were not fully formed in newly hatched larvae of **P.decipiens**, although she did not describe the cephalic morphology of this stage beyond the presence of the boring tooth. However, the mouth opening and excretory pore, at least, must develop within this individual life cycle stage as both of these structures are present in third stage larvae of **P.decipiens** from fish. It is possible that the mouth opening, sensory structures and excretory pore observed in **P.decipiens** at this stage by McClelland and Ronald (1974a) have not yet been manifested externally in the larvae examined during the present study.

Yoshinaga **et al.** (1987) observed a triangular process at the anterior end of newly hatched second stage larvae of **H.aduncum** (presumably the boring tooth), and this was the only cephalic feature noted by these authors. Moravec and Nagasawa (1986) observed distinct cephalic papillae in third stage larvae of **H.aduncum** recovered from amphipods in freshwater, but stated that the lip mass in third stage larvae of **H.aduncum** was largely undeveloped in smaller specimens and well developed in other specimens, and the caudal tail spike was not developed in the smallest larvae. This suggests that development of the larvae occurs within this single life cycle stage. It is therefore possible that the specimens of early third stage larvae of **P.decipiens**

examined during this study had not yet developed to a point where the mouth, sense organs and excretory pore were visible externally. Reports of these structures and lip formation in early larval stages of nematodes appear to vary, both within the same nematode species according to different authors, and between different nematode species. Grabda (1976b) observed no evidence of lip formation in second (= early third) stage larvae of **A.simplex**, and did not mention either the presence or absence of sense organs or a mouth opening. Crites (1980, in Kelly and Crites 1993) observed the first stage larvae of **Philometra** sp. as having no discernible lips. No amphids were observed in **Sulcascaris sulcata** at the early third stage (Berry and Cannon 1981). Smith (1984b) observed papillae, a mouth opening and the excretory pore in second stage larvae of **Raphidascaris acus**. Kelly and Crites (1993) observed amphidial pores on the cephalic ends of first stage **Philometra** sp. larvae. Huizinga (1967) found that second stage larvae of **Contracaecum multipapillatum** had a mouth. McClelland and Ronald (1974b) observed three rounded lip masses in newly exsheathed larvae of **C.osculatum**. Sprent (1959) found that second (and third) stage larvae of **Toxascaris leonina** had small lips, papillae and amphids. It must be remembered that, with the exception of Kelly and Crites (1993), all of these studies have been carried out using light microscopy, and as such these structures may have been seen under the cuticle. SEM would reveal whether they are manifested externally. In addition, it is not clear whether parasite growth and development within individual life cycle stages would affect the results obtained by individuals when examining the early stages of nematode species.

In terms of general external body form and structure, it appears that the early stages of many nematode species are very similar one to another and may only become distinct from one another as growth and development occurs.

#### 4.4.1.3 Growth and development within third larval stage

The gross and ultrastructural external morphology of newly hatched third stage larvae of **P.decipiens** is vastly different from that of the third stage larvae of this species recovered from fish. Therefore many morphological changes must occur within this single life cycle stage.

A vast increase in growth must occur, from approximately 200µm in length when newly hatched, to over 30mm at later stages in fish, with an associated increase in width. Such growth is presumably accompanied by production of cuticle to allow for this increase. Other authors have reported growth and development of early larval stages and growth of nematodes in invertebrates and fish. Rosenthal (1967) observed growth of **Contracaecum** sp. larvae in the body cavities of infected herring larvae which had been fed on wild plankton. Smith (1983b) found a wide range of lengths (7.7 to 23.6mm) of larvae of **A.simplex** in euphausiids, so presumably growth is occurring in the invertebrate host. Larvae of **A.simplex** from fish were 18 to 21.9mm long. Huizinga (1966) observed growth in second stage larvae of **C.spiculigerum** from 330µm in length (newly hatched\free living), to 370µm (in experimentally infected copepods), 753µm (in experimentally infected fish exposed directly to free living larvae) and a maximum of 1,492µm (in fish exposed to infected copepods). Similar size increases were noted by Huizinga (1967) for second stage larvae of **C.multipapillatum**, with a further increase in size noted after migration of second stage larvae into the body cavities of guppies, where they reached 5mm in size before moulting to the third stage.

In terms of the development of the cephalic region of third stage larvae of **P.decipiens**, the boring tooth is observed to increase in size; the mouth opening must expand, as it is quite distinct in the later stages; some development of lips must occur, as rudimentary structures are present in larvae of **P.decipiens** from fish. McClelland and Ronald

(1974b) found that larvae of **C.osculatum** exsheathed **in vitro** and grew immediately without further moults, to 6.5mm in length after 32 weeks. Three rounded lip masses were observed in newly exsheathed larvae of this species, with the dorsal one becoming very prominent as the larvae grew. Moravec and Nagasawa (1986) observed that the lip mass was poorly developed in the smallest third stage larvae of **H.aduncum** recovered from freshwater amphipods, but was well developed in others. Smith (1983b) found that larvae of **A.simplex** from euphausiids and teleosts were morphologically similar, even when examined under SEM, and it is possible that the fully developed cephalic region of the third stage of this species, and others, becomes fixed at a young age, and the only further development of these is growth. Moravec (1980) observed and illustrated continuing morphological developmental changes in all regions of **Philometra ovata** larvae from the first free living stage through to early and late second stages and the third stage all within copepods.

McClelland (1982) found that once newly exsheathed larvae of **P.decipiens** had penetrated into the haemocoel of copepods, they began to grow, and this growth was exponential throughout the period of the infection. Larvae grew an average of 60% in length in copepods, with a maximum length and width of 450µm and 31µm respectively being achieved; however, infected copepods only survived for a maximum of 7 days. McClelland stated that larvae grew without distinct morphological change, although the cephalic papillae and excretory pore became more pronounced. Under natural conditions, over a longer period of time, larvae grow to much greater lengths in invertebrate hosts, and therefore may develop further. McClelland and Ronald (1970) found that exsheathed larvae of **P.decipiens**, cultured **in vitro**, doubled in length from 200µm within a week, and increased tenfold after four months. In a similar study, McClelland and Ronald (1974a) observed that after 18 weeks cultured larvae of **P.decipiens** were 11.4mm in length and grew to an average of 31.3mm in length over 52 weeks. This falls within the length

range of larvae recovered from fish, and as such these larvae may be expected to possess the features of larvae recovered from fish **in vivo**, thus the parenchyma of three distinct bilobed lips was observed in larvae over 5mm in length. It is to be expected that such increases in growth are associated with further development of morphological features.

It would be of interest to examine **P.decipiens** larvae at different periods post-hatch, as free living larvae, and within invertebrate and fish hosts, to determine what, when, and in which habitat, external developmental changes occur.

### 4.4.2 Cephalic Region of Third Stage Larvae from Fish

#### 4.4.2.1 General Cephalic Structure

The lip masses in third stage larvae of all genera examined were, in general, poorly developed, although it is possible to distinguish the three lips, using the positions of the boring tooth and excretory pore to distinguish the dorsal, and sub-ventral lips. The three rudimentary lips could be seen in **P.decipiens** and **C.osculatum**. The specimens of **A.simplex** examined showed only undifferentiated quadrangular lip masses, although the positions of the future lips can be ascertained, in contrast to the findings of Grainger (1959), who stated that the positions of the future lips could not be discerned on the lip mass surrounding the mouth of third stage **Anisakis** larvae. Under SEM, Valter **et al.** (1982) noted poorly developed lips in this species, which were not morphologically separated but formed a quadrangular lip mass, as observed here. Weerasooriya **et al.** (1986) observed three low lip bulges in **A.simplex** third stage larvae, one dorsal and two subventral. Weerasooriya **et al.** also noted that the lip bulges in third stage larvae of **P.decipiens** were more prominent and well marked than those of third stage **A.simplex**, and certainly the three rudimentary

lips can clearly be seen here in **P.decipiens**, although distinct bulges marking the lips of **A.simplex** were not observed. Hurst (1984a) stated that in third stage larvae of **A.simplex** from New Zealand waters, the lip mass was differentiated into three inconspicuous lips, a term used also by Cannon (1977) and Smith (1983b) for this species at this stage. Grabda (1976b) observed three poorly developed lips in third stage larvae of **A.simplex** from fish. The lips are indistinct, compared to the complex and protruded ones reported from fourth stage larvae and adults of this species by, for example, Gibson (1970), Pippy and Van Banning (1975) and Grabda (1976b). Gibson (1970) and Beverley-Burton **et al.** (1977) also observed three lips in third stage larvae of **A.simplex**, although the former author did state that they did not protrude very much; however, these observations were made with the light microscope, and it seems likely that although three lips can be seen, they do not appear externally as separated structures.

The first form of third stage **A.simplex** larvae observed appears to be larger, both generally, and in terms of the cephalic structures, than the second form, although in the latter the rounded protrusions (double papillae) were slightly larger. The second form observed, although smaller, appears to be at a more advanced state of development. These two forms have apparently both been described previously, with the first form appearing most similar to **A.simplex** third stage larvae from fish and euphausiids examined by Smith (1983b) from Scottish waters, while the second form appears most similar to third stage larvae of **Anisakis** type I examined by various Japanese authors (eg. Weerasooriya **et al.** 1986, Tongu **et al.** 1990). Given that **Anisakis** type I = **A.simplex**, and that, during this study, specimens of **A.simplex** examined were similar to larvae of this species examined from both Scottish and Japanese waters, it seems that the two forms observed differ in cephalic appearance as a result of differences in development rather than in terms of sibling species or geographic differences. It seems likely

then, that the cephalic region of the larger worm is less well developed than that of the smaller worm, and the state of development may well be related to the age of the worm, rather than to its size. If this were the case, the cephalic regions of the third stage larvae of **A.simplex** examined by Smith (1983b) would not be fully developed for this stage, as stated previously, but Smith may have examined younger specimens, and older ones would show the features observed here in the second form examined. Smith (1983b) also examined small third stage larvae of **A.simplex** from euphausiids and found that their heads were similar to those of longer (and presumably older) third stage larvae. However, these were not examined by SEM so Smith did not comment on the possible presence of sense organs in these small larvae, although he did note that rectal glands were not seen, but were present in longer third stage larvae from euphausiids and fish, which suggests that development does occur within at least part of this stage. In addition, like **P.decipiens**, newly hatched third stage larvae grow in length from approximately 250µm (Grabda 1976b) to approximately 22.6mm (Gibson 1970) when recovered from fish, and such growth must include developmental changes. Immature larval **Anisakis** sp. from Australian petrels and albatrosses were observed to have three low lips and a distinct boring tooth. Some of the specimens appeared to be older as the lip morphology was more definite (Johnston and Mawson 1942). Johnston and Mawson (1941a) stated that some **Contracaecum** spp. larvae from Australian birds had boring teeth and no lips present, whilst others bore both a boring tooth and three low lips. McClelland and Ronald (1974b) stated that the dorsal lip mass of **C.osculatum** second (= third) stage larvae became very prominent as larvae grew. Deardorff and Overstreet (1981a) stated that larvae of **Hysterothylacium** spp. developed to advanced stages in fish intermediate hosts, as well as in invertebrates, although they did not discuss in what way they were advanced. Such observations suggest that larvae within the same life cycle stage do appear to differ in their state

of development, and this may account for the differences observed by different authors. Black and Lankester (1980) found that larvae of **Cystidicola cristivomeri** of equal length and recovered from different fish often differed in their state of development. This may indicate that development is related to age rather than length. The age of the specimens of third stage **A.simplex** examined in this study, by Smith (1983b), Weerasooriya **et al.** (1986) and Tongu **et al.** (1990) is not known, therefore it is impossible to comment conclusively on the question of cephalic development. It would be of interest to examine third stage larvae of **A.simplex** of known ages under SEM in order to clarify this matter.

Tongu **et al.** (1990) observed no differentiated lips, or lip bulges, in third stage larvae of **Terranova** type A (**sic**), whereas this study, and Weerasooriya **et al.** (1986), noted three differentiated lip bulges in third stage larvae of **P.decipiens**. Indeed, the **P.decipiens** examined here is similar to that illustrated by Weerasooriya **et al.** (1986). Valter **et al.** (1982) stated that the cephalic end of **P.decipiens** larvae was blunt and observed three lips separated from each other by grooves and both of these features were also observed here for this species. Myers (1960) found that third stage larvae of **P.decipiens** from fish had three bilobed lips. Whilst Myers did not illustrate this feature, three rudimentary lips were observed in **P.decipiens** third stage larvae here, and the margins of the subventral lips, at least, did appear to be bilobed. The anterior margins of the lips in later stages of **P.decipiens** bear a single projection which is bilobed, and it is possible that the two projections observed on each side of the margins of the lips at this stage may become manifested as a fused structure in the later stages. Valter **et al.** (1982) observed a denticulated edge on the lips of third stage larvae of **P.decipiens**, both under light and scanning electron microscopy. Such structures are present along the margins of the lips in later stages; however, they were not observed at the third larval stage during this study

and do not appear to have been noted by other authors. From apparently consisting only of a boring tooth in the early third stage, the cephalic region of larvae of **P.decipiens** recovered from fish hosts has increased in size, eg. the width of the head in **P.decipiens** has increased significantly from the early third stage, and developed a mouth opening, lips and excretory pore. Grainger (1959) found that the lip mass in third stage larvae of **P.decipiens** was undivided but indentations at the edge of the lip mass indicated three lips. However, his figure indicates that the cephalic region was similar to that observed here, although the indentations were slightly less demarcated. The **en face** illustration of **Terranova** type I (**sic**) in Cannon (1977) showed "spine armature"; it is unclear, however, where this is exactly and therefore it cannot be commented on here.

Fukuda **et al.** (1988) stated that the heads of **Contracaecum** type A (**sic**) and **Anisakis** types I (and II) third stage larvae were similar, and illustrated the third stage larvae of **Contracaecum** type A as having no clear differentiation of lip tissue into the three future lips; Tongu **et al.** (1990) also found this to be the case for **Contracaecum** type A. This was not found to be the case here for **A.simplex** and **C.osculatum**. Although both forms of **A.simplex** examined showed no lip differentiation, the cephalic region of **C.osculatum** was clearly differentiated into three lip bulges, with a transverse slit separating the dorsal lip from the two subventral lips. However, the description and photomicrograph of **Contracaecum** type B (**sic**) examined by Weerasooriya **et al.** (1986) is similar to the specimens of **C.osculatum** examined during this study, with **Contracaecum** type B third stage larvae having three well marked lip bulges also, and with the dorsal one being larger than the two subventrals. Thus it appears that **Contracaecum** larvae from fish in Scottish waters are similar to **Contracaecum** type B from Japanese waters. McClelland and Ronald (1974b) illustrate the dorsal lip of **C.osculatum** as a large "bulge", which their figure suggests is higher than

the subventral bulges. I did not find this to be the case; however, the dorsal lip in the specimen shown here does appear to show some shrinkage and it is possible that this lip may have been taller than the subventrals.

Although the third stage larvae of **H.aduncum** could not be examined during this study, third stage larvae of **Thynnascaris\Hysterothylacium** also have three inconspicuous lip bulges (Cannon 1977, Weerasooriya **et al.** 1986), although Mawson (1957) described these as low lips. However, Valter **et al.** (1982) stated that the lips of third stage **Thynnascaris** protruded markedly over the entire anterior of the head, and were separated from the rest of the body by a shallow groove. This groove was also noted by Kahl (1936). Valter **et al.** also found the dorsal lip to be slightly separated from the subventral lips. Moravec and Nagasawa (1986) observed interlabia in third stage larvae of **H.aduncum** recovered from freshwater amphipods, and presumably these would be found in larvae from fish also, although Valter **et al.** (1982) did not mention interlabia in their third stage specimens.

#### 4.4.2.2 Mouth Opening, Boring Tooth and Excretory Pore

A triangular mouth opening was observed in third stage larvae of **A.simplex** (both forms) and **P.decipiens**. Inglis (1960) stated that triangular mouth openings in some members of the Subuluridae may reduce the flexibility of the lip lobes. In **A.simplex** and **P.decipiens**, no lips are present at the third stage, but the form of the mouth opening may well change to triradiate in the fourth stage larvae and adults to allow increased flexibility of the lips. It is known that the lips in fourth larval and adult stages of some species are flexible, as they have been observed in both open and closed positions. A triangular mouth opening has previously been observed in third stage larvae of **A.simplex** and **P.decipiens** under light microscopy, eg. Grainger (1959) and

Gibson (1970), and SEM, eg. Valter **et al.** (1982), Fujino **et al.** (1984) and Weerasooriya **et al.** (1986). Grainger (1959) also illustrated the tri-radiate oesophageal lumen beneath the triangular mouth opening in third stage larvae of **Anisakis**. Under SEM, the oesophagus of **P.decipiens** could also be seen through the mouth opening, although it was not possible to discern the oesophagus through the opening of other specimens. The actual mouth opening of specimens of **C.osculatum** was obscured by the large lip bulges; however, a transverse slit was observed separating the dorsal lip from the subventral lips, and this slit was also observed by Weerasooriya **et al.** (1986) in third stage larvae of **Contracaecum** type B, and by Valter **et al.** (1982) for third stage larvae of **Contracaecum**.

The boring tooth in the third stage larvae of all genera examined was situated in the same position (between the subventral lips), was similar in size and appeared to derive from the cuticle. Myers (1960) also stated that, for **P.decipiens**, the boring tooth does not appear to be derived from the lip pulp, and this is likely to be true for **A.simplex** and **C.osculatum** also. While the boring teeth in third stage larvae of **P.decipiens**, **C.osculatum** and the first form of **A.simplex** examined have rounded ends, the tooth in the second form of **A.simplex** examined has a markedly sharp point, and, if this form is further developed than the first form of **A.simplex** examined, it also appears to have undergone a change in direction from projecting slightly upwards to being parallel with the anterior end of the larva. Grainger (1959) illustrated the boring tooth of **Anisakis** third stage larvae as being rounded. This was observed to be the case for the first form examined here. Grainger also stated that large numbers of small teeth were present at this stage, but his illustration shows these to be widely spread in a broad ring over the anterior tip of the head. Again, these were not observed during this study and do not appear to have been described by other authors, either under light or scanning electron microscopy. Denticles are present from the fourth

larval stage, but these are in a single ridge, and are on the anterior margins of each lip, and it does not seem likely that these would develop before the lips themselves. The tooth of **A.simplex** illustrated by Van Theil and Van Houten (1967) is similar to that observed in the second form of **A.simplex** examined during this study, where it is almost horizontal with the anterior end of the larva. The boring tooth in **C.osculatum** appears to point slightly inwards, towards the centre of the larva, rather than outwards; Weerasooriya **et al.** (1986) also noted this in **Contracaeum** type B third stage larvae, and added that this was different from the outwardly directed form of the tooth in **Anisakis** and **P.decipiens**. The size and rounded appearance of the boring tooth in specimens of **P.decipiens** examined during this study is strikingly similar to that illustrated by Weerasooriya **et al.** (1986). However, the boring tooth of **C.osculatum** observed in this study appears smaller than that found by Weerasooriya **et al.** for **Contracaecum** type B third stage larvae.

Hurst (1984a) found that the boring tooth in third stage larvae of **P.decipiens** from New Zealand waters was markedly smaller than that of **A.simplex**. In relation to the size of the head, the boring tooth is smaller in **P.decipiens** than in **A.simplex** third stage larvae, and Grainger (1959) and Ishii **et al.** (1989) illustrated this.

Fukuda **et al.** (1988) stated that the boring tooth of third stage larvae appeared to be too small to bore a hole into host tissue, although it is difficult to imagine another function for this organ, particularly since this tooth is lost after the fourth larval stage and the nematodes do not penetrate completely through the gut walls of the final hosts, as they do in the invertebrate and fish hosts. However, it may be, as suggested earlier, that the tooth is used for cutting through the cuticular sheath of the previous stage, or for penetration through invertebrate hosts only, and penetration through fish alimentary tracts may occur only by means of secretions\excretions from the excretory gland. Alternatively, and more likely, given the thickness of the

gut wall of teleost fish, the tooth may be used to penetrate the digestive systems of fish in combination with secretions\excretions from the excretory gland. The opening of the excretory pore near the boring tooth in larval **A.simplex**, **P.decipiens** and **C.osculatum** may thus be related to the penetration ability of the larvae (Fukuda **et al.** 1988).

Third stage larvae of **Thynnascaris\Hysterothylacium** also have a triangular mouth opening and a boring tooth between the subventral lips (Deardorff and Overstreet 1981a, Valter **et al.** 1982, Weerasooriya **et al.** 1986).

#### 4.4.2.3 Cephalic Sense Organs

Third stage larvae of **A.simplex** (both forms) and **C.osculatum** bore four rudimentary double papillae, two on the dorsal lip, and one on each subventral lip, manifested as raised, rounded protrusions. Valter **et al.** (1982) described "indefinite outlines" of papillae in these positions in third stage **Anisakis**, and observed papillae in these positions in third stage **Contracaecum**, the height of these being similar to those observed in this study for **C.osculatum**. Weerasooriya **et al.** (1986) also noted four elevations in these positions in third stage larvae of **A.simplex** and **C.osculatum**. Cannon (1977) observed four papillae in **Contracaecum** type I third stage larvae examined under the light microscope. Contradictions do, however, occur in the literature with regard to the presence of cephalic sensory structures at the third larval stage of these parasites, eg. Fukuda **et al.** (1988) gave no indications of possible sensory structures in third stage larvae of **Anisakis** types I and II and **Contracaecum** type A examined under SEM. Smaller single papillae\amphids were also observed in the first form of **A.simplex** examined in this study; however, the second form of **A.simplex** and the specimen of **C.osculatum** were of too poor a quality to observe small, fine structures such as single

papillae and amphids. Valter **et al.** (1982) observed amphidial openings in **Anisakis** larvae under SEM, and transmission electron microscopy studies (Chapter 5) have indicated that the underlying amphidial sensory processes are present in third stage larvae of **A.simplex**, although it is not known if this structure is fully developed internally at this stage. Smith (1983b) could not confirm the presence of amphids in third stage larvae of **A.simplex** examined under the SEM, owing to possible shrinkage of specimens or debris, and Tongu **et al.** (1990) also stated that it was difficult to locate the amphids in **Anisakis** third stage larvae under SEM. Fixation artifacts and shrinkage may both contribute to obscuring these small sense organs. However, it seems likely that the second form of **A.simplex** examined will also have single papillae\amphids, as these were seen in the apparently younger first form. Amphids have also been reported in larval **P.decipiens** and **C.osculatum** examined under the light microscope (McClelland and Ronald 1974a,b), but not by Valter **et al.** (1982) under SEM. No sensory structures were observed in the third stage larvae of **P.decipiens** examined during this study, but again, this may have been due to the poor quality of the specimens. Cannon (1977) noted four papillae in **Terranova** sp. type I third stage larvae, and Myers (1960) found **P.decipiens** third stage larvae to have two papillae on the dorsal lip, and one on each subventral lip. Valter **et al.** (1982) also noted the presence of four slightly elevated papilla- like structures in third stage **P.decipiens**. However, Weerasooriya **et al.** (1986) did not appear to find such papillae in the specimens of **P.decipiens** larvae which they examined, and which appear similar to the specimen illustrated in this study. Furthermore, it is possible that the single (and, in the case of **P.decipiens**, double) papillae and amphids can be seen underneath the third stage cuticle using light microscopy, but do not appear as external structures in specimens examined under SEM, or perhaps do not appear externally until a later stage of development is reached within this third stage.

Weerasooriya **et al.** (1986) noted no structures which resembled papillae in third stage larvae of **Hysterothylacium**. However, Moravec and Nagasawa (1986) did observe distinct papillae in third stage larvae of **H.aduncum** from amphipods, and Valter **et al.** (1982) observed minute amphids and four rounded and protruding papillae in third stage larvae of **Thynnascaris adunca** (= **H.aduncum**). Cannon (1977) also noted four papillae in **Thynnascaris** types I and III third stage larvae examined under the light microscope.

Most descriptions of the sense organs in third stage larvae of marine ascaridoids refer to the presence of the large papillae, which correspond to the positions of the double papillae in the adults. Although these structures are double papillae, they do appear externally as a single structure, at least at this stage. Cannon (1977) and Smith (1983b) appear to have observed the single papillae in third stage larvae of **A.simplex**, both authors noting six papillae at this stage, and Tongu **et al.** (1990) also observed six papillae on the cephalic region of third stage larvae of **Anisakis** and **Terranova** type A, examined under SEM. Of these six papillae, two are likely to be single papillae, whilst four are likely to be the future double papillae. Valter **et al.** (1982) suggested that Cannon (1977) may have observed amphids rather than the single papillae. From the observation of the double papillae being rounded protrusions in the third stage larvae of **A.simplex** and **C.osculatum**, and from the absence of fully formed lips in all three genera examined at this stage, we can see that, compared to structure in adults, the cephalic structures and sense organs are not fully formed, at least externally, at the larval stage.

It seems likely that as third stage larvae age in fish, the sense organs develop along with the lip mass, but only to a certain extent: the double papillae in third stage **A.simplex** and **C.osculatum** are not separated even partially from the rest of the head cuticle, as they are in later stages. The nervous system is one of the last systems to develop in ascaridoid nematodes, and this may explain why the

sense organs do not develop fully until a later stage. Myers (1960) stated that the nervous system of **P.decipiens** was poorly developed in third stage larvae and appeared to be confined to a nerve ring surrounding the oesophagus. The sense organs are also unlikely to be functional at this stage, as they would need the presence of a fully developed nervous system to work. The sense organs are likely to develop along with the nervous system, and along with the lips, and therefore will not become fully developed until these themselves develop.

It seems likely that, as the cephalic ends of the larvae do not show all of the adult features, they may not be useful in distinguishing any possible differences between potential sibling species, or between nematodes of the same species from different geographical regions; thus Fukuda **et al.** (1988) and Tongu **et al.** (1990) observed no clear differences between the cephalic regions of third stage **Anisakis** type I and type II larvae, although Fukuda **et al.** (1988) did state that the excretory pore was only clearly apparent in larvae of **Anisakis** type I (however, this may be a fixation effect, or the pore itself may have been closed during fixation in the **Anisakis** type II); Hurst (1984a) stated that third stage larvae of **P.decipiens** from New Zealand waters appeared similar to descriptions of this species from other waters, although there were differences in the length of the intestinal caeca; Grainger (1959) found that **Anisakis** larvae from fish in Icelandic and Greenland waters, and the Barents Sea were identical, as were third stage larvae of **P.decipiens** from fish collected in Icelandic waters, eastern Canadian waters, waters off Greenland and the west coast of Scotland and the Barents Sea. In addition, given the probability that development is still occurring in the larval stages, it seems sensible to concentrate on examination of the adult specimens, where fully developed cephalic features are present, to observe if any distinct structural differences occur in the cephalic region between individuals of the same species taken from different regions and\or which are sibling

species.

### 4.4.3 Cephalic Region of Fourth Stage Larvae and Adults

#### 4.4.3.1 Development of Cephalic Regions

The cephalic regions of the fourth stage larvae and adults of each species are very different in appearance from those of the third stage larvae. It can be seen that at the adult stage the lip masses of all four species have differentiated into three fully separated lips. This separation occurs at the fourth larval stage.

At the fourth stage, the three rounded rudimentary lips seen in third stage larvae of **C.osculatum** had developed into roughly square shaped lips similar to those observed in the adult. In addition, the three lips of the fourth stage were equal in size, whereas the dorsal lip bulge seen in the third stage larva was larger than that of the subventral lips. The lip bulges themselves have increased in size, from 70µm to 150µm in width for the dorsal lip, and from approximately 42µm to 150µm in width for the subventral lips. The lips present in the fourth larval stage did not, however, bear the distinct antero-lateral knobs seen in the adult, or the clear depression seen in the centre of the lips at the anterior end. This depression is likely to have formed from the posterior part of the shallow groove seen in the lips of the fourth stage larvae. The remainder of this groove forms the groove seen leading from the depression in the adult lips. Examination of the photographs of Fagerholm (1988a) also appears to show that fourth stage larvae of **C.osculatum** do not have the antero-lateral knob on the lips at this stage. McClelland and Ronald (1974b) did note lateral projections on the lips of fourth stage **C.osculatum**, although these were observed to be more prominent in the adults. It is possible that these authors examined these structures under the cuticle at the fourth larval stage, as they examined

specimens using light microscopy. Interlabia, although not fully developed, were also apparent in this species at the fourth larval stage but these were not observed in third stage larvae. However, Fagerholm (1988a) noted that fourth stage larvae of **C.osculatum** did not possess interlabia, and McClelland and Ronald (1974b) found that fourth stage larvae of **C.osculatum** obtained **in vitro** did not possess interlabia, but adults did have these structures. It seems that either these authors did not consider these incompletely developed structures as interlabia, or that the interlabia in this species show some development within the fourth larval stage. Huizinga (1966, 1967) observed that interlabia were not developed in third stage larvae of either **C.spiculigerum** or **C.multipapillatum**. By the adult stage, the anterior ends of the fourth stage interlabia of **C.osculatum** had also become fully separated from the underlying tissue, and had become hooked at their anterior ends. It seems likely that the interlabia in **C.osculatum** develop along with the lips as neither structure appears fully developed at the fourth larval stage. McClelland and Ronald (1974b) also noted a change in the mouth opening of **C.osculatum** from triangular at the third larval stage to triradiate in the fourth stage larvae and adults.

At the adult stage, the three straight-edged lips of the fourth stage larvae of **H.aduncum** have developed anterior auricles and cuticular flanges on their lateral margins. Smith (1984b) also observed that the dorsal lip of fourth stage larvae of **Raphidascaris acus** had a flat anterior margin, with straight sides, whereas lateral flanges were present in the adult. This observation appears very similar to that seen in **H.aduncum**, where the fourth stage larva has straight margins to the lips which bear no lateral flanges, but these flanges are present in the adult stage. The anterior auricles and lateral flanges of adult **H.aduncum** are likely to have extended, and differentiated from the narrow cuticular margin seen around the lips in the fourth stage larva, which is no longer present at the adult stage.

Yoshinaga **et al.** (1987) did however observe small flanges at the base of each lip of fourth stage **H.aduncum**.

Presumably the shallow V-shaped indentation seen on each lip of the adults of this species has formed as a result of the extension of the lateral margins to form the auricles. The semi-interlabia in this species appears similar at both the fourth larval and adult stage, although the interlabial grooves have extended in the adult stage, making the base of the lips, in the adult, narrower than at the fourth larval stage. Tongu **et al.** (1990) observed semi-interlabia in fourth stage **Thynnascaris** type B larvae. Soleim (1976b) and Soleim and Berland (1981) suggested that the interlabia of **Contracaecum** developed by anterior growth of interlabial tissue, which extended forwards to form full interlabia, while the semi-interlabia of **Thynnascaris** (= **Hysterothylacium**) have developed by backward growth of grooves between and towards the bases of the lips, and the semi-interlabia could be regarded as left over areas between the interlabial grooves.

The mouth opening of third stage **A.simplex** changed from being triangular to triradiate in the adult. This change has evidently occurred at the fourth larval stage, along with the development of separated lips, as Gibson (1970) illustrated the change from a triangular mouth opening in third stage larvae of **A.simplex** to a triradiate form in the fourth stage larvae and adults. After moulting to the fourth stage, an increase in head width of **A.simplex** occurs (Gibson 1970), and three distinct, separated lips are present (Van Theil and Van Houten 1967), with the dorsal lip being larger than the subventral lips (Fujino **et al.** 1984, Weerasooriya **et al.** 1986). The latter authors also noted that the mouth opening was triradiate at this stage. A further increase in head width is seen in the adult stage of **A.simplex** (Gibson 1970).

Weerasooriya **et al.** (1986) observed three lips equal in size and shape in fourth stage larvae of **Terranova** (= **P.decipiens**). The rudimentary lips of third stage **P.decipiens** have developed bilobed anterior projections and dentigerous

ridges on the lips. Adults of **A.simplex** have developed lips from the lip masses of the third stage, also with bilobed projections and denticles. Such structures have also been observed on the three lips of fourth stage larvae of **A.simplex** and **P.decipiens**, eg. by Grainger (1959), McClelland and Ronald (1974a), Carvajal **et al.** (1981), Fujino **et al.** (1984) and Weerasooriya **et al.** (1986).

The boring tooth has disappeared in the fourth stage larvae, presumably having been shed with the cuticle of the third stage. It seems that the lips of the fourth stage larvae and adults gradually develop under the cuticle of the previous stage, prior to moulting, rather than forming after the moult has taken place; however, it is not known whether the interlabia in **C.osculatum** and interlabial grooves in **H.aduncum** develop further in the fourth stage larvae. Van Theil and Van Houten (1967) and Grabda (1976b) observed developing lips of the fourth stage larvae under the cuticle of the third stage larvae in **A.simplex**. Kahl (1936) provided a figure of an apparent third stage larva of **Contracaecum clavatum** ( = **H.aduncum**), and illustrated the cephalic end of a larva with distinct lips underneath the cuticle. Other authors have also noted developing lips of fourth stage larvae within the third stage cuticle of various ascaridoids including **Raphidascaris anchoviellae** (Deardorff and Overstreet 1981b) and **Sulcascaris sulcata** (Berry and Cannon 1981). Gibson (1973) observed projections (present only in the adult) developing beneath the lips in larger specimens of apparent fourth stage larvae of **Pseudanisakis tricupola**. It is not known when the lips of the fourth stage begin to develop under the third stage cuticle, although Smith (1984b) stated that three masses of lip pulp were observed to be forming under the cuticle at the anterior end in larger third stage larvae of **Raphidascaris acus**.

The cephalic regions of each species can be seen to change with each moult and, for **C.osculatum** and **H.aduncum** at least, the lips are not fully developed until the adult stage. There is no reason to suppose that this is not the

case for **A.simplex** and **Pseudoterranova decipiens** also. Morphometric measurements of **Toxocara genettae** (Sanmartín **et al.** 1992) suggested that growth of all structures examined was continuous, and this may be true for the cephalic regions of the nematodes examined here also.

Given the rudimentary appearance of the lips in third stage larvae, compared to that of the adults, it does not seem likely that the lips function in any way at the third stage.

#### 4.4.3.2 General Cephalic Structure

The heads of fourth stage larvae and adult specimens of each species differ significantly from those of the third stage larvae, and also differ one from another within these latter life cycle stages. The most distinct differences between the morphology of the mouthparts of each species were observed in the adults. Whilst the dorsal lip of adult **A.simplex** is larger than the subventral lips, the lips of the other three species were seen to be approximately equal in size. However, the bilobed regions of the lips are proportionally larger in **P.decipiens** than in **A.simplex**. Van Theil and Van Houten (1967) and Gibson (1970) stated that the excretory pore was no longer distinctly visible in fourth stage larvae of **A.simplex**. In the adult specimens observed here, the pore was clearly visible. However, this pore may open and close, and if so, Van Theil and Van Houten (1967), and Gibson (1970) may have examined a specimen in which the pore was closed.

Myers (1960), using light microscopy, observed each lip of adult **P.decipiens** to be marked by a central indentation, giving a bilobed appearance. It can be seen under SEM that the lips of this species are indeed bilobed, and these lobes are separated not only from each other by a central indentation, but also from the rest of the lip by a

transverse continuation of this groove. Grainger (1959) cultured fourth stage larvae of **P.decipiens** from Atlantic waters, and observed the three lips with bilobed anterior portions and dentigerous ridges. Grainger also noted an additional posterior lobe on each of the lips between the two anterior lobes. Such a lobe was not noted in the adult specimens of **P.decipiens** examined here, although a small projection between the two lobes of this species was also observed by Carvajal **et al.** (1981). The general cephalic morphology of adult **A.simplex** and **P.decipiens** examined under SEM agrees with that described for these species, under light microscopy, by Davey (1971) and Myers (1959), and also that of fourth stage larvae of these species by Weerasooriya **et al.** (1986).

The width of the lips in fourth stage larvae of **C.osculatum** examined by Fagerholm (1988a) was approximately 53µm, with that of the adults being approximately 60µm. This is approximately half the width of the lips examined in fourth stage larvae and adults of **C.osculatum** examined during the present study.

The triangular flanges and semi-interlabia of **H.aduncum** have previously been documented from light microscopy studies (Berland 1991). The three well developed lips and semi-interlabia were also observed under light microscopy by Cannon (1977) in fourth stage larvae of **Thynnascaris** (= **Hysterothylacium**) type IV and **H.aduncum** respectively. Distinct lips were observed in fourth stage larvae of **Thynnascaris** by Mawson (1957), and Tongu **et al.** (1990) noted separate lips in fourth stage larvae of **Thynnascaris** type B. Fukuda **et al.** (1988) noted, under SEM, that fourth stage larvae of **Thynnascaris** A and B, from Japanese waters, had interlabia and from their photomicrographs it is observed that these are, in fact, semi- interlabia. These interlabia were similar in appearance to those observed here. The lateral edges of the lips of adult **H.aduncum** bear lateral flanges. Deardorff and Overstreet (1981b) described the variations in the positions of the cuticular flanges in

relation to the lips for several species of **Hysterothylacium**. In **H.aduncum** examined here, the flanges could be seen to end approximately ⅓ down from the anterior end of the lips, and were widest at their anterior ends. **Hysterothylacium eurycheilum** and **H.corrugatum** have flanges which are widest nearest the base (Deardorff and Overstreet 1980,1981b).

In the present study, indentations were also observed on the anterior edges of the lips of **C.osculatum** and **H.aduncum**.

The disappearance of the boring tooth at the fourth stage may indicate that the parasite no longer requires it to aid in penetrating through the gut walls of intermediate hosts, if this is indeed its function. **A.simplex**, **P.decipiens** and **C.osculatum** do attach intermittently to the walls of the digestive tract in mammalian hosts (Vik 1964, Young and Lowe 1969, McClelland 1980c, Fagerholm 1988a) but they do not penetrate through the entire wall. **A.simplex** and **C.osculatum** need to be attached to the stomach wall in order to feed (see Section 4.4.3.4). **A.simplex** and **P.decipiens** do, however, develop a row of small denticles along the anterior edges of the bilobed central portion of each lip, present from the fourth larval stage onwards, and the presence of these denticles may aid in the attachment to the stomach mucosa of the final hosts.

The general shapes and sizes of the lips of adult ascaridoids differ between species. In the four nematode species examined here, the lips were equal in size in all genera except **A.simplex**, where the dorsal lip was larger than the subventral lips. In **A.simplex** and **P.decipiens**, the lips appear rounded, whereas in **C.osculatum** the lips are roughly square in shape, and in **H.aduncum**, if the auricles were removed, the lips would be roughly diamond shaped.

#### 4.4.3.3 Structure of the Anterior Lip Margins

The anterior margins of the adult lips of all species examined differ in appearance one from another. Both **A.simplex** and **P.decipiens** have bilobed anterior projections in the centre of each lip. These bear dentigerous ridges, which are single, short, thin and stubby in **P.decipiens**, and are triangular and in the form of molar-like ridges in **A.simplex**. These lobes and ridges were also observed in **A.simplex** by, for example, Pippy and Van Banning (1975) and Davey (1971), in **P.decipiens** by Myers (1959) and in both species by Carvajal **et al.** (1981). According to Davey (1971), such structures are characteristic of the Anisakidae. Davey (1971) noted a distinct difference in shape between the dorsal lips of **A.simplex** and **A.typica**, whereby the anterior bilobed projection of the latter species was more "pinched off" from the rest of the lip. Other species of **Anisakis** also have toothed bilobed anterior projections on the lips, eg. **A.diomedeae** (Johnston and Mawson 1942).

**C.osculatum** has a shallow indentation, situated medially, and a single antero-lateral knob, positioned on each side of the lips. Baylis (1937) noted the two anterior lobes projected laterally into prominent points on the lips of adult **C.osculatum**, and stated that the medial indentation of the anterior lip margin formed the pair of lobes. However, the lobes are not as well marked as those of **A.simplex** and **P.decipiens**, and do not project from the main body of the lips. Other species of **Contracaecum** also bear these projections, eg. **C.ogmorhini** (Johnston and Mawson 1941b) and **C.pelagicum** (Johnston and Mawson 1942). Anterior cuticular lobes on the dorsal lip of **C.radiatum** were less distinct, had blunter points and were directed more posteriorly than those of **C.osculatum** (Baylis 1937).

The central part of the lips is shallowly V-shaped in adult **H.aduncum**, and bears a pair of rounded auricles at each of the lateral edges. Each species can therefore be seen to have some form of paired structure on their anterior lip

margins - either bilobed anterior projections (**A.simplex**, **P.decipiens**), antero-lateral knobs (**C.osculatum**) or lateral auricles (**H.aduncum**). Tongu **et al.** (1990) stated that the lips of **Thynnascaris** type B fourth stage larvae had several denticles on the anterior margins; however, examination of their photograph suggests that these are, in fact, the developing anterior auricles. Soleim and Berland (1981) stated that the pair of anterior auricles on each lip of **Thynascaris adunca** (=**H.aduncum**) can interlock, and Valter (1973) stated that the morphology of the lips in **C.aduncum** ( = **H.aduncum**) was suggestive of a closing apparatus or gripping organ, which would enable this parasite to attach to host tissue. However, neither Soleim and Berland (1981) nor I observed adult specimens of **H.aduncum** attached to the intestines of fish, although the lips have been observed in open and closed positions.

Ansel **et al.** (1974) observed a groove running down the centre of the anterior end of the lips in **Parascaris equorum**. Ansel and Thibaut (1973) also observed a groove running down each of the outer lips from the centre of the anterior edge in adult **Ascaris suum**. Such a groove was observed in specimens of **C.osculatum** and it may be that the groove separating the two lobes on the anterior projections of the lips of **Anisakis simplex** and **Pseudoterranova decipiens** is a modification of this. Ansel and Thibaut (1973) and Ansel **et al.** (1974) stated that these grooves may mark the join of the primitive lips, being the traces of the junctions where the lips joined each other, as the cephalic region of a nematode is theoretically made up of six lips, which have been joined two by two to form three lips in ascaridoids. These traces are not seen on all species, eg. no grooves were observed on the outer lip surface of **H.aduncum**, and Ansel and Thibaut (1973) did not observe these grooves in **Ascaris lumbricoides**. The two lobes in **Anisakis simplex** and **P.decipiens**, the shallow indentations on the anterior edges of **C.osculatum**, the V-shaped anterior margins of **H.aduncum**, and the paired antero-lateral knobs and auricles seen on the anterior lip

margins of the two last named species, may also be indications of the fusing of two lips at some point during their evolution.

#### 4.4.3.4 Structure of Lips in Relation to Feeding.

Inglis (1960) stated that the structure of the head in nematodes must be considered in relation to the uptake of food, and Sprent (1983) considered that head structures appear to show specific morphology adapted to a wide range of feeding processes.

Inglis (1960) suggested that the development of processes on the anterior edge of the lining of the buccal cavity in some members of the Subuluridae would enable food particles to be held in position against the mouth opening. It is possible that the structures seen at the anterior lip margins of the species examined during this study may also be involved in such a function. Lee (1965) stated that parasitic nematodes are microbivorous and saprophagous feeders, and Soleim and Berland (1981) suggested that the presence of auricles, with associated longitudinal axial ridges, in **T.adunca** (=**H.aduncum**) may aid in the uptake of liquid food; these authors also stated that the interlocking of the auricles when the mouth closes may aid in transporting food into the digestive tract of the nematode.

Third stage larvae in fish are not thought to feed, so well developed lips and sense organs are not required at this stage. A mouth is present at the third stage but it seems unlikely that it is used. Digestion in **Anisakis** is possibly extra-corporeal and secretions from the well developed dorsal oesophageal glands in this species are thought to be involved in the feeding process by liquifying stomach tissue (Hsü 1933). Fagerholm (1988a) noted that fourth stage larvae of **C.osculatum**, embedded in the stomach of rats, appeared to have consumed host blood. McClelland (1980c) observed that **P.decipiens** in seals fed on the stomach contents of the host.

The denticles of **A.simplex** and **P.decipiens**, and hooked interlabia of **C.osculatum** may be used to aid in attachment to the stomach wall, where at least **A.simplex** and **C.osculatum** are thought to feed.

#### 4.4.3.5 Denticles

Sprent (1952) suggested that denticle structure may be of taxonomic value, and used this feature to distinguish between specimens of pig and human **Ascaris**, which had not previously been distinguishable on morphological grounds.

Denticles, separating the outer and inner surfaces of the lips, were observed in the adults of **Anisakis simplex** and **P.decipiens** only, and differences in denticle form, shape and size were noted in these two species. The denticles of the former were manifested on molar-like ridges, approximately 5µm in height, and were triangular in shape. Those of **P.decipiens** were single structures, approximately 2µm in height, and were thin and stubby. However, the denticles on **P.decipiens** were observed to be severely worn in places. Carvajal **et al.** (1981) and Weerasooriya **et al.** (1986) also observed differences in denticle shape in adult **A.simplex** and **P.decipiens**, from Chilean and Japanese waters respectively, with those of the latter species being thinner. The distinct gap observed between the dentigerous ridges on each lobe of **A.simplex** does not appear to have been reported previously for this species.

Where present, denticles in nematode species are generally manifested in ridges on the lips - although in **Pseudanisakis** sp. the mouth is completely surrounded by a vellum with one or more rings of denticles (Gibson 1973).

The denticles in **Raillietascaris varani** were single projections, and were roughly of equal size (De and Dey 1992). The denticles in these specimens are more similar, in

terms of appearance, to those found in **Pseudoterranova decipiens** than in **Anisakis simplex**, being single projections of approximately equal size. The denticles observed in **P.decipiens** in the present study, appeared to be regularly spaced, but the worn appearance of these structures made it difficult to assess this with any certainty.

The teeth of adult **A.simplex** were distinct, but quite variable in number, size and shape (even within the same individual specimen), and were irregularly spaced. This variability may be due to wearing of the denticles. Denticles in nematodes are widely documented to vary in shape, size and form, both within genera, species and individuals, eg. in **Baylisascaris** spp. (Sprent 1968, Kazacos and Turek 1982), **Ascaris** spp. (Sprent 1952, Madden **et al.** 1970, Weise 1973, Maung 1973, Ansel and Thibaut 1973), **Porrocaecum ensicaudatum** (Barus **et al.** 1983) and **Pseudanisakis** spp. (Gibson 1973). Weerasooriya **et al.** (1986) noted that each dentigerous ridge in **Anisakis simplex** bore single or bifurcate denticles. It is difficult, from the specimens examined during the present study, to distinguish bicuspid denticles in **A.simplex**, the large molar-like ridge having the denticles manifested as points along its edge. However, some apparently bicuspid denticles were observed. A large variety of denticle forms have been observed in **Ascaris suum** and **A.lumbricoides**, including molar-like structures in **A.suum** (Ansel and Thibaut 1973, Maung 1973). Kazacos and Turek (1982) suggested that variation in size and shape of denticles may be due to general variation, age of individuals, or to wearing of denticles during feeding as suggested by Madden and Tromba (1976). Lysek (1980) found that denticle both size and shape, and distance between denticles was variable between individuals and within the same individual (even within the same lip) of **A.lumbricoides** adults, and stated that these structures could not therefore be used for taxonomic purposes.

Severe wearing of denticles was apparent in **Pseudoterranova decipiens**. Sprent (1952) observed evidence

of wear and tear in the denticles of some specimens of **A.suum**, which had rounded points, and in some specimens of **A.lumbricoides**, in which only the ridge remained. Madden and Tromba (1976) also observed wearing of the lip denticles of older specimens of **Ascaris suum** adults. Wearing was found to increase with age in terms of numbers of denticles affected and degree of wear. In the oldest worms examined, all the denticles in the central area were severely worn, appearing blunt and with a molar appearance. However, only the denticles in the central part of the lips were affected. Ansel and Thibaut (1973) also noted that the denticles in the centre of the lips of **A.suum** appeared to have been pointed originally, but never appear as such in the centre of the lips. This observation also appears to be true for **P.decipiens**, where the denticles in the centre of the projection were seen to be severely worn. In contrast, the denticles of **Anisakis simplex** did not appear to be worn in this area, but were generally smaller (and therefore perhaps worn) at the edges of the lobes. Madden and Tromba (1976) concluded that as wear could be related to age, the denticles of this species were functional and became worn through use, and further suggested that adult **Ascaris suum** wear down their denticles by attacking the intestinal mucosa of their hosts. This observation is also likely to be true for **P.decipiens** and **Anisakis simplex** as they too attach to the intestinal mucosa of their final hosts. The distinct wearing of the denticles in the specimen of **P.decipiens** illustrated in the present study suggests that this is likely to have been an old specimen. In younger specimens of **Ascaris suum** the denticles were observed to be triangular and evenly spaced, but as the specimens aged, slight wearing on some of the denticles gave them a molar appearance (Madden and Tromba 1976). The molar appearance of the dentigerous ridges in **Anisakis simplex** examined during the present study may also be a result of ageing. Madden and Tromba (1976) found the size of the denticles of **Ascaris suum** to differ between specimens of the same age and in individual specimens,

although average denticle size was directly related to the size, and therefore age, of the worm. Lysek (1980) observed no difference in denticle size of young and old **A.lumbricoides**.

The numbers of denticles present on the lips appear to have been documented for relatively few nematode species. Only the denticles on the lips of **Anisakis simplex** were counted in this study (those of **P.decipiens** being worn, and often absent in places, making a count of these structures difficult). 37 to 47 denticles were observed on each of the subventral lips of **A.simplex**, and 45 to 52 on the larger dorsal lip. The number of teeth recorded from the subventral lips of adult **A.simplex** examined during the present study are similar to that reported by Weerasooriya **et al.** (1986) for fourth stage **A.simplex** from Japanese waters examined under SEM; however, these authors recorded 35 to 45 denticles on each lip, whilst I counted 45 to 52 denticles on the larger dorsal lip. Further denticles may be formed in this species during the transition to the adult stage. Sprent (1959) observed an increase in denticle number from the fourth stage of **Toxascaris leonina** to that of the adult, and also noted that the appearance of the denticles had altered. Sanmartín **et al.** (1992) also observed a difference in the numbers of denticles between sexes of **Toxocara genettae**. The specimens of **A.simplex** and **P.decipiens** were not sexed during the present study, so no comment can be made in this respect. Differences in denticle number between sexes would have to be taken into consideration when examining specimens for differences within species. However, variations in lengths of the denticles may differ with the size of the lips, and Sanmartín **et al.** (1992) found lip length to differ relative to body length in **T.genettae**. This may also be the case here, and if so, then denticle number and shape may be better criteria for differentiation within species. However, differences in lip size may also affect the denticle number. Sanmartín **et al.** (1992) observed more denticles on the dorsal lip of **T.genettae**. It may be that, in general, there are more

denticles on the dorsal lip of all species. The dorsal lip of **A.simplex** is larger in any case, and is likely to bear more denticles. Weerasooriya **et al.** (1986) found 45 to 50 denticles in fourth stage larvae of **P.decipiens**.

The dentigerous ridges in **P.decipiens** can be seen clearly to form from, and be a continuation of, the inner lip surface, and this feature has also been reported for other nematode species, eg. **Ascaris** spp. (Madden **et al.** 1970, Madden and Tromba 1976, Lysek 1980), **Toxocara pteropodis** (Prociv 1989), **Baylisascaris procyonis** (Kazacos and Turek 1982), **Raillietascaris varani** (De and Dey 1992) and **Parascaris equorum** (Ansel **et al.** 1974). Sanmartín **et al.** (1992) described the denticles of **T.genettae** as being smaller at the sides of the lips. This feature was also observed in the **Anisakis simplex** examined during the present study, with more denticles being present in the ridges at the outer edges of the lips, and with these denticles observed to extend down the sides of the lobes. The denticles in **Paranisakis australis** (Johnston and Mawson 1943) and **Sulcascaris sulcatum** (Allison **et al.** 1972) were observed to extend along the anterior edge and the lateral margins. In **A.simplex** and **Pseudoterranova decipiens** the denticles were only observed on the bilobed projections. Baylis (1920) stated that the genus **Contracaecum** lacked dentigerous ridges, and denticles are also absent in **Hysterothylacium** sp.. Dentigerous ridges are also reported to be absent in species of **Phocascaris** (Johnston and Mawson 1941b, 1942).

#### 4.4.3.6 Interlabia

Interlabia and semi-interlabia were observed only in fourth stage larvae and adults of **C.osculatum** and **H.aduncum** and, indeed, interlabial structures are characteristic of these genera. The interlabia and semi-interlabia of **C.osculatum** and **H.aduncum** are both triangular in appearance, although the general forms of these structures are vastly

different. The interlabia in **C.osculatum** appear significantly thicker than those of **H.aduncum** (which appear flat), and have hooked anterior margins, with rounded ends. The semi-interlabia in **H.aduncum** taper concavely to a narrow point, and are not hooked. The interlabia in **C.osculatum** also protrude outwards from the cephalic region, whereas the semi-interlabia of **H.aduncum** project anteriorly, in line with the outer lip surfaces. However, it is not known if the interlabia in **C.osculatum** are flexible structures. If they are, then the outwardly angled positions in which the interlabia were observed here may not necessarily be the only position in which these structures occur. The sizes and shapes of interlabial structures differ between nematode species, but are generally triangular, and tapering in form. The width of the semi-interlabia in **H.aduncum** is approximately three times that of their height, although Soleim and Berland (1981) observed the interlabia in this species to be twice as wide as long. The interlabia of **H.eurycheilum** are approximately twice as wide as long (Deardorff and Overstreet 1981b). The size of the interlabia is seen to vary in species of **Hysterothylacium**. Deardorff and Overstreet (1980) noted that in most cases the height and width of the interlabia were approximately equal; however, in **H.incurvum** the interlabial height was two times greater than the width at the base. Not all species of **Hysterothylacium** have interlabial grooves (see Deardorff and Overstreet 1981b), although these were present in **H.aduncum** examined here, and in **H.incurvum** (Deardorff and Overstreet 1981b), where deep interlabial grooves were observed, with adjacent grooves nearly merging at base of lip. The interlabial grooves in adult **H.aduncum** are also observed to be deep, and as a result of this, the lip bases in this species are seen to be narrow. **C.spiculigerum**, **C.bancrofti**, **C.sinulabiatum**, **C.microcephalum** and **C.pelagicum** were observed to have interlabia with bifid tips (Johnston and Mawson 1941a,1942) but this was not seen here for **C.osculatum**. The only feature observed on the tips of this structure was the

distinct hooking. Inglis (1960) stated that interlabial lobes in some members of the Subuluridae were of little value as gripping structures , but may improve the flexibility of the lip lobes. The hooked interlabia of **C.osculatum** certainly look like gripping organs; however, it is not known whether or not they function as such.

The interlabial structures in both **C.osculatum** and **H.aduncum** are relatively smooth in appearance. Striated interlabia have been reported from other ascaridoids, eg. **Gedoelstascaris vandenbrandeni** (Sprent 1978b).

It seems likely that if any cephalic structures were to differentiate sibling species, or species from different geographical areas, it would be features of the dentigerous ridge, as suggested by some authors for distinguishing between human and pig **Ascaris** and by Sprent (1968) for species within **Baylisascaris**. This feature could only be used to potentially differentiate species within **Anisakis simplex** and **P.decipiens**, but it remains to be seen whether denticles can be used to distinguish between forms of these species, as the ultrastructure of the adults of these species has not been examined in detail by other authors, so there is little with which to compare the results obtained here. In addition, the variations observed in denticles of a number of species under SEM may prevent differentiation using these structures. Examination of a large number of specimens of these nematodes, from different geographical areas, and areas where sibling species are known to occur, would have to be carried out to ascertain the constancy and reliability of this feature, and to determine if any observed differences were significant, or simply due to natural variability within individual nematodes. Variations in age and overall size of the nematodes would also have to be considered in relation to variations observed in cephalic structure.

#### 4.4.3.7 Cephalic Sense Organs

The three distinct separated lips present in the adult stages of all four nematode genera bear prominent sensory papillae and amphids indicating that these will be required for chemo- and mechano- reception in the final host. Four double papillae are situated generally in the same position on the lips of all four genera, one near each lateral margin of the dorsal lip, and one near the ventral lateral margins of each subventral lip. Each subventral lip also bears a single papilla and an amphid, positioned close together, and on the dorsal sides of the subventral lips. The positions of the sense organs on the cephalic lips show bilateral symmetry, if the dorsal lip were to be bisected.

The function of the cephalic sense organs is unknown; however, as they only appear to become fully developed, at least externally, when the lips are fully formed, a feeding-related function may be indicated. That they are fully developed at the adult stage may also suggest that they are involved in reproduction eg. to detect sexual attractants.

##### 4.4.3.7.1 Development of Sense Organs

As the cephalic sense organs are situated on the lips, they are only likely to become fully developed when the lips themselves develop. Furthermore, Myers (1960) stated that the nervous system of **P.decipiens** is poorly developed at the third stage, and the development of the sense organs is likely to be related to the development of this system.

From the structure observed in the adults, it can be seen that the rounded protrusions of the double papillae in third stage specimens are rudimentary. The double papillae in **A.simplex** can be seen to have developed in both size and form from the third stage. The papillae have increased slightly in size, have become separated from the rest of the lip surface, and have differentiated into double structures.

The double papillae in **C.osculatum** have doubled in size, from the third stage to the fourth, and a groove has formed partially surrounding these structures. The papillae at the fourth stage appear to be incompletely developed, however, as they can be seen to remain attached to the rest of the lip surface at either end, whereas those of the adult are attached only by a single papillar stalk. The double papillae in fourth stage larvae of **H.aduncum** also appear to be incompletely developed at this stage - those of the adult being distinctly raised, and attached to the rest of the lip cuticle by a single stalk and have a deep papillar groove surrounding them. The single papillae and amphids can also clearly be seen in the adults of each species. Development of the sense organs of **P.decipiens** cannot be commented on as these structures were not observed in the third stage larvae examined in the present study.

#### 4.4.3.7.2 Double Papillae

The double papillae of **A.simplex**, **P.decipiens** and **C.osculatum** were approximately the same size as each other, whilst those of **H.aduncum** were approximately half this size. However, in relation to the size of the lips, which are smaller in **H.aduncum**, the size of the papillae in this species is similar to that of the other three species.

The papillae were similar in appearance in **P.decipiens**, **C.osculatum** and **H.aduncum**, and were observed externally as raised, single structures. Although these structures appear single externally, they are termed double papillae as they are seen by light microscopy to have double nerve endings. Elevated double papillae have also been observed in other ascaridoid species, eg. in **Ascaris suum** (Madden **et al.** 1970), **Sulcascaris sulcata** (Allison **et al.** 1972) and **Angusticaecum holopterum** (Sprent 1980). De and Dey (1992) noted that the four double papillae of **Raillietascaris varani** appeared as single structures under SEM, separated from the rest of the

cuticle by a groove. Their photographs also show these papillae to be raised structures, and they are strikingly similar in appearance to those observed in adults of **P.decipiens**, **C.osculatum** and **H.aduncum**. The double papillae were seen externally as double structures only in **Anisakis simplex**, and also appeared to be flat. However, it seems likely that the central elevations seen within the double papillae of **P.decipiens**, **C.osculatum** and **H.aduncum**, in addition to the main structure, are external indications of the double nature of the internal structure. The slight elevation of the smaller lobe of the double papillae in adult **A.simplex** may support the hypothesis that the elevated portions of the papillae in **P.decipiens**, **H.aduncum** and **C.osculatum** are indications of the underlying double structure. These elevations within the papillae do not appear to have been reported previously in these species.

In terms of shape, the double papillae in all species were seen to be roughly oval, although each had slight modifications of this shape.

Weerasooriya **et al.** (1986), using SEM, illustrated the large and small areas of the double papillae in fourth stage larvae of **A.simplex** but showed them to be distinctly raised, whereas the double papillae in the adults of **A.simplex** examined during this study were relatively flat structures, although the small region was slightly elevated. Grainger (1959) also showed the double papillae as raised structures in cultured fourth stage larvae of **A.simplex** using light microscopy. It may be that the papillae become flatter at the adult stage. Davey (1971), using light microscopy, observed the double papillae of the dorsal lip of **A.simplex** to have the smaller lobe of each papilla situated on the side of the papilla facing towards the centre of the lips. This feature was also observed during the present study for this species, with the smaller regions of the papillae on the subventral lips being situated towards the lip margins.

Sprent (1959) and Kazacos and Turek (1982) found that

the double papillae in **Toxocara leonina** and **Baylisascaris procyonis** respectively, consisted of two distinct parts, one large and flat and the other smaller and more rounded. Kazacos and Turek observed this feature externally, using SEM. Sprent, however, examined his specimens by light microscopy, and did not state whether this double structure was observed internally only or externally also. The double papillae in **A.simplex** also showed this pattern, with the small portion being slightly raised, and the larger part being flat.

Grainger (1959) observed only one papilla in the centre of each of the subventral lips in cultured fourth stage larvae of **P.decipiens**, and two papillae on the dorsal lip (although his illustration shows only one on the dorsal lip, again in the centre). These are likely to be the double papillae, but they are not present in the centre of the lips. Grainger also stated that they were often difficult to see. This may be true under light microscopy, but SEM does reveal them as distinct external structures. It is possible that these structures are not as apparent in the fourth stage of this species as they are in the adult. It is relevant that Weerasooriya **et al.** (1986), using SEM, found the double papillae in fourth stage larvae of **P.decipiens** to be flat. In the adult specimens of this species examined in the present study, the double papillae were raised. This species may be similar to **H.aduncum**, where the double papillae were seen to be level with the lip surface at the fourth stage, but raised in the adult. Weerasooriya **et al.** (1986) also stated that a slight depression in the middle of the double papillae showed them to be double structures. In the adults examined during the present study, a central elevation was seen, extending across half of the papilla. Again, this apparent difference may be a result of variation between the fourth stage and adult stage. Fourth stage larvae of **P.decipiens** were not examined during this study, so comparisons cannot be made with the results of Weerasooriya **et al.** (1986).

Baylis (1937) illustrated the double papillae of **C.osculatum** as seen by light microscopy, and noted the single external structure of these organs.

The description of Soleim and Berland (1981) for **T.adunca** (=**H.aduncum**) from Norwegian waters, agrees with that observed in the present study for **H.aduncum**, with regard to both the size and form of the single and double papillae. However, these authors did not note the central elevation within the double papillae, although, from their photographs, this structure does appear to be present.

The numerous processes within the papillar groove of adult **H.aduncum** may be the tips of the sensory dendrites of this sense organ. The double papillae of **Raphidascaris acus**, observed by Smith (1984a), appear similar to those of **H.aduncum**, both in terms of width (approx 18µm compared to 15µm for **H.aduncum**), and general form - the papillae of **R.acus** also being manifested as "flaps", with those on the dorsal lip having their papillar stalk towards the centre of the lip, while those on the subventral lips have their stalks towards the margins of the lips. **R.acus** is closely related to **H.aduncum**, both being raphidascaridines.

#### 4.4.3.7.3 Single Papillae and Amphids

Although Grainger (1959), Gibson (1970), Pippy and Van Banning (1975) and Grabda (1976b) mentioned the double papillae on the lips of fourth stage larvae and adults of **A.simplex**, they did not state whether they observed the single papillae or the amphids. In addition, Grainger (1959) and Myers (1960) did not appear to observe the single papillae or amphids in adult **P.decipiens**. However, the above authors examined specimens by light microscopy, and these structures may have been difficult to see. The single papillae and amphids have not previously been described in detail for the four species examined in the present study, either by SEM or light microscopy. The single papillae are

smaller than double papillae, and appear to vary slightly between species. The single papillae of **C.osculatum** and **P.decipiens** consist of flat, irregularly pitted areas, surrounded by a distinct groove. In **A.simplex**, the single papilla appears to consist of an area of cuticular pitting surrounding the amphid. **H.aduncum** was observed to have smooth, round, raised single papillae, although they were surrounded by an area of irregular cuticular grooves and pitting. Only in **P.decipiens** were the single papillae immediately adjacent to the double papillae on the subventral lips. Cuticular pitting observed in the adults of **A.simplex**, **P.decipiens**, **H.aduncum** and **C.osculatum** does not appear to have been described in light microscopy studies; the reticulated nature, the observation that, at least in **C.osculatum** and **P.decipiens**, the area is separated from the rest of the lip cuticle by a shallow groove, and the fact that the sensory organs are modifications of the cuticle combine to suggest that cuticular pitting forms part of the sensory complex, and is, in **C.osculatum** and **P.decipiens**, the single papilla. In **A.simplex** and **H.aduncum**, this pitting is observed around the amphids and small, rounded papillae respectively, and is also likely to be the single papilla in **A.simplex**, and part of the single papilla in **H.aduncum**. It is relevant that Fagerholm (1989) referred to this structure in **C.osculatum** as a papilla, and Kazacos and Turek (1982) described the single papilla of **Baylisascaris procyonis** as being of a highly sculptured nature, with numerous slits and creases. Although this pitting was not separated by a groove, it was heavily concentrated in one area. Cuticular pitting has been observed by SEM in association with the amphids and single papillae of other nematode species. Barus **et al.** (1983) observed fine and irregularly distributed pits in the cuticle of **Porrocaecum ensicaudatum** above the cephalic papillae which were separated from the rest of the lip cuticle by a clear groove. This structure can be seen adjacent to these sense organs in specimens of **Pseudanisakis baylisi** examined by Gibson (1973). In these cases, the

pitting is likely to perform a sensory function.

The structure of the amphids is similar to that reported by other authors for a variety of nematode species, eg. McLaren (1976a), Wright (1980) and Kazacos and Turek (1982). The openings of the amphids in nematodes are observed to vary from species to species; for example, Jilek and Crites (1982a) and Kazacos and Turek (1982) noted that the amphidial openings of **Spinitectus beaveri** (parasitic in marine fish) and **Baylisascaris procyonis** respectively, consisted of a single pore (like **Anisakis**), whereas those of **Angiostrongylus cantonensis** (Lian-Yin **et al.** 1984), **Toxocara pteropodis** (Prociv 1989) and **Pseudanisakis tricupola** (Gibson 1973) were manifested as slit-like openings (like **Pseudoterranova decipiens**, **C.osculatum** and **H.aduncum**). Anderson and Townshend (1980) found that the shape, size and position of amphidial pores varied slightly within specimens of **Pratylenchus penetrans** (root lesion nematodes) recovered from three different host plants. In the present study distinct variation in the shape and size of amphids was not observed between species, with the exception of **Anisakis simplex**, where the amphids were approximately half the size of those observed in **Pseudoterranova decipiens**, **C.osculatum** and **H.aduncum**; in addition **A.simplex** amphids had a large central pore (surrounded by a narrow rim) rather than a slit.

#### 4.4.3.7.4 Role of Sense Organs

It is generally thought that a sense organ with a gap or pore in the cuticle, such as the amphids in this case, is evidence of chemoreception (McLaren 1976a, Kazacos and Turek 1982), where molecules have direct access to the nerve cell through the pore leading to the nerve ending. Sense organs thought to be mechanoreceptors normally show modifications of the cuticle that allow mechanical triggering of the sensory cell; this may include elevation of the cuticle to form a prominent papilla (Wright 1980). From the external

raised structure of the double papillae in **P.decipiens**, **C.osculatum** and **H.aduncum**, and the small portion of the double papillae in **A.simplex**, one might envisage pressure on these structures directly stimulating the cells below. It has been suggested that the amphids in secernentean nematodes (which include **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum**) may be chemosensory, the double papillae mechanosensory and the single papilla may function as a combined chemo-mechanosensory receptor (Wright 1980). The present study (Chapter Five) has suggested that the amphids, at least in **H.aduncum** and **A.simplex,** are chemosensory, and the single papilla in **P.decipiens** appears to be mechanosensory. McLaren (1976a) stated that the cephalic papillae of nematodes were normally thought to function as mechanoreceptors. However, McLaren (1976a) and Wright (1980) noted two forms of papillae - one where the dendrite or dendrites are overlaid with cuticle at their distal ends and which function as mechanoreceptors, as observed in **Dipetalonema vitae** (McLaren 1972a) and **Nippostrongylus brasiliensis** (McLaren 1976a), and another form where the dendritic cilia open externally via a pore in the cuticle, similar to structures in arthropods which have been found to function as chemoreceptors (Dethier 1963, in Kazacos and Turek 1982). Kazacos and Turek (1982) suggested that the double papillae of **Baylisascaris procyonis** function as both mechano- and chemoreceptors, with the small papilla of this structure being mechanosensory, and the large papilla, with a pore, being chemosensory. It is possible that the double papillae of **A.simplex** are combined mechano- and chemoreceptors, with the raised, small part being mechanosensory, and the larger part chemosensory, although no pore was observed on this part of the structure. The double papillae in **P.decipiens**, **C.osculatum**, and **H.aduncum** are completely overlaid with cuticle and appear to fit the description of the first form of papillae described above by Mclaren (1976a) and Wright (1980), and which are mechanosensory. The deep grooves and pitting of the single

papillae in **A.simplex**, **P.decipiens** and **C.osculatum** may be a modified form of the second type of papilla observed by McLaren and Wright, where instead of a pore in the cuticle, the papillae opens externally via grooves and pits. If this is the case, these single papillae could be considered as chemosensors. The single papilla of **H.aduncum** is both raised and overlaid with cuticle, indicating a mechanosensory function; however, the cuticle surrounding these papillae is also irregularly pitted, and it is possible that the single papillae in this species are combined mechano- and chemosensors.

### 4.4.4 Internal Ultrastructure

Few authors appear to have examined the internal ultrastructure of nematodes using SEM. Ramakrishna and Burt (1991) illustrated a transverse section of a **P.decipiens** larva from a fish host using SEM, however, their paper dealt with host tissue response to the parasite, and no descriptions were made with regard to the parasite itself in this particular preparation. Soleim and Berland (1981) examined the internal structure of the oviduct and uterine horn of female **T.adunca** (= **H.aduncum**) and musculature of the body wall of adults of this species using SEM. In the latter examination, muscle cells were seen giving off processes (to a nerve) and processes from neighbouring cells were seen to fuse. The muscle wall was approximately 100µm thick. In the third stage larvae of **A.simplex** examined in the present study, the body musculature was approximately 75µm thick and only the strands of muscle fibres were observed; however, this may have been due to the preparation of the specimens. The specimens examined here were cut, whilst still alive, with a razor blade, which may have caused disruption to the internal structures, whereas Soleim and Berland fractured their specimens after processing for SEM had been completed. The presence of what appears to be food particles in the

oesophagus of third stage **A.simplex** is puzzling, as the larvae are not thought to feed at this stage.

Examination of such sections reveals information on the general gross structure and positions of various internal organs and tissues within the nematode. In general, however, this type of study gives less information than light microscopy sections but would be useful for gross examination of, for example, longitudinal sections through the head and digestive system, such as carried out by Gibbons (1984) with **Strongylus** spp..

The structure of the body walls, excretory glands and digestive systems is given in detail in Chapter Five.

## 4.5 CONCLUSIONS

During the life cycles of these species, the cephalic region undergoes external changes with each moult, and the different life cycle stages of the one species can be distinguished by cephalic morphology. The similar life cycles of these species, in particular **A.simplex**,**P.decipiens** and **C.osculatum**, might be expected to lead to similarities in external cephalic morphology, having been subjected to the same influences in adapting to hosts. However, each species, at different life cycle stages, varies with regard to cephalic structure. The morphology of the cephalic region is likely to be related to the behaviour and habitats of the nematode species; for example, third stage larvae in fish are thought not to feed, and lie free, or encapsulated in the body cavity, and this may be why the cephalic structures are not fully developed at this stage; in the final hosts the nematodes do feed, and fully developed lips are present. **A.simplex**,**P.decipiens** and **C.osculatum** also attach to the gut walls of their mammalian hosts and the presence of denticles or hooked interlabia may aid in this attachment. Sprent (1982) stated that **Anisakis**, **Terranova\Phocanema** (= **Pseudoterranova**) and **Contracaecum** were closely related

genera, and although **P.decipiens** and **C.osculatum** are both found in seal final hosts and might be expected to be similar morphologically at the adult stage, **P.decipiens** appears to be most similar to **A.simplex**. Although there are differences between the third stage larvae, each has a boring tooth at this stage.

The presence or absence of interlabia and dentigerous ridges in adult nematodes are of generic value and have been used for diagnostic and systematic purposes (Barus **et al.** 1983). The adults of **P.decipiens** and **A.simplex** have bilobed projections bearing dentigerous ridges, but no interlabia are present, whereas **C.osculatum** and **H.aduncum** have interlabial structures but no dentigerous ridges. The adults of **P.decipiens** and **A.simplex** can be distinguished by the form of the dentigerous ridges, and the presence of a large dorsal lip in **A.simplex**. **H.aduncum** differs from **C.osculatum** in the form of the lips, and interlabial structures. The form of the sense organs, particularly the double and single papillae, also differs between species.

No conclusions can be made with regard to cephalic morphology and possible differentiation of sibling species, or between the same species from different geographic areas. This was due mainly to the fact that the areas of the adult cephalic region potentially useful for showing differentiation (eg. denticle size\number\distribution; form of sense organs) had generally not been described in detail, or at all, by other authors. In cases where detailed descriptions have been made, from an area of water other than the North Atlantic, and where differences have been found, no firm conclusions are possible, as a large number of species from both areas would have to be examined to ascertain variability of the features within individuals, and whether such differences are significant. Differences would also have to be considered in relation to variations in general size of the nematodes, and this could only be ascertained by examining nematodes of equal size. Indeed, it seems likely that natural individual variation within species

will preclude the use of differences in cephalic structures to differentiate forms of the same species.

# CHAPTER FIVE : TRANSMISSION ELECTRON MICROSCOPY OBSERVATIONS OF INTERNAL ULTRASTRUCTURE.

## 5.1 INTRODUCTION

The general ultrastructure of nematodes does not appear to have been studied in detail, although Bird (1971), McLaren (1976a,b) and Wright (1980) did review the structure and function of nematode sense organs, using ultrastructural studies. Many authors have examined nematodes under transmission electron microscopy (TEM), however, such studies have generally concentrated on one particular species, often with regard to a single structural feature. Such TEM studies of nematodes include those of Smith (1970) and Bruce (1970), who examined the ultrastructure of the body wall of **Haemonchus placei** and **Trichinella spiralis** respectively; Rosenbluth (1965), who examined the fine structure of the somatic muscle fibres in **Ascaris lumbricoides** and Jenkins and Erasmus (1969), who examined the intestinal epithelium of **Metastrongylus** sp..

The excretory systems of various species of nematodes have been examined using TEM eg. **Nippostrongylus brasiliensis** (Lee 1970), **Stephanurus dentatus** (Romanowski **et al.** 1971).

Several authors have examined the internal ultrastructure of nematode sense organs eg. McLaren (1972a) described the ultrastructure of the sense organs of **Dipetalonema viteae** (Filarioidea), and compared her findings to the ultrastructure of the sense organs of three other species of filarial worms.

With regard to the early life cycle stages of nematodes, McLaren (1972b) used TEM to examine the internal ultrastructure of five species of microfilariae, Berry and Cannon (1981) briefly described the internal ultrastructure of first, second and early third stage larvae of **Sulcascaris sulcata**, and Kelly and Crites (1993) studied the ultrastructure of the first stage larvae of **Philometra** sp..

Few TEM studies have been carried out on marine ascaridoid nematodes. Soleim and Berland (1981) made a detailed study of **Thynnascaris adunca** ( = **H.aduncum**), using both SEM and TEM, which included the digestive tract (in particular the oesophagus and glandular tissue), the excretory and nervous systems and the body wall. Lee **et al.** (1973) examined the ultrastructure of the excretory system of an undetermined species of **Anisakis** larvae, and Fukuda **et al.** (1990) described ultrastructural changes in the oesophagus, intestine and excretory organ of larval **Anisakis** after incubation in artificial gastric juice. Fredericksen and Specian (1981) found that the fine structure of the cuticle could be used to identify juvenile anisakine nematodes.

In particular, there are apparently no studies of any aspects of the internal ultrastructure of **C.osculatum**, and no studies of early third stage larvae of **A.simplex**, **P.decipiens**, **C.osculatum** or **H.aduncum** have been made, although Grabda (1976b), McClelland and Ronald (1974a,b) and Yoshinaga **et al.** (1987) did discuss the internal structure of early larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** as examined under light microscopy. In addition to this, no detailed studies on the internal ultrastructure of the cephalic sense organs of marine ascaridoids have been made.

Attempts were made to obtain sections through newly hatched third stage larvae of **P.decipiens**, to examine the internal ultrastructure of the parasite at this stage, and through the cephalic regions of third stage **A.simplex**, **P.decipiens**, **C.osculatum** and **H.aduncum** larvae from fish, and adult **P.decipiens** from seals, with the aim of obtaining views of the sensory organs. Such observation would aid in characterising the internal structure of these structures, and help to ascertain whether the amphids and papillae act as chemo- or mechanosensors.

Sections through the cephalic regions of the nematodes examined also revealed the structure of the anterior

oesophagus (= preventriculus), and further studies were undertaken to examine the ultrastructure of different regions of the digestive tract of these nematodes. Such cross sections also gave views of the excretory gland and body wall structures. Comparisons could then be made between different species at the same life cycle stage and between different life cycle stages of the same parasite, to note any morphological changes during development. It has been suggested that the larval stages of these nematodes in fish do not feed, and it was hoped that examination of various regions of the digestive systems of these nematodes at different stages may reveal differences in the ultrastructure of the gut. In addition the fine structure of the digestive system of these nematodes has not been studied in great detail, although several descriptions of the structure of this system have been reported using light microscopy (see below).

### 5.1.1 Cephalic Sense Organs

The position and nature of the cephalic sense organs as observed externally in marine ascaridoids has been discussed in Chapter 4. However, it is of relevance to this chapter to briefly discuss the internal nature of these organs in nematodes in general.

The sense organs of nematodes comprise modifications of the cuticle associated with an underlying nerve process (Wright 1980). Each sensory unit is composed of three parts - cuticle, sensory dendrites and processes of non-neuronal cells (Wright 1980).

The neural component consists of one or more tips of dendrites of sensory neurones. From the tip of the dendrite, one or more dendritic processes derive and these are modified cilia (McLaren 1976a, Wright 1980). The dendritic processes contain, almost exclusively, microtubules (Wright 1980). Both

the cilia and microtubules in sense organs show diversity in number and arrangement (Bird 1971, McLaren 1976a).

Two non-neuronal cells are generally associated with each sense organ, and these enclose the neuronal parts. A support cell (also known as a socket cell) is present around the anterior region of the organ, and below this a sheath cell (or gland cell) is present (Wright 1980) - the morphology of which is indicative of a secretory capacity (McLaren 1976b). Supporting cells and gland cells are not, however, associated with the sense organs of all nematodes eg. in trichuroids the nerve cells are ensheathed by extensions of the hypodermal tissue (McLaren 1976).

The cell bodies of neurones from the cephalic sense organs occur in the ganglia of the central nervous system (Wright 1980). Myers (1960) described papillar nerves in **P.decipiens** which connected to the nerve ring and extended forwards to the papillae.

#### 5.1.1.1 Amphids

The amphids communicate with the exterior through a small pore in the cuticle (McLaren 1976a,b; Wright 1980). This pore is thought to allow molecules from the external environment direct access to the sensory dendritic processes, and thus such organs are presumed to be chemosensory (McLaren 1972a, Wright 1980). McLaren (1976a) stated that amphids generally differ from papillae in that each amphid usually contains several modified cilia. In amphids, the bundles of modified cilia project into the amphidial canal, which is lined by cuticle (McLaren 1972a,b; Wright 1980), continuous with, but structurally different, from the outer cuticle of the worm (McLaren 1976b). The amphidial pore opens into this channel (McLaren 1976a). The number of modified cilia\dendritic processes associated with the amphid varies in different nematode species, and variations also occur with regard to the number and form of microtubules (McLaren 1976a,

Wright 1980), even along the length of the same process (Wright 1980).

The amphidial sheath cell (= amphidial gland\secretory cell) surrounds the base of the amphidial canal, and in a number of nematode species this cell is documented as containing vesicles and membranes (Wright 1975; McLaren 1972a,1976).

#### 5.1.1.2 Papillae

McLaren (1976a) stated that all nematode papillae examined were innervated by one or more nerve axons which usually terminate as modified cilia. Bird (1971) suggested that there may be only one to three cilia in papillae, whereas in amphids cilia are more numerous. In most papillae, the distal tip of the modified cilium is overlaid by cuticle and is not therefore exposed to the external environment, suggesting that these particular sense organs are sensitive to mechanical pressures (McLaren 1972a,1976a,b). The distal end of the cilium is often either spatulate, bulbous or discoidal, a structural modification which probably serves to increase the area of sensitivity at the stimulus site (McLaren 1972a, 1976a,b). The number of cilia within individual papillae varies considerably between species (McLaren 1976b). McLaren (1976b) stated that the papillar glands do not achieve the degree of development recorded in the amphids, and gland cells containing membrane bound secretory granules have yet to be identified in association with nematode papillae.

### 5.1.2. Digestive System

In contrast to the majority of other endoparasites, nematodes possess a complete digestive tract, comprising mouth, oesophagus, intestine, rectum and anus (see Figure 1,

in Chapter 1). Examinations of the digestive systems of **Anisakis** sp., **P.decipiens**, **C.osculatum** and **H.aduncum** under light microscopy are well documented eg. Myers (1960), Cannon (1977), Smith (1983b).

#### 5.1.2.1 Oesophageal Region

Sections through the oesophageal region of marine ascaridoids show a circular oesophagus with a triradiate lumen, lined with cuticle. The oesophagus is divided into two distinct areas -the anterior preventriculus, which is muscular and the posterior ventriculus, which is glandular (Myers 1960, Grabda 1976b, Cannon 1977, Soleim and Berland 1981, Smith 1983b). These regions vary in length between nematode species.

Myers (1960) described both radial and marginal muscle fibres in the oesophagus of **P.decipiens**, with the former running from the lumen to the sides of the oesophagus, and the marginals originating at the arms of the lumen. The radial muscle fibres in **A.simplex** also originate at the arms of the lumen (Ishii **et al.** 1989).

Oesophageal glands are also present in this region of the digestive tract. In **A.simplex** the dorsal oesophageal gland extends from the base of the oesophagus to just anterior to the nerve ring, and two small subventral glands occur near the posterior end of the oesophagus, extending into the ventriculus and opening into the posterior oesophageal lumen (Hsü 1933, Beverley-Burton **et al.** 1977). Matthews (1984) also stated that the dorsal oesophageal gland of **A.simplex** has three lobes. The dorsal gland in **P.decipiens** arises in the ventriculus, and extends forward into the preventriculus, to the level of the nerve ring, where it opens into the lumen of the oesophagus (Myers 1960). **Thynnascaris adunca** (= **H.aduncum**) also has one dorsal oesophageal gland and two sub ventral glands (Soleim and Berland 1981). In this species, the nucleus of the dorsal

gland is situated in the ventricular appendix.

Both **C.osculatum** and **H.aduncum** have a posteriorly directed ventricular appendix arising from the region of the ventriculus (eg Smith and Wootten 1984c, Soleim and Berland 1981). The ventricular appendix in **Thynnascaris adunca** (= **H.aduncum**) is filled with extensions of the oesophageal glands (Soleim and Berland 1981). Miyazaki **et al.** (1988) found the oesophageal caecum (presumably = ventricular appendix) of **H.dollfusi** to contain albuminous granules, with the same histochemical characteristics as those of the oesophageal glandular cells.

### 5.1.2.2 Intestinal Region

Immediately posterior to the ventriculus lies the intestine. **P.decipiens**, **C.osculatum** and **H.aduncum** also have an anteriorly directed intestinal caecum (eg. Myers 1960, Smith and Wootten 1984c, Soleim and Berland 1981), which arises from the anterior end of the intestine and extends along the lateral margins of the oesophagus. Myers (1960) found the intestinal caecum of **P.decipiens** in cross section to be composed of palmate structures without definite cellular boundaries, arranged radially to form an irregular lumen. Ishii **et al.** (1989), however, described the intestinal caecum of this species as having approximately 50 single columnar epithelial cells.

The intestinal wall itself, as reported for **P.decipiens**, **Anisakis** sp. and **Thynnascaris adunca** (= **H.aduncum**), is composed of a single layer of tall columnar cells, with a basement membrane (Myers 1960, Grabda 1976b, Soleim and Berland 1981, Ishii **et al.** 1989). Soleim and Berland (1981) observed approximately 80 epithelial cells in the intestinal wall of **Thynnascaris adunca** (= **H.aduncum**), Ishii **et al.** (1989) approximately 100 in **P.decipiens** and 60-80 in **A.simplex**. Grabda (1976b), Soleim and Berland (1981) and Ishii **et al.** (1989) also noted nuclei situated at the base

of the intestinal cells. The intestinal cells bear a brush border of microvilli eg. **A.simplex** (Grabda 1976b), **Thynnascaris adunca** (= **H.aduncum**) (Soleim and Berland 1981); and the intestinal lumen is multi-radiate in cross-section eg. **Anisakis** sp. (Grabda 1976b), **H.aduncum** (Soleim and Berland 1981), although Grabda (1976b) and Ishii **et al.** (1989) stated that the intestine in **A.simplex** was tri-radiate prior to the fourth larval stage. Miyazaki **et al.** (1988) described both the intestine and intestinal caecum of **H.dollfusi** as being lined by columnar epithelial cells, with a brush border and the intestine with a folded outer wall and irregular lumen.

#### 5.1.2.3 Rectal Area

The intestine leads to the rectum which opens at the anus. The rectum is lined by cuticle eg. **A.simplex** (Grabda 1976b), **P.decipiens** (Myers 1960) and **Thynnascaris adunca** (= **H.aduncum**) (Soleim and Berland 1981).

The rectum of **H.aduncum** is surrounded by three unicellular rectal glands (Soleim and Berland 1981, Moravec and Nagasawa 1986). Three rectal glands have also been reported for **C.osculatum** (McClelland and Ronald 1974b), **P.decipiens** (McClelland and Ronald 1974a) and **A.simplex** (Van Theil and Van Houten 1967, Grabda 1976b, Cannon 1977, Smith 1983b, Ishii **et al.** 1989), although Smith (1983b) stated that rectal glands were not visible in small specimens of **A.simplex** (4.2-5.9mm).

Cannon (1977) described two bands of circular muscle in the rectum of larval **Anisakis**. The rectum in **Thynnascaris adunca** (= **H.aduncum**) is surrounded by a number of muscles - the most prominent of which is the H-shaped depressor ani muscle (Soleim and Berland 1981); this muscle, and other structures such as the anal sphincter and rectal ligament, were reported in **P.decipiens** by Myers (1960).

### 5.1.3 Excretory System

The structure of the excretory system is considered to be significant in terms of nematode taxonomy (Gibson 1983, Sprent 1983). The excretory system of nematodes opens to the exterior via the excretory pore, with a terminal duct opening to the pore being common to all nematode excretory systems (Bird 1971).

The excretory system in **A.simplex**, **P.decipiens** and **C.osculatum** lies in the left ventral side of the pseudocoelom, and runs alongside, and parallel to, the anterior region of the digestive tract (eg. Mueller 1927, Cannon 1977). In these species, the excretory system is composed of a long ribbon-like single-celled excretory gland, with a large nucleus; a terminal cuticle-lined excretory duct opens the excretory canal of the gland cell to the exterior through an excretory pore at the anterior extremity of the worm - between the subventral lips (Mueller 1927, Myers 1960, Davey and Kan 1968, Lee **et al.** 1973, Beverley-Burton **et al.** 1977, Cannon 1977, Smith 1983b, Smith and Wootten 1984a, Fukuda **et al.** 1990). The excretory canal passes longitudinally through the entire gland and terminates blindly at the posterior tip of the gland (Lee **et al.** 1973). The excretory cell in **A.simplex** and **P.decipiens** is approximately one third of body length (Mueller 1927, Myers 1960, Davey and Kan 1968, Lee **et al.** 1973), and are wide in the middle, and tapered anteriorly and posteriorly (Davey and Kan 1968, Lee **et al.** 1973, McClelland and Ronald 1974b).

Mueller (1927), Lee **et al.** (1973) and Fukuda **et al.** (1990) described drainage tubules and secretory granules in the excretory gland of **Anisakis** sp..

The excretory system in **Hysterothylacium** sp. is also comprised of a duct and excretory canal, however, the excretory pore opens at the level of the nerve ring (Deardorff and Overstreet 1980, 1981a, Soleim and Berland 1981). The excretory system in **H.aduncum** has both left and right lateral ducts, however, only remnants of the right hand

side of the system are present; the excretory system consisting of a narrow tube running for almost its entire length in the left lateral cord (Soleim and Berland 1981).

### 5.1.4 Body wall

The outer body wall of nematodes is composed of a cuticle, hypodermis and a layer of longitudinal muscle (Lee 1965). Bird (1971) stated that nematode cuticle can usually be divided into two or three layers - an inner basal, a median layer and an outer cortical layer. Depending on the complexity of the cuticle each layer may be further subdivided. Each muscle cell is divided into a contractile, and a non-contractile portion, the latter of which contains the nucleus and which sends processes to the nervous system (Lee 1962). The muscle layer is divided into quadrants by lateral and median chords (eg. Ishii **et al.** 1989). Ishii **et al.** noted 60-90 muscle cells per quadrant in **A.simplex**, and approximately 70 per quadrant in **P.decipiens**.

The muscle cell structure is similar for all nematodes, however, variations may occur in the observed structure of the cuticle. Myers (1960) described the body wall of **P.decipiens** as examined by light microscopy, and observed a thin hypodermal layer between the cuticle and the muscle cells. In cross-section, the cuticle was found to be composed of external and internal cortical layers, an oblique fibre layer, an internal matrix and a basal lamella. The cuticle of **Anisakis**-like larvae from man appeared to be composed of three layers - an external, a middle and an internal layer (Asami **et al.** 1965). Fredericksen and Specian (1981) examined the cuticles of juvenile **Thynnascaris** (= **Hysterothylacium**), **Anisakis** and **Phocanema** under TEM, but could only reliably divide that of **Anisakis** and **Phocanema**, and then only into two major layers - basal and cortical. The cuticles of each species of nematode appeared to consist of layered bands of homogeneous material which varied only in electron density.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Conventional TEM Processing

Due to concerns over the thickness of the nematode cuticle and doubts whether the glutaraldehyde and resin would penetrate fully over the normal fixation time, some modifications were made to the normal process.

(1) Fixation in Karnovskys' fixative (see Appendix 1b ) - normally 4-6 hours, however, this was found to be insufficient to kill the nematodes. Specimens were therefore left in Karnovskys' from anything between overnight to six days (see different parasite stages for details) to ensure that specimens were completely fixed.

(2) Rinse in cacodylate buffer (see Appendix 1c) - brief shake, then placed in fresh change of buffer and left overnight.

During processing, the above stages were carried out in a refrigerator at 8°C. The following stages were carried out in a fume cupboard.

(3) Post fixation in 0.5% osmic acid in cacodylate buffer - normally one hour, however, this was often found to be insufficient to turn the specimens black. Specimens were therefore left in osmium from 1-1½ hours, depending on the extent of osmium penetration. It was found that sections from the body of larval **A.simplex** never turned completely black, even after 1½ hours in osmium. Only the ends of the sections changed colour, giving a striped appearance to the pieces of **A.simplex** being processed. Only the black areas, indicative of good fixation, were cut from the resulting block for examination.

(4) Dehydration in an acetone series

30% Acetone - 15 minutes

60% Acetone - 15 minutes

90% Acetone - 15 minutes

100% Acetone - 2 x 30 minutes

(5) Impregnation in resin mix (see Appendix 1d) - samples placed in a rotator.

100% Acetone + Resin Mix 1:1 - 45 minutes

100% Acetone + Resin Mix 1:3 - 45 minutes

Resin Mix - 1½ hours

(6) Embedding

Specimens were then transferred to either pre-heated vertical B.E.E.M. capsules or horizontal plastic moulds, into which a small amount of fresh resin mix had been placed. After transferring the specimen, the mould was filled with 100% resin, and a small coded tag placed in the mould to allow identification of the specimen. All specimens were placed in the resin in such a position as to allow cross sections of the tissue to be taken. Blocks were transferred to a 60°C oven to polymerise for 48 hours.

### 5.2.2 Microwave method of fixation

Due to concern regarding complete fixation of the nematode tissue, given that the cuticle presents a tough barrier to penetration, some specimens of third stage larvae were also fixed using a microwave method (J.T.Jones pers.comm.) previously used to prepare plant parasitic nematodes for T.E.M.. Such nematodes are known to be notoriously difficult to prepare for electron microscopy. Some modifications were made to the method of Jones as it was observed that these ascaridoid nematodes were not killed after the suggested fixation time for plant parasitic nematodes, probably due to their larger size.

The method used was as follows:-

(1) The anterior ends of live nematodes were cut off and fixed in glass vials containing 3% glutaraldehyde in seawater buffer, in a Proline powerwave 800 microwave oven fitted with a temperature probe. The temperature probe was placed in a separate vial containing 3% glutaraldehyde in seawater only. The oven was set to a medium power setting and was allowed to operate until the temperature probe reached 50°C. This is the end point for fixation of plant parasitic nematodes. However, the ascaridoid nematode sections were still observed to be actively moving at this point, even although Khalil (1969) previously reported that **Anisakis** larvae died after exposure to 55°C for ten seconds. The oven was then set on a "heat and hold" function, to maintain the temperature at 50°C. Nematodes were kept in the oven for 2-3 minutes, until they had stopped moving. A further 30 second burst of 50°C processing was carried out, to ensure that the sections were adequately fixed.

A beaker containing 250ml of water was also placed in the oven during the fixation process, to act as a "heat sink", preventing the small volume of fixative from heating up too quickly.

(2) Rinse specimens in seawater buffer for 15-20 minutes.

(3) Post fixation in 0.5% osmic acid in seawater, in the oven on medium power setting until the temperature probe in the vial of osmic acid reached 50°C. Again this is the end point for post-fixation of plant parasitic nematodes, and again this was not found to be successful for the ascaridoid nematodes which had turned only slightly black. The oven was again set on the "heat and hold" function at 50°C, and specimens were kept in the oven for a further 2-3 minutes, until they had turned completely black.

The heat sink was kept in the oven for this stage.

Specimens were transferred directly to 30% acetone, and

processed conventionally from Step 4 onwards.

Comparisons between conventionally fixed and microwaved fixed material were made under the TEM and it was found that microwave-fixed material gave no better results than conventionally fixed material. Conventional fixation was therefore used to fix further specimens.

### 5.2.3 Early third stage larvae of Pseudoterranova decipiens

Third stage larvae of **P.decipiens** were cultured from eggs as described in Chapter Four. For the purposes of TEM, these larvae did not have to be artificially exsheathed from the second stage cuticle, as sectioning would reveal the structure of the third stage larvae within both the second and third stage cuticles. However, due to the small size of the larvae (approximately 200µm in length by 15µm width), conventional TEM processing could not be carried out, as they are almost impossible to see with the naked eye and difficulties would arise when changing the fixation and processing solutions, in ensuring that the specimens remained in their vials. An alternative method, using agar jelly, was used to fix and process these samples :-

#### 5.2.3.1 Agar Jelly Method

Several pipettefuls of ensheathed third stage larvae and seawater were removed from the culture dishes and placed in a plastic tube with a conical base (Figure 29). The larvae were left to settle overnight. The following day, as much seawater as possible was removed from the tube with a pipette and with care being taken to remove the seawater from the top of the column and to avoid agitation of the base of the tube where the larvae lay. The seawater was then replaced with Karnovskys' and left overnight. The following day the process

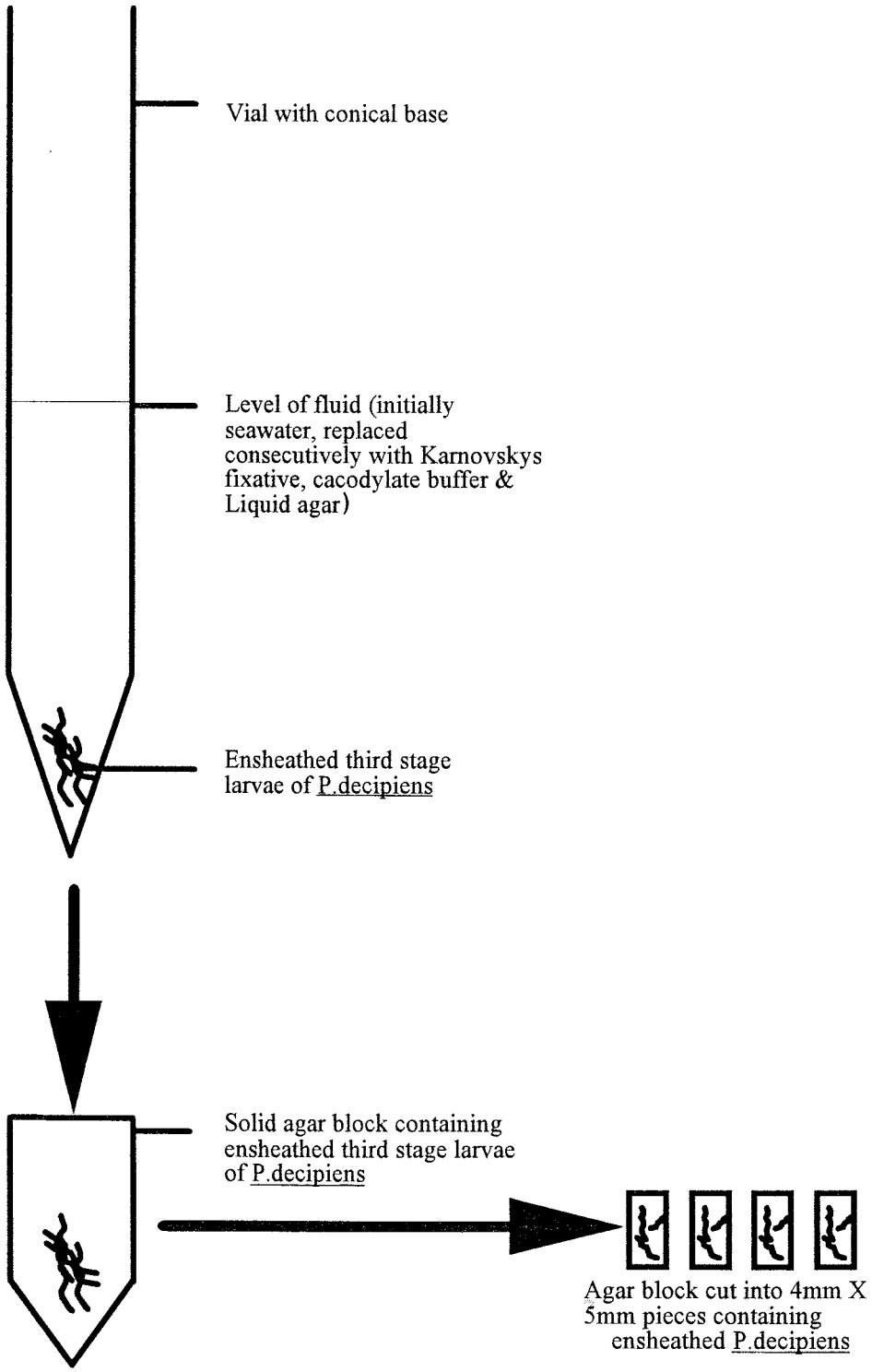



FIGURE 29 : Initial preparation of early third stage larvae of P.decipiens for T.E.M. studies.

was repeated; removing the Karnovskys and replacing it with cacodylate buffer. This was again left overnight. The next day as much of the buffer as possible was pipetted out of the tube, leaving the larvae at the bottom. Liquid agar was then poured into the tube and left to set. This solid agar contained the third stage larvae within it. Once the agar had solidified, it was removed from the tube by gently teasing the sides of the block with a fine seeker, until the whole block was released. The agar block was then examined under a dissecting microscope to ensure that it did contain larvae, and the areas which contained larvae were then cut, still under the microscope, into small oblong sections, approximately 5mm by 4mm (Figure 29). These were then processed conventionally for TEM from Step 3 onwards.

### 5.2.4 Third Stage Larvae From Fish, Fourth Stage Larvae and Adults

Cleaning of specimens was not required prior to processing for TEM as only the internal structure of the parasites were being examined.

#### 5.2.4.1 Structure of the Cephalic End

Third stage larvae of **A.simplex**, **P.decipiens**, **C.osculatum** and third and fourth stage larvae and adults of **H.aduncum** for use in this part of the study were collected from freshly dissected fish. Adult **P.decipiens** were recovered from the stomachs of grey seals (**H.grypus**) from the west coast of Scotland. Chapter Four gives details of the recovery of these nematodes. In addition, for comparative purposes, specimens of **A.simplex** were divided into two groups - encapsulated **A.simplex**, which represented specimens recovered from fish viscera, within a capsule of host tissue (released from the capsule prior to fixation), and "free" **A.simplex**,

representing specimens which were recovered moving freely in the viscera of the fish. Fish were examined as soon as possible after collection, and it is considered that "free" larvae represent specimens which entered the fish relatively recently, although it must be noted that some "free" larvae may have been specimens which had in fact excapsulated shortly after death of the fish. Encapsulated larvae were assumed to have been present in the body cavity for a longer period of time. Specimens were initially fixed in their entirety in Karnovskys' fixative. Specimens were left in Karnovskys' fixative for at least three days, and in most cases, this seemed enough to kill and fix them. However, it was found that specimens of **A.simplex** remained alive in the fixative after this time. For these specimens, the larvae were removed from the vials and the cephalic ends cut off, halfway along the length of the oesophagus (Figure 30) and they were then replaced in fresh Karnovskys' fixative for another three days. For the other specimens, the cephalic ends were cut off as above after three days in Karnovskys' fixative, and were not further processed in this fixative. After the initial attempts at this type of fixation, it was felt that in order to obtain complete fixation from the outset, it was better to cut off the cephalic ends while the nematodes were still alive, before placing them in Karnovskys' fixative. Smith (1970) stated that third stage larvae of **Haemonchus placei**, in common with many other third stage larvae, such as **Trichostrongylus colubriformis** (Smith 1969), proved highly impermeable to fixatives when the cuticle was intact. Smith (1969,1970) therefore cut nematodes into pieces, in the fixative, prior to fixation.

Once the cephalic ends of the nematodes had been fixed in the Karnovskys' fixative, they were processed conventionally for TEM.

### 5.2.4.2 Structure of the Digestive System

Sections from the cephalic end of the nematode (see above), for examination of sense organs, included a portion of the preventriculus, from which thin sections could be cut for examination under the TEM.

Further specimens of nematodes (as detailed above) were cut into four sections for TEM fixation and processing (Figure 30):-

(a) Ventriculus - the worms were cut immediately above and below the ventriculus. Such sections also included portions of the ventricular appendix and\or intestinal caecum where present.

(b) Anterior Intestine - an approximately 4mm long piece of tissue, cut from immediately below the ventriculus.

(c) Posterior Intestine - an approximately 4mm long piece of tissue cut from immediately above the rectum (see below).

(d) Rectum - the posterior region of the worm, cut approximately 1mm above the anal opening. However, on dissection of this region, it was found that the rectum of many of the specimens often became completely released from the body of the worm, or was flattened and distorted as a result of the dissection. Examination of this region was therefore not be carried out, due to the damage often caused to the tissue.

Each section of the digestive tract, from individual nematode species, was placed in a separate marked vial, and processed conventionally for TEM.

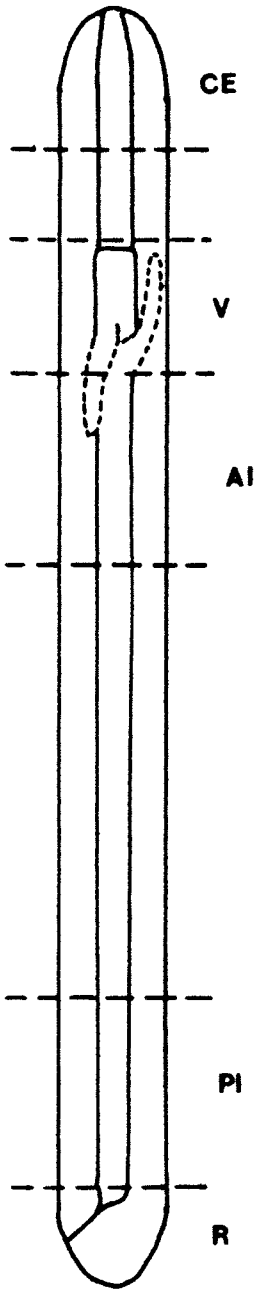



FIGURE 30 : Regions of nematodes sectioned for T.E.M. studies. CE = cephalic end (including sense organs and preventriculus), V = ventricular region (including ventriculus, plus ventricular appendix and\or intestinal caecum where present), AI = anterior intestine, PI = posterior intestine, R = rectal region.

### 5.2.5 Cutting of Blocks

Blocks were cut on a Reichert Ultracut-E ultramicrotome using glass knives, and two thicknesses of sections were cut :-

(a) Semi-thin Sections

Semi-thin sections for identification of the level at which the thin sections were to be cut, were cut at 1µm, and transferred to a drop of distilled water on a slide, using a modified glass pipette tube. The slide was then placed on a hot plate and the water allowed to evaporate, leaving the sections firmly attached to the slide itself. Sections were then stained as described below.

(b) Thin Sections

Thin sections were cut at approximately 80nm, and ribbons of sections transferred to copper grids which were placed on filter paper in a petri dish. Grids were then left in the covered dish at least overnight to completely dry out and were then stained as described below.

Pieces of third and fourth stage larvae and adults were cut as far as possible in cross-section, however, because the second stage larvae were embedded within agar it was impossible to initially orientate them in any way.

The ultrastructure of marine invertebrate tissues has often proved difficult to preserve well (see Eisenman and Alfert 1982). During this study, difficulties were encountered with regard to fixation and section cutting. Some specimens proved impossible to cut, and, in those that were, some did not appear to be adequately preserved; therefore not all life cycle stages of each nematode species which were processed could be adequately described. In particular, good sections of **H.aduncum** proved almost impossible to obtain; with the exception of the sections taken through the amphid, in all other cases the tissues were degenerated. The method of processing, with extended fixation time, is likely to have

caused such degeneration. Unlike the other species of nematodes examined, **H.aduncum** generally does not appear to survive for long periods, after removal from fish (personal observation).

Due to the small size of early third stage larvae of **P.decipiens** and the visual obstruction by agar and resin, it was impossible to ascertain the orientation of the nematodes during sectioning. Similarly, it was difficult to ascertain the position of the sense organs on the head of the nematodes due to the entire tissue being blackened by the fixation in osmium and being surrounded by resin.

### 5.2.6 Staining of Sections

(a) Semi-thin Sections

Once the slides were completely dry, a drop of warmed 1% toluidine blue in 1% borax was placed over the sections, and the slide allowed to dry out over a hot plate. The dried stain was then thoroughly washed off with distilled water and slides placed again on the hot plate to allow any remaining water to evaporate.

(b) Thin Sections

Copper grids containing thin sections were stained as follows:-

(1) 20% uranyl acetate in absolute methanol - two minutes, grids submerged with sections face up, in individual drops of uranyl acetate on a wax sheet.
(2) Rinse grids in stream of distilled water.
(3) Blot grids on filter paper.
(4) Lead citrate (see Appendix 1e) - two minutes, grids submerged sections face down, in individual drops of lead citrate on a wax sheet.
(5) Rinse grids in stream of distilled water.
(6) Blot grids on filter paper.

### 5.2.7 Examination of Specimens

Semi-thin sections on slides were mounted with a cover slip and examined under an Olympus CH compound microscope.

Copper grids containing thin sections were examined under a Philips 301 Transmission Electron Microscope, operating at 80kV, and photographs taken using a camera attached to the Transmission Microscope.

Abbreviations to the plates are given facing Plates 5.1a-d.

## 5.3 RESULTS

### 5.3.1 Early third stage larvae of Pseudoterranova decipiens

Figure 31 shows a whole early third stage larva of **P.decipiens** taken under a light microscope. The boring tooth is distinct and the developing oesophagus and intestine can be seen. Granular material is present running down one side of the mid-region of the body.

TEM sections taken through an early third stage larva of **P.decipiens** revealed a partially developed gut in the centre of the larva marked only by small elongated fibres, approximately 31nm thick (Plate 5.1a). These fibres are surrounded by vesicular tissue (see also Plate 5.1a), which is also commonly found scattered throughout the mid-region of the larval body (Plate 5.1b). Plate 5.1c shows a longitudinal section, taken through part of an ensheathed larva. The wrinkled second stage cuticle can be seen overlying the third stage cuticle. The cuticles are each approximately 133nm in thickness. The structure of the two cuticles appears similar, with no layers being observed; however, the third stage cuticle is of a lower electron density. A wide variety of material completely fills the body of the larva. Large electron dense vesicles appear to enclose

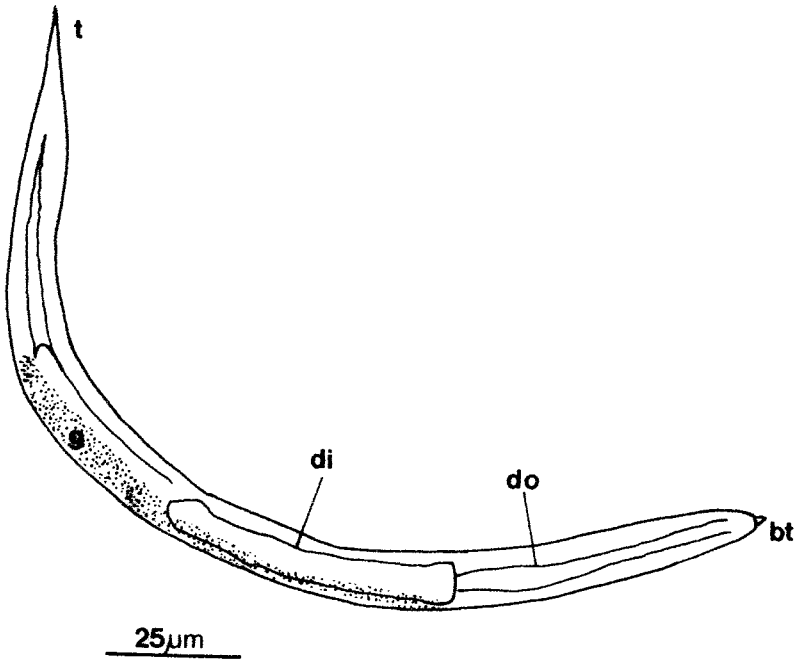
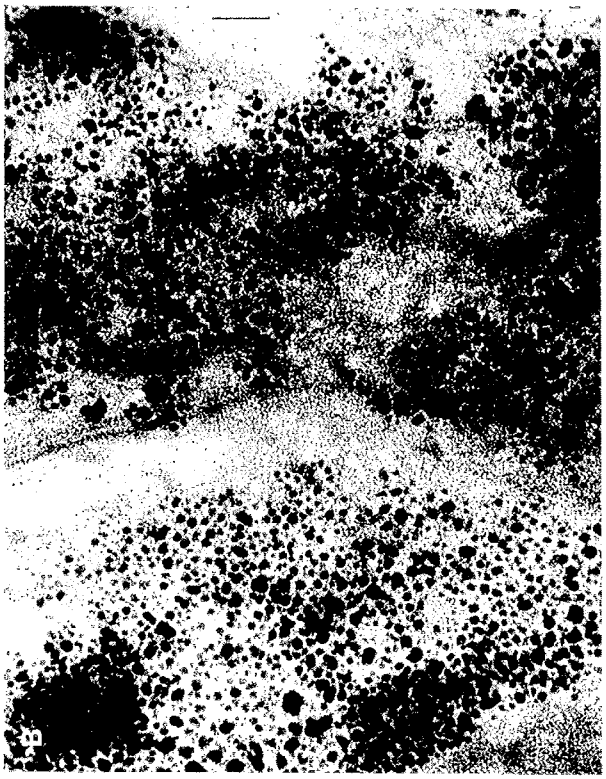
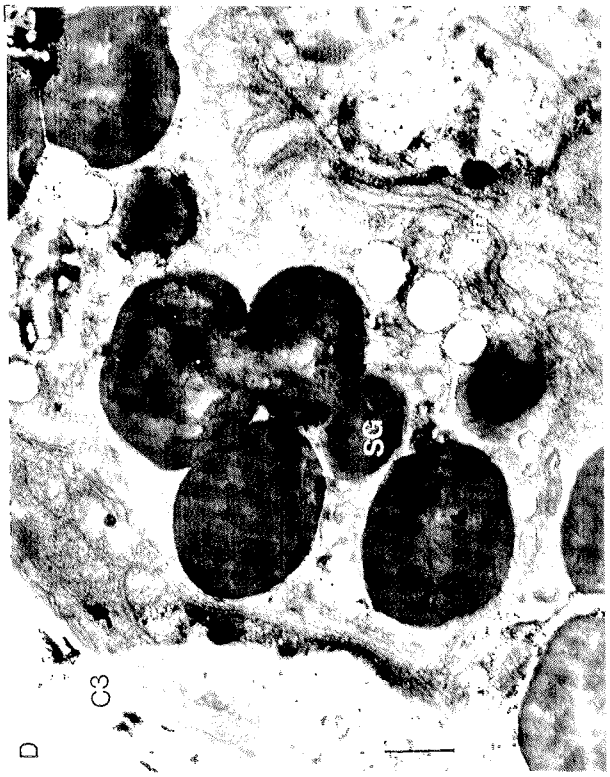



FIGURE 31 : Early Third Stage Larva of P.decipiens
(bt = boring tooth, di = developing intestine,
do = developing oesophagus, g = granular material,
t = tail).

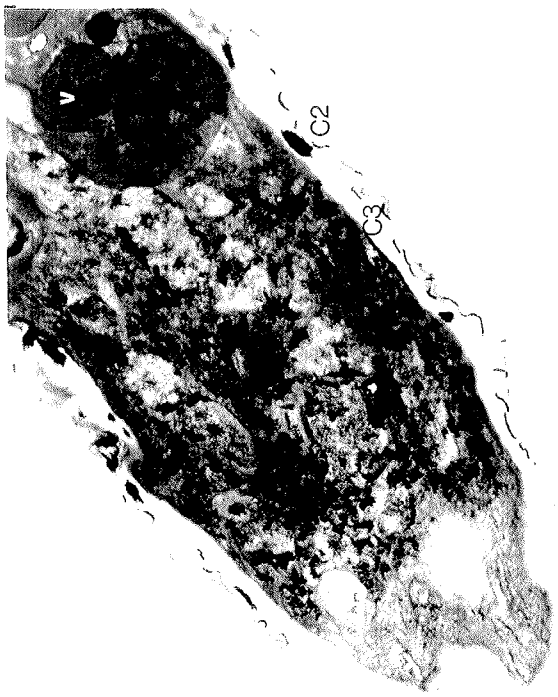
ABBREVIATIONS TO PLATES 5.1 - 5.8

C\c = cuticle
CC = contractile components
CoM = convoluted membrane
C2 = second larval stage cuticle
C3 = third larval stage cuticle
dm = double membrane
dv = drainage vessel
DP = dendritic processes
EC = excretory canal
EG = excretory gland
er = endoplasmic reticulum
F = fibres
G = glycogen
GM = granulated material
H = hypodermis
HedM = high electron dense material
IC = intestinal cell
icf = intracytoplasmic filaments
Ins = intercellular space
IrC = irregularly shaped cells
L = lumen
LLV = lipid-like vesicle
m = membrane
mc = membranous channel
MC = modified cilium
MI\mi = mitochondria
ML = microvilli
MMF = marginal muscle fibres
MS = muscle cells
MT\mt = microtubules
mv = membrane partially surrounding vesicle
MY = myofibril
NA = nerve axon
NCR = non-contractile region
OGN = oesophageal gland nucleus
OM = outer membrane
R = microtubule "rosette"
RMF = radial muscle fibres
SG = secretory granules
ShC = sheath cell
SuC = supporting cell
t = tubules
tb = terminal bar
TJ = tight junction
V = vesicles
VE = ventriculus



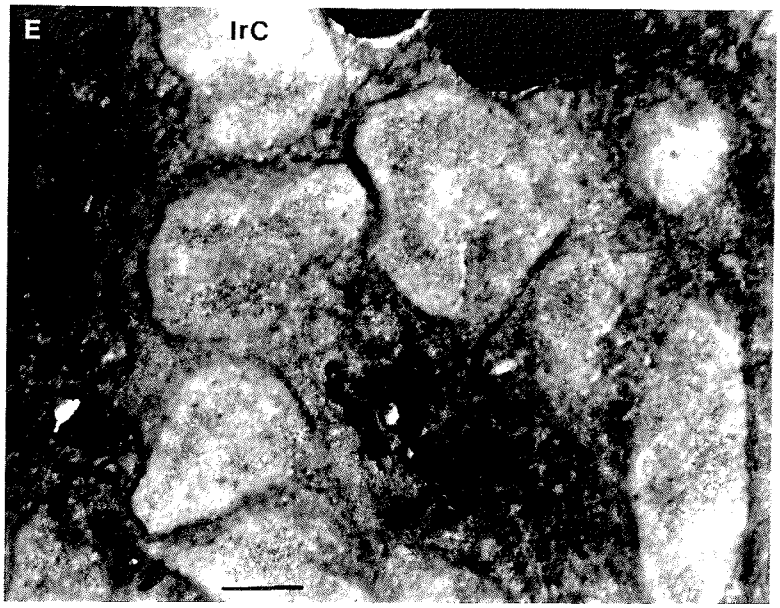








**PLATES 5.1(a-e): Longitudinal sections through an early third stage larva of <u>P.decipiens</u>**

(a) Fibres of partially developed gut, surrounded by vesicular tissue (scale bar = 100nm)
(b) Vesicular tissue (scale bar = 100nm)
(c) Low power micrograph of part of an ensheathed larva (scale bar = 1μm)
(d) Membrane-bound structure containing electron dense granules (scale bar = 500nm)
(e) Irregular cells within membrane-bound structure (scale bar = 500nm) (OVERLEAF)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secondary ones, and there are numerous cell membranes and irregularly shaped areas of granular tissue.

There is an area at the edge of the body wall (Plate 5.1d) which is clearly surrounded by a double membrane and contains large circular electron dense granules, of varying sizes - the largest being approximately 1.2µm in diameter- which may be secretory granules, and larger irregularly shaped cells (Plate 5.1e), containing a homogeneous substance. The small size of the larvae made differentiation between anterior and posterior ends virtually impossible whilst cutting sections, and this section passes through neither end so it is impossible to ascertain the relative position of this structure within the worm.

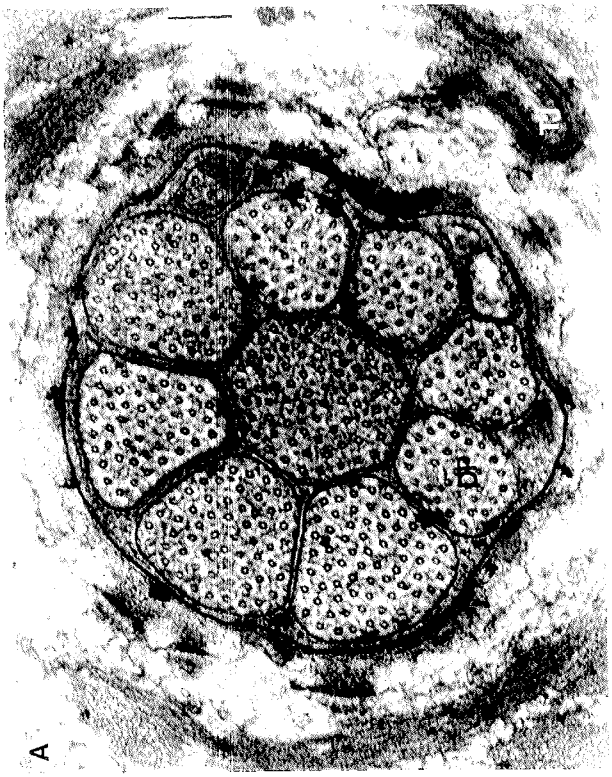
### 5.3.2 Cephalic Sense Organs

Due to the relatively small size of the sense organs compared to the size of the head, it proved extremely difficult to cut sections of the sense organs, although sections were obtained from a fourth stage specimen of **H.aduncum**, a third stage larva of **Anisakis** and from an adult **P.decipiens**.

#### 5.3.2.1 Amphids

Plate 5.2a shows a cross-section through the base of an amphidial canal of a fourth stage larvae of **H.aduncum**. At least ten, and possibly twelve, membrane bound dendritic processes (= modified cilia) can be seen within a roughly circular receptor cavity, approximately 1.4µm in diameter. The section appears to have been taken at a slightly oblique angle. These processes were evenly spaced from one another, and the diameter of the largest process observed was 0.5µm. The processes mostly contain numerous regularly spaced single microtubules, although some appear to be double. A loose






**PLATES 5.2(a-c): Amphids**

(a) Cross section through an amphidial canal of a fourth stage larva of H.aduncum (scale bar = 200nm)

(b) Longitudinal section through an amphidial canal of a third stage larva of A.simplex (scale bar = 1μm)

(c) Microtubules within dendritic processes of a third stage larva of A.simplex (scale bar = 200nm)

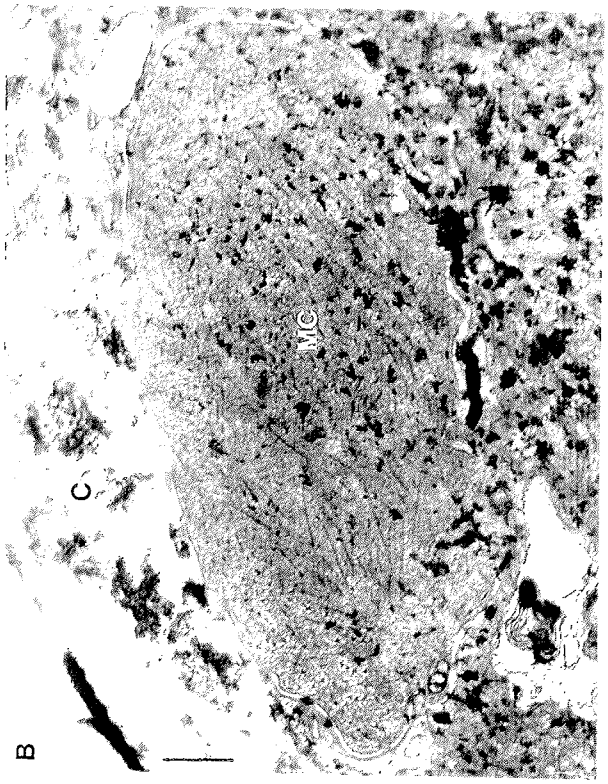
granular material of low electron density surrounds the microtubules in each dendritic process. Apparently similar material is present in the receptor cavity surrounding the dendritic processes. A tight junction is present between the dendrites and the surrounding sheath cell.

Longitudinal sections taken from the side of the amphidial canal in a third stage larvae of **A.simplex** (Plate 5.2b) show the elongated nature of the dendritic processes, and reveal the microtubules to be regularly spaced, aligned longitudinally and to run throughout the entire dendritic process. Plate 5.2c shows the microtubules at a higher power, the limiting membranes of the cilia are apparent and loose granular material can be seen between the microtubules. The arrow in Plate 5.2b points to the direction of the amphidial pore, although unfortunately, this section does not pass through it; it has been cut from the side of the amphidial canal and at a slightly oblique angle, revealing two "layers" of processes. The cuticle surrounding the exterior of the pore and the amphidial canal can be seen. A cell membrane transversely crosses the dendritic processes, approximately 5µm beneath the cuticle, separating the two non-nervous cells of the sensory structure - the anterior supporting cell, surrounding the amphidial canal and extending to the cuticle, and the sheath cell below. These cells do not appear to be separated from the rest of the head tissue by a membrane, but they are distinguished by their appearance - the supporting cell with thick electron dense longitudinal bands of fibres, and the sheath cell also appearing to contain some fibrous tissue - though not to the extent seen in the supporting cell - and the cytoplasm appearing slightly more dense and granular than that of the supporting cell. Where the dendritic processes are enclosed by the supporting cell, they are tightly packed, and the diameter of the amphidial canal is approximately 1.5µm, but in the area of the sheath cell, the processes begin to splay out. The maximum diameter of a single process observed was approximately 0.5µm. At the base of two of the processes, the nerve axons can be discerned,

containing spherical bodies and small scattered microtubules. The surrounding head tissue is variable in nature and appears to be composed randomly of several different substances, irregular in shape and enclosed by membranes. Its general appearance is granular.

#### 5.3.2.2 Papillae

Plate 5.3a shows a cross section through the side of a single papilla of an adult **P.decipiens**. Part of the tip of a single large modified cilium can be seen lying immediately under the cuticle. The cilium is irregularly oval in shape in this particular section, and is approximately 3.3µm in width. The cilium is immediately surrounded by a thin layer of cuticle and is separated from the surrounding tissue. The form of the cilium suggests that this section has been taken through the side of the papilla rather than the centre, as the base of the cilium cannot be seen. This in itself suggests that the tip of the cilium has extended under the cuticle. The cilium contains randomly distributed and irregularly orientated microtubules, within slightly granular homogeneous material. Plate 5.3b is a section from the same specimen which was taken closer to the centre of the cilium. Here the cilium is still oval in shape, but is larger (approximately 7.5µm wide), and lies along the base of the cuticle. Longitudinal strands of microtubules can be seen in the centre of the cilium, and cross-sections of microtubules occur towards the top, suggesting that these sections have been cut at a slightly oblique angle. The dark material scattered over the section is artifact from the staining procedure. Plate 5.3c is a higher power micrograph of the microtubules shown in Plate 5.3b. The longitudinal arrangement of the microtubules can be seen, and the cross-sections of the microtubules reveal that the microtubules occur in groups of two to four, those of four in the form of "rosettes".





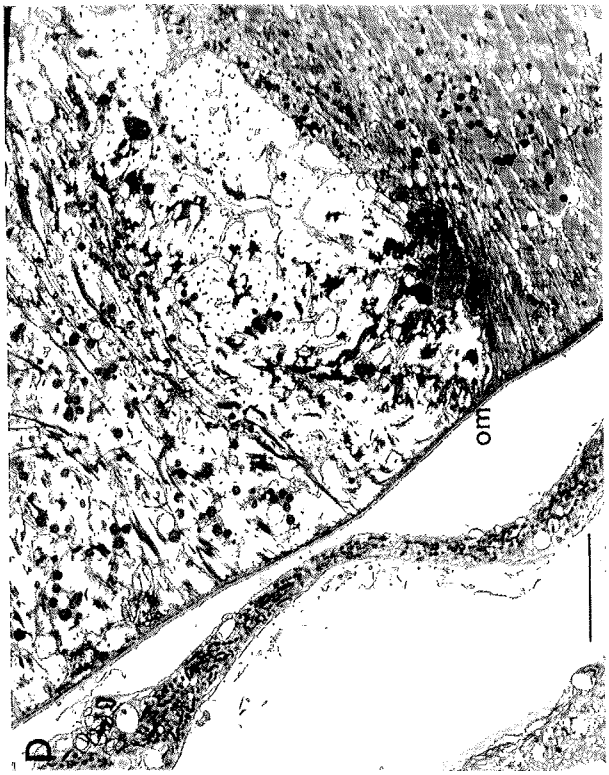

**PLATES 5.3(a-c): Single papilla of adult <u>P.decipiens</u>**
(a)&(b) Tip of a modified cilium, lying under the cuticle (scale bar = 1μm and 500nm respectively)
(c) Microtubules within a cilium (scale bar = 200nm)

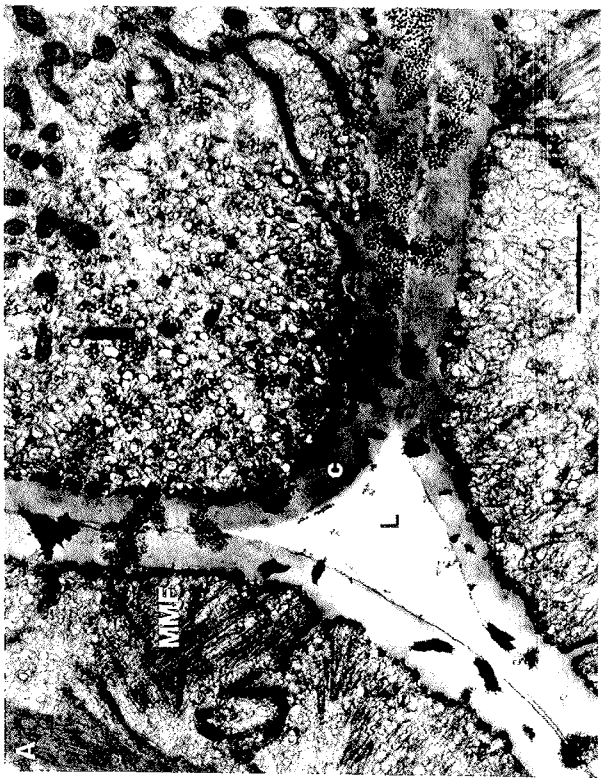
### 5.3.3 Digestive System

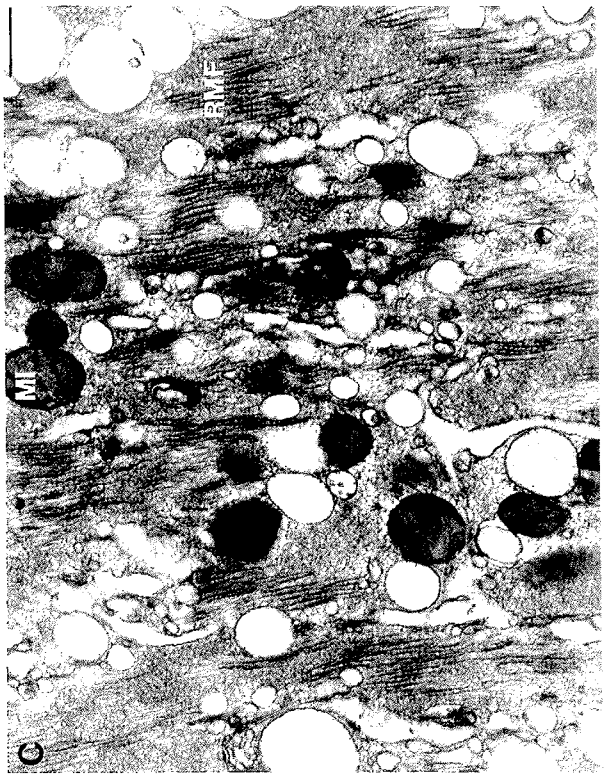
#### 5.3.3.1 Preventriculus

Plate 5.4a shows a cross-section taken through the preventricular region of a third stage larva of **C.osculatum**. The lumen of the preventriculus is lined with a single thick cuticular layer, approximately 400nm thick. Short bundles of marginal muscle fibres run from under the cuticular lining, and numerous mitochondria are present around the edges of the lumen, although many of these appear to be degenerated. There are also large numbers of small vesicles of varying electron densities. The cytoplasm appears to be homogeneous and granular. Examination of the outer edge of the preventriculus, from the same section (Plate 5.4b), taken at a higher power, reveals the radial muscle fibres, which take the form of long thin strands. Between these fibres are what appear to be degenerated structures, bound by a double membrane and filled with a homogeneous low electron dense material. Some of these appear to have been mitochondria, however, others may be vesicles. This might be a result of poor fixation, however other parts of this section showed good cell preservation.

A section of the same area of the same magnification from an adult **P.decipiens** (Plate 5.4c), also shows the strands of muscle fibres, however, numerous vesicles of varying sizes and electron densities are present amongst the fibres. Some are filled with a homogeneous electron dense material, and many appear empty, or partially so. Mitochondria are also present. Although the cytoplasm appears similar in both cases, the structures in the preventriculus of adult **P.decipiens** do not appear to be degenerated. Plate 5.4d shows the edge of the preventriculus in adult **P.decipiens**, and with the outer limiting membrane. Plate 5.4c is taken from the area at the bottom right of Plate 5.4d, showing closely packed bundles of muscle fibres, interspersed with vesicles and mitochondria. Plate 5.4e is a higher power


**PLATES 5.4(a-f): Cross sections through preventricular region**

(a) Third stage larva of C.osculatum; region surrounding the lumen of the preventriculus (scale bar = 1μm)

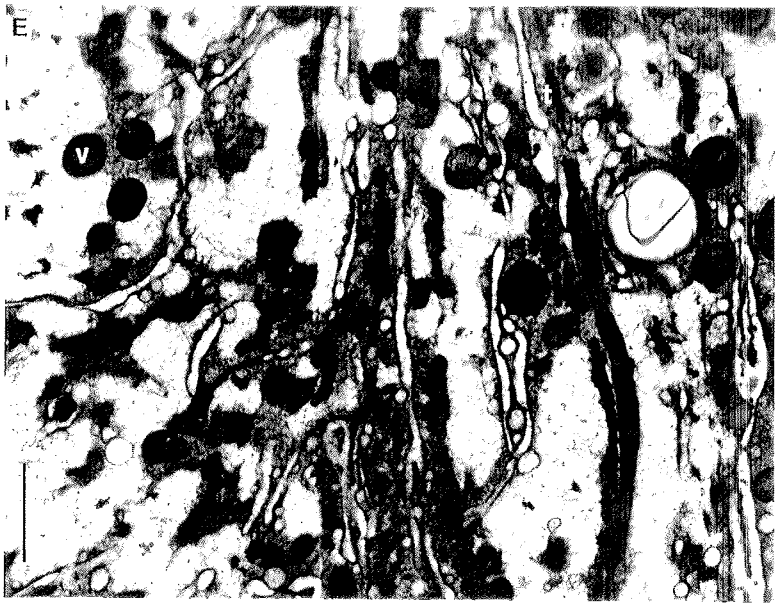
(b) Third stage larva of C.osculatum; preventriculus wall (scale bar = 500nm)

(c) Adult P.decipiens; preventriculus wall (scale bar = 500nm)

(d) Adult P.decipiens; edge of preventriculus (scale bar = 5μm)

(e) Adult P.decipiens; oesophageal gland within preventriculus (scale bar = 1μm) (OVERLEAF)

(f) Adult P.decipiens; region surrounding the lumen of the preventriculus (scale bar = 4μm)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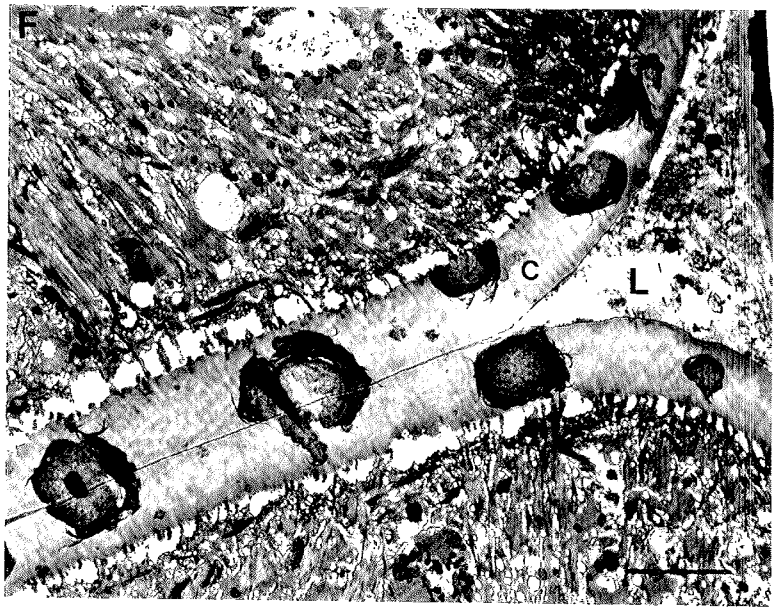
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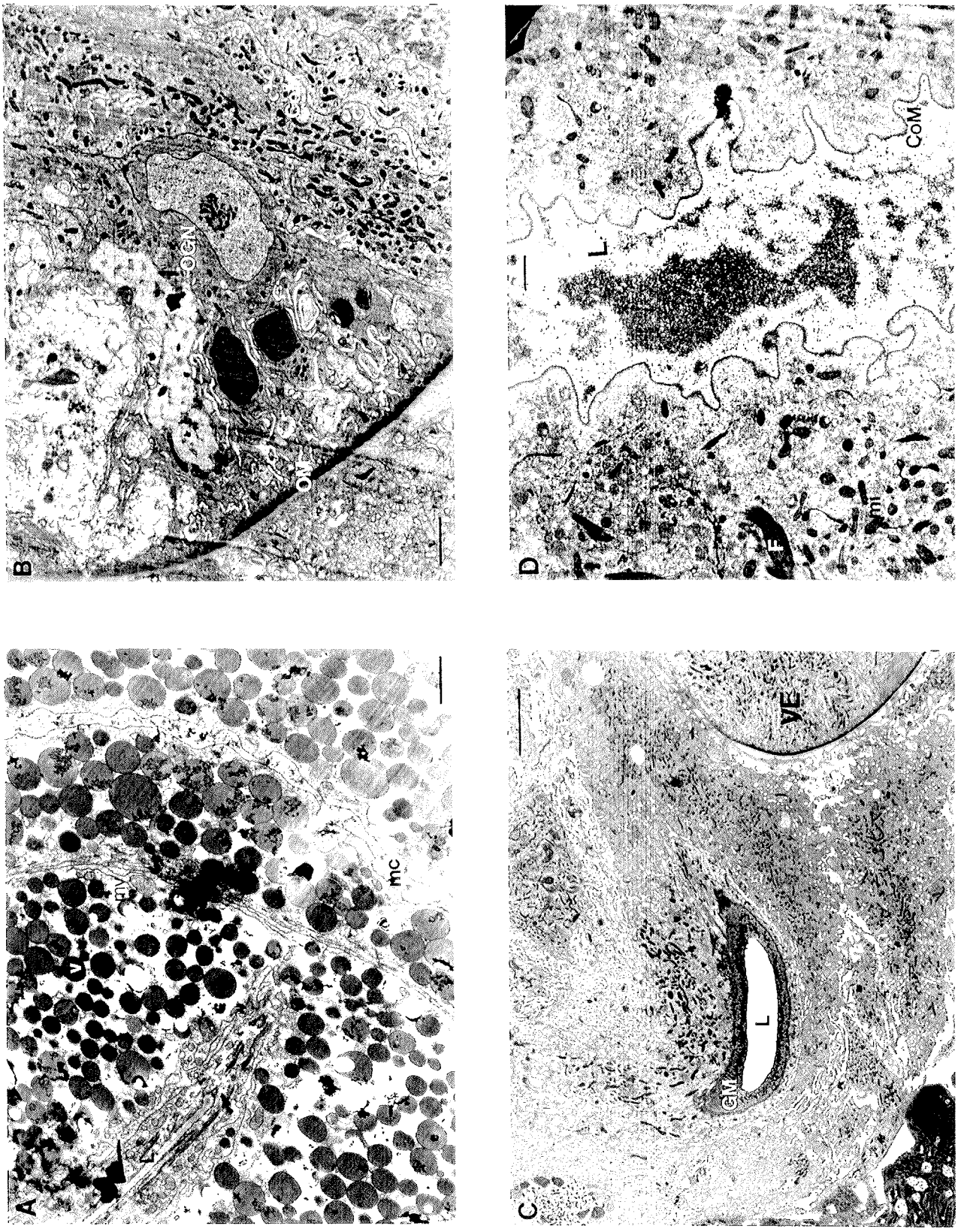
micrograph from the area at the top left of Plate 5.4d. The nature of the tissue here is very different from that shown in Plate 5.4c, and the two types of tissue are clearly demarcated (Plate 5.4d). The region of the preventriculus shown in Plate 5.4e is likely to be part of an oesophageal gland. Many long thin tubules can be seen branching throughout the tissue in this region, and numerous circular vesicles of varying sizes are present, the majority filled with a highly electron dense material. The cytoplasm is granular and of a low electron density. Plate 5.4f shows the preventricular lumen of an adult **P.decipiens**. The cuticular lining is approximately 2.6µm thick and the lumen is filled with a granular material. Although, the preventricular tissue has pulled away from the cuticle lining the lumen, the bundles of marginal muscle fibres can be seen, as can the numerous mitochondria in the tissue around the lumen. Vesicles similar to those found amongst the radial muscle fibres are present, and there are several larger areas mainly filled with granular material of varying electron densities, several of which have conspicuous aggregations of mitochondria surrounding them.

Sections taken through the preventriculus of third stage larvae of **P.decipiens** and **A.simplex** showed similar features to those seen in the third stage larva of **C.osculatum**, with bundles and strands of muscle fibres between which lay large areas of what appeared to be degeneration of membrane bound structures. In addition, no distinct differences in the structure of the preventriculus were observed between "free" and encapsulated specimens of third stage **A.simplex** larvae.

#### 5.3.3.2 Ventriculus

Plate 5.5a shows a section through the centre of the ventriculus in a third stage larva of **A.simplex** which had been found "free" in the viscera of a fish. Numerous large circular membrane bound vesicles can be seen throughout the




**PLATES 5.5(a-d):**

(a) Ventriculus of a third stage larva of A.simplex (scale bar = 2μm)

(b) Ventriculus of a third stage larva of C.osculatum (scale bar = 2μm)

(c) Ventricular appendix of a third stage larva of C.osculatum (scale bar = 5μm)

(d) Intestinal caecum of an adult P.decipiens larva (scale bar = 1μm)

tissue, and although these are of varying sizes - the largest being approximately 1.8μm, they contain solid homogeneous material and appear to be of similar electron densities. Two concentric channels, composed of membranes, divide these vesicles into separate regions. The first of these appears to be composed of several blind ending membranes. One of these membranes can be seen to partially surround a vesicle, which is significantly lower in electron density than the rest of the vesicles in that area, and the tissue inside appears to be granular rather than solid. The second of these membraneous channels appears to consist of only two membranes, and these are separated by gap filled with granular material. A closed arm of the lumen, with a cuticular lining approximately 100nm thick, can be seen towards the top left of the micrograph, and this is immediately surrounded by a complex of convoluted membranes, apparently blind-ending and generally lying parallel to the arm of the lumen. Scattered amongst these membranes lie several short bundles of fibres and membrane-bound vesicles which are significantly smaller in size than those vesicles found outwith this area. The cytoplasm generally appears to be granular, and of a low electron density.

Plate 5.5b shows a section through the ventriculus of a third stage larva of **C.osculatum**. The outer membrane of the ventriculus is approximately 330nm thick. The large nucleus seen in the centre of the cell is that of an oesophageal gland cell. The boundary of this cell is indistinct in some regions, however, it is similar in structure to that of the oesophageal gland seen in the preventricular region of adult **P.decipiens** (see Plate 5.4e), being composed of branches of thin elongated tubules, vesicles of varying sizes and electron densities, many of a high electron density, and a granular cytoplasm. The remainder of the ventriculus is structurally different in appearance from that of the third stage **A.simplex** larva. Although many membrane bound vesicles of a high electron density are present, these are generally irregular in shape and many are elongated in appearance. A

few thick short bundles of fibres are also present in clusters. A convoluted membrane, which appears to double back on itself, is situated radially in the wall of the ventriculus, enclosing material which does not differ in appearance from the surrounding tissue. The cytoplasm appears granular.

#### 5.3.3.3 Ventricular Appendix

Plate 5.5c is a low power shot taken at the level of the ventriculus of a third stage larva of **C.osculatum**, showing the ventricular appendix. This is similar in structure to the adjacent ventriculus, and is made up of patches of tissue packed with numerous irregular vesicles of high or low electron density; the latter being apparently empty. The vesicles are of differing sizes, and again, many are elongated. A few larger vesicles containing granular material are also scattered through the tissue. The appendix partially surrounds the ventriculus, and has a central lumen (elongated in this section). The lumen is immediately surrounded by an approximately 1µm layer of granulated material composed of several substances of high electron density. This layer is clearly demarcated from the rest of the tissue.

#### 5.3.3.4 Intestinal Caecum

Plate 5.5d shows the centre of the intestinal caecum of an adult **P.decipiens**, taken at the level of the ventriculus. The lumen is filled with a granular substance, comprising both high and low electron dense material. The convoluted membrane lining the irregularly-shaped lumen can be seen. The caecum itself is composed of a granular material, with scattered mitochondria, and other granular vesicles, of a similar size to the mitochondria and generally of an electron density similar to that of the cytoplasm. Several thick,

short bundles of fibres are also present, occurring randomly through the tissue. No cell boundaries were observed in the wall of the caecum. The appearance of the caecum is generally similar to that of the intestinal cells (see below).

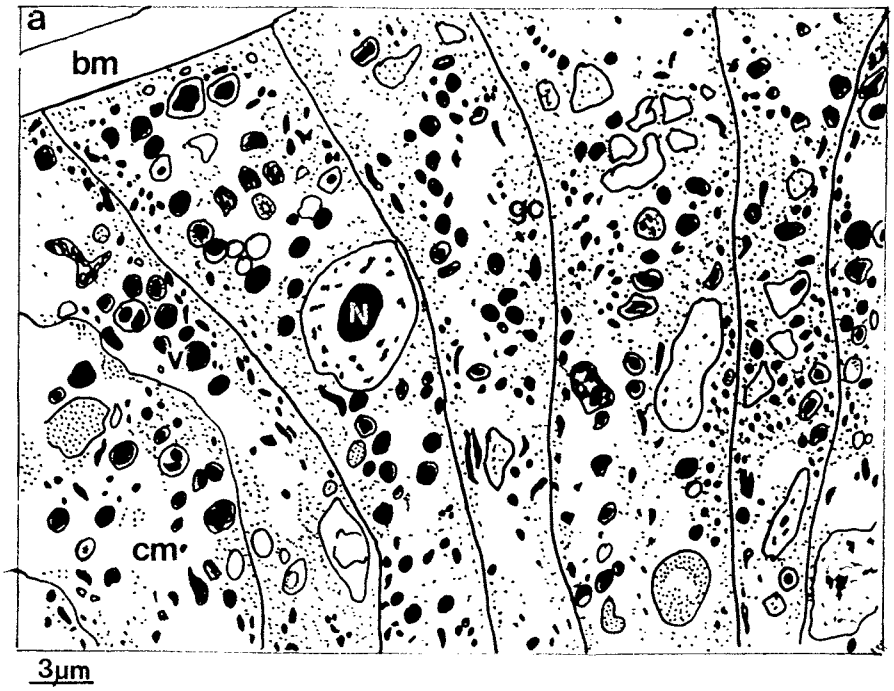
#### 5.3.3.5 Intestine

The structure of the intestine was generally similar in adult and larval **P.decipiens** and "free" and encapsulated larvae of **A.simplex**; however, detailed structural observations will be presented under the individual specimens examined.

The structure of the anterior and posterior intestine was also found to be generally similar, and thus these two regions will not be treated separately.

The intestinal epithelium is composed of a single layer of closely packed columnar cells, with a brush border of microvilli. Figure 32a is a diagrammatic representation of the outer edge of the intestine, in this case from an adult **P.decipiens**. The thick basement membrane surrounding the intestinal epithelium is homogeneous and approximately 2.5µm in thickness. The elongated intestinal cells are closely packed and there is a large nucleus near the base of the cell. These cells contain many small circular vesicles, some granular and some homogeneous, of varying sizes and of high electron densities, and which appear to be more prevalent towards the basal region of the intestine. The remaining cytoplasm appears to be granular.

Figure 32b shows a diagrammatic representation of the anterior region of the intestinal cells, in a "free" **Anisakis** third stage larvae. The cells are again closely packed and are elongated. The cytoplasm is granular, and there are only a few scattered circular vesicles. The cell edges lining the intestinal lumen have a layer of high electron dense material (= plasma cap) immediately under the cell membrane. This layer also extends down the sides of each cell, around the



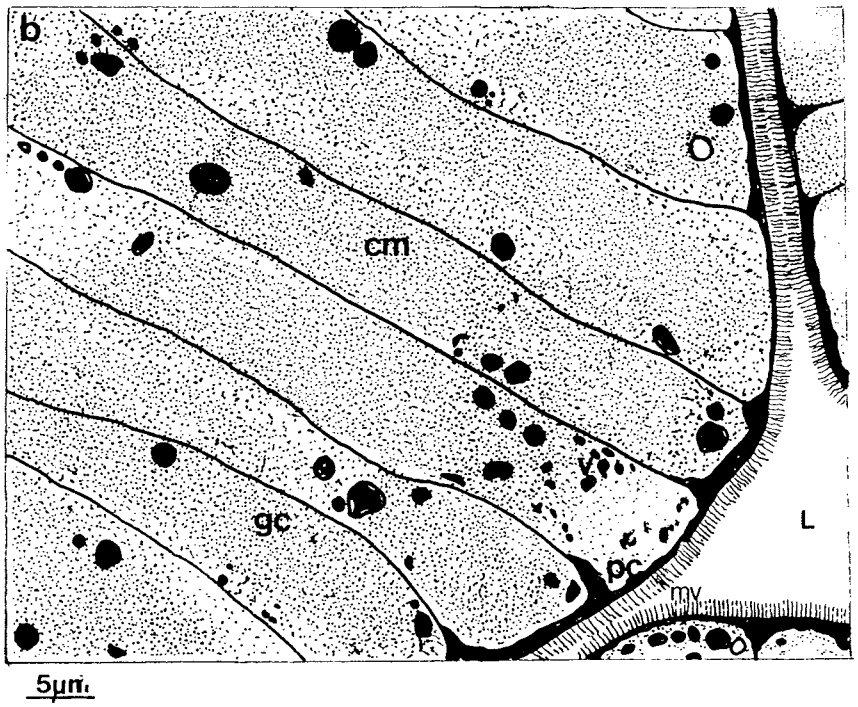




FIGURE 32: Diagrammatic representations of the ultrastructure of intestinal cells.

(a) Adult P.decipiens - basal region of intestinal cells.
bm = basement membrane, cm = columnar cells,
gc = granular cytoplasm, N = nucleus,
v = vesicles

(b) "Free" third stage larvae of A.simplex - anterior region of intestinal cells.
cm = columnar cells, gc = granular cytoplasm,
L = intestinal lumen, mv = microvilli,
pc = plasma cap, v = vesicles

lateral cell membranes, although the layer is significantly thinner in this area. The entire lumen is lined by a layer of microvilli.

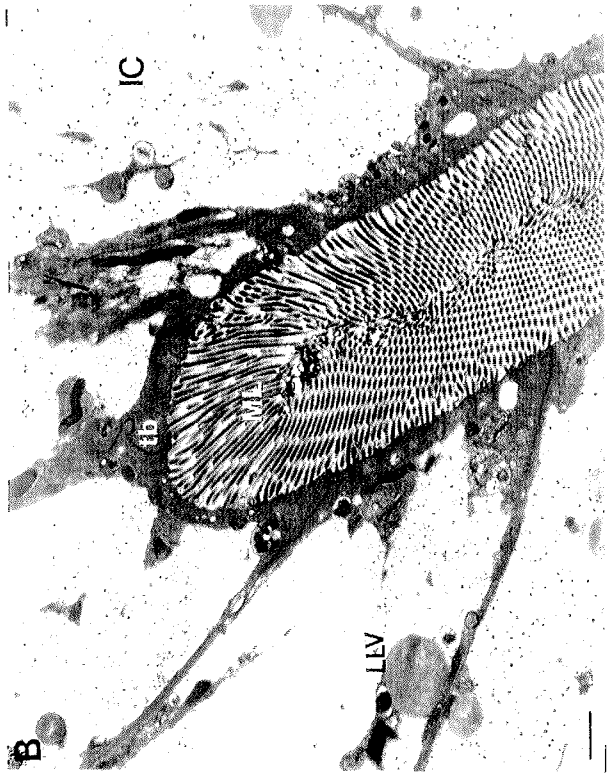
### Third Stage Larvae of Contracaecum osculatum

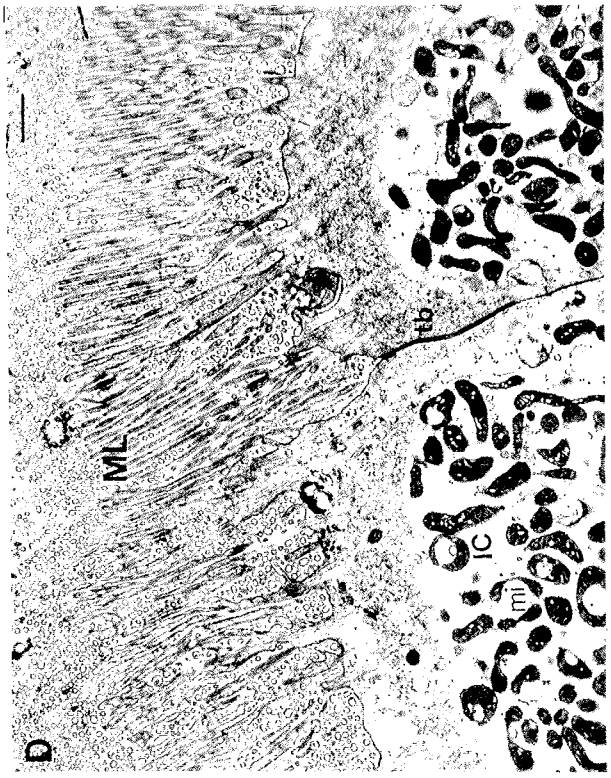
Plate 5.6a shows the fine structure of the intestinal cells in a third stage larva of **C.osculatum**. The fine, granular nature of the cytoplasm can be seen, and a few scattered circular vesicles of varying sizes, and of similar electron density. The largest vesicle seen here is approximately 1.3µm. The thin layers of high density material are apparent around the junction of the adjoining intestinal cells.

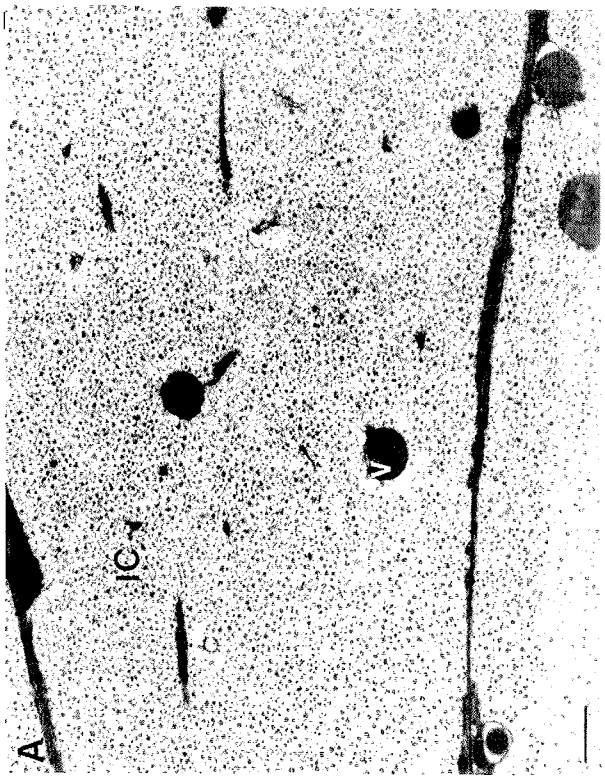
The intestinal lumen and surrounding cells of this specimen is shown in Plate 5.6b. This particular arm of the lumen is closed, and the microvillar layer has drawn closely together. The microvillar layer is approximately 2.2µm in thickness. The layer of high electron dense material at the apical edge of the cells (approximately 700nm in thickness) - and extending down the lateral cell membranes is distinct. There are scattered vesicles, one of which in particular appears to be lipid-like, and is approximately 1.7µm in diameter. The junction of the lateral cell membranes, at the anterior end of the cell, is thickened to form a dense terminal bar.

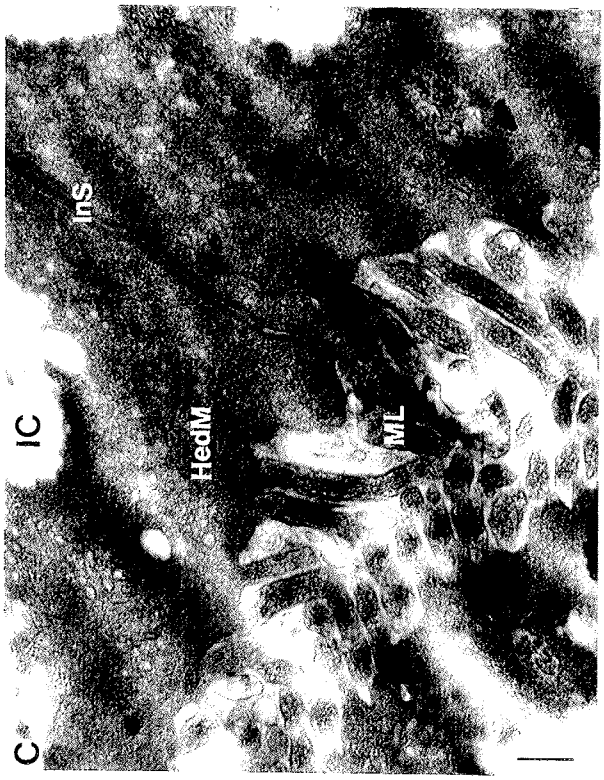
### Third Stage Larvae of Anisakis simplex

The fine structure of the apical regions of the intestinal cells is similar to that shown in Plate 5.6b, for **C.osculatum**. Little internal structure was observed, other than the granular cytoplasm, and a few scattered circular vesicles of high electron density, the largest being approximately 1.9µm. The lateral cell membranes were defined




**PLATES 5.6(a-e): Cross sections through intestine**

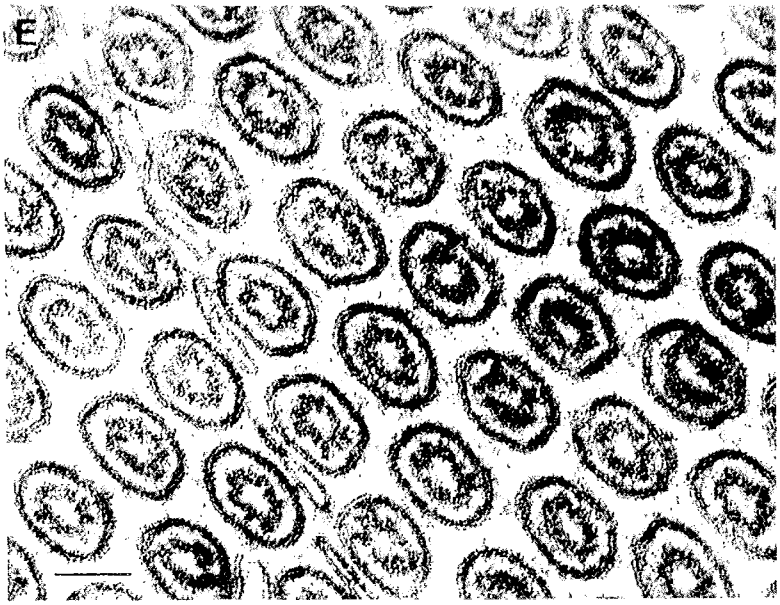
(a) Intestinal cell of a third stage larva of C.osculatum (scale bar = 1μm)

(b) Intestinal lumen and apical regions of intestinal cells in a third stage larva of C.osculatum (scale bar = 1μm)

(c) Microvilli and apical regions of intestinal cells of a third stage larva of A.simplex (scale bar = 200nm)

(d) Intestinal lumen and apical regions of intestinal cells in an adult P.decipiens (scale bar = 1μm)

(e) Intestinal microvilli of an adult P.decipiens (scale bar = 100nm) (OVERLEAF)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by the thin layer of high electron dense material which lines each membrane. Plate 5.6c shows a high power micrograph of the microvilli in the intestinal lumen of a third stage **A.simplex** larva. The microvilli appear to have been produced from the layer of high electron dense material lining the leading edge of the intestinal cells. This layer is approximately 800nm thick. Filamentous strands of material can be observed within some of the microvilli. The two adjoining intestinal cell membranes can also be seen at the apex of the cell, and these are dense and thickened, and separated by an intercellular space. The microvillar layer in third stage **A.simplex** larvae is approximately 1µm thick, and the basement membrane, again homogeneous, is approximately 1.1µm thick.

**Third Stage Larvae of Pseudoterranova decipiens**

The structure of the intestine of third stage larvae of **P.decipiens** was similar to that described above for **C.osculatum** and **A.simplex**, with the size of the circular vesicles being the only difference. In general, larger vesicles were observed in third stage larvae of **P.decipiens**, up to approximately 2.5µm in diameter, and the majority of these appeared to contain lipid. The electron dense layer at the anterior regions of the cells, approximately 1.6µm thick.

**Adult Pseudoterranova decipiens**

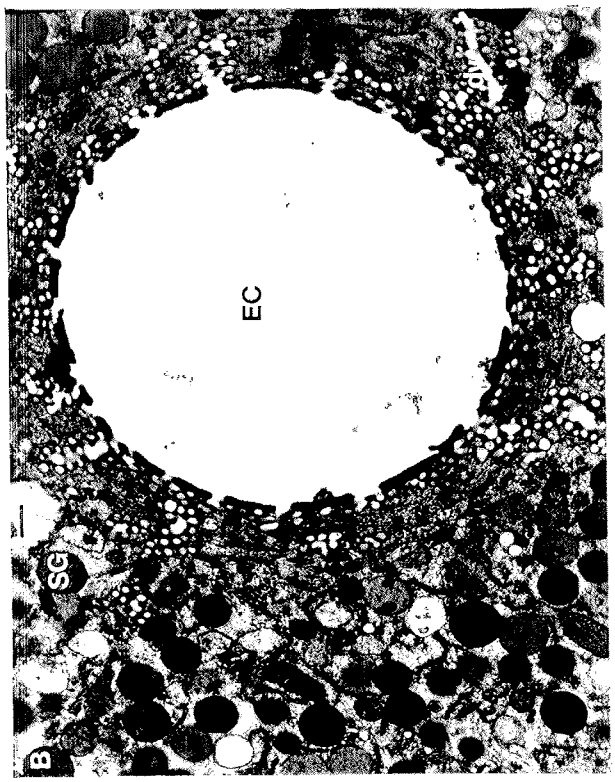
Plate 5.6d shows the intestinal lumen and apical regions of the surrounding cells in an adult **P.decipiens**. The distinct dense terminal bar can be seen at the junction of the adjacent lateral cell membranes in the anterior region of the cell. Numerous microvilli are present in the lumen and can be seen orientated in different planes - some lying longitudinally and some in cross section. The microvilli are

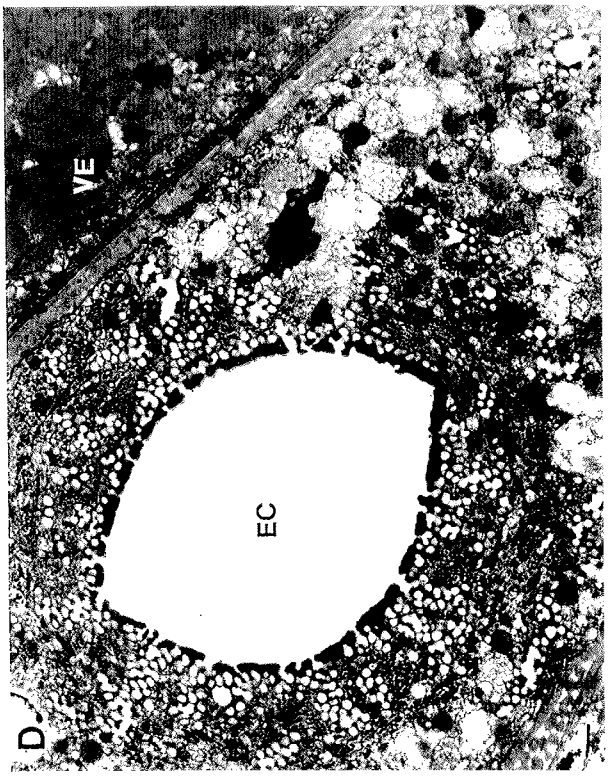
clearly being produced from the layer of material at the leading edge of the cells lining the lumen. This layer is approximately 2µm thick. The intestinal cell membrane lining the lumen is convoluted into spreading branches, which contain this material, and these branches are seen to form the microvilli, the layer of which is approximately 5µm in thickness in this case. Small strands of filamentous material can also be seen within these branches and at their bases, leading into the main body of the cell. The cells lining the lumen are also packed with mitochondria just beneath the point where the layer of denser material begins and the microvilli are produced. Such accumulations of mitochondria were not observed in the intestinal cells of any of the species of third stage larvae examined. Plate 5.6e shows a higher power cross-section of the intestinal microvilli in an adult **P.decipiens**, revealing regularly spaced microvilli in rows, and which are of equal size. The lumen of each microvillus is irregular in shape.

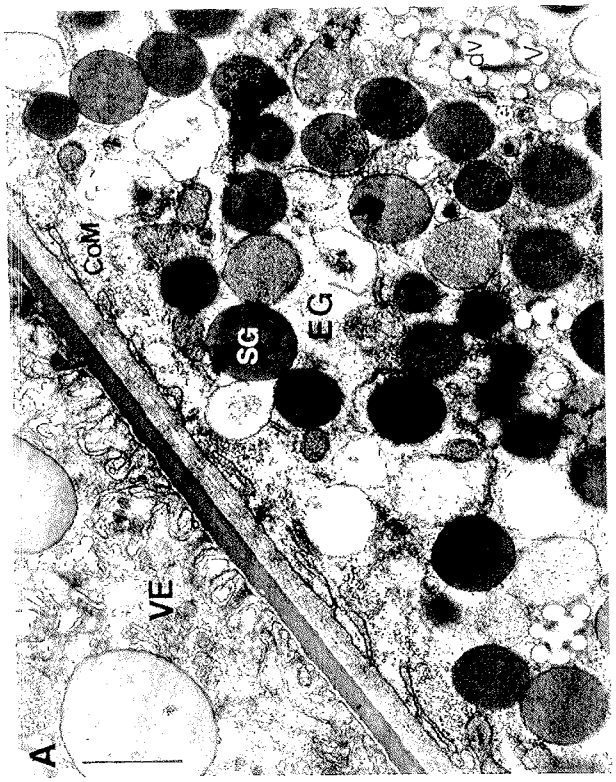
### 5.3.4 Excretory gland

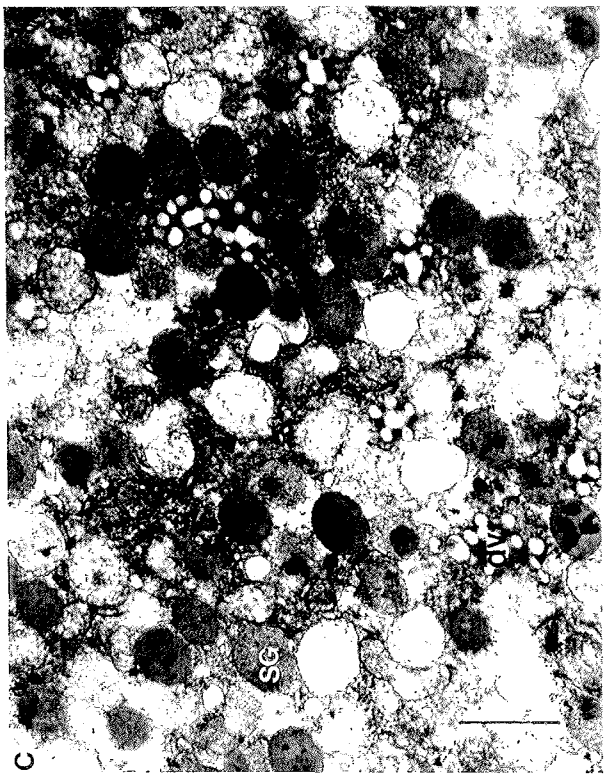
Sections through the excretory gland of a "free" and an encapsulated third stage larva of **A.simplex** were compared (Plates 5.7a,b and 5.7c,d). In both specimens, the sections were taken at the level of the ventriculus.

Plate 5.7a shows the excretory gland adjacent to the ventriculus in a "free" **A.simplex** larva. The excretory gland contains membrane bound circular secretory granules of varying sizes and electron densities, although the material within most individual granules is homogeneous, and appears granular in those of lower electron density and solid in those of higher electron density. The granules in this micrograph are approximately 150 - 900nm in diameter. Some empty, or partially empty granules can also be seen. The cell membranes surrounding some granules appear to have lysed. The cytoplasm between the granules is filled with a granular




**PLATES 5.7(a-e): Cross sections through excretory gland**

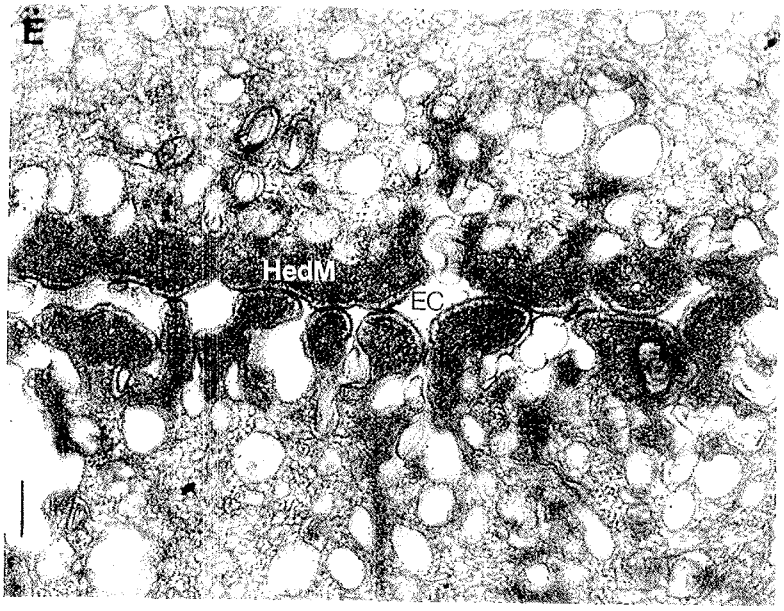
(a) Edge of excretory gland of a "free" third stage larva of A.simplex (scale bar = 1µm)

(b) Excretory canal of a "free" third stage larva of A.simplex (scale bar = 500nm)

(c) Edge of excretory gland of an encapsulated third stage larva of A.simplex (scale bar = 1µm)

(d) Excretory canal of an encapsulated third stage larva of A.simplex (scale bar = 500nm)

(e) Closed excretory canal of a "free" third stage larva of A.simplex (scale bar = 200nm) (OVERLEAF)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material and scattered strands of endoplasmic reticulum. Numerous small drainage vessels can be seen within the cytoplasm, lined with a high electron dense material and into which smaller empty vesicles are merging. These smaller vesicles are circular in shape and are of a similar size, being approximately 150nm in diameter. Some vesicles can be seen free in the cytoplasm, and have often fused and formed a single large irregularly shaped vesicle. The excretory gland lies in close contact with the ventriculus, and the inner plasma membrane of the outer lamella is convoluted, bearing elongated lobed protuberances which extend into the cytoplasm of the gland cell. Plate 5.7b shows the centre of the excretory gland in the same specimen, taken at a lower power. The large, round excretory canal can be seen, approximately 7µm in diameter. The boundary of the canal is composed of a high electron dense substance similar to that seen in the smaller vessels. This too contains perforations where drainage vessels and associated vesicles empty into the excretory canal. Another large drainage vessel can be seen in the bottom left of the micrograph. Numerous large circular granules of varying sizes and electron densities, can be seen, however, these are not present immediately around the area of the canal. This area is occupied by the drainage vessels and associated empty vesicles, and small bundles of filaments arranged parallel to the walls of the excretory canal.

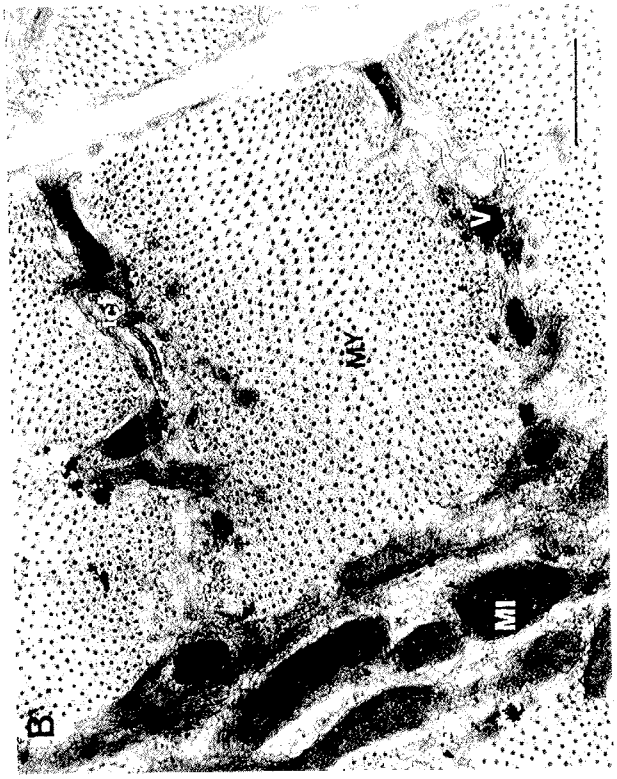
Plates 5.7c and 5.7d, from an encapsulated **A.simplex** larva, show the gland tissue and excretory canal at the same magnifications respectively as those from the "free" **A.simplex** larva. Although the excretory gland appears similar in appearance in both types of larvae, there is a general absence of high electron dense granules in the encapsulated larva, and many more granules appear to be empty, or partially empty and to have lysed. In addition, the granules appear to be slightly smaller in the encapsulated larva (observed range = approximately 150 - 700nm in diameter).

Plate 5.7e shows a closed excretory canal in a "free"

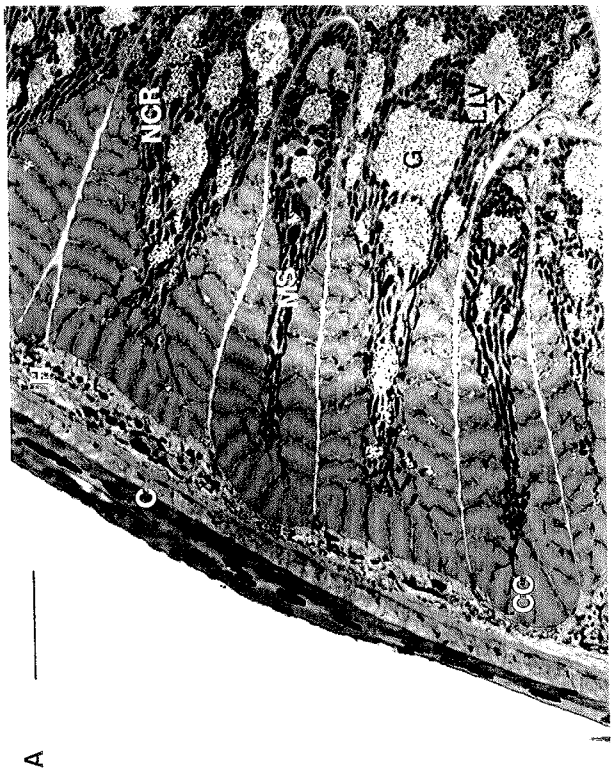
third stage larva of **A.simplex**. The edges of the canal have drawn together, and the perforated nature of the wall can be seen. The wall itself is lined by a convoluted membrane, under which the layer of high electron dense material can be seen to extend to the bases of the perforations.

### 5.3.5 Body Wall

Plate 5.8a shows a cross-section through the body wall of a third stage larva of **C.osculatum**. The cuticle consists of a number of homogeneous, with denser layers situated towards the outer surface, and a thin layer of high electron dense material immediately on the exterior. The entire cuticle is non-cellular and is approximately 4µm thick. The hypodermis is cellular, and is situated between the cuticle and the muscle cells. This layer is approximately 1.6µm thick, and contains numerous irregularly shaped vesicles of varying electron densities. The individual muscle cells of the body wall are closely packed, and although every second muscle cell appears to be isolated, this is likely to be a result of the plane in which the section was cut. The contractile components of these cells form a distinct U-shape around the outer edges of the base of the cells, towards the outer edge of the body. The contractile components are composed of individual approximately square-shaped myofibrils. These myofibrils are packed with myofilaments which run longitudinally down the body, appearing circular in cross-section. The non-contractile part of the muscle cell (sarcoplasmic region) extends into the cavity formed by the contractile components, and contains numerous mitochondria which are elongated in the area surrounded by the myofibrils. Areas of homogeneous granular material of varying sizes, but roughly circular, are present among the mitochondria, generally above the level of the contractile region, towards the centre of the body, and these are thought to contain glycogen. Several scattered circular droplets of lipid can





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**PLATES 5.8(a-c): Cross sections through the body wall**
(a) Third stage larva of C.osculatum (scale bar = 5µm)
(b) Body wall myofibril of a third stage larva of C.osculatum (scale bar = 500nm)
(c) "Free" third stage larva of A.simplex (scale bar = 2µm)

also be observed in the non-contractile region of the muscle cell. Plate 5.8b shows a myofibril from the contractile region at higher power. This particular myofibril is approximately 2µm by 1.5µm. Thick regularly spaced myofilaments can be seen throughout the entire block, and each of these is surrounded by thinner myofilaments, arranged in a circular pattern around them. The individual myofibrils are separated by a thin layer of dense tissue containing vesicles of varying sizes and electron densities, and intracytoplasmic filaments. These structures often extend into part of the muscle block itself.

Plate 5.8c shows a section through the body wall of a "free" third stage larvae of **A.simplex** . The composition of the muscle cells is similar to that described above, however, in this case the muscle cells appear to be in a greater state of contraction. The hypodermis, although showing slight processing damage, appears very dense and is approximately 0.8µm thick. The cuticle again appears slightly damaged, and the structure is not distinct, but several layers are indicated, although no difference in electron density is apparent between the outer and inner layers. The cuticle is approximately 3.9µm thick.

## 5.4 DISCUSSION

### 5.4.1 Early third stage larvae of Pseudoterranova decipiens

Examination of early third stage larvae of **P.decipiens** under TEM revealed the wrinkled cuticle of the second stage larvae loosely surrounding the cuticle of the third stage. Smith (1970) and Wharton (1986), also noted the loose second stage cuticle surrounding third stage larvae in TEM studies of **Haemonchus placei** and **Trichostrongylus colubriformis,** respectively. Berry and Cannon (1981) found early third stage larvae of **Sulcascaris sulcata** to be ensheathed in both the first and second stage cuticles, however, no evidence of the

first stage cuticle was observed around early third stage larvae of **P.decipiens** examined under TEM. McClelland (1982) found early larvae of **P.decipiens** to be enclosed in sheaths 1-2µm thick, in the present study, the cuticles of the second and third stage were approximately equal in size and were approximately 133nm in thickness. Such a difference in cuticular thickness between the results obtained in the present study, and those of McClelland (1982), may indicate that the larvae examined during the present study were not as well developed as the larvae examined by McClelland. This may also explain the absence of a mouth, excretory pore and sensory structures in early third stage **P.decipiens** larvae examined here, under SEM (Chapter 4), as compared to those examined by McClelland and Ronald (1974a). Third stage larvae of **C.multipapillatum** also retained the cuticle of the second stage, which was 9µm thick, around the underlying cuticle of the third stage which was well developed, and approximately 13µm thick (Huizinga 1967). In the ensheathed third stage larvae examined during this study, the structures of the two cuticles appeared similar, varying only in electron density.

The excretory gland in early larvae of **T.decipiens** (= **P.decipiens**) and **C.osculatum** has been noted by McClelland and Ronald (1974a,b) as the most prominent internal structure at this stage and is seen as granular under the light microscope. The structure seen in **P.decipiens** in this study, bound by the double membrane, containing vesicles and granular cells, and adjacent to the body wall may be the excretory gland.

Early third stage larvae of **P.decipiens** examined under light microscopy revealed the presence of a developing oesophagus and intestine, and granular material concentrated to one side of the mid-body. Newly exsheathed larvae of **T.decipiens** (= **P.decipiens**) and **C.osculatum** cultivated **in vitro** and examined under the light microscope were observed to have a rudimentary oesophagus, intestine and rectum (McClelland and Ronald 1974a,b), and McClelland (1982) found the rudimentary gut of early **P.decipiens** larvae to consist

of a distinct oesophagus and an indistinct intestine. Kelly and Crites (1993), examined first stage larvae of **Philometra** sp. under TEM, and observed a cuticle lined oesophagus with a lumen, which led into an area that was not lined with cuticle and had no apparent lumen, and which they stated probably represented the beginning of the intestine. Second stage larvae of **H.aduncum** from experimentally infected mysids also had little internal structure (Yoshinaga **et al.** 1987). Grabda (1976b) found the internal organs of early larvae of **A.simplex** to be poorly differentiated, with the oesophagus visible as a thin canal and the intestine poorly developed. Oesophageal differentiation in early larval stages of nematodes apparently occurs before that of the intestine, and the partially developed gut observed during this study was probably part of the intestine.

Berry and Cannon (1981) found little internal differentiation in second stage larvae of **Sulcascaris sulcata**, although gut development was observed at both ends of the worm. Organ development was observed in early third stage larvae, with the gut being clearly distinguished. From their diagrams, the majority of the body of the early larval stages of this species appeared to contain large rounded granules of varying sizes. Huizinga (1967) examined the digestive tract of hatched second stage larvae of **C.multipapillatum**, and found the oesophagus to be incompletely developed and the intestine filled with granular material. The intestine of early larvae of **Philometra ovata** was filled with numerous small granules, and the rectum surrounded by elongated rectal granules (Moravec 1980). In addition, Sprent (1959) found the body of second stage larvae of **Toxascaris leonina** to be filled with granules. Examination of early larvae of **P.decipiens** under TEM showed the gut to be surrounded by vesicular tissue, which may appear granular under light microscopy. This tissue was also observed scattered in patches throughout the body. Such tissue may well give rise to the gut itself, or act as an energy source for the development of body organs. The longitudinal section

through the posterior end of a **Philometra** sp. first stage larvae, illustrated by Kelly and Crites (1993), appears very similar to the longitudinal section illustrated at low power of the early third stage larvae of **P.decipiens**. Presumably, the wide variety of structures and materials observed within the body of the early third stage larvae of **P.decipiens** are the raw materials required for both growth of the larva and associated development of the various body organs and structures.

Rosenthal (1967) observed larvae of **Contracaecum** sp. to increase in size within the body cavity of herring larvae which had been fed with wild plankton. However, third stage larvae of the species examined during this study are not thought to feed, and therefore, activity and increases in growth may be due to utilization of energy stores within the body of the larvae. Indeed, the large difference in size between early third stage larvae of **P.decipiens** and those third stage larvae recovered from fish indicates that a large amount of growth occurs within this individual stage alone. The early third stage larvae were also very active within the culture dish. Under natural conditions, a large amount of larval activity is likely to occur, both at the free living stage and in the invertebrate and fish hosts. The larvae must therefore harbour energy reserves in order for such activity to occur, and such stores might have to last over a potentially long non-feeding stage in fish. Crites (1980, in Kelly and Crites 1993) stated that philometrid embryos increase in size as they develop and appear to store lipids and glycogen. First stage larvae of **Philometra** sp. examined under TEM were found to have a partially developed digestive system, filled with a yolklike material and storage cells (Kelly and Crites 1993), and these authors stated that larvae may rely on such material as an energy source.

Few TEM studies of the morphology of early larval stages of nematodes have been carried out, however, several light microscopy studies have documented the development of marine

anisakids. As newly hatched larvae grow, development and differentiation of internal structures occurs. The **in vitro** growth and development of the early stages of **T.decipiens** (= **P.decipiens**) and **C.osculatum** was described by Davey (1969) and McClelland and Ronald (1970,1974a,b). McClelland (1982) observed that as larvae of **P.decipiens** experimentally infected into copepods grew, the intestine and excretory system became more distinct.

Davey (1969) studied the early development of **C.osculatum**, and found temperature to influence development with the gut and oesophagus present after approximately 10 days at 16°C but 2-3 months at 8°C (the temperature at which the early larvae of **P.decipiens** were cultivated during this study).

It is therefore possible that the early third stage larvae here were fixed at the beginning of development and differentiation of internal organs. It would be of value to examine the ultrastructure of these larvae at different time periods post-hatching to discern any changes in the internal structure of this stage over time, and also to examine the ultrastructure of third stage larvae from invertebrates, for comparative purposes.

### 5.4.2 Cephalic Sense Organs

Bird (1971) stated that nematode papillae may only contain one to three cilia whereas in amphids, cilia are more numerous, and there may be as many as 23. In the specimens examined here, the amphids of fourth stage **H.aduncum** and third stage **A.simplex** contained many cilia (10-12 in the case of **H.aduncum**), whereas the single papilla of an adult **P.decipiens** apparently contained a single cilium. In the amphids of **H.aduncum** and **A.simplex**, the microtubules were aligned longitudinally, and were regularly spaced and single, at least in **H.aduncum**, although there was evidence that some were double. In the cephalic papillae of **P.decipiens**, the

microtubules were orientated randomly and spaced irregularly, and examination of the microtubules towards the top of the cilium revealed that they occurred in groups of 2 - 4.

The cuticle lining surrounding the modified cilia may have a protective and\or supportive function (Wright 1980).

### 5.4.2.1 Ultrastructure of Amphids

The modified cilia within nematode amphids lie within a canal. This canal was found to be approximately 1.4µm in diameter in fourth stage larvae of **H.aduncum**, and 1.5µm in diameter in third stage larvae of **A.simplex**. The maximum diameter of the amphidial canal in **Dipetalonema viteae** was approximately 1.7µm (McLaren 1972a). The amphidial canal opens to the amphidial pore (McLaren 1976a), and the dendritic processes end just below the amphidial pore, although this pore was not apparent in the longitudinal section through the amphid of **A.simplex**. McLaren (1972a) found the cilia of **Dipetalonema viteae** to terminate at different levels, so that only three to five of the nine present reached the orifice. This may also be the case in **A.simplex**, where different levels of cilia were observed, although this was likely to be due to the slightly oblique plane in which the section was cut.

Ten to twelve cilia were observed in the amphid of fourth stage **H.aduncum**. The numbers of cilia associated with the amphids varies between species. Wright (1980) stated that amphids in secernentian nematodes (which includes the marine ascaridoids) have 6 - 14 cilia, and adenophoreans (mainly found in marine sediments) 10 - 36. Numerous regularly spaced, longitudinally arranged microtubules were observed in the amphidial cilia of **H.aduncum** and **A.simplex**. In **H.aduncum**, these microtubules generally appeared single, although a few did appear to be double structures. Variations in the form of the microtubules along the length of the same cilia have previously been reported (Wright 1980). Wright

also stated that tight junctional complexes occurred between the dendrites and the sheath cells; the extracellular space enclosed by sheath cell is then sealed from the extracellular spaces of the rest of the body, and is designated the receptor cavity. The cross-section taken through the amphid in fourth stage **H.aduncum** was thus taken at the level of the sheath cell, however, the manifestation of the dendritic processes as modified cilia suggests that the section was taken at the anterior region of the sheath cell, as more posterior sections would show the processes as nerve axons. McLaren (1972b) found the microtubules in the amphidial cilia of five species of microfilaria to be double at the base of cilium but single in the shaft. This may also be the case for **H.aduncum**, if this section was taken from the anterior end of the sheath cell, immediately above the base of the cilia, thus revealing single microtubules with some doublets apparently visible.

The cilia in **Dipetalonema viteae** tapered anteriorly, and varied in diameter along their length from approximately 0.1 - 0.38µm. The maximum observed diameters of cilia in **H.aduncum** and **A.simplex** were the same - 0.5µm. In **Dipetalonema viteae**, the cilia were surrounded by dense granular material, and the cytoplasm between the microtubules had a filamentous appearance (McLaren 1972a). Such material was observed around the cilia in **H.aduncum**, and granular material of a looser nature was also observed around the microtubules within the cilia in both **H.aduncum** and **A.simplex**. A supporting cell surrounded the anterior region of the amphidial canal in third stage **A.simplex**, and extended to the cuticle. This cell was seen to contain bundles of longitudinally arranged fibres. McLaren (1972a) also observed a similar cell around the anterior region of the amphidial canal and extending to the cuticle in **Dipetalonema viteae**, also with bundles of longitudinally arranged fibres. Fibre bundles are apparently typically contained in supporting cells, and are known to assist in laying down new cuticle around the amphidial canal at moulting.

The basal region of the cilia leading to the nerve axon in **A.simplex** was surrounded by a sheath cell, containing a few bundles of fibres and with a slightly denser and granular cytoplasm than the supporting cell. McLaren (1972a) also observed a similar cell around the basal regions of the cilia and associated nerve axons in the amphid of **Dipetalonema viteae**, and termed it the amphidial gland, as it was found to be multivesicular and contain a variety of other structures as well as small clusters of dense fibres. This cell has been reported from a wide variety of nematode species and the internal morphology suggests a secretory capacity (McLaren 1976b). The sheath cell in **A.simplex** was not particularly glandular in appearance, however, increases in the secretory activity of this cell is known to occur in other nematodes after physiological activity such as the moult to the fourth stage and migration to the intestine in **Necator americanus** (McLaren 1976a) which may reflect an increased requirement for amphidial gland secretions in the intestinal environment, or attachment to the host tissues and the onset of feeding (**Syngamus trachea** Jones 1975, in McLaren 1976b). An increase in secretory cell activity may be also occur in **A.simplex**, after the moult to the fourth stage in the digestive tract of the final mammalian host, where both feeding and attachment to the gut wall will occur.

As observed in **A.simplex**, the amphidial nerve axons of **Dipetalonema viteae** contained spherical bodies and small microtubules, in addition to other structures (McLaren 1972a). Wright (1980) stated that vesicles of various sizes and densities described in dendrite tips are of unknown function, but their presence may be related to the state of firing of receptors.

The internal structure of the amphid in the third stage larva of **A.simplex** appeared well-developed, although it is not known if this organ develops further in later life stages. However, the amphid in this species may well be functional at the third stage, and may be used to monitor the worms external environment within the intermediate hosts, or

to detect aggregation attractants (see Chapter 3). McLaren (1976a) stated there were few structural differences between the amphids of microfilaria, larval or adult filarial worms.

### 5.4.2.2 Ultrastructure of Papillae

The single cephalic papilla in **P.decipiens** apparently contains a single large cilium within a cuticle-lined canal. Groups of irregularly spaced and orientated microtubules were observed in the single papilla of adult **P.decipiens**. Those in groups of four formed "rosette"-like structures. McLaren (1972a) observed rosettes of microtubules in the basal regions of the amphidial cilia in adult **Litmosoides carinii**. The cephalic papillae of adult **Dipetalonema viteae** also consist of a single large cilium within a cuticle-lined canal, which contains large, randomly distributed microtubules, these usually occurring as single structures (McLaren 1972a). The cilium in **Dipetalonema viteae** was found to lie within a mushroom-shaped canal, which spread into the surface cuticle to form a flattened circular cavity, appearing lenticular in section; the terminal region of the cilium extended to fill this cavity, and was thus in the form of a flattened disc, with the microtubules arranged around the periphery. The supporting cell surrounded the stalk part of the canal and did not extend around the terminal region of the cilium. The cilium of the single papilla in **P.decipiens** may be morphologically similar to that of **Dipetalonema viteae**, as the description by McLaren would agree with the assumption that the sections from **P.decipiens** were obtained from the sides of the structure. The sections of the cilium seen in **P.decipiens** would therefore represent the side of the flattened end of the cilium. This is supported by the presence of microtubules -which McLaren observed at the edges of the cilium, and the apparent absence of the supporting cell - which according to McLaren surrounded the stalk part of the cilium. However, unlike the

cilium in **Dipetalonema viteae**, the terminal end of the cilium in **P.decipiens** does not extend into the surface cuticle, but lies immediately underneath it.

No sections were obtained through the double papillae of any of the marine ascaridoids examined.

### 5.4.2.3 Function of Cephalic Sense Organs

The ultrastructure of the amphids, and the direct exposure of the cilia to stimuli from the external environment via the amphidial pore, suggests that they are chemoreceptors; in cephalic papillae the cilia are modified to produce an area of greater sensitivity and they are not exposed to the external environment, suggesting that they probably function as mechanoreceptors (McLaren 1972a, 1976a,b). In view of the morphology of the amphid observed in fourth stage larvae of **H.aduncum** and third stage **A.simplex**, (and its' association with a cuticular pore), it is suggested that the amphid in these two species of marine ascaridoids does indeed function as a chemoreceptor. The amphids in **P.decipiens** and **C.osculatum** are also likely to have a similar amphidial structure and, therefore, function. McLaren (1972a) regarded the cephalic papillae in **Dipetalonema viteae** to be mechanoreceptors, with the complex arrangement of microtubules (also observed in **P.decipiens**) functioning to increase the area of sensitivity of the papilla. Although only sections from the side of the single papilla in **P.decipiens** were obtained, this organ appears to be a mechanoreceptor, and again, this may be true for the single papillae in other marine ascaridoids. The fine structure of the double papillae in marine ascaridoids remains to be described, and until such studies are carried out the possible function\s of these organs cannot be elucidated. It may however be of relevance to mention the findings of Goldschmidt (1903, in Bird 1971), who found that sections through the double papillae in **Ascaris** revealed one

papillar nerve to terminate as a fibre which projected through a canal in the cuticle, and the other nerve to terminate as a bulb under the cuticle. Such morphology suggests that the double papillae in this species is a combined chemo- and mechanoreceptor.

The sense organs may respond to a variety of environmental stimuli, and Bird (1971) stated that it has been suggested by various workers that amphids respond to a number of chemicals, including gases such as carbon dioxide, as well as to physical stimuli such as electric currents and heat. It seems highly probable that the amphids of nematodes also function to detect any aggregation, and - at the adult stage - sexual, attractants produced by other worms of the same species. Other biological activities such as feeding, orientation, hatching and moulting may also result from stimulation of the sense organs. Physical pressure on the single papillae will trigger the mechanoreceptor.

McLaren (1976b) stated that the close relationship between some nematode amphids with what appears to be a secretory cell (ie. gland cell = sheath cell), may suggest that these organs do not function solely as receptors of external stimuli but also monitor and control the secretory activity of this cell. McLaren described the morphological features of the sense organ\secretory cell, presented experimental evidence for active secretion by nematodes and speculated on the mechanics of secretion.

McLaren (1976a) considered the functions of nematode sense organs in detail, and also stated that it should be considered that stimuli detected by nematodes may be transmitted directly to the nerve ring.

### 5.4.3 Digestive System

The digestive tracts of third stage larvae from fish were generally similar in terms of ultrastructure, and, in addition, resemble that of adult **P.decipiens**. Myers (1960)

also found that the structure of the digestive tract of larval stages of Anisakinae in fish, and invertebrates, resemble the adult stages.

In the case of **P.decipiens**, at least, the digestive system must develop from the mass of cells and materials observed in the early third stage larvae. This development must occur within the third stage itself, as the organs of the digestive tract are differentiated in third stage larvae recovered from fish.

A change in the form of the intestine does occur at later stages eg. Ishii **et al.** (1989) had stated that the intestinal lumen of **A.simplex** was tri-radiate at the third stage, and multi-radiate at the fourth stage; Grainger (1959) observed a similar change in fourth stage larvae of **Porrocaecum**, but found no changes in the rest of the gut.

### 5.4.3.1 Preventriculus

In the marine ascaridoids examined during the present study, the triangular, or triradiate mouth (see Chapter 4) opens into a tri-radiate preventricular lumen, which is lined by cuticle. The preventriculus is muscular, and the muscles are used for pumping (Lee 1965), which aids in feeding, as described in **T.decipiens** (=**P.decipiens**) by Townsley **et al.** 1963). The radial muscles in the preventriculus aid in dilating the oesophageal lumen (Lee 1965).

Soleim and Berland (1981) found that the oesophageal portions of the digestive tract in **Thynnascaris aduncа** (= **H.aduncum)** did not differ significantly from those of other nematodes. In all species examined during this study, the preventriculus - appears to be fully developed in third stage larvae from fish and appears similar both between species at the third stage, and to the adult specimen of **P.decipiens** examined. However, the preventriculus in third stage larvae contained apparently degenerated structures, including mitochondria; whereas the preventriculus in adult **P.decipiens**

had the appearance of an active tissue, with well developed vesicles and mitochondria. It seems probable that differences observed between the preventriculus of adults and third stage larvae, are related to the active feeding of the adult worm, whereas the third stage larvae are not thought to feed, and the preventriculus may thus be non-functional at this stage. This, and the general similarity of the preventriculus in all third stage larvae, would also account for the similarity in structure of "free" and encapsulated **A.simplex**. The lumen in the preventriculus of the adult **P.decipiens** examined was full of granular material, which may suggest that this particular worm had been actively feeding prior to fixation.

#### 5.4.3.2 Ventriculus

The ventriculus is glandular, and in **A.simplex** was found to be composed of large, circular membrane-bound vesicles of varying sizes, but of the same electron density and containing a solid homogeneous material. Grabda (1976b), using light microscopy, described the ventriculus of third stage **A.simplex** as having a wall of large glandular cells filled with a granular substance. Fukuda **et al.** (1990) found the cuticular lumen of the ventriculus in larval **A.simplex** to be approximately 80nm thick, this compares to 100nm found during this study. Fukuda **et al.** (1990) also found the ventriculus of larval **A.simplex** to be composed of columnar cells, partially occupied by dense granules. The ventriculus of larval **A.simplex** examined during this study was not found to contain columnar cells, although complexes of membranes were observed in the wall of the structure, and the ventriculus was also filled by granules.

After 10 hours incubation in artificial gastric juice, no changes were noted by Fukuda **et al.** (1990) in the granules of the ventriculus of larval **A.simplex**, and they concluded that these granules did not appear to be involved in larval penetration into humans. The function of the ventriculus is

unclear, however, it is presumably involved in the digestion process, and the vesicles thus probably contain enzymes for secretion.

A large nucleus was present in the oesophageal gland of third stage **C.osculatum**, at the level of the ventriculus. This gland was similar in morphology to the section through the oesophageal gland in the preventriculus of adult **P.decipiens**. This, and the appearance of the tissue, suggests that at least in **C.osculatum**, although the preventriculus in third stage larvae does not appear to be functional, the oesophageal glands apparently are. Matthews (1984) reported proteolytic enzyme activity within the oesophageal glands of third stage larvae of **A.simplex** from fish.

Hsü (1933) stated that ascarids with a ventriculus digest extra-corporeally by secretions from the dorsal oesophageal gland and added that there is a relationship between the development of the oesophageal glands in Ascaroidea and the method of feeding. Of the nematodes examined by Hsü, **Anisakis** showed well developed oesophageal glands, and digestion was extra-corporeal in this species, as also in **C.osculatum** and **P.decipiens**. Other species of ascaridoids which ingested contents of the host gut exhibited less well developed glands. The glands are thought to contain enzymes and secretions from this gland would liquify stomach wall tissue and allow the nematodes to feed on it (Hsü 1933). Hoeppli (1933, in Riley 1972) stated that tissue around the buried anterior ends of **Anisakis** sp. in the stomach of a walrus had been liquified by secretions from the oesophageal glands of the nematodes. Soleim and Berland (1981) suggested that **Thynnascaris adunca** (= **H.aduncum**) may possess enzymes - possibly produced from the dorsal gland - which carry out some extracorporeal digestion, as worms were often found boring into undigested fish in the stomach of hosts. In third stage **C.osculatum**, the apparently functional oesophageal glands may secrete substances which are the same as those used for digestion in the adult, but in the third stage larvae aid in the penetration of the larvae through tissues

of the fish host, as suggested by Matthews (1984) for **A.simplex**. Matthews (1984) considered it probable that oesophageal gland secretions will also ease the passage of **Anisakis** larvae into mammalian host tissues even if their primary function is to prepare those tissues for ingestion. This is also likely to be true for other marine anisakids, such as **P.decipiens**.

The vesicles observed in the oesophageal glands of both third stage **C.osculatum** and adult **P.decipiens** are therefore likely to contain enzymes.

#### 5.4.3.3 Ventricular Appendix

The morphology of the ventricular appendix of third stage larvae of **C.osculatum** was similar to that of the ventriculus in this species, and its general appearance suggests that the tissue is glandular in marine ascaridoids. McClelland and Ronald (1974b) also found the ventriculus and ventricular appendix to be homogeneous in fourth stage larvae and adults of **C.osculatum**, although they did state that the ventricular appendix changed in appearance after the moult from the third to the fourth stage. Jägerskiöld (1894, in Hsü 1933) stated that the oesophageal appendix in certain species of nematodes is filled with a portion of the oesophageal glands.

The ventricular appendix of **Thynnascaris adunca** (= **H.aduncum**) was filled with extensions of the oesophageal glands (Soleim and Berland 1981). These authors observed the ventricular appendix as an apparently double structure, the two parts being separated by a thin incomplete septum. The ventricular appendix in third stage **C.osculatum** did not appear to be a double structure but was composed of patchy areas of vesicles of either low or high electron density, those of low electron density being apparently empty.

Huizinga (1967) stated that the ventricular appendix of **C.multipapillatum** serves as pumping organ to aid in ingestion

of blood - pumping fluid from the oesophagus to the intestine. A similar function may also be true for **C.osculatum** (and **H.aduncum**). However, no muscle tissue, which would be indicative of a pumping function, appears to be associated with the appendix in third stage **C.osculatum**, although it is not known if muscle develops in this organ in later stages, when the nematode begins to feed. The morphology of the tissue in third stage larvae is indicative of a glandular function, and any secretions may aid in digestion. It may be relevant to mention that Miyazaki **et al.** (1988) found the oesophageal caecum (presumably = ventricular appendix) in **H.dollfusi** to contain albuminous granules, with the same histochemical characteristics as those of the oesophageal gland cells.

#### 5.4.3.4 Intestinal Caecum

Myers (1960) observed the intestinal caecum in **P.decipiens** to have no distinct cell boundaries, however, Ishii **et al.** (1989) stated that the intestinal caecum of **P.decipiens** was composed of approximately 50 single columnar cells. Miyazaki **et al.** (1988) found that the intestinal caecum, as well as the intestine, in **H.dollfusi** was lined by columnar epithelial cells and a brush border. Examination of the intestinal caecum of an adult **P.decipiens** during this study agreed with the findings of Myers (1960), who also described the lumen to be irregular. The caecum did, however, bear a resemblance to intestinal cells, containing granular material and scattered vesicles. Myers (1960) stated that the function of the caecum was unknown, and the findings of the present study are also inconclusive with respect to this organ. It is possible that the caecum aids in digestion, providing an increased absorptive surface.

### 5.4.3.5 Intestine

The intestinal cells of the third stage larvae were similar in appearance both to each other and to those of the adult **P.decipiens** examined. The similarity in morphology between the larval and adult stages, suggests that the intestine is fully developed in third stage larvae from fish. However, although microvilli were present in all species examined, only in adult **P.decipiens** was an accumulation of mitochondria observed, in the anterior region of the intestinal cells, below the microvillar layer. This suggests that the cells were highly active, and the morphology of the apical region of the intestinal cells also indicates that microvilli are being actively produced. In non-feeding third stage larvae, mitochondria may be absent from the intestinal cells as the latter are not active. However, Fukuda **et al.** (1990) noted many mitochondria, mainly at the periphery of the intestinal cell, in larval **A.simplex**. The mitochondria in the intestinal cells of adult **P.decipiens** may have a dual function - to provide energy for the active uptake and absorption of food, and also for the production of microvilli, which may be abraded and which therefore have to be replaced. The position of these mitochondria - in the region of the cell immediately lining the lumen - supports this suggestion. In addition, Grabda (1976b) stated that the intestine in **A.simplex** becomes active at the fourth stage. Jenkins and Erasmus (1969) noted that the cytoplasm of the intestinal cells of **Metastrongylus** sp. was rich in mitochondria.

As was distinct in adult **P.decipiens**, the apical plasma membrane of **Metastrongylus** sp. was elevated to form numerous microvilli (Jenkins and Erasmus 1969). Microvilli, and the associated increase in surface area, perform the basic function of absorption. The layer of dense material producing the microvilli, observed in all specimens in this study, in the apical region of the cells, has been noted by other authors, and termed the plasma cap eg. in **H.aduncum** (Soleim

and Berland 1981), **Ascaris lumbricoides** (Lee 1962), or submicrovillar layer eg. in **Metastrongylus** sp. (Jenkins and Erasmus 1969). Jenkins and Erasmus found this layer to be approximately 0.1µm in thickness. In the specimens examined during this study, this layer varied in thickness from approximately 0.7µm in third stage **C.osculatum**, to 2µm in adult **P.decipiens**.

Terminal bars were observed at the apical junction of the lateral plasma membranes in the intestinal cells of the nematodes examined during this study. Jenkins and Erasmus (1969) also observed such structures in **Metastrongylus** sp., and stated that the intercellular space in this region was occupied by a substance believed to have cementing properties (Porter and Bonneville 1964, in Jenkins and Erasmus 1969).

Numerous spherical vesicles of varying sizes and electron densities were found within the intestinal cells of all nematode species examined during this study. Mueller (1927) noted numerous granules in the intestine of **A.simplex**, similar in size and shape to those in the excretory gland, but they stained differently. Fukuda **et al.** (1990) observed electron dense granules in the intestine of larval **A.simplex**, with a maximum diameter of 2.4µm. The largest electron dense granules observed in this species during the present study was approximately 1.9µm in diameter. Fukuda **et al.** observed no changes in these dense granules after 10 hours incubation in artificial gastric juice, although glycogen particles in the cells did appear to be utilised. Fukuda **et al.** concluded that the dense granules did not appear to be involved in the act of larval penetration into human tissue. Soleim and Berland (1981) described spherical cell inclusions in the intestinal cells of **Thynnascaris adunca** (= **H.aduncum**), and Miyazaki **et al.** (1988) reported that the intestinal cells of larval **H.dollfusi** contained, among other substances, glycogen. Jenkins and Erasmus (1969), under TEM, observed small scattered glycogen granules, and large electron dense secretory granules in the intestinal cells of **Metastrongylus**

sp.. The secretory granules were spherical, membrane bound, variable in appearance and distributed throughout the cell, but, unlike those observed during this study, were particularly numerous just below the submicrovillar area at the apex of the cell. Many of the membrane-bound vesicles observed during this study were also electron dense. Lee (1962) found small granules of esterase concentrated in the basal two thirds of the intestinal cells throughout the intestine in **Ascaris lumbricoides**, and large globules of esterase were also present in many cells, which apparently moved through the cell and discharged the enzyme into the lumen of the intestine. The gut contents were also strongly positive for esterase. Jenkins and Erasmus (1969) had also observed secretory granules in the intestinal cells of **Metastrongylus** sp. to discharge their material into the gut lumen, via the microvilli. Similar to the observation by Lee (1962), vesicles were more numerous towards the basal regions of the intestinal cells of the nematodes examined during this study, and were also observed both in the anterior and posterior regions of the intestine. Lee (1965) stated that the intestinal cells appear to be both secretory and absorptive in function, but added that the intestine of nematodes in which digestion was carried out extracorporeally (as the specimens examined during this study are believed to do), the intestine was wholly absorptive in function. If the vesicles present in the intestine do not contain enzymes utilised for the process of digestion, they may contain stored nutrients for activity or growth eg. Chitwood and Chitwood (1950) stated that the intestines of several nematodes contain globules of protein material, and these may be used to form structural proteins as the nematodes increase in size. A few of the vesicles observed during the present study also appeared to contain lipids. Jenkins and Erasmus (1969) also observed large isolated lipid droplets in the intestinal cells of **Metastrongylus** sp. and Lee (1962) reported fat globules in the intestinal cells of **Ascaris lumbricoides**, and demonstrated (1960) that fat globules were

utilised by nematodes during starvation.

The outer basement membrane surrounding the intestine is thick, being approximately 1.1µm in third stage **A.simplex** and 2.5µm in adult **P.decipiens**. The intestine of **Thynnascaris adunca** (= **H.aduncum**) is also surrounded by a thick basement membrane (Soleim and Berland 1981). The basement layer in **Metastrongylus** sp. consisted of an outer homogeneous fibrous zone approx 0.11µm thick, and an inner less electron dense region (Jenkins and Erasmus 1969). In adult **P.decipiens** and third stage **A.simplex**, the basement membrane was observed to be homogeneous.

### 5.4.3.6 Feeding and Energy Stores

Third stage larvae of marine ascaridoids apparently do not feed within the fish host eg **A.simplex** (Grabda 1976b, Smith 1983b), or **in vitro** eg. **Anisakis** sp. (Sommerville and Buzzell 1974). Third stage larvae of marine ascaridoids may pass through several fish hosts before the final mammalian host is reached, and thus the non-feeding stage of these larvae may potentially be long. Although metabolism may be low within encapsulated larvae, third stage larvae will require energy for both growth, and activity when migrating through the stomach wall and viscera of the fish hosts. Sommerville and Buzzell (1974) showed that **in vitro** development of **Anisakis** larvae occurred without the presence of conventional nutrients. It is therefore to be expected that these larvae will harbour internal food reserves and energy stores.

In third stage larvae, vesicles were observed in the intestinal cells; some of these contained lipids, and others may consist of carbohydrates such as glycogen or glucose. Stores of glycogen and lipids were also observed in the non-contractile region of the somatic muscle cells in third stage **C.osculatum**. Fukuda **et al.** (1990) found that after 10 hours' incubation in artificial gastric juice, glycogen particles

in the intestinal cells of larval **A.simplex** became hollow, and they suggested that these particles may be a stored energy source consumed when the larvae move actively in the artificial gastric juice. Glycogen is the chief food reserve in nematodes but considerable amounts of fat are also stored, mostly located in the hypodermis and the non-contractile part of the muscle cells, but also in the intestine (Lee 1965), as observed during this study. Smith (1970) considered that the large amounts of lipids present in the intestinal cells of **Haemonchus placei** could possibly act as energy reserves for the third stage larvae and Von Brand (1952, in Lee 1965) considered that nematodes used fats as a source of energy for movement. Fairbairn (1958) recorded carbohydrates as constituting more than 60% of the dry weight of **P.decipiens** larvae in fish. Glycogen content was 55% of dry weight, with lipid, trehalose and glucose accounting for 3.7%, 6% and 0.6% of dry weight respectively. Fairbairn suggested that the large carbohydrate accumulations occurred during the early larval stages and were used to maintain larval metabolism during the potentially long non-feeding stage in fish or until the final host is reached. Fairbairn supported this with the observation that **P.decipiens** larvae survive for several weeks **in vitro** in 30% seawater. In addition to **P.decipiens**, third stage larvae of **A.simplex** and **C.osculatum** also survive in seawater, or physiological saline for several weeks (personal observation), and in the case of **A.simplex**, often for several months. In relation to this, it would be of interest to compare the food stores of third stage **A.simplex**, **C.osculatum** and **P.decipiens** larvae, with those of third stage **H.aduncum**, which does not appear to survive for long after removal from the fish host. **H.aduncum** matures in the intestines of fish, and if a fish infected with third stage larvae of this species is ingested by a larger fish, then the parasites develop to the fourth stage. The potential non-feeding stage may thus be greatly reduced in this species and therefore third stage larvae of **H.aduncum** may harbour fewer energy reserves than **A.simplex**, **P.decipiens** and

**C.osculatum.**

### 5.4.4 Excretory System

#### 5.4.4.1 Structure of Excretory System

The excretory gland of third stage **A.simplex** contained numerous drainage vessels throughout the cytoplasm and surrounding the excretory canal. Lee **et al.** (1973) and Fukuda **et al.** (1990) also observed drainage tubules branched around the excretory canal in larval **Anisakis** sp. which extended into the cytoplasm. The vessels surrounding the excretory canal empty into the canal itself.

Smaller empty vesicles opened into the drainage vessels of the excretory gland in **A.simplex**, and Mueller **et al.** (1927) and Lee **et al.** (1973) also noted these in larval **Anisakis** sp.. Mueller (1927) regarded them as secretory passages of the cell.

The excretory canal and drainage tubules of third stage **A.simplex** were lined with a highly electron dense material, which was interrupted where drainage tubules (in the case of the excretory canal), and small empty vesicles (in the case of the drainage tubules) emptied into them. Lee **et al.** (1973) observed an interrupted layer of dense material lining the main canal and drainage tubules in larval **Anisakis** sp.. and suggested that as the vesicles in the gland open into the lumen of the canal and drainage tubules through areas not lined by this material, the latter is likely to be a cytoskeletal element maintaining the shape of the canal and tubules.

The excretory gland in third stage **A.simplex** larvae was filled with numerous membrane bound spherical secretory granules of varying sizes and electron densities. Mueller (1927), under light microscopy, and Lee **et al.** (1973) and Fukuda **et al.** (1990), under TEM, had also reported these in **Anisakis** sp. larvae, with Lee **et al.** (1973) describing the

contents as granular. Mueller (1927) found these granules to measure 500-800nm in diameter, Lee **et al.** (1973) approximately 550-1000nm in diameter. In this study, the granules in third stage **A.simplex** were from approximately 150-900nm in diameter. Lee **et al.** (1973) stated that it was not established whether the secretory granules in larval **Anisakis** sp. represented more than one population of granules with different properties, or if they were various stages in the formation of the same type of granule. Mueller **et al.** (1927) considered that variations in granule size indicated that they may be deposited and enlarged. Lee **et al.** (1973) suggested that the granules in larval **Anisakis** sp. arose from granular endoplasmic reticulum and Golgi complexes within the excretory cell. Although many of the secretory granules are adjacent to drainage vessels, there is no morphological evidence to suggest their contents are secreted into these vessels. Lee **et al.** (1973) stated that the appearance of the secretory granules suggested that they contained protein-rich substances, possibly histolytic enzymes. Ruitenberg and Loendersloot (1971a,b) carried out enzyme histochemical tests on **Anisakis** sp. larvae and found that the excretory organ showed slight to strong activity by enzymes, particularly non-specific esterase. The secretory granules in adult **Nippostrongylus brasiliensis** contained nonspecific esterase, cholinesterase and aminopeptidase (Lee 1970).

The excretory canals, drainage vessels and vesicles observed during this study were apparently empty, however, Lee **et al.** (1973) noted moderately dense material in the lumen of the canal and drainage vessels in the **Anisakis** larvae which they examined, and presumed that substances for excretion or the contents of the secretory granules were discharged into the canal and drainage tubules through the small vesicles. Romanowski **et al.** (1971) postulated that the secretory granules in **Stephanurus dentatus** ruptured or lysed at the membrane surface and released their contents, with the material moving into the excretory duct and out of the excretory pore. The apparent lysing of the cell membrane in

some of the granules observed during this study in third stage **A.simplex** also suggests that the contents of these granules are released into the cytoplasm, where they may then enter the small vesicles, and pass to the drainage tubules, and ultimately to the excretory canal for release via the excretory pore. A similar route of secretion was suggested by Lee (1970) for the contents of the secretory granules in **Nippostrongylus brasiliensis**.

The small bundles of fine filaments arranged around the excretory canal in the third stage **A.simplex** examined were reported previously by Lee **et al.** (1973), and they suggested that the fibres may have a cytoskeletal or a contractile function. These fibres may thus reinforce the canal and\or are involved in the opening and closing the canal. Lee **et al.** (1973) stated that the excretory canal in **Anisakis** larvae varied in shape from round to a partly closed Y-shape. In the specimens of third stage **A.simplex** examined during this study, the excretory canal was observed in both open (round) and completely closed positions. The closed position of the excretory canal may be a result of fixation, however the open and closed positions may also suggest that pulsation of the canal may be used as a method of emptying the contents. Weinstein (1960) in Lee 1965) described pulsations in parts of the excretory system of several nematodes, and noted that in **Spironoura** the excretory canal widens slowly then contracts to such an extent that the lumen is obliterated. When the tube contracts, fluids move forward and are carried along the length of the tube by a peristaltic wave.

Invaginations of the cell membrane surrounding the excretory gland were observed in third stage **A.simplex**, and were also noted by Lee **et al.** (1973) and Fukuda **et al.** (1990). Tubular invaginations of plasma membranes are generally a surface specialization of cells involved in ion transport and absorption, and may serve to increase the absorptive surface area (Lee et al 1973).

**P.decipiens** and **C.osculatum** are closely related to **A.simplex**, and their excretory systems are the same

anatomically. This, and the apparent similarity between the excretory glands of a wide variety of nematode species, indicates that their fine structure is not likely to differ from that of **A.simplex**. This is confirmed by light microscopical examinations of the excretory gland of these nematodes, such as Mueller (1927), who observed secretory granules similar to those in **A.simplex**, in the excretory gland of **Contracaecum** sp.; Myers (1960), who reported a layer of dark staining material lining the excretory canal in **P.decipiens**; and Davey and Kan (1968) reported the excretory gland of **P.decipiens** to contain vacuoles which were connected by a system of fine tubules and canals leading into the canal. The vacuoles they observed are no doubt the vesicles surrounding the drainage vessels.

#### 5.4.4.2 Function of Excretory System

Lee **et al.** (1973) suggested that the excretory gland of **Anisakis** is involved not only in excretion, but also in secretion of enzymes, present within the granules, which are released through the excretory pore. Ruitenberg and Loendersloot (1971) proposed that the system was secretory, since the lumen of the excretory organ of **Anisakis** sp. larvae was sometimes filled with material having significant activities of phosphatases, oxidative enzymes and esterases. Lee **et al.** (1973) further stated that the excretory gland of **Anisakis** sp., as that of **Stephanurus dentatus** (Romanowski **et al.** 1971) is comprised of many of the structural components seen in secretory cells specialised in the production and temporary intracellular storage of a protein or glycoprotein rich secretion, and Romanowski **et al.** found that electron dense granules in the excretory gland cells of adult **Stephanurus dentatus** were similar to the secretory granules of exocrine and endocrine glands in a variety of mammals, and they therefore concluded that the excretory gland cells had a secretory function.

Ruitenberg and Loendersloot (1971) and Lee **et al.** (1973) suggested a histolytic or digestive role for the secretions of the excretory gland, although no experimental evidence was presented to support this. Lee (1970) considered the enzymes within the secretory granules of **Nippostrongylus brasiliensis** to be histolytic, and play an important role in feeding. Lee suggested that enzymes attacked the mucosa of the host and prepared cells for ingestion in a similar manner to the oesophageal gland secretions, and as the excretory gland is larger it was likely to play a more important role in extra-corporeal feeding activity than the oesophageal glands (Lee 1969, in Lee 1970). As feeding in **A.simplex** is also thought to be extra-corporeal, the enzymes may play a similar role. However, third stage larvae are not thought to feed, and the presence of an apparently active excretory gland at this stage suggests a different function for the secretions. Bird (1971) stated that the function of the excretory system in many parasitic forms of nematodes appears different in the larval and adult stages. Sommerville and Buzzell (1974) noted that material was discharged from the excretory pore of third stage **Anisakis in vitro**, and considered that, if the parasite was not feeding at this time, it was unlikely that these secretions contained enzymes for extra-corporeal digestion. They considered the secretions to be involved in moulting, as shown by Davey and Kan (1968) in **P.decipiens**. However, Sommerville and Buzzell (1974) were studying **in vitro** development, and as such, the parasites may have been about to moult to the fourth stage, whereas third stage larvae in fish would not be in such a pre-moult stage. The excretory system of third stage larvae of **A.simplex** has been suggested as a source of invasive enzymes. These enzymes may be involved in aiding penetration of the larvae into host tissue (Ruitenberg and Loendersloot 1971, Matthews 1984), and the position of the excretory pore at the level of the lips, and the presence of a boring tooth at the third larval stage, in **A.simplex**, **P.decipiens** and **C.osculatum** may also support this hypothesis.

Ruitenberg and Loendersloot (1971) and Lee **et al.** (1973) indicated that little was known regarding changes in the excretory organ when the worm has penetrated either fish or mammalian hosts. Fukuda **et al.** (1990) observed changes in the excretory gland of **Anisakis** larvae after incubation in artificial gastric juice; many of the granules became less electron dense, their membranes disappeared and granules fused together. They concluded that the excretory organ appeared to play an important role when the nematode penetrated into human tissues. This assumption was based on the location of the excretory pore and the suggestion that the granules may be involved in the formation of the histolytic enzymes necessary for the worm to penetrate into human tissues.

"Free" third stage larvae of **A.simplex** were compared to encapsulated larvae to examine if any differences in internal ultrastructure were apparent between the former which were assumed to have entered the body cavity of the fish relatively recently, and the latter which were assumed to have been present in the body cavity for longer. No distinct differences were seen in the ultrastructure of the digestive system. However, since the larvae do not appear to feed at the third stage, the digestive system was considered to be non-functional and similarities in morphology were to be expected. Differences were expected in the excretory gland, as this organ is suggested to aid in the penetration of the larvae through host tissue. In retrospect, it would have been advantageous to have also examined larvae found free in the stomach lumen, prior to penetration, as both classes of larvae examined during the present study had penetrated the stomach; examination of "unpenetrated" larvae would have given a better basis for comparison of ultrastructure and therefore insight into the possible role of the gland during penetration of tissue. It must also be noted that although encapsulated larvae were assumed to represent older infections, and "free" larvae recent infections, the actual age of these infections was not known, and it is also not

known if these larvae had previously infected a fish, perhaps even several, or if they came directly from an invertebrate host. Slight differences were, however, observed - including a general absence of high electron dense secretory granules in the encapsulated larva, along with the appearance of more numerous empty - or partially empty - and lysed granules, and the secretory granules were generally slightly smaller in size. It is tempting to suggest that if the "free" larva has indeed penetrated relatively recently, then the differences observed may be due to the fact that the gland in the "free" larva has only recently ceased its activity, whereas that of the encapsulated larva may have remained quiescent for some time, and as such the structures have degenerated to an extent. However, factors such as the ease of penetration, and number and type of previous hosts may have had an effect on the appearance of the gland.

Lee **et al.** (1973) suggested that the appearance of the vesicles surrounding and opening into the excretory canal and drainage vessels in larval **Anisakis** sp. indicated that the excretory gland is also associated with osmoregulation and\or excretion. Soleim and Berland (1981) also suggested that the excretory system of **Thynnascaris adunca** (= **H.aduncum**) may have an excretory and\or osmoregulatory function.

It seems likely that the central excretory canals observed here collect fluid which is then expelled via the excretory pore, however, analysis of the contents of this fluid would have to be carried out to ascertain its' constituents and determine if the system is involved in osmoregulation or excretion, in addition to secretion. It may be relevant that Waddell (1968) stated that the change from a free-living habit to a parasitic habit may lessen the need for osmoregulation in the excretory system of **Stephanurus dentatus**.

The similar life cycles of **A.simplex**, **P.decipiens** and **C.osculatum** would imply that the function of the excretory system in these two latter species is the same as that of **A.simplex**. If the excretory gland does indeed aid in

penetration of the third stage larvae through fish tissue, it must develop during this life cycle stage, as, at least in **P.decipiens**, the excretory system in early third stage larvae appears to be at a rudimentary stage of development.

### 5.4.5 Body Wall

The cuticles of third stage **A.simplex** and **C.osculatum** larvae were approximately 3.9µm and 4µm in thickness, respectively. That of **Anisakis** being approximately half that reported by other authors eg. Beverley-Burton **et al.** (1977), Kliks (1983). Fredericksen and Specian (1981) however, found the cuticle of juvenile **Anisakis** sp. to be approximately 1.5µm in thickness. Kliks (1983) observed that the cuticle of fourth stage larvae of **P.decipiens** recovered from humans was 10-20µm in thickness. and Soleim and Berland (1981) found the cuticle of **Thynnascaris adunca** (= **H.aduncum**) to vary in thickness from 5-11µm. Soleim and Berland (1981) described the structure of the cuticle of **Thynnascaris adunca** (= **H.aduncum**) in detail, as did Fredericksen and Specian (1981) for juvenile **Anisakis**, **Phocanema** and **Thynnascaris** sp.. Fredericksen and found the cuticles of these species, like that of **C.osculatum** examined here, to apparently consist of variously layered bands of homogenous material that differed only in electron opacity. A thin sheet of dense material covered the cuticle in each of the species examined by Fredericksen and Specian; this was also observed in the specimens of third stage **A.simplex** and **C.osculatum** examined during this study.

The hypodermis in **Thynnascaris adunca** (= **H.aduncum**) was 0.8-1.5µm in thickness (Soleim and Berland 1981); this compares to approximately 0.8µm and 1.6µm in thickness for third stage **A.simplex** and **C.osculatum** respectively. The hypodermis in **C.osculatum** was observed to contain numerous irregularly shaped vesicles of varying sizes and densities. Bruce (1970), Smith (1970) and McLaren (1972b) also observed

numerous vesicles in the hypodermis of **Trichinella spiralis**, third stage larvae of **Haemonchus placei** and microfilaria respectively. Bruce (1970) and McLaren (1972b) described a wide variety of other structures in the hypodermis. Hypodermal cells are known to be associated with the formation of cuticle, and such metabolically active cells may contain a variety of materials relating to the production of cuticular tissue (Smith 1970). Bruce (1970) described possible formation of the cuticle in **Trichinella spiralis** and suggested secretion from the hypodermis as the probable mode of development. Wisse and Daems (1968, in Smith 1970) also suggested that the hypodermal layer is involved in antagonistic action with the somatic muscles during locomotion. The hypodermis in nematodes is also known to contain reserves of fat and glycogen (Lee 1965), although such substances were not apparent in the third stage **C.osculatum** examined during this study.

Both thick and thin myofilaments were observed within the myofibrils of the muscle cells of **C.osculatum**, each thick filament being surrounded by a number of thin filaments. This has also been observed in **Haemonchus placei** (Smith 1970), **Ascaris lumbricoides** (Rosenbluth 1965) and microfilaria (McLaren 1972b). Rosenbluth (1965) observed a narrow band of thick filaments only in the centre of the myofibrils of **Ascaris lumbricoides**, on either side of this there was a wider strip of thick and thin filaments, then a narrow strip at the edge of the myofibril containing thin filaments only. The myofibrils observed in **C.osculatum** did not show this pattern, being composed throughout of thick fibres surrounded by thinner ones. Rosenbluth (1965) observed thin dense bands between the myofibrils in **Ascaris lumbricoides** and these were complex and irregular in structure, consisting of several different constituents including dense bodies and deep plasma membrane invaginations. Irregular vesicles and filaments were also observed in the dense bands separating the myofibrils in **C.osculatum**.

The sarcoplasmic (non-contractile) region of each muscle

cell in the specimens examined during this study contained numerous mitochondria and large areas containing glycogen, with lipid droplets also being observed in **C.osculatum**. These features are apparently typical for nematode muscle cells (Lee 1965), and have also been observed by Soleim and Berland (1981) in **Thynnascaris adunca** (= **H.aduncum**), Bruce (1970) in **Trichinella spiralis**, Smith (1970) in third stage **Haemonchus placei** larvae, Rosenbluth (1965) in **Ascaris lumbricoides**, and McLaren (1972b) in microfilaria. Mueller (1929, in Lee 1962) regarded the clusters of fat globules in the muscle cells of **Ascaris** as food stored in a region of great metabolic activity.

The ultrastructure of the somatic musculature in the specimens examined during the present study was similar. Ishii **et al.** (1989) also found the muscle cells of **A.simplex** and **P.decipiens** to be similar. The form of the muscle cells does not appear to differ in nematode species, and the morphology examined here has also been reported from, among others, **Thynnascaris adunca** - = **H.aduncum** (Soleim and Berland 1981), **Haemonchus placei** (Smith 1970), **Ascaris lumbricoides** (Rosenbluth 1965) and **Trichinella spiralis** (Bruce 1970a).

## 5.5 CONCLUSIONS

Early third stage larvae of **P.decipiens** show no apparent differentiation of the body organs, with the exception of a rudimentary digestive tract, and a possible rudimentary glandular structure. It is presumed that the wide variety of materials observed in the body of these larvae will be used both for differentiation of internal organs and as energy reserves for activity and growth.

Sections through the sense organs of marine ascaridoids proved difficult to obtain, however, the internal structure of the sense organs which were examined was similar to that reported for these organs in other species of nematodes, and it is concluded that the amphids in both **H.aduncum** and

**A.simplex** are chemoreceptors, and the papillae in **P.decipiens**, mechanoreceptors. The amphid in the third stage specimen of **A.simplex** appeared well developed, suggesting that it is functional at this stage, and it may function to monitor the external environment and\or to detect aggregation attractants.

The close taxonomic relationship, and similar life cycles of **A.simplex**, **P.decipiens** and **C.osculatum**, suggested that similarities in the ultrastructure of internal organs were to be expected. This was found to be the case for the preventriculus and intestine, where the general morphology did not differ significantly between either different nematode species at the same larval stage, or, as compared to the adult of **P.decipiens**, between the larval and adult stage; suggesting that the digestive tract is fully developed in third stage larvae from fish. However, although the general appearance of these regions was similar, there were differences which indicated that the digestive system does not function at the third larval stage; these included the presence of apparently degenerated structures in the preventriculus of third stage larvae, as compared to the well developed vesicles and mitochondria in the preventriculus of adult **P.decipiens**, and the presence of mitochondria in the intestinal cells of adult **P.decipiens**. That the regions of the digestive tract appear active at the adult stage, but not the third larval stage, agrees with the hypothesis that third stage larvae in fish do not feed. Stores of glycogen and lipids were observed in the non-contractile region of the somatic muscle cells in third stage **C.osculatum**, and vesicles in the intestinal cells were also presumed to contain nutrient reserves. Such stores are thought to act as energy reserves during the non-feeding third stage in fish.

The ventriculus, oesophageal glands and ventricular appendix of the specimens examined contain what appear to be secretory vesicles, and it is suggested that these contain enzymes which aid in digestion. The oesophageal glands of third stage **C.osculatum** and adult **P.decipiens** were similar

in appearance, and appeared active. Secretions produced from these glands at the third larval stage may aid in penetration, rather than digestion.

The ultrastucture of the excretory gland in third stage **A.simplex** agreed with that previously reported from larval **Anisakis** sp. by Lee **et al.** (1973) and Fukuda **et al.** (1990), and a secretory function is suggested. The secretions are thought to aid in penetration of host tissue at the third larval stage. An excretory and\or osmoregulatory function is also possible.

The general morphology of the digestive tract, excretory gland and body wall is similar to that observed in a wide variety of nematode species.

## CHAPTER SIX : SUGGESTIONS FOR FUTURE WORK

When considering the site distribution of nematodes in fish, differences with age of the fish should be examined further, in relation to the suggested optimum distance over which larvae can migrate. The length ranges of the fish examined during the present study were not divided into different groups, as data from all fish species examined were pooled. Division into different length classes may reveal variations in the location of nematodes with increasing size of fish, as indeed McClelland **et al.** (1983b) observed for **P.decipiens** in cod. Detailed studies of the migration pathways of each nematode should be undertaken to determine the route from the alimentary tract, and to ascertain what determines the final position of the nematode within the body of the fish eg. if the final position of **C.osculatum** and **H.aduncum** within the fish does indeed depend on the site of penetration through certain regions of the digestive tract, and if these two species do have limited distances for migration. The biological factors influencing migration must also be investigated eg it is possible that **P.decipiens** and **A.simplex** larvae have a larger distance of migration because they may contain more energy stores than **C.osculatum** or **H.aduncum**. In the case of **C.osculatum**, it should be determined whether these larvae do show a site preference for the liver. In addition, **C.osculatum** larvae migrate into the parenchyma of the liver, whereas nematodes of other species are typically found on the surface of this organ. The reasons for this difference are not known, but would be of interest to examine.

Further studies relating to competition between these nematode species in fish should be carried out, as the investigation for interactions between species in the present study was only preliminary. In particular, any future work should include examinations for competition between nematodes in individual organs of fish to ascertain if competition

occurs between species in the same microhabitat, and if competition for space occurs in individual organs when these organs are heavily infected.

With respect to further work regarding the pathology of fish stomach lesions, it would be of interest to examine the ultrastructure of the stomach lesions found in fish, to determine the fine formation of the lesion and the cell types involved in the reaction, and to examine the host-parasite interface for evidence of cellular reactions occurring here.

To determine if a mass infection does indeed cause the formation of stomach ulcers, experimental infection of fish, both large and small, with a large number of **A.simplex** larvae, should be carried out to observe if a similar reaction occurs, and if these lesions can also occur in small fish.

Stomach lesions associated with larval **C.osculatum** and **H.aduncum** do not appear to have previously been reported from fish, and experimental infections, using large numbers of these larvae, may reveal whether these species can invoke formation of stomach ulcers. **Contracaecum** sp. have been associated with lesions in the stomachs of natural final hosts, and gastro-intestinal pathology caused by other **Hysterothylacium** spp. has been documented from fish, and mammals, so it is possible that, like **A.simplex** and **P.decipiens**, these too can cause lesions to form in the stomach of fish. In the case of **H.aduncum**, larvae from invertebrates would have to be used for experimental infections, as larvae of this species recovered from fish would develop into fourth stage larvae and adults in the alimentary tracts of new fish hosts.

Although experimental studies have been made on chemical attraction,communication and aggregation on free-living and parasitic species of nematodes, none have been carried out on **A.simplex**,**P.decipiens**, **C.osculatum** or **H.aduncum**. Such studies would determine if **A.simplex** are aggregatory and if other species are not.

With regard to future SEM work, it would be of interest to conduct a preliminary examination of adults of the four species from different waters worldwide in order to investigate possible ultrastructural differences between them. Furthermore, an investigation of the cephalic morphology of third stage larvae at different times post hatch, encompassing free-living larvae and larvae from invertebrates and fish, may ascertain when, and to what level, development occurs within this stage, and would indicate age variations in morphology and possibly account for differing observations by authors. Moreover, a full examination of **A.simplex** and **P.decipiens** adults under SEM does not appear to have been carried out - only the fourth larval stages have been examined in detail. Beverley-Burton **et al.** (1977) stated that larval **A.simplex** and **A.typica** may be morphologically similar, and it would be of value and of taxonomic interest to examine the external structure of **A.typica** under SEM, and also that of **A.[illegible]seteris** (**Anisakis** type II), for comparison with **A.simplex**.

The potential for further study of marine ascaridoids, in relation to the ultrastructural, comparative and functional morphology of their internal organs is large. Further TEM examination of marine ascaridoids are needed to examine the development of organ systems throughout the life cycle. This would require examination of early third stage larvae at different time periods post-hatching, and also third stage larvae from invertebrate hosts, and of known age from fish hosts; **in vitro** culture of larvae and adults would also enable parasites to be studied throughout their developmental cycle. A more detailed examination of adult worms is also necessary, as only sections through the preventriculus, intestinal caecum and intestine of adult **P.decipiens** were obtained for examination. The ventriculus and excretory gland were therefore not studied, and examination of these organs may give further insights into their functions. In addition, neither sections through the

excretory gland and intestinal caecum of third stage **P.decipiens** and **C.osculatum**, or sections through the ventriculus of third stage **P.decipiens** were obtained for examination. Again, for comparative purposes, it would be of interest to examine the ultrastucture of early larvae and adults of **A.simplex** and **C.osculatum**. Examination of **H.aduncum** at both larval and adult stages would also be useful for comparative purposes, particularly as the this species is a raphidascarine - rather than an anisakid, as the above mentioned species are, and also, the life cycle in this species is slightly different from that of the above mentioned species - **H.aduncum** having fish as their final hosts, rather than marine mammals.

The rectum, and rectal area, of the marine ascaridoids examined was not studied during the present work, and examination of this area would be necessary to ascertain its ultrastructural characteristics.

Due to the limited sections obtained through the sense organs of these nematodes, further TEM studies are required with regard to the sense organs of marine ascaridoids, in particular the single papillae - of which only a section from the side of the cilium was obtained - and also the double papillae, in order to examine the morphology of this organ and determine its' function\s. Sections through the papillae from larvae and adult worms would be of value for comparison, to investigate whether these organs appear to be functional at the third larval stage, as the amphid in third stage **A.simplex** does.

Histochemical studies would determine the nature of the vesicles in the different regions of the digestive tract, and histochemical studies of the digestive and excretory system through different life cycle stages may reveal changes in enzyme composition and activity and distribution throughout the development of the nematodes.

An analysis of the carbohydrate, lipid and protein composition in early and late third stage larvae and adults, may be useful in indicating changes during development eg to

determine if stores of glycogen and lipid are higher in the non-feeding third stage than in the adult; and for comparisons of anisakid larvae with **H.aduncum**, which, unlike anisakids, does not generally survive for long **in vitro**.

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## APPENDIX 1

## Preparation of Chemicals for Light and Electron Microscopy

### (a) McKnights Fixative (Buffered Formal Saline)

| | |
|---|---|
| Formalin | 500ml |
| Sodium dihydrogen orthophosphate | 22.75g |
| Sodium Chloride | 22.5g |
| Disodium hydrogen orthophosphate | 32.5g |
| Distilled water | 2 litres |

**(b) Karnovskys' Fixative**

1) Dissolve 2g Paraformaldehyde in 25ml distilled water by heating to 60-70°C and shaking the flask constantly (this must be done in a fume cupboard).

2) Add approximately 3 drops N NaOH until solution is clear or still slightly turbid.

3) Allow to cool before adding 10ml of stock solution of Glutaraldehyde (this is usually 25%) and 25mg Anhydrous Calcium Chloride.

4) Make up to 150ml with cacodylate buffer (0.1M Sodium Cacodylate - [21.4g/litre] adjusted to pH 7.4 with concentrated HCl).

This solution contains 1.3% Paraformaldehyde 1.6% Glutaraldehyde

**(c) Cacodylate Rinse**

0.1M Sodium Cacodylate (21.4g/litre) + 0.1M Sucrose (34.2g/litre). Adjust pH to 7.4 with concentrated HCl.

## (d) Resin Mix for Transmission Electron Microscopy

| | |
|---|---|
| Araldite CY212 | 20ml or 23g |
| DDSA | 22ml or 22g |
| BDMA | 1.1ml or 1.2g |

## (e) Lead Citrate Stain for Thin Sections

Venable,J.H. and Coggeshall,R. (1965) J.Cell Biology 17: 208

Method B

Add 0.01 - 0.04g of lead citrate to 10ml of distilled water in a centrifuge tube.

Add 0.1ml of ion sodium hydroxide (carbonate free) and seal the tube. Shake until the lead citrate is dissolved then centrifuge before use. (Lead citrate takes approximately 15 minutes to dissolve).