Proteases in the Atlantic salmon, *Salmo salar* L.
Physiological and biological aspects.

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DECLARATION.

I declare that this thesis represents, except where note is made to the contrary, work carried out by myself. The text was composed by myself.

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December, 1993.

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ABSTRACT:

The digestive tract of the Atlantic salmon, *Salmo salar* L., was studied on a histological and an ultrastructural basis and was found to be fairly similar to the digestive tract of the rainbow trout, *Oncorhynchus my*kiss. Endocrine cells containing granules, hypothetically with a cholecystokinin-like (CCK-like) hormone, were identified in the pyloric caecal region of the intestine. Supportive evidence is presented for the hypothesis that digestive enzymes from the pancreatic tissue are conveyed by many small ducts into the pyloric caeca and the pyloric caecal region of the intestinal wall.

Pepsinogen was found to be stored in the oxynticopeptic cells of the stomach glands, in the cardiac- and the transitional area of the stomach. The number of oxynticopeptic cells were gradually reduced in the caudal direction in the transitional area. The results support the hypothesis that pepsinogens have not undergone much change during vertebrate evolution. Trypsinogen and chymotrypsinogen were found to be stored in the secretory granules of the acinar cells, which were embedded in the fatty tissue surrounding the pyloric caeca.

Optimal assay conditions, and optimal processing and storage of samples for pepsin, trypsin and chymotrypsin analysis were determined. These enzymes were found to follow the kinetics of single substrate reactions.

Purified, natural, porcine CCK, sulphated, stimulated the secretion of trypsinogen and chymotrypsinogen both *in vivo* and *in vitro*. The effect of the CCK was found to be temperature dependent *in vivo*. It was also found to cause the discharge of the gallbladder *in vivo*. These findings support the hypothesis that the CCK-like peptides were established early in the evolution of vertebrates.

Starvation for 2 days resulted in a slight increase of the stored pepsinogen, trypsinogen and chymotrypsinogen, but no reduction in these activities was found after at least 16 days. Starvation caused a drastic reduction of secreted trypsin and chymotrypsin activities to a minimum within 16 days, but not in secreted pepsin activity, which remained roughly the same. Ingested food stimulated immediate secretion and resynthesis of pepsinogen. Digesta from the

stomach entering the pyloric caecal region of the intestine stimulated secretion of trypsinogen and chymotrypsinogen, caused the discharge of the gallbladder and probably peristalsis of the intestine, since trypsin and chymotrypsin activities appeared in all parts of the intestinal lumen at the same time. The relevance of these results for the identification of a CCK-like hormone is reviewed. The results may suggest that the pancreas secretion is under a feedback control. The intestinal lumen as well as the pancreas appear to be under a strict homeostatic control with regard to its content of trypsin and chymotrypsin.

The arrest of growth of the lower modal group (LMG) fish during their first winter was not caused by a lack of digestive capacity, since quantities of pepsin, trypsin and chymotrypsin were no less than in the upper modal group (UMG) fish. A challenge with a pureified, natural, porcine CCK, sulphated, demonstrated that the pancreas of both groups was able to secrete both trypsinogen and chymotrypsinogen. The response to starvation and the response to CCK demonstrated that the stomach mucosa and the pancreas were secreting pepsinogen, trypsinogen and chymotrypsinogen more actively, in the UMG fish than in the LMG fish. This was also reflected in greater feeding activity of the UMG fish, which contained greater amounts of stomach digesta during the winter months. The suppression of appetite and the thresholds of response to plasma thyroxine in the LMG are reviewed.

Chapter I.

General introduction.

1. The biology of the Atlantic salmon.

The Atlantic salmon (*Salmo salar* L.) is a member of the *Protacanthopterygii*, one of the most primitive superorders of the teleosts, which is mainly made up of the family *Salmonidae*. This family contains the *Salmoninae* (the Atlantic and Pacific salmon, the trout and the charr), the *Thymallinae* (the grayling) and the *Coregoninae* (the whitefishes; Norden, 1961). The Atlantic salmon's habitat is the Northern Atlantic and the Baltic seas and most rivers flowing into these (MacCrimmon and Gots, 1979). It is an anadromous animal, reproducing in freshwater with subsequent feeding and growth of the new generation occurring in the sea.

Spawning occurs in late autumn or winter, i.e. from November to February, the salmon preferring a clean, silt-free, aerated gravel for their redds. Size distribution of the gravel has been found to vary greatly, but more than 20 % of sand caused a low permeability, i.e. water flowing through the redd (Peterson, 1978). A minimal permeability (1 m/h) appears to be necessary for successful emergence of fry (*Ibid.*). Spawning salmon appear to prefer certain water velocities and depths (Beland *et al.*, 1982). A relationship between the fork length of the salmon and the number of eggs produced has been found (Pope *et al.*, 1961). Females which had developed more rapidly in fresh water produce more but smaller eggs at any given body size, than slower developers (Thorpe *et al.*, 1984). Almost no males and only a small proportion of the females survive spawning. Surviving females can return to spawn a second time.

The eggs, buried in the gravel, hatch in March / April, 2-5 months after laying of the eggs, depending on the water temperature. Large eggs result in large first-feeding fry, but this size advantage is not necessarily maintained (Thorpe *et al.*, 1984).

In northern countries and in high altitude streams, the water temperature appears to be the most decisive factor in salmon survival. Low water temperature may inhibit the development of the egg and delay the hatch of alevins. This in turn shortens the feeding time before winter, when feeding stops (Niemela *et al.*, 1985). In countries further south, low pH values and high concentrations of suspended solids are probably the factors limiting egg survival.

The alevins, ca. 2 cm long, possess a large yolk sac. Their movements in the gravel are guided by photokinesis and geo-and rheotaxes (Dill, 1969, 1977; Brannon, 1965, 1972; Mason, 1976), being first downwards and then upwards. When the fish have emerged from the gravel, their yolk sac has been absorbed and they are ready to start feeding. These are now known as fry. Through positive thigmotaxis and positive rheotaxis the fry are able to keep their location in the river.

The fry are subject to high mortality rates in the first week after hatching, caused by starvation, predation and competition for territories, and these decide the carrying capacity of the stream (Mills, 1964, 1965, 1969a, 1969b; Egglishaw and Schackley, 1977, 1980). When the fry have reached a fork length of ca. 6.5 to 7.0 cm, they develop dark blotches along their sides, the parr marks. They are now known as parr. The fry stage ends after dispersal from the redd (Allan and Ritter, 1975).

Young salmon are territorial (Kalleberg, 1958; Keenleyside and Yamamoto, 1962; Leániz, 1988). The density of the fry and the parr in the stream depends on this territoriality and on water temperature, gross production of the stream, other fish species, stream bed stability, water depth and water velocity (Egglishaw and Schackley, 1982, 1985).

The juvenile salmon selects areas of bed where at least 10 % of the substrate is of particle size greater than 10 cm (Gray *et al.*, 1986), in streams of moderate size (Kennedy, 1984). They prefer a riffle habitat in summer, but move into deeper water in winter, when feeding ceases. This movement appears to be temperature-dependent (Allen, 1940a, 1940b, 1941a, 1941b; Gardiner and Geddes, 1980; Gibson, 1978; Rimmer *et al.*, 1984).

Growth of fry and parr has been found to take place from early April to October in the River Eden, England (Allen, 1940a, 1941a). It is known that populations of juvenile Atlantic salmon in their first year of growth develop a bimodal distribution with respect to the fork length. The upper modal fish (which become S1 smolts) smoltify 1 year earlier than the lower modal fish (which become S2 smolts). Lower modal group fish become smolts in their 2nd to 4th year of life (Mills, 1989). The age of smolt migration increases towards the north, and can become as high as 7 years (Dahl, 1910; Sømme, 1941). This is probably the result of a slower growth in the northern areas, caused by lower temperature, but it has been hypothesised that a certain size threshold must be obtained in the spring, before migration can take place (Elson, 1957). Results showing that a higher smolt rate is correlated with a higher growth rate in juvenile Atlantic salmon support this hypothesis (Thorpe *et al.*, 1980).

The proportion of juveniles becoming upper modal fish is determined mainly by inheritance (Simpson and Thorpe, 1976). This is apparently caused by internal suppression of appetite in the destined lower modal fish, commencing in midsummer and continuing during the winter (Metcalfe *et al.*, 1986). Factors in favour of growth will tend to increase the proportion becoming upper modal fish, such as increased water flow (*Ibid.*) or °Cxdaylight hours (Thorpe *et al.*, 1989). Factors which decrease growth, such as crowding (Simpson and Thorpe, 1976), tend to reduce the proportion of fish becoming upper modal.

Smoltification encompasses a number of changes, morphological, physiological and behavioral, which occur whilst the fish is in fresh water, preadapting it for life in the sea (Hoar, 1976). Smoltification is likely to be triggered when parr have reached a certain size, towards the end of one growing season (Elson, 1957). Photoperiod is considered to be the environmental cue, acting through the endocrine system, triggering the changes which result in smoltification (Saunders and Sreedharan, 1977). The timing of migration appears however to be mostly influenced by water temperature (Berry, 1932, 1933; Mills, 1964; Solomon, 1978; Einarsson, 1987), or by a combination of rate of increase in temperature and absolute water temperature in spring (Jonsson and Ruud-Hansen, 1985).

Salmon usually spend 1 to 4 winters at sea before returning to their river to spawn. Those which return from the sea after 1 winter are termed grilse. The way in which the salmon finds its way back to its home river is reviewed by Mills (1989).

2. Feeding of salmon in the wild, in culture and in relation to the digestive enzymes.

In freshwater juvenile Atlantic salmon feed mainly on aquatic larvae of insects, while at sea adults feed mainly on crustaceans and small fishes, such as herring, sprat, sand-eels, capelin and small gadids (Whitehead *et al.*, eds., 1986).

Salmonid fish show a high degree of prey selection based on body size (Ricker, 1932; Allen, 1941a; Martin, 1952; Lindstrom, 1956; Nilsson, 1957, 1958; Zordidi, 1970; Metz, 1974; Moore and Moore, 1974). In the juvenile Atlantic salmon moth breadth and gill raker spacing have been proposed as morphometric limitations to the range of prey sizes available, which remains constant at 0.06 x fish fork length (Wankowski, 1979). This is the upper size threshold since, from observations, salmon ingest prey whole and do not bite pieces out of large items. Maximum breadth of prey animals have in various teleosts been found to correspond closely to predator mouth breadth (Northcote, 1954; Lawrence, 1958; Yasuda, 1960; Okada and Taniguchi, 1971; Wong and Ward, 1972; Werner, 1974). Gill raker spacing has been associated with minimum prey size in some fish (Nilsson, 1958, 1965; Ivlev, 1961; Luzanne, 1970; Nakamura, 1968). Abrupt lower prey size threshold found in rainbow trout, *Oncorhynchus mykiss*, was however due to selection of individual prey (Galbraith, 1967). A high degree of prey size selection exists within these morphometrically determined limits.

Prey size of 0.025 x fish fork length elicited a maximum feeding and a maximum growth response in the juvenile Atlantic salmon (Wankowski, 1979). A close correlation in the pattern of prey size selection from laboratory work and in fish from natural environment has been demonstrated (*Ibid.*). Further studies demonstrated maximum growth of the juveniles on particle diameters of 0.022 to 0.026 x fish fork length during spring (Wankowski and Thorpe, 1979b). But a progressive decrease in mean relative prey size from early summer to autumn has been found (*Ibid.*; Allen, 1940a, 1940b, 1941a), reflecting the animals reduced energy needs during falling water temperatures. It has also

been suggested that decreased food abundance in autumn leads to reduced specialisation in feeding, i.e. a comparatively wider range of prey sizes (Wankowski, 1981). Consumption and prey accessability may no longer be critical factors at this time of year.

Predator-prey models predict that successful predators are able to maximise their net energy gain during predation (MacArthur and Pianka, 1966; Emlen, 1968; Pulliam, 1974). The prey size reflecting the maximum feeding and growth in juvenie Atlantic salmon gives probably the highest net energy gain during growth in early summer. Larger prey is scarcer causing increased energy costs in search. Smaller prey costs proportionally more in energy terms to catch. The optimal prey size with respect to the net energy gain of the animal should be somewhere between these limits.

It is tempting to suggest that the selection of prey/particle size is a learned activity as observations of alevin behaviour indicate that the capture of particulate material irrespective of its size and composition is characteristic of the initial stages of feeding (Allen, 1941a; Wankowski and Thorpe,1979b). In support of this view are studies done on rainbow trout, where this animal acquired a "searching image", based on familiarity with a prey item facilitating future searches for that prey (Ware, 1971). Wild Atlantic salmon parr may possess a greater variety of prey searching images than do hatchery parr, as a result of their more varied diet, but greater number of taxa have been identified in wild parr stomachs than in hatchery parr (Sosiak *et al.*, 1979). It was also suggested that hatchery-reared parr feed less effectively than do wild parr, for at least 2 months after release in streams (*Ibid.*).

Juvenile salmon are territorial and feed while holding station in water current. In salmonids feeding is accomplished by visual location of prey (Polyak, 1957; Ali, 1959; Protasov, 1968; Ware, 1973; Wankowski, 1977). Prey can be substrate associated, drifting on the water surface or drifting suspended. Atlantic salmon juveniles make use of all three types of prey (Allen, 1941a, 1941b; Műller, 1954; Thomas, 1962; Egglishaw, 1967), but drift feeding was found to be the predominant method of food acqisition (Wankowski and Thorpe, 1979a). The utilisation of drift prey is independent of local productivity and eliminates the energy expenditure which results from the hunting and actively searching for the prey (Schoener, 1969). Atlantic salmon juveniles sometimes avoided maintainance

of a station against a current flow by remaining in low-velocity conditions immediately adjacent to areas of high-velocity and high drift abundance within which feeding took place (Wankowski and Thorpe, 1979a), thus avoiding the high energy costs connected with stations in high stream waters.

The maximum capture distance for the juveniles was linearly related to fish body length (*Ibid.*) implying a close relationship with swimming ability (Bainbridge, 1958). Larger fish were also found to keep a position in faster currents (Wankowski and Thorpe, 1979a), which reflected greater food requirements. Th fish are thus segregated into different territories with respect to size, which at least partially explains greatest aggression between individuals of the same size (*Ibid.*). The space within which a visul predator, such as the Atlantic salmon responds to prey depends in the first instance of the maximum distance at which the prey is detected. This distance is determined by the following:

i) Environmental factors such as light intensity (Hemmings, 1966; Protosov, 1968), turbidity (Tyler and Preisendorfer, 1962; Moore and Moore, 1976) and the contrast of prey and its background (Duntley, 1962; Hemmings, 1966).

ii) The predators visual system, where the probability of detection is highest in the binocular part of the visul field (Tamura, 1957; Hester, 1968).

iii) Prey characteristics such as size.

Atlantic salmon do not feed during night. Greatest appetite, swimming speed and food intake is highest in early morning and evening (Hoar, 1942; Kadri *et al.*, 1991). Considerable depression in feeding activity occurs in early afternoon (*Ibid.*).

Appetite in salmonids has been found to depend on stomach fullness, which in turn depended on the stomach evacuation rate and up to a certain temperature level, ca. 15 to 18 °C, the stomach evacuation rate was found to increase (Brett and Higgs, 1970; Brett, 1971; Elliot, 1975b; Doble and Eggers, 1978; Grove *et al.*, 1978). It was also demonstrated for rainbow trout that appetite depended on ingested calorific value rather than the stomach fullness (Grove *et al.*, 1978). A kaolin diluted food increased the feeding rate and the stomach evacuation rate in this animal (*Ibid.*). Pre-prandial starvation of Atlantic salmon caused a larger test meal to be consumed (Talbot *et al.*, 1984) further aiding the hypothesis that food intake is dependent on the animals need for calories and

nutrition such as vitamins, essential amino- and fatty acids. Incoming nutrition is somehow able to signal its presence to the feeding center in the animals hypothalamus, which processes this information to ballance the feeding activity with its requirements.

No studies on the feeding of adult Atlantic salmon at sea have been undertaken with regard to net energy acquisition through predation. It is however likely that the animals behaviour at sea is directed towards a maximal net energy gain through this activity, just as it is for the juveniles.

In aquaculture, the progress in feed formulation, feeding methods and management have made it possible to realise more of the growth potential of the fish (Austreng *et al.*, 1987). These and other factors of genetic origin, are constantly increasing the growth potential and growth rate of farmed salmonids such as the Norwegian ones (*Ibid.*).

As a predator feeding on protein, i.e. a carnivore, the Atlantic salmon has a well developed set of proteolytic digestive enzymes. Few studies have been done on the relationship of feeding habits of fish with their composition of the digestive enzymes. In a study done on some freshwater fishes (Hsu and Wu, 1979), it was found that carnivores with shortest gut had highest pepsin level and lowest chymotrypsin level, while microphages with longest gut had highest chymotrypsin level and no pepsin activity. The herbivores had intermediate chymotrypin level, with intermediate gut length, but with no pepsin activity. Pepsin activity is apparently related to predation. Increase in the chymotrypsin activity of the herbivore- and the microphage fish, may supplement loss of pepsin activity. These results however, do not confirm in this respect, i.e. increased chymotrypsin activity for the herbivore- and the microphage fish, with results from another study, where proteolytic activity in the digestive tract was related to the gut length (inversely) and the feeding habits of the fish (Hofer and Schiemer, 1981). The concentration of proteolytic activity was higher in carnivorous and omnivorous species than in herbivorous species. Herbivorous species compensated lower concentration in proteolytic activity with longer gut length, i.e. longer exposure time of ingested food to digestive enzymes. These species have often lower digestion- and assimilation efficiencies than carnivorous species (Fischer and Layokhnovich, 1973; Mironova, 1974; Moriarty and Moriarty, 1973), which they compensate with greater intake of plant material, which has

a lower nutritive quality than nutrition from animals. Carnivorous species such as the Atlantic salmon have specialised on fast and complete processing of their food, i.e. their prey, as hunting takes time. Here proteolytic enzymes play a central role, where pepsin activity in the stomach speeds up digeston. Herbivores on the other hand do not have to hunt, so more time can be spent on feeding itself.

In aquaculture, Atlantic salmon is of great importance for countries such as Norway, Scotland and Iceland. Feed and labour are the greatest running cost factors. Improving the feed is of vital importance with respect to the economy of the aquaculture stations. Basic knowledge on how this animal digests its food, with respect to digestive enzymes, is needed, if the feed is to be improved.

3. The digestive enzymes pepsin, trypsin and chymotrypsin.

As the Atlantic salmon (*Salmo salar* L.) is a carnivorous species, the major aspect of its digestion will be the breakdown of protein. This is achieved mainly by the following proteolytic digestive enzymes: Pepsin in the stomach and trypsin and chymotrypsin in the intestine. Pepsin from Atlantic slamon has been purified and characterised (Norris and Elam, 1940). Trypsin and chymotrypsin activity has previously been identified in this animal (Pringle *et al.*, 1992). These are endopeptidases and convert most of the ingested protein to smaller and soluble peptides. The peptides are in turn broken down to amino acids by exopeptidases. The amino acids are absorbed by the intestinal epithelium and enter the blood stream of the animal.

3.1. Endopeptidases of the stomach.

For most teleostei, pepsin is undoubtedly the major, if not the only endopeptidase in the stomach. Pepsin is a proteinase of broad side chain specificity (Hill, 1965). It breaks the bond between the dicarboxylic acid and the aromatic acid in the substrate. Pepsin splits peptide bonds on the amino side of aromatic amino acids or other amino acids with bulky side chains (Barrett, 1977). It is an aspartic proteinase, a class of endopeptidases active at acid conditions. Chymosin, rennin, cathepsin D and E and some microbial proteinases belong to this group (Gildberg, 1988). The active site of these enzymes contains two

aspartate residues. The one aspartate group makes a nucleophilic attack against the carboxyl carbon of the peptide binding, while the other supplies a proton for the "leaving" amide end of the polypeptide chain.

Only one isozyme of pepsinogen has been idntified in rainbow trout (Twining *et al.*, 1983), while one isozyme was found in the juveniles and two isozymes in the adult salmon fish Oncorhynchus keta (Sánches-Chiang *et al.*, 1987).

The aspartic proteinases are inhibited reversibly by the pentapeptide pepstatin, a very specific inhibitor (*Ibid.*). Pepstatin is probably a transition state analogue of the substrate during peptic catalysis (Tang, 1976).

The presence of pepsin in fish stomachs is already well established. Pepsin has been purified and characterised from various teleostei, such as the sardine (Noda and Murakami, 1981), the capelin (Gildberg and Raa, 1983) and the Atlantic cod (Brewer *et al.*, 1984; Haard, 1986; Martinez and Olsen, 1989). Gastric proteases have also been characterised in the Greenland cod (Squires *et al.*, 1986) and the Dover sole (Glass *et al.*, 1989).

Pepsin has also been characterised in salmonids. As early as 1940, Norris and Elam had purified pepsin by crystallisation from the Pacific Coast king salmon (*Oncohrynchus tschawytscha*). Proteases from the rainbow trout stomach have been characterised (Kitamikado and Tachino, 1960). Pepsinogen, the inactive precursor of pepsin, has also been characterised in this fish (Twining *et al.*, 1983) and the process of its activation studied. The conversion of pepsinogen to pepsin occurs in the stomach lumen and is known in mammals to be caused by acid or active pepsin (Stryer, 1981). It has been shown that many fish secrete at least two pepsins with different pH optima (Noda and Murakami, 1981; Gildberg and Raa, 1983; Martinez and Olsen, 1986). These pepsins are usually referred to as Pepsin I (pH optimum 3-4 when assayed with hemoglobin) and Pepsin II (pH optimum 2-3 when assayed with hemoglobin).

Partial purification of pepsins from the Pacific salmon (*Oncorhynchus keta*), revealed that juveniles only contained pepsin II, whereas adult fish had both pepsin I and II (Sánches-Chiang *et al.*, 1987). It was also shown for this anadromous fish that pepsin I was

activated by NaCl, whereas pepsin II was not. An interesting question arises, as to whether smoltification of the Atlantic salmon triggers transcription of a new pepsin, which is salt activated.

3.2. Endopeptidases of the intestine.

The endopeptidases of the intestine are trypsin, chymotrypsin and elastase. These are secreted from the pancreatic tissue as their respective inactive zymogens and are activated in the intestine by enterokinase and/or trypsin. These enzymes are closely related and have probably evolved from a common ancestral enzyme (Stryer, 1981). These are serine proteases.

At least 3 isozymes of trypsin have been identified and studied in the Atlantic salmon (Torrissen and Torrissen, 1985; Torrissen, 1991; Torrissen and Shearer, 1992). Various isozymic forms of chymotrypsin and elastase have not been identified in the Atlantic salmon. Three isozymes of trypsin and 4 of chymotrypsin have been purified from chinook salmon (Croston, 1965). Two pancreatic proelastases from the African lungfish have been isolated and partially sequence analysed (de Haën and Gertler, 1974).

The following description of the active site and the substrate binding site is taken mainly from Stryer (1981), reviews of serine proteases by Hess (1971) and by Kraut (1977) and a review by Stroud (1974).
The active site comprises an "oxyanion hole", containing a serine residue and a proton shuttle. The proton shuttle consists of a histidine (Schoellmann and Shaw, 1963; Ong *et al.*, 1965; Shaw *et al.*, 1965; Smillie and Hartley, 1966; Shaw and Springhorn, 1967) and an aspartic residue side chain (Stewart *et al.*, 1963).
After binding of the substrate to the enzyme, the proton shuttle removes the proton from the hydroxyl group of serine. The O- of the serine now makes a nucleopilic attack against the carboxyl carbon of the peptide binding, while the proton shuttle supplies a proton for the leaving amide end of the polypeptide chain. The carboxyl end of the polypeptide chain is then cleared off, with the hydrolysis of one water molecule as a result.
Trypsin, chymotrypsin and elastase differ with respect to their substrate binding site and hence their substrate specificity. The substrate binding site of trypsin contains an ionized

carboxyl group from aspartate, and hence it cleaves the peptide chain on the carboxyl side of basic amino acids, such as lysine and arginine.
The substrate binding site of chymotrypsin is uncharged, hence it cleaves the peptide chain on the carboxyl side of amino acids with aromatic or bulky nonpolar side chains. The specificity of elastase is directed towards the smaller uncharged side chains, as its substrate binding site is mainly blocked by valine and threonine.

It is well established that trypsin and chymotrypsin are inhibited by the Bowman-Birk inhibitor (Cohen *et al.*, 1981; Birk, 1985; Hanlon and Liener, 1986). The soybean trypsin inhibitor (SBTI) has been proven to be an inhibitor of trypsin from mammals, birds and fish (Krogdahl and Holm, 1983) and a weak inhibitor of bovine chymotrypsin (Kunitz, 1947). SBTI has also been shown to inhibit tryptic activity in Chinook salmon (Croston, 1960).

Trypsin and chymotrypsin from various fish species have been inactivated by N*a*-p-tosyl-L-lysine chloromethyl ketone (TLCK) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) respectively. Examples are trypsin and chymotrypsin from the Atlantic cod (Raae and Walther, 1989), trypsin and chymotrypsin from anchovy (Martínez and Serra, 1989), chymotrypsin from herring and capelin (Kalac, 1978b) and trypsin and chymotrypsin from herring and capelin (Kalac, 1978a).

Trypsin and chymotrypsin are stored in the pancreas as their respective zymogens. These are activated by enterokinase and/or trypsin upon entering the intestinal lumen. This process of activation has been studied in the catfish (Yoshinaka *et al.*, 1981b).

Trypsin, chymotrypsin and elastase have been purified and characterized from the Atlantic cod (Raae and Walther, 1989) and from carp (Cohen *et al.*, 1981). Trypsin from rainbow trout (Genicot *et al.*, 1988) and from Chum salmon (Uchida *et al.*, 1984) has been purified and characterised. Proteases from the pyloric caeca of herring and capelin have been partially purified and characterized (Kalac, 1978a), while two trypsin-like enzymes from the pyloric caeca of the salmon *Oncorhynchus keta* have been purified and characterised (Ushiyama, 1968). Three trypsin like enzymes have been identified in the Atlantic salmon by electrophoretic zymograms (Torrisen and Torrisen, 1984). Trypsin was

assayed with N*a*-benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA) as a substrate (Erlanger *et al.*, 1961), and chymotrypsin with N-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide (SAAPP*p*NA) as a substrate (Del Mar e*t al.*, 1979).

Proelastase, the inactive precursor of elastase, has been purified and characterised, from pig (Gertler and Birk, 1970), and elastase from the Atlantic cod (Gildberg and Øverbø, 1990), and from catfish (Yoshinaka *et al.*, 1982). The general pattern from these studies is that the characteristics of these enzymes are roughly the same for all vertebrates. The major difference between these enzymes from mammals and from fish, lies in the fact that mammals are homeothermic and fish poikilothermic. The fish enzymes are adapted to a colder environment, which is reflected in higher catalytic efficiency (k_{cat}/K_M) at lower temperatures, but faster inactivation at higher temperatures. k_{cat} is the turnover number of an enzyme, i.e. the number of substrate molecules converted into product per unit time, when the enzyme is fully saturated with substrate. K_M is the Michaelis constant, and is the substrate concentration giving a reaction rate equal to half of the maximal reaction rate, which in turn is at infinately high concentration of substrate, i.e. the saturatin of the enzyme with the substrate. k_{cat} reflects how fast the enzyme can work during saturation of the substrate, and K_M represents the binding affinity of the enzyme overfor the substrate.

4. Digestive physiology of teleostei.

4.1. Digestive physiology of the stomach.

The stomach mucosa secretes acid and pepsinogen, in response to ingested food. The same cells, termed the oxynticopeptic cells, secrete both H^+ and pepsinogen in nonmammalian species (Sedar, 1962; Smit, 1968; Helander, 1981). This had already been indicated by histological studies (Weinreb and Bilstad, 1955; Western and Jennings, 1970; Yasutake and Wales, 1983). These have been located by indirect immunofluorescence in the stomach mucosa of the rainbow trout (Yasugi, 1988). Indirect immunofluorescence is based on primary antibodies binding to the antigen and then secondary antibodies binding to the primary antibody. The secondary antibody contains some detectable marker. The

secretion is known in mammals to be triggered via neuronal and humoral mechanisms (Bell *et al.*, 1980).

Gastric acid secretion in the teleostei has mostly been studied in the Atlantic cod (Holstein, 1975, 1976, 1977, 1979a, 1979b, 1982, 1983, Holstein and Cederberg, 1980; Holstein and Humphrey, 1980). A summary of gastric acid secretion is given by Jönsson and Holmgren (1989).

Gastrin (Little gastrins, gastrin-17: Gastrin II: Glu-Gly-Pro-Try-Met-Glu-Glu-Glu-Glu-Glu-Ala-Tyr(SO_3H)-Gly-Try-Met-Asp-Phe-NH_2; gastrin I is identical, except it lacks the sulphate group on the Tyr; Gregory *et al.*, 1964. Big gastrin, gastrin-34, sulphated: Gln-Leu-Gly-Leu-Gln-Gly-His-Pro-Pro-Leu-Val-Ala-Asp-Leu-Ala-Lys-Lys-Gln-Gly-Pro-Trp-Met-Glu-Glu-Glu-Glu-Glu-Ala-Tyr(SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2; gastrin-34 can also exist nonsulphated; Vigna, 1979) is known to be the humoral factor causing the secretion of pepsinogen in mammals (Hirschowitz, 1984). Gastrin in mammals also causes stimulation of gastric acid secretion, antral contraction, and the growth of the gastric mucosa (Grossman, 1977). Pentagastrin (N-t-butyloxycarbonyl-β-Ala-Trp-Met-Asp-Phe-NH_2; Vigna and Gorbman, 1977), the active component of gastrin, has been found to induce hypertrophy of the fundic mucosa in mammals and an increase in DNA, RNA and protein synthesis; the variants of gastrin, G34 and G17 I (not sulphated) and II (sulphated), have been shown to have similar actions (Johnson and Guthrie, 1976). Sulphation of G17 had no significant effect on its trophic activity. The humoral factor(s) responsible for these actions in fish has not been identified.

Gastrin (G; Sulphated: -Glu-Glu-Glu-Ala-Tyr(SO_3H)-Gly-Trp-Met-Asp-Phe-NH_2. The nonsulphated form is without the SO_3H on the Tyr; Larsson and Rehfeld, 1977) and cholecystokinin (CCK; Sulphated: -Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2; *Ibid.*) share a common pentapeptide sequence at the carboxyl end (-Gly-Trp-Met-Asp-Phe-NH_2; *Ibid.*), containing the biological activity of the molecules, and this sequence is very immunogenic (Nilsson and Holmgren, 1989). Immunocytochemical methods are therefore unable to discern between G and CCK, hence the term G/CCK. G/CCK immunoreactive cells have been reported in the antral mucosa of the stomach and the intestine of the cod. The molecule causing G/CCK immunoreactivity is suggested to be

a caerulein (Sulphated: Pyroglutamic acid-Gln-Asp-Tyr(SO_3H)-Thr-Gly-Trp-Met-Asp-Phe-NH_2. Nonsulphated caerulein lacks the SO_3H of Tyr; *Ibid.*) or caerulein-like peptide (Larsson and Rehfeld, 1978). G/CCK like immunoreactivity has been reported in the stomach mucosa of two teleostei, the perch and the catfish (Noaillac-Depeyre and Hollande 1981). No G/CCK immunoreactivity was found in the stomach of the rainbow trout (Holmgren *et al.*, 1982), but endocrine cells showing G/CCK like immunoreactivity were found in the lower part of the cardiac and in the pyloric stomach of the Atlantic cod (Jönsson *et al.*, 1987). Gastrin-related peptides have a weak inhibitory effect, if any, on pepsin secretion (Holstein and Cederberg, 1986).

The humoral mediator of pepsin secretion in fish is presently not known. Serotonin, i.e. 5-hydroxytryptamine (5-HT), has been found to cause a secretion of pepsin in the Atlantic cod and it has been suggested that 5-HT may be a physiological regulator of acid and pepsin secretion in the teleostean fish (Holstein and Cederberg, 1984). The combined effect of 5-HT and histamine was found to produce a maximal effect.
Tachykinins, which are a family of peptides with widespread distribution in vertebrate tissue (Erspamer, 1981; Pernow, 1983), have been found to be extremely powerful pepsigogues, i.e. causing stimulation of pepsin secretion (Holstein and Cederberg, 1986). Physalaemin (an amphibian tachykinin: Pyr-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met-NH_2; Erspamer, 1981) and eledoisin (a molluscan tachykinin: Pyr-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2; *Ibid.*) were the most potent, but substance P (a member of this family, found in the gut of mammals and fish: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2; *Ibid.*) and eledoisin-related peptide less so. It has been suggested (*Ibid.*), that some tachikinins may be physiological stimulators of pepsin secretion and that the effect of acid secretion results from activation of both stimulatory and inhibitory pathways.

Substance P has been suggested as a candidate gastrointestinal hormone, since elevation of substance P immunoreactivity in the blood stream, following a protein meal or bombesin (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2; Melchiorri, 1978) infusion, has been demonstrated in dogs (Jaffe *et al.*, 1982; Martensson, *et al.*, 1984). Bombesin is a potent gastrin-releasing agent in mammals, the gastrin in turn stimulating pepsinogen and acid secretion (Walsh *et al.*, 1981). Bombesin is known to stimulate acid secretion in the Atlantic cod (Holstein and Humphrey, 1980).

Substance P-like immunoreactivity has been found in the endocrine cells of the gastric mucosa of the Rainbow trout (Holmgren *et al.*, 1982). For various teleostei, substance P has been found in the upper midgut or in the pyloric caeca, but not in the stomach (Langer *et al.*, 1979). Studies on gastrointestinal peptides in fish have been reviewed by Holmgren *et al.*, (1986).

Distension of the stomach has been found in the bluegill to cause both secretion of H^+ and pepsin from its mucosa (Smit, 1967; Norris *et al.*, 1973). The mechanism by which this occurs in fish is not known.

4.2 Digestive physiology of the pancreas.

A thorough review of the exocrine pancreas in mammals has been provided by Go *et al.* (1986).

Most teleostei, including salmonids, have a dispersed pancreas. In rainbow trout the pancreatic tissue is spread over the surface of fatty tissue surrounding the pyloric caeca (Weinreb and Bilstad, 1955; Yasutake and Wales, 1983).

The pancreas in mammals is known to secrete a cocktail of digestive enzymes, together with water and electrolytes. The gut hormone secretin (His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Asp-Ser-Ala-Arg-Leu-Gln-Arg-Leu-Leu-Gln-Gly-Leu-Val-NH_2; Bodansky *et al.*, 1973b) causes the centroacinar and duct cells to secrete water and electrolytes, containing bicarbonate anion, which neutralises the acid coming from the stomach (Bell *et al.*, 1980). Acetylcholine, released from the vagus, and cholecystokinin, released from the anterior intestinal gut wall, upon food entering the intestine, stimulate the pancreatic acinar cells to secrete digestive enzymes and the gallbladder to discharge gall (*Ibid.*). Duodenal acidification and intra arterial injection of CCK(8), sulphated, have been found to increase gallbladder motility in the rainbow trout, *Oncorhynhus mykiss* (Aldman *et al.*, 1992). Trypsin and chymotrypsin are among the secreted enzymes. An overview of pancreatic exocrine secretion on a cellular basis is given by Grossman (1984).

Not many studies have been made on secretion of digestive enzymes from the teleostean

pancreas. Intestinal extracts from the river lamprey and the marine lamprey have been found in mammals to cause an increase in the flow of pancreatic secretion, with an increase in its protein concentration in the rat (Barrington and Dockray, 1970). Intestinal extract from the eel has been found to cause a gallbladder contraction *in vitro* in the rabbit and a pancreatic secretion of fluid and protein in the rat (Barrington and Dockray, 1972). These studies have revealed that some factor(s), residing in the intestinal gut wall of these fish, cause the secretion of digestive enzymes from the pancreas and the discharge of gall from the gallbladder. Cholecystokinin octapeptide (CCK(8), sulphated: Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH2; Vigna and Gorbman, 1977), caerulein and nonsulphated caerulein (cholecystokinin analogues), have been found to stimulate secretion of lipase from the pancreas in the killifish during *in vivo* conditions (Honkanen *et al.*, 1988). This study gives strong support to the hypothesis that a gut hormone, with a structure common to CCK(8) or caerulein, probably sulphated, causes the secretion of digestive enzymes from the teleostean pancreas. This secretion was not inhibited by atropine, indicating that the hormones are acting directly on the exocrine pancreas in the killifish (*Ibid.*).

Studies on various teleosts have revealed that sulphated cholecystokinin or cholecystokinin octapeptide, sulphated and desulphated, caused a discharge of the gallbladder in the Coho salmon (Vigna and Gorbman, 1977), the rainbow trout (Aldman and Holmgren, 1987; Aldman *et al.*, 1992) and the bluegill, killifish and bowfin (Rajjo *et al.*, 1988a). Sulphation of CCK or caerulein increased potency. These studies support the hypothesis that some cholecystokinin-like hormone triggers the discharge of gall from the gallbladder. It has been found for the Atlantic salmon, that food entering the intestine triggers the discharge of gall from the gallbladder (Talbot and Higgins, 1982), presumably via some cholecystokinin-like factor.

Many studies have been carried on the localisation of the various gut hormones in fish, and a review on gastrointestinal peptides in fish has been published by Holmgren *et al.* (1986).

The intestinal mucosa in the rainbow trout has been found to contain endocrine cells reactive to G/CCK antiserum (Holmgren *et al.*, 1982) and CCK-like immunoreactivity extracted from the small intestine and the pyloric caeca of this fish exhibited marked

molecular heterogeneity (Vigna *et al.*, 1985). Gastrin/CCK immunoreactive endocrine cells have also been found in the proximal part in the intestine of the Atlantic cod (Jönsson *et al.*, 1987). CCK immunostained cells were found in the anterior and midintestine of the bowfin, a holostean fish, and in the anterior and the mid intestine and the pyloric caeca of the bluegill, a teleostean fish (Rajjo *et al.*, 1988b). Interestingly, the immunoreactive cells were open in appearance, i.e. making contact with the gut lumen.
These studies support the hypothesis that CCK-like peptides, present in the proximal intestinal gut wall, are released into the blood stream in response to food entering the intestine and hence acting on the pancreas and the gallbladder.

In mammals, cholecystokinin, sulphated, is known to stimulate protein synthesis (Reggio *et al.*, 1971) and growth (Go, 1978) of the pancreas. The factors serving this role in fish have not been identified.

5. Workplan.

Many studies have been undertaken on feeding of the Atlantic salmon (Hoar, 1942; Talbot and Higgins, 1983; Talbot *et al.*, 1984; Higgins, 1985; Usher *et al.*, 1988; Usher *et al.*, 1990; Kadri *et al.*, 1991; Carter *et al.*, 1992), especially with respect to behaviour (Metcalfe *et al.*, 1986; Metcalfe *et al.*, 1988; Metcalfe *et al.*, 1992; Stradmeyer, 1992; Erikson and Alanärä, 1992; Holm, 1992). How well this animal utilises its food, i.e. the weight gain in flesh per weight of consumed feed, is of vital importance for the aquaculture industry. Progress in feed formulation, feeding method and management has made it possible to realise more of the growth potential of the Atlantic salmon and the rainbow trout (Austreng *et al.*, 1987), but the growth potential and the growth rate of these animals farmed in Norway are continuously increasing (*Ibid.*). Obviously, digestion plays a great part in the utilisation of ingested food. As the Atlantic salmon is a carnivore, the major component of its food will be protein. The first step in the digestion of protein is executed by the endopeptidases of the gut, where pepsin, trypsin and chymotrypsin are the most common. Few studies exist on digestive enzymes in fish, with respect to biology or physiology. Hardly any such studies have been undertaken in the Atlantic salmon.

A systematic study on the histology and ultrastructure of the gut of the rainbow trout

(*Oncorhynchus mykiss)* has been undertaken (Weinreb and Bilstad, 1955; Yasutake and Wales, 1983; Ezeasor, 1978), but none on the Atlantic salmon. Species differences in this respect, might exist between the rainbow trout and the Atlantic salmon. Such a study will be appropriate in the beginning for the thesis work, as a basis for further studies. Knowledge of the localisation of the oxynticopeptic cells of the stomach, which synthesise pepsinogen, and the acinar cells of the pancreas, which synthesise trypsinogen and chymotrypsinogen, is therefore a necessary basis for the experimental study of digestive physiology. The localisation of endocrine cells is also of interest.

The main theme of this thesis will revolve around the enzymes pepsin, trypsin and chymotrypsin, with respect to the biology and digestive physiology of the salmon. These enzymes will be characterised biochemically and the validity of their assay methods tested and improved if necessary, with respect to accuracy, precision and pH. Elastase will not be included in this study. Elastase and other endopeptidases, apart from trypsin and chymotrypsin, are generally either absent from fish alimentary canals (Nilsson and Fänge, 1970; Jany, 1976), or only found at low concentrations (Yoshinaka *et al.*, 1978; Nilsson and Fänge, 1969; Buddington and Doroshov, 1986).

Pepsinogen has been localised in the rainbow trout (Yasugi *et al.*, 1988) and trypsin and chymotrypsin in the Atlantic cod (Overnell, 1973). A histological or an ultrastructural study can not be taken as a definite proof of the localisation, with respect to storage of pepsin, trypsin and chymotrypsin. Pepsin, trypsin and chymotrypsin will be localised on a morphological and a cellular basis, and trypsin and chymotrypsin on an ultrastructural basis. Pepsinogen will be localised with an immunocytochemical method, using a monoclonal antibody. If CCK causes secretion of trypsin and chymotrypsin, it will be used to localise the site of release of these enzymes on an ultrastructural basis.

Food in the stomach of teleostei probably stimulates secretion of pepsinogen via distension of the stomach (Norris *et al.*, 1973), where substance P is likely to play a role (Holmgren *et al.*, 1985). The secretion of trypsinogen and chymotrypsinogen in mammals is initiated when food in the duodenum stimulates secretion of CCK from endocrine cells in the duodenal mucosa (Dockray, 1989).

The UMG fish is known to retain its appetite better into the autumn (Metcalfe *et al.*, 1986) and to have higher levels of food intake and growth during the winter months, than the LMG fish (Higgins, 1985). How does pepsin, trypsin and chymotrypsin, stored and secreted, conform with the intake of food in the upper and the lower modal fish during the winter months?

The digestive tract of the Atlantic salmon will be studied with respect to histology and ultrastructure with special emphasis on the localisation of oxynticopeptic-, acinar-and endocrine cells (Chapter II).

Pepsin, trypsin and chymotrypsin will be studied with respect to kinetics, pH and substrate specificity (Chapter III).

The role of CCK in the secretion of trypsin and chymotrypsin will be studied *in vivo* at two temperature levels, and also by *in vitro* methods (Chapter IV).

The exact and definite localisation of pepsinogen, trypsinogen and chymotrypsinogen will be found (Chapter V).

The synthesis and secretion of pepsin, trypsin and chymotrypsin and the discharge of the gallbladder in response to food will be studied (Chapter VI).

The LMG and the UMG fish will be studied with respect to their feeding and their stored and secreted pepsin, trypsin and chymotrypsin (Chapter VII).

Chapter II.

Morphology and ultrastructure of the digestive tract.

1. Introduction.

In this chapter a survey of the histology and the ultrastructure of the gut of the Atlantic salmon will be undertaken to aid in the understanding of its function. The histology of the digestive tract, with its related structures, has been described in the rainbow trout (*Oncorhynchus mykiss*) by Weinreb and Bilstad (1955) and Yasutake and Wales (1983). Ezeasor (1978) has also undertaken a histological and ultrastructural study of the gut of this animal.

The ultrastructure of the gastric epithelium and the stomach glands in the perch (*Perca fluviatilis*) has been described by Noaillac-Depeyre and Gas (1978). The exocrine pancreas in the Atlantic salmon (Munro *et al.*, 1984) and the intestinal absorptive cells in the rainbow trout (Bauermaeister *et al.*, 1979; Ezeasor and Stokoe, 1981) have also been studied.

Pepsin is found in all vertebrates, except the stomachless fish (Vonk, 1937). It is known to be in the stomach glands of teleostean fish (Kapoor *et al.*, 1975). Pepsin has also been shown to be in the stomach glands of rainbow trout (Yasugi *et al.*, 1988). Trypsin has been found in the pyloric caeca of rainbow trout (Stevens and McLeese, 1988) and the pyloric caeca and intestine of the Atlantic salmon (Torrissen and Torrissen, 1985). The main emphasis will therefore be on the tissues containing these enzymes, i.e. the stomach glands and the pancreatic tissue with its ductal system.

In mammals endocrine cells located in the gut epithelium play a role in the control of digestion. Gastrin and cholecystokinin are regulatory peptides, secreted by both neurons and gastrointestinal endocrine cells, and play a role in the regulation of digestion.

Endocrine cells showing gastrin-/cholecystokinin-like immunoreactivity (G/CCK-like IR), have been demonstrated in the intestinal wall of the rainbow trout (Holmgren *et al.*, 1982), while no immunoreactivity was found in the stomach. CCK-li IR has been found in the walls of, the antral region of the stomach and the intestine of the rainbow trout (Vigna *et al.*, 1985). G/CCK-li IR cells have been reported in the mucosa of the antral region of the stomach and in the intestinal mucosa of the Atlantic cod (Larsson and Rehfeld, 1977). It has also been reported in the lower part of the cardiac and the pyloric regions of the stomach, and in the proximal part of the intestine (Jönsson *et al.*, 1987). Endocrine cells will therefore be localised, to shed some light on the possible role they may have in digestion.

2. Materials and methods.

2.1. The fish.

The experimental animals were hatchery-reared fish from the River Almond and had been kept on a commercial salmon food (Freshwater smolt 1, commercial pellets, size 3 mm, BP Nutrition) in excess, *ad libitum*, at least 3 months prior to the experiment. They were killed by a blow to the head.

The animals used for light microscopy (LM) and transmission electron microscopy (TEM) were immature lower modal group fish in their 1st year, 13.5-14.5 cm in fork length (ca. 30-40 g fresh weight). The fish were starved for 15 days at 4.5-14.0 °C prior to sampling. Sampling was executed at the end of September 1991 and 3 fish were sampled for the LM-, and 4 fish were sampled for the TEM studies.

The two fish used for the scanning electron microscopic studies were upper modal in their 2nd year (post smolts), 29.1 and 29.5 cm in fork length (242.9 and 242.6 g fresh weight). The fish were starved for 13 days at 4.5-12.8 °C prior to sampling. Sampling was executed in April 1992.

2.2. Light microscopic studies.

The fish were opened ventrally and the gut with the gallbladder was removed and put on ice. Some digesta remained in the descending intestine. The gallbladder was extended and dark green. Transverse sections of the gut , ca. 1.5 mm in width, were then removed with scissors as follows:

i) Stomach, cardiac area: One specimen immediately posterior to the airbladder duct and one specimen ca. one third from the transitional area (See Fig. 1).

ii) Stomach, transitional area: One specimen from approximately the middle of this region.

iii) Stomach, pyloric area: One specimen from approximately the middle of this region.

iv) Intestine, pyloric caecal region: One specimen from approximately the middle of this region. This was a section going through the intestine, the pyloric caeca and surrounding fatty tissue (See Fig. 1).

v) Mid-intestinal region: One specimen from approximately the middle of this region.

vi) Rectum: One specimen from approximately the middle of this region.

The specimens were immediately fixed in Bouins fluid at 0 °C and kept in the fixative for 3 days at 2-5 °C.

Dehydration, clearing and primary waxembedding was executed with a Histokine Automatic Tissue Processor (2 L Processor Mk II), as follows:

1. 70, 70 and 90 % alcohol for 3 hrs each.
2. 8 % phenol and 95 % alcohol for 2 hrs.
3. Absolute alcohol I and II for 2 hrs each.
4. Histoclear I and II for 2 hrs and 1 hr respectively.
5. Paraffin wax(BDH: Prod. no. 361078 F) 1 and 2 at 56-60 °C for 3 hrs each.

Tissue specimens were finally embedded in a paraffin wax(BDH: Prod. no. 361078 F) block at 56-60 °C and stored at 2-5 °C.

Specimens were sectioned (4-8 μm). The slides were kept at 60 °C for 40 minutes and

stained with haematoxylin and eosin (H and E). The staining procedure was modified from Bancroft and Stevens (1990: pp. 109, 112 and 700) as follows:

1. Histoclear I for 10 minutes.
2. Absolute alcohol I for 3-5 minutes.
3. Sections to water through graded alcohols:
    a. Absolute alcohol II for 3 minutes.
    b. 90, 70, 50 and 30 % alcohol for 3 minutes each.
    c. Distilled water for 3 minutes.
4. Harris's haematoxylin for 2 minutes.
5. Wash in running water.
6. Differentiate in acid alcohol; 3-4 dips.
7. Wash in running water.
8. Scott's tap water until blue for 10-15 seconds.
9. Wash in running water.
10. Examination under the microscope: Nuclei should appear blue, mucosa light blue and muscle transparent.
11. Differentiate nuclear stain further if necessary: Into haematoxylin, if the stain is too weak or into acid alcohol if the stain is too strong.
12. Counterstain in Putt's eosin for 4 minutes.
13. Differentiate in running water.
14. Examination under the microscope if necessary: Muscle and mucosa should appear fairly red.
15. Differentiate further if necessary: Into water, if the stain is too strong, or into Putt's eosin if the stain is too weak.
16. Sections dehydrated through graded alcohols:
    a. 30 % alcohol for 10 seconds.
    b. 50 % alcohol for 20 seconds.
    c. 70 and 90 % alcohol for 30 seconds each.
    d. Absolute alcohol III for 30 seconds.
17. Histoclear II for 5 minutes.
18. Histoclear III for 5 minutes.
19. Sections mounted with DPXmountant (BDH: Prod. no. 36029).

Slides were left for 3 days and then photographed on Fujicolor DXfilm, 160 ASA.

2.3. Transmission electron microscopic studies.

The protocol for the specimen sampling was identical to the one used for light microscopic studies
(See section 2.2. i-vi), except for the pyloric caecal region, which was sampled as follows:
Three specimens were taken from this region:

1. Pancreatic tissue and pyloric caecal fat. This was gently scraped off the gut wall and the pyloric caeca.
2. Pyloric caeca, a transverse section. All fat was carefully cleaned away.
3. Intestinal wall, a transverse section. All fat was carefully cleaned away.

The fixation procedures, dehydration, embedding and sectioning were adapted from Hayat, (1989).

After removal from the gut , the specimens were fixed at 0 °C, for 2 hrs. The fixative solution was made up as follows:

25 % glutaraldehyde (Sigma: G-5882)	10 ml
0.2 M phosphate buffer, pH = 7.4	50 ml
0.1 % calcium chloride	2 ml
distilled water	38 ml
sucrose	1 g

Osmolarity of this solution was 300 mOsm.

The phosphate buffer was made up as follows:

0.2 M KH_2PO_4.....19 ml.
0.2 M Na_2HPO_4.....81 ml.

The specimens were trimmed to a diameter of less than 1 mm in the fixative solution (the prefixsoln) and left at 0 °C for 2-3 hrs. These were then washed overnight at 2-5 °C

in a buffer rinse solution made up as follows:

0.2 M phosphate buffer, pH = 7.4	50 ml
0.1 % calcium chloride	2 ml
distilled water	48 ml
sucrose	1 g

After the rinse was changed 3 times, 5 minutes each, the specimens were postfixed for 1 hr in a solution comprising equal volumes of 2 % OsO_4 (in distilled water) and the buffer rinse. After this the following procedure for the specimens was executed:

1. Distilled water, 3x10 minutes.
2. 0.5 % uranyl acetate, 1 hour in the dark.
3. Quick rinse in distilled water.
4. 30, 50 and 70 % alcohol, 2x5 minutes each.
5. 1 % p-phenylenediamine in 70 % alcohol, for 1 hour under rotation .
6. 70 and 90 % alcohol, 2x5 minutes each.
7. Absolute alcohol, 3x10 minutes.
8. Dried absolute alcohol, 2x10 minutes.
9. Epoxypropane, 3x5 minutes.
10. 1:1 araldite embedding mixture: epoxy propane, under rotation overnight.
11. Araldite embedding mixture under vacuum for 2 hrs and then rotation for 5 hours.
12. Araldite vacuum embed for 4 hours.
13. Polymerise at 60 °C for 48 hours.

The embedded samples were left for ca. 24 hours after the polymerisation. Sections (80-100 nm) were cut with a glass knife on an ultra microtome (Reichart OME 3) and mounted on grids coated with 1 % formvar (Agar Scientific: Prod. no. R 1202).

The sections were stained as follows:

1. 2 % methanolic uranyl acetate in the dark < 5 minutes

2. Distilled water	1x30 seconds
3. Distilled water, gentle agitation	3x10 seconds
4. 0.02 N NaOH	1x30 seconds
5. Reynold's lead citrate in a Na OH chamber	< 5 minutes
6. 0.02 N NaOH	1x30 seconds
7. Distilled water	1x30 seconds
8. Distilled water, gentle agitation	3x10 seconds

After the staining, the grids were dried and the sections examined in a transmission electron microscope (Zeiss 902). Photographs were taken using Agfa Scientia: 23D56 film.

2.4. Scanning electron microscopic studies.

The fish were opened ventrally and transverse sections of 4-5 mm were removed from the pyloric caeca and the intestinal wall in the pyloric caecal region of the intestine (See Fig. 1). The tube-like specimens were placed in 0.2 M phosphate buffer, pH = 7.4 and opened by a single longitudinal incision. They were then pinned down on a piece of a cork with the luminal side uppermost. The pyloric caeca was jet washed 10 times and the intestinal wall 20 times with the phosphate buffer, from a 1ml pipette.

The specimens were then fixed, post fixed, stained with uranyl acetate and quickly rinsed in distilled water, as in the transmission electron microscopic studies (See section 2.3.). The dehydration was done with 30, 50, 70 and 90 % acetone, 2x5 minutes each, and subsequently 100 % and 100 % dried acetone, 2x10 minutes each. Specimens were now processed according to the criticl point drying method (Anderson, 1951) as described by Hayat (1978) and stored desiccated.

The specimens were mounted with carbon tape on aluminium stubs. The carbon tape was covered with a conductive silver paint. Finally the specimens were gold coated by sputtering (Polaron E 5000 sputtercoater) for 30 minutes. The specimens were then examined with a scanning electron microscope (Philips SEM 500) and photographed with Kodak Plus-Xfilm.

3.Results.

The gross anatomy of the salmon gut is displayed in Figure 1 (a). The terminology presented in Figure 1 (b) is similar to that presented by Weinreb and Bilstad (1955).

3.1. The cardiac and the transitional area.

The cardiac area, stretching from the airbladder duct to the transitional area, contains the mucosa, with its surface epithelium and its stomach glands, the submucosa, the muscularis externa and serosa (Fig. 2 (a) and 2 (b)). The submucosa became less prominent as one moved down the cardiac area and the primary folds become more prominent and the stomach glands occupy a proportionally greater area. The gastric pits lead from the lumen and frequently extend into one third of the mucosa. The stomach glands gradually disappear in the transitional area (Fig. 2 (d)) and the stratum granulosum appears somewhat thinner than in the cardiac area (Fig. 2 (c)).

The lumen of the cardiac area is covered by a tall columnar epithelium, with a poorly developed apical brush border. Junctional complexes are frequently seen between the columnar epithelial cells at the level of the terminal web. The terminal web is a cytoskeletal network right basalt to the bruh border (Fig. 5(c)) giving mechanical strength to the epitelial cells. This network is connected between cells at the desmosomes. The epithelium of the gastric mucosa contains cells with numerous fine granules, which are mainly situated on the basal laminal side of the nucleus. These cells are found throughout the cardiac area of the stomach, but also in the pyloric area, where they are more frequent. These cells are similar in appearance to the so called " type II endocrine cells " of rainbow trout (Ezeasor, 1978) and the endocrine cells of perch (Noaillac-Depeyre and Gas, 1978).

The stomach glands are situated beneath the epithelium (Fig. 2 (a) and 2 (b)). These are simple, sometimes branched tubular glands, and open into the gastric pits (Fig. 2 (c)). The gland cells are situated radially around a narrow central lumen (Fig. 3). Their apical surface contains scattered and sinusoidal microvilli. The apical cytoplasm of these cells consists of a tubulovesicular network of smooth membranes, whereas the supra nuclear

Figure 1: Gross anatomy of the gut of the Atlantic salmon. (**a**) Overall view of the gut and its related components, such as the airbladder, gallbladder and liver.


(a)

Figure 1: Gross anatomy of the gut of the Atlantic salmon. (**b**) Schematic map explaining the terminology of the gut. **EP** oesophagus posterior; **AD** airbladder duct; **S** stomach: **CA** cardiac area, **TA** transitional area, **PA** pyloric area; **PT** pancreatic tissue; **PC** pyloric caeca; **I** intestine: **AI** ascending intestine (**PCA** pyloric caecal region and **MI** mid intestinal region) and **DI** descending intestine (rectum).

(b)

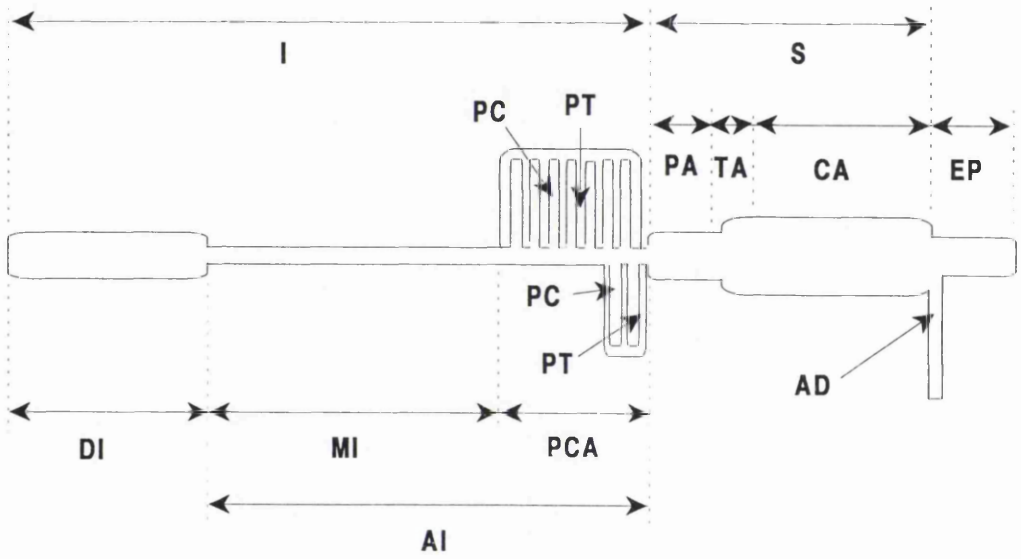


Figure 2: Sections from the cardiac and transitional area of the stomach (Haematoxylin and Eosin). (**a**) Transverse section from the whole upper cardiac area. Bar equals 200 μm. **L** lumen; **PF** primary fold; **GP** gastric pits; **E** epithelium; **GG** stomach glands; **SM** submucosa; **M** muscularis externa; **S** serosa. (**b**) Transverse section from the whole lower cardiac area. Bar equals 200 μm. Abbreviations as for (a).

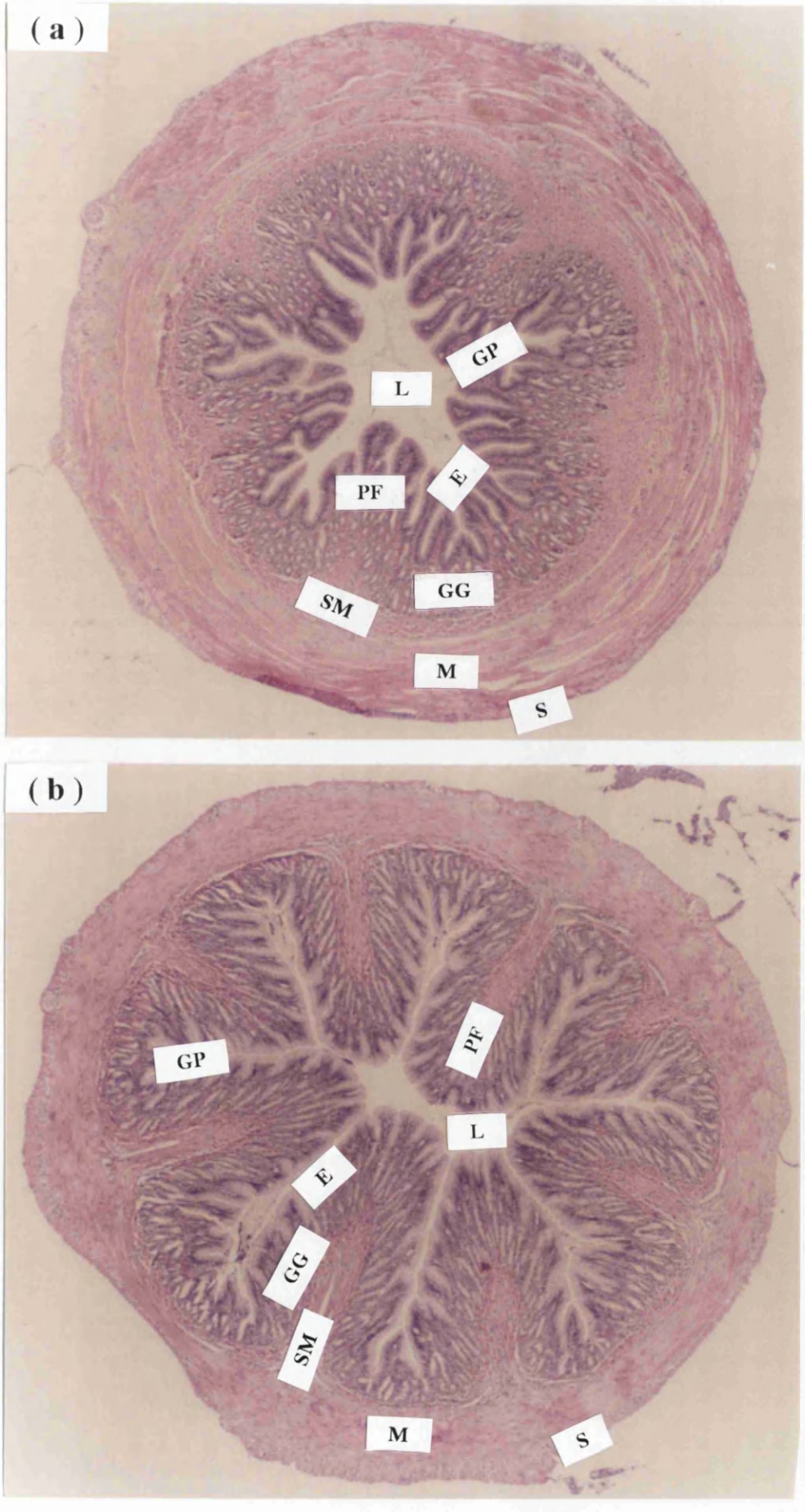

(a)
GP
L
E
PF
GG
SM
M
S
(b)
PF
GP
L
E
GG
SM
M
S

Figure 2: Sections from the cardiac and transitional area of the stomach (Haematoxylin and Eosin). (**c**) Section showing the stomach glands in the cardiac area. Note the apparent tubular shape of the glands. Bar equals 30 μm. **GP** gasric pits; **E** epithelium; **GG** stomach glands; **SG** stratum granulosum; **SC** stratum compactum.

SC
SG
GG
GG
GP
GP
E
(c)

Figure 2: Sections from the cardiac and transitional area of the stomach (Haematoxylin and Eosin). (**d**) Section from the transitional area of the stomach. Note the disappearing stomach glands and the thin stratum granulosum. Bar equals 30 µm. **GP** gasric pits; **E** epithelium; **GG** stomach glands; **SG** stratum granulosum; **SC** stratum compactum; **M** muscularis externa.

M
SG
SC
GG
GG
E
GP
(d)

Figure 3: T.E.M. section from the cardiac area of the stomach; transverse section from gastric gland. Bar equals 10 µm. **L** lumen; **MV** microvilli; **TVN** tubulovesicular network; **G** granules; **M** mitochondria; **RER** rough endoplasmic reticulum.

M
RER
G
M
RER
M
RER
TVN
TVN
MV
L
TVN
G
M
G
M
G

area, i.e. the area of the cytoplasm immediately on the luminal side of the nucleus, contains round and electron dense granules and mitochondria. Frequently in this area, and invariably in the basal cytoplasm, a rough endoplasmic reticulum is seen.

3.2. The pyloric area.

The pyloric area stretches from the transitional area to the pyloric sphincter. It is basically similar to the cardiac area, except that it lacks stomach glands and the stratum granulosum appears somewhat thinner (Fig. 4 (a) and 4 (b)). The muscularis circularis is also thicker. Endocrine cells are found throughout this region. These are similar in appearance to those of the cardiac area (See section 3.1.), but occur more frequently.

3.3. The intestine.

The pyloric caeca appear as a numerous finger-like projections from the pyloric caecal region (Fig. 1), surrounded by fatty tissue. The walls of the caeca and the remaining part of the intestine appear histologically fairly similar in structure (Fig. 5 (a) and 5 (b)). These comprise mucosa, muscularis externa and serosa. The intestine lacks a true submucosa and a muscularis mucosa. The mucosa is thrown into large primary folds. It is in turn composed of an epithelium, stratum granulosum and stratum compactum. The epithelium is tall columnar (Fig. 5 (c) and 5 (d)). In passing from the ascending to the descending intestine, the following observations may be made:

i) The brush border becomes shorter: 2.89-3.16 μm in length in the ascending intetine and 0.79-1.45 μm in length in the descending intestine.

ii) The smooth endoplasmic reticulum becomes finer: 0.53-1.46 μm appearing in length in the ascending intetine and 0.09-0.39 μm appearing in length in the descending intestine.

iii) The epithelial cells containing electron dense granules increase from being nonexistent in the pyloric caecal region to becoming ca. 95-100 % in the rectum. The granules within these cells also inceased, having an apparent diameter of 0.26-0.66 μm in the mid-intetinal area and 0.46-2.51 μm in the rectum (Fig. 5 (d)).

It is likely, that the electron dense granules (EDG) seen in Fig. 5 (c) are within a secretory duct coming from the pancreatic tissue (See later), because epithelial cells in this

Figure 4: Sections from the pyloric area of the stomach. (Haematoxylin and Eosin). (**a**) Transverse section through the whole pyloric antrum. Bar equals 200 µm. **PF** primary fold; **M** mucosa; **SM** submucosa; **ME** muscularis externa; **S** serosa.

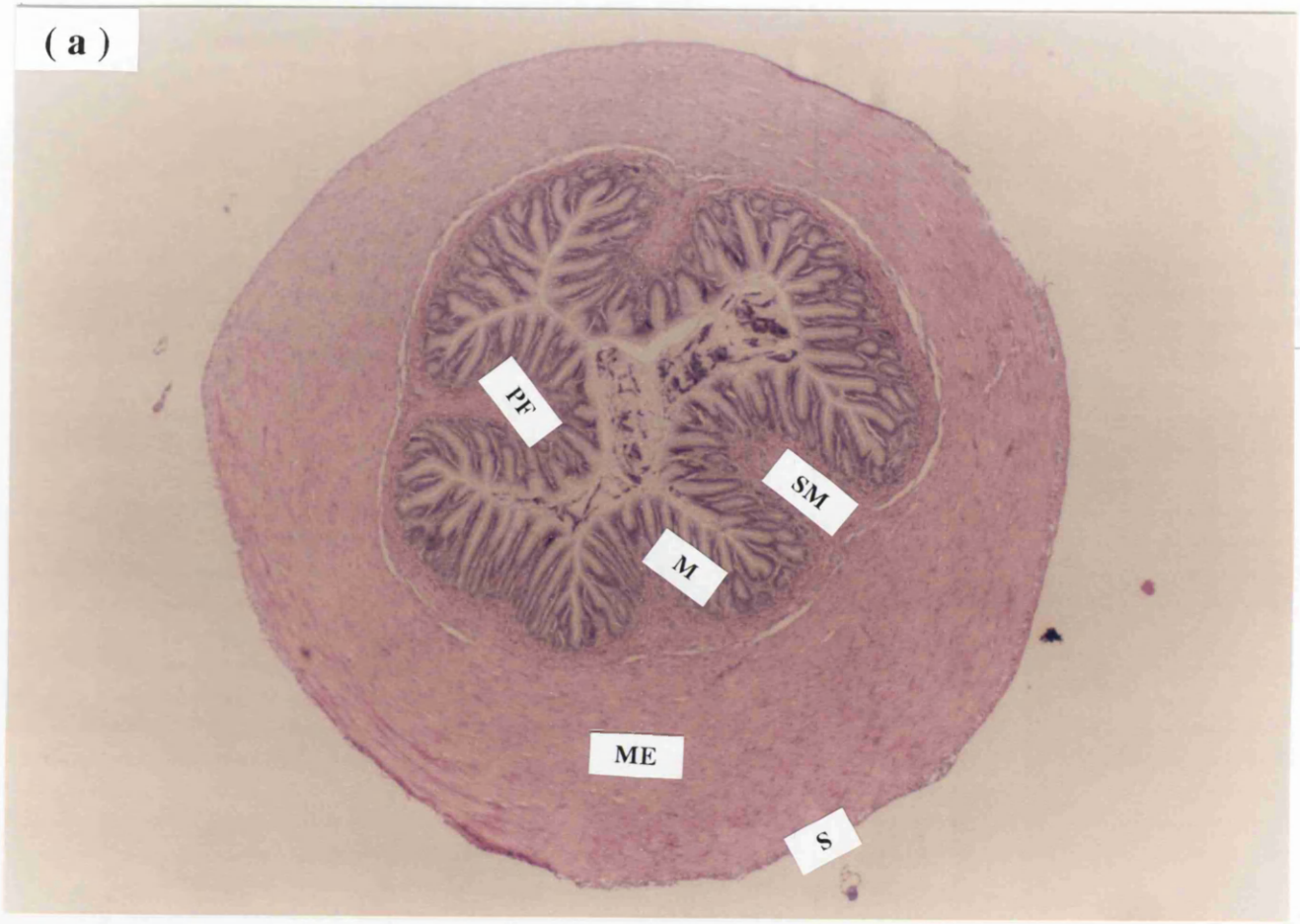

(a)
PF
SM
M
ME
S

Figure 4: Sections from the pyloric area of the stomach. (Haematoxylin and Eosin). (**b**) Section from the mucosa of the pyloric area. Bar equals 30 μm. **GP** gastric pit; **E** epithelium; **PG** pyloric glands; **SG** stratum granulosum; **SC** stratum compactum.

GP
E
E
PG
PG
SG
SC
SC
(b)

Figure 5: Sections from the intestine. (**a**) Transverse section from the pyloric caeca and the surrounding fat and pancreatic tissue. (Haematoxylin and Eosin). Bar equals 100 μm. **PC** pyloric caeca; **E** epithelium; **SG** stratum granulosum; **M** mucosa; **ME** muscularis externa; **PT** pancreatic tissue; **FC** fat cells; **D** duct; **A** arteriole.

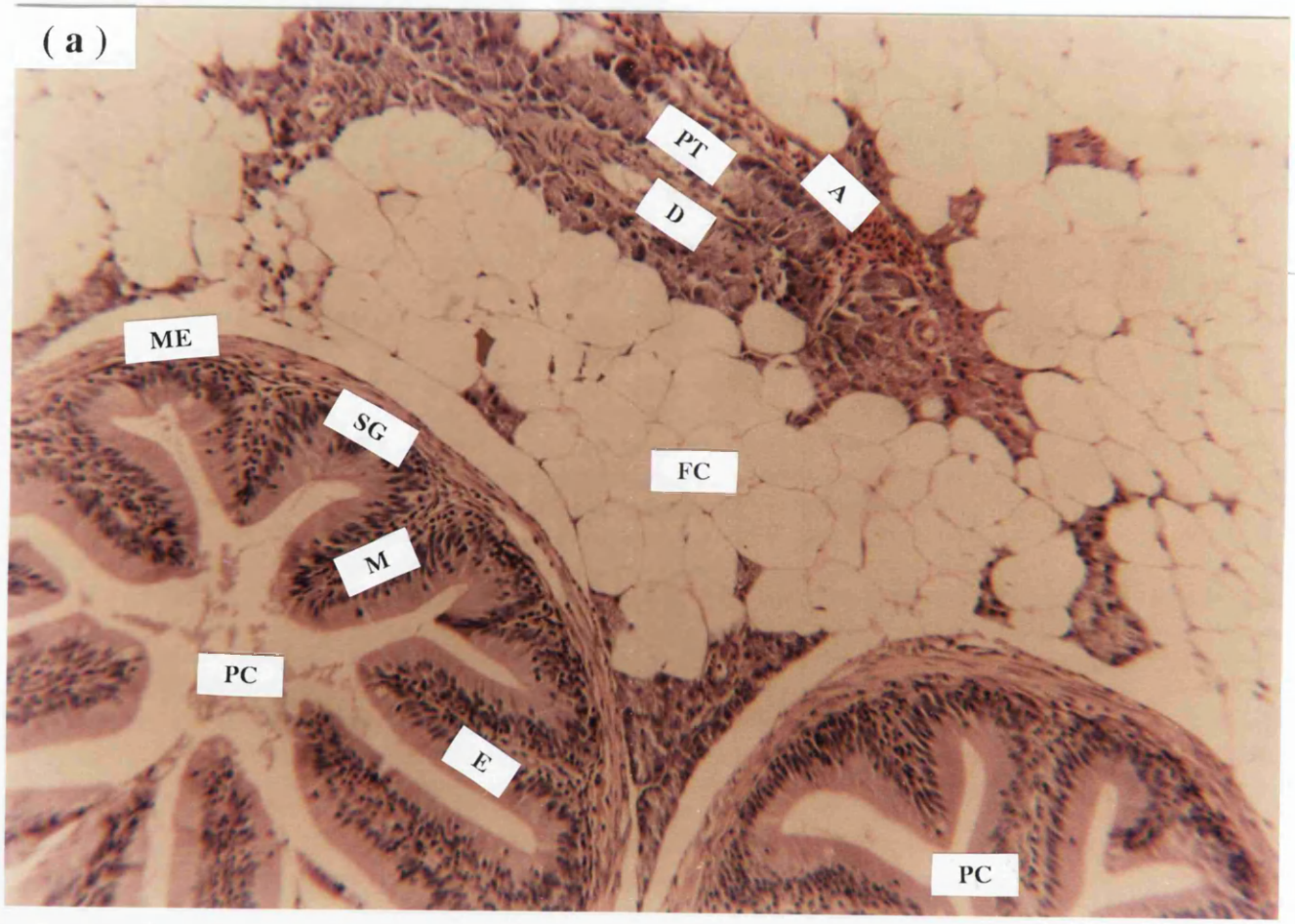

(a)
PT
A
D
ME
SG
FC
M
PC
E
PC

Figure 5: Sections from the intestine. **(b)** Section from the ascending intestinal wall. (Haematoxylin and Eosin). Bar equals 30 μm. **E** epithelium; **GC** goblet cell; **BM** basement membrane; **SG** stratum granulosum; **SC** stratum compactum; **ME** muscularis externa; **S** serosa.

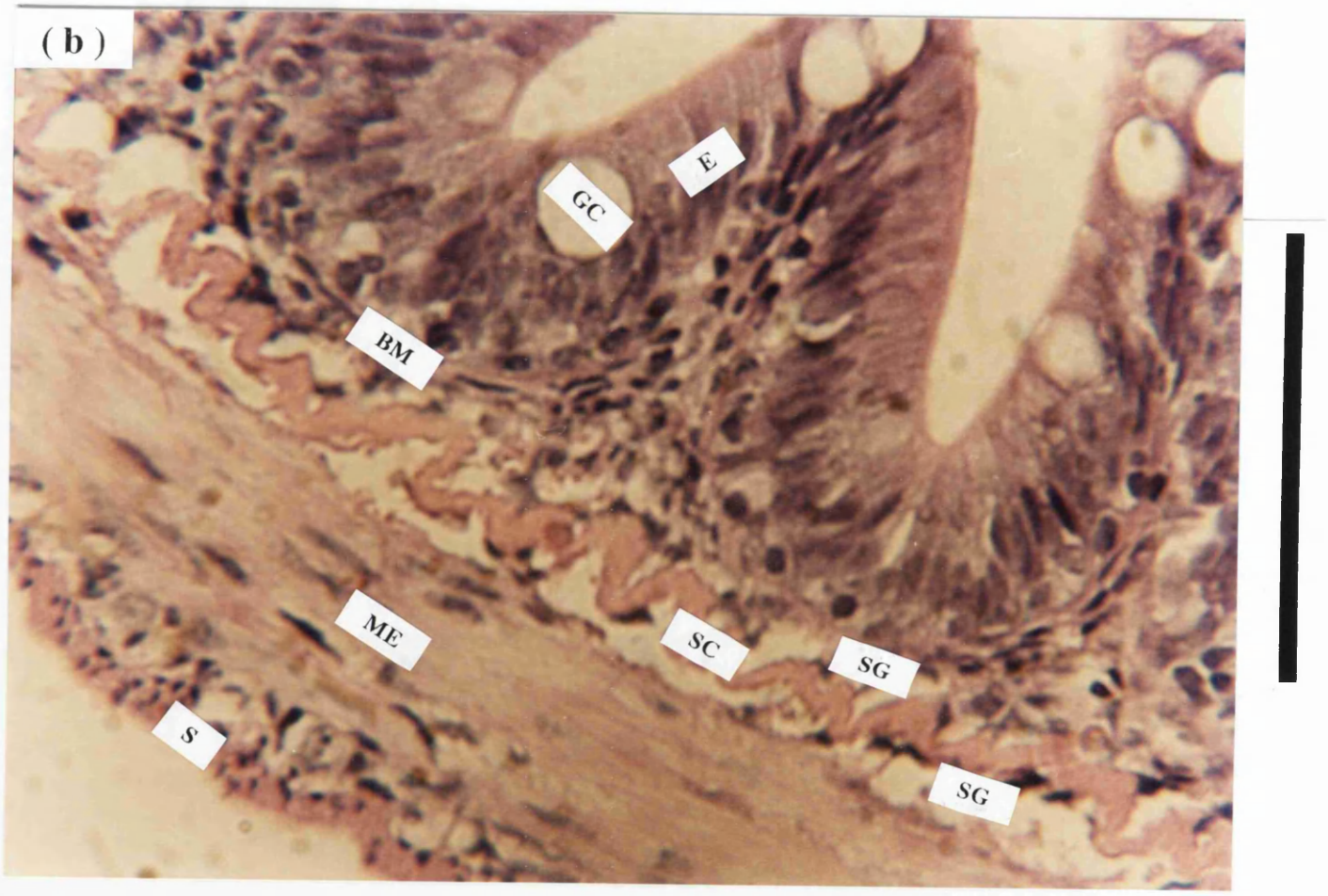

(b)
GC
E
BM
ME
SC
SG
S
SG

Figure 5: Sections from the intestine. **(c)** T.E.M. section from the epithelium of the pyloric caecal region. Bar equals 10 μm. **BB** brush border; **TW** terminal web; **SMR** smooth endoplasmic reticulum; **GC** goblet cell; **EDG** electron dense granules.

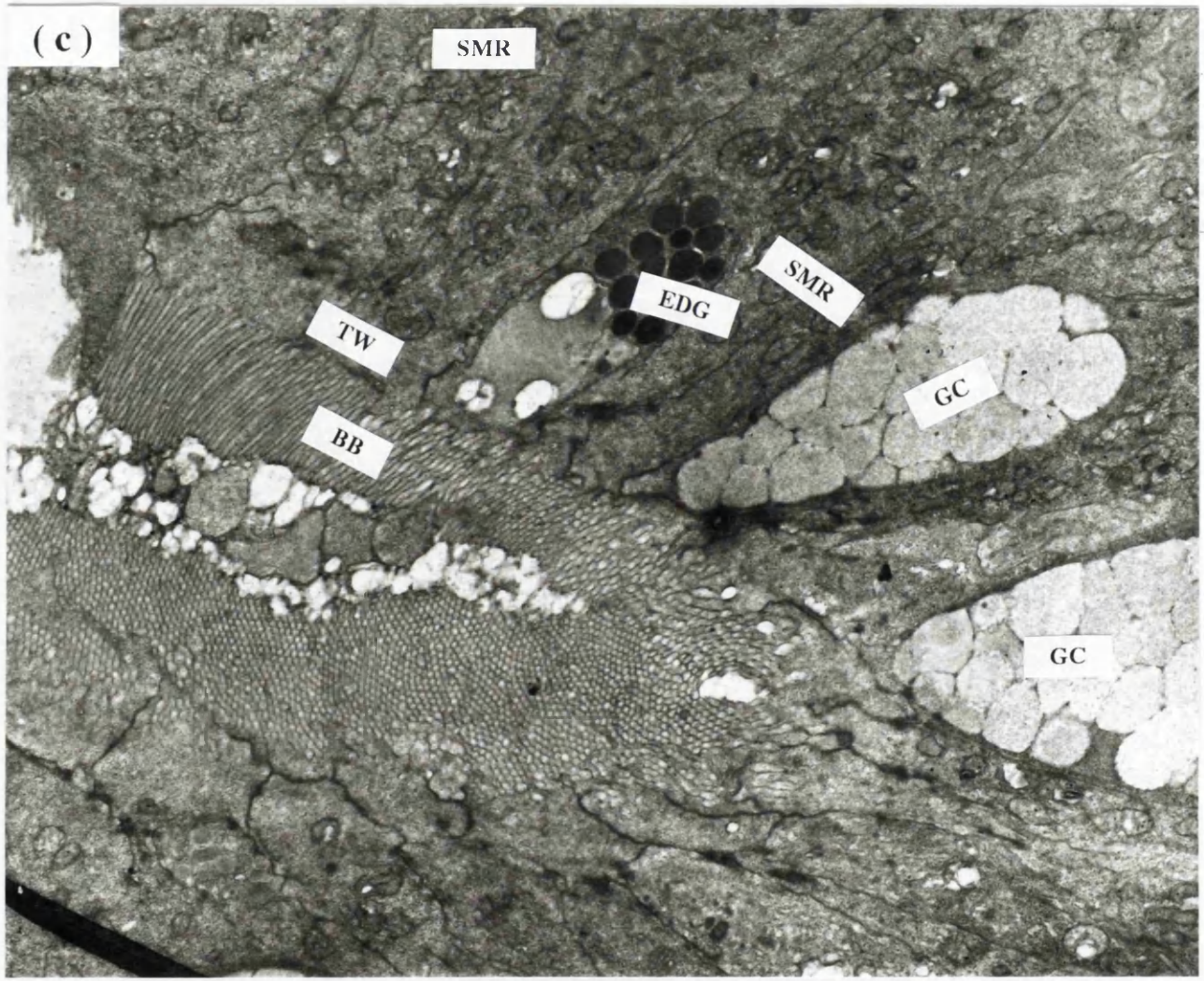

(c)
SMR
SMR
EDG
TW
BB
GC
GC

Figure 5: Sections from the intestine. (**d**) Section from the epithelium of the rectum. Bar equals 10 μm. **BB** brush border; **TW** terminal web; **SMR** smooth endoplasmic reticulum; **GC** goblet cell; **EDG** electron dense granules.

TW
BB
TW
SMR
EDG
GC
BB
SMR
EDG
(d)

area, i.e. the pyloric caecal area were not found to contain such electron dense granules. Epithelial cells in the mid intestine were clearly observed to contain electron dense granules. These cells are identical in appearance to comparable cells in the rectum (Fig. 5 (d)), but with fewer and smaller granules (See before). Goblet cells, frequently discharging, were observed throughout the intestine.

Endocrine cells of a similar structure to the so called "type II endocrine cells" found in the rainbow trout intestine (Ezeasor, 1978) were observed in the ascending intestine (Fig. 6 (a) and 6 (b)). These frequently occur in groups and appear to be more frequent in the pyloric caecal region, where they are usually situated at the base of the intestinal folds. These cells are long and thin and appear to stretch from the lumen down to the basal lamina. They have long thin nuclei (Fig. 6 (a)), and have distinctive fine electron-dense granules, which are invariably located on the basal laminar side of the nucleus (Fig. 6 (a) and 6 (b)).

The muscularis is somewhat thinner in the pyloric caeca, than in the remaining part of the intestine (Fig. 5 (a) and 5 (b)) and also thinner than in the stomach.

The luminal surface of the pyloric caecal region of the intestine is characterised by duct openings of various sizes. These were observed on the primary folds of the pyloric caeca (Fig. 7 (a)). These duct openings also occur on the primary folds in the intestinal wall in the pyloric caecal region (Fig. 7 (b)) and were either seen as singular (Fig. 7 (b)), or composed of 2 or 3 smaller openings.

3.4. Pancreatic tissue.

The pancreatic tissue can be seen as strands embedded in the fat surrounding the pyloric caeca (Fig. 5 (a)). The tissue was occasionally observed as acini. The cytoplasm of the haematoxylin and eosin stained pancreatic cells was seen to become somewhat brighter towards the acinar lumen, i.e. the apical region, indicating the presence of secretory granules. The typical pancreatic acinar cell could be described as follows: From the apical region to the nucleus, numerous granules, condensing vacuoles and mitochondria were observed (Fig. 9 (a)). The endoplasmic reticulum appeared vesicular here. Basally to the

Figure 6: T.E.M. sections from the epithelium of the intestinal mucosa in the pyloric caecal region, showing endocrine cells. (**a**) Section showing how the granules are situated on the basal laminal side of the nucleus. Bar equals 10 μm. **L** to lumen; **N** nuclei; **G** granules.

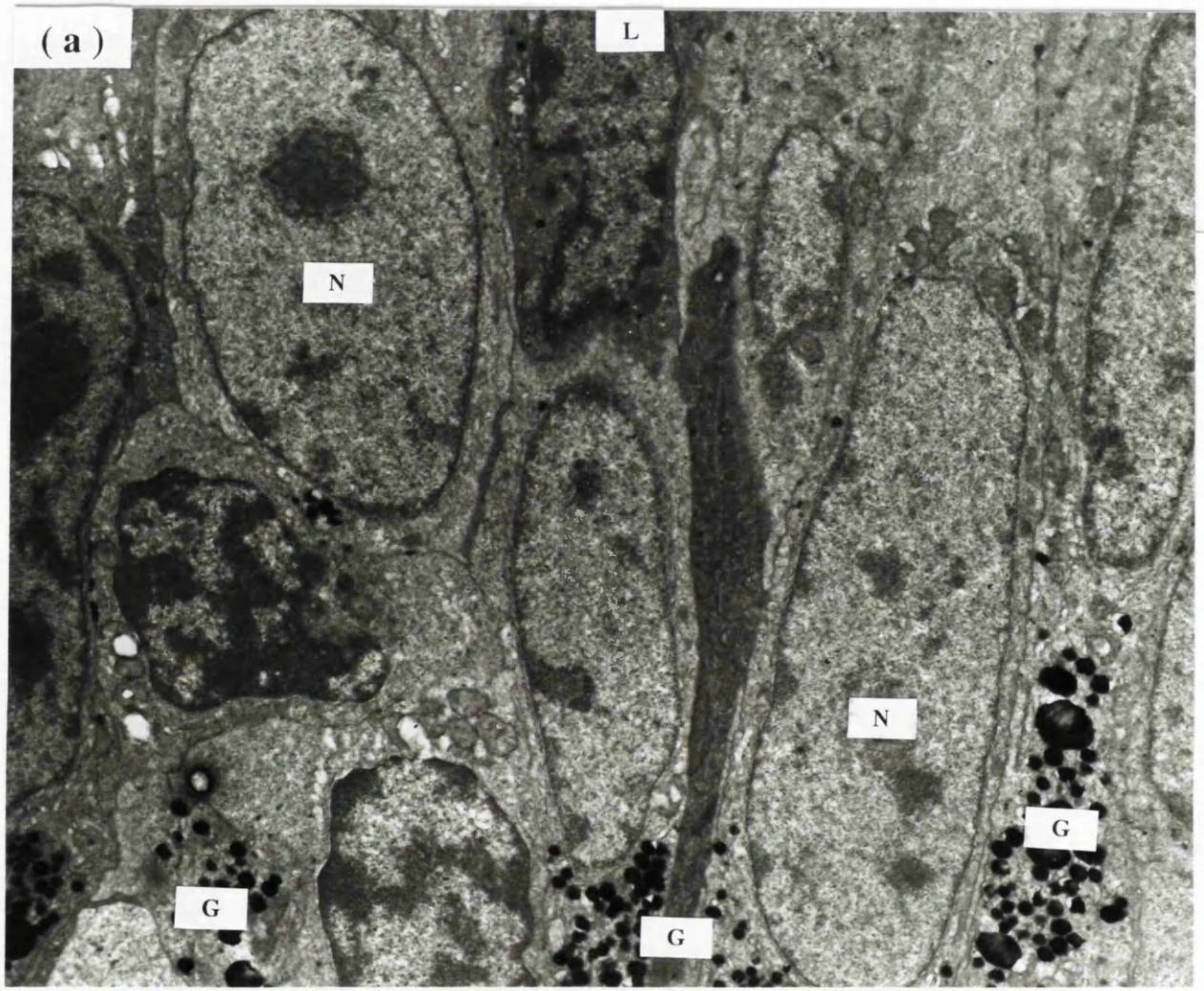
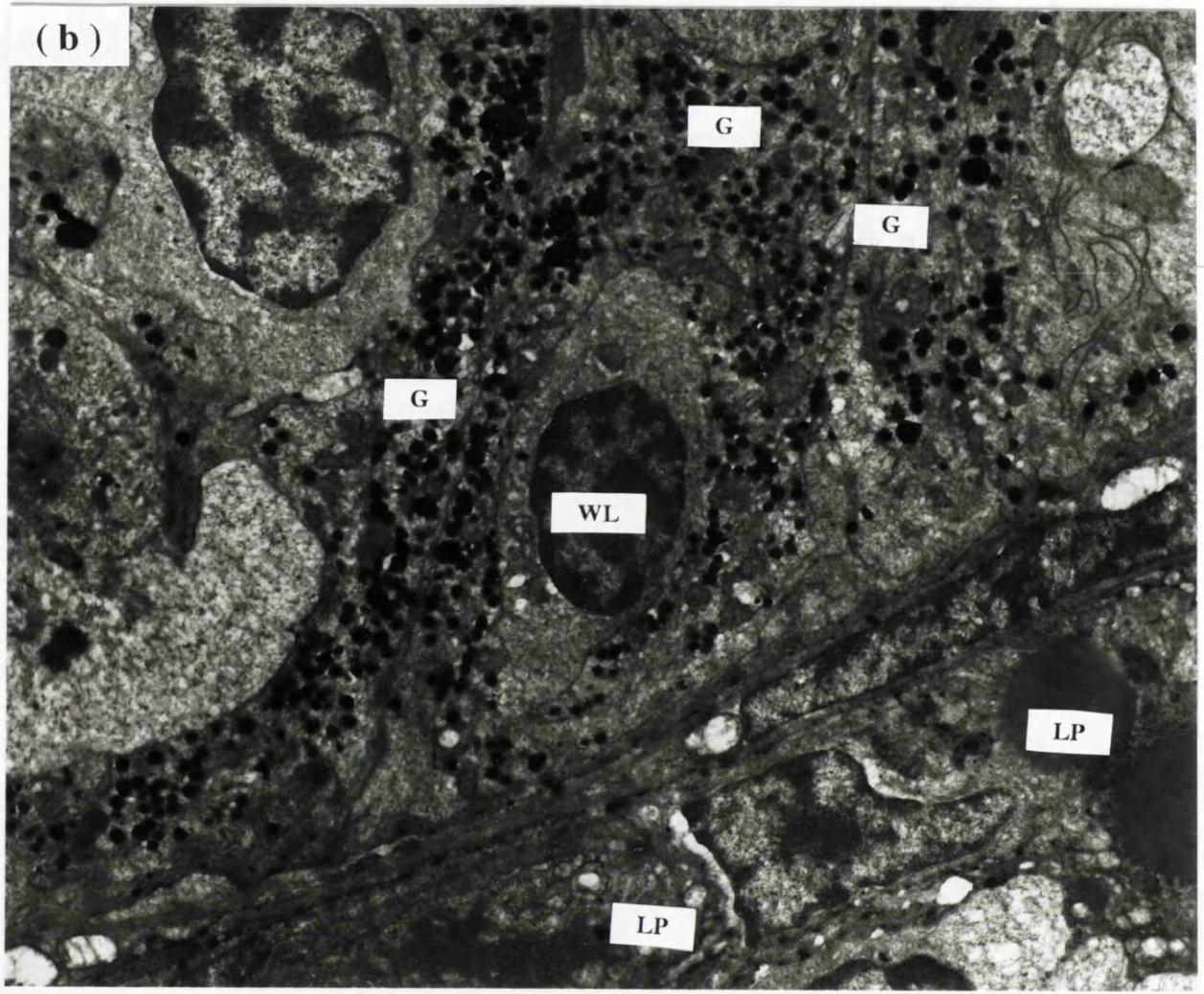

(a)
L
N
N
G
G
G

Figure 6: T.E.M. sections from the epithelium of the intestinal mucosa in the pyloric caecal region, showing endocrine cells. (**b**) Section showing the cytoplasm of the endocrine cells packed with granules, where it makes contact with the lamina propria. Bar equals 10 μm. **G** granules; **LP** lamina propria; **WL** wandering lymphocyte.


(b)
G
G
G
WL
LP
LP

nucleus, the only structure observed was an extensive lamellar endoplasmic reticulum. Occasionally, pancreatic acinar cells were encountered, where the endoplasmic reticulum was thrown into extensive sheets and whorls and occupied the whole cytoplasm.

Ducts and arteries are frequently encountered in the strands of acinar cells embedded in the fatty tissue surrounding the pyloric caeca (Fig. 5 (a)). Duct-like structures were also observed, going from the pancreatic tissue into the muscularis of the pyloric caeca (Fig. 8 (b)) and also directly into the intestinal wall in the pyloric caecal region. The duct openings observed on the luminal surface of the pyloric caeca (Fig. 7 (a)) and on the luminal surface of the intestine in the pyloric caecal region (Fig. 7 (b)) are probably the openings of these ducts.

Islets of Langerhans tissue (Brockmann bodies) are frequently seen scattered in the pancreatic tissue
(Fig. 8 (a)). The islet cells have irregular nuclei and their cytoplasm contains electron dense tiny granules (Fig. 9 (b)).

4. Discussion.

4.1. The cardiac stomach and the transitional area.

The stomach epithelium is similar to the surface mucous cells found in the perch (Noaillac-Depeyre and Gas, 1978). In the rainbow trout, stubby microvilli are found only on the cell boundaries in the stomach epithelium (Ezeasor and Stokoe, 1980). In the present study similar results to those found for the rainbow trout were observed: either small and stubby microvilli, or none at all. The results suggest that very limited absorption of nutrients occurs in the stomach.

The mucous secreting cells in the epithelium of the cardiac area have the same appearance as comparable cells described for the perch (Noaillac-Depeyre and Gas, 1978). The absence of goblet cells in this area is in accordance with findings on pike (Bucke, 1971).

The existence of endocrine cells in the cardiac area confirms findings on the perch

Figure 7: A scanning electron micrograph of duct openings of the luminal surface of the pyloric caecal area of the intestine. (a) Duct openings on the surface of the primary fold of a pyloric caeca. Note the polygonal pattern on the surface. Bar equals 30 μm.

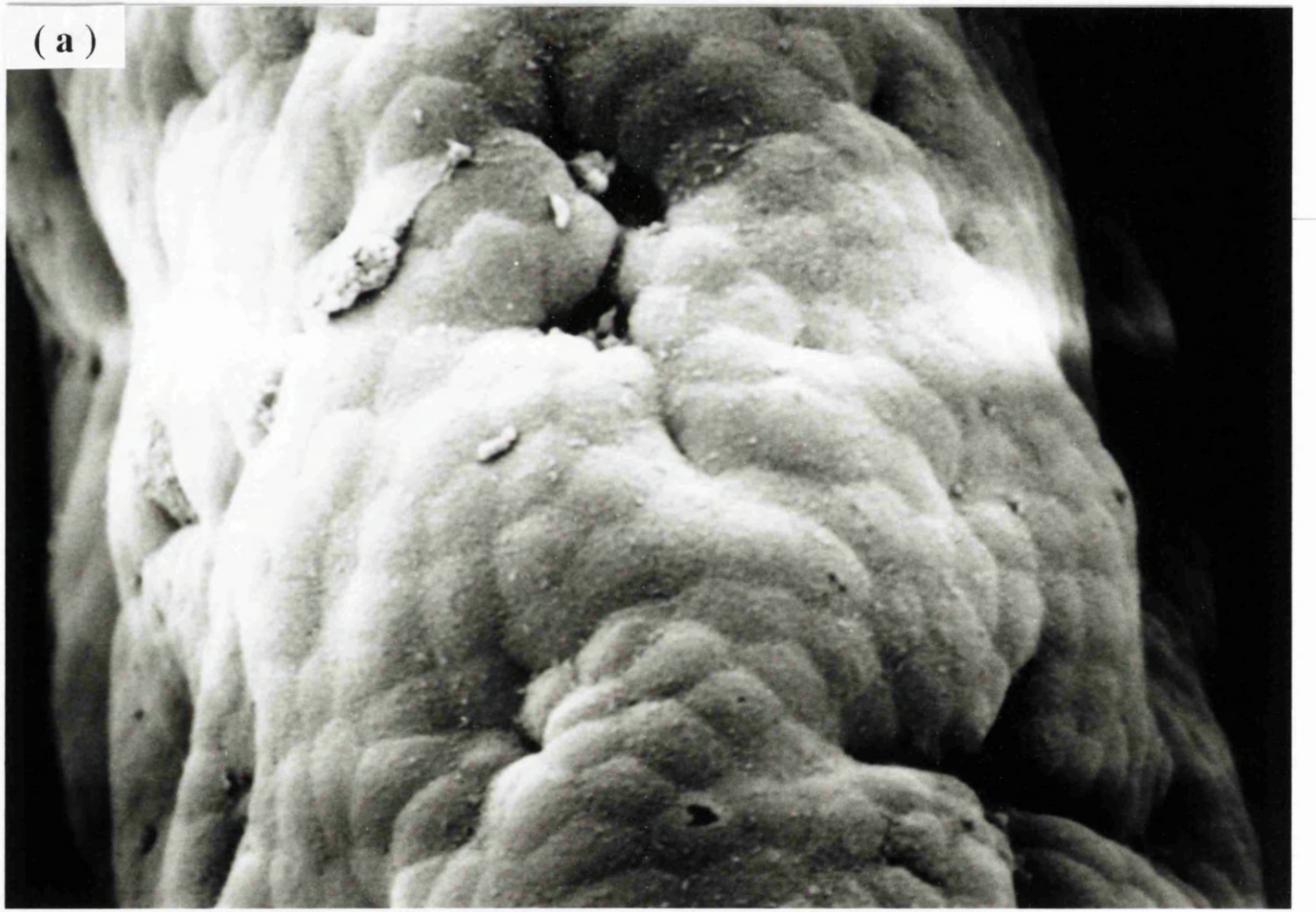

(a)

Figure 7: A scanning electron micrograph of duct openings of the luminal surface of the pyloric caecal area of the intestine. (**b**) Duct opening on the surface of the intestinal wall lumen, partially clogged by mucus. Bar equals 5 μm.


(b)

Figure 8: Sections from the pancreatic tissue (Haematoxylin and Eosin). (a) Pancreatic tissue containing Islet of Langerhans. Bar equals 30 μm. **IL** islet of Langerhans; **PT** pancreatic tissue; **FC** fat cells.

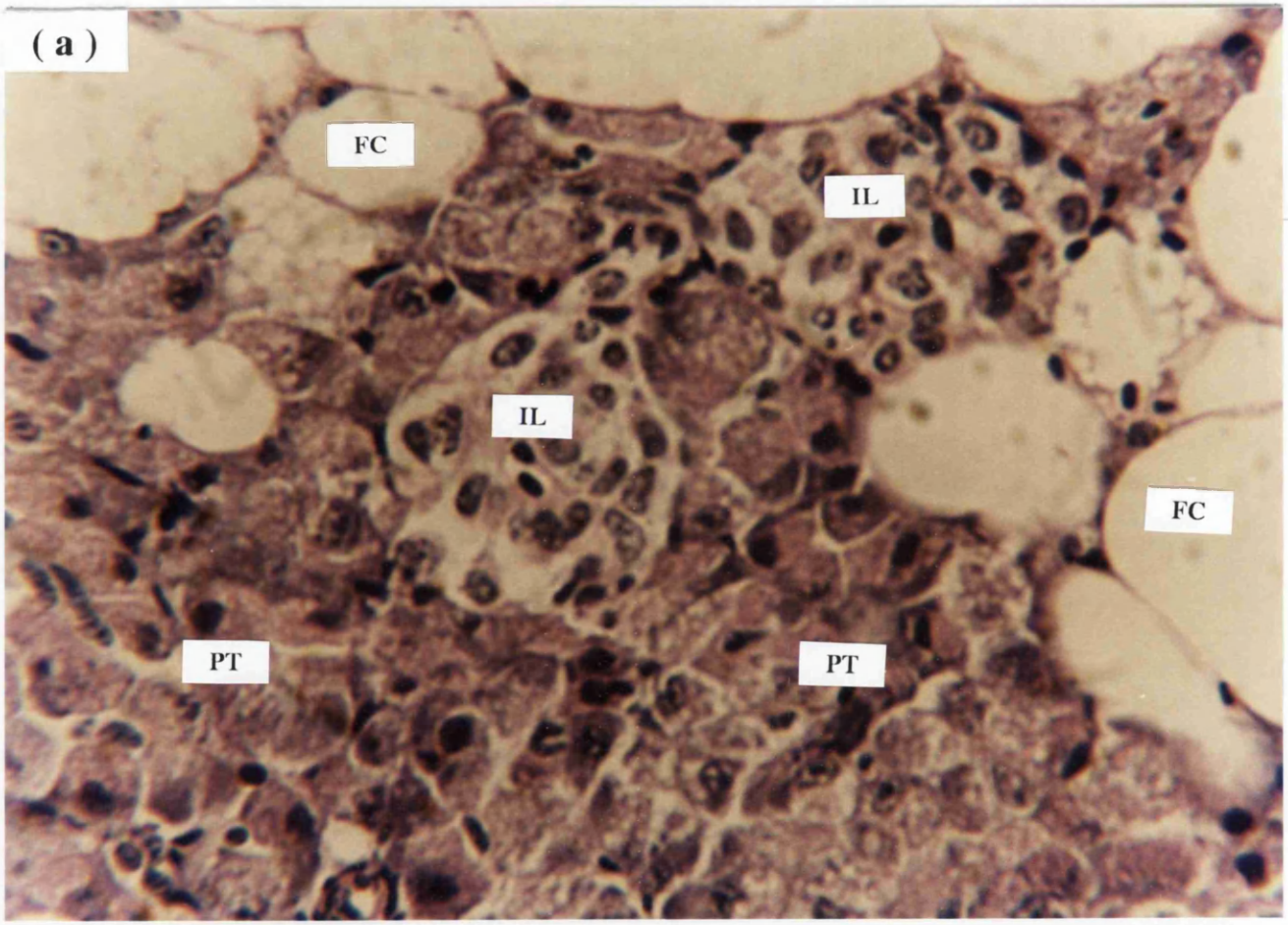

(a)
FC
IL
IL
FC
PT
PT

Figure 8: Sections from the pancreatic tissue (Haematoxylin and Eosin). **(b)** Pancreatic duct. Bar equals 30 μm. **PT** pancreatic tissue; **PD** pancreatic duct; **PC** pyloric caeca.

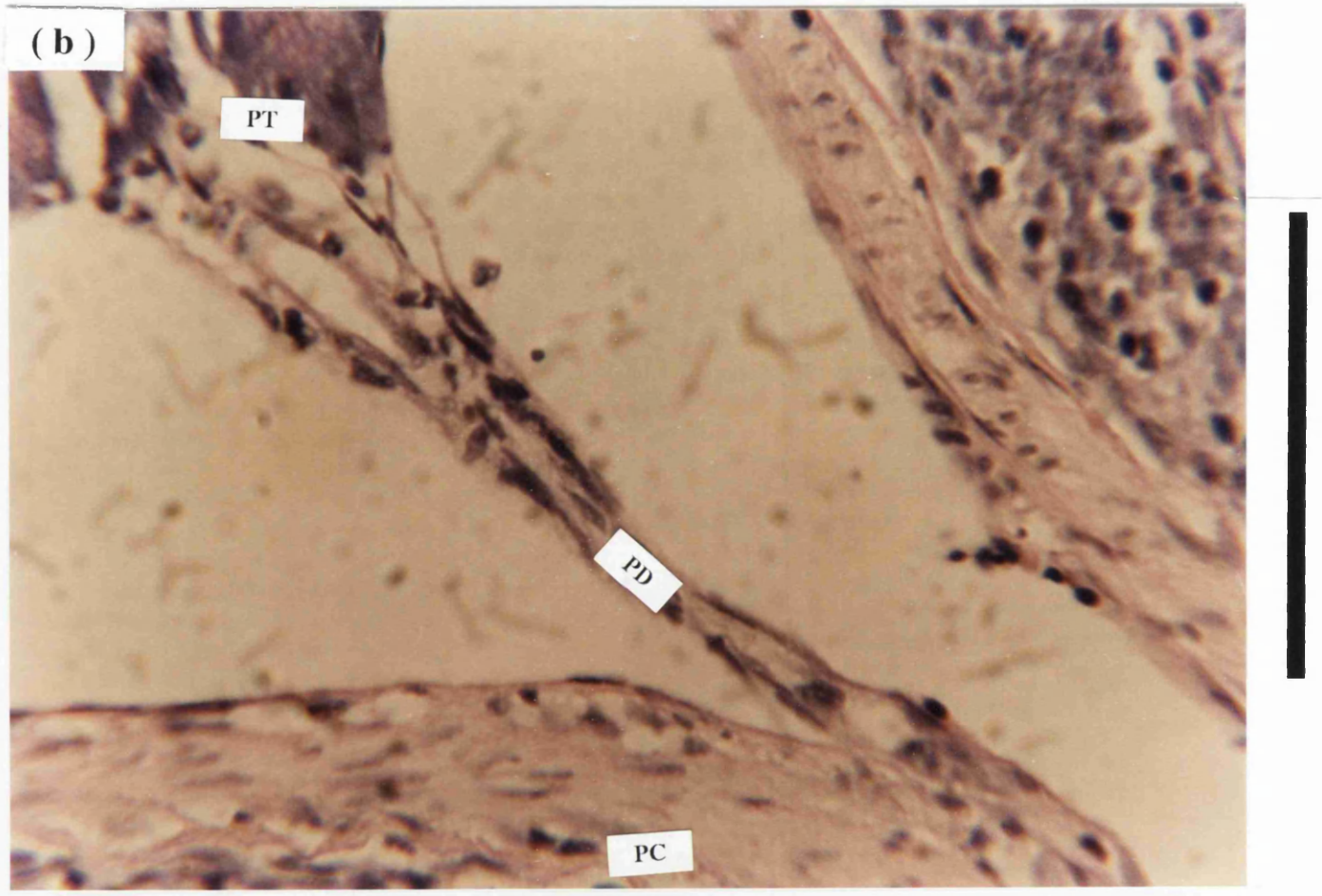

(b)
PT
PD
PC

Figure 9: T.E.M. sections from the pancreatic tissue. (a) Exocrine pancreatic cells. Bar equals 5 μm. **N** nucleus; **G** granules; **CV** condensing vacuoles; **VSER** vesicular smooth endoplasmic reticulum; **M** mitochondria.

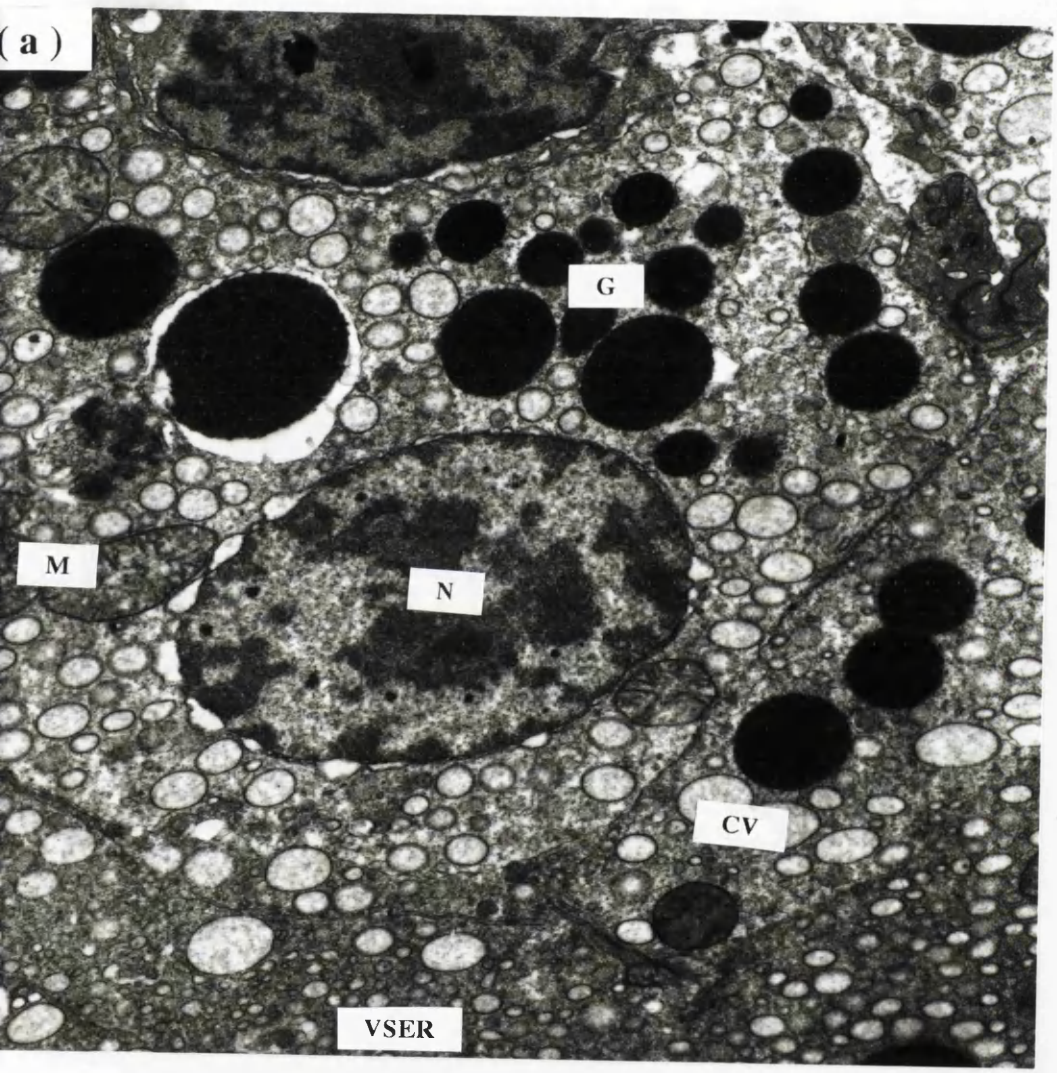
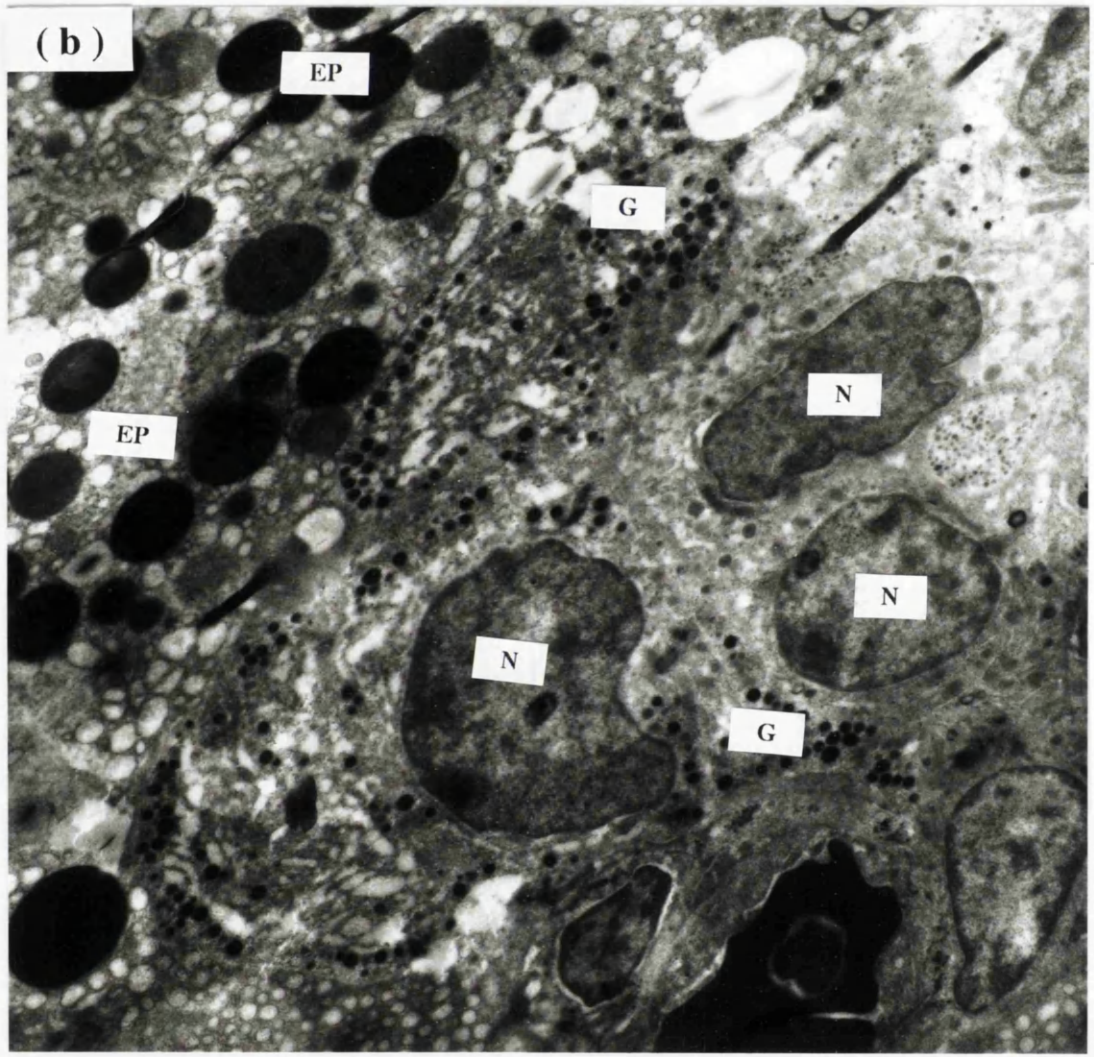

(a)
G
M
N
CV
VSER

Figure 9: T.E.M. sections from the pancreatic tissue. (b) Endocrine pancreatic cells (Islet of Langerhans cells). Bar equals 10 μm. **N** nucleus; **G** granules; **EP** exocrine pancreas.


(b)
EP
G
N
EP
N
N
G

(Noaillac-Depeyre and Gas, 1978). These cells are similar in structure to the so called " type II endocrine cells" found in a comparable region in rainbow trout (Ezeasor, 1978). Gastrin/CCK-like immunoreactive endocrine cells have been found in the lower part of the cardiac area in the Atlantic cod (Jönsson *et al.*, 1987).

The location and the characterisation of the stomach glands of the Atlantic salmon was in good agreement with previous studies on rainbow trout (Weinreb and Bilstad, 1955; Yasutake and Wales, 1983). These glands appear to be simple tubular, and open into the gastric pits. The glandular tissue appears to increase as one goes from the anterior cardiac area to the posterior cardiac area. This would predict that the highest stored pepsinogen concentration would be in the posterior cardiac area, where the stomach is most extensible.

The transitional area shows the gradual disappearance and appearance of the characteristics of the cardiac and the pyloric area respectively. These results were in good agreement with findings on the rainbow trout (Weinreb and Bilstad, 1955).

The gland cells in the rainbow trout were found to be similar in appearance to the chief cells in mammals (*Ibid.*) and similar to those described for the king salmon (Greene, 1912). The glandular cells in the stomach of the teleost are thought to secrete both acid and pepsinogen (Barrington, 1957; Iro, 1967; Smit, 1968). A histochemical demonstration of hydrochloric acid in the gastric tubules of fingerling brown trout (Western and Jennings, 1970) and ultrastructural studies of the gastric gland cells in the perch (Noaillac-Depeyre and Gas, 1978) support this. The present study of the ultrastructure of the stomach gland cells is also in good agreement with their description. These cells have the structure of protein secreting cells: The rough endoplasmic reticulum is located basally and at the level of the nucleus, whilst electron-dense granules are found in the supra nuclear region of these cells. These granules are similar in structure and cytochemical properties to the chief cell granules of mammals (*Ibid.*). They are probably the source of pepsin activity in fish gastric juice (Norris and Mathies, 1953; Kitamikado and Tachino, 1960; Kitamikado *et al.*, 1965). The similarity of these cells to protein secreting cells, suggests that the granules are being synthesised in the same manner as protein, and are being secreted.

The tubulovesicular network is found in the apical region of these cells. This is a well developed network of smooth membranes. A similar system appears in the parietal cells of mammals, but also in chloride cells of fish (Pilpott and Copeland, 1963). These tubulovesicles are believed to migrate towards the apical cell surface, fuse with the apical plasma membrane and release their acid into the gastric lumen, during acid secretion (Noaillac-Depeyre and Gas, 1978). Stimulated gland cells in rainbow trout showed the tubulovesicular system making contact with the luminal surface (Ezeasor, 1978). The hypothesis that the gland cells of the teleostei stomach secrete both acid and pepsinogen is well founded. The present results give support to this hypothesis.

The stomach can be regarded as a processing tank, where proteins are made accessible to proteolytic breakdown by acid denaturation. Proteolytic breakdown commences in the stomach. Stomachless fish are known to have a lower apparent K_M value for their trypsins, than fish with a stomach (Hofer *et al.*, 1975). The trypsins in the stomachless fish have to attack native protein, in contrast to fish with a stomach. These enzymes must therefore have a greater catalytic efficiency, which is partially reflected in a lower apparent K_M value. The low pH of the stomach also serves as a defence mechanism against infection (Barrington, 1942).

4.2. The pyloric area.

This area was found to be basically similar to the cardiac area of the stomach, except that stomach glands are lacking and the muscularis circularis is thicker here, than in the cardiac area. The thickening of the muscularis circularis reflects the role of this area, which is the pumping of the chyme into the intestine.

The endocrine cells found here are similar in appearance to the so called "type II endocrine cells", found in the comparable region of the rainbow trout (Ezeasor, 1978). G/CCK-like IR endocrine cells have been found in the pyloric area of the Atlantic cod (Jönsson, *et al.*, 1987) and a CCK like immunoreactivity has been found in the pyloric area of the rainbow trout (Vigna, *et al.*, 1985).

4.3. The intestine.

Apart from the goblet cells, the cells of the intestinal epithelium show morphological characteristics of absorption, i.e. the microvillous brush border and smooth endoplasmic reticulum in the apical cytoplasm. The pyloric caecal region of the intestine displays a greater surface area, resulting from longer microvilli, and has more elaborate smooth endoplasmic reticulum, than the descending intestine. This indicates greater absorption of nutrients in this area.

The smooth endoplasmic reticulum plays a key role in lipid assimilation. Greater lipid absorption was shown to be in the ascending intestine of the carp (Noaillac-Depeyre and Gas, 1974). Lipid absorption has been studied in the pyloric caeca of the rainbow trout (Bauermeister *et al.*, 1979). Similar patterns emerged from these studies as for other vertebrates (Trier and Rubin, 1965): free fatty alcohols and free fatty acids are absorbed across the intestinal brush border and resynthesized into triacylglycerols in the smooth endoplasmic reticulum. These are then transferred to and processed in the Golgi region and finally discharged into the intercellular space.

Electron dense granules in the epithelial cells of the mid intestine and the rectum are somewhat unexpected in fish starved for 15 days. The possible explanation is that this is pinocytosed indigestible material in the feed, and some digestal remains were observed in the descending intestine upon dissection of the fish. Pinocytosis of protein in the descending intestine has been established in the goldfish (Gauthier and Landis, 1972) and in the rainbow trout (Ezeasor and Stokoe, 1981). These granules may again be the result of a progressive accumulation of undigested lipoid material (*Ibid.*).

The finding of endocrine cells in the ascending intestine is in agreement with previous findings in rainbow trout (Ezeasor, 1978). These cells were similar to the " type II endocrine cells " found in the comparable area of this animal (*Ibid.*), with two exceptions: i) These cells were seen to make contact with the lumen. ii) The secretory granules of these cells were invariably on the basal laminal side of the nucleus. If these cells are comparable, then the effect of starvation might explain the slight difference in the location

of the secretory granules. G/CCK-li IR endocrine cells have been found in the intestine of the Atlantic cod (Jönsson *et al.*, 1987) and the rainbow trout (Holmgren *et al.*, 1982), with a higher concentration of these cells in the ascending intestine, especially in the pyloric caeca. This was in agreement with the present observations of endocrine cells in the intestine. These appeared to be highest in number in the pyloric caecal region.

The stratum granulosum and stratum compactum layers are situated in the lamina propria throughout the intestine as well as muscularis externa and the serosa.

4.4. The pancreatic tissue.

The histological structure of the pancreatic tissue is in fairly good agreement with previous studies on pancreatic tissue in rainbow trout (Weinreb and Bilstad, 1955; Yasutake and Wales, 1983). The presence of active and non active pancreatic cells was also observed. These have previously been identified in the Atlantic salmon and called cells A and B, respectively (Munro *et al.*, 1984). An active cell (type A) has the typical appearance of a protein-secreting cell. The electron dense granules probably contain the zymogens, secreted by the pancreas. The increase of non active cells (type B) in the pancreatic tissue of the Atlantic salmon, is known to be the first sign of exocrine pancreas disease of the pancreas in this animal (*Ibid.*).

Islets of Langerhans (The Brockmann bodies) are also frequently observed in the pancreatic tissue. This is an endocrine tissue, and is found in all vertebrates, except the cyclostomes and is generally recognised to produce the following hormones at least: glucagon, insulin and islet hormone (Epple, 1969). Secretory granules were indeed observed in the cells of this tissue. These granules probably contain some hormone(s), but immunoreactivity to somatostatin, insulin and glucagon has been found in the Brockmann bodies of the teleost species *Carassius auratus*, *Salmo trutta*, *Oncorhynchus mykiss* and *Gadus morhua* (Jönsson, 1991).

The observation of many duct-like structures, passing from the pancreatic tissue into the pyloric caeca or the intestinal wall of the pyloric caecal region, suggests the route taken by the pancreatic zymogens. The frequent ductal openings found on the luminal surface

of the pyloric caeca and intestinal wall of the pyloric caecal region support this hypothesis. Peculiar electron dense granules, having the appearance of pancreatic zymogen granules, were seen in the epithelium of the pyloric caeca. The epithelial cells in this region do not appear to have granules of this kind in their apical cytoplasm. The possible explanation is that these granules are within a small duct, leading from the pancreatic tissue into the intestine. This possible duct may then be one of many of a similar structure and may convey secretion from the pancreas and into the intestine. Weinreb and Bilstad (1955) mention "the large pancreatic duct", giving an indication of a collective ductal system in the rainbow trout. Munro *et al.* (1984) found the ductal system of the Atlantic salmon to be collective, i.e. leading into a single large duct, which entered the duodenum in close proximity to the bile duct. No results regarding this were however presented. Yasutake and Wales (1983) present compelling evidence of tiny ducts, leading from the pancreatic tissue into the pyloric caeca, in the rainbow trout. The possibility exists that the ductal system in the Atlantic salmon comprises a collective system, draining into one single duct, and also many smaller ducts entering the intestine directly.

Chapter III.

Assay procedures for digestive enzymes.

1. Introduction.

The Atlantic salmon (*Salmo salar*) is a carnivorous species, and its diet is therefore mainly constituted of protein. Proteolytic enzymes play a major role in the necessary breakdown of protein, before assimilation of amino acids and peptides can take place. These are classified as endopeptidases (cleaving the protein chain into peptides and amino acids) and exopeptidases (removing one amino acid at a time from either end of the chain). Endopeptidases play a central role in initiating the breakdown of protein, i.e. turning these into peptides, and solubilizing structural proteins. Pepsin is the major protease in the stomach of mammals (Stryer, 1981). Peptic hydrolysis has been found to lead to a significant change of the type of protein in the rainbow trout (*Oncorhynchus mykiss*) from soluble polypeptides to di-and oligopeptides during intestinal digestion (Grabner and Hofer, 1989). So there is little doubt that pepsin hydrolysis in the stomach aids intestinal digestion. A study of proteolytic digestive enzymes from carnivorous (*Silurus glanis* L.), herbivorous (*Hypophthalmichthys molitrix*Val.) and omnivorous (*Cyprinus carpio* L.) fish found that in all three the largest part of the proteolytic enzymes in the intestine consisted of seryl proteinases (Jónás *et al.*, 1983). Of these trypsin, chymotrypsin and elastase are regarded as by far the most common in mammals (Sryer, 1981). Elastase and other endopeptidases, apart from trypsin and chymotrypsin, are generally either absent from fish alimentary canals (Nilsson and Fänge, 1970; Jany, 1976) or occurs at low concentrations (Yoshinaka *et al.*, 1978; Nilsson and Fänge, 1969 ; Buddington and Doroshov, 1986). It is obvious that trypsin and chymotrypsin are the major endopeptidases in fish intestine. Pepsin in the stomach and trypsin and chymotrypsin in the intestine are the major endopeptideses in mammals and probably also in fish. The optimalisation of the assays and the identification of pepsin, trypsin and chymotrypsin comprises the contents of this chapter.

Substrate saturation of pepsin, trypsin and chymotrypsin will be studied, in order to find an appropriate substrate concentration for the assays and to establish if these enzymes follow single substrate kinetics, i.e. if the Briggs and Haldane modification (Briggs and Haldane, 1925) of the Michaelis Menten equation (Stryer, 1981) applies. If the Briggs and Haldane modification applies, a direct linear relationship (Enzyme concentration = constant x Rate) between enzyme activity, i.e. rate, and enzyme concentration, is valid under the conditions of substrate saturation (Palmer, 1981).

Profiles of pepsin, trypsin and chymotrypsin activities, at varying pH, will be found, to establish an optimal pH for the assays.

Pepstatin A is an effective inhibitor of aspartic proteinases, including pepsin (Gildberg, 1988). It is well established that trypsin and chymotrypsin are inhibited by the Bowman-Birk inhibitor (Cohen *et al.*, 1981; Birk, 1985; Hanlon and Liener, 1986). The soybean trypsin inhibitor has been proven to be an inhibitor of trypsin from mammals, birds and fish (Krogdahl and Holm, 1983) and a weak inhibitor of bovine chymotrypsin (Kunitz, 1947). SBTI has also been shown to inhibit tryptic activity in Chinook salmon (Croston, 1960). Trypsin and chymotrypsin from various fish species have been inactivated by TLCK and TPCK respectively (specific inactivators for trypsin and chymotrypsin, respectively). These include trypsin from the Atlantic cod (Raae and Walther, 1989), trypsin from anchovy (Martínez and Serra, 1989) and chymotrypsin from herring and capelin (Kalac, 1978b) as well as trypsin and chymotrypsin from herring (Kalac, 1978a). These inhibitors and inactivators will be used to identify the pepsin, trypsin and chymotrypsin activities.

In assaying an enzyme, one is always concerned with how specific the assay is, i.e. the accuracy of the assay. The specificity of the substrates used for assaying pepsin, trypsin and chymotrypsin towards their respective enzymes will be studied.

If pepsin was assayed with hemoglobin (Anson and Mirsky, 1932) cathepsin D, which is the major lysosomal proteinase in fish muscle (Reddi *et al.*, 1972), might be expected to interfere with the assay in samples from homogenised stomachs. Neither pepstatin nor diazoacetyl compounds could be used to differentiate between pepsin and cathepsin D,

because of the relatedness of this enzyme to pepsin (See before). However, cathepsin D from squid mantle muscle did not digest azocasein (Sakai-Susuki *et al.*, 1983). Similar results were obtained with enzyme extracts from crawfish waste, which showed no ability in digesting casein at pH 4, although rapid tissue autolysis was detected at this pH (Chen and Meyers, 1983). There are no apparent differences between cathepsin D from invertebrates, fishes and mammals. Most data on the molecular properties and the catalytic activity of cathepsin D are consistent regardless of the animal source (Gildberg, 1988). It is therefore highly unlikely that cathepsin D from the Atlantic salmon will digest azocasein. Trypsin will be assayed with BA*p*NA as a substrate (Erlanger *et al.*, 1961), and chymotrypsin with SAAPP*p*NA as a substrate (Del Mar e*t al.*, 1979).

2. Materials and methods.

2.1 The kinetics of pepsin, trypsin and chymotrypsin activity.

An analysis of velocity data using Linweaver-Burk plots allows direct comparisons of kinetic constants with data published elsewhere.

The experimental protocol and the preparation of the samples:

The experimental animals used were a mixture of lower-and upper modal fish from the River Spey, fresh weight 9-33 g. The fish had been kept on a commercial salmon food (See Chapter II, section 2.1.).

The fish were killed by a blow on the head, and thereafter all samples were kept on ice, until frozen. The abdomen was opened ventrally and the stomach and intestine, together with the fat surrounding the pyloric caeca, removed. The stomachs were sampled together with their digesta and the remaining part of the gut was sampled as a whole. All samples were frozen at-20 °C.

Samples from the stomachs were homogenised in an ice cold Johnson Lindsay-buffer (Johnson and Lindsay, 1939), pH = 4.25 and 5 % CCl_4 (Hjelmeland and Raa, 1982). Buffer volume was ca. 10xthe volume of the samples on a v/w (ml/g) basis. The final pH was adjusted to pH = 4.25.

The samples from the intestine were homogenised in ice cold saline, 0.9 % NaCl, 20 mM $CaCl_2$ (Del Mar *et al.*, 1979) and 20 % CCl_4 (Hjelmeland and Raa, 1982). Buffer volume

was ca. 15xthe volume of the CCl_4 on a v/v (ml/ml) basis.
All samples were left overnight at 2-5 °C and centrifuged at 9,000 *g* for 4 minutes. They were kept on ice, and assayed as follows:

The pepsin assay:

Initial studies had shown the following, which gave guidelines for the assay:
Pepsin activity in the extract remains stable for 6 days at 2-5 °C and pH = 3.75. It should be left overnight after the homogenisation, at these conditions, to secure full activation. Freezing may reduce the pepsin activity by almost 7 %. The delta absorbance, i.e. the difference in the absorbance values of the samples and the blanks, should never exceed a value of 0.97, due to a deviation from linearity for the assay of pepsin as executed below.

Pepsin activity was measured in the samples from the stomachs with azocasein as a substrate (Charney and Tomarelli, 1947; Tomarelli *et al.*, 1949; Brock *et al.*, 1982). 200 μl 5 % (w/v) azocasein (Sigma: A 2765), dissolved in distilled water, was added to Johnson Lindsay-buffer (Johnson and Lindsay, 1939), pH = 4.25. The supernatant added was 200 μl and the buffer volume was adjusted to make the total volume of the incubation mixture 1 ml, giving final azocasein concentrations of 0.00, 0.15, 0.29, 0.56, 1.00 and 1.67 % (w/v). Incubation time was 0.5, 2.0, 4.0 and 6.0 hours at 25 °C. The respective reaction rates were calculated in milliunits (mU). One milliunit (mU) was defined as one microgram of azocasein broken down in one hour. The reaction was stopped with 600 μl of 20 % (w/v) TCA (trichloroacetic acid). The blanks were incubated with the TCA and a pepstatin A concentration of 0.17 mM. The pepstatin A (Sigma: P 4265) was dissolved in the buffer by stirring for ca. 2-4 hrs at room temperature and then aliquoted into the incubation medium, and incubated with the stomach extract for 20 minutes at room temperature, prior to the assay. After stopping the reaction, the incubation medium was left for at least 50 min at room temperature and then centrifuged for 6 min at 9,000 *g*. 1ml of NaOH was mixed with 1ml of the supernatant. The spectrophotometer was zeroed at 450 nm with 3.75 % (w/v) TCA. The initial reaction rate, V_o, was found by a backward extrapolation at 0.0 hours.

The trypsin assay:

Initial studies had shown the following, which gave guidelines for the assay:
Trypsin activity in the extract remains stable for 4 days at 2-5 °C and pH ca. 7. It should be left overnight after the homogenisation, at these conditions, to secure full activation. Freezing may reduce the trypsin activity by almost 17 %. At 20 °C and pH ca. 7 trypsin activity in the extract is reduced by less than 1 % over a period of 5 minutes. The delta absorbance should never exceed a value of 0.21, due to deviation from linearity, for the assay of trypsin as executed below.

The trypsin activity was measured in the samples from the intestine, with DL-BA*p*NA as a substrate (Erlanger *et al.*, 1961). The DL-BA*p*NA (Sigma: B 4875) was dissolved in DMFA (dimethylformamide), with a resulting concentration of 12 mM. 200 μl of this solution were mixed with 200 mM tris buffer (Fasman, ed., 1976), pH = 7.8, in the cuvette at 25 °C. An aliquot of the sample supernatant, 100 μl, was added and mixed with the buffer substrate solution. The volume of the buffer was adjusted to give a total volume in the cuvette of 2.2 ml. The DL-BA*p*NA concentration in the cuvette was 0.00, 0.03, 0.05, 0.11, 0.22, 0.33, 0.44, 0.55, 0.65, 0.76, 0.87, 0.98, 1.09 and 1.64 mM. The absorbance of the released *p*-nitroaniline (*p*NA) was measured at 405 nm at 6, 16, 26, 46 and 66 seconds, after the commencement of the reaction. The respective reaction rates were calculated in milliunits (mU). One milliunit (mU) was defined as one nanomol of *p*NA released in one minute. The initial reaction rate, V_o, was found by a bacward extrapolation at 0 seconds.

The chymotrypsin assay:

Initial studies had shown the following, which gave guidelines for the assay:
Chymotrypsin activity in the extract remains stable for 6 days at 2-5 °C and pH ca. 7. It should be left overnight after the homogenisation, at these conditions, to secure a full activation. Freezing may reduce the chymotrypsin activity by almost 8 %. At 20 °C and pH ca. 7 chymotrypsin activity in the extract is reduced by less than 0.6 % over a period of 5 minutes. The delta absorbance should never exceed a value of 0.34, due to deviation from linearity, for the assay of chymotrypsin as executed below. The intestinal extract

should be incubated with the specific trypsin inactivator, TLCK, for 2 minutes at ca. 20 °C prior to the chymotrypsin assay, with the TLCK at 30 mM in the incubation mixture, but this was done in chymotrypsin assays presented in chapters IV, V, VI and VII, but not in chapter III.

The chymotrypsin activity was measured in the samples from the intestine, with SAAPP*p*NA as a substrate (Del Mar *et al.*, 1979). The SAAPP*p*NA (Sigma: S 7388) was dissolved in DMFA, with a resulting concentration of 20 mM. 50 μl of this solution were mixed with 100 mM tris buffer (Fasman, ed., 1976), pH = 9.0, in the cuvette at 25 °C. 25 μl aliquot of the samples supernatant, was added and mixed with the buffer substrate solution. The volume of the buffer was adjusted to give a total volume in the cuvette of 2.0 ml. The SAAPP*p*NA concentrations in the cuvette was 0.00, 0.03, 0.05, 0.10, 0.15, 0.25, 0.35, 0.50, 0.63, 0.75, 0.88, 1.00, 1.25 and 1.50 mM. The absorbance of the released *p*-nitroaniline (*p*NA) was measured at 405 nm at 6, 16, 26, 46 and 66 seconds after the commencement of the reaction.

Chymotrypsin activity was presented in milliunits (mU). One milliunit was defined as one nanomole of *p*NA released in one minute. The initial reaction rate, V_o, was found by a bacward extrapolation at 0 seconds.

Statistics:

By testing the regression lines of the Lineweaver-Burk plots of pepsin-, trypsin-and chymotrypsin activities for linearity (Zar, 1984: p. 278), one will be able to establish if the activities of these enzymes follow single substrate kinetics. A regression line will be fitted to the Lineweaver-Burk plot of each enzyme activity and this line then tested for linearity.

2.2. pH profiles of pepsin, trypsin and chymotrypsin.

The experimental protocol, the preparation of the samples and the pepsin assay was done as before (Section 2.1.), with the following exceptions:

Samples from the stomach used for constructing pH-profiles for pepsin were homogenised in ice-cold distilled water, instead of the buffer, containing 5 % CCl_4 (Hjelmeland and

Raa, 1982). The pH-profiles were constructed with a glycine-HCl buffer (Fasman, ed., 1976) at pH: 1.0, 1.5, 2.0, 2.5 and 3.0 and with a Johnson Lindsay buffer (Johnson and Lindsay, 1939) at pH: 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5. The final azocasein concentration was 1 % (w/v) and the incubation time was 2 hours. Pepsin was also assayed with hemoglobin as a substrate.(See Chapter V, section 1.2.). The pepsin inhibitor, pepstatin A, was added to the blanks as before (See section 2.1.).

Samples from the intestine, used for constructing the pH profiles of trypsin and chymotrypsin, were prepared as before (Section 2.1.).

The pH profile of trypsin:

The pH-profiles were constructed with a Johnson Lindsay buffer at a pH of 4.95, 5.95, 6.49, 6.95, 7.45, 7.93, 8.48, 8.96, 9.43, 9.83, 10.21, 10.69 and 11.03 and a glycine-NaOH buffer (Fasman, ed., 1976) at a pH of 11.31, 11.79 and 12.97. The specific trypsin inactivator, TLCK (Sigma: T 7254), was dissolved in distilled water and incubated with the intestinal extract, prior to the trypsin assay as follows: at 6.45 mM for 15 seconds at ca. 20 °C, at 6.45 mM for 2 minutes at ca. 20 °C, at 10.03 mM for 2 minutes at ca. 20 °C and at 10.03 mM for 2 hours at 2-5 °C. The soybean trypsin inhibitor, SBTI, (Sigma: T 9003), was dissolved in distilled water and incubated with the intestinal extract at a concentration of 1.19 mg/ml for 15 seconds, prior to the trypsin assay.

The pH profile of chymotrypsin:

The pH-profiles were constructed with a Johnson Lindsay buffer at a pH of 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0 and a glycine-NaOH buffer (Fasman, ed., 1976) at a pH of 11.5, 12.0, 12.5 and 13.0. The Bowman-Birk inhibitor, a specific inhibitor of both trypsin and chymotrypsin, (Sigma: T 9777) was dissolved in distilled water and incubated with the intestinal extract at a concentration of 1.67 mg/ml for 30 seconds at ca. 20 °C, prior to the chymotrypsin assay. The specific chymotrypsin inactivator, TPCK (Sigma: T 4376) was dissolved in dimethylformamide (DMFA) and incubated with the intestinal extract at a concentration of 71.04 mM for 3 hours at 2-5 °C. A white precipitate formed when the TPCK was mixed with the intestinal extract.

Trypsin and chymotrypsin activities were assayed as before (Section 2.1.). The absorbance

of released paranitroaniline (*p*NA) was measured in the interval of 6-66 seconds, after the commencement of the reaction, for both the trypsin and the chymotrypsin assays and the activity was calculated as the average release of *p*NA per minute over this time interval.

2.3. Specificity of DL-BA*p*NA and SAAPP*p*NA towards trypsin and chymotrypsin, respectively.

The bovine trypsins, trypsin type I (Sigma: T 8003) and trypsin, type III (Sigma: T 8253) and bovine α-chymotrypsin, type I-S (Sigma: C7762) were dissolved in ice-cold saline, 0.9 % NaCl, 20 mM $CaCl_2$, with resulting concentrations at the assays of 50.0 μg/ml, 50.0 μg/ml, 1.00 μg/ml and 12.5 μg/ml, respectively. Activity was expressed as mU per milligram weighed enzyme.

Trypsin and chymotrypsin activities were assayed as before (Section 2.1.). Results are presented as means and standard deviations of 6 measurements in Figure 16.

2.4. The effect of trypsin on the chymotrypsin assay.

Bovine α-chymotrypsin, type I-S (Sigma: C 7762) and intestinal extract from the previous experiment (Section 2.2.) were assayed with the chymotrypsin assay (Section 2.1.), before and after an incubation for 2 minutes at ca. 20 °C with the specific trypsin inactivator, TLCK, at the incubation concentration of 96.77 mM. The incubation with the TLCK abolished almost all trypsin activity in the intestinal extract, and a further increase in the concentration of TLCK during the incubation, did not significantly reduce the trypsin activity any further. The concentration of the bovine α-chymotrypsin in the assay was 0.2 μg/ml.

Trypsin and chymotrypsin activities were assayed as before (Section 2.1.). Results are presented as means and standard deviations of 6 measurements in Figure 17.

Statistics:

The following comparisons using a multiple comparisons test, the Tukey's test (Zar, 1984), were done in order to reveal the effect of the TLCK on the α-chymotrypsin and

the chymotrypsin activity in the intestinal extract from the salmon: 1) the bovine α-chymotrypsin without and with the TLCK treatment, 2) the intestinal chymotrypsin activity without and with the TLCK treatment.

3. Results.

3.1. Characteristics of pepsin.

3.1.1. Kinetics of pepsin activity.

Substrate saturation of pepsin activity measured against azocasein can be seen in Figure 10 (a). The initial reaction rate, V_o, increases with the increase in the substrate concentration. Theoretically, the initial reaction rate is given by the equation

$$V_o = (V_{max} \times [\ S_o\ ]) / (K_M + [\ S_o\ ]) \qquad (1)$$

valid at constant enzyme concentration. This is the Briggs and Haldane modification of the Michaelis and Menten equation (Palmer, 1981). At high levels of the initial substrate concentration, $[\ S_o\ ]$, the following holds: $V_o \approx V_{max}$. An azocasein concentration of 1 %, can be seen to be almost at the level of substrate saturation (Figure 10 (a)). Hypothetically, substrate saturation is the condition when all the enzyme molecules are fully occupied in converting the substrate into product(s). By rearranging equation (1) the Lineweaver-Burk equation can be arrived at

$$1 / V_o = K_M /(V_{max} \times [\ S_o\ ]) + 1 / V_{max} \qquad (2)$$

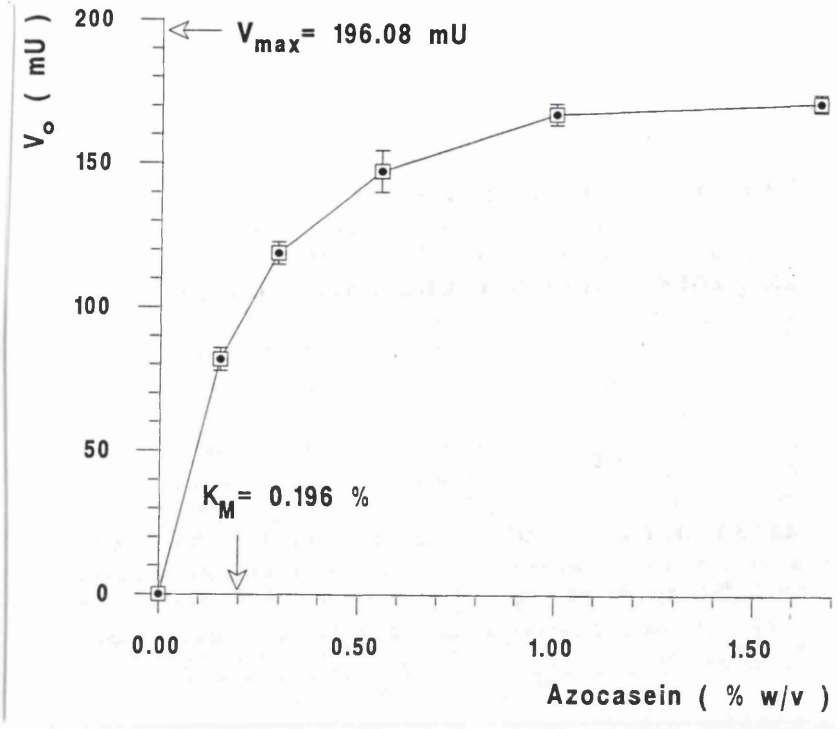
A Lineweaver-Burk plot of data presented in Figure 10 (a) can be seen in Figure 10 (b). The equation for the regression line was found to be

$$1/V_o = 0.00503 + 0.00107 \times 1/[\ S_o\ ]; \quad r^2 = 0.980.$$ (X.Appendices. Table III.1-3)

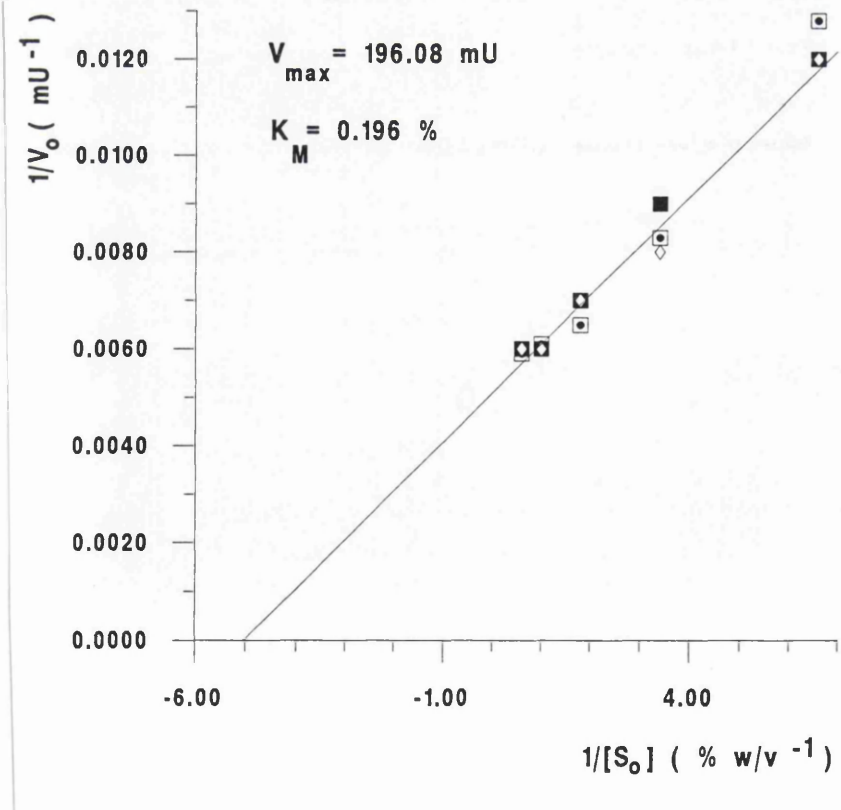
This gives V_{max}= 196.08 mU and K_M = 0.196 % (w/v). A test for linearity (Zar, 1984: p. 278) showed the following: The hypothesis H_o: the population regression is linear, could

Figure 10: Kinetics of pepsin activity: **(a)** Substrate saturation of pepsin activity. The initial reaction rate, V_o, is plotted against the initial substrate (azocasein) concentration, [S_o]. Each data point on the graph represents the average from 3 sets of values, where the initial reaction rate, V_o, was found by interpolation of the reaction rate at a reaction time of 0 minutes. The initial reaction rate, V_o, is expressed in milliunits (mU) and the initial azocasein concentration in percent, i.e. weight per volume (%). One milliunit (1 mU) is one microgram (1 µg) of azocasein broken down in one hour. Note how an azocasein concentration of 1 % has almost reached a substrate saturation. Error bars are standard deviations. **(b)** Lineweaver-Burk plot of data from a). The inverse of the initial reaction rate, $1/V_o$, is plotted against the inverse of the initial substrate concentration, $1/$ [S_o]. The equation for the regression line was found to be $1/V_o = 0.0051 + 0.0010 \cdot 1/$ [S_o]; $r^2 = 0.9821$. This equation gives $V_{max} = 196.08$ mU and $K_M = 0.196$ %.

(a)


V_o (mU)
200
150
100
50
0
V_{max}= 196.08 mU
K_M= 0.196 %
0.00
0.50
1.00
1.50
Azocasein (% w/v)

(b)


1/V_o (mU^{-1})
0.0120
0.0100
0.0080
0.0060
0.0040
0.0020
0.0000
V_{max} = 196.08 mU
K_M = 0.196 %
-6.00
-1.00
4.00
1/[S_o] (% w/v^{-1})

not be rejected (p = 0.2335. X.Appendices. Table III.4).

3.1.2. pH-profiles of pepsin.

The pH-profiles of pepsin activity can be seen in Figure 11, where either azocasein or hemoglobin are used as substrates. The pH-optimum for pepsin activity varies with the substrate. When pepsin is assayed with hemoglobin as a substrate, maximal activity is obtained at pH = 2.0, whereas when azocasein is used as a substrate, maximal activity is obtained at pH = 4.0. The inhibition of the pepsin activity by pepstatin A, when azocasein is used as the substrate, reveals the following: The residual breakdown of the azocasein is seen to be fairly constant throughout the range of pH : 1.00-6.25.

3.2. Characteristics of trypsin.

3.2.1. Kinetics of trypsin activity.

Substrate saturation of trypsin activity measured against DL-BApNA can be seen in Figure 12 (a). The same theory applies here as before (See section 3.1.1.). A DL-BApNA concentration of 1.09 mM, can be seen to be almost at the level of substrate saturation. A Lineweaver-Burk plot of data presented in Figure 12 (a) can be seen in Figure 12 (b). The equation for the regression line was found to be

$1/V_o = 0.0117 + 0.00191 \times 1/[S_o]$; $r^2 = 0.972$. (X.Appendices. Table III.5-7)

This gives V_{max}= 85.30 mU and K_M = 0.161 mM. Linearity was tested as before (Section 3.1.1.). The hypothesis, H_o: the population regression is linear, could not be rejected (p = 0.7424. X.Appendices. Table III.8).

3.2.2. Trypsin activity as a function of pH.

The pH-profile of trypsin activity in the intestinal extract and its inhibition and inactivation can be seen in Figure 13. Maximal trypsin activity is seen in the range of pH: 8.0-11.5. The TLCK, an inactivator specific for trypsin, can be seen to cause a gradual reduction of trypsin activity over the entire range of pH: 5.0-13.0. This reduction of

Figure 11: Pepsin activity as a function of pH for the substrates hemoglobin and azocasein. Pepsin activity was measured with hemoglobin as a substrate (◊), azocasein as a substrate (⊡) and azocasein with added pepstatin A (▩). Hemoglobin concentration was 3 % (w/v), azocasein concentration 1 % (w/v) and pepstatin A concentration 0.17 mM. The pepstatin A concentration was the concentration where further increase did not give an increase in the inhibition of pepsin activity. The pepsin inhibitor, pepstatin A, was incubated with the stomach extract for 20 minutes at ca. 20 °C, prior to the assay. Pepsin activity is expressed as the difference in the absorbance (dA) between samples and blanks, incubated without and with TCA, respectively. Each data point is the mean of 2 measurements. Error bars are differences between the 2 values.

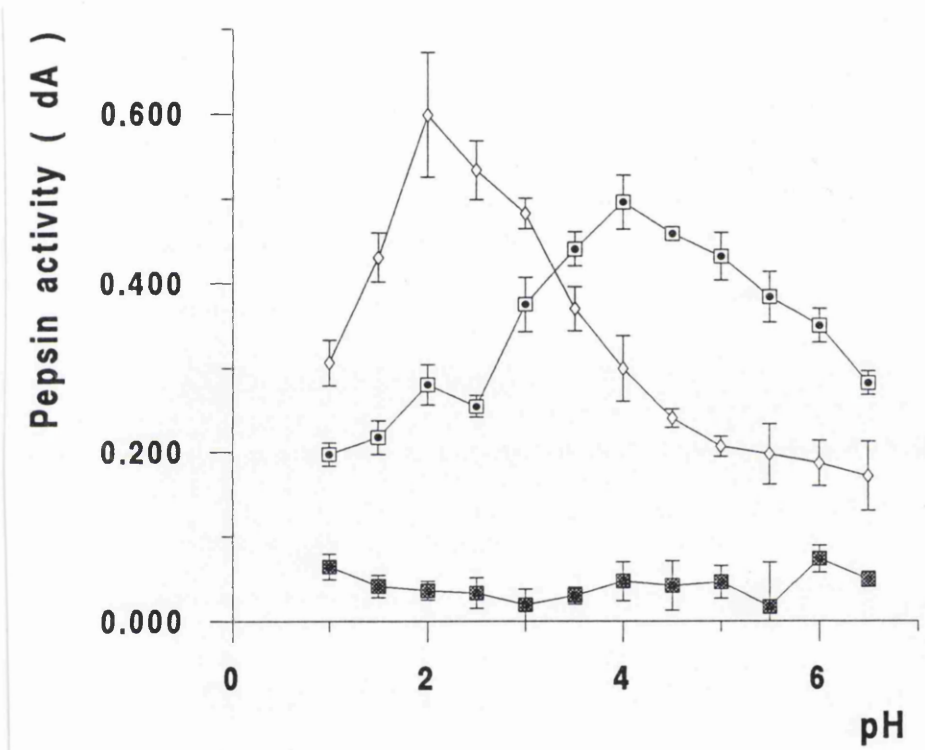
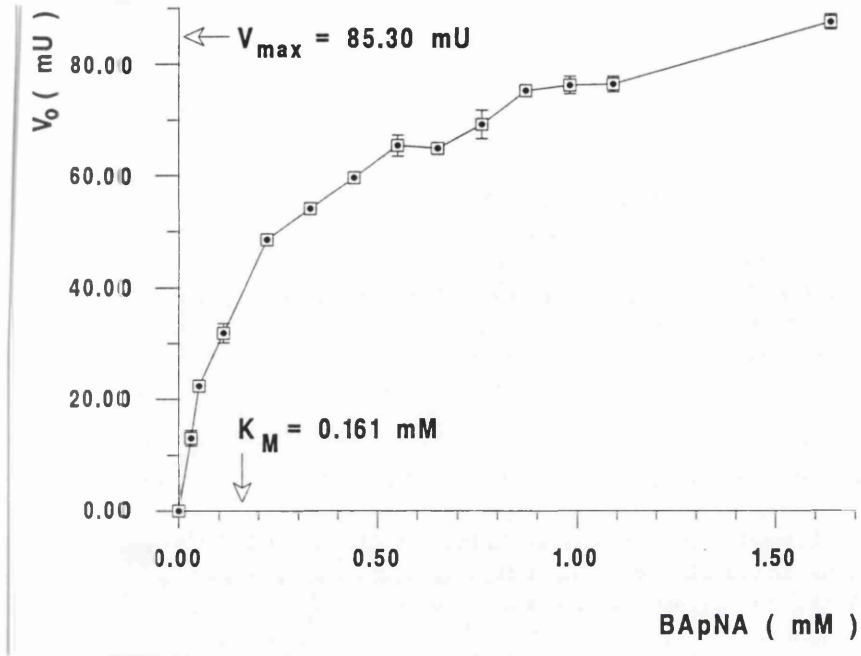

Pepsin activity (dA)
0.600
0.400
0.200
0.000
0
2
4
6
pH

Figure 12: Kinetics of trypsin activity. **(a)** Substrate saturation of trypsin activity. The initial reaction rate, V_o, is plotted against the initial substrate (DL-BA*p*NA) concentration, [S_o]. Each data point on the graph represents the average from 2 sets of values, where the initial reaction rate, V_o, was found by interpolation of the reaction rate at a reaction time of 0 seconds. The initial reaction rate, V_o, is expressed in milliunits (mU) and the initial DL-BA*p*NA concentration in mM. One milliunit (1 mU) is one nanomol of *p*NA (1 nmol) formed in one minute. Note how a DL-BA*p*NA concentration of 1.09 mM has almost reached a substrate saturation. Error bars are differences between the 2 values. **(b)** Lineweaver-Burk plot of data from a). The inverse of the initial reaction rate, $1/V_o$, is plotted against the inverse of the initial substrate concentration, $1/[S_o]$. The equation for the regression line was found to be $1/V_o = 0.011723 + 0.001887 \cdot 1/[S_o]$; $r^2 = 0.9902$. This equation gives $V_{max} = 85.30$ mU and $K_M = 0.161$ mM.

(a)


V_{max} = 85.30 mU
V_o (mU)
80.00
60.00
40.00
20.00
0.00
K_M = 0.161 mM
0.00
0.50
1.00
1.50
BApNA (mM)

(b)

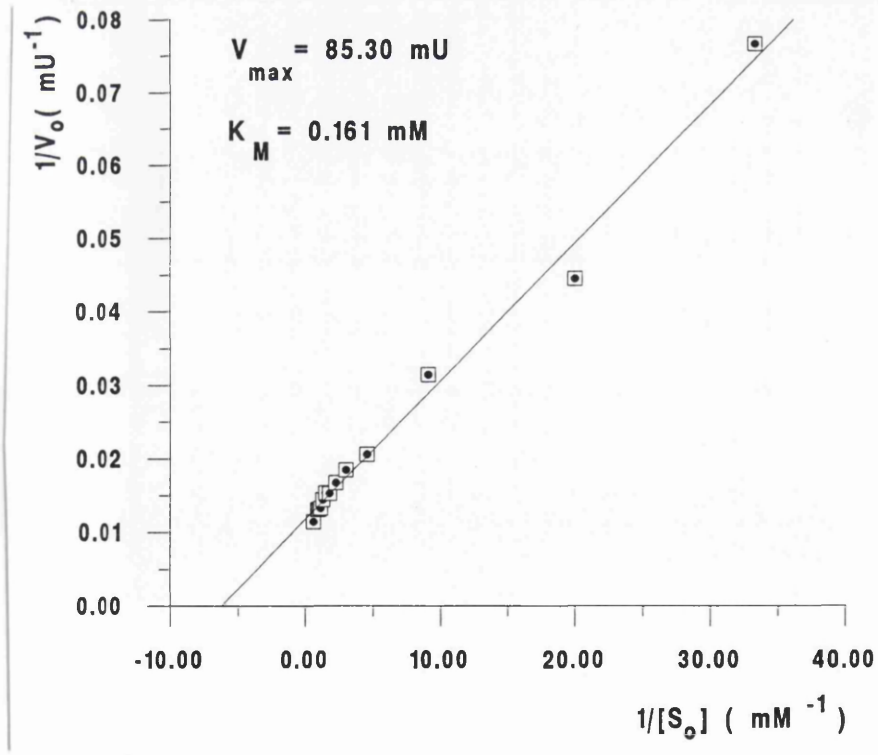
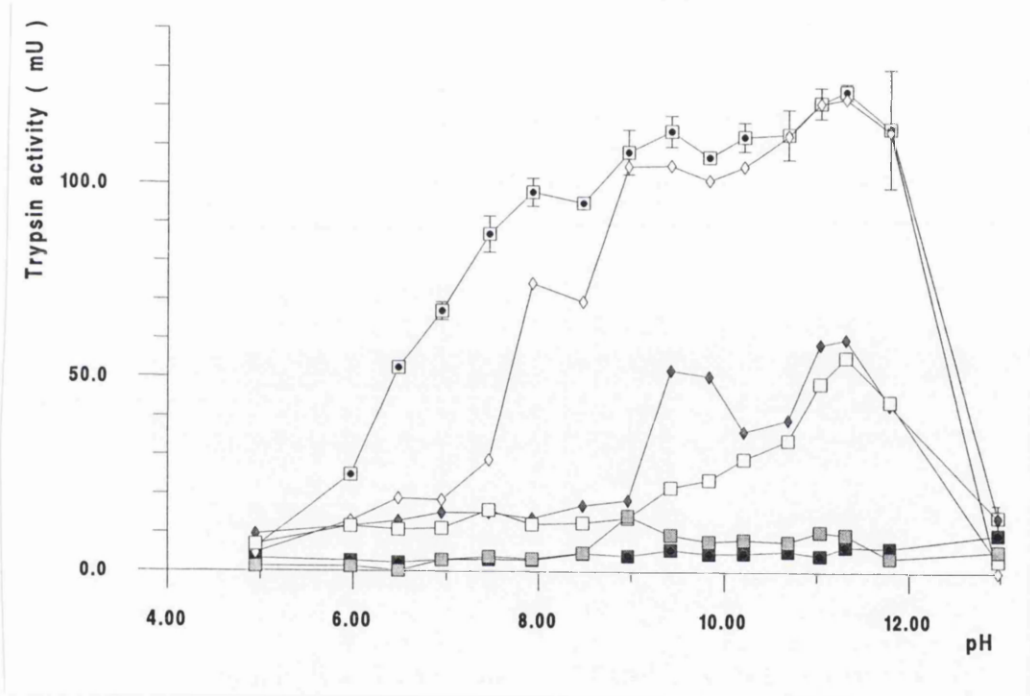

$1/V_o$ (mU^{-1})
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
V_{max} = 85.30 mU
K_M = 0.161 mM
-10.00
0.00
10.00
20.00
30.00
40.00
$1/[S_o]$ (mM^{-1})

Figure 13: Trypsin activity as a function of pH. Each data point for the trypsin activity is the average of 3 measurements, except for data points showing inactivation for TLCK and inhibition by SBTI, which were based on one measurement. Error bars are standard deviations (⊛). Trypsin activity was inactivated by TLCK, and in the variants where this was done, each data point was one measurement. TLCK was incubated with the intestinal extract prior to the assay as follows: for 15 seconds at ca. 20 °C and a TLCK concentration of 6.45 mM (◊), for 2 minutes and at ca. 20 °C and a TLCK concentration of 6.45 mM ( ), for 2 minutes at ca. 20 °C and a TLCK concentration of 10.03 mM (□) and for 2 hours at 2-5 °C and a TLCK concentration of 10.03 mM (▨). Note how the TLCK always inactivates the trypsin activity first at lower pH values. Trypsin activity was inhibited with a soyabean trypsin inhibitor, type I-S, at a concentration of 1.19 mg/ml, for 15 seconds at ca. 20 °C (■). The inhibitor concentration was the concentration giving almost a maximal inhibition during these conditions. Trypsin activity was expressed in milliunits (mU). Further details as in Figure 12 (a).


Trypsin activity (mU)
100.0
50.0
0.0
4.00
6.00
8.00
10.00
12.00
pH

trypsin activity is enhanced, either by an increased incubation time of TLCK with the intestinal extract prior to the assay, or by an increase in the TLCK concentration during this incubation. Interestingly, the reduction in the trypsin activity, always occurs first at the lower end of the range of pH: 5.0-13.0 for a given incubation time and incubation concentration of TLCK, prior to the assay.

Either an incubation prior to the assay for 2 hours at 2-5 °C with TLCK at a concentration of 10.03 mM, or an incubation prior to the assay for 15 seconds at ca. 20 °C with soyabean trypsin inhibitor, type I-S, at a concentration of 1.19 mg/ml, is seen to almost completely abolish all trypsin activity over the entire range of pH: 5.0-13.0.

3.3. Characteristics of chymotrypsin.

3.3.1. Kinetics of chymotrypsin activity.

The substrate saturation of chymotrypsin activity measured against SAAPP*p*NA can be seen in Figure 14 (a). The same theory applies here as before (See section 3.1.1.). A SAAPP*p*NA concentration of 0.50 mM, can be seen to be almost at the level of substrate saturation. A Lineweaver-Burk plot of data presented in Figure 14 (a) can be seen in Figure 14 (b). The equation for the regression line was found to be

$$1/V_o = 0.00746 + 0.000218 \times 1/[S_o]; \quad r^2 = 0.948.$$ (X.Appendices. Table III.9-11)

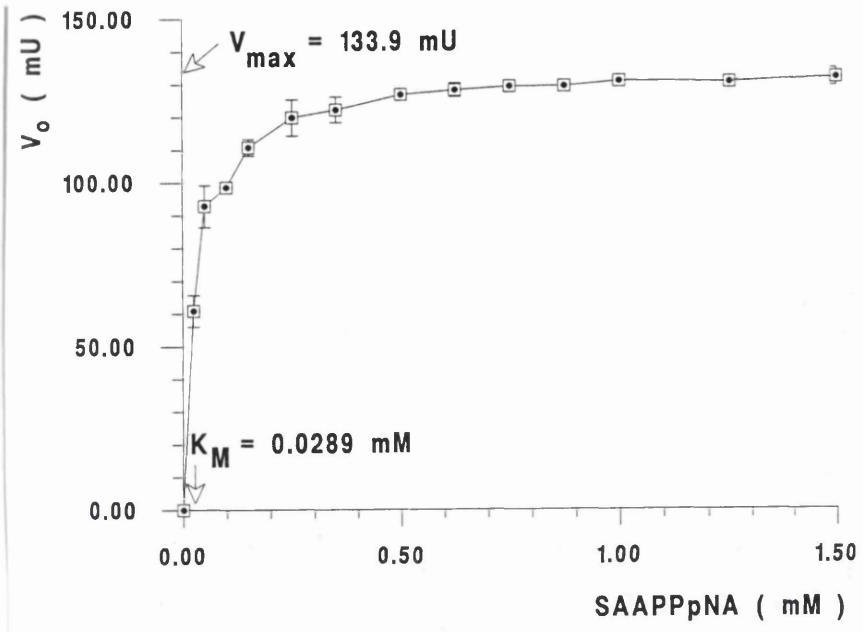
This equation gives V_{max}= 133.9 mU and K_M = 0.0289 mM. Linearity was tested as before (Section 3.1.1.). The hypothesis, H_o: the population regression is linear, could not be rejected (p = 0.7645. X.Appendices. Table III.12).

3.3.2. Chymotrypsin activity as a function of pH.

The pH-profile of chymotrypsin activity in the intestinal extract and its inhibition and inactivation can be seen in Figure 15. Maximal chymotrypsin activity is seen in the range of pH: 9.0-12.0. The Bowman-Birk inhibitor, a specific inhibitor of trypsin and chymotrypsin, totally abolished all chymotrypsin activity, while TPCK only partially

Figure 14: Kinetics of chymotrypsin activity. (a) Substrate saturation of chymotrypsin activity. The initial reaction rate, V_o, is plotted against the initial substrate (SAAPP*p*NA) concentration, [S_o]. Note how a SAAPP*p*NA concentration of 0.50 mM has almost reached a substrate saturation. Further details as in Figure 12 (a). (**b**) Lineweaver-Burk plot of data from a). The inverse of the initial reaction rate, $1/V_o$, is plotted against the inverse of the initial substrate concentration, $1/[S_o]$. The equation for the regression line was found to be $1/V_o=0.007467 + 0.000216 \cdot 1/ [S_o]$; $r^2=0.9801$. This equation gives $V_{max}= 133.9$ mU and $K_M=0.0289$ mM.

(a)


150.00
100.00
50.00
0.00
V_o (mU)
V_{max} = 133.9 mU
K_M = 0.0289 mM
0.00
0.50
1.00
1.50
SAAPPpNA (mM)

(b)

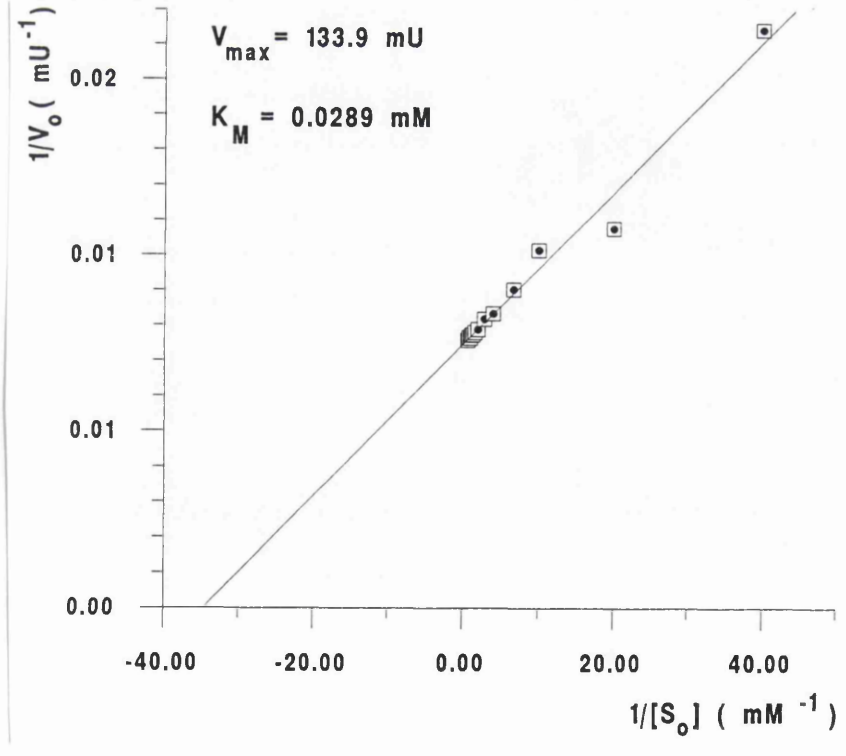
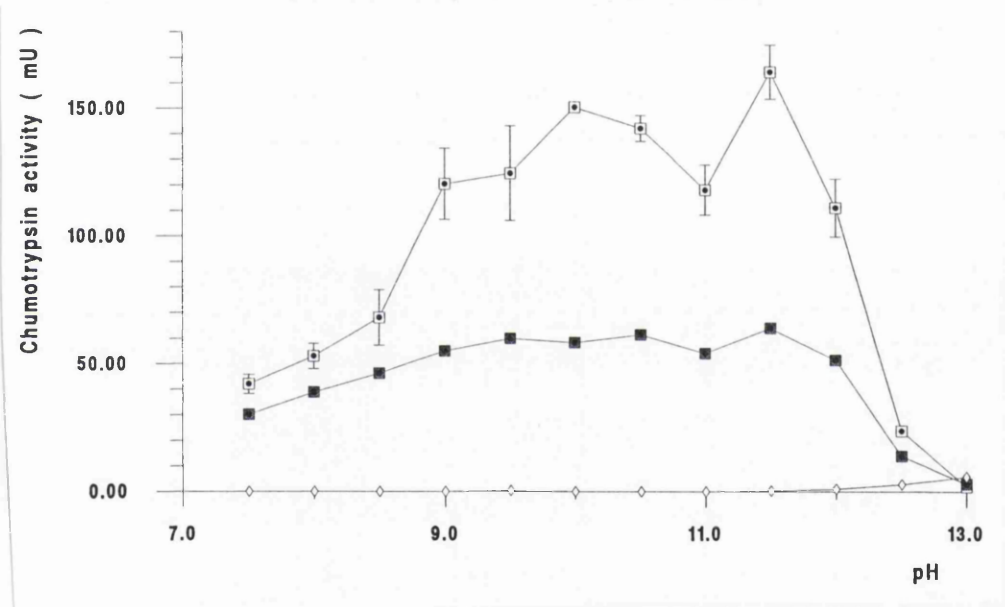

$1/V_o$ (mU^{-1})
0.02
0.01
0.01
0.00
V_{max} = 133.9 mU
K_M = 0.0289 mM
-40.00
-20.00
0.00
20.00
40.00
$1/[S_o]$ (mM^{-1})

Figure 15: Chymotrypsin activity as a function of pH, where each data point for the chymotrypsin activity is the average of 3 measurements and error bars are standard deviations (⊙). Chymotrypsin activity was inactivated with TPCK, where each data point is 1 measurement (■); note the great residual chymotrypsin activity after the inactivation with TPCK; TPCK at a concentration of 71.04 mM was incubated with the intestinal extract for 2 hours at 2-5 °C prior to the assay. The TPCK concentration was the concentration giving almost a maximal inactivation during these conditions. Chymotrypsin activity was inhibited with the Bowman Birk inhibitor, where each data point is 1 measurement (◊); the Bowman Birk inhibitor at a concentration of 1.67 mg/ml was incubated with the intestinal extract for 30 seconds at ca. 20 °C prior to the assay.


Chumotrypsin activity (mU)
150.00
100.00
50.00
0.00
7.0
9.0
11.0
13.0
pH

inactivated chymotrypsin activity over the range of pH: 7.5-13.0.

3.4. Specificity of DL-BA*p*NA and SAAPP*p*NA, towards trypsin and chymotrypsin, respectively.

How the purified enzymes, bovine trypsin type I and type II (50 μg/ml) and bovine-chymotrypsin, type I-S (1 μg/ml) proportionally break down DL-BA*p*NA, the trypsin substrate, and SAAPP*p*NA, the chymotrypsin substrate can be seen in Figure 16. It can be seen that-chymotrypsin was not capable of breaking down DL-BA*p*NA, the trypsin substrate. Both trypsin I and III are however capable of breaking down SAAPPpNA, the chymotrypsin substrate. While trypsin may interfere with the chymotrypsin assay, by breaking down SAAPP*p*NA, chymotrypsin is not capable of interfering with the trypsin assay, by breaking down DL-BA*p*NA.

3.5. The effect of trypsin on the chymotrypsin assay.

How TLCK affects the activity of purified bovine-chymotrypsin and chymotrypsin activity in the intestinal extract can be seen in Figure 17. TLCK had no significant effect on the SAAPP*p*NA breakdown of the purified bovine-chymotrypsin (Tukey test: $p > 0.50$. X.Appendices. Table III.13), but significantly reduced the chymotrypsin activity, i.e. the SAAPP*p*NA breakdown, in the intestinal extract by 4.0 % (Tukey test: $0.005 > p > 0.001$. X.Appendices. Table III.14). Trypsin activity in the intestinal extract is clearly interfering with the assay of chymotrypsin activity in the intestinal extract, by breaking down SAAPP*p*NA. This interference appears to be small, however.

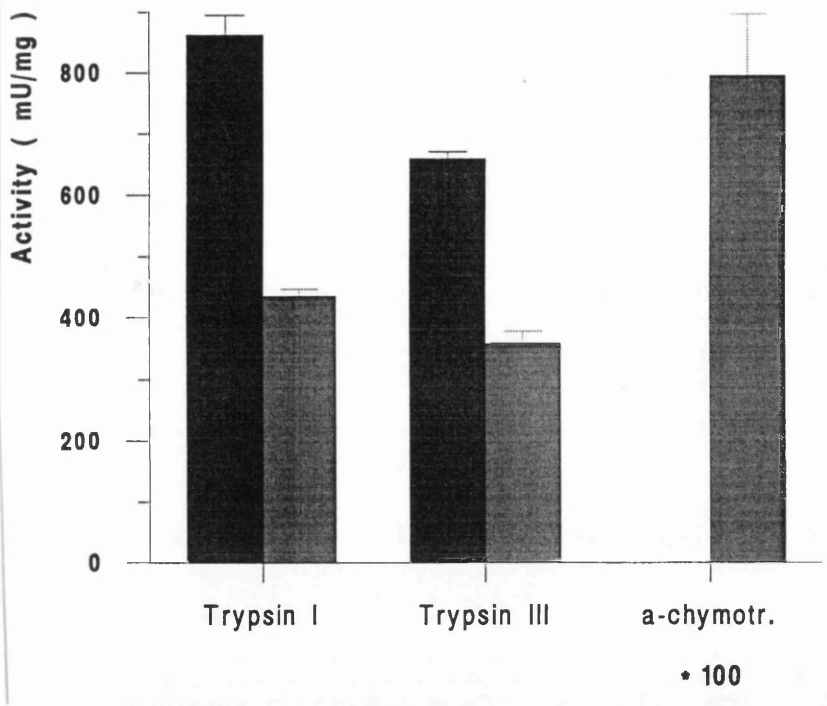
4. Discussion.

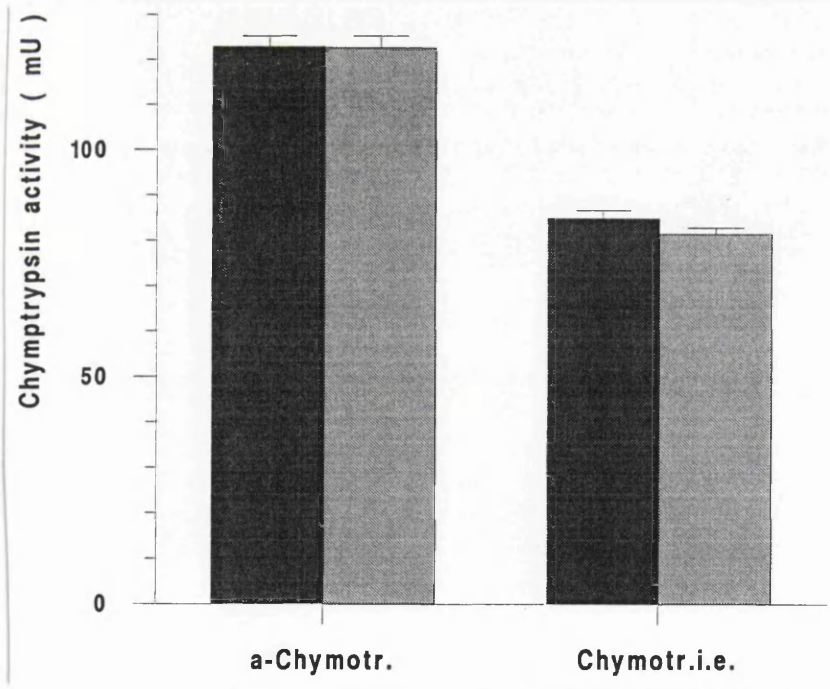
4.1. Pepsin.

Azocasein is regarded by some as being unsuitable as a substrate for peptic analysis, because of its poor solubility at low pH (Charney and Tomarelli, 1947; Tomarelli *et al.*, 1949). My use of azocasein in assaying pepsin, showed both reproducibility and precision (The coefficient of variation for the assay was almost always lower than 5 %). The

Figure 16: Breakdown of the trypsin substrate, DL-BA*p*NA (■) and the chymotrypsin substrate, SAAPP*p*NA (▒) by bovine trypsin I (Trypsin I) and III (Trypsin III) at 50 µg/ml each and a bovine α-chymotrypsin, type I-S (a-chymotr.), at 1 µg/ml. Note how the trypsins are able to break down the chymotrypsin substrate, SAAPP*p*NA, and how the-chymotrypsin is unable to break down the trypsin substrate, DL-BA*p*NA. The breakdown of SAAPP*p*NA by α-chymotrypsin is 100 times that displayed. All enzyme activities are expressed as milliunits per milligram of weighed enzyme (mU/mg). Each column is the

Figure 17: The effect of TLCK on bovine α-chymotrypsin, type I-S (a-Chymotr.), at a concentration of 0.2 µg/ml and on chymotrypsin activity in the intestinal extract (Chymotr.i.e.). Not inactivated (■), and inactivated by TLCK (▒), chymotrypsin activity, where TLCK was incubated with the α-chymotrypsin and the intestinal extract at a concentration of 96.77 mM for 2 minutes at ca. 20 °C, prior to the assay. Chymotrypsin activity is expressed in milliunits (mU). Each column is the average of 6 measurements. Error bars are standard deviations.


Activity (mU/mg)
800
600
400
200
0
Trypsin I
Trypsin III
a-chymotr.
* 100


Chymptrypsin activity (mU)
100
50
0
a-Chymotr.
Chymotr.i.e.

abolition of cathepsin D interference on the assay more than compensates for the possible loss in sensitivity.

Pepsin follows the kinetics of single substrate reactions (Fig. 10 (b)): Once the enzyme substrate complex, i.e. pepsin azocasein complex, is produced, it will be maintained in a steady-state (Briggs and Haldane, 1925). This assumption produces the modified Michaelis-Menten equation (Fig. 10 (a)). Inverting this equation gives the Lineweaver-Burk equation (Fig. 10 (b); Lineweaver and Burk, 1934). This is the equation of a straight line. As linearity in the data (Fig. 10 (b); section 3.1.1.) could not be rejected it must be concluded that the pepsin activity follows the Briggs and Haldane modification of the Michaelis-Menten equation, meaning that at substrate saturation, a linear relationship exists between enzyme concentration and activity. This assumption is approximately valid for an azocasein concentration of 1 % (w/v). The calculated K_M value is likely to be an integrated value of more than one pepsin, as at least two pepsins, pepsin I and II, are common in fish (Gildberg, 1988).

It is well established that fish pepsins are inhibited by pepstatin A (*Ibid.*), so it is not surprising to find that pepstatin A inhibits pepsin from the Atlantic salmon (Fig. 11). Pepsin is almost completely inhibited at a pepstatin A concentration of ca. 10^{-4} M (Fig. 11). Similar results have been presented for pepsin from dogfish, *Scyliorhinus canicula* (Guerard and Gal, 1987) and sardine (Noda and Murakami, 1981). The inhibition pattern of pepstatin A at varying pH values (Fig. 11), implies a small nonenzymatic breakdown of azocasein over the range of pH: 1.00-6.25.

Pepsin assayed with hemoglobin has an optimum at pH = 2.0, but assayed with azocasein an optimum of pH = 4.0 (Fig. 11). Similar results were obtained with pepsin from the Atlantic cod (*Gadus morhua*), when assayed with hemoglobin and casein (Martínez and Olsen, 1989), but azocasein is a chemically modified casein. It is well known that proteinases may have different pH optima on different substrates (Gildberg, 1988).

4.2. Trypsin.

Trypsin follows the kinetics of single substrate reactions (Fig. 12 (b)). As for pepsin, linearity in the data could not be rejected (Section 3.2.1.). It must be concluded that the trypsin activity follows the Briggs and Haldane modification of the Michaelis-Menten equation, meaning that at substrate saturation, a linear relationship exists between enzyme concentration and activity. This assumption is approximately valid for a DL-BA*p*NA concentration of 1.09 mM.

The K_M values of trypsin in the crude intestinal extract obtained for the brook trout (*Salmo trutta)* and the rainbow trout (*Oncorhynchus mykiss*), with respect to DL-BA*p*NA and measured at both 15 °C and 30 °C (Hofer *et al.*, 1975) were almost identical to the K_M value obtained for the Atlantic salmon, *Salmo salar* (Section 3.2.1.).
It has been demonstrated that trypsins from exothermic animals possess a lower K_M value than trypsins from endothermic animals (Ásgeirsson *et al.*, 1989; Cohen *et al.*, 1981). K_M has also been demonstrated to be lower for trypsins in vertebrates with a low temperature preference than in vertebrates with a high temperature preference (Hofer *et al.*, 1975). The likely explanation is the need to compensate for lower temperature, and therefore lower reaction rates, by a lower K_M value. This is in accordance with the hypothesis stating that enzymes from exothermic species are better catalysts than homologous enzymes isolated from endothermic species (Somero, 1977). This similarity in the K_M values from brook trout and rainbow trout on one hand and juvenile Atlantic salmon in the present study on the other hand is not surprising as the ambient temperature for these animals is fairly similar.
These studies were done with the amide substrate BA*p*NA, which is more relevant to the hydrolysis of peptide bonds, than are the ester substrates (Mihalyi, 1978; Simpson and Haard, 1984).

The inactivation of trypsin by TLCK is thoroughly established for trypsins from fish and almost a standard procedure in the identification of trypsin. Previous studies on trypsins from capelin (*Mallotus villosus*) and anchovy (*Engraulis encrasicholus*) have shown this (Hjelmaland and Raa, 1982; Martínez *et al.*, 1988). In these studies trypsin was purified

before the inactivation, but the inactivation proved 100 % in both cases. The residual trypsin activity (Fig. 13) is probably caused by a protecting mechanism of protein in the intestinal extract. While the binding site of the trypsin is occupied by protein molecules, TLCK has no access. Supporting this is the finding that the inhibitor benzamidine protects trypsin from the inactivation of TLCK (Shaw *et al.*, 1965). The same may apply to SBTI.

The pH-profiles show maximal activity ranging from pH: 8-12 (Fig. 13). The profile is however, almost abolished, with both SBTI and TLCK (Fig. 13), so this profile must be formed by trypsin in the intestinal extract. Trypsin activity from carp (*Cyprinus carpio*) has been inhibited with SBTI (Cohen *et al.*, 1981). A somewhat similar profile was found for trypsin from rainbow trout (*Oncorhynchus mykiss*) and Antarctic fish (*Paranotothemia magellanica* F.) assayed against L-BA*p*NA (Genicot *et al.*, 1988).

The reason for a reduction in trypsin activity as pH is lowered is the protonation of Asp, clogging the proton shuttle (Fersht, 1977). From the pH-profile (Fig. 13) it can be seen that the effective pK of the Asp carboxyl group is approximately 7.

The reason for a reduction in trypsin activity as the pH is raised is the deprotonation of the-amide group ($-NH_2$) Ile. This amino terminal group interacts electrostatically with Asp in the interior of the molecule, stabilising the active form of trypsin (Stryer, 1981). The fact that trypsin retains its maximal activity up to a pH of 12 (Fig. 13) is possibly based on the ability of the molecule to preserve the positively charged form of Ile and the negatively charged form of Asp. This is possibly obtained by a better shielding from the solution of this amino acid pair within the molecule. This amino acid pair might then be buried deeper within the trypsin molecules of the Atlantic salmon, than within the bovine trypsin molecules. But there is another more likely explanation: Sigler *et al.*, (1968) conclude that substrate binding in chymotrypsin prevents the disruption of the ion pair in Asp-Ile, even at high pH values, thus keeping the molecule in an active conformation. As trypsin is known to have a comparable ion pair between Asp and Ile , it is highly likely that an active form of the molecule at high pH values is preserved, because of the presence of a high concentration of substrate, i.e. protein in the intestinal extract. With this in mind then, an assay of trypsin done at pH = 7.8, would give more reproducible results, than this assay carried out at higher pH values.

The stepwise inactivation of trypsin from a lower to a higher pH (Fig. 13) is probably

caused by the partial deprotonation of the lysine group in the TLCK molecule (Palmer, 1981). Supporting this is the fact that the side chain-NH_3^+ group of lysine has a pK = 10.8 at 25 °C (Lehninger, 1975). It is necessary for the lysine group in the TLCK molecule to have a positive charge, i.e. protonised, for the inactivator to be active. The reason for this is that trypsin has a ionised carboxyl group, sidechain from Asp, at the bottom of its substrate binding site, which only positively charged lysine or arginine side chains can bind to. At higher pH values TLCK becomes neutralised and therefore unable to dislodge water molecules from the bottom of the substrate binding site.

4.3. Chymotrypsin.

Chymotrypsin follows the kinetics of single substrate reactions (Fig. 14 (b)). As for both pepsin and trypsin, linearity in the data could not be rejected (Section 3.3.1.), it must be concluded that the chymotrypsin activity follows the Briggs and Haldane modification of the Michaelis-Menten equation, meaning that at substrate saturation, a linear relationship exists between enzyme concentration and activity. This assumption is approximately valid for a SAAPP*p*NA concentration of 0.50 mM.

.

The apparent K_M value (0.0289 mM) is distinctively lower than a K_M value of purified bovine chymotrypsin, 0.043 mM (Del Mar *et al.*, 1979) and far lower than a K_M value (0.203 mM) of nonpurified chymotrypsin from humans (Kaspar *et al.*, 1984). All K_M values were found at 25 °C. It is obvious from this that the chymotrypsin activity from the exothermic Atlantic salmon displays a considerably lower K_M, than chymotrypsin activity from the endothermic cattle or humans. Just as for trypsin the likely explanation is the need to compensate for lower temperature by a lower K_M value.

The Bowman-Birk inhibitor (BB) inhibits chymotrypsin completely (Fig. 15). This is in accord with previous findings (Kunitz, 1947; Birk, 1985) and also in agreement with findings on chymotrypsin activity from carp (Cohen *et al.*, 1981) and the Atlantic cod (Raae and Walther, 1989).

The standard inactivator of chymotrypsin, TPCK, was shown to inactivate chymotrypsin activity only partially (Fig. 15). It was observed that, as soon as the TPCK solution was

mixed with the intestinal extract or the bovine α-chymotrypsin solution, a white precipitate was formed. It is possible that the TPCK precipitates out of solution before completely inactivating the α-chymotrypsin. Dissolving the TPCK in ethanol or in DMFA made no difference.

The chymotrypsin pH-profile had a maximal activity ranging from pH: 9-12 (Fig. 15). The carboxyl group of Asp in the proton shuttle has a pK_a of approximately 8.75 (Fig. 15), so this group has to be more shielded from solution by the chymotrypsin molecule, i.e. have a more hydrophobic environment, than the comparable group in trypsin (Section 4.3), which has a pK_a=7.0. The extension of the maximal chymotrypsin activity to a pH = 12, is probably the cause of substrate stabilisation, as for trypsin (Section 4.2.). Assaying chymotrypsin at pH = 9.0, would therefore give more reproducible results.

Considerable residual activity was observed, when the chymotrypsin pH-profile was "inactivated" by TPCK (Fig. 15). This profile was completely abolished by the Bowman-Birk inhibitor. Birk (1985) states in his review on the Bowman-Birk inhibitor, that this inhibitor does not inhibit elastase, but a slight inhibition with this inhibitor was observed for elastase from carp (Cohen *et al.*, 1981). The residual activity after the inactivation of TPCK in the chymotrypsin pH-profile (Fig. 15), is then likely to stem only from chymotrypsin-(meaning the TPCK is not efficient) and / or trypsin activity, as elastase is known to be scarce in fish (see below).
It was found that TPCK inactivates elastase from the Atlantic cod (Gidberg and Øverbø, 1990). As has been shown (Fig. 17), the trypsin activity in the same intestinal extract affects the chymotrypsin assay by breaking down SAAPP*p*NA. This amounted to 4 % of the total breakdown of SAAPPpNA (Section 3.5). The trypsin interference can not account for the great residual activity in the chymotrypsin pH-profile, after the inactivation by TPCK (Fig. 15). This residual activity is then mostly caused by the chymotrypsin and/or elastase activity in the intestinal extract.
The data does not allow any further assessment, but elastase and other endopeptidases (apart from trypsin and chymotrypsin) are generally either absent from fish alimentary canals (Nilsson and Fänge, 1970; Jany, 1976) or at low concentrations (Yoshinaka *et al.*, 1978; Nilsson and Fänge, 1969 ; Buddington and Doroshov, 1986). It is therefore likely that the effect of elastase in the intestinal extract on the SAAPP*p*NA breakdown is

minimal. Therefore the residual activity after the TPCK inactivation of the chymotrypsin pH-profile (Fig. 17) is probably caused by the precipitation of TPCK out of solution, before it can completely inactivate the chymotrypsin activity in the intestinal extract. It has been observed for various fish species, that TPCK almost completely abolishes the chymotrypsin activity (Kalac, 1978b; Martínez and Serra, 1989; Raae and Walther, 1989). It is possible that the precipitation of TPCK from the supernatant can be prevented by increasing the proportion of the DMFA in the mixture, but that was done on purified elastase from the Atlantic cod (Gildberg and Øverbø, 1990).

4.4. Specificity of BA*p*NA, SAAPP*p*NA, towards trypsin and chymotrypsin, respectively.

While trypsin (bovine, type I and III) breaks down BA*p*NA as well as SAAPP*p*NA, chymotrypsin (bovine α-chymotrypsin, type I-S) only breaks down SAPP*p*NA (Fig. 16). This supports the previous conclusion (Section 4.2.), that no other proteolytic enzyme is interfering with the trypsin assay, when DL-BA*p*NA is used as a substrate (Fig. 13). Gildberg and Øverbø (1990) arrived at the same finding, where neither cod-and porcine elastase nor bovine chymotrypsin broke down DL-BA*p*NA, but bovine trypsin did. It can be safely concluded that DL-BA*p*NA is totally specific for trypsin.

SAAPP*p*NA is not only broken down by chymotrypsin, but by trypsin as well (Fig. 16). The effect of trypsin on SAAPP*p*NA is in harmony with previous findings. Trypsin is known to split certain aromatic bonds in high molecular weight peptides (Maraux *et al.*, 1966) and also known to split SAAPP*p*NA as well (Kaspar *et al.*, 1982).

4.5. The effect of trypsin on the chymotrypsin assay.

TLCK was shown to have no effect on purified chymotrypsin (bovine α-chymotrypsin), while reducing the SAAPP*p*NA breakdown in the crude intestinal extract significantly by 4 % (Fig. 17). This is in accordance with previous studies of proteases in tuna, which have shown that TLCK only inactivates trypsin, while leaving chymotrypsin intact (Zendzian and Barnard, 1967). It is also well established in the previous studies, that TLCK inactivates trypsin (*Ibid.*; Nilsson and Fänge, 1969; Martínez *et al.*, 1988; Uchida *et al.*, 1984; Hjelmeland and Raa, 1982). It must therefore be concluded that trypsin is

interfering with the chymotrypsin assay. As the proportion of trypsin to chymotrypsin may vary from sample to sample it is recommended that trypsin activity be inactivated by excess TLCK (2 min at room temperature and 30 mM), before the chymotrypsin assay with SAAPP*p*NA.

Chapter IV.

Control of secretion of trypsin and chymotrypsin from the pancreas.

1. Introduction.

Secretion of proteolytic digestive enzymes from the pancreas in vertebrates is controlled by neuronal and humoral factors, including acetylcholine (ACh) and cholecystokinin (CCK), respectively. Mammalian CCK has a COOH-end of 8 amino acids, containing its biological activity (Ondetti *et al.*, 1970; Tracy and Gregory, 1964).

CCK is known to stimulate gallbladder discharge, secretion of digestive enzymes from the pancreas, to augment secretin stimulation on pancreatic bicarbonate secretion, and inhibits gastric emptying in mammals (Grossman, 1977; Dockray, 1989). CCK has been proposed to cause the pancreatic enzyme secretion via cholinergic vagovagal reflexes (Dockray, 1982). It is also known to have a trophic effect on the pancreas (Barrowman and Mayston, 1973) and to regulate pancreatic cell proliferation (Logson and Williams, 1986) in mammals. It has been found in mammals to be released into the blood stream by fat in the duodenum (Shiratori *et al.*, 1986) and in the jejunum and ileum (Fujimura *et al.*, 1984). CCK controls small intestinal digestion by matching the supply of incoming nutrients with that of pancreatic enzymes and bile salts (Dockray, 1989). This is achieved on the one hand by stimulation of exocrine pancreatic secretion and gallbladder contraction, and on the other hand by controlling rates of gastric emptying and food intake. Food intake appears to be controlled by CCK via a satiety effect and a modification of behaviour. Exogenous CCK is also known to have a satiety effect in the rat (Gibbs *et al.*, 1973). Vagotomy abolished the satiety effect of CCK, indicating a peripheral site of action (Smith *et al.*, 1981). Exogenous CCK has also been found to cause a decrease in exploratory behaviour, which is also inhibited by vagotomy (Crowley *et al.*, 1981).

Considerable studies on the localisation of CCK in fish have been undertaken with immunocytochemical methods. Gastrin and CCK have a common C-terminal pentapeptide, which is highly antigenic, so studies using antisera towards this sequence, can not differentiate between the two (Nilsson and Holmgren, 1989). The same antisera recognise the amphibian peptide caerulein, also having the identical C-terminal pentapeptide (Anastasi *et al.*, 1967). Gastrin/CCK-like activity has been found in every vertebrate species, where sought (Vigna, 1979). CCK-like immunoreactivity has been found in the brain, intestine and pyloric caeca of the rainbow trout, but lesser amounts were found in the pyloric area and the rectum of this animal (Vigna *et al.*, 1985). A marked molecular heterogeneity of CCK-like activity in the various regions of the rainbow trout gut was found (*Ibid.*). Endocrine cells reactive to gastrin/CCK antiserum were demonstrated in the intestinal mucosa of the rainbow trout (*Oncorhynchus mykiss)*, while no immunoreactivity was found in the stomach (Holmgren *et al.*, 1982). CCK immunoreactive cells have also been detected in the anterior-and mid-intestine in the bowfin, *Amia calva*, and in the pyloric caeca, anterior-and mid-intestine in the bluegill, *Lepomis macrochirus* (Rajjo *et al.*, 1988b). Gastrin/CCK immunoreactive cells in the antral mucosa and intestine of cod (*Gadus morhua*) were found to contain a molecule more closely related to CCK than to gastrin (Larsson and Rehfeld, 1977), which was later suggested to be caerulein or a caerulein-like peptide (Larsson and Rehfeld, 1978). Many variants of CCK-like/caerulein-like molecules have been found in the gut and the brain of rainbow trout (Vigna *et al.*, 1985).

The stimulation of pancreatic exocrine secretion by either ACh or CCK is well founded in mammals (Grossman, 1984). CCh (an acetylcholine agonist), caerulein and CCK were found to cause secretion of digestive enzymes from the pancreas in the Guinea pig (Scheele and Palade, 1975; Tartakoff *et al.*, 1975). Intestinal extract from lampreys (*Lampetra fluviatilis* and *Petromyzon marinus*) stimulated pancreatic secretion in the rat, both in terms of an increase in flow and an increase in protein concentration (Barrington and Dockray, 1970). Intestinal extract from eel was also found to stimulate release of fluid and protein from the pancreas and a discharge of the gallbladder of the rabbit (Barrington and Dockray, 1972). CCK-related peptides were shown to cause secretion of lipase from the pancreas in the killifish, but no discharge of the gallbladder (Honkanen *et al.*, 1988). The discharge of bile from the gallbladder caused by CCK is well founded in mammals

(Behar and Biancani, 1980; Shiratori *et al.*, 1986), as well as in fish. Porcine CCK has been shown to cause contraction of the Coho salmon gallbladder (Vigna and Gorbman, 1977). Cholecystokinin related peptides have been found to cause gallbladder contraction in teleosts, such as the bluegill, killifish and bowfin (Rajjo *et al.*, 1988a) and rainbow trout (Aldman and Holmgren, 1987).

Many studies have found that ACh, or its agonists, stimulated secretion of digestive enzymes from the mammalian pancreas (Nevalainen, 1970; Scheele and Palade, 1975; Tartakoff *et al.*, 1975). In the killifish (*Fundulus heteroclitus*) lipase secretion from the pancreas was not induced by carbachol (Honkanen *et al.*, 1988), but ACh stimulated gallbladder contraction in the rainbow trout, *Oncorhynchus mykiss* (Aldman and Holmgren, 1987).

Natural, puified and sulphated CCK from pork (Mutt and Jorpes, 1968) stimulated indirectly the contraction of the smooth muscle of the Guinea pig ileum, as atropine was found to block this effect (Hedner *et al.*, 1967; Hedner and Rorsman, 1968). Natural, puified and sulphated CCK from pork was also found to stimulate smooth muscle contraction in the jejunum of conscious dogs, where it increased the intraluminal pressure of a jejunal loop (Ranirez and Farrar, 1970). Jönsson *et al.* (1987) concluded that one or several gastrin/CCK-like peptides, present in the gut of the Atlantic cod (*Gadus morhua*), may be involved in the nervous and the endocrine control of its gut motility. Caerulein, sulphated, was found to be more potent than CCK(8), sulphated or desulphated, gastrin, desulphated, or pentagastrin, sulphated. Exogenous CCK, CCK(8), sulphated or desulphated, and caerulein, sulphated, more consistently than gastrin have an excitatory effect on the motility of the intestinal and rectal smooth muscle preparations from the spiny dogfish, *Squalus acanthias* (Aldman *et al.*, 1988). It is highly likely that in fish a gut hormone, related to CCK, plays a role in the evacuation of digesta from the intestine.

The rate of stomach evacuation in salmonids has been found to be dependent on the water temperature (Brett and Higgs, 1970; Elliott, 1972; Doble and Eggers, 1978). As the rate of intestinal evacuation is expected to be dependent on the stomach evacuation, it is obvious that it also is dependent on the water temperature. It is therefore to be expected that the effect of CCK on gut motility in salmonids and therefore the amount of trypsin

and chymotrypsin in the intestinal lumen, is dependent on the water temperature.

Water temperature has been found to affect the secretion rate of pepsin in the brown bullhead, *Ictalurus nebulosus* (Smit, 1967). As the rate of enzyme secretion and the rate of evacuation of digesta from the intestine in fish is expected to be dependent on the water temperature, it is to be expected that the effect of CCK on trypsin and chymotrypsin secretion and possibly the discharge of the gallbladder is temperature dependent.

The aim of the work presented in this chapter is to study the possible role of ACh and CCK in the control of the secretion of the digestive enzymes trypsin and chymotrypsin from the pancreatic tissue and the role of CCK in the discharge of gall from the gallbladder. A porcine cholecystokinin will be used, which is slightly contaminated with secretin. Secretin is a gut hormone, which stimulates secretion of HCO_3^--rich fluid from the pancreas (Case, 1978) and is known in mammals to act synergistically with CCK (*Ibid.*; Fölsch and Wormsley, 1973). The effect of CCK will be studied at two temperature levels *in vivo* in order to be able to assess the effect of temperature on secretion of trypsin and chymotrypsin and the discharge of the gallbladder. The effect of CCK and effect of ACh and CCh on the *in vitro* secretion of trypsin and chymotrypsin will also be studied.

2. Materials and methods.

The experimental animals used were hatchery-reared fish, approximately 300 individuals. The fish had been maintained throughout in a 2-m radial flow tank. For details of tank design see Thorpe (1981). These had been kept on a commercial salmon food (freshwater smolt 1, commercial pellets, size 3 mm, PB size F1, BP Nutrition). Feeding 15 days prior to starvation was in excess, *ad libitum*.

2.1. Cholecystokinin *in vivo*.

The fish were taken from a sibling population, 10.6-14.8 cm in fork length (13.24-35.25 g fresh weight).

Two experiments were executed in the beginning of December, 1990. The fish were taken

from a sibling population and had been starved for 4 days. In one of the experiments the fish were kept for a fortnight at 1.3-8.8 °C and in the other experiment the fish were kept for a fortnight at 8.0-10.0 °C. Two different temperature regimes were used in order to determine how the secretion of trypsin and chymotrypsin is affected by the acclimation to different water temperatures and to different water temperatures during the experiment.

Anaesthesia:

The fish, 3 at a time, were kept 3-4 minutes in 5 litres of freshwater with 10 ml of benzocaine solution. The benzocaine solution was made up as follows: 4 g of benzocaine were dissolved in 100 ml of absolute alcohol. An adequate anaesthesia was obtained when the fish were lying upside down.

Intraperitoneal injection:

Purified, natural CCK from pork, sulphated (Sigma: P 4429), was dissolved in 0.1 M ammonium bicarbonate buffer, containing 1 % (v/v) acetonitrile (Honkanen *et al.*, 1988) and 2.5 % (w/v) BSA. This mixture was now stirred for 30 minutes at room temperature. The CCK-product is a mixture of CCK-33 (Mutt and Jorpes, 1967) and CCK-39 (Mutt, 1976). There are reasons to suspect that the CCK exists in more forms than the ones mentioned bove (Rehfeld, 1978). All these forms are sulphated. CCK-33 has the following structure: Lys-Ala-Pro-Ser-Gly-Arg-Val-Ser-Met-Ile-Lys-Asn-Leu-Gln-Ser-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe (Mutt and Jorpes,1971). CCK-39 is identical to CCK-33, except it has 6 additional amino acids at its N-terminus: Tyr-Ile-Gln-Gln-Ala-Arg (Go, 1978). A doubled volume of Cortland saline (Wolf, 1963) was added and the pH adjusted to 7.5 with conc. HCl. The injections were done roughly 1.5 mm behind the pelvic fin, 1.5-2.0 mm deep into a 25 g fish, fresh weight (Average thickness of the body wall of a 25 g fish is 1 mm). Injected volume was 500 μl for a 25 g fish (20 μl/g). Sixfish were injected for each of the following doses: 0 (the control), 0.001, 0.01, 0.1 and 0.5 Crick unit/g fresh weight of the fish. A few extra fish were injected with 0.5 Crick unit/g and two fish were killed after each hour and the gallbladders inspected. This was done for both temperature regimes. When the gallbladders had been found to have discharged their gall, the rest of the injected fish were sampled. One Crick unit, i.e. Crick, Harper and Raper unit, is the amount of active CCK in 1 g of fresh weight of porcine intestinal mucosa (Bodanzky *et al.*, 1973a). One Ivy dog unit equals 4 Crick units (*Ibid.*), or 6 Crick units (Jorpes and Mutt, 1962). The porcine CCK was slightly contaminated with secretin, containing 2-4 Crick units of CCK and 0.4-0.7

Units of secretin per milligram solid. After the intraperitoneal injections the fish acclimatised at 1.3-8.8 °C were kept at a water temperature of 4.6 °C, and the fish acclimatised at 8.0-10.0 °C at 8.0 °C, until killed.

Dissection:

The fish were killed by a blow to the head 4 hours after the injections of CCK and kept on ice. They were opened ventrally and the intestine, together with fat surrounding the pyloric caeca, removed. The state of the gallbladder was scored as follows: If it had more than 50 % of its maximal volume, it was classified as not having discharged. If it had less than 50 % of its maximal volume, it was classified as having discharged. This was done visually and proved easy, as the gallbladders were almost invariably full or empty. Slight digesta existed only in the rectum. A transverse cut was made behind the pyloric caeca. Luminal contents in the pyloric caecal part and in the remaining part of the intestine were squeezed out, mixed and frozen at-20 °C. The pyloric caeca with the surrounding fatty tissue was also frozen at-20 °C. The reason for assaying trypsin and chymotrypsin activities in the pancreatic tissue and the pyloric caeca as well as in the intestinal digesta is as follows: a reduction of trypsin and chymotrypsin activities in the pyloric caeca and the pancreatic tissue may not be detected because of resynthesis. An increase of these enzymes in the digesta may not be detected because of evacuation out of the animal. The fish were dried to a constant weight at 80 °C and weighed to the nearest 0.1 mg.

The tissue and digestal samples were prepared and assayed for trypsin and chymotrypsin activities as before (Chapter III, section 2.1.), with the following exception: for the chymotrypsin assay the samples were incubated with TLCK (See Chapter III, section 2.1.) at 30 mM for 2 minutes at room temperature immediately prior to the chymotrypsin assay, to guarantee no interference of trypsin activity on the chymotrypsin assay. Trypsin and chymotrypsin activities were expressed as milliunits per gram of dry weight fish (mU/g).

Statistics:

One way analysis of variance (One way ANOVA) will be applied to find if the CCK affects the activities of trypsin and chymotrypsin in the pancreatic tissue and pyloric caeca, and in the intestinal digesta, i.e. find if secretion of these enzymes has occurred. A multiple comparisons test, the Tukey's test, will be used to determine at which doses of

CCK the change in trypsin and chymotrypsin activities, i.e. secretion, becomes significant (Zar, 1984).

As the data for the gallbladder discharge are counts, having only integer values from 0 to 6, they are probably not normally distributed. Therefore the nonparametric Kruskal Wallis's test (Zar, 1984) was used to reveal if the CCK caused a significant discharge of the gallbladder and at what doses of CCK the gallbladder discharge became significant.

2.2. Cholecystokinin, *in vitro*.

The fish were post smolt from a River Spey stock, lower mode 2+. They were 26.2-27.1 cm in fork length (201.6-229.3 g fresh weight) and starved for 3 days prior to the experiment, which was executed in early October, 1990.

A modified Cortland saline was put into a double chambered organ bath, 20 ml in each. As the original saline (Wolf, 1963) frequently precipitated, the saline was modified by a reduction of the $NaH_2PO_4 \cdot 2H_2O$ and an addition of N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Good *et al.*, 1966), resulting in the following recipe:

NaCl	7.25 g/l	(124.06 mM)
KCl	0.38 g/l	(5.10 mM)
$CaCl_2 \cdot H_2O$	0.23 g/l	(1.56 mM)
$MgSO_4 \cdot H_2O$	0.13 g/l	(0.93 mM)
$NaHCO_3$	3.00 g/l	(35.70 mM)
$NaH_2PO_4 \cdot 2H_2O$.	0.01 g/l	(0.06 mM)
Glucose	1.00 g/l	(5.55 mM)
HEPES	2.38 g/l	(10.00 mM)

The saline was equilibrated with a mixture of 97 % O_2 and 3 % CO_2 at a pressure slightly above the atmospheric pressure and the pH adjusted to 7.5 at 10.0 °C.

One single fish was killed by a blow on the head and the pyloric caeca with the

surrounding fat promptly removed. The tissue was cut into ca. 5-8 pieces, washed in 10.0 °C saline and ca. 500 mg wet weight suspended into each chamber of the organ bath. Aliquots of 500 μl of saline were taken from each chamber of the organ bath after 0, 4, 14, 24 and 44 minutes. After 15 min, 800 l of distilled deionized water and 800 μl of porcine CCK (Sigma: P 4429; see section 2.1.2.), dissolved in distilled deionized water, were added to the organ bath chambers of the control and the CCK-treated tissue, respectively, with the resulting concentration in the saline for the CCK-treated tissue, 8.0 Crick units/ml of CCK and 1.2 Units/ml of contaminating secretin. For definition of Units, see Bodanzky *et al.* (1973a). The product porcine cholecystokinin (Sigma: P 4429) is contaminated with some secretin. 500 μl of the modified Cortland saline were added to the organ bath chambers, each time an aliquot was removed. The aliquots were promptly and thoroughly mixed with CCl_4 (20 % v/v) and centrifuged at 10,000 *g* for 4 min. The supernatants were kept at 2-5 °C for 24 hrs, and trypsin and chymotrypsin assayed as described in section 2.1. Activities were expressed as milliunits per milligram dry weight of tissue (mU/mg). The contents of the organ bath chambers were dried at 80 °C to a constant weight (2-3 days).

The above procedure was repeated for 6 fish, but aliquots were then only removed after 44 minutes.

Each individual gave rise to pairwise data for both trypsin and chymotrypsin, originating from the CCK treated tissue and from the control. The difference in the trypsin-or the chymotrypsin activities in the medium, i.e. the buffered saline, between the CCK treated tissue and the control can be tested against 0 with the *t*-test for pairwise data (Zar, 1984). If this difference is significantly greater than 0, then the CCK will have caused a transfer of trypsin-or chymotrypsin activity into the medium.

2.3. Acetylcholine and carbamylcholine, *in vitro.*

The *in vitro* stimulation of trypsin and chymotrypsin secretion by ACh and CCh was carried out using the procedure of the previous section (Section 2.2.). The tissue was challenged with 10^{-7}, 10^{-6},10^{-5},10^{-4},10^{-3},10^{-2} and 10^{-1} M concentration of ACh and 10^{-7}, 10^{-6},10^{-5},10^{-4},10^{-3},10^{-2} and 10^{-1} M concentration of CCh.

The same statistical tests, i.e. one way ANOVA and the Tukey's test, were planned here as before (See section 2.2.), and with the same objectives in mind.

3. Results.

3.1. The *in vivo* effect of cholecystokinin.

The effect of intraperitoneally injected porcine cholecystokinin (CCK) on the secretion of trypsin (Fig. 18 (a)) and chymotrypsin (Fig. 18 (b)) at 4.6 °C for fish acclimatised in the temperature range of 1.3-8.8 °C was found to decrease these activities in the pancreatic tissue and the pyloric caeca and increase these activities in the intestinal digesta.

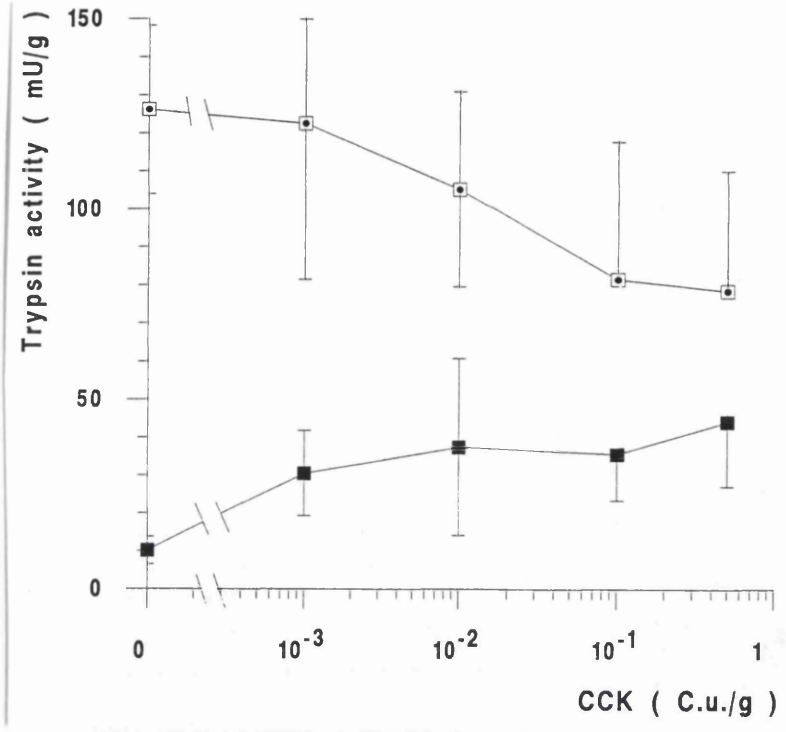
Trypsin activity in the pyloric caeca and the pancreatic tissue was seen to fall, as the dose of CCK was increased (Fig. 18 (a)). This was however not significant (One way ANOVA: p = 0.044. Tukeys test: 0.0 C.u./g, i.e. the control versus 0.5 C.u./g: $p > 0.05$. X.Appendices. Table IV.1-2). Trypsin activity in the intestinal digesta was seen to rise significantly, as the dose of CCK was increased (One way ANOVA: p = 0.007. X.Appendices. Table IV.3). A dose of 10^{-2} C.u./g caused a significant increase of trypsin activity in the intestine (Tukeys test: $p < 0.05$. X.Appendices. Table IV.4).

Chymotrypsin activity in the pyloric caeca and the pancreatic tissue was seen to fall, as the dose of CCK was increased (Fig. 18 (b)). This was however not significant (One way ANOVA: p = 0.273. X.Appendices. Table IV.5). A significant rise in chymotrypsin activity in the intestinal digesta, caused by an increase in the dose of CCK was found (One way ANOVA: p = 0.020. X.Appendices. Table IV.7). A dose of 10^{-1} C.u./g caused a significant increase of chymotrypsin activity in the intestinal digesta (Tukeys test: $p <$ 0.05. X.Appendices. Table IV.8). By comparing Fig. 18 (a) and Fig. 18 (b), it can be seen, that the porcine CCK apparently causes a greater secretion of trypsin, than of chymotrypsin. This is also reflected in the fact that in the intestinal digesta a significant rise in trypsin activity, is observed at a lower dose of CCK, i.e. at a dose of 10^{-2} C.u./g, than a significant rise in chymotrypsin activity at a dose of 10^{-1} C.u./g (See above).

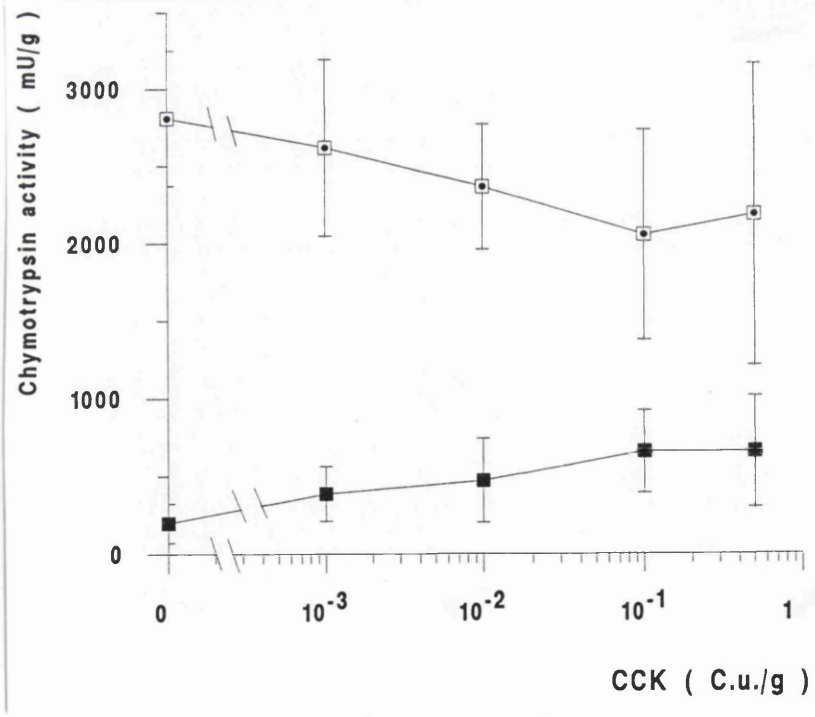
With an increasing dose of CCK, the number of fish having their gallbladders discharged

Figure 18: The effect of intraperitoneally injected natural, purified, porcine cholecystokinin, sulphated, on trypsin (**a**) and chymotrypsin (**b**) activity in the pyloric caeca and the pancreatic tissue (◙) and in the intestinal digesta (■) at 4.6 °C, for fish acclimatised at 1.3-8.8 °C. The fish were starved for 4 days and then injected intraperitoneally with modified Cortland saline, containing various amounts of porcine CCK. Trypsin (a) and chymotrypsin (b) activity is expressed as milliunits per dry weight fish in grams (mU/g) and cholecystokinin dose in Crick units per gram fresh weight in fish (C.u./g). The control is the modified Cortland saline, without the CCK. Data points are mean values (n=6), and error bars are standard deviations.

(a)


Trypsin activity (mU/g)
150
100
50
0
0
10^{-3}
10^{-2}
10^{-1}
1
CCK (C.u./g)

(b)


Chymotrypsin activity (mU/g)
3000
2000
1000
0
0
10^{-3}
10^{-2}
10^{-1}
1
CCK (C.u./g)

increased. A CCK dose of 10^{-1} C.u./g was shown to cause a significant discharge of the gallbladder in all the 6 fish injected (Kruskal Wallis's test: $p < 0.001$. X.Appendices. Table IV.9-12).

The effect of intraperitoneally injected porcine cholecystokinin (CCK) on the secretion of trypsin (Fig. 19 (a)) and chymotrypsin (Fig. 19 (b)) at 8.0 °C for fish acclimatised in the temperature range of 8.0-10.0 °C was found to decrease these activities in the pancreatic tissue and the pyloric caeca and only slightly increase these activities in the intestinal digesta..

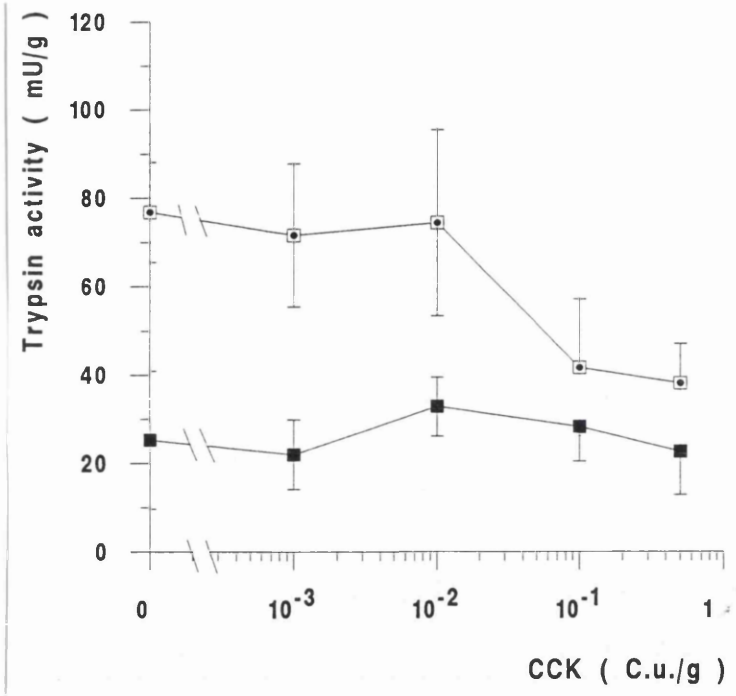
Trypsin activity in the pyloric caeca and the pancreatic tissue (Fig. 19 (a)) was seen to fall, as the dose of CCK was increased from 10^{-2} C.u./g to 10^{-1} C.u./g. This was significant (One way ANOVA: $p < 0.0005$. X.Appendices. Table IV.13). A dose of 10^{-1} C.u./g caused a significant fall of trypsin activity in the pyloric caeca and the pancreatic tissue (Tukeys test: $p < 0.01$. X.Appendices. Table IV.14). No significant rise in trypsin activity was found in the intestinal digesta (One way ANOVA: $p = 0.344$. X.Appendices. Table IV.15).

Chymotrypsin activity in the pyloric caeca and the pancreatic tissue (Fig. 19 (b)) was seen to fall, as the dose of CCK was increased from 10^{-2} C.u./g to 10^{-1} C.u./g.. This was significant (One way ANOVA: $p < 0.0005$. X.Appendices. Table IV.17). A dose of 10^{-1} C.u./g caused a significant fall in chymotrypsin activity in the pancreatic tissue and pyloric caeca (Tukeys test: $p = 0.001$. X.Appendices. Table IV.18). No significant rise in the chymotrypsin activity was observed in the intestinal digesta (One way ANOVA: $p = 0.288$. X.Appendices. Table IV.19).

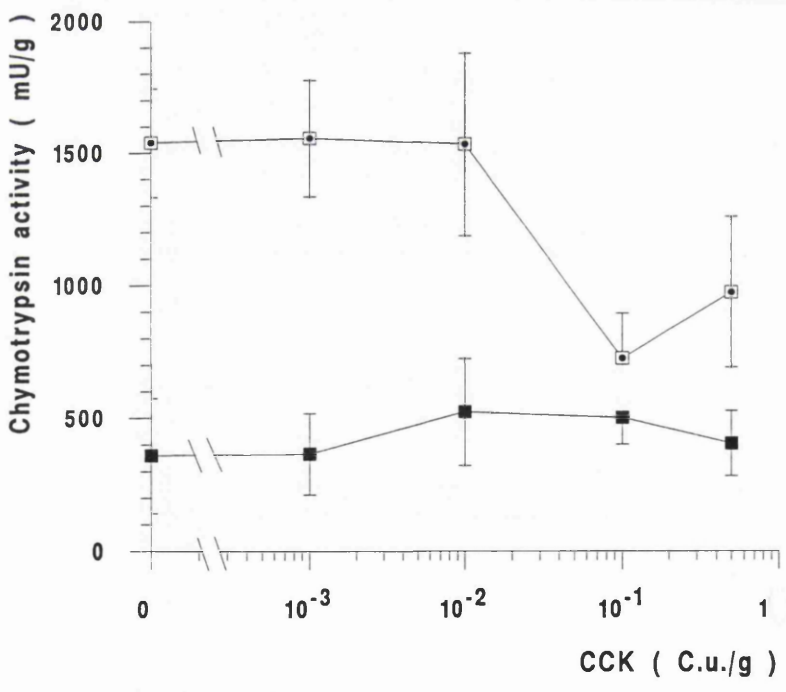
With an increasing dose of CCK, the number of fish having their gallbladders discharged increased. A CCK dose of 10^{-1} C.u./g was shown to cause a significant discharge of the gallbladder in 4 of the 6 fish injected (Kruskal Wallis's test: $p < 0.05$. X.Appendices. Table IV.21-24). At a dose of 0.5 C.u./g the gallbladder in all 6 of the 6 fish injected discharged (Kruskal Wallis's test: $p < 0.001$. X.Appendices. Table IV.21-24).

Figure 19: The effect of intraperitoneally injected natural, purified, porcine cholecystokinin, sulphated, on trypsin (**a**) and chymotrypsin (**b**) activity in the pyloric caeca and the pancreatic tissue (⊡) and intestinal digesta (■) at 8.0 °C for fish acclimatized at 8.0-10.0 °C. Further details as in Figure 18.

(a)


Trypsin activity (mU/g)
120
100
80
60
40
20
0
0
10^{-3}
10^{-2}
10^{-1}
1
CCK (C.u./g)

(b)


Chymotrypsin activity (mU/g)
2000
1500
1000
500
0
0
10^{-3}
10^{-2}
10^{-1}
1
CCK (C.u./g)

3.2. The *in vitro* effect of cholecystokinin.

Pyloric caeca and pancreatic tissue from each fish was split into two: The control and the CCK treated tissue. This gave rise to pairwise data for trypsin (Fig. 20) and chymotrypsin (Fig. 21) activities.

CCK caused a release of trypsin from pyloric caeca and pancreatic tissue, suspended in the organ bath (Fig. 20). Trypsin activity in the organ baths for the CCK-treated tissue and the control from a single fish differed after addition of CCK (Fig. 20 (a)). Considerable washout from the pyloric caeca and the pancreatic tissue is observed within the first 4 minutes. A definite rise in trypsin activity is seen to occur in the organ bath for the CCK treated tissue between 14 and 24 minutes, but the tissue was challenged with CCK after 15 minutes in the organ bath. The trypsin activity in the organ bath of the control, rose only slightly after 4 minutes. A greater trypsin activity was observed in the saline surrounding the CCK treated tissue than in the control. CCK caused a significant rise in released trypsin activity (*t*-test for pairwise data, tested against 0: $p < 0.00005$. X.Appendices. Table IV.25).

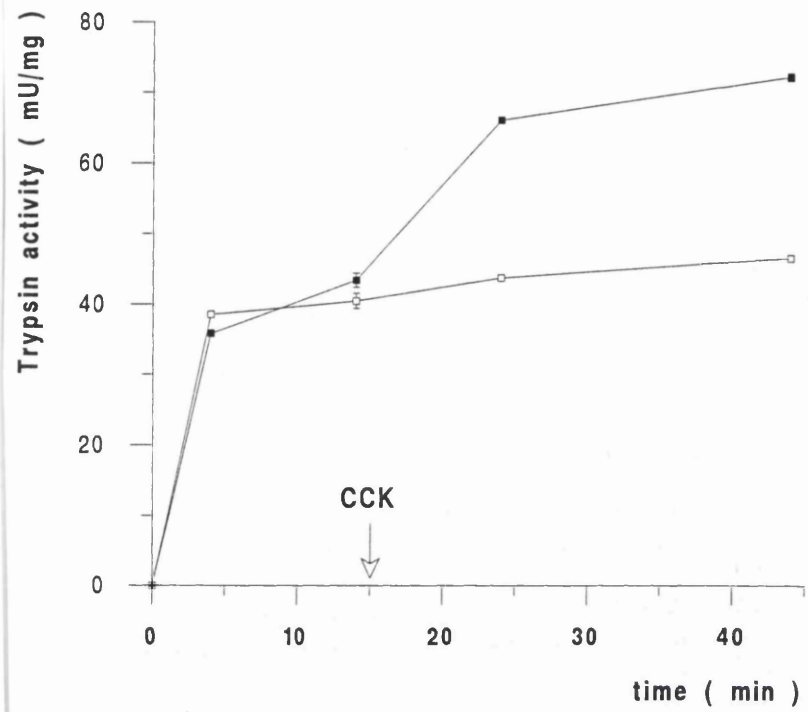
CCK caused a release of chymotrypsin from pyloric caeca and pancreatic tissue, suspended in the organ bath (Fig. 21). Chymotrypsin activity in the organ baths for the CCK-treated tissue and the control, from a single fish is shown in Fig. 21 (a). This activity has a fairly similar pattern to the trypsin activity (Fig. 20 (a)). A greater chymotrypsin activity was found in the saline surrounding the CCK treated tissue, than in the control (Fig. 20 (b)). CCK caused a significant rise in released trypsin activity (*t*-test for pairwise data, tested against 0: $p = 0.0002$. X.Appendices. Table IV.25).

3.3. The *in vitro* effect of acetylcholine and carbamylcholine.

The *in vitro* stimulation of trypsin and chymotrypsin secretion by ACh and CCh proved unsuccessful at 10^{-7}, 10^{-6},10^{-5},10^{-4},10^{-3},10^{-2} and 10^{-1} M concentration in the organ bath (Results are not presented).

Figure 20: Effect of natural, purified, porcine cholecystokinin, sulphated, on trypsin secretion *in vitro* by the pyloric caeca and the pancreatic tissue. (**a**) Released trypsin activity from a single fish in the control (□) and in the CCK-treated tissue (■) as a function of time. The tissue was challenged with a mixture of 8.0 Crick units/ml of CCK and 1.2 Units/ml of secretin after 15 minutes. Trypsin activity was expressed in milliunits per dry weight of tissue in milligrams (mU/mg). Each data point is the average of two trypsin assays. (**b**) Released trypsin activity from 6 fish in the control and CCK-treated tissue after 44 minutes. Data points are mean values (n=6), and error bars are standard deviations. Further details as in legend to (a).

(a)


Trypsin activity (mU/mg)
80
60
40
20
0
CCK
0
10
20
30
40
time (min)

(b)

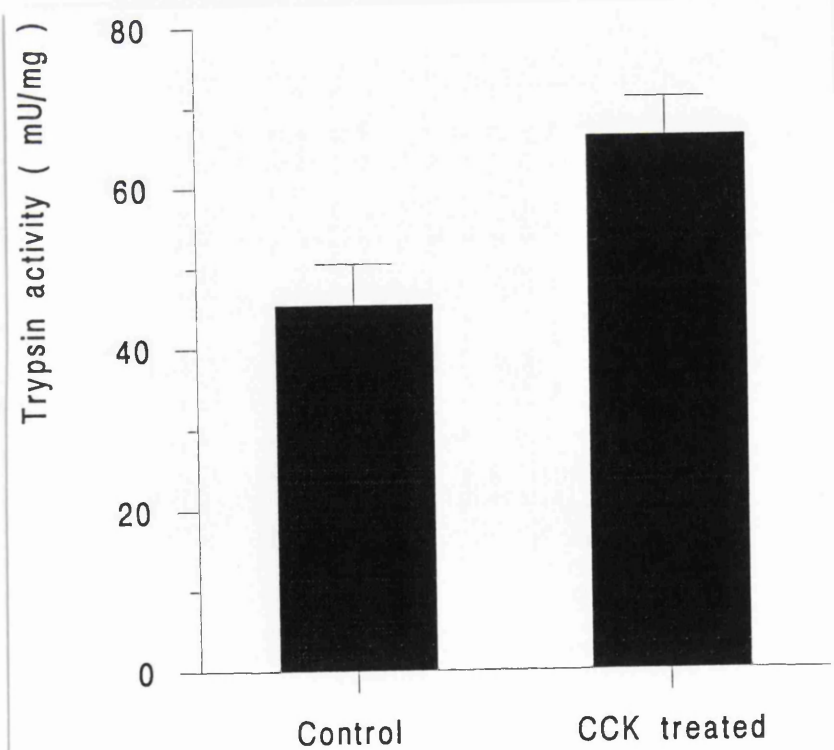
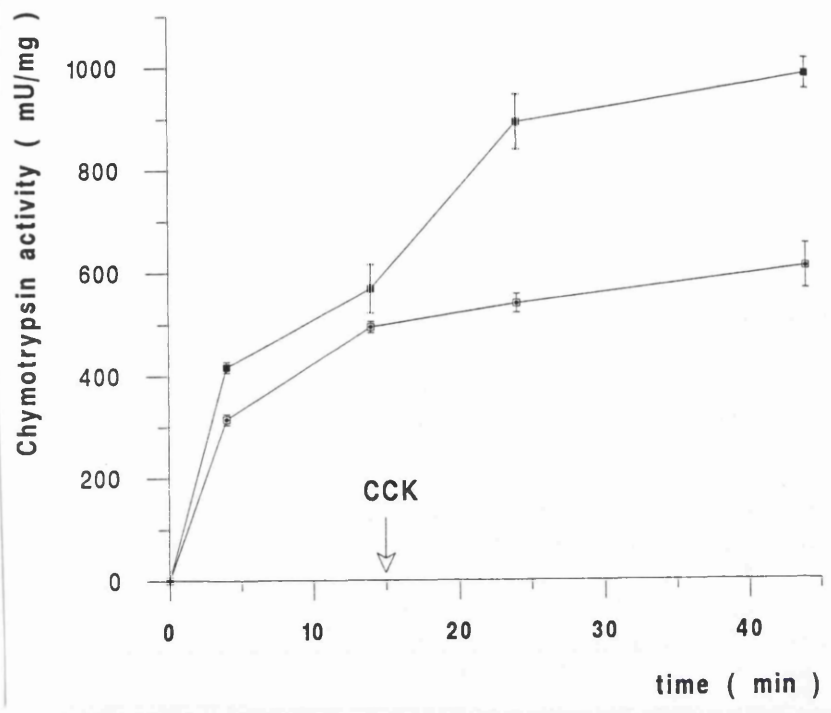
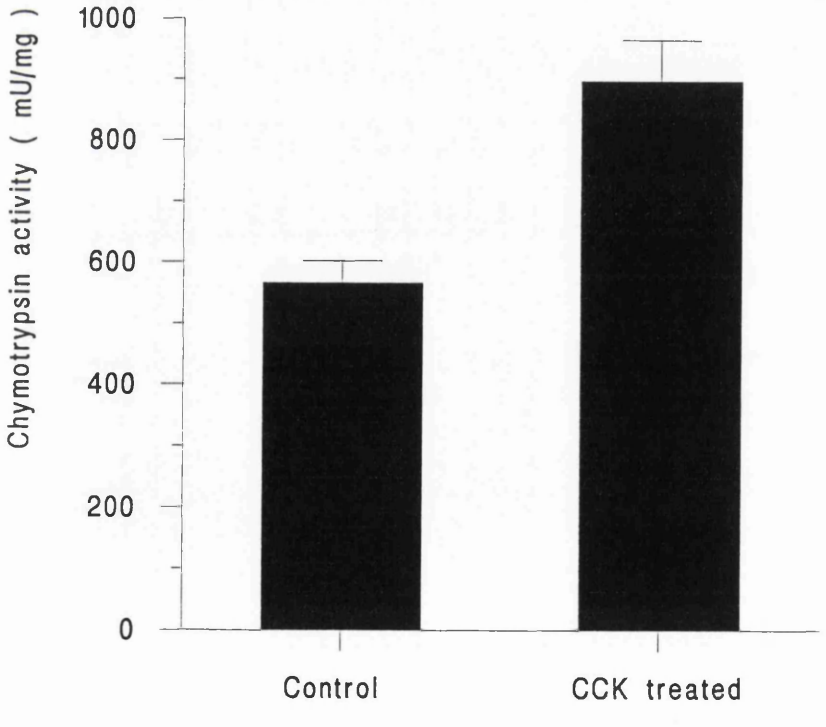

Trypsin activity (mU/mg)
80
60
40
20
0
Control
CCK treated

Figure 21: Effect of natural, purified, porcine cholecystokinin, sulphated, on chymotrypsin secretion *in vitro* by the pyloric caeca and the pancreatic tissue. (**a**) Released chymotrypsin activity from a single fish in the control (⊡) and in the CCK-treated tissue (■) as a function of time. Further details as in Figure 4 (a). (**b**) Released chymotrypsin activity from 6 fish in the control and CCK-treated tissue after 44 minutes. Further details as in legend to Figure 20 (b).

(a)


Chymotrypsin activity (mU/mg)
1000
800
600
400
200
0
CCK
0
10
20
30
40
time (min)

(b)


Chymotrypsin activity (mU/mg)
1000
800
600
400
200
0
Control
CCK treated

4. Discussion.

Porcine cholecystokinin causes secretion of trypsin and chymotrypsin *in vivo* (Fig. 18 and Fig. 19), and this is in agreement with previous studies in other vertebrates. *In vivo* stimulation of the rat pancreas by a mixture of natural, purified porcine CCK, sulphated, and secretin resulted in a 40 % reduction in its amylase activity after 4 hours (Reggio *et al.*, 1971). This was roughly the same for trypsin and chymotrypsin in the present study, especially at the higher temperature regime (Fig. 19), but the fish had been killed 4 hours after the injection. Sub maximal stimulation of secretion of digestive enzymes from the rat pancreas by natural, purified porcine CCK, sulphated, is potentiated by secretin (Fölsch and Wormsley, 1973), but secretion of amylase and trypsin commenced immediately with the infusion of CCK and secretin. *In vivo* studies on pancreatic secretion in humans revealed that chymotrypsin, amylase and lipase were secreted from the pancreas during challenge with CCK (Dagorn *et al.*, 1977). Chymotrypsin, amylase and lipase were found to be secreted from the rat pancreas during *in vivo* conditions by a mixture of natural, purified porcine CCK, sulphated, and secretin, pilocarpine (ACh agonist) and secretin, but not by secretin alone (Dagorn, 1978). Secretion commenced almost instantly upon challenge. Secretin was in these studies found only to potentiate secretion, but CCK initiated it.

Intraperitoneally injected CCK(8), sulphated, or caerulein, sulphated and desulphated, stimulated *in vivo* secretion of lipase in the killifish (*Fundulus heteroclitus*), where caerulein was found to be the most potent (Honkanen *et al.*, 1988). The fish had been killed 20 minutes after the injection. Intraperitoneal injection will probably work as an infusion: the CCK plasma concentration will gradually rise up to a certain point, but then gradually fall. It is possible that the CCK was taken up into the blood stream at a slower rate from the intraperitoneal cavity in the salmon, than in the killifish. It is also possible that digestive enzyme secretion may have commenced immediately after the CCK injection in the salmon. Supporting this is the finding in the present study that *in vitro* challenge with CCK (Fig. 20 (a) and Fig. 21 (a)) did show that the pancreatic tissue secreted trypsin and chymotrypsin almost immediately.

Intestinal extracts from lampreys were found to stimulate pancreatic secretion in the rat

during *in vivo* conditions (Barrington and Dockray, 1970) and identical findings were obtained for intestinal extract from eel during *in vivo* stimulation in the rabbit (Barrington and Dockray, 1972). Some factor in the intestine of fish is capable of causing stimulation of pancreatic secretion in mammals. The above studies suggest that this factor has remained unchanged during vertebrate evolution. Vigna (1979) suggests after reviewing the studies of Holmquist *et al.* (1979) and Larsson and Rehfeld (1977) that a CCK-like peptide or peptides were established early in vertebrate evolution. The present study supports this, as CCK from a mammal, i.e. the pig, is able to stimulate the secretion of digestive enzymes in the salmon. Indeed, a CCK immunoreactivity has been found in the various teleostei (Rajjo *et al.*, 1988b), including rainbow trout (*Oncorhynchus mykiss*) where gastrin/CCK-like immunoreactivity was found in the intestine (Holmgren *et al.*, 1982). Further studies on the rainbow trout found the highest concentrations of CCK-like immunoreactivity in the gut of this animal were in the small intestine and the pyloric caeca (Vigna *et al.*, 1985). A further support for the existence of a CCK-like factor in the Atlantic salmon is apparent, as endocrine cells containing conspicuous granules, having a location distal from the lumen, were found in the pyloric caecal region of this animal (Chapter II).

The fact that the secretion of trypsin and chymotrypsin is also initiated by porcine cholecystokinin acting on isolated pancreatic tissue *in vitro*, prooves that the CCK effect on the tissue is not dependent on extrinsic nerves. Dockray (1982) expresses the view that vagovagal cholinergic reflexes are important in the normal control of pancreatic enzyme secretion, as the plasma CCK concentrations after a normal light meal are unlikely to be sufficient to account for the response of the pancreas with respect to the secretion of digestive enzymes. It is a possibility that the CCK in the present study is causing secretion of trypsin and chymotrypsin at a "pharmacological", rather than a physiological level. This must at least be the case for the *in vitro* studies (Fig. 20 and Fig. 21), as the concentration of the CCK in the organ bath was at least 10 times greater than the dose in the *in vivo* study. This difference might be because of insufficient saline flow to every acinar cell in the tissue lumps, or proteolytic breakdown of the CCK-receptors, thus decreasing their affinity towards the porcine CCK. This difference may also be explained by vagovagal reflexes (*Ibid.*) being destroyed upon the removal of the pancreatic tissue from the animal and the cutting of this tissue into smaller pieces. *In vitro* experiments usually require

higher concentrations of secretogogues for stimulation, than the comparable plasma concentration during *in vivo* conditions (Case, 1978). *In vitro* studies of pancreatic secretion in mammals have confirmed that natural, purified porcine CCK, sulphated, is able to stimulate secretion of digestive enzymes from the pancreas (*Ibid.*; Scheele and Palade, 1975; Tartakoff *et al.*, 1975), which is in agreement with the present study, confirming that CCK is able to stimulate the pancreas directly. It is well established for many mammals that CCK stimulates the acinar cell directly, i.e. via its own receptors (Jensen *et al.*, 1980; Sankaran *et al.*, 1980; Bommelaer *et al.*, 1981; Sankaran *et al.*, 1982; Susini *et al.*, 1986; Fourmy *et al.*, 1987; Gardner and Jensen, 1987). Studies on the killifish (*Fundulus heteroclitus*) also support this, as atropine (muscarinic antagonist) was unable to inhibit the stimulation of lipase secretion from the pancreas, initiated by CCK-like peptides during *in vivo* conditions (Honkanen *et al.*, 1988). In this study, however it was not demonstrated that atropine blocked a cholinergic *in vivo* stimulation of the pancreas, which had resulted inthe secretion of trypsin and chymotrypsin

Natural, purified porcine CCK, sulphated (section 2.1), caused the discharge of the gallbladder *in vivo* and this is in agreement with previous studies in mammals (Jorpes and Mutt, 1973; Walsh, 1978) and in teleosts (Vigna and Gorbman, 1977; Aldman and Holmgren, 1987; Rajjo *et al.*, 1988a). Caerulein, sulphated and desulphated, and CCK(8), sulphated, at doses effective in stimulating lipase secretion failed however to cause discharge of the gallbladder *in vivo* in the killifish (Honkanen *et al.*, 1988). The present study showed that the gallbladder did not discharge its gall until 4 hours after the intraperitoneal injection for both temperature regimes. It is highly likely that in the killifish, which was sampled only 20 minutes after injection with caerulein, sulphated and desulphated, and CCK(8), sulphated, that the gallbladder responds much later to intraperitoneally injected CCK, than the pancreas (*Ibid.*). This could happen during rising plasma concentrations of the CCK. Studies on this animal during *in vitro* conditions, found that CCK(8), sulphated, or caerulein, sulphated, caused dose-related contractions of its gallbladder and were not affected by atropine (Rajjo *et al.*, 1988a). The CCK receptors on the gallbladder are likely to have a lower affinity towards CCK, than CCK receptors on the acinar cells. In support of this is the conclusion that regulation of pancreatic enzyme secretion is a primitive action of CCK-like peptides, and that the sensitivity of the gallbladder muscle to these peptides evolved later, possibly in the ancestral lineage that

led to the gnathostomes (Vigna, 1983).

The effect of temperature on CCK stimulation of trypsin and chymotrypsin secretion was as follows:

i) A significant fall in the trypsin and chymotrypsin activities in the pancreatic tissue and the pyloric caeca was only observed at the higher temperature regime (Fig. 19 (a) and Fig. 19 (b)). This is explained by faster secretory rates of trypsin and chymotrypsin from the pancreas, as the water temperature rises, as the rate of resynthesis should also be higher at the higher temperature regime. This agreed with a previous study on pepsin secretory rate in the brown bullhead (*Ictalurus nebulosus*), which was found to rise with rising temperature in the range of 10 to 25 °C (Smit, 1967).

ii) A significant rise in the trypsin and chymotrypsin activities in the intestinal digesta was only observed at the lower temperature regime (Fig. 18 (a) and Fig. 18 (b)). It is highly likely that secreted trypsin and chymotrypsin had already been evacuated from the fish at the time of sampling for the higher temperature regime. This is in agreement with previous studies, which have established that evacuation rate from the stomach increases with temperature (Brett and Higgs, 1970; Elliott, 1972; Doble and Eggers, 1978), and is probably partially at least under the control of some CCK-like peptide (Section 1). An increase in the evacuation rate from the stomach calls for an increase in the evacuation rate from the intestine.

Stimulation of secretion with ACh or CCh proved unsuccessful (Results are not presented). The fact that, porcine CCK was able to cause secretion of trypsin and chymotrypsin *in vitro*, proves that the pancreatic acinar cells were viable during these conditions. Apparently, ACh or CCh are either unable to reach the necessary receptors on the acinar cell for the stimulation (perfusion required) or secretion of trypsin and chymotrypsin is not initiated by neuronal stimuli in the Atlantic salmon. Supporting the second suggestion is the fact, that CCh (ACh agonist) did not cause secretion of lipase from the pancreatic tissue of the killifish *in vivo* (Honkanen *et al.*, 1988). Indeed initial studies had found that *in vivo* stimulation of trypsin and chymotrypsin secretion and discharge of the gallbladder with either ACh or CCh proved unsuccessful. Wang and Grossman (1951) suggested that in the dog hormones play a dominant role in the regulation of the pancreatic activity in normal digestion. Recent studies on herring larvae

did however indicate that secretion of trypsin is initiated by neuronal stimulation (Pedersen, 1993).

Chapter V.

Localisation of pepsinogen, trypsinogen and chymotrypsinogen.

1. Introduction.

The histology and the ultrastructure of the stomach gland cells and the pancreatic tissue have already been surveyed with light and transmission electron microscopy (TEM) in Chapter II. In this chapter the location of the storage forms, i.e. the zymogens of pepsin, trypsin and chymotrypsin, will be studied. These are pepsinogen, trypsinogen and chymotrypsinogen, respectively, and are the inactive precursors of these enzymes. Pepsinogen is activated, i.e. turned into pepsin, by a low pH in the stomach, while trypsinogen and chymotrypsinogen are activated, i.e. turned into trypsin and chymotrypsin, respectively, by trypsin in the intestine. In mammals trypsinogen is also activated by enterokinase (Lehninger, 1975). Enterokinase is secreted by the mucosa of the intestinal wall and has the primary purpose of activating trypsinogen in the intestinal lumen.

In all vertebrates, the stomach gland cells are found to produce pepsinogens (Giraud and Yeomans, 1982; Vonk and Western, 1984). Pepsinogen has been demonstrated in the stomach gland cells of some lower vertebrates, including rainbow trout, by indirect immunufluorescence (Yasugi, 1987; Yasugi *et al.*, 1988). No systematic study of pepsinogen location in the various parts of the stomach of the teleostei appears to have been undertaken, using this method.

It was shown in Chapter II, that the pancreatic tissue is embedded in the fat surrounding the pyloric caeca and that the pancreatic acinar cells, contained granules, probably zymogen granules, containing trypsinogen and chymotrypsinogen. A thorough study of the synthesis, intracellular transport and discharge of secretory proteins has been undertaken in the pancreatic exocrine cells from Guinea pigs (Jamieson and Palade, 1971). However, little is known about the pancreas in the teleostean fish. Trypsinogen and

chymotrypsinogen were found in the Atlantic cod to be present in the extracts of the fatty tissue surrounding the pyloric caeca (i.e. the pancreas with its surrounding fat), but not in the caeca themselves (Overnell, 1973). This is in agreement with the qualitative histological observations on the pancreatic tissue in this animal (Bishop and Odense, 1966).

In this chapter, the exact location of pepsinogen, trypsinogen and chymotrypsinogen, will be determined.

The pepsinogen location with respect to the various areas of the stomach will be investigated by an assay of pepsin activity in the stomach wall, after the activation of the pepsinogen, for these respective regions, i.e. the posterior oesophagus, the anterior and the posterior cardiac area and the pyloric area. The pepsinogen location with respect to the aforementioned areas and with respect to the chief cells of the stomach, identified in Chapter II, will also be investigated by indirect immunofluorescence.

Porcine cholecystokinin (CCK) was found to cause a secretion of trypsin and chymotrypsin from the pyloric caeca and their surrounding fatty tissue (Chapter IV). The trypsinogen and the chymotrypsinogen location, with respect to the pancreatic tissue, the pyloric caeca and the intestine, will be investigated by an assay of trypsin and chymotrypsin activities, after activation of their zymogenic forms. Activation is the conversion of pepsinogen into pepsin, trypsinogen into trypsin and chymotrypsinogen into chymotrypsin. After homogenisation of the samples a full activation of these enzymes was always obtained after an overnight storage in the fridge, i.e. at 2-5 °C (Chapter III, section 2.1.). The effect of CCK on the secretion of trypsin and chymotrypsin, will also be monitored by these assays. A reduction in both trypsin and chymotrypsin activities, within either the pancreatic tissue, the pyloric caeca or the intestine, can be taken as a strong support of their secretion from that tissue, hence their localisation. The question arises whether porcine CCK simultaneously causes a reduction in the granules already observed in the pancreatic acinar cells (Chapter II). This will be investigated from T.E.M. sections, with an evaluation of the proportional granular area in the acinar cells.

2. Materials and methods.

2.1. Localisation of pepsinogen.

The fish: Hatchery reared, lower modal group salmon in their 2nd year, and a stock from the River Almond, had been fed *ad libitum*, before starvation of 5 days, prior to the experiment. The fish had been kept at 10.0-16.3 °C a fortnight before the experiment. The experiment was conducted in August, 1992.

2.1.1. Light microscopy and immunolocalisation of pepsinogen.

Five fish (fresh weight 13.1-22.7 g) were killed by a blow to the head , opened ventrally and the stomachs removed. The guts had the characteristics of starving fish: Empty stomachs and only slight digesta in the rectum. The gallbladders were full and green. Four transverse slices, ca. 1.5 mm in thickness, were removed from the posterior oesophagus and the stomach in the following way: i) Immediately anterior to the airbladder duct. ii) Immediately posterior to the airbladder duct. iii) Immediately anterior to the transitional area of the stomach. iv) Immediately posterior to the transitional area of the stomach (See Chapter II).

Histological treatments were adapted from Yasugi (1987) and Sainte-Marie (1962).

Fixation: Samples were immediately placed in ice-cold 95 % ethanol in stoppered vials for 4 hours and thereafter at 2-4 °C for 22 hours.

Dehydration: 4 changes of ice-cold absolute alcohol, 1.5 hrs each, under gentle motion.

Clearing: 3 changes of ice-coldxylene, 1.5 hrs each, under gentle agitation and then stored inxylene (histoclear contains pyridine, which damages the sample with respect to the binding of the primary antibody) ca. 60 hrs at 0 °C.

Embedding: The samples were embedded in a low melting point (m.p. 37 °C) polyester wax (Steedman, 1957) as follows: The samples were allowed to come to room

temperature before entering 5 changes of molten polyester wax(TAAB: W 005), at 38.0-39.5 °C, for 1 hr each. Samples were then embedded, and finally stored in the fridge for 9 days.

Sectioning: Blocks and knife were chilled with an ice cube during the sectioning. 5 μm sections were cut and floated on to a 20-22 °C water bath for 5-10 min. Sections were mounted on glass slides covered with poly-L-lysine, which had been prepared as follows: Glass slides were washed in 1 % HCl in 70 % ethanol by a gentle agitation for 4 hrs, dried at 60 °C, dipped into a diluted poly-L-lysine solution (Poly-L-lysine, Sigma: P 8920, diluted 1/10 in distilled and deionized water) for 5 min and dried at 60 °C for 1 hr.

Every other 4 sections from all samples underwent haematoxylin and eosin staining and photography (See Chapter II, section 2.2), in order to correlate the location of immunofluorescent staining with specific areas of the stomach mucosa.

The remaining sections for the immunolocalisation were handled at 4 °C, as follows:

Deparaffinization: Under a very gentle motion.

xylene: 2 changes, 10-15 sec each.
95 % ethanol: 3 changes, 10-15 sec each.
PBS: 3 changes, 1 min each.

The PBS (phosphate buffered saline) was made up as follows: 0.8 % NaCl, 0.05 M phosphate buffer (See Chapter II, section 2.3) and pH = 7.0.

Primary antibody and controls:

Primary antibody: Lyophilised monoclonal antibody, Y 37, raised against embryonic chicken pepsinogen (Yasugi *et al.*, 1987), was kindly donated by Professor Sadao Yasugi. It was reconstituted with 0.5 ml of deionized water, containing 0.1 % Na azide and stored at -75 °C in 20 μl aliquots.

Control 1: A solution of a monoclonal mouse IgG against *Trypanosoma brucei* was kindly donated by Dr. C.M.R. Turner.

The primary antibody and the control 1 solutions were now diluted 1/10 in an antibody medium, which was made up as follows:

PBS (See before)	10 ml
BSA (Sigma: A 4503)	0.5 mg
Thyroglobulin (Sigma: T 1001)	0.5 mg
Na azide	25 mg
Triton-X100	50 μl

Sections were now incubated in the primary antibody solution, control 1 solution (Control no. 1) or the antibody medium (Control no. 2) in a humid chamber for 20 hrs (Ca. 50 μl on each slide).

Washing: PBS 3 changes, 5 min each.

Secondary antibody: Goat anti-mouse IgG (Sigma: F 0257), labelled with fluoroisothicyanate (FITC), was diluted 1/10 in the antibody medium. Sections were incubated in this solution for 2 hrs.

Washing: PBS 2 changes, 5 min each.

Mounting and photography: Sections were mounted in polyvinyl alcohol (Sigma: P 8136) mounting medium (Rodriguez and Deinhardt, 1960), stored in the dark overnight at 2-4 °C and sealed with nail lacquer. They were then photographed ca. 2-4 hrs later using Ectachrome ASA 400 film with a Leitz Wetzler fluorescence microscope at λ=495 nm using filters for FITC-labelled antibodies.

2.1.2. Assay of pepsin activity in the stomach.

Five fish (fresh weight 20.5-31.3 g) were killed by a blow to the head . Dissection was as described in section 1.1, except that the sections removed from the stomach, were ca. 2 mm thick. These were opened up lengthwise and the luminal mucus gently removed with a blunt scalpel and put into ice-cold PBS, pH = 7.0 (See section 1.1). The sections

underwent 5 changes of PBS under a gentle agitation for a total of 6 hrs at 0 °C. They were slightly dried on absorbent paper and frozen at -75 °C.

The samples were subsequently thawed and homogenised with a Potter homogeniser for 45 sec in 1 ml ice-cold Johnson Lindsay (JL) buffer (See Chapter III, section 2.1.), pH = 2.0 and 200 μl of CCl_4. The final pH was adjusted to 2.0 and the samples left overnight at 2-4 °C. The assay was carried out on the supernatant, following centrifugation at 11,000 *g* for 4 min.

Pepsin activity was assayed (Barrett, 1972) using dialysed hemoglobin (Sigma: H 2625) as a substrate:

> Samples: 0.5 ml of JL-buffer, pH = 2.0, and 4 μl of sample soln.
> Blanks: 0.5 ml of JL-buffer, pH = 2.0, and 4 μl of sample soln. and 10 μl of pepstatin A (Sigma: P 4265) giving a final conc. of 0.17 mM.

The difference in the absorbance values between the samples and the blanks, inhibited by pepstatin A, is the proteolytic activity of all aspartate proteases of the stomach. This is probably almost only pepsin activity, as pepstatin A inhibits aspartic proteinases (See Chapter III, section 1.), but pepsins (in the stomach mucosa) and cathepsins (in the stomach walls smooth muscle) are the members of this enzyme family, which can be expected to be encountered in the stomach wall. The pepsin activity is likely to exceed the cathepsin activity by far. The samples and the blanks were then left at room temperature for 20 min. The incubation was started by adding 10 % (w/v) hemoglobin to the samples and the blanks, giving a final hemoglobin conc. of 3.0 % (w/v) and run at 25 °C and pH = 2.0. The incubation was stopped after 1 hr by adding 5 ml of 6 % (w/v) TCA (trichloroacetic acid) and left at room temperature for 10 min. Following centrifugation at ca. 7,000 *g* for 10 min, folin positive material was assayed as follows (Lowry *et al.*, 1951): 4 ml of copper-reagent (See later) were added to 1 ml of the supernatant of sample and blank and to 1 ml of a standard, containing 0.1 μmol tyrosine/ml in 5 % (w/v) TCA. They were then left at room temperature for 30 min. Folin and Ciocalteu's phenol reagent (Sigma: F 9252), 0.25 ml, was added to the samples, the blanks and the standard during powerful vortexing. The absorption was measured at 700 nm 30 min later.

The copper-reagent was made up as follows:

Solution A:	Na_3 Citrate·2 H_2O	1.0 g
	Cu SO4·5 H_2O	0.5 g
	Distilled water	100 ml
Solution B:	NaOH	16.0 g
	Na_2CO_3	50.0 g
	Distilled water	500 ml

Before use, 1 ml of solution A is added to 20 ml of solution B and distilled water added to make the final volume of 100 ml.

Proteolytic activity was defined as follows:

1 unit (U) = 1 μmol tyrosine per hour (μmol/hr)

The samples were finally dried to a constant weight at 80 °C. Proteolytic activity was expressed as units per dry weight tissue (U/mg).

Statistics:

One way ANOVA will be used to find if the pepsin activity is unevenly distributed in the stomach. Pepsin activity is expected to be found only in the cardiac area, and possibly in greater quantyties in the posteriour than in the anteriour cardiac area (Chapter II). A rise in the pepsin activity is therefore expected to occurr between the oesophagus posterior (OP) and the cardiac area anterior (CA), and between the cardiac area anterior (CA) and the cardiac area posterior (CP). A fall in the pepsin activity between the cardiac area posterior (CP) and the pyloric area (PA) is likewise expected. A one tailed multiple comparisons test is therefore appropriate. A difference in the varinces of the pepsin activity from the various areas of the stomach wall, i.e. OP, CA, CP and PA, is not suitable for the Tukey's test, as this requires roughly even variances. A one step Bonferroni, i.e. a *t*-test with a raised significance level, is applied here, because of its resistance to difference in varinces between the groups tested (Hochberg and Tamhane,

1987). A one step Bonferroni (i.e. the significance level is raised to 0.05/number of tests) is more conservative, i.e. safer with regard to committing type I error, than a sequential step Bonferroni (i.e. with the significance level for the first test 0.05, the second test 0.05/2, .., the n-th test 0.05/n).

2.2. Localisation of trypsinogen and chymotrypsinogen.

The fish: Hatchery reared fish from a river Almond stock (Fresh weight 28.9-245.2 g), post smolts, upper modal fish, in their 2nd and 3rd year, 1+ and 2+ respectively. The biggest fish available were used so that the separation of the pancreatic tissue from the pyloric caeca would prove easy. As only 8 fish in their 3rd year were available, 8 fish in their 2nd year were added. This gave 8 fish for the control: 4 fish in their 2nd year and 4 fish in their 3rd year and 8 fish for the CCK treatment: 4 fish in their 2nd year and 4 fish in their 3rd year.

The fish were kept at 11.5-20.5 °C and were starved for 8 days prior to the experiment, which was executed in June, 1992.

The anaesthesia and the intraperitoneal injections were carried out as before (See Chapter IV, section 2.1.). 8 fish were injected with porcine CCK (See Chapter IV, section 2.1.), a dose of 1.4 C.u./g (the CCK treated fish) and 8 fish were injected with the carrier solution, without the CCK (the control fish). The porcine cholecystokinin (Sigma: P 4429) contained also small amounts of secretin (For each 2-4 Crick units of cholecystokinin, the product contained 0.4-0.7 Units of secretin; see Chapter IV). The fish were kept at 18.0 °C for 3 hours and then killed with an excessive dose of anaesthetics.

Dissection: The fish were opened ventrally and the gut removed. The surrounding fat and pancreatic tissue was scraped off the pyloric caeca with a blunt scalpel and the weight of the fat and the pancreatic tissue registered to the nearest 0.1 mg. 2 samples were randomly removed from the fat and the pancreatic tissue of each fish for TEM and put into fixative (See Chapter II, section 2.3.). The fat and the pancreatic tissue (termed the pancreas) were reweighed to the nearest 0.1 mg and frozen at-20 °C.

The intestinal wall in the pyloric caecal region (See Fig. 1 (b), Chapter II) was opened by a lengthwise incision. This and the pyloric caeca were cut into approximately 40 smaller pieces (termed the pyloric caeca). The remaining part of the intestine were opened with a lengthwise incision and cut into ca. 4 pieces (termed the intestine).
The pyloric caeca and the intestine underwent 3 changes of ice-cold saline (0.9 % NaCl and 20 mM $CaCl_2$), 10 min each time during agitation. The tissue was finally removed from the saline and frozen at-20 °C. The samples for the TEM were treated as before (See Chapter II, section 2.3).

The following procedure is partially based on the methods given by Reith and Mayhew (1988): The TEM grids containing sections for photography were randomly chosen. The location for photography on each chosen grid was chosen randomly and only 1 photograph was taken from each chosen grid. Five photographs were taken from each sample (block), but 2 samples had been taken randomly from each fish. A transparent film with marked points, located at regular intervals, was laid over the photographs. The points overlaying the granules were counted and called the granule point counts. The points overlaying the acinar tissue were counted and called acinar tissue point counts. The calculated fraction of the granule to acinar tissue point counts, was then the number of points counted over granules, divided by the number of points counted over acinar tissue.

Statistics:
A three factor ANOVA with respect to the CCK treatment, the sample from each fish, (i.e. the block) and the fish, will reveal not only differences in the granule to acinar tissue point counts with respect to the treatement, but also demonstrate if differences exist between fish or sample in this respect. If sampling of the fish and of the samples (i.e. the blocks) has been random as well as photography of the acinar tissue, then these factors should not contribute to a significant difference in the granule to acinar tissue point counts.

The samples for the trypsin and chymotrypsin assays were kept on ice and handled as follows:

Fish	Tissue	Saline (ml)	CCl_4 (ml)	Ultra Turrax(sec)
Large, 3rd year	Pancreas	10	2.0	10
	Pyloric caeca	4	0.8	50
	Intestine	4	0.8	50
Small, 2nd year	Pancreas	4	0.8	10
	Pyloric caeca	4	0.8	30
	Intestine	4	0.8	30

The saline was made up as before (See above) and the Ultra-Turraxwas run at a medium speed.

All samples were now homogenised with a Potter homogenizer for 30 sec each, kept overnight at 2-4 °C and centrifuged at 11,000 *g* for 4 min. Trypsin and chymotrypsin were assayed as before (See Chapter IV, section 2.1.1.). The fish were dried to constant weight at 80 °C.

The one step Bonferroni, one tailed, is chosen here for the same reasons as before (See section 2.1.2.).

3. Results.

3.1. Localisation of pepsinogen.

3.1.1. Immunolocalisation of pepsinogen.

The pattern of immunoreactivity of Y 37 on an anatomical scale was found to be positive for the same area as the stomach glands as identified by haematoxylin and eosin staining using light microscopy, i.e. the part of the stomach from the airbladder duct to the transitional area. In the transitional area, the area of the immunoreactivity of Y 37 receded gradually. No immunoreactivity was found in the pyloric area of the stomach. This pattern of immunoreactivity was found in all of the 5 fish examined. No immunoreactivity was found in the controls.

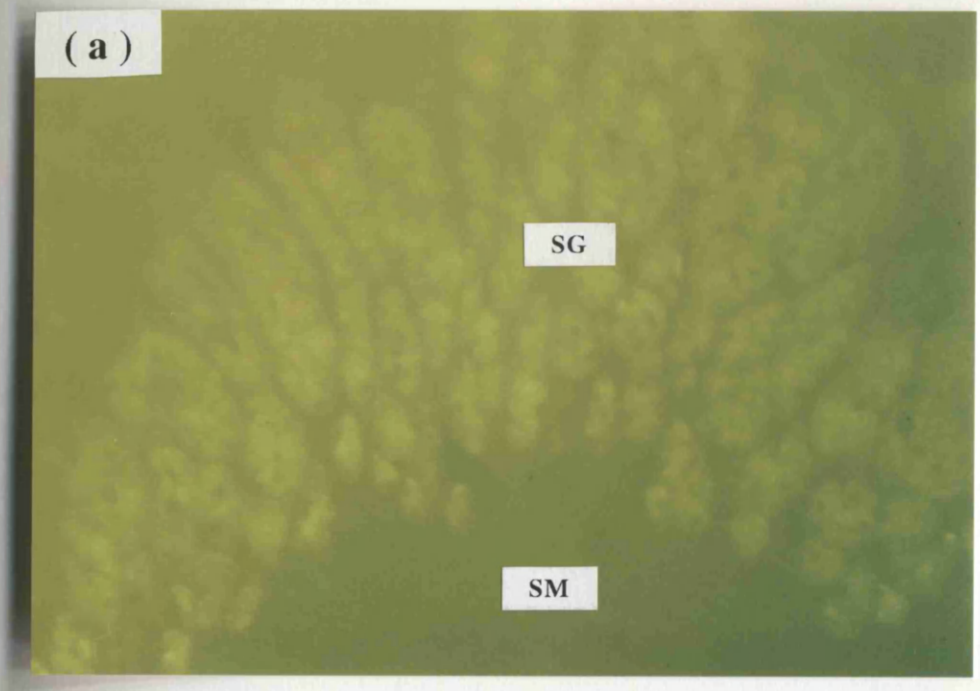
The pattern of immunoreactivity of Y 37 on a cellular scale was found to match the location of the stomach gland cells in the stomach mucosa (Fig. 22 (a) and Fig. 22 (b)). Figure 22 (a) shows the immunoreactivity of Y 37, taken from the anterior cardiac area of the stomach. Comparing Figure 22 (a) with Figure 2 (a) in Chapter II, it can be seen that the immunoreactivity of Y 37 clearly matches the stomach glands in the stomach primary folds. The immunoreactivity has the same appearance as the stomach glands in one primary fold (Fig. 2 (a) and Fig. 2 (c) in Chapter II), giving an indication of their tubular structure. Immunoreactivity was always found to match the shape of the stomach glands. Above the immunoreactivity of Y 37 (Fig. 22 (a)) and beneath, it can be seen that the epithelium and the submucosa respectively, show no immunoreactivity.

Figure 22 (b) shows the immunoreactivity of Y 37, taken from the posterior cardiac area of the stomach. The tubular formation of the stomach glands (Fig. 2 (c), Chapter II) is clearly apparent here. The nonreactive nuclei of the stomach gland cells, are seen as dark spots in the periphery of the glands and the lumen is visualised in the centre.

3.1.2. Pepsin activity in the stomach.

Pepsin activity is unevenly distributed in the stomach wall (Fig. 23. One way ANOVA: $p < 0.0005$. X.Appendices. Table V.1). Almost no activity is seen in the posterior oesophagus, taken from the area in front of the airbladder duct (**OP**) and from the pyloric area of the stomach, taken behind the transitional area (**PA**). A significant increase ($p < 0.0035 < p = 0.05/3 = 0.0167$; Bonferroni 3 tests; one tailed. X.Appendices. Table V.2) in pepsin activity is seen to occur between posterior oesophagus (**OP**), and the cardiac area anterior (**CA**). No significant change ($p > 0.23 > p = 0.05/3 = 0.0167$; Bonferroni 3 tests; one tailed. X.Appendices. Table V.2) in pepsin activity occurred between the anterior cardiac area (**CA**) and the posterior cardiac area (**CP**). A significant fall ($p = 0.0006 < p = 0.05/3 = 0.0167$; Bonferroni 3 tests; one tailed. X.Appendices. Table V.1) occurred between the posterior cardiac area (**CP**) and the pyloric area (**PA**) of the stomach.

Figure 22: Indirect immunofluorescence of sections from the cardiac area of the stomach. 5 μm sections were stained with monoclonal antibody, Y 37 (Yasugi *et al.*, 1987) and goat anti-mouse IgG. **(a)** Section from the anterior cardiac area. Bar equals 400 μm. The stomach glands **(SG)** are seen radiating away from the primary folding of the submucosa **(SM)**. **(b)** Section from the posterior cardiac area. Bar equals 200 μm. The tubular formation of the glands is apparent, with a centrally located lumen **(L)** and peripherally located nuclei **(N)**.


(a)
SG
SM

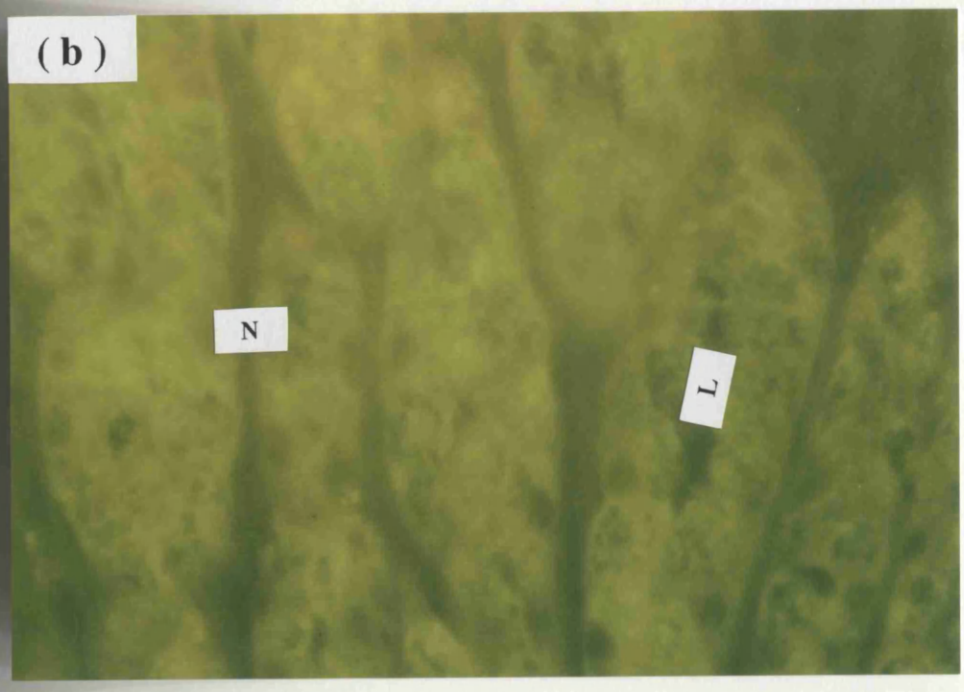
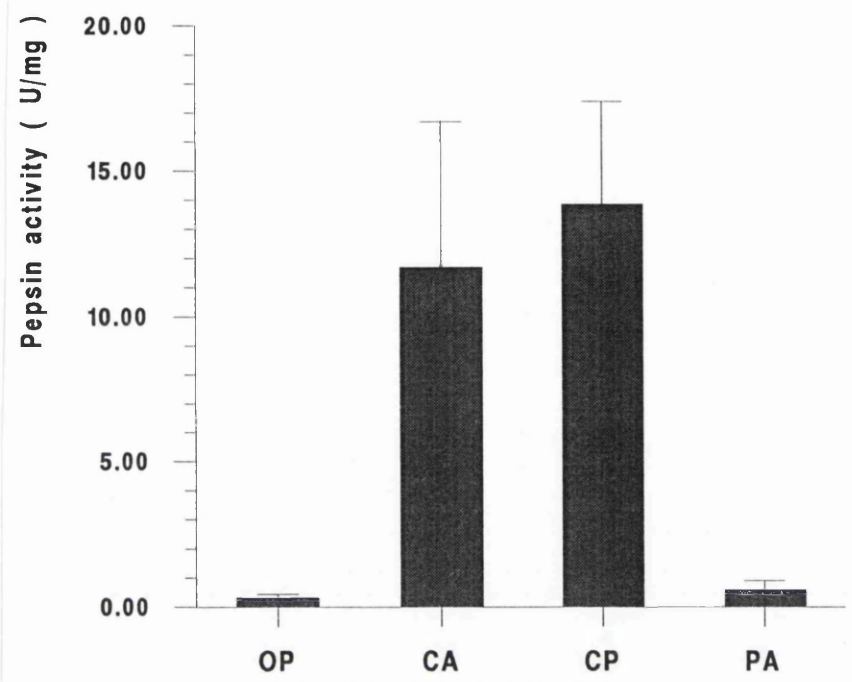
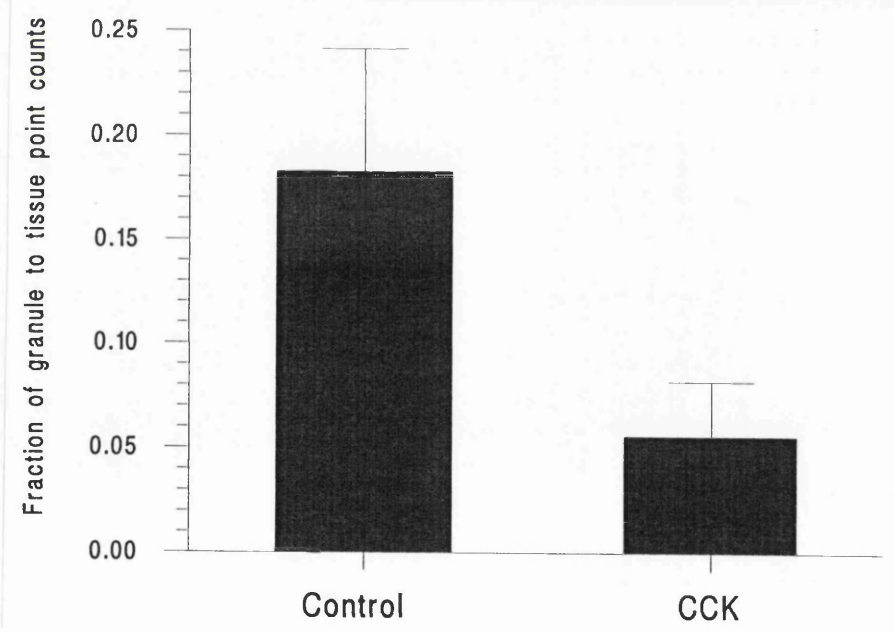

(b)
N
L

Figure 23: Pepsin activity in the posterior oesophagus (**OP**), anterior cardiac area (**CA**), posterior cardiac area (**CP**) and pyloric area (**PA**) of the stomach. Pepsin activity was assayed with hemoglobin as a substrate. Incubated for 1 hr at 25 °C and pH=2.0. Pepsin activity in units per dry weight tissue (U/mg), where 1 unit (1 U) is defined as 1 µmol tyrosine released per hour. Data points are mean values from 5 fish (n=5), and error bars are standard deviations.

Figure 24: The fraction of granule to acinar tissue point counts in the pancreas in TEM sections for control and CCK treated fish. The CCK used was a natural, purified, porcine cholecystokinin, sulphated. Data points are mean values from 8 fish (n=8), and error bars are standard deviations.


Pepsin activity (U/mg)
20.00
15.00
10.00
5.00
0.00
OP
CA
CP
PA


Fraction of granule to tissue point counts
0.25
0.20
0.15
0.10
0.05
0.00
Control
CCK

3.2. Localisation of trypsinogen and chymotrypsinogen.

3.2.1. Mobilisation of pancreatic granules by CCK.

The fraction of granule to acinar tissue point counts in the pancreatic tissue is shown in Figure 24 for the control fish and the CCK treated fish. It is clearly seen that the porcine CCK reduces the granule to tissue point count; this eduction was on the average 69 %. The difference in the amount of zymogenic granules between cells appeared to be greater in the CCK treated fish, than in the control fish. The acinar cells in the CCK treated fish showed one of two following patterns: i) they usually appeared to be empty of zymogenic granules, or ii) they, i.e. the acinar cells, sometimes appeared intact, when compared to cells in the control fish.

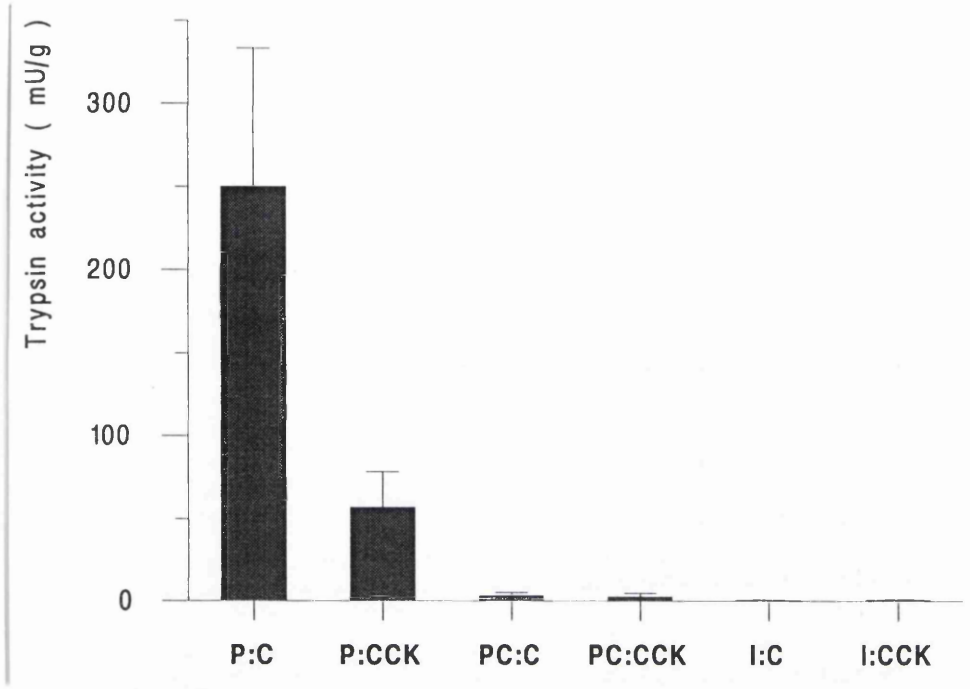
After arcsine transformation of the data presented in Figure 24 (Zar, 1984: p. 239) a three factor ANOVA (X.Appendices. Table V.3) showed that treatment with the porcine CCK resulted in a significant reduction of the granule to acinar tissue point counts ($p < 0.0005$). No significant difference in the response to the CCK treatment was observed between the larger and the smaller fish ($p = 0.977$), nor between the samples ($p = 0.253$). No significant interaction was observed, either between fish and sample ($p = 0.151$), fish and CCK treatment ($p = 0.578$), sample and CCK treatment ($p = 0.586$), nor between fish, sample and treatment ($p = 0.202$).

3.2.2. Trypsin and chymotrypsin in pancreas, pyloric caeca and intestine.

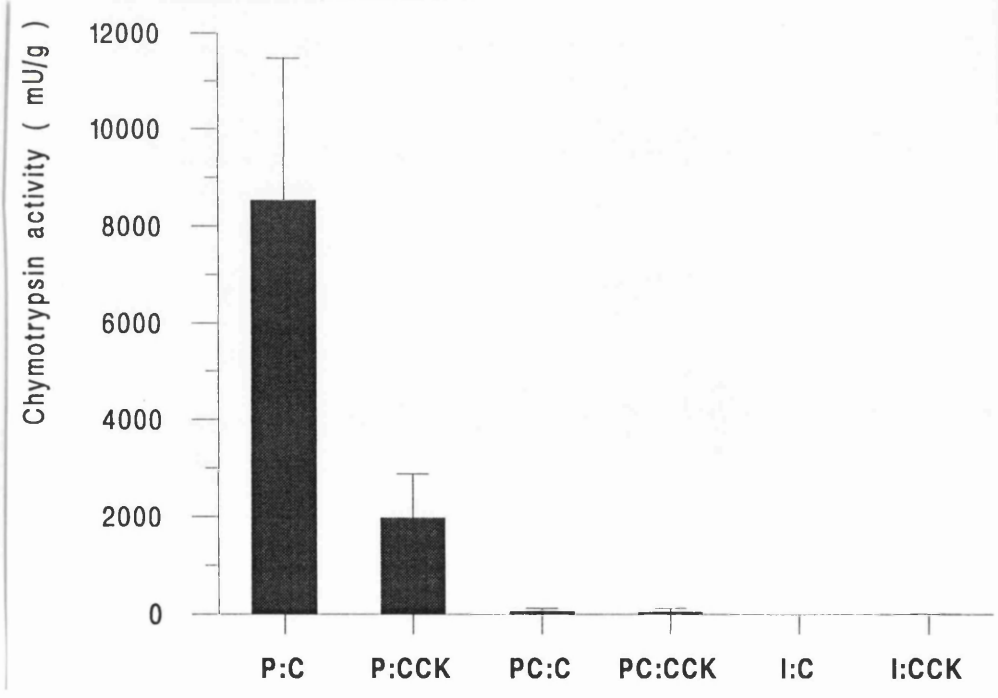
Both trypsin and chymotrypsin activities are low or negligible in the pyloric caeca and the intestine, compared to the pancreatic tissue (Fig. 25 (a) and Fig. 25 (b)). Both trypsin and chymotrypsin activities are significantly reduced in the pancreatic tissue ($p = 0.0002 < p = 0.05/6 = 0.00833$ and $p = 0.0001 < p = 0.05/6 = 0.00833$, respectively; Bonferroni 6 tests; one tailed. X.Appendices. Table V.4) by the CCK treatment. This reduction was 77 +/-1 % for both trypsin and chymotrypsin. No significant difference was found between the control fish and the CCK treated fish in the pyloric caeca and in the intestine ($p > 0.05 > p = 0.05/6 = 0.00833$ in all cases; Bonferroni 6 tests; one tailed.

Figure 25: Trypsin and chymotrypsin activities, in the pancreatic tissue of control fish (**P:C**) and the CCK injected fish (**P:CCK**), in the pyloric caeca of control fish (**PC:C**) and CCK injected fish (**PC:CCK**), and in the intestine of control fish (**I:C**) and CCK injected fish (**I:CCK**). The CCK used was a natural, purified, porcine cholecystokinin, sulphated. Trypsin and chymotrypsin activities were expressed in milliunits per dry weight of fish (mU/g). Data points are mean values from 8 fish (n=8), and error bars are standard deviations. (**a**) Trypsin activity. (**b**) Chymotrypsin activity.

(a)


Trypsin activity (mU/g)
300
200
100
0
P:C
P:CCK
PC:C
PC:CCK
I:C
I:CCK

(b)


Chymotrypsin activity (mU/g)
12000
10000
8000
6000
4000
2000
0
P:C
P:CCK
PC:C
PC:CCK
I:C
I:CCK

X.Appendices. Table V.4).

4. Discussion.

Y 37 has been shown to work well on pepsinogen in rainbow trout (Yasugi and Jönsson, pers. comm.). It is therefore not surprising to find that this antibody binds to pepsinogen by indirect immunofluorescence in the Atlantic salmon (Fig. 22 (a) and 22 (b)), as these species are closely related. The results from the pepsin assay (Fig. 23) were in good agreement with the results from the indirect immunofluorescence. Moreover, both the results from the indirect immunofluorescence and the pepsin assay were in perfect harmony with the expectation from the light microscopic studies (Chapter II): In the regions of the stomach where stomach glands were found, pepsinogen was located by indirect immunofluorescence with Y 37 and a considerable pepsin activity was found as well. This was in the cardiac and the transitional area of the stomach, but the glands gradually disappeared in the transitional area. This is in good agreement with findings in rainbow trout (Weinreb and Bilstad, 1955).

The fact that Y 37, raised against embryonic chicken pepsinogen, reacts positively with pepsinogen of the Atlantic salmon supports the hypothesis that the pepsinogens have been conserved well during the vertebrate evolution (Yasugi, 1987). Further studies have revealed that stomachs from the 6 major classes of vertebrates, possess at least one immunologically related molecular species of pepsinogen (Yasugi *et al.*, 1988). A similar study carried out on four species of teleosts, found that only the oxynticopeptic cells showed immunoreactivity, while surface mucous epithelium showed no immunoreactivity (Reifel *et al.*, 1985). This was in agreement with previous (Yasugi, 1987; Yasugi *et al.*, 1988) and present results.

The somewhat greater pepsin activity in the posterior cardiac area, than in the anterior cardiac area (although not significant), gives support to the earlier observations, that the stomach gland cells appeared to be more numerous in the posterior cardiac area, than in the anterior cardiac area (See Chapter II).

The fact that trypsin and chymotrypsin activities are greatly reduced in the pancreatic

tissue, as a result of challenge with CCK, gives further support to the hypothesis that these enzymes are stored in this tissue, which is embedded in the fat surrounding the pyloric caeca. The fact that the zymogen granules of the acinar cells are reduced in approximately the same ratio, and at the same time as, trypsin and chymotrypsin activities with the CCK treatment gives strong support for the hypothesis that trypsin and chymotrypsin are stored in these granules.

It is already well established that stimulation of the pancreatic acinar cells in mammals with CCK causes exocrine secretion of digestive enzymes into the intestine. This occurs as a result of the content of the secretory granules of the acinar cells being discharged into the acinar lumen (Grossman, 1977). Cells from Guinea pig pancreas were almost completely degranulated after 5 hours of intense stimulation during *in vitro* conditions (Scheele and Palade, 1975). This was accompanied by an increase in trypsin, chymotrypsin, carboxypeptidase A and B, amylase, lipase and ribonuclease activities in the incubation medium. The enzymes were found to be secreted in the same proportions over a shorter period of time, irrespective of the type of stimulus. Further studies confirmed these results (Tartakoff *et al.*, 1975). The disappearance of secretory granules from the pancreatic acinar cell and the increase of amylase activity in the incubation medium, during stimulation of the pancreatic acinar cell by CCK at *in vitro* conditions, had been found in previous studies (Jamieson and Palade, 1971). An intensive stimulation may reduce the zymogen granulation population to almost zero, at least during *in vitro* conditions (Case, 1978).

A reduction of zymogenic granules by 69 % and the trypsin and chymotrypsin activity by 77 % for the pancreatic tissue in the present study, caused by CCK , is in good agreement with previous studies. *In vivo* stimulation of the pancreas has revealed the following:

In the pigeon, stimulation with carbamylcholine reduced the amylase activity by about 40 %, within 1 hour after the injection (Fernandes and Junqueira, 1955).

In the rat stimulation by pilocarpine or carbamylcholine, reduced the amylase activity by 64 % and the proteolytic activity by 67 % (Farber and Sidransky, 1956) about 3 hours after the injection. In the same animal species stimulation with pilocarpine caused 90 % of the zymogenic granules to be discharged within 2 hours (Geuze and Poort,

1973), while in another study (Geuze and Kramer, 1974) pilocarpine caused 70 % reduction in the zymogenic granules during the first hour after the injection of the drug. Stimulation with a mixture of CCK and secretin during *in vivo* conditions reduced acinar zymogenic granules markedly and also pancreatic amylase activity by approximately 60 %, in the pancreas of the rat (Reggio *et al.*, 1971).

Some acinar cells in the CCK treated fish appeared to be devoid of zymogenic granules, while others were still replete. This is in agreement with previous studies on mammals (Martin *et al.*, 1969; Tardini *et al.*, 1971; Geuze and Poort, 1973) and is probably caused by a difference in accessibility and/or sensitivity of the acinar cells to the stimulus.

Practically no trypsin or chymotrypsin activity was found in the pyloric caeca or the intestine (Fig. 25). These findings are in good agreement with previous studies on the Atlantic cod (Overnell, 1973). Trypsin, chymotrypsin, carboxypeptidase A and B activities were only found in the fatty tissue surrounding the pyloric caeca of the Atlantic cod, but none in the pyloric caeca themselves. Most of the ribonuclease and the amylase activity was also found in the fatty tissue, surrounding the caeca (Overnell, 1973). The very same enzymes as the ones mentioned above, have been found to be secreted from the Guinea pig pancreas (Scheele and Palade, 1975). Trypsinogen and chymotrypsinogen, i.e. the storage forms of trypsin and chymotrypsin respectively, were also only found in the fatty tissue surrounding the caeca of the cod (Overnell, 1973). The residual activity of trypsin and chymotrypsin in the pyloric caeca probably either results from pancreatic tissue remaining after dissection, or residual trypsin and chymotrypsin activities remaining on the inside of the caeca.

Chapter VI.

Effect of feeding on the secretion of pepsin, trypsin and chymotrypsin.

1. Introduction.

The main objective of this chapter is to study the effect of ingested food upon the secretion of pepsin, trypsin and chymotrypsin, and the discharge of the gallbladder. Since the fish have to be starved before such a study can be undertaken, the effects of starvation on stored pepsin, trypsin and chymotrypsin are also investigated, since this has not been systematically studied in fish.

Starvation for 5 days of the common Malayan toad (*Bufo melanostictus*) caused a significant fall in the amount of pepsin in the stomach mucosa, but proteolytic activity in the pancreas was unaffected after 2 weeks of starvation (Theo *et al.*, 1990). Starvation may have caused an increase in the pepsin activity in the gastric mucosa of the blackfish (*Gadopsis marmorcadus*) due to storage of pepsinogen within the oxynticopeptic cells (Buddington and Doroshov, 1986).

The immediate effect of starvation on pancreatic hydrolases in a mammal, such as the rat, was a slight increase (Girard-Globa *et al.*, 1980). A 2 month starvation of catfish caused an increase in both trypsin and chymotrypsin activity in the pancreas (Yoshinaka *et al.*, 1981a). It was not clear if this change was significant, since only 2-3 individuals were used in each group in the experiment. A 1.5 month starvation of eel (*Anguilla japonica*) caused a reduction in trypsin and chymotrypsin activity in the pancreas, which was probably significant (Yoshinaka *et al.*, 1984).

Starvation will probably cause a reduction in trypsin and chymotrypsin activities in the intestinal digesta, since starvation causes a reduction in digestive enzyme synthesis and secretion in carp (Onishi *et al.*, 1973) and in cod (Overnell, 1973). Trypsin and

chymotrypsin activities were found to disappear from the intestinal digesta of catfish upon starvation for 2 months (Yoshinaka *et al.*, 1981b).

Studies on the effect of food on the secretion of pepsin, trypsin and chymotrypsin in fish are both limited and fragmentary. Food in the intestine is known to stimulate secretion of digestive enzymes and also pancreatic protein synthesis in mammals (Corring, 1980; Girard-Globa *et al.*, 1980) and in amphibians (Scapin, 1982). Total proteolytic activity in the pancreas of carp decreased at the early stage of feeding, then more or less recovered 5 hours later (Onishi *et al.*, 1973), resulting in an increased proteolytic activity in the gut.

The effect of starvation and feeding on the gallbladder in the Atlantic salmon has been studied (Talbot and Higgins, 1982). Starvation caused the accumulation of gall in the gallbladder and food entering the hindgut caused its release. The question arises whether trypsin and chymotrypsin are concurrently secreted from the pancreas. It was found in Chapter IV, that CCK from pig caused a simultaneous secretion of trypsin and chymotrypsin from the pancreas and a concurrent discharge of gall from the gallbladder. In Chapter II, endocrine cells were found in the epithelium of the pyloric caecal area of the intestine, containing conspicuous granules, possibly containing a CCK-type hormone. It was suggested that these endocrine cells, would release this CCK-type gut hormone into the blood stream, when food from the stomach entered the pyloric caecal region.

CCK in the gut of mammals is mostly situated in mucosal endocrine cells in the duodenum and the jejunum, and it has been found in studies on the dog, that fat and protein or their digestion products cause a release of CCK into the blood stream (Dockray, 1989). It has been demonstrated for mammals, that the three main physiological functions of CCK is to stimulate pancreatic enzyme secretion, gallbladder contraction and relaxation of the sphincter of Oddi (Jorpes and Mutt, 1973; Walsh, 1978).

In this chapter the following questions will be answered:

I. How does starvation affect the following factors:
i) The pepsin activity in the stomach mucosa, i.e. stored pepsin activity, and in the stomach lumen, i.e. secreted pepsin activity?

ii) The trypsin and chymotrypsin activity in the pancreatic tissue, i.e. the stored trypsin and chymotrypsin activities, and trypsin and chymotrypsin activity in the intestinal digesta, i.e. the secreted trypsin and chymotrypsin activities?

Feeding fish will be starved and pepsin in the stomach mucosa and in the stomach lumen, and trypsin and chymotrypsin in the pancreatic tissue and in the intestinal digesta, will be assayed as before (See Chapter III and IV). The state of the gallbladder will be described and the digesta in the stomach and in the intestine will be measured as the dry weight.

II. How does ingested food affect the pepsin activity in the stomach mucosa, in the stomach lumen/digesta and in the stomach as a whole?

Starved fish will be fed for 1 hour and, during the passage of food through the stomach, the pepsin activity in the stomach mucosa and in the stomach lumen/digesta will be assayed as before (See Chapter III), the food/digesta will be measured as the dry weight, and the pepsin activities in the stomach mucosa and in the stomach digesta will be added to give the total pepsin activity of the stomach.

III. How does ingested food affect the discharge of the gallbladder, the trypsin and chymotrypsin activity in the pancreatic tissue, and the trypsin and chymotrypsin activity in the intestinal digesta?

Starved fish will be fed for 1.25 hours and during the passage of the food through the gut, the trypsin and chymotrypsin activity in the pancreatic tissue and in the intestinal digesta will be assayed as before (See Chapter V), the food/digesta will be measured as the dry weight, and the gallbladder index(the fresh weight of the gallbladder/the fresh weight of the liver) will be measured.

The digesta and trypsin and chymotrypsin activities, will be measured in the pyloric caecal area, the mid intestinal area and the rectum. This is done to be able to evaluate, how the trypsin and chymotrypsin activities are distributed throughout the intestinal lumen, during secretion of these enzymes, by peristalsis of the intestinal wall etc.

2. Material and methods.

Three consecutive experiments were executed: i) the effect of starvation on pepsin, trypsin and chymotrypsin, and the gallbladder, ii) the effect of food on pepsin and iii) the effect of food on trypsin and chymotrypsin and the gallbladder.

2.1. The effect of starvation on pepsin, trypsin and chymotrypsin, and the gallbladder.

This experiment was executed from the 18th of April to the 8th of May, 1990.

The fish:

The experimental animals used were a mixture of lower modal and upper modal fish, in their 2nd year (1+), from the River Spey. The fork length was 12.7 cm to 16.8 cm (Fresh weight: 22.80 g to 44.99 g). The fish were either starved or fed (the control) for 20 days. Sixfish from the fed group and 6 fish from the starved group were sampled after 0, 2, 4, 6, 8, 10, 13, 16 and 20 days.

Feeding history:

The fish had been kept on a commercial salmon food (Freshwater smolt 1, commercial pellets, size 3 mm, BP Nutrition), *ad libitum*, 15 days prior to the starvation.

Assay of feed:

It is to be expected that the feed might contain some activity from digestive enzymes or other enzymes, acting on the substrates for pepsin, trypsin and chymotrypsin. The feed pellets were crushed into a fine powder and 1.1955 g added to 20 ml of 2 % lauryl trimethylammonium chloride, containing 500 mM NaCl and 100 mM $CaCl_2$ (Kaspar *et al.*, 1982). This was stirred at 2-5 °C in a sealed container, and 600 μl aliquots were removed after 6, 12, 24, 48 and 72 hours. These were centrifuged for 4 min at 11,000 ***g*** and the supernatant frozen at -20 °C. Pepsin, trypsin and chymotrypsin activities were assayed as described in Chapter IV. The residual mixture containing the feed was dried to a constant weight at 80 °C. The activity in the feed was calculated as milliunits per milligram feed (mU/mg). The maximal extractable pepsin, trypsin and chymotrypsin activity was defined as the activity, which was not increased by further extraction. This value was multiplied by the dry weight of the respective digesta (mg), to find the respective value of pepsin, trypsin and chymotrypsin activities in the stomach and intestinal lumen of the fish,

originating from the feed. These were finally subtracted from the respective activities in the stomach and intestinal digesta.

Water temperature:

The water temperature in the 15 days before the experiment, ranged from 3.0 °C to 12.0 °C and during the experiment from 5.3 °C to 21.8 °C.

Dissection:

The fish were always sampled at 10 a.m. in the morning after 0, 2, 4, 6, 8, 10, 13, 16 and 20 days. Each time sixfish were sampled from the feeding population and 6 fish from the starving population. They were killed by a blow to the head and put on ice. The abdomen was opened ventrally and the digestive tract removed, together with the fat surrounding the pyloric caeca. The state of the gallbladder was recorded.

The stomach was opened by a longitudinal incision and the digesta in the feeding fish and the luminal mucus in the starving fish, gently removed with a blunt scalpel. These and the stomachs were then frozen at -20 °C.

The intestine, together with the fat surrounding the pyloric caeca, were divided by a cut posterior to the pyloric caeca. The luminal contents of the pyloric caeca, were added to the luminal contents of the remaining intestine. This was termed the intestinal digesta.

The pancreatic tissue and the pyloric caeca (i.e. the part of the gut, comprising the pyloric caecal region, but devoid of intestinal digesta, see Chapter II, Fig. 1 (b)), as well as the intestinal digesta, were frozen at -20 °C.

The fish were dried to a constant weight at 80 °C and weighed to the nearest 0.1 mg.

Processing and the assay of samples:

Samples were kept on ice during the processing. The stomachs, and the pyloric caeca and the pancreatic tissue, were weighed to the nearest 0.1 mg. 2 ml of Johnson and Lindsay buffer (Johnson and Lindsay, 1939), pH = 4.25, were added to the stomachs, and the luminal contents of the stomachs, while 5 ml of distilled water, containing 0.9 % (w/v) NaCl and 20 mM $CaCl_2$ were added to the pyloric caeca and the pancreatic tissue and to the intestinal digesta.

The stomachs were homogenised in an Ultra Turrax (Ystral GmbH, Type:x-120) at maximum speed for 40 seconds, while the pyloric caeca and the pancreatic tissue were homogenised for 20 seconds.

All samples were then homogenised in a Potter homogeniser (ComtexLtd. Type GL CSO

12860) as follows: samples from stomachs were homogenised for 30 seconds, after the addition of CCl_4 (Hjelmeland and Raa, 1982), at a final concentration of 5 % (v/v). Stomach digesta were homogenised for 20 seconds. Samples from the pancreatic tissue and the pyloric caeca were homogenised for 30 seconds, after the addition of CCl_4, at a final concentration of 20 % (v/v). The intestinal digesta were homogenised for 20 seconds, after the addition of CCl_4, at a final concentration of 10 % (v/v).

All samples were kept in the fridge overnight, at 2-4 °C and then centrifuged at 9,000 *g* for 4 minutes. Pepsin, trypsin and chymotrypsin activities were assayed as described in Chapter IV. The stomach digesta and the intestinal digesta were dried to a constant weight at 80 °C and weighed to the nearest 0.1 mg. The weight of added salts was subtracted.

Statistics:

Two way ANOVA will be used to reveal any overall differences in the stored pepsin-, trypsin-and chymotrypsin activities between the starved and the fed fish.

For reasons mentioned previously (Section 2.1.2. Chapter V), then a single step Bonferroni test was applied (Hochberg and Tamhane, 1987: p. 58). The significance level was raised to p = 0.05 / No. of tests = 0.05 / 10 = 0.005. Of interest is the immediate change in the stored enzyme activities (i.e. after 2 days), upon withdrawal of the stimulus (i.e. the food) from the oxynticopeptic-and the acinar cells. Also of interest is the change over longer term (i.e. after 16 and 20 days), because it is necessary to know if the stored pepsin, trypsin and chymotrypsin are preserved during starvation. A one tailed test is chosen, because starvation is expected to affect the quantities of the stored and the secreted pepsin, trypsin and chymotrypsin, both over longer and shorter periods. The starving and the fed group were always compared with the Bonferroni tests at identical times.

2.2. The effect of food on pepsin.

This experiment was executed from the 8th to the 12th of May, 1990.

The fish:

The experimental animals used were from the same population as the ones used before (Section 2.1.). The fork length was 12.6 cm to 17.2 cm (Fresh weight: 18.64 g to 45.65 g).

Feeding history and experimental protocol:

The fish had been fed as before, prior to starvation (See section 2.1.). After 20 days of

starvation the population was divided into 2 groups. One group was fed *ad libitum* for 1 hour (fed group). The control group continued their starvation (starved group). Sixfish from each group were sampled at 0, 1, 2, 4, 7, 13 and 25 hours, after the feeding of the fed group. Each time 6 fish were sampled from the starving population and 6 fish from the fedpopulation.

Assay of feed:

As in section 2.1.

Water temperature:

The water temperature 20 days before the experiment, ranged from 5.3 °C to 21.8 °C and during the experiment from 11.0 °C to 16.0 °C.

Dissection, processing and assaying of samples:

The fish were dissected, and the samples processed and assayed as before (Section 2.1), but only the stomachs and the stomach contents were sampled. The stomach digesta were dried to constant weight at 80 °C and weighed to the nearest 0.01 mg. Calculated weights of the added salts were subtracted.

Statistics:

The same tests were applied as before and for the same reasons (Section 2.1.). The significance level was raised to $p = 0.05$ / No. of tests = 0.05 / 14 = 0.0036. The objective of the Bonferroni tests is to reveal when the fed group differs significantly from the starving group, and when these groups cease to be significantly different with respect to the stomach digesta, pepsin activity in the stomach mucosa, pepsin activity in the stomach digesta and total pepsin activity in the stomach. The starving and the fed group will always be compared with the Bonferroni tests at identical times. The family of inferences will be defined as the number of Bonferroni tests required to detect when the fed group differs significantly from the starving group, and when significant differences between these groups cease to exist with respect to aforementioned parameters. As food enters the stomach of the fed group these will be expected to differ significantly from the starving group with respect to these parameters. These groups will be compared with a Bonferroni test at each sampling time until a significant difference between them is found with respect to stomach digesta, pepsin activity in the mucosa, pepsin activity in the stomach digesta and total pepsin activity. Pepsin activity in the stomach mucosa, in the stomach digesta and total pepsin activity will also be compared for the aforementioned groups with the

Bonferroni test to reveal when these grops cease to be significantly different with respect to the aforementioned factors. These criteria will require 14 Bonferroni tests, i.e. the stomach digesta 1 test, the pepsin in the stomach mucosa 5 tests, the pepsin in the stomach digesta 3 tests and the total pepsin 5 tests.

2.3. The effect of food on trypsin and chymotrypsin and the gallbladder.

This experiment was executed from the 14th to the 16th of September, 1992.

The fish:

The experimental animals used were from a sibling population of lower modal fish (the only fish available), in their 2nd year (1+), from a River Almond stock. The fresh weight was 10.53 g to 34.81 g.

Feeding history and experimental protocol:

The fish had been fed as before, prior to starvation (Section 2.1.). After 25 days of starvation, the population was divided into 2 groups. One group was fed *ad libitum* for 1.25 hours (fed group). The control group continued their starvation (starved group). Twenty fish from the fed group and 10 fish from the starved group were sampled after 0.00, 1.25, 3.73, 13.65, 25.32 and 54.07 hours, after feeding of the fed group.

Assay of feed:

As before (Section 2.1).

Water temperature:

The water temperature 25 days before the experiment, ranged from 8.0 °C to 15.8 °C and during the experiment from 9.0 °C to 11.8 °C.

Dissection:

The fish were prepared as before (Section 2.1.). The intestine, together with the fat surrounding the pyloric caeca, were divided into 7 parts as follows:

1. The pancreatic tissue: The fat surrounding the pyloric caeca was scraped off these with a blunt scalpel.
2. The pyloric caeca: Removed from the digestive tract by one cut immediately in front and one cut right behind the pyloric caeca.
3. The pyloric caecal digesta: This was gently squeezed out of the pyloric caeca.
4. The mid intestine: This was removed from the remaining intestine

by a cut in front of the rectum.

5. The mid intestinal digesta: This was gently squeezed out of the intestine.

6. The rectum: This was the remaining part of the intestine.

7. The rectum digesta: This was gently squeezed out of the rectum.

All these samples were frozen at -20 °C and stored at -75 °C.

The gallbladders and the livers were weighed to the nearest 0.1 mg and the gallbladder indexcalculated (Fresh weight of the gallbladder / Fresh weight of the liver). The fish were dried to a constant weight at 80 °C and weighed to the nearest 0.1 mg.

Processing and the assay of samples:

Samples were homogenised with a Potter homogeniser (Citenco.F.H.P. motors) in saline, containing 0.9 % NaCl, 20 mM $CaCl_2$ and 0.25 % Bovine Serum Albumin, fraction V, protease free (ICN Biomedicals, Inc.: Cat. no. 82-045) as follows:

Sample name	**Saline** (ml)	**CCl_4** (ml)	**Homogenising time** (sec)
The pancreatic tissue	25.00	5.00	40
The pyloric caeca	10.00	1.00	40
The pyloric caecal digesta	1.60	0.10	15
The mid intestine	5.00	0.50	40
The mid intestinal digesta	1.60	0.10	15
The rectum	5.00	0.50	40
The rectum digesta	1.60	0.10	15

All samples were kept in the fridge overnight at 2-4 °C and thereafter at 0 °C until analysed. They were then centrifuged at 11,000 *g* for 4 minutes. Pepsin, trypsin and chymotrypsin activities were assayed as described in Chapter IV.

The mid intestinal digesta and the rectum digesta were combined. The digesta were dried to constant weight at 80 °C and weighed to the nearest 0.01 mg. Calculated weights of the added salts and the bovine serum albumin were subtracted. Intestinal digesta is the sum of the pyloric caecal digesta, the mid intestinal digesta and the rectum digesta.

Observations on mid intestinal and the rectum digesta will not be presented separately.

Statistics:

As before (Section 2.1. and 2.2.). The significance level was raised to $p = 0.05$ / No. of tests = 0.05 / 29 = 0.00172. As before Bonferroni will be used to reveal when the fed group becomes significantly different from the starving group with respect to digesta in the pyloric caecal region of the intestine, total intestinal digesta, gallbladder index, trypsin and chymotrypsin activity in the pancreas, trypsin and chymotrypsin activity in the pyloric caecal region of the intestine, mid intestinal region of the intestine and the rectum. The objective here to find if the aforementioned parameters respond simultaneously to digesta appearing in the pyloric caecal region of the intestine, i.e. the gallbladder indexand pancreatic enzyme activities being reduced and enzyme activities in the intestine increased. This requires two Bonferroni tests for each parameter. Also of interest is a knowledge of how resynthesis of trypsin and chymotrypsin in the pancreas occurrs after the secretion of these enzymes. This requires a Bonferroni test for each enzyme activity at all sampling times, except 0.00 hours. One extra test is needed to estalish when evacuation from the intestine begins. To fulfill these requirements the number of tests needs to be 29, i.e. digesta in the pyloric caecal region of the intestine needs 2 tests, total intestinal digesta needs 3 tests, gallbladder indexneeds 2 tests, trypsin and chymotrypsin activities in the pancreas need 5 tests each, trypsin and chymotrypsin activity in the pyloric caecal region of the intestine need 2 tests each, mid intestinal region of the intestine needs 2 tests each and the rectum needs 2 tests each.

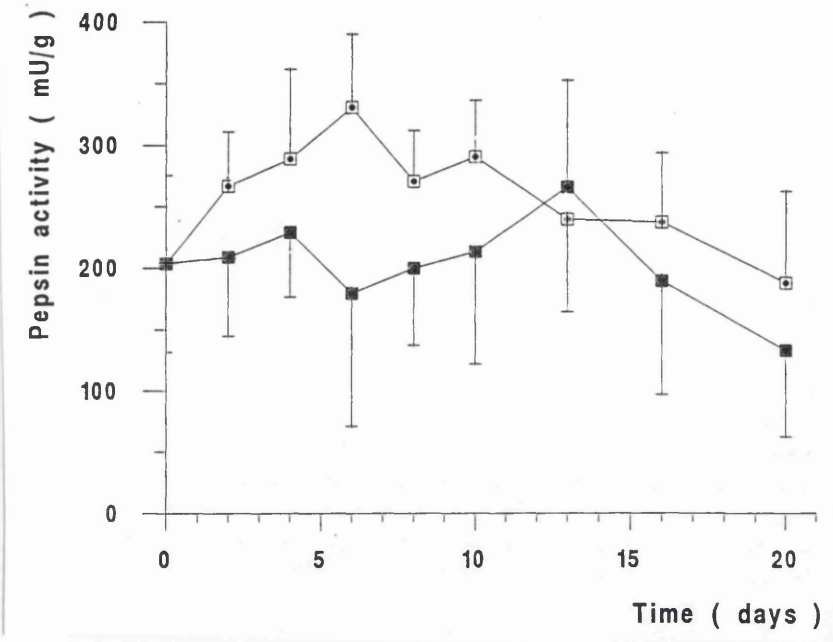
3. Results.

3.1. The effect of starvation on pepsin, trypsin and chymotrypsin, and the gallbladder.

The pepsin activity in the stomach mucosa, is seen to rise in the starving fish, within 2 days of starvation (Fig. 26 (a)). The overall difference between the starving and the fed fish is significantly different (Two way ANOVA: $p < 0.0005$. X.Appendices. Table VI.1). However no significant difference was found after 2 days ($p = 0.054 > p = 0.005$; Bonferroni: 10 tests; one tailed tests. X.Appendices. Table VI.2). The pepsin activity in the stomach mucosa was always higher in the starving fish, than in the feeding fish

Figure 26: Pepsin activity in the stomach mucosa (**a**) and in the stomach digesta/mucus (**b**) of feeding (■) and starving (⊡) fish. The maximally extractable pepsin activity in the feed, was none. The pepsin activity was presented in milliunits per dry weight fish (mU/g). Each data point was a mean value from 6 fish (n=6) and error bars were standard deviations.

(a)


Pepsin activity (mU/g)
400
300
200
100
0
0
5
10
15
20
Time (days)

(b)

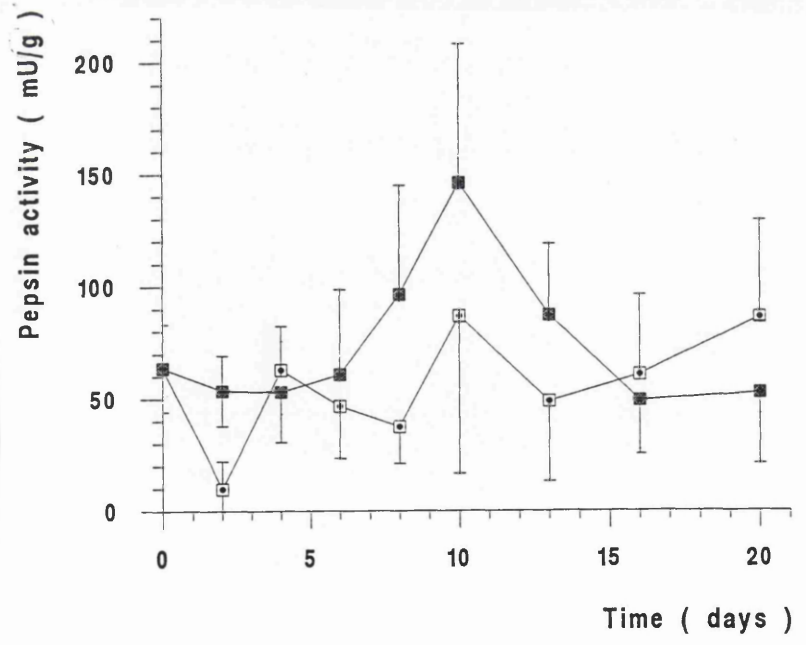
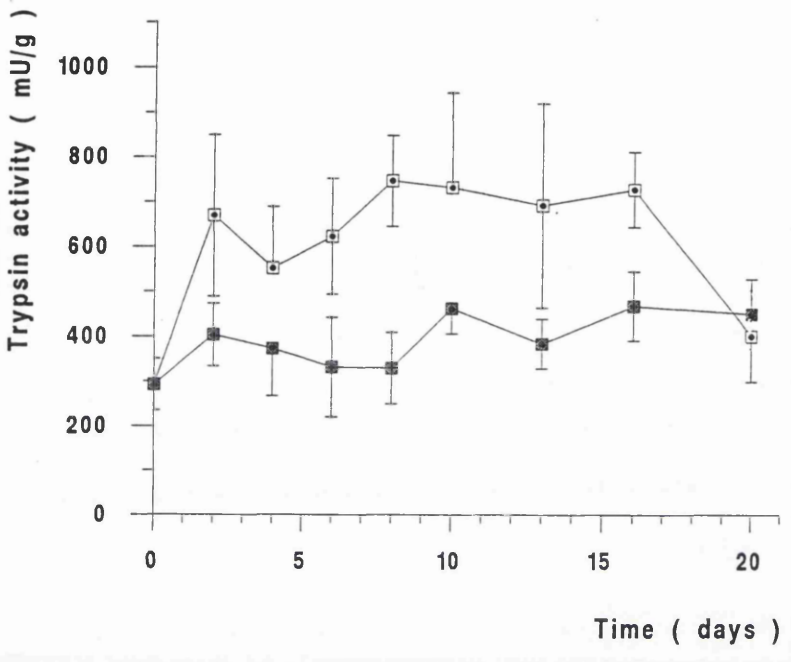

Pepsin activity (mU/g)
200
150
100
50
0
0
5
10
15
20
Time (days)

Figure 27: Trypsin activity in the pancreatic tissue and the pyloric caeca (**a**) and in the intestinal digesta (**b**) of feeding (■) and starving (⊡) fish. The maximally extractable trypsin activity in the feed, was subtracted from the respective trypsin activity in the intestinal digesta. Trypsin activity was presented as milliunits per dry weight fish (mU/g). Further details as in Figure 26.

(a)


Trypsin activity (mU/g)
1000
800
600
400
200
0
0
5
10
15
20
Time (days)

(b)

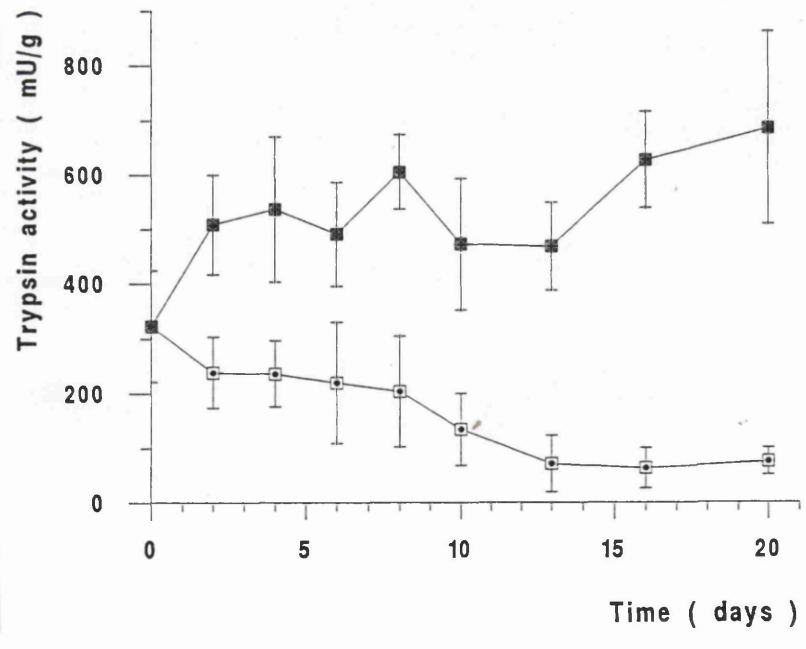
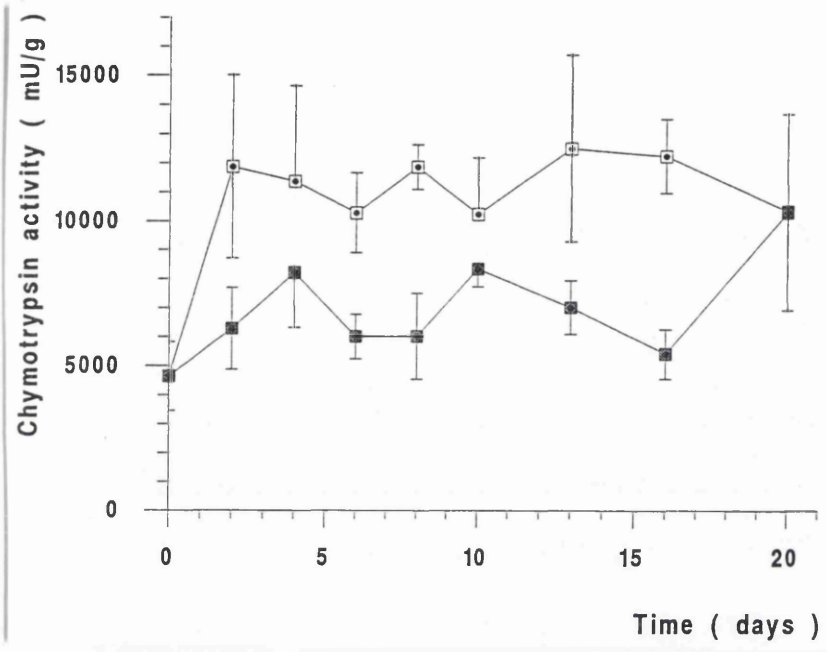
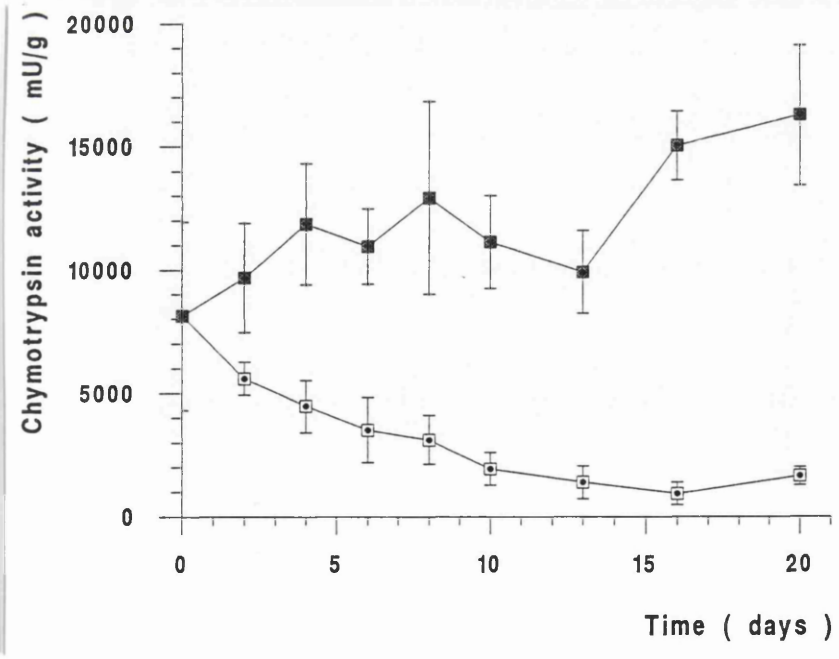

Trypsin activity (mU/g)
800
600
400
200
0
0
5
10
15
20
Time (days)

Figure 28: Chymotrypsin activity in the pancreatic tissue and the pyloric caeca (**a**) and in the intestinal digesta (**b**) of feeding (■) and starving (▣) fish. The maximally extractable chymotrypsin activity in the feed, was subtracted from the respective chymotrypsin activity in the intestine. Chymotrypsin activity was presented as milliunits per dry weight fish (mU/g). Further details as in Figure 26.

(a)


Chymotrypsin activity (mU/g)
15000
10000
5000
0
0
5
10
15
20
Time (days)

(b)


Chymotrypsin activity (mU/g)
20000
15000
10000
5000
0
0
5
10
15
20
Time (days)

(except at 13 days). This was however never significant.

The pepsin activity in the stomach lumen (Fig. 26 (b)) was only significantly lower in the starving fish after 2 days ($p = 0.0002 < p = 0.005$; Bonferroni: 10 tests; one tailed tests.). No digesta, but some mucus, was observed in the stomach of the starving fish at and after 2 days, in contrast to the feeding fish, which constantly had digesta in their stomach, throughout the experiment.

The trypsin activity in the pancreatic tissue and pyloric caeca, is seen to rise in the starving fish, within 2 days of starvation (Fig. 27 (a)). The overall difference between the starving and the fed fish is significantly different (Two way ANOVA: $p < 0.0005$. X.Appendices. Table VI.3). However no significant difference was found after 2 days ($p = 0.0076 > p = 0.005$; Bonferroni: 10 tests; one tailed test. X.Appendices. Table VI.4). The trypsin activity in the pancreatic tissue and the pyloric caeca, was always higher in the starving fish, than in the feeding fish, except after 20 days. This difference was sometimes significant and sometimes not. It was significantly different after 16 days ($p = 0.0002 < p = 0.005$; Bonferroni: 10 tests; one tailed. X.Appendices. Table VI.4), but not after 20 days ($p = 0.18 > p = 0.005$; Bonferroni: 10 tests; one tailed. X.Appendices. Table VI.4).

The trypsin activity of the intestinal digesta (Fig. 27 (b)) fell gradually in the starving fish, reaching a minimum after 16 days. A significant difference in the intestinal trypsin activity, between the feeding and the starving fish, was observed after 2 days ($p = 0.0001 < p = 0.005$; Bonferroni: 10 tests; one tailed test.)

The chymotrypsin activity in the pancreatic tissue and the pyloric caeca, is seen to rise in the starving fish, within 2 days (Fig. 28 (a)). The overall difference between the starving and the fed fish is significant (Two way ANOVA: $p < 0.0005$. X.Appendices. Table VI.5). The difference was also significant after 2 days ($p = 0.0037 < p = 0.005$; Bonferroni: 10 tests; one tailed test. X.Appendices. Table VI.6). The chymotrypsin activity in the pancreatic tissue and the pyloric caeca, was always higher in the starving fish, than in the feeding fish, except after 20 days. This difference was usually significant. It was significantly different after 16 days ($p < 0.00005 < p = 0.005$; Bonferroni: 10 tests; one

tailed test. X.Appendices. Table VI.6), but after 20 days the values were the same (Fig. 28 (a)).

The chymotrypsin activity in the intestinal digesta (Fig. 28 (b)), fell gradually in the starving fish, reaching a minimum after 16 days. A significant difference in the intestinal chymotrypsin activity, between the feeding and the starving fish, was found after 2 days ($p = 0.0038 < p = 0.005$; Bonferroni: 10 tests; one tailed test.).

The gallbladder in the feeding fish was usually empty or contained small amounts of light yellow gall. In the starving fish the gallbladder became distended, reaching its maximal volume after 2 days of starvation. It was then a pale green colour. However, the colour changed to dark green after 4 days.

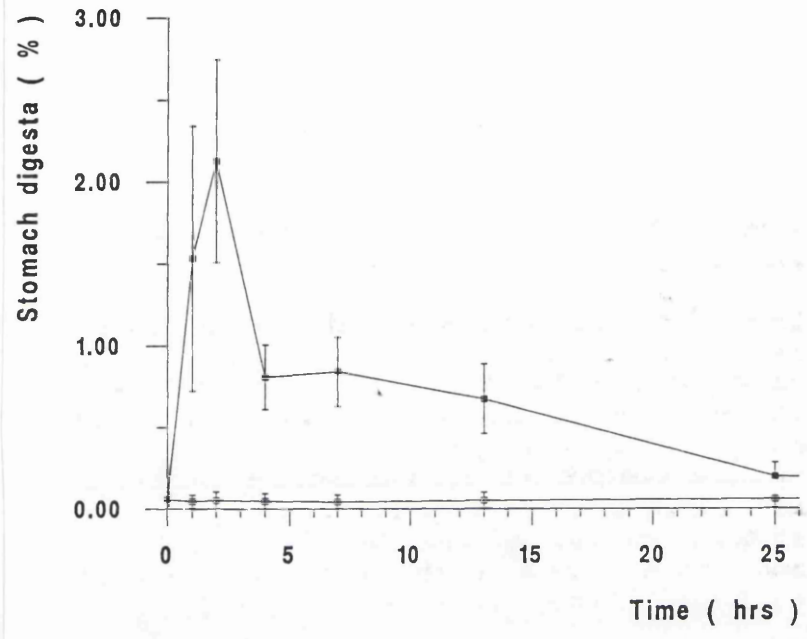
3.2. The effect of food on pepsin.

The dry weight of the stomach digesta was seen to rise and the overall difference between the fed fish and the starving fish is significant (Two way ANOVA: $p < 0.0005$. X.Appendices. Table VI.7), as well as the difference in the first hour ($p = 0.0032 < p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.8) after feeding began (Fig. 29 (a)). Evacuation from the stomach began between 2 and 4 hours.

Pepsin activity in the stomach mucosa, was seen to rise, and the overall difference between the fed fish and the starving fish is significant (Two way ANOVA: $p = 0.020$. X.Appendices. Table VI.9), but not the difference during the one hour ($p = 0.011 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.10) in which the fish were fed (Fig. 29 (b)). It was seen to fall in the interval of 1 to 7 hours, but is not significantly lower in the fed fish, than in the starving fish (the control), either after 7 hours ($p = 0.015 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.10) or 25 hours ($p = 0.0044 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.10). The difference in the pepsin activity of the stomach mucosa between the fed and the starving fish was never significant for single comparisons, throughout the experiment.

Figure 29: (a) Stomach digesta in the fed fish (▩) and mucus in the starving fish, i.e. the control (⊡). The fish had been starved for 20 days, and then fed *ad libitum* in the first 1 hour, whereafter the feed was withdrawn. The stomach digesta/mucus was expressed as dry weight digesta/mucus in percent of dry weight fish. Further details as in Figure 26. (b) Pepsin activity in the stomach mucosa of the fed fish (▩) and the starving fish, i.e. the control (⊡). Other details as in (a).

(a)


Stomach digesta (%)
3.00
2.00
1.00
0.00
0
5
10
15
20
25
Time (hrs)

(b)

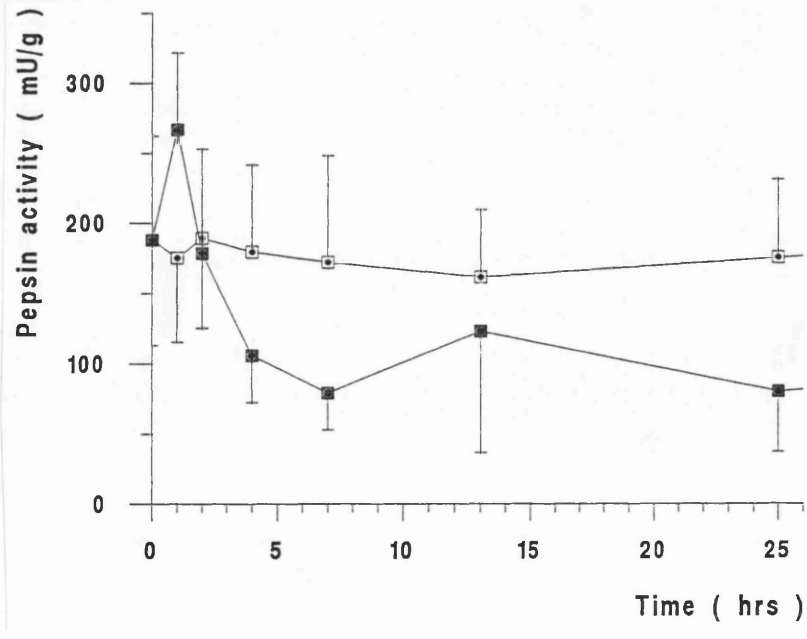
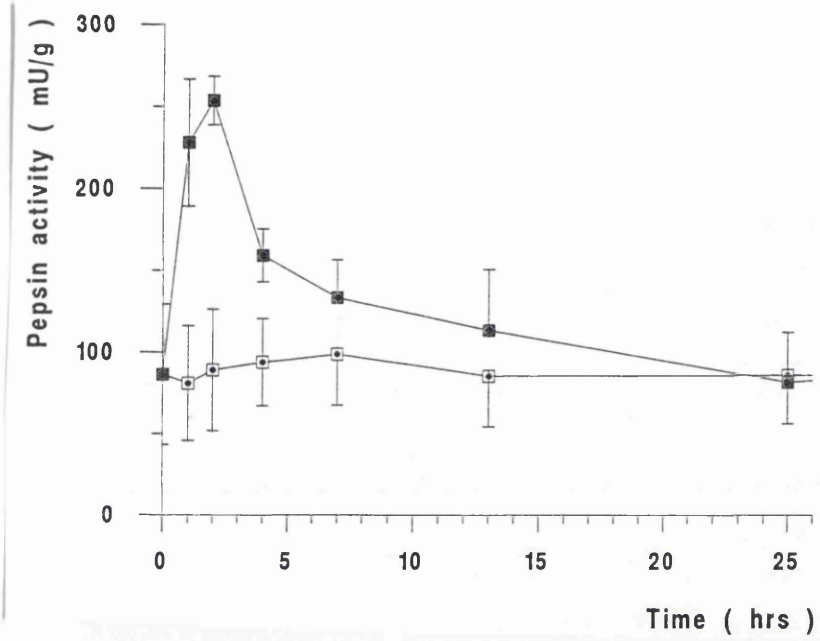
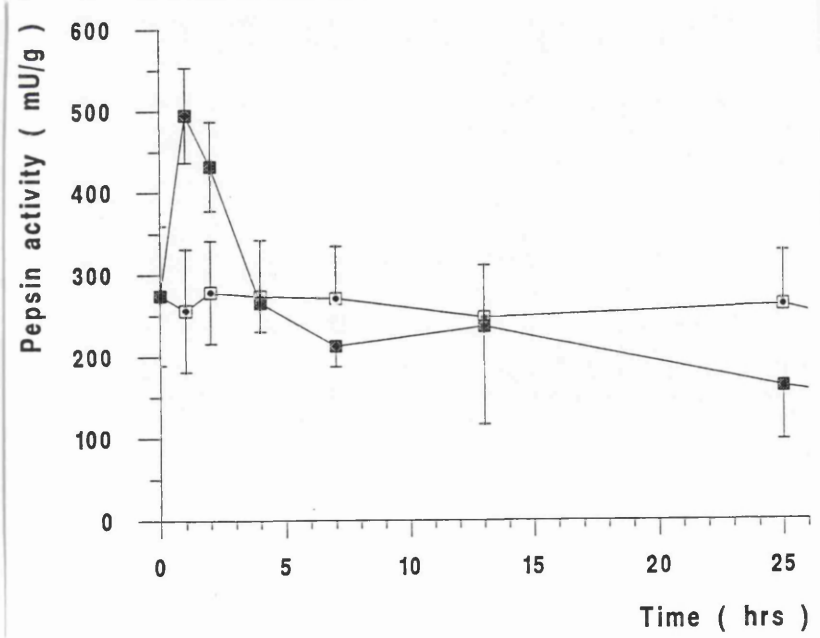

Pepsin activity (mU/g)
300
200
100
0
0
5
10
15
20
25
Time (hrs)

Figure 29: (c) Pepsin activity in the stomach digesta of the fed fish (▩) and the stomach lumen of the starving fish, i.e. the control (⊡). Other details as in (b). (d) Total pepsin activity, i.e. the activity of the mucosa, was added to the respective activity of the digesta/mucus in the fed fish (▩), or added to the respective activity in the lumen of the starving fish, i.e. the control (⊡). Other details as in (b).

(c)


Pepsin activity (mU/g)
300
200
100
0
0
5
10
15
20
25
Time (hrs)

(d)


Pepsin activity (mU/g)
600
500
400
300
200
100
0
0
5
10
15
20
25
Time (hrs)

The pepsin activity in the stomach digesta of the fed fish (Fig. 29 (c)) was found to rise and the overall difference between the fed fish and the starving fish is significant (Two way ANOVA: $p < 0.0005$. X.Appendices. Table VI.11), during the 1 hour of feeding ($p < 0.00005 < p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.12). The pepsin activity remained significantly higher in the fed fish, than in the starving fish (the control) from 1 to 4 hours. It was seen to fall, in the interval of 2 to 4 hours. It is significantly higher in the fed fish, than in the starving (the control) fish, after 4 hours ($p = 0.0005 < p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.12), but not after 7 hours ($p = 0.028 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.12).

The total pepsin activity, i.e. the activity in the stomach mucosa added to the comparable activity in the stomach digesta/mucus (Fig. 29 (d)), in the stomach of the fed fish, rose in the interval of 0 to 1 hours. It was overall significantly greater, than the comparable activity in the starving fish (Two way ANOVA: $p = 0.047$. X.Appendices. Table VI.13), as well as after 1 hour ($p = 0.0001 < p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.14) and after 2 hours ($p = 0.0007 < p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.14). The total pepsin activity in the fed fish fell in the interval of 1 to 4 hours, but was not significantly different from the comparable activity in the starving fish after 4 hours ($p = 0.40 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.14), after 7 hours ($p = 0.042 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.14), or after 25 hours ($p = 0.013 > p = 0.0036$; Bonferroni: 14 tests; one tailed test. X.Appendices. Table VI.14).

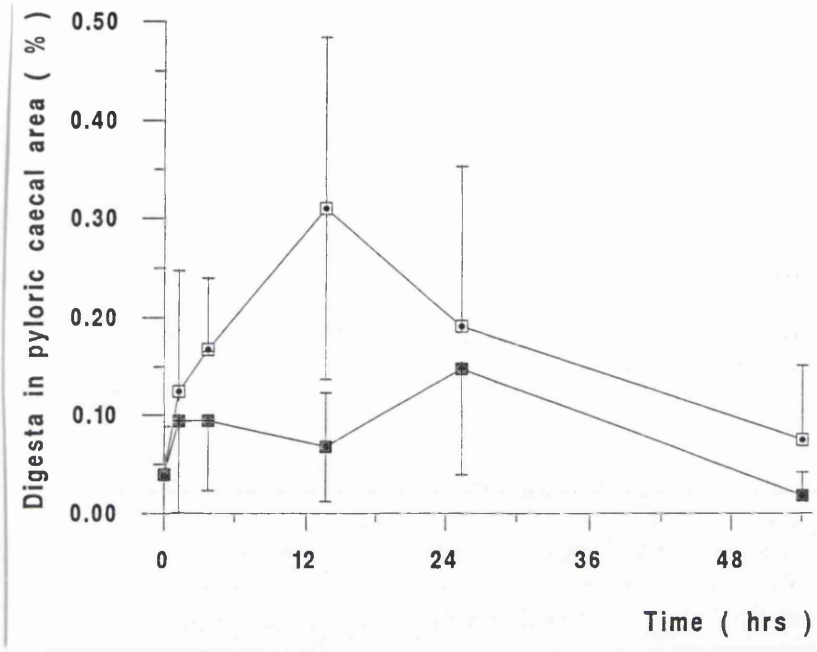
3.3. The effect of food on trypsin and chymotrypsin and on the gallbladder.

3.3.1. Digesta.

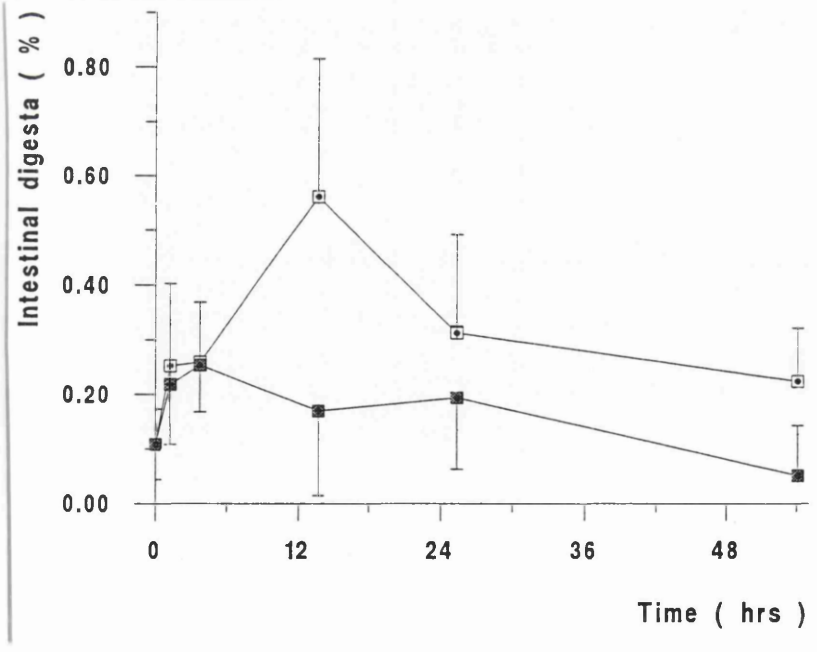
The digesta in the pyloric caecal region and in the intestine, i.e. the pyloric caecal digesta, the mid intestinal digesta and the rectum digesta (Fig. 30 (a) and Fig. 30 (b), respectively) was overall greter in the fed, than in the starved fish (Two way ANOVA: $p < 0.0005$ and $p < 0.0005$, respectively. X.Appendices. Table VI.15 and 17). It was not significantly greater in the fed fish, than in the starved fish after 3.73 hours ($p = 0.0082 > p = 0.00172$

Figure 30: Digesta in (**a**) the pyloric caecal area and (**b**) the intestine of the fed (▣) and the starving fish, the control (■). The fish had been starved for 25 days and then the fed fish were fed *ad libitum* for 1.25 hours. The digesta was expressed as dry weight in percent (%) of dry weight fish. Time was expressed in hours (hrs). Each data point was a mean value from 20 fish for the fed fish (n=20) and 10 fish for the starving fish (n=10). Error bars are standard deviations.

(a)


Digesta in pyloric caecal area (%)
0.50
0.40
0.30
0.20
0.10
0.00
0
12
24
36
48
Time (hrs)

(b)


Intestinal digesta (%)
0.80
0.60
0.40
0.20
0.00
0
12
24
36
48
Time (hrs)

and $p = 0.44 > p = 0.00172$, respectively; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.16 and 18), but became significantly greater after 13.65 hours ($p < 0.00005 < p = 0.00172$ and $p < 0.00005 < p = 0.00172$, respectively; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.16 and 18), meaning that the evacuation from the stomach began sometime after 3.73 hours and before 13.65 hours.

The intestinal digesta was not significantly greater in the fed fish, than in the starving fish, after 25.32 hours ($p = 0.025 > p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.18), meaning that the evacuation of the intestinal digesta out of the fish, began before 25.32 hours.

3.3.2. Discharge of the gallbladder and secretion of trypsin and chymotrypsin.

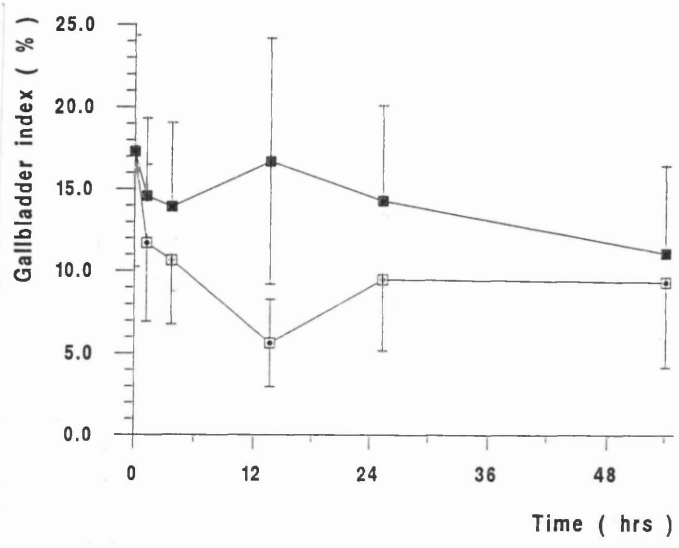
The gallbladder index (Fig. 31 (a)) was overall significantly smaller in the fed fish, than in the starving fish (Two way ANOVA: $p < 0.0005$. X.Appendices. Table VI.19). It was not significantly smaller in the fed fish after 3.73 hours ($p = 0.049 > p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.20), but became significantly smaller by 13.65 hours ($p = 0.0006 < p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.20).

There was overall significant difference in the trypsin activity in the pancreatic tissue (Fig. 31 (b)) of the fed fish and the starving fish (Two way ANOVA: $p = 0.024$. X.Appendices. Table VI.21). No significant difference between the fed and the starving fish was found after 3.75 hours ($p = 0.45 > p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.22), but the trypsin activity for the fed fish became sificantly lower, than the trypsin activity for the starving fish, after 13.65 hours ($p = 0.0001 < p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.22). The difference in the trypsin activity, between the fed and the starved fish, either at 25.32 or 54.07 hours, was not significant.

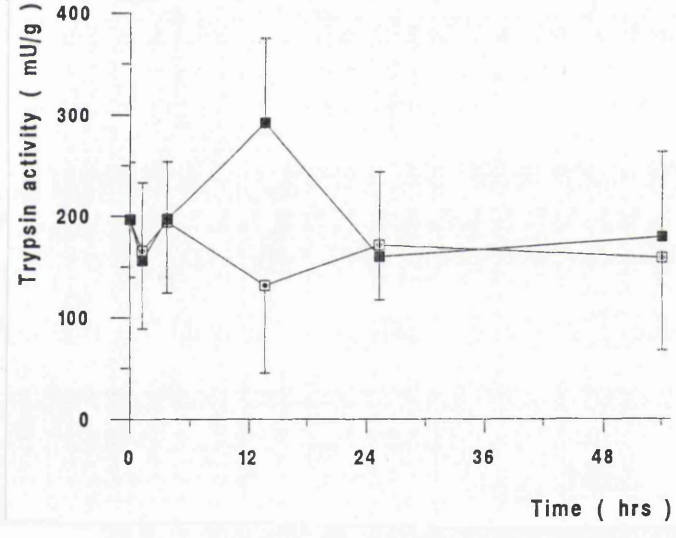
The overall difference between the fed and the starving fish was not significant (Two way ANOVA: $p = 0.083$. X.Appendices. Table VI.23) for chymotrypsin activity in the pancreatic tissue (Fig. 31 (c)). The pattern was however similar to that of trypsin (See

Figure 31: The gallbladder index(**a**) and trypsin (**b**) and chymotrypsin (**c**) activity in the pancreas of the fed fish (⊡) and the starving fish, i.e. the control (■). The gallbladder indexis the wet weight of the gallbladder per wet weight of the liver in percent (%). Trypsin and chymotrypsin are expressed in milliunits per dry weight fish (mU/g). Further details as in Figure 30.

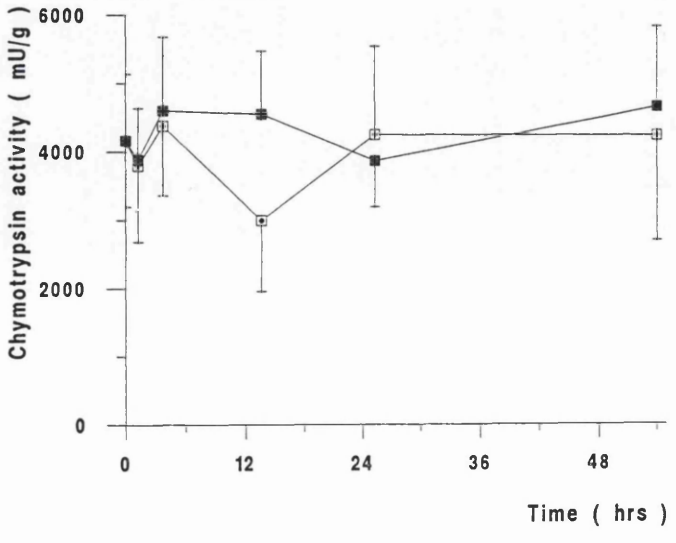
(a)


Gallbladder index (%)
25.0
20.0
15.0
10.0
5.0
0.0
0
12
24
36
48
Time (hrs)

(b)


Trypsin activity (mU/g)
400
300
200
100
0
0
12
24
36
48
Time (hrs)

(c)


Chymotrypsin activity (mU/g)
6000
4000
2000
0
0
12
24
36
48
Time (hrs)

above). Chymotrypsin activity became significantly lower in the fed fish, than in the starving fish, after 13.65 hours ($p = 0.0002 < p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.24).

The reduction in the trypsin and chymotrypsin activity in the pancreatic tissue at 13.65 hours, caused by feeding, was 160.6 mU/g and 1,554 mU/g (Fig. 31 (b) and Fig. 31 (c)), equivalent to 55.0 % and 34.2 % of the control, respectively.

3.3.3. Trypsin and chymotrypsin activities in the intestinal digesta.

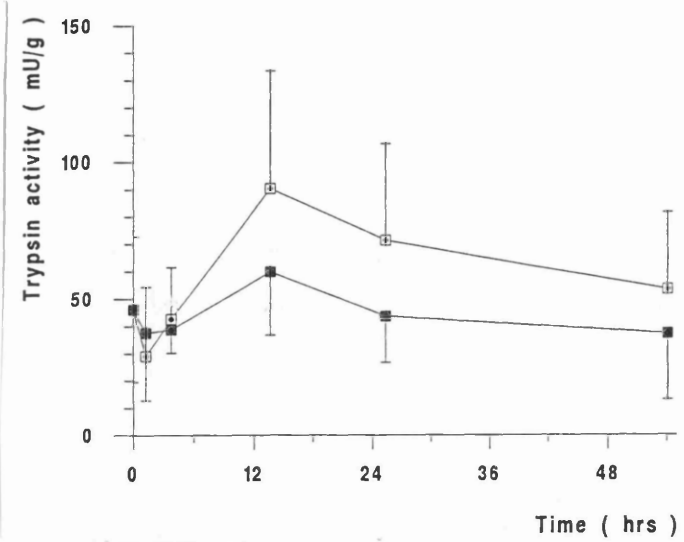
The trypsin and chymotrypsin activities in the intestinal digesta are presented in Figure 32 and Figure 33, respectively.

The overall trypsin and chymotrypsin activity in the pyloric caecal region of the intestine (Fig. 32 (a) and Fig. 33 (a), respectively) was significantly greater in the fed fish, than in the starving fish for trypsin (Two way ANOVA: $p = 0.009$. X.Appendices. Table VI.25), but not for chymotrypsin (Two way ANOVA: $p = 0.086$. X.Appendices. Table VI.31). These activities are not significantly greater in the fed fish, than in the starving fish, at 3.75 hours ($p = 0.23 > p = 0.00172$ and $p = 0.44 > p = 0.00172$, respectively; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.26 and 32), or at 13.65 hours ($p = 0.009 > p = 0.00172$ and $p = 0.018 > p = 0.00172$, respectively; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.26 and 32), although the activity in the fed fish, compared to that of the starving fish, is seen to rise in the interval of 3.75 to 13.65 hours. The difference in the trypsin and chymotrypsin activity, between the fed and the starving fish, is greater at 13.65 and 25.32 hours (and for trypsin at 54.07 hours), than at 1.25 and 3.73 hours. The difference in the trypsin and chymotrypsin activity, between the fed and the starving fish, remained fairly constant at 13.65 and 25.32 hours (and for trypsin at 54.07 hours).

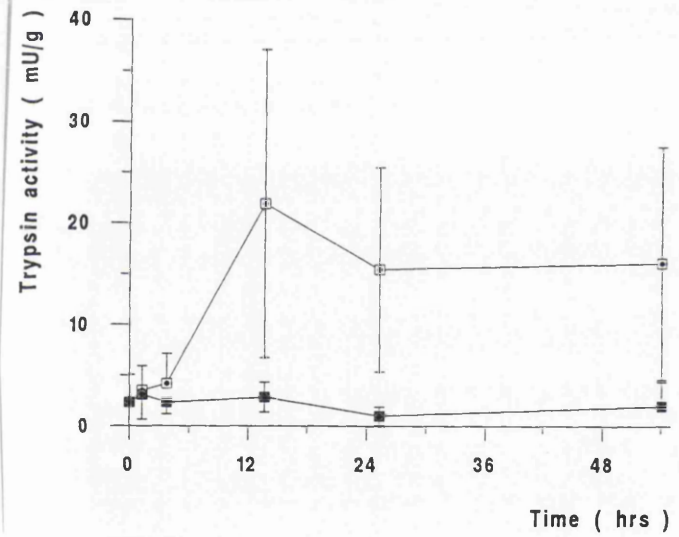
The overall trypsin and chymotrypsin activity in the mid intestinal area (Fig. 32 (b) and Fig. 33 (b), respectively) was significantly greater in the fed fish, than in the starving fish (Two way ANOVA: $p < 0.0005$ and $p < 0.0005$, respectivly. X.Appendices. Table VI.27 and 33). These activities are not significantly greater in the fed fish, than in the starving

Figure 32: Trypsin activity in the digesta from the pyloric caecal region (**a**), the mid intestinal region (**b**) and the rectum (**c**), in the fed fish (▣) and the starving fish, i.e. the control (▩). Maximal extractable trypsin activity in the feed, was subtracted from the trypsin activity. Further details as in Figure 30.

(a)


Trypsin activity (mU/g)
150
100
50
0
0
12
24
36
48
Time (hrs)

(b)


Trypsin activity (mU/g)
40
30
20
10
0
0
12
24
36
48
Time (hrs)

(c)

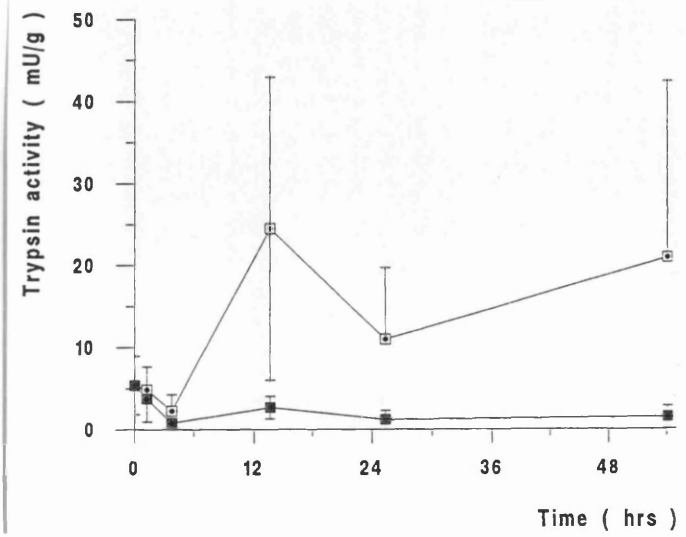
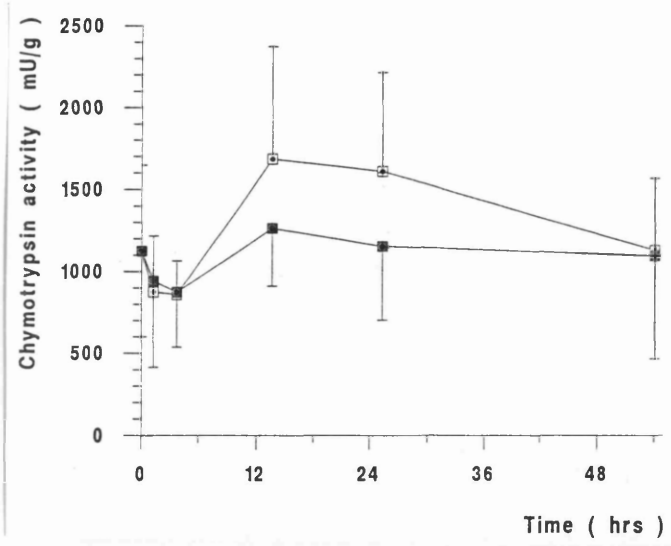
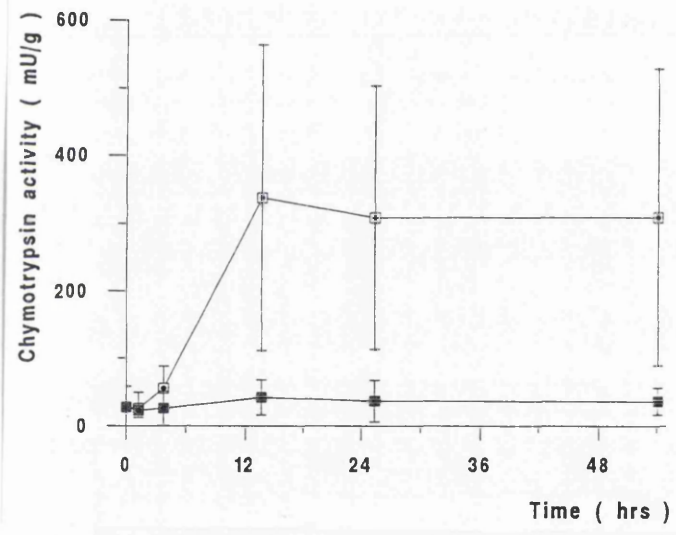

Trypsin activity (mU/g)
50
40
30
20
10
0
0
12
24
36
48
Time (hrs)

Figure 33: Chymotrypsin activity in the digesta from the pyloric caecal region (**a**), the mid intestinal region (**b**) and the rectum (**c**), in the fed fish (▣) and the starving fish, i.e. the control (■). Maximal extractable chymotrypsin activity in the feed, was subtracted from the chymotrypsin activity. Further details as in Figure 31.

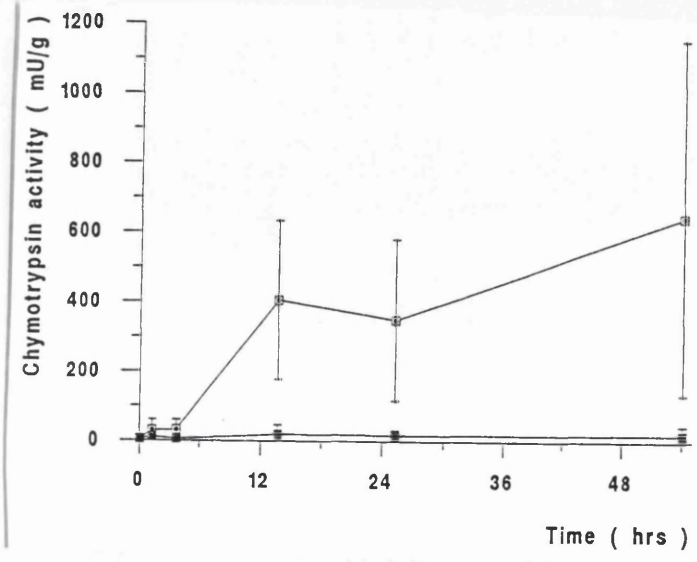
(a)


Chymotrypsin activity (mU/g)
2500
2000
1500
1000
500
0
0
12
24
36
48
Time (hrs)

(b)


Chymotrypsin activity (mU/g)
600
400
200
0
0
12
24
36
48
Time (hrs)

(c)


Chymotrypsin activity (mU/g)
1200
1000
800
600
400
200
0
0
12
24
36
48
Time (hrs)

fish at 3.75 hours ($p = 0.011 > p = 0.00172$ and $p = 0.0056 > p = 0.00172$, respectively; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.28 and 34), but significantly greater at 13.65 hours ($p < 0.00005 < p = 0.00172$ and $p < 0.00005 < p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.28 and 34). The difference in the trypsin and the chymotrypsin activity, between the fed and the starving fish, remained fairly constant at 13.65, 25.32 and 54.07 hours.

The overall trypsin and chymotrypsin activities in the rectum (Fig. 32 (c) and Fig. 33 (c)) was significantly greater in the fed fish, than in the starving fish (Two way ANOVA: $p < 0.0005$ and $p < 0.0005$. X.Appendices. Table VI.29 and 35). These activities were not significantly greater in the fed fish, than in the starving fish, at 3.75 hours ($p = 0.0024 > p = 0.00172$ and $p = 0.005 > p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.30 and 36), but significantly greater at 13.65 hours ($p < 0.00005 < p = 0.00172$ and $p < 0.00005 < p = 0.00172$; Bonferroni: 29 tests; one tailed test. X.Appendices. Table VI.30 and 36). The difference in the trypsin and the chymotrypsin activity, between the fed and the starving fish, remained roughly constant at 13.65, 25.32 and 54.07 hours.

4. Discussion.

4.1. The effect of starvation on pepsin, trypsin and chymotrypsin, and the gallbladder.

Starvation caused a slight rise, in the 2 first days, in the activity of stored pepsin, trypsin and chymotrypsin. As secretion of these enzymes is mostly switched off, caused by the withdrawal of food, the likely explanation for this is that basal synthesis of these enzymes is slightly higher than the basal secretion in this period. This is in agreement with previous observations, on the rat (Girard-Globa *et al.*, 1980) and with studies on catfish starved for 2 months (Yoshinaka *et al.*, 1981a), but apparently contradicts studies with the eel (Yoshinaka *et al.*, 1984). The data from the eel (*Ibid.*) were from 3 fish. Basal synthesis and basal secretion are known in mammals to occur at all times in the pancreas (Case, 1978; Girarad-Globa *et al.*, 1980).

Starvation caused a gradual reduction of trypsin and chymotrypsin activities in the

intestinal digesta, demonstrating that secretion of these enzymes is almost abolished upon withdrawal of food. This agrees with previous studies (Yoshinaka *et al.*, 1981a; Onishi *et al.*, 1976; Overnell, 1973).

The reduction of pepsin activity in the stomach lumen was only found after 2 days of starvation, but increased after that, and was henceforth not significantly different from the comparable activity in the feeding fish (Fig. 26 (b)). This activity could stem from already secreted pepsinogen, residing in the mucus, which covers the stomach lumen. A basal secretion of pepsinogen (the inactive precursor of pepsin, which is activated by HCl or active pepsin in the stomach lumen) may result in an accumulation of pepsin activity in the stomach lumen, i.e. the stomach secretion, if the stomach evacuation is switched off during starvation. It could also be coming from pepsinogen squeezed from the oxynticopeptic cells, during the separation of mucus from the stomach mucosa. It is unlikely that oxynticopeptic cells themselves were removed in the separation, as these are buried deep in the mucosa.

The response of the gallbladder was in accordance with previous studies (Talbot and Higgins, 1982).

Starvation of 16 to 20 days did not reduce the stored amount of pepsin, trypsin and chymotrypsin, while the secreted amount of trypsin and chymotrypsin was reduced to a minimum.

4.2. The effect of food on pepsin.

Secreted pepsin activity, i.e. activity in the stomach digesta, correlated fairly well with the stomach digesta itself. The ingestion of food causes secretion of pepsinogen from the stomach mucosa. The secretion may be triggered by the distension of the stomach, thus stimulating mechano, i.e. stretch, receptors resulting from the ingestion of food. Pepsin secretion from the stomach mucosa of the brown bullhead (*Ictalurus nebulosus*), has been triggered by the distension of the stomach with a sponge (Smit, 1967). The secretion may also be triggered by food coming into contact with biochemical receptors, situated in the lumen in the pyloric area of the stomach (Bell *et al.*, 1980).

Secretion of pepsin in mammals is initiated by polypeptides and amino acids, which cause a release of gastrin into the blood stream, and gastrin stimulates pepsin secretion from the chief cells (Dockray, 1978).

Recent studies suggest that serotonin, i.e. 5-hydroxytryptamine (Holstein and Cederberg, 1984), and/or some tachykinins (Holstein and Cederberg, 1986) may be physiological stimulators of pepsin secretion in fish, but these studies have been done on the Atlantic cod (Chapter I). Substance P (SP) is a member of the tachykinin family and was found to be an effective pepsigogue (*Ibid.*). Interestingly, studies on the rainbow trout have shown that SP acts by releasing serotonin (5-HT) from enteric neurons in the stomach of this animal (Holmgren *et al.*, 1985). SP was shown to be indirectly excitatory on the stomach wall muscle, acting via a neuron, as tetrodotoxin (abolishes Na^+-mediated action potentials) blocked the stimuli. This neuron was shown to be non-adrenergic and non-cholinergic, as no reduction of the response was obtained with atropine (muscarinic cholinoceptor antagonist), chlorisondamine (nicotinic cholinoceptor antagonist) or phentolamine (α-adrenoceptor antagonist). This neuron released 5-HT during stimulation with SP, but 5-HT was found to stimulate the stomach muscle directly, as tetrodotoxin did not abolish this effect. SP-like immunoreactivity has been found in endocrine cells of the rainbow trout gastric mucosa (Holmgren *et al.*, 1985). Histaminergic and/or cholinergic mediation of pepsin secretion probably does not exist in the Atlantic cod (Holstein and Cederberg, 1986) and may not exist in other teleostei.

The total pepsin activity, i.e. the activity in the stomach mucosa and in the digesta, increased immediately upon ingestion of food. Ingested food triggers the synthesis of pepsinogen. This synthesis may be under a homeostatic regulation, but the pepsin activity in the stomach digesta/lumen became the same for the fed and the starving fish after 25 hours (Fig. 29 (c)) and the total pepsin activity of the stomach (Fig. 29 (d)), became the same in the fed and the starving fish after 4 hours, and remained like that till the end of the experiment. The pepsin activity of the stomach mucosa of the fed fish, however remained lower (not significantly) between 4 and 25 hours, than the comparable activity in the starving fish. The synthesising capacity of the oxynticopeptic cell, with respect to pepsin, was apparently not great enough to obtain homeostasis of the mucosal pepsin activity, within 25 hours (Fig. 29 (b)). In agreement with this are studies on pepsin

secretion in the bluegill (*Lepomis macrochirus*), which suggested that a food mass (volume) greater than 1.25 % of the fish body weight, is too great a volume to be adequately processed, resulting in an exhaustion of the secretory capabilities of the stomach (Norris *et al.*, 1973). Incompletely digested food would be passed into the intestine and would appear in the faeces. The ingested food mass far exceeded 1.25 % in the present study (Fig. 29 (a)), but species differences with respect to the stomach size in terms of bodymass are likely to exist. The fish used in the present study, ca. 19-46 g freh weight, were bigger than the fish, which Norris *et al.* (1973) used, i.e. 6-18 g fresh weight. For juvenile Atlantic salmon of 10 to 100 g fresh weight, the organ indicator for the stomach (fresh weight stomach/fresh weight fish x 100 %) was found to fall only slightly, i.e. from 0.92 to 0.86 % (unpublished observations). Size differences alone between *Salmo salar* and *Lepomis macrochirus* should predict that the limit of food intake in *Salmo salar* with respect to adequate processing should be lower than 1.25 % of the fish body weight.

Pentagastrin (a gastrin analogue) and G34 and G17 (variants of gastrin) are known in mammals to cause a trophic action on the fundic mucosa, an increase in DNA, RNA and protein synthesis in the chief cells (Johnson and Guthrie, 1976). The factor causing the pepsin synthesis, during stimulation of the oxynticopeptic cells by food, is unknown in fish.

4.3. The effect of food on trypsin and chymotrypsin and the gallbladder.

The evacuation from the stomach begins between 3.73 and 13.65 hours, as digesta had appeared in the pyloric caecal region at 13.65 hours (Fig. 30 (a)). Occurring concurrently with the digesta appearing in the pyloric caecal region of the intestine at 13.65 hours, are the following events:

1. The gallbladder discharges (Fig. 31 (a)).
2. Trypsin and chymotrypsin are secreted from the pancreas
(Fig. 31 (b) and Fig. 31 (c), respectively).
3. Trypsin and chymotrypsin appear in all parts of the intestine
(Fig. 32 (b), Fig. 32 (c)) and Fig. 33 (b), Fig. 33 (c), respectively).

There is no doubt from the above, that digesta appearing in the intestine, i.e. the pyloric

caecal area of the intestine, triggers the secretion of trypsin and chymotrypsin from the pancreas, and the discharge of the gall from the gallbladder, and probably peristalsis of the intestinal wall. Hypothetically, endocrine cells, situated in the pyloric caecal region, would be stimulated to secrete a CCK-like gut hormone into the blood stream. Cells fitting this role, have been found (See Chapter II). This CCK-like hormone would then stimulate the pancreas to secrete trypsin and chymotrypsin, and the gallbladder to discharge its gall. CCK from pig has been found to have these actions in the Atlantic salmon (See Chapter IV). These results are in good agreement with previous studies on the Atlantic salmon, where food entering the intestine was found to cause gallbladder discharge (Talbot and Higgins, 1982), and a ca. 50 % reduction of trypsin activity in the pancreatic tissue and the pyloric caeca (Pringle *et al.*, 1992).

Intestinal extract from the river lamprey (*Lampetra fluviatilis*) and from the marine lamprey (*Petromyzon marinus*) evoked during *in vivo* conditions an increased flow of secretion, with heightened protein concentration, from the rat pancreas (Barrington and Dockray, 1970). Intestinal extract from eel (*Anguilla anguilla* L.) stimulated contraction of the gallbladder of the rabbit *in vitro* and caused release of fluid and protein from the pancreas of the rat during *in vivo* conditions (Barrington and Dockray, 1972). Obviously in fish, some factor, residing in the intestinal mucosa, is able to stimulate secretion from the pancreas and discharge from the gallbladder. This factor has been identified, isolated and analysed as CCK in mammals (Jorpes and Mutt, 1973).

Previous studies showed definitely that a gastrin/CCK peptide exists in the teleostean intestine, having at least the biologically active part, i.e. the pentapeptide sequence from the COOH end, in common with mammalian CCK (Chapter IV). Fat in the upper small intestine of dogs, has been found to cause gallbladder emptying via an increase in circulating endogenous CCK (Shiratori *et al.*, 1986) and in a separate study, to cause a release of both CCK and secretin into the portal circulation (Fujimura *et al.*, 1984). HCl, milk, corn oil, 5 % peptone solution, protein hydrolysate or 5 % solution of essential amino acids, perfused through the duodenum, increased pressure in the bile duct, i.e. stimulating bile discharge, in conscious dogs (Hong *et al.*, 1956; Hong, 1960). It was concluded that substances causing release of CCK from the duodenum, do so by

stimulating procaine (anaesthetic) sensitive receptors, which are connected to the cells producing CCK, by a pathway, that can be blocked by hexamethonium chloride (nicotinic cholinoceptor antagonist). Peptone and acid in the duodenum caused secretion of liquid and digestive enzymes from the pancreas in pigs (Hong and Magee, 1970). Atropine and pentolinium (nicotinic cholinoceptor antagonist) significantly reduced the secretion of digestive enzymes. It was concluded that the release of CCK depends on a cholinergic mechanism, with at least one synapse. Different rsults were found in the dog, where atropinisation did not abolish the effect of peptone introduced in the intestinal lumen on pancreatic secretion, indicating that a cholinergic nervous mechanism is not likely to be involved (Wang and Grossman, 1951). Intraduodenal perfusion with a solution of amino acids stimulated the secretion of lipase from the pancreas in man (Gamble, 1970), in subjects which had undergone vagotomy (cutting of the vagus). Other studies in mammals have revealed, that lipolytic split products or peptones and various amino acids in the duodenum, caused release of CCK from the intestinal mucosa (Wang and Grossman, 1951; Sircus, 1958). Essential amino acids or beef hydrolysate in the duodenum stimulated secretion of lipase, trypsin and amylase from the pancreas (Go *et al.*, 1970) in humans, and essential amino acids in the upper duodenum of the rabbit, stimulated secretion of protein from its pancreas (Rothman, 1972). L-isomers of various amino acids, fatty acids and Ca^{+2} are all potent releasers of CCK (Go, 1978). The present study is in harmony with studies on mammals (See above) and it is highly likely, that a similar mechanism for the triggering of secretion of trypsin and chymotrypsin and the discharge of the gallbladder, exists in the Atlantic salmon and probably in teleostei in general. Both duodenal acidification and intra arterial injection of CCK(8), sulphated, increased gallbladder motility in the rainbow trout, *Oncorhynchus mykiss* (Aldman *et al.*, 1992).

The digestive enzymes trypsin, chymotrypsin, carboxypeptidase A and B, amylase, lipase and ribonuclease have been found to be secreted in fixed proportions from the Guinea pig pancreas, during *in vitro* stimulation with carbamylcholine (acetylcholine (ACh) analogue), synthetic caerulein, sulphated, (CCK analogue) or purified, porcine CCK, sulphated (Scheele and Palade, 1975). *In vivo* stimulation of the pancreas in the pigeon by carbamylcholine caused a ca. 40 % reduction in its stored amylase activity (Fernandes and Janqueira, 1955), in the rat by pilocarpine or carbamylcholine causing a reduction of 64 % in amylase and 67 % loss of total proteolytic activity (Farber and Sidransky, 1956). *In*

vivo stimulation of the rat pancreas by a mixture of purifie CCK, sulphated, and secretin was found to reduce amylase activity by 60 % after 4 hours (Reggio *et al.*, 1971), of the sheep pancreas by CCK(8), sulphated (CCK active fragment) causing secretion of juice and protein (Harada *et al.*, 1986), of the pancreas in the killifish by CCK(8), sulphated, caerulein, sulphated and desulphated (CCK analogues) causing secretion of lipase (Honkanen *et al.*, 1988). The present study is in agreement with studies on mammals and teleostei (See above), and the enzyme depletion and the secretion time appear also to be in agreement.

Purified, porcine CCK, sulphated, and CCK(8), sulphated, have been found to cause a contraction of the gallbladder and a relaxation of the sphincter of Oddi during *in vivo* conditions in the cat (Behar and Biancani, 1980). Purified porcine CCK, sulphated, CCK(8), sulphated and desulphated, and ACh have been found to cause a contraction *in vitro* in the coho salmon gallbladder (Vigna and Gorbman, 1977). Caerulein, sulphated and desulphated, CCK(8), sulphated and desulphated, have been found to cause gallbladder contractions *in vitro* in the bluegill, killifish and bowfin (Rajjo *et al.*, 1988a) and in the rainbow trout (Aldman and Holmgren, 1987; Aldman *et al.*, 1992). CCK(8), sulphated, caused contractions in the rainbow trout gallbladder, which were inhibited by VIP (Aldman and Holmgren, 1992) and it was suggested that VIP finetuned the effects of CCK. Duodenal acidification and intra arterial CCK(8), sulphated, was also found to increase gallbladder motility in this animal (Aldman *et al.*, 1992).

Food in the intestinal lumen causes a stimulation of trypsin and chymotrypsin secretion and gallbladder discharge, probably via some CCK factor or possibly via a neural pathway.

It was demonstrated in the present study for trypsin and chymotrypsin, which had been secreted between 3.75 and 13.65 hours (Fig. 32 and Fig. 33), that the activities in the intestinal digesta of these enzymes remain constant after 13.65 hours. Something appears to switch off the secretion of these enzymes, as the intestinal digesta, i.e. the stimulus, still remain in the intestine (Fig. 30 (b)).

CCK plays a central role in control of digestion, by matching the supply of food and the amount of the pancreatic enzymes and bile salts, but apart from causing secretion from the pancreas and discharge of the gallbladder, it also controls rates of gastric emptying and food intake (Dockray, 1989). An interesting hypothesis on the regulation of secretion of digestive enzymes has been proposed (*Ibid.*): A certain CCK-releasing peptide, secreted from the pancreas and degraded by proteolytic enzymes, regulates this secretion. Proteolytic enzymes apparently inhibited CCK secretion as a part of a negative feedback loop, but protease inhibitors depressed this action in man (Owyang *et al.*, 1986). Protease inhibitors are postulated to increase intestinal digestal concentrations of this CCK-releasing peptide in the rat and hence, release CCK (Iwai *et al.*, 1987). Another not less likely hypothesis on the regulation of the effects of CCK on the pancreas has been suggested by Aldman and Holmgren (1992): vasoactive intestinal peptide (VIP) may finetune the effects of CCK by cancelling its effect.

The secretion and consequently resynthesis of both trypsin and chymotrypsin in the pancreas (Fig. 31 (b) and Fig. 31 (c)), is in agreement with similar findings in the carp (Onishi *et al.*, 1973), where proteolytic activity in the hepatopancreas of this animal was first found to decrease, before it increased again. This is also in agreement with similar findings in mammals (See below). Resynthesis of trypsin and chymotrypsin begins only after the secretion of these enzymes has begun. This resynthesis is finished at 25.32 hours, and must be under a strict homeostatic control, as both trypsin and chymotrypsin activities in the pancreas of the fed fish became almost identical to the comparable activities in the starved fish at 25.32 hours and remained so till 54.07 hours (Fig. 31 (b) and Fig. 31 (c)).

In salmon smolts, starved and fed one meal, trypsin activity in the pancreatic tissue and the pyloric caeca appeared to have returned to normal 47 hours after commencement of feeding, the fish having been kept at a water temperature of 12.5 °C (Pringle *et al.*, 1992), which was similar to the temperature in the present study. It is however not possible to state exactly when resynthesis was completed, as no proper control was applied, but the results appear to be in harmony with the present study. The way of sampling, i.e. collecting the pancreatic tissue with the pyloric caeca, in this study is not an ideal one. Trypsin activity residing in the lumina of the caeca themselves may be taken for stored activity. Expressing the trypsin activity as activity per wet weight of caecal tissue is not

good, when secretion from the pancreatic tissue is studied, because secretion is known to affect the wet weight of the organ (Farber and Sidransky, 1956). In studying protein synthesis in the pancreas then data should be normalised to a constant denominator (Jamieson and Palade, 1971).

It is interesting to note how the resynthesis of trypsin and chymotrypsin in the pancreas appears to be under some sort of a homeostatic control, as resynthesis is seen to match the trypsin and the chymotrypsin in the fed fish to that of the control after 25.32 hours (Fig. 31 (b) and Fig. 31 (c)). Some studies have suggested that secretogogues acting on the pancreas increase synthesis of digestive enzymes (Rabinovitch *et al.*, 1952; Allfrey *et al.*, 1953; Daly *et al.*, 1955; Fernandes and Junqueira, 1955; Farber and Sidransky, 1956), hence providing a model for the control of synthesis of digestive enzymes (Case, 1978). A definite increase in synthesis of digestive enzymes in the pancreas after stimulation with food, has been demonstrated in the frog (Poort and Geuze, 1969; Van Venrooij and Poort, 1971) and in the snake (Alcon and Bdolah, 1975). An elevation in plasma amino acid concentration resulting from absorption from the intestinal digesta at 13.65 hours (passive supply) and/or an increase of cellular uptake (active supply) are possible causes for stimulation of protein synthesis in the pancreatic acinar cell (Girard-Globa *et al.*, 1980). The increase in cellular uptake of amino acids may be stimuted by CCK.

Resynthesis of digestive enzymes in the course of stimulation has been studied in vertebrates, mainly in mammals. *In vivo* stimulation of pigeon pancreas by carbamylcholine (ACh analogue) resulted in a complete resynthesis of amylase 4-6 hours later (Fernandes and Junqueira, 1955). The increased glycine turnover, resulting from the stimulation, in both pancreatic protein and pancreatic RNA, may indicate the participation of nucleic acid in the resynthesis of amylase. *In vivo* stimulation of the rat pancreas by pilocarpine (ACh analogue), resulted in a complete resynthesis at least 7-8 hours later (Farber and Sidransky, 1956). *In vivo* stimulation of the pancreas in the same animal species with a mixture of CCK and secretin, resulted in an 8-10 times increase in the rate of biosynthesis of pancreatic enzymes, 2 hours later (Reggio *et al.*, 1971). CCK did not appear to act through transcription of DNA, since actinomycin D did not affect the rate increase caused by CCK, indicating that mRNA is not the rate-limiting factor in the resynthesis of pancreatic enzymes after stimulation (*Ibid.*). Further studies on the same

species, using identical stimulants, indicated independent regulation of resynthesis for amylase, lipase and chymotrypsin, as non-parallel variations of the rates of biosynthesis for these enzymes was observed (Dagorn and Mongeu, 1977). This is in agreement with the hypothesis that pancreatic adaptation to diet could occur through successive non-parallel changes between synthetic rates of the various digestive enzymes, resulting from a specific hormonal stimulation related to the diet (*Ibid.*).

Further *in vivo* studies on the rat pancreas, using caerulein (CCK analogue) as a stimulant, showed that synthetic rates of the various digestive enzymes, were either increased (trypsin form 1 and 2, chymotrypsin form 1 and 2, elastase form 1, ribonuclease and carboxypeptidase A and B), decreased (amylase form 1 and 2) or unaffected (trypsin form 3, elastase form 2 and lipase; Schick *et al.*, 1984). Food entering the intestine can either stimulate synthesis of digestive enzymes in the pancreas via neuronal or humoral stimuli. The food composition can then initiate a certain composition of stimuli, both neuronal and humoral, affecting the pancreas, and hence cause the various digestive enzymes to be synthesised at various rates, resulting in a definite proportion of these. A combined *in vivo* and *in vitro* study on the rat pancreas indicated that nutritional regulation of pancreatic enzyme synthesis and hence enzyme composition is mediated through the composition of active cytoplasmic mRNA (Wicker *et al.*, 1984). An *in vitro* study on the rat pancreas further indicated that caerulein exerts predominantly translational control on the biosynthesis of digestive enzymes, but additional control at a transcriptional or post-transcriptional level (i.e. via mRNA stability) may well take place as well (Wicker *et al.*, 1985). An increase in pancreatic DNA-dependent RNA polymerase activity has been demonstrated, following in vivo stimulation with bethanechol (muscarinic cholinoceptor agonist) or CCK (Black and Webster, 1974). CCK may regulate pancreatic enzyme levels, i.e. the synthesis as well as the secretion, in teleostei. Such observations have been made on cultured exocrine cells from pike pancreas (Pilz and Plantikow, 1992).

The trypsin and chymotrypsin activities, secreted from the pancreas at 13.65 hours (160.6 mU/g and 1,554 mU/g, respectively), are considerably greater than comparable activities appearing in the intestine at this time (71.4 mU/g and 1,101 mU/g, respectively). Some of the secreted activity must therefore have been evacuated from the fish, probably dissolved in the gut fluids and not bound to the digesta, as digesta was not evacuated from

the fish, until after 13.65 hours. This could well be the case, as at 13.65 hours, both trypsin and chymotrypsin activities, are roughly evenly distributed throughout the intestine, showing that the secretion from the pancreatic tissue is rapidly mixed with the digesta throughout the whole intestinal lumen. It is probable that the same factor causing secretion of trypsin and chymotrypsin from the pancreas, also causes peristalsis in the intestine. This has been suggested to be a CCK-like gut hormone (See before). Previous studies have indicated that CCK plays a role in intestinal motility (Chapter IV, section 1).

Chapter VII.

Differences in digestive enzyme activity in upper and lower modal group salmon.

1. Introduction.

Bimodality has been shown to occur in the frequency distribution of the fork length in sibling juvenile Atlantic salmon at the end of their first growing season (Simpson and Thorpe, 1976; Thorpe, 1977; Thorpe and Morgan, 1978). Bimodality has also been reported in Canadian (Bailey *et al.*, 1980; Saunders *et al.*, 1982) and Norwegian (Knutsson and Grav, 1976) stocks. A good deal of studies on this bimodal distribution has been undertaken (Thorpe *et al.*, 1980; Huntingford *et al.*, 1988; Thorpe *et al.*, 1989). The underlying reason responsible for this distribution is that the lower modal group (LMG) of juvenile salmon do not grow during the winter, as a result of internal suppression of appetite, commencing in midsummer (Metcalfe *et al.*, 1986). In the winter months, i.e. from October to March, the upper modal group (UMG) fish were found to take significantly larger meals, as percentage of body weight, than the LMG fish, and fed for a longer proportion of each 24 hour period (Higgins, 1985; Higgins and Talbot, 1985).

Earlyer studies on digestive proteases in the Atlantic salmon demonstrated that these increased, as the fish became mature (Torrissen and Torrissen, 1984). The activity of the digestive proteases in the digestive tissues was found to be higher in early maturation and lower in the later stages of maturity (Torrissen and Torrissen, 1985). An association between certain trypsin isozymes, the homozygote variant *TRP-2 (92/92)* and the heterozygote variant *TRP-2 (92/100)*, and growth has been established in this animal (Torrissen, 1991) and individuals possessing these isozymes seemed to be able to utilise the feed better than individuals without it (Torrissen and Shearer, 1992). Trypsin and chymotrypsin activities have also been studied in the Atlantic salmon in relation to the pancreas disease of this animal (Pringle *et al.*, 1992). No studies have however, been done comparing the storage and the release of digestive enzymes, such as pepsin, trypsin and chymotrypsin in the LMG and UMG salmon. Knowledge on digestive enzymes in relation to

growth in fish is scarce. Proteolytic activities in the tissues of the digestive tract of the rainbow trout, have been found to correspond to the growth rate (Rungruangsak and Utne, 1981) and amylase activity in the pancreas of the frog *Rana esculenta* has been found to undergo seasonal fluctations, where the amylase activity was correlated with the feeding of the animal (Scapin, 1982).

The main objective of this work was to study how the activities of pepsin, trypsin and chymotrypsin, both stored and secreted, related to food intake and growth of juvenile Atlantic salmon, in the winter and the spring after their first growing season (Section 2.1 and 3.1). A separation of the population into LMG and UMG fish, had already occurred, before the study. This study was undertaken to shed some light on how the above mentioned parameters varied in two different length groups, chosen from the population as follows:

1. The LMG fish: approximately 5 % of the population having the shortest fork length.
2. The UMG fish: approximately 2 % of the population, having the longest fork length.

Cholecystokinin (CCK) is known to cause secretion of digestive enzymes from the pancreas (Dockray, 1989) and it has already been demonstrated that a porcine CCK, slightly contaminated with secretin, stimulates secretion of trypsin and chymotrypsin (Chapter IV and V). As the LMG fish eats proportionally less and does not grow during the winter months, the question arises whether its pancreatic tissue becomes less capable of secreting trypsin and chymotrypsin than the pancreatic tissue of the UMG. It is also of interest to know in this context, how starvation, i.e. no stimulus, affects the stored and secreted amounts of pepsin, trypsin and chymotrypsin in the LMG and in the UMG fish. An increase in the stored and a decrease in the secreted activities of these enzymes, as a result of starvation, is to be expected (Chapter VI). The presence of food increases the demand for secretion, lowering the concentration of stored forms of pepsin trypsin and chymotrypsin, but increasing their concentration in the intestinal digesta. As the LMG fish is expected to feed proportionally less during the winter months then its concentration of stored pepsin, trypsin and chymotrypsin should be higher and its concentration of secreted pepsin, trypsin and chymotrypsin should be lower than for the UMG fish.

To address these questions of the response to i) starvation, ii) starvation and porcine CCK (intraperitoneally injected) and iii) feeding, i) to iii) were compared, with respect to the above mentioned digestive enzymes (Section 2.2 and 3.2) for LMG and UMG fish.

2. Materials and methods.

2.1. Lower and upper modal group salmon in their first year.

This experiment was executed from the 13th of December, 1991, to the 14th of July, 1992.

The fish:

The experimental animals used were a sibling population, containing LMG and UMG fish from a River Almond stock in their first year (0+).

Feeding history:

The fish had been kept on a commercial salmon food (Freshwater smolt 1, commercial pellets, size 3 mm, BP Nutrition) in excess, *ad libitum*, at least 3 months prior to and during the experiment.

Assay of feed:

Pepsin, trypsin and chymotrypsin activities were assayed in the feed as before (See Chapter VI, section 2.1), for each sampled month. These were related to the dry weight of the feed. The total activity, resulting from the feed, was calculated in the respective digesta and subtracted from the respective enzyme activity.

Water temperature:

The water temperature in the aquarium tanks for the duration of the experiment are shown in Figure 34.

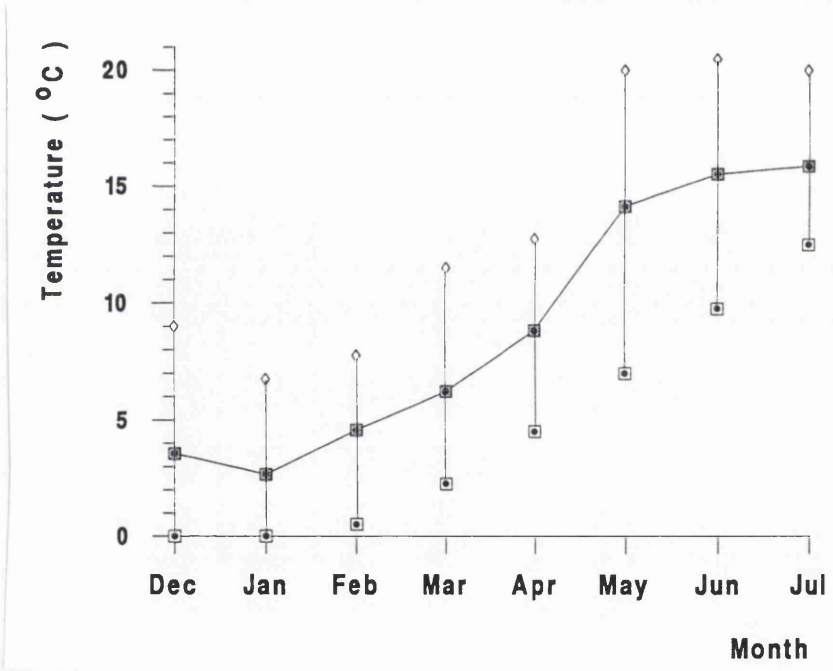
Experimental protocol:

The fork length of 700 fish, randomly chosen from the population, was measured during anaesthesia (See Chapter IV, section 2.1.1) to the nearest 0.5 mm, which were then returned to the population. Sampling was executed 2-4 days later as follows:

The LMG fish: approximately the 5 % of the population, having the shortest fork length; 20 fish were sampled.

The UMG: approximately the 2 % of the population, having the longest fork length; 20 fish were sampled.

Figure 34: The average (▣), the maximal (◇) and the minimal (⊡) water temperature in °C from December, 1991, to July, 1992.


Temperature (°C)
20
15
10
5
0
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

The following table shows the dates of sampling and the sampled fork lengths:

Month	Fork length measured	LMG and UMG Date	sampled LMG (mm)	UMG (mm)
December '91	13th	16th	< 51	> 89
January '92	14th	16th	< 51	> 89
February '92	14th	17th	< 52	> 90
March '92	13th	16th	< 55	> 101
May '92	15th	18th	< 64	> 128
July '92	10th	13th	< 97	> 139

Dissection:

The fish were killed by an excessive dose of anaesthetic, then weighed to the nearest 0.1 mg, and put on ice, but 20 fish were sampled from each group each month. The abdomen was opened ventrally and the digestive tract removed, together with the fat surrounding the pyloric caeca.

Luminal contents (termed the stomach digesta), were gently squeezed out of the stomach with foreceps and frozen , together with the stomachs (termed the stomach mucosa), at -20 °C.

The intestine was opened by an incision behind the pyloric caeca and the luminal contents from both parts pooled (termed the intestinal digesta), and frozen at -20 °C. The pyloric caeca with their surrounding fat (termed the pancreatic tissue and the pyloric caeca), and the remaining part of the intestine (termed the intestine), were frozen , separately, at -20 °C. All samples were stored at -75 °C.

The fish were dried to a constant weight at 80 °C and weighed to the nearest 0.1 mg.

Processing of samples:

Samples were kept on ice during processing. The stomach mucosas and the stomach digesta were homogenised with a Potter homogeniser (Citenco. F.H.P.motors) in a citrate buffer, pH = 4.25, and CCl_4, equal to 10 % of the buffer volume, for 30 and 20 seconds respectively.

The pancreatic tissue and the pyloric caeca, and the intestine, from May and July, were prehomogenised in saline (0.9 % NaCl and 20 mM $CaCl_2$) and CCl_4 with an Ultra Turrax(Ystral GmbH, Type:x-200) for 10 seconds. The pancreatic tissue and the pyloric caeca, the intestinal digesta and the intestine, were subsequently homogenised with a potter homogeniser in the saline and CCl_4 for 30, 20 and 30 seconds, respectively. Added CCl_4 was 20 % of the saline volume for the pancreatic tissue and the pyloric caeca, and the intestine. It was 10 % of the saline volume for the intestinal digesta.

The following table gives the volume of the buffer or saline in milliliters (ml), which were added to the samples:

Sample	**Fish group**	**December '91 to March '92**	**May '92**	**July '92**
Stomach mucosa	LMG	0.25	0.30	0.60
and stomach digesta	UMG	0.50	0.80	1.60
Pancreatic tissue	LMG	4.00	4.00	8.00
and pyloric caeca	UMG	8.00	16.00	32.00
Intestine	LMG	2.00	4.00	6.00
	UMG	2.00	16.00	24.00
Intestinal digesta	LMG	1.80	1.80	1.20
	UMG	1.80	1.80	1.20

All samples were kept in the fridge overnight, at 2-4 °C and then centrifuged at 9,000 *g* for 4 minutes. Pepsin, trypsin and chymotrypsin activities were assayed as before (Chapter IV). Supernatants of intestinal digesta from May '92 were diluted for the chymotrypsin assay in the saline as follows: the lower modal fish 1/5 and the upper modal fish 1/20. Supernatants of the intestinal digesta from July '92 were diluted to 1/40 for both the trypsin and the chymotrypsin assay, for both groups of fish. Trypsin and chymotrypsin

activities in the intestine were added to the respective activities in the digesta.

The stomach digesta and the intestinal digesta were dried to a constant weight at 80 °C and weighed to the nearest 0.01 mg (Added salts subtracted).

Statistics:

A single step Bonferroni test was applied (Hochberg and Tamhane, 1987: p.58) to reveal when growth of LMG and UMG commenced during late winter or early spring. One taile test is applied as a rise in bodymass is predicted. This was obtained by compairing consecutive sampling times, i.e. months. Five such conparisons are possible for each group, making the total number of comparisons 10. The significance level was raised to p = 0.05/No. of tests = 0.05/10 = 0.005. One way ANOVAs were executed to reveal a change with time for the measured parameters for the LMG and the UMG fish. Two way ANOVAs were executed to reveal differences between the LMG and the UMG fish. The ANOVAs were done as described by Zar (1984).

2.2. The effect of starvation, CCK and feeding.

The experiment was executed on the 26th of February, 1992. The fish, feeding history, assay of feed and water temperature are described in section 2.1.

Experimental protocol:

The "starved fish" had been starved for 5 days prior to the experiment, and 10 fish were sampled. The "starved and injected" fish were starved for 5 days and then injected with porcine cholecystokinin (See Chapter IV, section 2.1), and 14 fish were sampled for each variant. The fed fish were fed *ad libitum*. The fish were killed in excess anaesthetics, 5 hrs after the injection. Both groups had the same length criteria, as comparable fish sampled in the middle of February, 1992 (See section 2.1).

Dissection:

The fish were dissected as before (See section 2.1), with the following exceptions: i) The stomach and the stomach digesta were sampled together. ii) The intestine and the intestinal digesta were sampled together. The same terms for the samples were applied as before

(See section 2.1).

Processing of samples:

The samples were processed and assayed as before (See section 2.1), but the added millilitres of buffer/saline was as follows:

Sample	LMG fish	UMG fish
Whole stomachs	0.25	0.75
Pancreatic tissue and pyloric caeca	4.00	8.00
Intestine with digesta	4.00	8.00

Homogenisation time was 30 seconds for all samples.

Statistics:

A single step Bonferroni (See section 2.1) was applied to reveal differences between treatments, i.e. starvation, CCK and feeding, for both the LMG and the UMG fish. The significance level was raised to $p = 0.05/14 = 0.0036$. The first family of inference will be all possible comparisons of treatments for total pepsin, and trypsin and chymotrypsin in the pancreatic tissue and the pyloric caeca, for each group of fish, i.e. the LMG and the UMG fish, which is 14 tests. The second family of inference will be all possible comparisons of tratments for trypsin and chymotrypsin in the intestinal digesta for each group of fish, which is 12 tests, but the same significans level will be used as before, i.e. the one taking 14 tests into account. As each of the treatments, i.e. starvation, CCK and feeding, may well cause a change in different directions for the measured paramters, for each of the groups of fish, i.e. the LMG and the UMG fish, a two tailed test is chosen. One treatment in comparison with another treatment might reduce a certain measured parameter in one group of fish, while increasing the same measured parameter in the other group of fish.

3. Results.

3.1. Lower and upper modal salmon in their first year.

3.1.1. Changes with time in body weight and gut digesta.

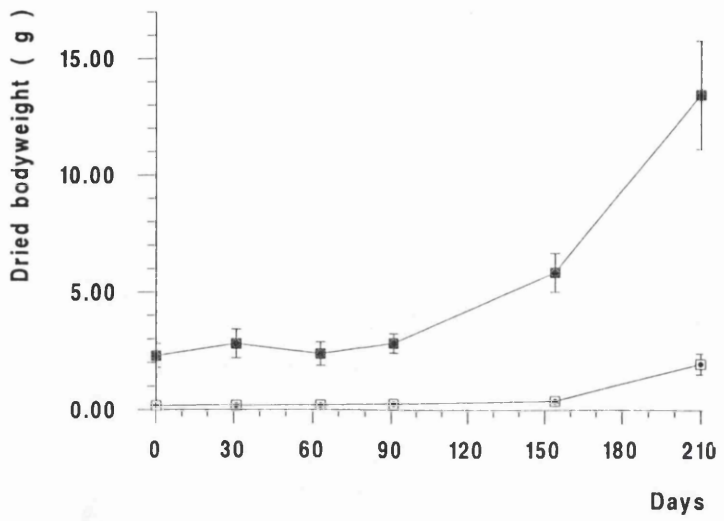
Rather few individuals became UMG fish, between 1 and 6 % of the population.

The bodymass, as dry weight, from the middle of December, 1991, to the middle of July, 1992, for the LMG fish and the UMG fish can be seen in Figure 35 (a).
For the LMG fish, a significant rise in the bodymass was found between March and May ($p < 0.00005 < p = 0.005$. Bonferroni: 10 tests; one tailed test. X.Appendices. Table VII.1) and May and July ($p < 0.00005 < p = 0.005$. Bonferroni: 10 tests; one tailed test. X.Appendices. Table VII.1). However, the rise in bodymass is negligible, except between May and July.
For the UMG fish, a significant rise in the bodymass was found between March and May ($p < 0.00005 < p = 0.005$. Bonferroni: 10 tests; one tailed test. X.Appendices. Table VII.1) and between May and July ($p < 0.00005 < p = 0.005$. Bonferroni: 135 tests; one tailed test. X.Appendices. Table VII.1).
There was no growth in either of the groups in the winter months, i.e. from the middle of December, 1991, to the middle of March, 1992. Growth for the LMG fish and for the UMG fish commenced in March. The growth of the LMG fish was however small until May.

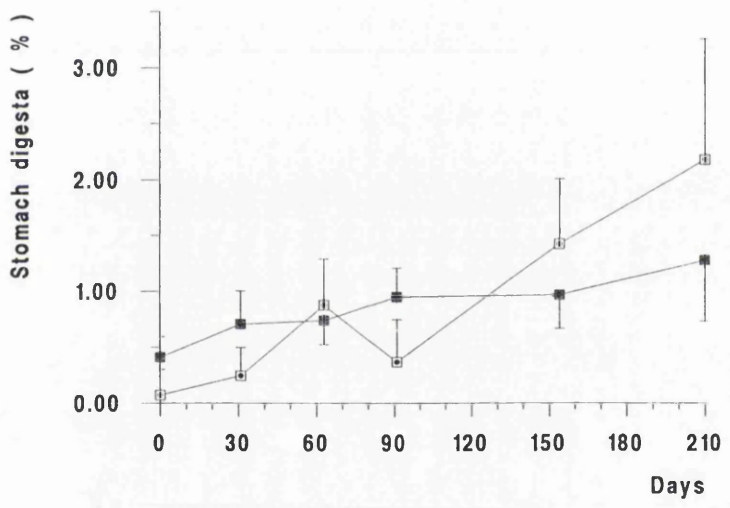
The dry weight of the digesta in the stomach per dry weight fish in percent, from the middle of December, 1991, to the middle of July can be seen in Figure 35 (b).
The stomach digesta, as a percent of the body weight, rose significantly for the LMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.2) and the UMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.2). The stomach digesta of the LMG fish was usually lower in the winter months (December, January and March), but higher in the summer months (May and July), than for the UMG fish. This was reflected in the results of a two way analysis of variance, which found an interaction between days and the group

Figure 35: Bodyweight (**a**), stomach digesta (**b**) and intestinal digesta (**c**) for the LMG fish (▣), and the UMG fish (▩), as a function of time (Days). Sampling was executed in the middle of each month: December, 1991 (0 days), January, 1992 (31 days), February, 1992 (63 days), March, 1992 (91 days), May, 1992 (154 days) and July, 1992 (210 days). The bodyweight was expressed in grams (g) and the stomach and intestinal digesta in percent of bodyweight (%). These had been dried to a constant weight. The LMG fish were chosen from 5 % of the population, having the shortest fork length and the UMG fish were chosen from 2 % of the population, having the longest fork length. Data points were average values from 20 individuals (n=20) and error bars, sometimes engulfed by the point markers, were standard deviations.

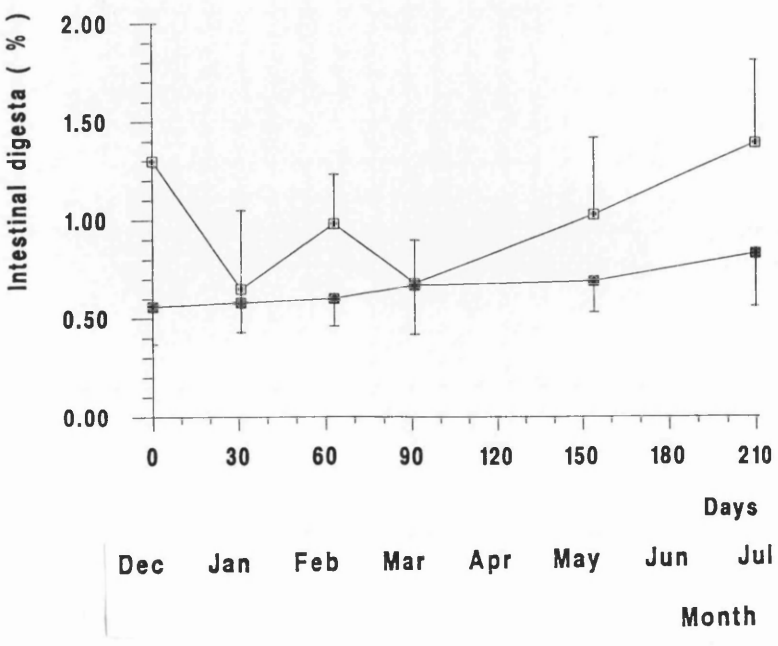
(a)


Dried bodyweight (g)
15.00
10.00
5.00
0.00
0
30
60
90
120
150
180
210
Days

(b)


Stomach digesta (%)
3.00
2.00
1.00
0.00
0
30
60
90
120
150
180
210
Days

(c)


Intestinal digesta (%)
2.00
1.50
1.00
0.50
0.00
0
30
60
90
120
150
180
210
Days
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

of fish ($F_{Interaction}$ = 16.02 > $F_{0.001(2),5,228.}$ = 4.64. X.Appendices. Table VII.4), i.e. the difference in the stomach contents of the LMG and the UMG fish, depends on the time of year. No overall difference was found between the two groups (F_{Mode} = 0.10 > $F_{0.005(2),1,228.}$ = 5.10. X.Appendices. Table VII.4).

The dry weight of the digesta in the intestine per dry weight fish in percent, from the middle of December, 1991, to the middle of July can be seen in Figure 35 (c).
The intestinal digesta, as a percent of the body weight, varied significantly for the LMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.5) and the UMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.6). A two way analysis of variance did show an interaction between days and the group of fish ($F_{Interaction}$ = 3.80 > $F_{0.01(2),5,228.}$ = 3.47. X.Appendices. Table VII.7), i.e. the difference in the intestinal contents of the LMG and the UMG fish, depends on the time of year. An overall difference was found in the intestinal contents, between the two groups (F_{Mode} = 35.62 > $F_{0.001(2),1,228.}$ = 12.5. X.Appendices. Table VII.7), i.e. the intestinal contents of the LMG fish were proportionally greater.

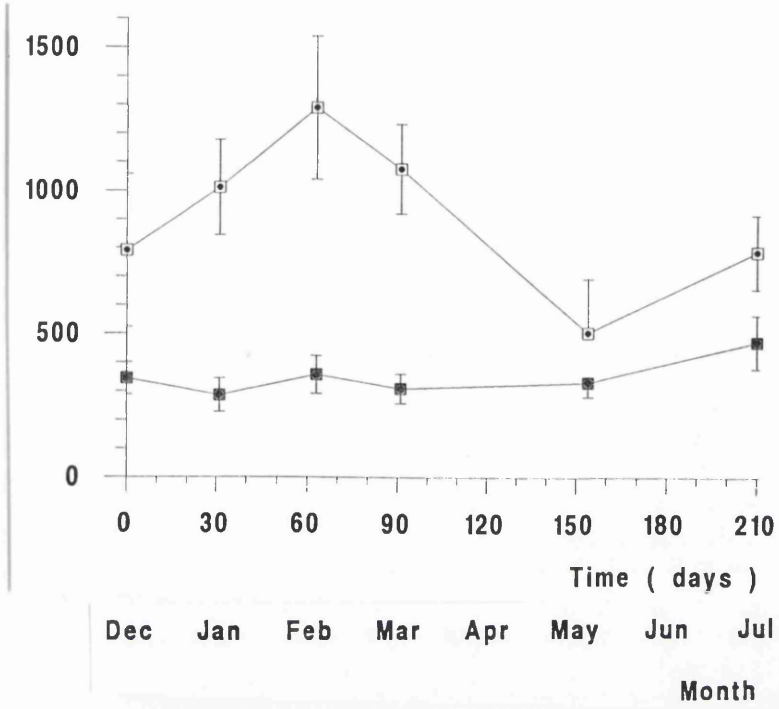
3.1.2. Changes with time in the digestive enzyme activity.

The pepsin activity in the stomach mucosa per dry weight fish in milliunits per gram (mU/g), from the middle of December, 1991, to the middle of July can be seen in Figure 36 (a).
The pepsin activity per dry weight fish varied significantly for the LMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.8) and the UMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.9). It was higher in the winter months (December to March), than in the summer months (May to July) for the LMG fish, but a two way analysis of variance found an interaction between days and the group of fish ($F_{Interaction}$ = 39.33 > $F_{0.001(2),5,228.}$ = 4.64. X.Appendices. Table VII.10), i.e. the difference in the mucosal pepsin activity between the LMG and the UMG fish, depends on the time of year. A clear overall difference was found in the mucosal pepsin activity, between the two groups (F_{Mode} = 855.41 >> $F_{0.001(2),1,228.}$ = 12.5. X.Appendices. Table VII.10).

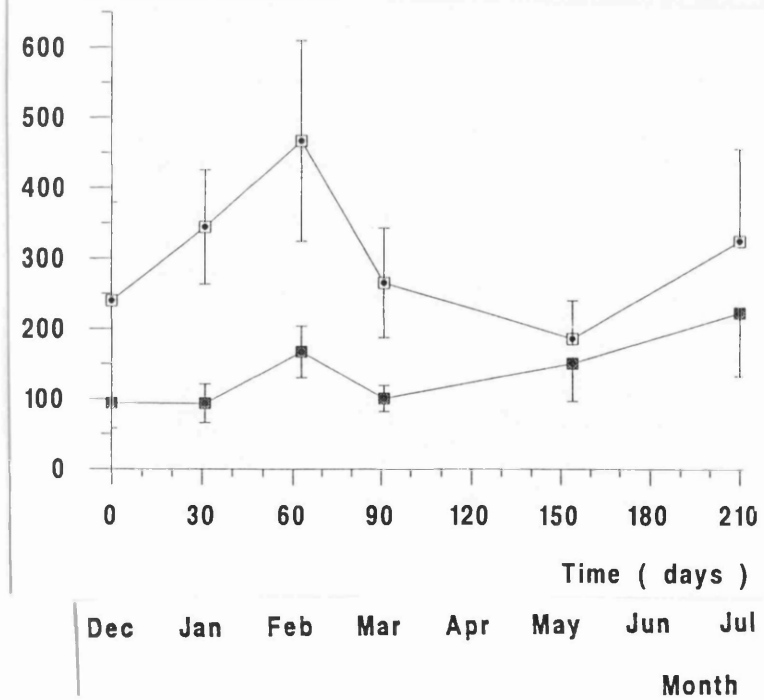
The pepsin activity in the stomach digesta per dry weight fish in milliunits per gram

Figure 36: Pepsin activity in the stomach mucosa (**a**), and in the stomach digesta (**b**) for the LMG fish (▣) and the UMG fish (▩), as a function of time (Days). The pepsin activity was expressed in milliunits per gram bodyweight (mU/g). Further details as in Figure 35.

(a)


1500
1000
500
0
0
30
60
90
120
150
180
210
Time (days)
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

(b)


600
500
400
300
200
100
0
0
30
60
90
120
150
180
210
Time (days)
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

(mU/g), from the middle of December, 1991, to the middle of July can be seen in Figure 36 (b).

The pepsin activity per dry weight fish varied significantly for the LMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.11) and the UMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.12). A two way analysis of variance found an interaction between days and the group of fish ($F_{Interaction} = 12.84 > F_{0.001(2),5,228.} = 4.64$. X.Appendices. Table VII.13), i.e. the difference in the digestal pepsin activity between the LMG and the UMG fish, depends on the time of year. A clear overall difference was found in the digestal pepsin activity, between the two groups ($F_{Mode} = 228.15 >> F_{0.001(2),1,228.} = 12.5$. X.Appendices. Table VII.13). This difference is however not significant in the summer months (May and July).

The trypsin activity in the pancreatic tissue and pyloric caeca per dry weight fish in milliunits per gram (mU/g), from the middle of December, 1991, to the middle of July can be seen in Figure 37 (a).

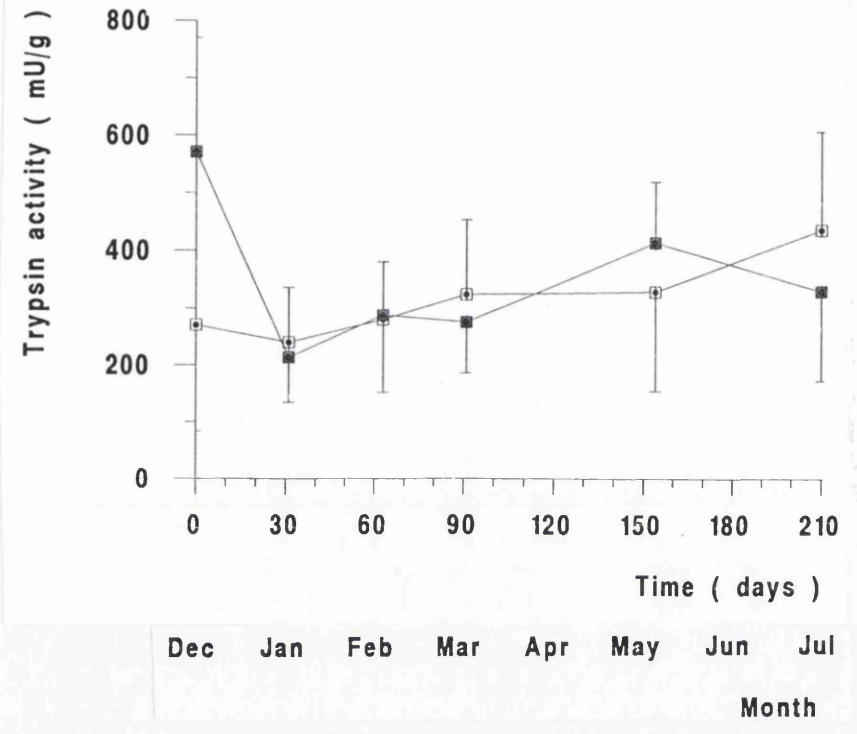
The trypsin activity per dry weight fish varied significantly for the LMG fish (One way ANOVA: $p = 0.002$. X.Appendices. Table VII.14) and the UMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.15). Apart from December, the trypsin activity is almost identical for the two groups. A two way analysis of variance found an interaction between days and the group of fish ($F_{Interaction} = 10.73 > F_{0.001(2),5,228.} = 4.64$. X.Appendices. Table VII.16). No overall difference was found in the pancreatic tissue and pyloric caecal trypsin activity, between the two groups ($F_{Mode} = 3.94 < F_{0.05(2),1,228.} = 5.10$. X.Appendices. Table VII.16).

The trypsin activity in the intestinal digesta per dry weight fish in milliunits per gram (mU/g), from the middle of December, 1991, to the middle of July is shown in Figure 37 (b).

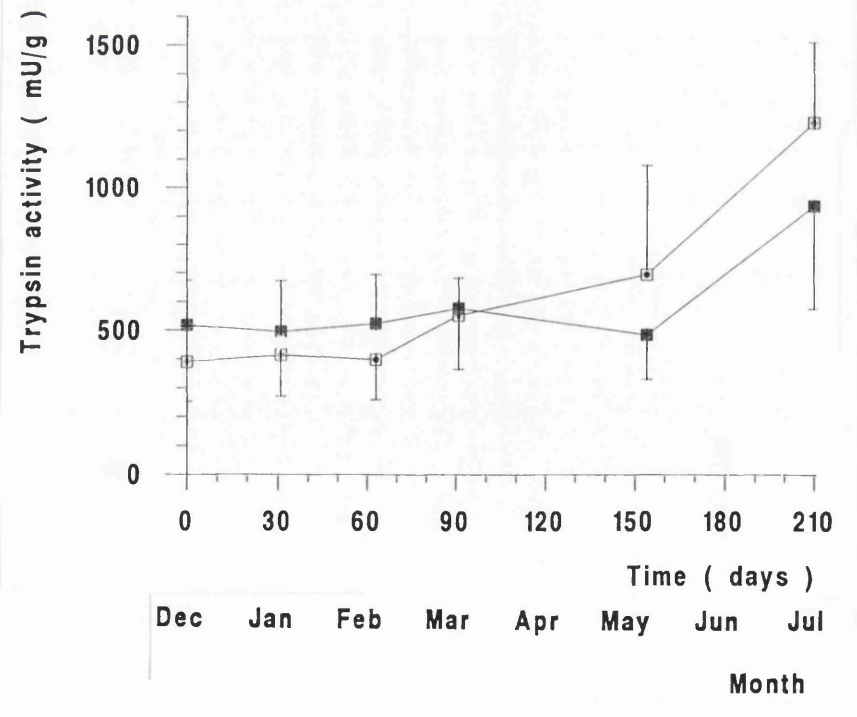
The trypsin activity per dry weight fish varied significantly for the LMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.17) and the UMG fish (One way ANOVA: $p < 0.0005$. X.Appendices. Table VII.18), but a sharp rise for both groups was observed between May and July. It was higher in the winter months (December to March) and lower in the summer months (May and July) for the UMG fish than for the LMG fish, but a two way analysis of variance found an interaction between days and the group of fish

Figure 37: Trypsin activity in the pancreatic tissue and the pyloric caeca (**a**), and in the intestinal digesta (**b**) for the LMG fish (▣) and the UMG fish (▩), as a function of time (Days). The trypsin activity was expressed in milliunits per gram bodyweight (mU/g). Further details as in Figure 35.

(a)


Trypsin activity (mU/g)
800
600
400
200
0
0
30
60
90
120
150
180
210
Time (days)
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

(b)


Trypsin activity (mU/g)
1500
1000
500
0
0
30
60
90
120
150
180
210
Time (days)
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

($F_{Interaction}$ = 6.93 > $F_{0.001(2),5,228.}$ = 4.64. X.Appendices. Table VII.19), i.e. the difference in the intestinal digestal trypsin activity, between the LMG and the UMG fish, depends on the time of year. No overall difference was however found in the intestinal digestal trypsin activity, between the two groups (F_{Mode} = 0.77 < $F_{0.05(2),1,228.}$ = 5.10. X.Appendices. Table VII.19).

The chymotrypsin activity in the pancreatic tissue and pyloric caeca per dry weight fish in milliunits per gram (mU/g), from the middle of December, 1991, to the middle of July is shown in Figure 38 (a).

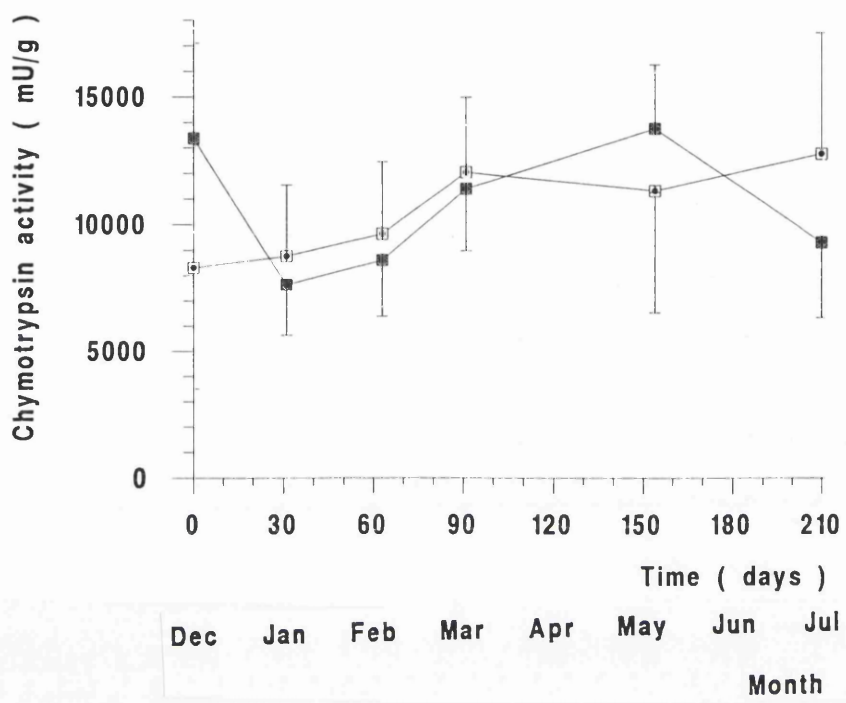
The chymotrypsin activity per dry weight fish varied significantly for the LMG fish (One way ANOVA: p = 0.001. X.Appendices. Table VII.20) and the UMG fish (One way ANOVA: p < 0.0005. X.Appendices. Table VII.21). Apart from December, then the chymotrypsin activity is almost identical for the LMG and the UMG fish. A two way analysis of variance found an interaction between days and the group of fish ($F_{Interaction}$ = 8.12 > $F_{0.001(2),5,228.}$ = 4.64. X.Appendices. Table VII.22). No overall difference was found in the pancreatic tissue and pyloric caecal chymotrypsin activity, between the two groups (F_{Mode} = 0.22 < $F_{0.005(2),1,228.}$ = 5.10. X.Appendices. Table VII.22).

The chymotrypsin activity in the intestinal digesta per dry weight fish in milliunits per gram (mU/g), from the middle of December, 1991, to the middle of July can be seen in Figure 38 (b).

The chymotrypsin activity per dry weight fish varied significantly for the LMG fish (One way ANOVA: p < 0.0005. X.Appendices. Table VII.23) and the UMG fish (One way ANOVA: p < 0.0005. X.Appendices. Table VII.24). It was higher in the winter months (December to March) and lower in the summer months (May and July) for the UMG fish, but a two way analysis of variance found an interaction between days and the group of fish ($F_{Interaction}$ = 23.10 > $F_{0.001(2),5,228.}$ = 4.64. X.Appendices. Table VII.25), i.e. the difference in the intestinal digestal chymotrypsin activity, between the LMG and the UMG fish, depends on the time of year. No overall difference was however found in the intestinal digestal chymotrypsin activity, between the two groups (F_{Mode} = 1.54 < $F_{0.05(2),1,228.}$ = 5.10. X.Appendices. Table VII.25), but this difference is probably significant in July.

Figure 38: Chymotrypsin activity in the pancreatic tissue and the pyloric caeca (**a**), and in the intestinal digesta (**b**) for the LMG fish (⊡) and the UMG fish (▩), as a function of time (Days). The trypsin activity was expressed in milliunits per gram bodyweight (mU/g). Further details as in Figure 35.

(a)


Chymotrypsin activity (mU/g)
15000
10000
5000
0
0
30
60
90
120
150
180
210
Time (days)
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

(b)

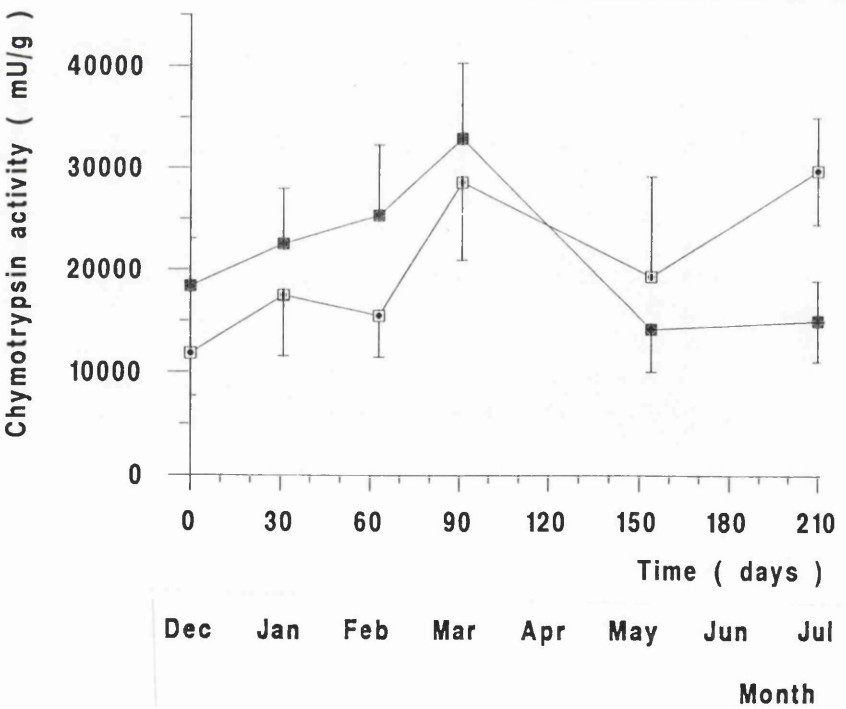
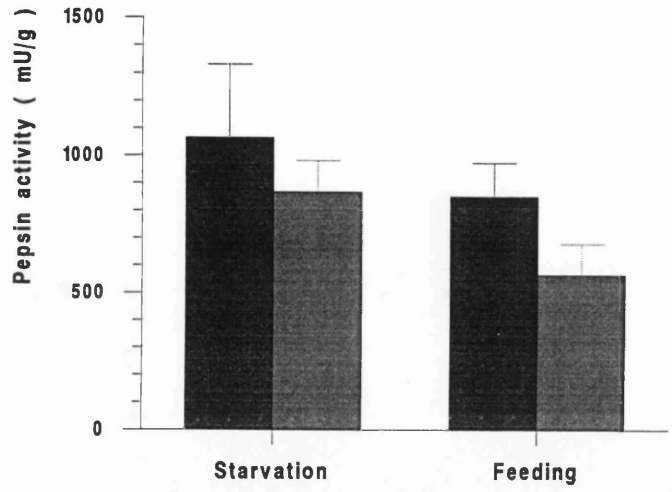
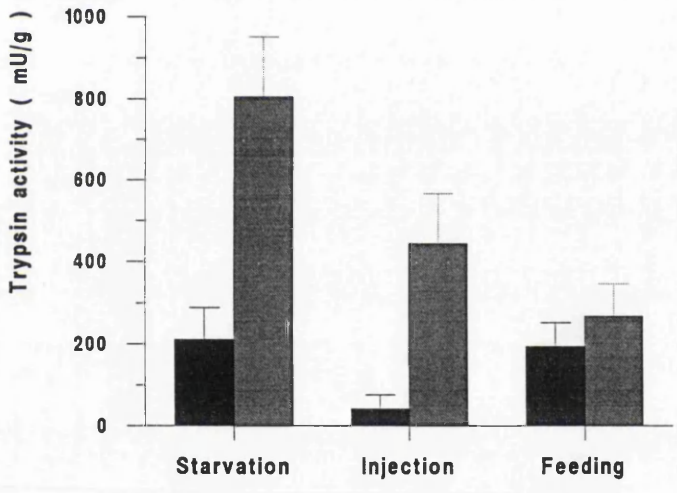

Chymotrypsin activity (mU/g)
40000
30000
20000
10000
0
0
30
60
90
120
150
180
210
Time (days)
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Month

Figure 39: Pepsin (a), trypsin (b) and chymotrypsin (c) activities for the LMG fish (■) and the UMG fish (▨), for starved fish (Starvation: n=10), starved and CCK injected fish (Injection: n=14) and feeding fish (Feeding: n=15). The pepsin activity was the total activity per fish, while trypsin and chymotrypsin activities were the total activities in the pancreatic tissue and the pyloric caeca per fish. All of these activities were expressed in milliunits per gram bodyweight (mU/g). Starved fish had been starved for 5 days and CCK injected fish had been starved for 5 days and injected intraperitoneally with porcine cholecystokinin (CCK). The CCK used was a natural, purified, porcine cholecystokinin, sulphated. Columns are average values and error bars are standard deviations.

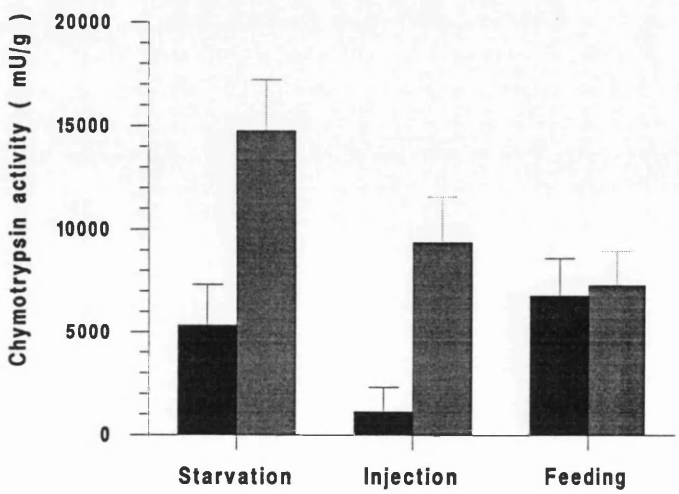
(a)


Pepsin activity (mU/g)
1500
1000
500
0
Starvation
Feeding

(b)


Trypsin activity (mU/g)
1000
800
600
400
200
0
Starvation
Injection
Feeding

(c)


Chymotrypsin activity (mU/g)
20000
15000
10000
5000
0
Starvation
Injection
Feeding

3.2. The effect of starvation, cholecystokinin and feeding.

How the total pepsin activity of the stomach in the lower modal fish and the upper modal fish was affected by starvation and feeding can be seen in Figure 39 (a).
In comparison to starved fish, then feeding caused a slight reduction of this activity. This was observed for both groups.
The only significant difference in pepsin activity, between treatments, were observed for the UMG fish: starved fish versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.26).

How trypsin activity in the pancreatic tissue and the pyloric caeca of the LMG fish and the UMG fish, was affected by starvation, starvation and CCK injection, and feeding can be seen in Figure 39 (b).
In comparison to starved fish, then intraperitoneal injection with CCK caused a considerable reduction in the trypsin activity. This was most pronounced for the LMG fish. In comparison to starved fish, then feeding caused only a slight reduction in the trypsin activity for the LMG fish, but a considerable reduction in the trypsin activity for the UMG fish. Significant difference between treatments in the pancreatic and pyloric caecal trypsin activities, were found to be as follows:

i) The LMG fish: Starved versus starved and CCK injected fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.27) and starved and CCK injected fish versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.27).

ii) The UMG fish: Starved versus starved and CCK injected fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.27)., starved and CCK injected fish versus feeding fish ($p = 0.0001 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.27) and starved versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.27).

How chymotrypsin activity in the pancreatic tissue and the pyloric caeca of the LMG fish and the UMG fish was affected by starvation, starvation and CCK injection, and feeding is shown in Figure 39 (c). A fairly similar pattern was found here, as for trypsin activity

(Fig. 39 (b)).

Significant differences between treatments in the pancreatic and pyloric caecal chymotrypsin activities were found to be as follows:

i) The LMG fish: Starved versus starved and CCK injected fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.28), and starved and CCK injected fish versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.28).

ii) The UMG fish: Starved versus starved and CCK injected fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.28) and starved versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.28).

Figure 40 shows how the intestinal digestal trypsin (Fig. 40 (a)) and chymotrypsin (Fig. 40 (b)) activities of the LMG fish and the UMG fish, were affected by starvation, starvation and CCK injection, and feeding. A fairly similar pattern is observed for the trypsin and chymotrypsin activities: In comparison to starved fish, then starvation and intraperitoneal injection with CCK, approximately doubled these, but feeding increased these greatly. This was valid for both groups of fish.

Significant differences between treatments in the intestinal digestal trypsin activities (Fig. 40 (a)) were found to be as follows:

i) The LMG fish: Starved and CCK injected fish versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.29) and starved versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.29).

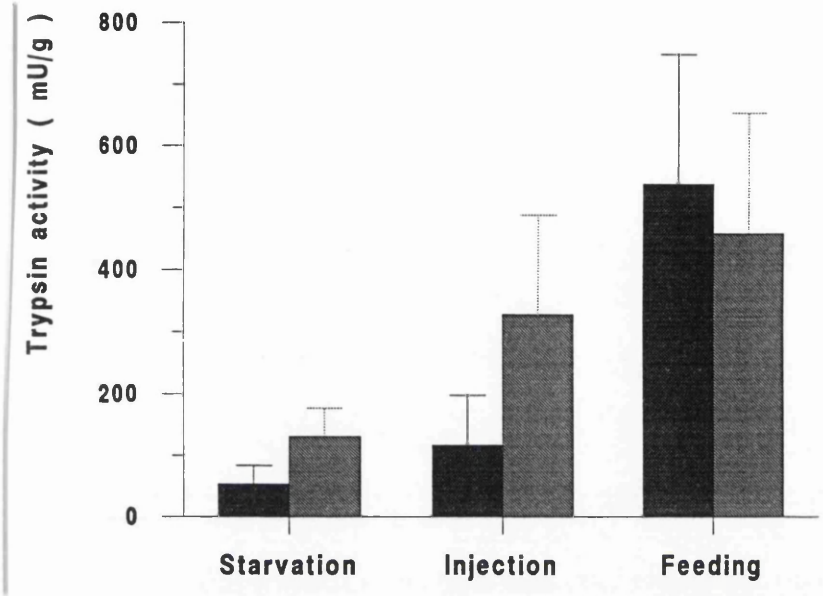
ii) The UMG fish: Starved versus starved and CCK injected fish ($p = 0.0006 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.29) and starved versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.29).

Significant differences between treatments in the intestinal digestal chymotrypsin activities (Fig. 40 (b)) were found to be as follows:

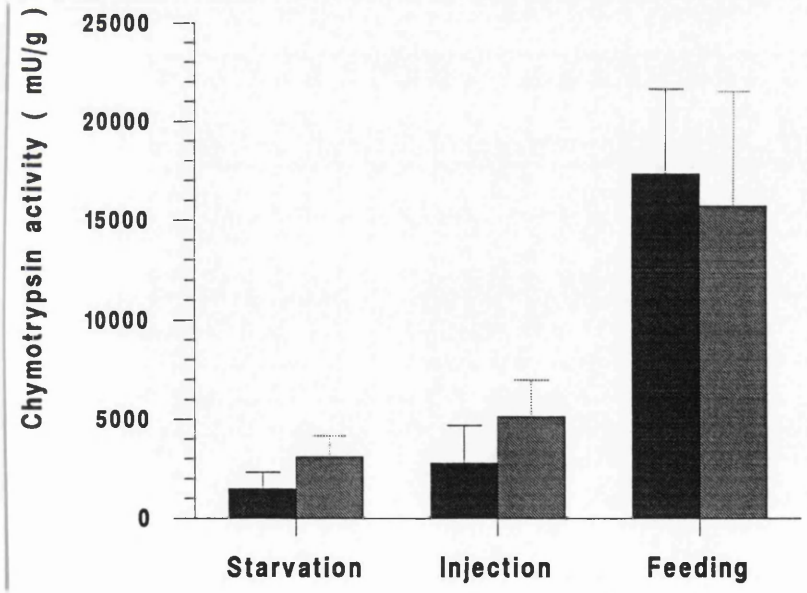
i) The LMG fish: Starved and CCK injected fish versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test. X.Appendices. Table VII.30) and starved versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test.

Figure 40: Trypsin (a) and chymotrypsin (b) activities for the LMG fish (■) and the UMG fish (▨), in relation to starved fish (Starvation), starved and CCK injected fish (Injection) and feeding fish (Feeding). The CCK used was a natural, purified, porcine cholecystokinin, sulphated. Further details as in Figure 39.

(a)


Trypsin activity (mU/g)
800
600
400
200
0
Starvation
Injection
Feeding

(b)


Chymotrypsin activity (mU/g)
25000
20000
15000
10000
5000
0
Starvation
Injection
Feeding

X.Appendices. Table VII.30).

ii) The UMG fish: Starved and CCK injected fish versus feeding fish ($p = 0.0028 < p = 0.0036$. Bonferroni: 14 tests; two tiled test.), starved and CCK injected versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test.) and starved versus feeding fish ($p < 0.00005 < p = 0.0036$. Bonferroni: 14 tests; two tailed test.).

4. Discussion.

4.1. Lower and upper modal salmon in their first year.

Growth in terms of body weight (Fig. 35 (a)) began only after the middle of March for the UMG fish and the LMG fish. Growth was small for the LMG fish until after the middle of May. The fact that growth commences later in the LMG fish in the spring, is probably caused by a factor of genetic origin. Thorpe and Morgan (1978) have shown that the proportion of potential 1-year smolts is primarily influenced by genetic factors. Simpson and Thorpe (1976) pointed out that differences in growth must have resulted from differences in biochemical rates under endocrine control. These authors found that in January (1976), the LMG fish had higher cortisol and lower thyroxine blood levels, than the UMG fish. Lower blood thyroxine levels indicate slower metabolic rates in the LMG fish.

Growth in terms of body weight was dormant during the winter months, in spite of a rise in temperature (Fig. 34) from January and an increase in daylight hours from December. The UMG fish have been found to grow in length during the winter months, albeit at a reduced rate, while the LMG fish ceased to grow during this period (Thorpe, 1977; Bailey *et al.*, 1980; Higgins, 1985). The finding of the present study, that UMG fish did not increase in weight during the winter months, reflects a reallocation of resources within the fish, enhancing skeletal relative to muscular growth. This agrees with previous studies, which have shown that the rate of increase of weight with length was significantly less for the UMG fish than the LMG fish (Wankowski and Thorpe, 1979a). Apparently, when certain temperature levels and/or daylight x hours are obtained, growth commences. Hypothetically, increase in daylength stimulates somatotroph activity (Gross *et al.*, 1965; Saunders and Henderson, 1970; Komourdjian *et al.*, 1976). This occurs presumably via

heightened blood growth hormone levels.

The increase in growth in weight during the spring, for the UMG fish, was distinctively greater than for the LMG fish. This may reflect higher blood levels of thyroxine or growth hormone, or lower response thresholds to these hormones, at the target sites in the UMG fish (Simpson and Thorpe, 1976). Obviously, the growth of the UMG commenced earlier in the spring and was by far greater, than that of the LMG (Fig. 35 (a)). These fish are smoltifying, as a result of a physiological decision to become S1 smolts, taken in July (Metcalfe *et al.*, 1989). The probability of an individual fish becoming an S1 smolt, i.e. entering the UMG, depends on the dominance rank and the length of the fish at this time (*Ibid.*). Fish becoming S1 smolts are not affected by a competitor, when feeding, in contrast to fish becoming S2 smolts (Metcalfe, 1990).

The LMG fish had, in terms of body weight, a lower stomach digestal content during the winter months (except for February, which did not differ significantly from the UMG fish), and a higher stomach content in the summer months, than the UMG fish (Fig. 35 (b)). These findings are in agreement with previous studies on feeding in LMG and UMG fish, which took place from October 1983 to March 1984, where the UMG fish were found to take more food in terms of body weight and to feed for longer time within the 24 hours than the LMG fish (Higgins, 1985; Higgins and Talbot, 1985). Studies on brown trout (*Salmo trutta* L.) fed maximum ration showed that over a wide size and temperature range, the smaller the fish the higher its food intake in terms of body weight (Elliott, 1975a; Elliott, 1975b). Studies on feeding in juvenile sockeye salmon (*Oncorhynchus nerka*) also showed that with increasing fish size the daily food intake in terms of body weight fell (Doble and Eggers, 1978). The previous study is in an agreement with these findings during the summer months (May to July), but contradicts these for the winter months (See above).

From March to May the LMG fish increased their food intake, from being less than that of the UMG fish in terms of body weight, to that of being greater than that of the UMG fish in May. With increasing temperature (Fig. 34) and increasing daylength, the rise in food intake in terms of body weight was greater for the LMG fish than the UMG fish (Fig. 35 (b)). This agrees with the previous study undertaken between October 1983 and

March 1984, where LMG fish were found to increase its food intake faster than the UMG, as the water temperature rose and days became longer (Higgins and Talbot, 1985).

Stomach fullness is a ballance between feeding rate, i.e. appetite, and digestion rate (Elliott and Persson, 1978). Stomach evacuation increases appetite (Miner, 1955). A rise in water temperature to 18.4 °C was found to increase appetite in terms of food intake in the brown trout (Elliott, 1975b), but an increase in temperature caused an increase in the evacuation rate of food from the stomach (Brett and Higgs, 1970; Elliott, 1972). Studies on LMG and UMG Atlantic salmon revealed that the evacuation rate from the stomach was found to be unrelated to body size (LMG and UMG fish) irrespective of feeding history (Talbot *et al.*, 1984). This study was undertaken at a water temperature of 9 to 13 °C, but the time of year was not specified (April-May ?). As the proportional feeding activity was lower in the winter months for the LMG, the evacuation rate from the stomach is likely to be lower for the LMG than the UMG at this time. The accumulation of pepsin activity in the stomach digesta / mucus of the LMG fish during the winter months supports this (See later).

Studies on feeding behaviour in juvenile Atlantic salmon have shown that bimodality in the length frequency distribution, arising in late autumn of their first year, was a result of a reduction in the appetite of the LMG fish in relation to the UMG fish after August (Metcalfe *et al.*, 1988). Previous studies had indicated that this was an internal suppression of appetite in the LMG fish, which commenced in midsummer, and caused an arrest of growth in the LMG fish during the winter (Metcalfe *et al.*, 1986). Further studies were in agreement with this, where appetite was found to decline from July to September in both modal groups, but more so in the LMG fish (Thorpe *et al.*, 1991). It is highly likely that the results in the present study (Fig. 35 (b)), show the removal of the suppression of appetite in the LMG fish somewhere between winter and spring, but this suppression was found to commence from July (*Ibid.*).

Simpson and Thorpe (1976) pointed out that differences in the rates of growth and maturation resulted from differences in rates of biochemical processes, which in turn are under endocrine control. They suggested that bimodality in juvenile Atlantic salmon was caused by high (LMG fish) and low (UMG fish) threshold of response to plasma

thyroxine. In support of this were findings on plaice (Osborn and Simpson, 1973) and on rainbow trout (Osborn *et al.*, 1978), which indicated that in response to falling water temperatures, metabolic rates start to drop, but are partially compensated by a photoperiod induced increase in the secretion of thyrotropin (TSH), causing an increase in plasma thyroxine levels. Irrespective of the exact mechanisms for the appetite control in the Atlantic salmon, the LMG fishes appetite is apparently more sensitive to changes in water temperature and light period: appetite is reduced faster during fall in water temperature and a reduction in light period (Higgins and Talbot, 1985; Thorpe *et al.*, 1991) and increases faster during rise in water temperature and longer days (Fig. 35 (b)). Hypothetically, the thresholds of response to plasma thyroxine are not only higher and lower in the LMG and UMG fish, respectively. They are also more and less sensitive to environmental change, i.e. water temperature and photoperiod, for the LMG and the UMG fish, respectively.

It is likely that not only thyroxine, but also growth hormone, are among the underlying factors playing a role in the differences in appetite during the winter months between the UMG and the LMG fish. Previous studies found that, during the winter months, UMG fish had greater food intake in relation to body weight, greater growth rate and greater metabolic rate than the LMG fish (Higgins and Talbot, 1985). Higgins and Talbot (1985) suggested that seasonal changes in photoperiod may stimulate growth hormones through the neuroendocrine system, increasing growth rate and appetite. Longer days have been found to coincide with an increase in number and apparent activity of pituitary somatotrophs in the Atlantic salmon (Komourdjian *et al.*, 1976).

The intestinal digesta was slightly and significantly greater in the LMG than in the UMG fish (Fig. 35 (c)). Adding the stomach (Fig. 35 (b)) and the intestinal (Fig. 35 (c)) digesta gives values in total digesta (not presented as such), which are in good agreement with previous studies (Talbot and Higgins, 1982), where LMG and UMG fish had a total digesta of ca. 3 and 2 % in relation to body mass, respectively, for fish sampled at 10 a.m. in June, but sampling was always done around that time in the present study. LMG fish had always higher total digesta, than the UMG fish, irrespective of a time of day (*Ibid.*) and is that in agreement with the previous study (Fig. 35 (b) and Fig. 35 (c)).

Pepsin activity, expressed in terms of body weight, in the stomach mucosa (Fig. 36 (a)) and in the stomach digesta (Fig. 36 (b)) was significantly higher in the LMG than in the UMG fish during winter, but the difference was smaller and probably barely significant between the two groups in summer (May to July). It is somewhat unexpected to find that the pepsin activity in the stomach mucosa and especially in the stomach digesta / mucus, is higher for the LMG than the UMG fish in the winter months (December to March), as the UMG fish were found to contain more stomach digesta in terms of body weight during this period (Fig. 35 (b)). One would expect the amount of pepsin activity in the stomach digesta to increase in the same proportion as the digesta itself, as digesta in the stomach was found to stimulate secretion of pepsin (Chapter VI).

The pepsin activity in the stomach mucosa of the LMG fish is seen to fall in the period of February to May (Fig. 36 (a)), but this fall coincides with rising water temperature (Fig. 35). Pepsin activity was found to be 30 % greater in cold acclimated brook trout (*Salvelinus fontinalis*), compared to that of warm acclimated fish (Owen and Wiggs, 1971). Pepsin activity was also found to decrease with an increase in water temperature for Atlantic salmon from 4.5-14.2 °C (Torrissen and Torrissen, 1984). This was not observed for the UMG fish, however. This activity is lower in the summer months (May to July) when the LMG fish have resumed active feeding. The great difference in this activity between the LMG and the UMG fish in the winter months may possibly be explained by a difference in feeding activity. The LMG fish feed proportionally less, than the UMG fish in this period, i.e. the oxynticopeptic cells are under less demand to secrete pepsin so this tends to accumulate in the cells. In support of this is the stable amount of pepsin activity in the stomach mucosa of the UMG fish, which retained its feeding activity during the winter months. It has been demonstrated in the bluegill (*Lepomis macrochirus*) that distension of the stomach wall (Norris *et al.*, 1973), and digesta in the stomach (Chapter VI) stimulated pepsin secretion.

The pepsin activity in the stomach digesta was greater for the LMG, than for the UMG fish during the winter months (December to March), but similar for both groups during summer (May to July). This may be explained by a greater evacuation rate in the actively feeding fish, i.e. the UMG fish, during the winter months, but the rate of digestion was found to be proportional to the mass of food remaining in the stomach of the fingerling

sockeye salmon (Brett and Higgs, 1970). The high pepsin activity found in the stomach mucosa of starving fish, probably resulting from the evacuation from the stomach being "switched off", is in agreement with this (Chapter VI). The pepsin activity of the UMG in the stomach mucosa and the stomach digesta, remained roughly constant during winter and the summer months (Fig. 36 (a) and Fig. 36 (b)). This is in agreement with previous studies on rainbow trout (*Oncorhynchus mykiss*), which demonstrated that changes in pepsin activity paralleled the growth rate (Kitamikado and Tachino, 1960; Kawai and Ikeda, 1973).

The rate of gastric evacuation (Brett and Higgs, 1970; Elliott, 1972) and the rate of pepsin secretion (Smit, 1967) has for fish been found to increase with an increase in water temperature. These factors will increase during the summer months for both groups of fish, but probably proportionally more so for the LMG fish, as the present study indicates.

Stored trypsin (Fig. 37 (a)) and chymotrypsin (Fig. 38 (a)) activity, i.e. activities in the pancreatic tissue and the pyloric caeca, as related to body weight, was not significantly different between the LMG and the UMG fish and was found to rise slightly from January to July. The small change in trypsin activity for both groups from January to July is in agreement with previous studies. No difference was found in trypsin activity in rainbow trout acclimated to 10 and 15 °C (McLeese and Stevens, 1982) and total protease activities in the pyloric caeca and intestine of Atlantic salmon were roughly the same in the temperature range of 4.5-14.2 °C (Torrissen and Torrissen, 1984). This is at least partially explained by a thermal compensation of trypsins in fish, but the trypsin K_M (a measure of the enzyme affinity for its substrate) has been found to be temperature independent over a wide range in various fish species, including rainbow trout and brown trout (Hofer *et al.*, 1975). Trypsin and chymotrypsin activities are both expressed as activities per body weight and the small change in these activities from December to July (Fig. 37 (a) and Fig. 38 (a)), reflects the fact that these activities are roughly in direct proportion to the body weight. Previous studies have also found this, but protease activities in tissues of the digestive tract of the rainbow trout, especially in the pyloric caeca, corresponded to the growth rate of the fish (Rungruangsak and Utne, 1981).

The fact that no significant difference was found in the pancreatic tissue and the pyloric caecal trypsin and chymotrypsin activities between the two groups (Fig. 37 (a) and Fig. 38 (a)) may be explained by the similarity in the specific growth rate between the two groups during this period (Higgins, 1985; Higgins and Talbot, 1985). These studies found a significant difference, however small, in the specific growth rate between the two modal groups, but trypsin and chymotrypsin activities in the salmon gut are prone to greater fluctuations than the body mass of this animal.

Both the trypsin and the chymotrypsin activities were conspicuously higher for the UMG than the LMG fish in December, but this was however not significant. A possible explanation for this is that the UMG fish retain their appetite better in the autumn than the LMG fish (See above).

Secreted trypsin (Fig. 37 (b)) and chymotrypsin (Fig. 38 (b)) activities, i.e. activities in the intestinal digesta as related to body weight, were lower in the winter months (December to March) and higher in the summer months (May to July) for the LMG fish, and vice versa for the UMG fish. This pattern is similar to the pattern for the stomach digesta (Fig. 35 (b)). The slightly higher trypsin and chymotrypsin activities for the UMG fish in the winter months is probably caused by a higher feeding activity of this modal group. The LMG was however found to have somewhat higher intestinal digesta (Fig. 35 (c)), but as intestinal digesta caused secretion of trypsin and chymotrypsin (Chapter VI), it might be expected that the LMG fish would have proportionally more of these activities in their intestinal digesta during the winter, than the UMG fish. This discrepancy may be explained by a higher evacuation rate from the intestine of the UMG fish during this period. This is likely to be the case, as these fish, i.e. the UMG, were found to eat proportionally more and for a longer time within 24 hours in winter (Higgins, 1985; Higgins and Talbot, 1985). More feeding activity will probably increase peristalsis of the intestine via CCK, but previous studies had indicated this (Chapter VI, section 4.3.). CCK has been found to indirectly stimulate contraction of the smooth muscle of the Guinea pig ileum, as atropine was found to block this effect (Hedner *et al.*, 1967; Hedner and Rorsman, 1968). CCK was also found to stimulate smooth muscle contraction in the jejunum of conscious dogs, where it increased the intraluminal pressure of a jejunal loop (Ranirez and Farrar, 1970).

The trypsin activity was roughly constant for both groups until July, when a sharp rise was observed. This may be explained by the increase in growth rate during summer (Higgins, 1985; Higgins and Talbot, 1985). It is also likely that the increased temperature causes an increase in the rate of secretion of the digestive enzymes such as trypsin; an increase in pepsin secretion in the brown bullhead (*Ictalurus nebulosus*) with rising temperature from 10 to 25 °C has been demonstrated (Smit, 1967).

4.2. The effect of starvation, CCK and feeding.

Starvation of UMG fish, resulted in a significant rise of total pepsin activity (Fig. 39 (a)) and pancreatic and pyloric caecal trypsin (Fig. 39 (b)) and chymotrypsin (Fig. 39 (c)) activities in comparison to feeding fish. This may reflect the greater capacity of the storage sites in the UMG fish, for pepsin, trypsin and chymotrypsin, already by the end of February, as starvation presumably cuts off secretion before synthesis of digestive enzymes (Chapter VI). The UMG fish are apparently prepared for the coming of the great increase in the stored pepsin, trypsin and chymotrypsin activities. A more probable explanation is that the oxynticopeptic cells of the UMG fish are actively secreting pepsinogen and the acinar cells of this group of fish are actively secreting trypsinogen and chymotrypsinogen. Starvation is bound to increase these enzyme activities in their storage sites, as the UMG fish are actively feeding. This is in agreement with previous results (Chapter VI, section 4.1.). Starvation caused no significant change in the stored pepsin, trypsin and chymotrypsin activities, for the LMG fish. This indicates that the feeding activity of these fish during winter is almost none, i.e. the fish are almost starving.

The response of the UMG fish to CCK was different to the one for the LMG fish: CCK treated UMG fish had greater trypsin (Fig. 39 (b)) and chymotrypsin (Fig. 39 (c)) activities, than feeding UMG fish, respectively. The LMG fish showed the greatest responsiveness to CCK. The possible explanation may be the smaller size of the LMG fish, or more likely that the pancreas is under less stimulation, i.e. demand, than the pancreas of the UMG fish during the winter months. It is obvious that the pancreas of the LMG fish is quite capable of trypsin and chymotrypsin secretion during winter. This and previous findings (See Section 3.1.2. and 4.1.) demonstrate that a lack of the digestive enzymes pepsin, trypsin and chymotrypsin, was in no way responsible for the lower

growth rate in the LMG fish, compared to the UMG fish, previously demonstrated (Higgins, 1985; Higgins and Talbot, 1985).

The response of the LMG and UMG fish, with respect to stored enzymes, to starvation (pepsin, trypsin and chymotrypsin) and to a challenge with CCK (trypsin and chymotrypsin) gave strong support for the suggestion that the oxynticopeptic and the acinar cell were more actively secreting in the UMG fish than in the LMG fish.

The starvation was found to decrease trypsin (Fig. 40 (a)) and chymotrypsin (Fig. 40 (b)) digestal activities for both groups. This demonstrated that food in the intestine causes secretion of trypsin and chymotrypsin for both groups.

The digestal trypsin (Fig. 40 (a)) and chymotrypsin (Fig. 40 (b)) activities for the feeding fish are fairly similar between the two groups of fish. The activities are related to the bodyweight (Rungruangsak and Utne, 1981) and represent the demand for nutrients per unit body weight, which in turn is an indication of the metabolic rate per body weight. This metabolic rate must represent the cost of maintainance in terms of nutrients, as growth in terms of body weight has not begun. This cost of maintenance then, will be directly proportional to the body weight, i.e. enzyme activities per body weight will not vary between the two groups.

Chapter VIII.

Conclusion and prospects.

Pepsin, trypsin and chymotrypsin activities in the Atlantic salmon, *Salmo salar* L., were characterised and studied with respect to their respective assays and the respective sample processing. This was a necessary prerequisite for further studies.

Pepsin activity can reliably be measured with azocasein as a substrate, optimally at a pH = 4, and with hemoglobin at a pH = 2. Pepsin activity was inhibited with pepstatin A.

The kinetics of these enzymes follows the Briggs and Haldane modification of the Michaelis-Menten equation, meaning that at substrate saturation, a linear relationship exists between enzyme concentration and activity. This assumption is approximately valid for the pepsin assay with an azocasein concentration of 1 % (w/v), the trypsin assay with a DL-BA*p*NA concentration of 1.09 mM and the chymotrypsin assay with a SAAPP*p*NA concentration of 0.50 mM.

Both trypsin and chymotrypsin were found to have K_M values characteristic of exothermic animals having the same temperature preferendum. Trypsin was effectively inhibited by SBTI. It was also completely inactivated by TLCK and hence identified by this highly specific inactivator. DL-BA*p*NA is totally specific for trypsin. Trypsin should be assayed at pH = 7.8 and not higher pH values. Chymotrypsin was effectively inhibited by the BB inhibitor, but was not completely inactivated by TPCK. SAAPP*p*NA is apparently not totally specific for chymotrypsin, but interference by trypsin can be abolished by TLCK. Other interference is probably minimal. Chymotrypsin should be assayed at pH = 9.0. Purification of the various isozymes of pepsin may reveal new isozymes being synthesised during some biological and physiological changes in the animal, such as smoltification.

Histological and ultrastructural studies revealed that the digestive tract of the Atlantic

salmon together with its pancreatic tissue was fairly similar to that of the rainbow trout. The location and the length of the microvillous brush border indicated greatest absorption of nutrients in the pyloric caecal region. Some spatial distribution occurs along the duodenum and the jejunum with respect to the absorption of the various nutritive components, but absorption in mammals occurs mostly here (Bell *et al.*, 1980). A strong support for the hypothesis that digestive enzymes are conveyed from the pancreas into the intestinal lumen via many small ducts is presented. This will aid in the mixing of the enzyme cocktail with the incoming digesta. This is probably also assisted by peristalsis, presumably initiated by some CCK-like hormone, released as digesta from the stomach entered the pyloric caecal region. The fact that trypsin and chymotrypsin activities were found to rise in the pyloric caecal region, the mid intestinal region and the rectum, all at the same time as digesta from the stomach entered the pyloric caecal region (Chapter VI) supports the hypothesis that a CCK-like hormone initiates peristalsis of the intestine. Endocrine cells, likely to be releasing a CCK-like hormone into the blood stream, were identified in the pyloric caecal region (Chapter II).

Oxynticopeptic cells were identified in the stomach, containing a tubulovesicular network and secretory granules, supporting the hypothesis that in fish these cells secrete both H^+ and pepsinogen. Indirect immunofluorescence and an assay of pepsin by hemoglobin were in agreement with the light microscopic studies: Pepsinogen and pepsin activity was localized in the stomach glands, which were found in the cardiac and in the transitional area of the stomach, but these gradually disappeared in the transitional area in the caudal direction. A somewhat greater immunoreactivity towards pepsinogen and a somewhat greater pepsin activity were found in the posterior cardiac than the anterior cardiac area agreeing with earlier light microscopic studies. The immunoreactivity of Y 37, raised against embryonic chicken pepsinogen and identified in the stomach of the Atlantic salmon, supports the hypothesis that pepsinogens have been conserved well during vertebrate evolution (Yasugi, 1987).

The presence of active and non-active pancreatic acinar cells was identified. CCK which already had been found to stimulate secretion of trypsin and chymotrypsin (Chapter IV), greatly reduced the activities of these enzymes in the pancreatic and the fatty tissue, surrounding the pyloric caeca. Zymogen granules of the acinar cells were reduced in

approximately the same ratio and at the same time as the trypsin and chymotrypsin activities, giving strong support for the hypothesis that these enzymes are stored in, and secreted from, these granules. These granules had been identified earlier in an ultrastructural investigation (Chapter II). The greater reduction in trypsin and chymotrypsin activities (Chapter V) as compared to previous results (Chapter IV) is at least partly due to a difference in the dissection. Trypsin and chymotrypsin are not stored in the pyloric caeca themselves or the intestinal wall. An exact location of pepsinogen, trypsinogen and chymotrypsinogen, will further aid *in vitro* studies, regarding secretion of these enzymes.

Islet of Langerhans (The Brockmann Bodies) were also identified in the pancreatic tissue, and contained granules presumably with insulin and glucagon. Measuring these hormones as well as the various nutrients in the blood plasma during a passage of a single meal through the digestive tract is a tempting step in resolving the role of these hormones in regulating the flow of the various nutrients from the digestive tract to the various tissues of the animal.

Starvation was found to cause a slight rise in the stored pepsin, trypsin and chymotrypsin. It greatly reduced the trypsin and the chymotrypsin activities in the intestinal digesta, but not the pepsin activity in the stomach mucus. It is likely that pepsin accumulates in the stomach lumen because starvation switches off the evacuation from the stomach. Starvation of 16 to 20 days did not reduce the stored amount of these enzymes.

Ingested food immediately triggers the secretion and the synthesis of pepsinogen. If this is mediated by the same humoral factor, then the measurement of possible candidates in the blood plasma, during the consumption of a meal, might aid in its further identification. It was previously demonstrated that pepsinogen was stored in the stomach glands of the cardiac and the transitional area of the stomach. Pepsin digestion in the stomach will only aid maximally in the breakdown of ingested protein if the fish consumes its feed evenly.

Digesta from the stomach, appearing in the pyloric caecal region, was found to stimulate secretion of trypsin and chymotrypsin from the pancreas and to trigger the discharge of the gallbladder (Chapter VI, section 2.1). It is hypothesised that the digesta stimulates endocrine cells in the epithelium of the pyloric caecal region to release a CCK-like

hormone into the blood stream. Cells potentially fitting this role were previously identified (Chapter II). Trypsin and chymotrypsin appeared in all parts of the intestine at the same time (Chapter VI), suggesting a stimulation of peristalsis of the intestine. A CCK-like hormone is likely to be the mediator of the these effects on the pancreas, gallbladder and the intestinal musculature. A CCK from pig was found (Chapter IV) to cause secretion of trypsin and chymotrypsin under *in vivo* and *in vitro* conditions, and a discharge of the gallbladder during *in vivo* conditions. This further supports the hypothesis that a CCK-like hormone is the mediator of these changes, and also the hypothesis that CCK-like peptides were established early in vertebrate evolution. Stimulation with ACh or CCh did not cause secretion of trypsin and chymotrypsin and it remains to be seen, in fish how great a role cholinergic nerves play in the secretion of these enzymes from the pancreas. Results from the previous study also indicated that a CCK-like hormone plays a role in intestinal motility, i.e. peristalsis and evacuation (Chapter IV, section 4.). An indication of this was found when trypsin and chymotrypsin appeared in all parts of the intestinal lumen at the same time following stimulation by food. Some factor must have assisted in the distribution of trypsin and chymotrypsin throughout the intestine, as these only enter the intestine in its pyloric caecal region. This occurred as digesta from the stomach entered the pyloric caecal region of the intestine (Chapter VI).

Something appears to "switch off" the secretion of trypsin and chymotrypsin (Fig. 32 and Fig. 33), and this is in agreement with a recent hypothesis regarding a certain CCK-releasing peptide, secreted by the pancreas and degraded by proteolytic enzymes, such as trypsin and chymotrypsin (Dockray, 1989). Another hypothesis (Aldman and Holmgren, 1992) suggests that VIP finetunes the effects of the CCK.

The resynthesis of trypsinogen and chymotrypsinogen in the pancreas began only after the secretion of these enzymes had begun. Conversely the resynthesis of pepsinogen commenced concurrently with its secretion. The possible explanation is that resynthesis of trypsinogen and chymotrypsinogen was triggered by internal, i.e. inside the cell, processes, resulting from the secretion of these enzymes. This could be accomplished by an end-product inhibition. The resynthesis of pepsinogen on the other hand would be triggered by an external, i.e. external to the cell, factor,such as a receptor, possibly the same as that which caused its secretion. A rise in plasma amino acid concentrations is also

a possible cause for the stimulation of protein synthesis in the pancreas (Girard-Globa *et al.*, 1980). Initation of zymogen discharge *in vivo*, by feeding or secretogogue administration, has generally, but not consistently, been found to result in apparently increased rates of protein synthesis measured as the ability of the acinar cells to incorporate amino acids (Jamieson and Palade, 1971). It is a worthy objective to determine how great a role a rise in plasma amino acid concentrations, resulting from digestion, play in stimulating digestive enzyme synthesis in the pancreas. As shown before, the pancreas appears to be under a strict homeostatic control, which probably operates internally with respect to the acinar cells.

Neither the LMG nor the UMG fish increased their body weight during the winter months, but the weight gain in spring appeared distinctivly greater for the UMG fish. The greater increase in food intake of the LMG fish with increase in daylength and water temperature appears to indicate a removal of a suppression of appetite, occurring somewhere between winter and spring. This suppression was found to commence from July the year before (Thorpe *et al.*, 1991). The thresholds of response to plasma thyroxine are more and less sensitive to environmental change for the LMG and the UMG, respectively (Chapter VII, section 4.1.).

The arrest of growth of the LMG, in contrast to the UMG, which had been found by earlier studies to continue growth during the winter months(Chapter VII, section 4.1.), does not appear to be as a result of a lack of either pepsin, trypsin or chymotrypsin. These enzymes are present in similar amounts in the LMG fish, as in the UMG fish, during the winter months. The pancreas in the LMG fish responded to CCK during this time. This indicated that the pancreas was able to secrete both trypsin and chymotrypsin. However, starvation and challenge with CCK did indicate that the pancreas of the UMG fish was more actively secreting trypsinogen and chymotrypsinogen than the pancreas of the LMG fish. The reason why these enzyme activities were not higher in the UMG fish than the LMG fish might be explained as follows: a higher secretion rate and a higher evacuation rate exists in the UMG fish, since they feed more actively during the winter months.

Interestingly, trypsin and chymotrypsin activities in the intestinal digesta show a similar pattern to that of the stomach digesta: they were greater for the UMG fish in the winter

months, but lower for this group in the summer months (Fig. 37 (b) and Fig. 38 (b)). This may probably be explained by the hypothesis that CCK matches the amount of the digestive enzymes with the amount of incoming nutrients, i.e. the digesta appearing in the intestine (Dockray, 1989).

IX. References.

Alcon, E. and Bdolah, A. (1975). Increase of proteolytic activity and synthetic capacity of the pancreas in snakes after feeding. *Comp.Bichem.Physiol.* 50A, 627-631.

Aldman, G. and Holmgren, S. (1992). VIP inhibits CCK-induced gallbladder contraction involving a β-adrenoceptor mediated pathway in the rainbow trout, *Oncorhynchus mykiss. in vivo. Gen.Comp.Endocrinol.* 88, 287-291.

Aldman, G. and Holmgren, S. (1987). Control of gallbladder motility in the rainbow trout, *Salmo gairdneri. Fish Physiol.Biochem.* 4(3), 143-155.

Aldman, G.; Grove, D. and Holmgren, S. (1992). Duodenal acidification and intra-arterial injection of CCK8 increase gallbladder motility in the rainbow trout, *Oncorhynchus mykiss. Gen.Comp.Endocrinol.* 86, 20-25.

Aldman, G.; Jönsson, A-C.; Jensen, J. and Holmgren, S. (1988). Gastrin/CCK-like peptides in the spiny dogfish, *Squalus acanthias*; concentration and action in the gut. *Comp.Biochem.Physiol.* 92C, 103-118.

Ali, M.A. (1959). The ocular structure, retino-motor and photo-behavioral responses of juvenile Pacific salmon. *Can.J.Zool.* 37, 965-995.

Allan, I.R.H. and Ritter, J.A. (1975). Salmonid terminology. *J.Cons.Perm.Int.Explor.Mer*, 37, 293-299.

Allen, K.R. (1940a). Studies on the biology of the early stages of the salmon (*Salmo salar*). 1. Growth in the River Eden. *J.Anim.Ecol.* 9, 1-23.

Allen, K.R. (1940b). Studies of the ecology of the early stages of salmon (*Salmo salar*): 1. Growth in the River Eden. *J.Anim.Ecol.* 10, 47-46.

Allen, K.R. (1941a). Studies of the ecology of the early stages of salmon (*Salmo salar*): 2. Feeding habits. *J.Anim.Ecol.* 10, 47-46.

Allen, K.R. (1941b). Studies of the ecology of the early stages of salmon: 3. Growth in Thurso river system, Caithness. *J.Anim.Ecol.* 10, 273-295.

Allfrey, V.; Daly, M.M. and Mirsky, A.E. (1953). Synthesis of protein in pancreas. II. The role of ribonucleoprotein in protein synthesis. *J.Gen.Phys.* 37, 157-175.

Anastasi, A.; Erspamer, V. and Endean, R. (1967). Isolation and structure of caerulein, an active decapeptide from the skin of *Hyla caerula*. *Experimentia*, 23, 669-670.

Anderson, T.F. (1951). Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. *Trans.N.Y.Acad.Sci.* 13, 130.

Anson, M.L. and Mirsky, A.E. (1932). The estimation of pepsin with hemoglobin. *J. Gen.Physiol.* 16, 59-63.

Ásgeirsson, B.; Fox, J.W. and Bjarnason, J.B. (1989). Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. *Eur. J. Biochem.* 180, 85-94.

Austreng, E.; Storebakken, T. and Åsgård, T. (1987). Growth rate estimates for cultured Atlantic salmon and rainbow trout. *Aquacultre*, 60, 157-160.

Bailey, J.K.; Saunders, R.L. and Buzeta, M.I. (1980). Influence of parental smolt age and sea age on growth and smolting of hatchery-reared Atlantic salmon (*Salmo salar*). *Can.J.Fish.Aquat.Sci.* 37, 1379-1386.

Bainbridge, R. (1958). The speed of swimming of fish as related to size and to the frequency and amplitude of the tail beats.*J.Exp.Biol.* 35, 109-133.

Bancroft, J. D. and Stevens, eds. (1990). *Theory and practice of histological techniques.* 3 rd edition. Churchill Livingstone. N. Y.

Barrett, A.J. (1977). Cathepsin D and other carboxyl proteinases. In *Proteinases in mammalian cells and tissues.* A.J. Barrettt, ed., 209-248. North Holland. Amsterdam.

Barrett, A.J. (1972). Lyzosomal enzymes. In Lysosomes-*A Laboratory Handbook* (Dingle, J.T. ed.), pp. 46-136. North-Holland. Amsterdam.

Barrington, E.J.W. (1942). Gastric digestion in the lower vertebrates. *Biol. Rev.* 17, 1-27.

Barrington, E.J.W. and Dockray, G.J. (1970). The effect of intestinal extracts of lampreys (*Lampetra fluviatilis* and *Petromyzon marinus*) on pancreatic secretion in the rat. *Gen.Comp.Endocrinol.* 14, 170-177.

Barrington, E.J.W. and Dockray, G.J. (1972). Cholecystokinin-pancreozymin-like activity in the eel (*Anguilla anguilla* L.). *Gen.Comp.Endocrinol.* 19, 80-87.

Barrington, E. J. W. (1957). The alimentary canal and digestion. In *The Physiology of Fishes*, (M. E. Brown, ed.), Vol. 1, pp. 109-161. Academic Press. N. Y.

Barrowman, J.A. and Mayston, P.D. (1973). The trophic influence of cholecystokinin on the rat pancreas. *Proc.Physiol.Soc.* December.

Bauermeister, A. E. M.; Pirie, B. J. S. and Sargant, J. R. (1979). An electron microscopic study of lipid absorbtion in the pyloric caeca of Rainbow trout (*Salmo gairdneri*) fed waxester-rich zooplankton. *Cell Tissue Res.* 200, 475-486.

Behar, J. and Biancani, P. (1980). Effect of cholecystokinin and the octapeptide of cholecystokinin on the feline sphincter of Oddi and gallbladder. *J.Clin.Invest.* 66, 1231-1239.

Beland, K.F.; Jordan, R.M. and Meister, A.L. (1982). Water depth and velocity preferences of spawning Atlantic salmon in Maine rivers. *North Am. J. Fish. Mgmt*, 2, 11-13.

Bell, G.H.; Peterson, C.R. and Smith, D.E. eds. (1980). *Textbook of Physiology*. 10th edition. Churchill Livingstone. New York. London.

Berry, J. (1932). Report on an investigation of smolts in the River Tay during spring, 1931. *Fisheries Scotland, Salmon Fisheries*, 1931, **IV**, 21 pp.

Berry, J. (1933). Notes on the migration of smolts from Loch Ness, summer, 1932. *Fisheries Scotland, Salmon Fisheries*, 1933, **I**, 12 pp.

Birk, Y. (1985). The Bowman-Birk inhibitor. *Int J. Peptide Protein Res.* 25, 113-131.

Bishop, C. and Odense, P.H. (1966). Morphology of the digestive tract of the cod, *Gadus morhua. J. Fish. Res. Bd. Can.* 23, 1607-1615.

Black, O. and Webster, P.D. (1974). Nutritional and hormonal effects on RNA polymerase enzyme activities in pancreas. *Am.J.Physiol.* 227, 1276-1280.

Bodansky, M.; Klausner, Y.S. and Said, S.I. (1973b). Biologicl activities of synthetic peptides corresponding to the fragment of and to the entire sequence of the vasoactive intestinal peptide. *Proc.Nat.Acad.Sci.USA*, 70(2),382-384.

Bodanzky, M.; Corratù, R.; Dreiling, D.A.; Fussgänger, R.; Janowitz, H.D.; Jamieson, J.D.; Jorpes, J.E.; Mutt, V.; Pfeiffer, E.F.; Plessier, J.; Ramonino, M.L.; Raptis, S.; Torsoli, A. and Zimmerman, M. (1973a). *Secretin, Cholecystokinin, Pancreozymin and Gastrin.* Springer-Verlag New York Inc. New York.

Bommelaer, G.; Rozenthal, G.; Bernier, C.; Vaysee, N. and Ribet, A. (1981). Action of secretogogues on amylase release from dog pancreatic acini. *Digestion*, 21, 248-254.

Brannon, E.L. (1965). The influence of physical factors on the development and weight of sockey salmon embryos and alevins. *Internat.Pacif.Salmon Fish.Comm*, Prog.Rep. 12, 1-26.

Brannon, E.L. (1972). Mechanisms controlling migration of sockey salmon fry. *Internat.Pacif.Salmon Fish.Comm.Bull.*, 21, 86 pp.

Brett, J.R. (1971). Satiation time, appetite, and maximum food intake of sockeye salmon (*Oncorhynchus nerka*). *J.Fish.Res.Bd.Can.* 28, 409-415.

Brett, J.R. and Higgs, D.A. (1970). Effect of temperature on the rate of gastric digestion in fingerling sockeye salmon, *Oncorhynchus nerka*. *J.Fish.Res.Bd.Can.* 27(10), 1767-1779.

Brewer, P.; Helbig, N. and Haard, N.F. (1984). Atlantic cod pepsin characterization and use as a rennet substitute. *Can. Inst. Food Sci. Technol. J.* 17(1), 38-43.

Briggs, G.E. and Haldane, J.B.S. (1925). A rate on the kinetics of enzyme action. *Biochem J.* 19, 338-339.

Brock, F.M.; Forsberg, C.W. and Buchanan-Smith, J.G. (1982). Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Appl. Env. Microbiol.* 44(3), 561-569.

Bucke, D. (1971). The anatomy and histology of the alimentary tract of the carnivorous fish the pike *Esoxlucius* L. *J. Fish. Biol.* 3, 421-431.

Buddington, R.K. and Doroshov, S.I. (1986). Digestive enzyme complement of white sturgeon (*Acipenser transmontanus*). *Comp. Biochem. Physiol.* 83A(3), 561-567.

Carter, C.G.; Houlihan, D.F. and McCarthy, I.D. (1992). Feed utilization efficiencies of Atlantic salmon (*Salmo salar* L.) parr: Effect of a single supplementary enzyme. *Comp.Biochem.Physiol.* 101A(2), 369-374.

Case, R.M. (1978). Synthesis, intracellular transport and discharge of exportable proteins in the pancreatic acinar cell and other cells. *Biol.Rev.* 53, 211-354.

Charney, J. and Tomarelli, R.M. (1947). A colorimetric method for the determination of the proteolytic activity of the duodenal juice. *J. Biol. Chem.* 171, 501-505.

Chen, H.M. and Meyers, S.P. (1983). Ensilage treatment of crawfish waste for improvement of astaxanthin pigment extraction. *J. Food Sci.* 48, 1516-1520.

Cohen, T.; Gertler, A. and Birk, Y. (1981). Pancreatic proteolytic enzymes from carp (*Cyprinus cyprio*)-II. Kinetic properties and inhibitor studies of trypsin, chymotrypsin and elastase. *Comp. Biochem. Physiol.* 69 B, 647-653.

Corring, T. (1980). The adaptation of digestive enzymes to the diet: it's physiological significance. *Reprod.Nutr.Devl.* 20, 1217-1235.

Croston, C.B. (1960). Tryptic enzymes of Chinook salmon. *Arch. Biochem. Biophys.* 89, 202-206.

Croston, C.B. (1965). Endopeptidases of salmon caeca: Chromatographic separation and some properties. *Arc.Biochem.Biophys.* 112, 218-223.

Crowley, J.N.; Hays, S.E. and Paul, S.M. (1981). Vagotomy abolishes the inhibitory effects of cholecystokinin on rat exploratory behaviours. *Eur.J.Pharmacol.* 73, 379-380.

Dagorn, J.-C. and Mongeu, R. (1977). Different action of hormonal stimulation on the biosynthesis of three pancreatic enzymes. *Biochim.Biophys.Acta*, 498, 76-82.

Dagorn, J.C.; Sahel, J. and Sarles, H. (1977). Nonparallel secretion of enzymes in human duodenal juice collected by endoscopic retrograde catheterization of the papilla. *Gastroenterology*, 73, 42-45.

Dagorn, J-C. (1978). Non-parallel enzyme secretion from rat pancreas: *In vivo* studies.

J.Physiol. 280, 435-448.

Dahl, K. (1910). *The Age and Growth of Salmon and Trout in Norway as Shown by their Scales* (translated by I. Baillie, eds. J.A. Hutton and H.T.Sheringham), Salmon and Trout Association, London.

Daly, M.M.; Allfrey, V.G. and Mirsky, A.E. (1955). Synthesis of protein in the pancreas. III. Uptake of glycine-N^{15} by the trypsinogen and chymotrypsinogen of the mouse pancreas. *J.Gen.Physiol.* 39, 207-210.

de Haën, C. and Gertler, A. (1974). Isolation and amino-terminal sequence analysis of two dissimilar pancreatic proelastes from the African lungfish, *Protopterus aethiopicus*. *Biochemistry* 13, 2673-2677.

Del Mar, E.G.; Largman,C.; Brodrick, J.W. and Geokas, M.C. (1979). A sensitive new substrate for chymotrypsin. *Anal. Biochem.* 99, 316-320.

Dill, L.M. (1969). The sub gravel behaviour of Pacific salmon larvae. In *Symposium on Salmon and Trout in Streams* (ed. T.G. Northcode), H.R. MacMillan Lectures in Fisheries, 1968, University of British Columbia, Vancouver, pp. 89-99.

Dill, P.A. (1977). Development behaviour in alevins of Atlantic salmon (*Salmo salar*) and rainbow trout (*S. gairdneri*). *Animal Behaviour*, 25, 116-121.

Doble, B.D. and Eggers, D.M. (1978). Diel feeding chronology, rate of gastric evacuation, daily ration, and prey selectivity in Lake Washington juvenile sockeye salmon (*Oncorhynchus nerka*). *Trans.Am.Fish.Soc.* 107(1), 36-45.

Dockray, G.J. (1982). The physiology of cholecystokinin in the brain and gut. *Brit.Med.Bull.* 38, 253-258.

Dockray, G.J. (1978). Gastrin overview. In *Gut Hormones* (S.R. Bloom, ed.). Churchill Livingstone. Edinburgh. London. New York.

Dockray, G.J. (1989). The integrative functions of CCK in the upper gastrointestinal tract. In *The Neuropeptide Cholecystokinin (CCK): anatomy and biochemistry, receptors, pharmacology and physiology* (J. Hughes, G. Dockray, G. Woodruff, eds.). Ellis Horwood Ltd. Chichester.

Duntley, S.Q. (1962). Underwater visibility. In The Sea (M.N. Hill, ed.), pp. 452-455. John Wiley. London.

Egglishaw, H.J. and Schackley, P.E. (1977). Growth, survival and production of juvenile salmon and trout in Scottish streams, 1966-1975. *J.Fish.Biol.* 11, 647-672.

Egglishaw, H.J. and Schackley, P.E. (1980). Survival and growth of salmon, *Salmo salar* L., planted in a Scottish stream. *J.Fish.Biol.* 16, 565-584.

Egglishaw, H.J. (1967). The food, growth and population structure of salmon and trout in two streams in the Scottish Highlands. *Freshw.Salmon.Res.Scotland*, 38, 32p.

Egglishaw, H.J. and Schackley, P.E. (1982). Influence of water depth on dispersion of juvenile salmonids, *Salmo salar* and *S. trutta* L. in a Scottish stream. *J.Fish.Biol.* 21, 141-155.

Egglishaw, H.J. and Schackley, P.E. (1985). Factors governing the production of juvenile Atlantic salmon in Scottish streams. *J.Fish Biol.* 27A, 27-33.

Einarsson, S.M. (1987). Utilisation of Fluvial and Lacustrine Habitat by a Wild Stock of Anadromous Atlantic Salmon (*Salmo salar* L.) in Icelandic Watershed. MPhil Thesis, University of Edinburgh, 187 pp.

Elliott, J.M. (1975a). The growth rate of brown trout, *Salmo trutta* L., fed on maximum rations. *J.Anim.Ecol.* 44, 805-821.

Elliott, J.M. (1975b). Weight of food and time required to satiate brown trout, *Salmo*

trutta L. *Freshwat.Biol.* 5, 51-64.

Elliott, J.M. (1972). Rates of gastric evacuation in brown trout, *Salmo trutta* L. *Freshwat.Biol.* 2, 1-18.

Elliott, J.M. and Persson, L. (1973). The estimation of daily rates of food consumption for fish. *J.Anim.Ecol.* 47, 977-991.

Elson, P.F. (1957). The importance of size in the change from parr to smolt in Atlantic salmon. *Can.Fish Cult.* 21, 1-6.

Emlen, J.M. (1968). Optimal choice in animals. *Am.Nat.* 102, 385-389.

Epple, A. (1969). The endocrine pancreas. In *Fish Physiology* (W. S. Hoar and D. J. Randall, eds.). Vol. 2, pp. 275-319. Academic Press. New York.

Eriksson, L.-O. and Alanärä, A. (1992). Timing of feeding behaviour in salmonids. In *The Importance of Feeding Behaviour for the Efficient Culture of Salmonid Fishes* (J.E. Thorpe and F.A. Huntingford, eds.). *World Aquaculture Workshops*, Number 2. The World Aquaculture Society. Louisiana State University. Pp: 41-48.

Erlanger, B.F.; Kokowsky, N. and Cohen, W. (1961). The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* 95, 271-278.

Erspamer, V. (1981). The tachykinin peptide family. *Trends Neurosci.* 4, 267-269.

Ezeasor, D. N. (1978). *The gut of the rainbow trout (Salmo gairdneri): A gross, histological and ultrastructural study with some aspects of functional significance.* Ph. D. thesis. University of Edinburgh.

Ezeasor, D. N. and Stokoe, W. M. (1980). Scanning electron microscopic study of the gut mucosa of the rainbow trout *Salmo gairdneri* Richardson. *J. Fish. Biol.* 17, 529-539.

Ezeasor, D. N. and Stokoe, W. M. (1981). Light and electron microscopic studies on the absorptive cells of the intestine, caeca and rectum of the adult rainbow trout, *Salmo gairdneri*, Rich. *J. Fish. Biol.* 18, 527-544.

Farber, E. and Sidransky, H. (1956). Changes in protein metabolism in the rat pancreas on stimulation. *J.Biol.Chem.* 222, 237-248.

Fasman, G.D., ed. (1976). *Handbook of biochemistry and molecular biology*. CRC Press, Inc. Cleveland Ohio.

Fernandes, J.F. and Janqueira, L.C.U. (1955). Protein and ribonucleic acid turnover rates related to activity of digestive enzymes of pigeon pancreas. *Arch.Biochem.Biophys.* 55, 54-62.

Fersht, A. (1977). *Enzyme structure and mechanism*. W.H. Freeman and Co. Reading. San Fransisco.

Fischer, Z. and Layokhnovich, V.P. (1973). Biology and bioenergetics of grass carp (*Ctenopharyngodon idella* Val.). *Pol.Arch.Hydrobiol.* 20, 521-557.

Fölsch, U.R. and Wormsley, K.G. (1973). Pancreatic enzyme response to secretin and cholecystokinin-pancreozymin in the rat. *J.Physiol. (Lond.)*. 234, 79-94.

Fourmy, D.; Zahid, A.; Fabre, R.; Pradayrol, and Ribet, A. (1987). Receptors for cholecystokinin and gastrin peptides display specific binding properties and are structurally different in guinea pig and dog pancreas. *Eur.J.Biochem.* 165, 683-692.

Fujimura, M.; Sakamoto,T.; Khalil, T.; Greeley, G.H., jr.; Townsend, C.M., jr., and Thompson, J.C. (1984). Role of the jejunum and ileum in the release of secretin and cholecystokinin by acid or fat in conscious dogs (Abstract). *Gastroenterology*, 86, 1083.

Galbraith, M.G. (1967). Size selective predation on *Daphnia* by rainbow trout and yellow perch. *Trans.Am.Fish.Soc.* 96, 1-10.

Gamble, W.S. (1970). Impaired pancreozymin (CCK-PZ) secretion after vagotomy and pyloroplasty. *J.Lab.Clin.Med.* 76, 871.

Gardiner, W.R. and Geddes, P. (1980). The influence of body composition on the survival of juvenile salmon. *Hydrobiologia*, 69, 67-72.

Gardner, J.D. and Jensen, R.T. (1987). Secretagogue receptors on pancreatic acinar cells. In *Physiology of the Gastrointestinal Tract*, 2nd edn. pp. 1109-1127. Raven Press. New York.

Gauthier, G. F. and Landis, S. C. (1972). The relationship of ultrastructural and cytochemical features to absorptive activity in the goldfish intestine. *Anat. Rec.* 172, 675-702.

Genicot, S.; Feller, G. and Gerday, Ch. (1988). Trypsin from Antarctic fish (*Paranotothenia magellanica* Forster) as compared with trout (*Salmo gairdneri*) trypsin. *Comp. Biochem. Physiol.* 90 B(3), 601-609.

Gertler, A. and Birk, Y. (1970). Isolation and characterization of porcine proelastase. *Eur. J. Biochem.* 12, 170-176.

Geuze, J.J. and Kramer, M.F. (1974). Function of coated membranes and multivesicular bodies during membrane regulation in stimulated exocrine pancreas cells. *Cell Tiss.Res.* 156, 1-20.

Geuze, J.J. and Poort, C. (1973). Cell membrane resorption in the rat exocrine pancreas cell after *in vivo* stimulation of the secretion, as studied by *in vitro* incubation with extracellular space markers. *J.Cell.Biol.* 57, 159-174.

Gibbs, J.; Young, R.C. and Smith, G.P. (1973). Cholecystokinin elicits satiety in rats with open gastric fistulas. *Nature (London)*, 245, 323-325.

Gibson, R.J. (1978). Recent changes in the population of juvenile Atlantic salmon in the Matamek River, Quebec, Canada. *J.Cons.Perm.Int.Explor.Mer*, 38(2), 201-207.

Gildberg, A. and Øverbø, K. (1990). Purification and characterization of pancreatic elastase from Atlantic cod (*Gadus morhua*). *Comp. Biochem. Physiol.* 97 B(4), 775-782.

Gildberg, A. and Raa, J. (1983). Purification and characterization of pepsins from the Arctic fish capelin (*Mallotus villosus*). *Comp. Biochem. Physiol.* 75 A, 337-342.

Gildberg, A. (1988). Aspartic proteinases in fishes and aquatic invertebrates. *Comp. Biochem. Physiol.* 91 B (3), 425-435.

Girard-Globa, A.; Bourdel, G. and Lardeux, B. (1980). Regulation of protein synthesis and enzyme accumulation in the rat pancreas by amount and timing of dietary protein. *J.Nutr.* 110, 1380-1390.

Giraud, A.S. and Yeomans, N.D. (1982). Comparative distribution of pepsinogens in the chordate gastric mucosa from representatives of five classes. *Comp. Biochem. Physiol.* 72 B, 145-147.

Glass, H.J.; Mac Donald, N.L.; Moran, R.M. and Stark, J.R. (1989). Digestion of protein in different marine species. *Comp. Biochem. Physiol.* 94 B(3), 607-611.

Go, V.L.W. (1978). The physiology of cholecystokinin. In *Gut Hormones* (S.R. Bloom, ed.). Churchill Livingstone. Edinburgh. London. New York.

Go, V.L.W.; Gardner, J.D.; Brooks, F.P.; Lebenthal, E.; DiMagno, E.P. and Scheele, G.A. eds. (1986). *The Exocrine Pancreas. Biology, Pathobiology and Diseases.* Raven Press. New york.

Go, V.L.W.; Hoffman, A.F. and Summerskill, W.H.J. (1970). Pancreozymin bioassay in man based on pancreatic enzyme secretion: potency of specific amino acids and other digestive products. *J.Clin.Invest.* 49, 1558-1564.

Good, N.E.; Winget, G.D.; Winter, W.; Connolly, T.N.; Izawa, S. and Singh, R.M.M. (1966). Hydrogen ion buffers for biological research. *Biochemistry*, 5(2), 467-477.

Grabner, M. and Hofer, R. (1989). Stomach digestion and its effect upon protein hydrolysis in the intestine of rainbow trout (*Salmo gairdneri* Richardson). *Comp.Biochem.Physiol.* 92A(1) 81-83.

Gray, R.W.; Cameron, J.D. and Jefferson, E.M.J. (1986). Stream characteristics and assessment of Atlantic salmon spawning and nursery habitat in the Le Have River, N.S. *Can.Tech.Rep.Fish.Aquat.Sci.* 6, 23-27.

Greene, C. W. (1912). Anatomy and histology of the alimentary tract of the king salmon. *Bull. U. S. Bur. Fish.* 32, 75-101.

Gregory, H.; Hardy, P.M.; Jones, D.S.; Kenner, G.W. and Sheppard, R.C. (1964). The antral hormone gastrin. *Nature*, 204, 931-933.

Gross, W.L.; Roelofs, E.W. and Fromm, P.O. (1965). Influence of photoperiod on growth of green sunfish *Lepomis cyanellus. J.Fish.Res.Board Can.* 22, 1379-1386.

Grossman, A. (1984). An overview of pancreatic exocrine secretion. *Comp.Biochem.Physiol.* 78B(1), 1-13.

Grossman, M.I. (1977). Physiological effects of gastrointestinal hormones. *Fed. Proc.* 36, 1930-1932.

Grove, D.J.; Loizides, L.G. and Nott, J. (1978). Satiation amount,frequency of feeding and gastric emptying rate in *Salmo gairdneri. J.Fish.Biol.* 12, 507-516.

Guerard, F. and Le Gal, Y. (1987). Characterization of a chymosin-like pepsin from the dogfish *Scyliorhinus Canicula. Comp. Biochem. Physiol.* 88 B(3), 823-827.

Haard, F.H. (1986). Atlantic cod gastric protease. Characterization with casein and milk substrate and influence of sepharose immobilization on salt activation, temperature characteristics and milk clotting reaction. *J. Food Sci.* 51(2), 313-316,326.

Hanlon, M.H. and Liener, I.E. (1986). A kinetic analysis of the inhibition of rat and bovine trypsins by naturally occurring protease inhibitors. *Comp. Biochem. Physiol.* 84 B(1), 53-57.

Harada, E.; Nijyama, M. and Syuto, B. (1986). Hepatic bile and pancreatic exocrine secretions evoked by gastrointestinal peptides in sheep. *Comp.Biochem.Physiol.* 85A(4), 729-734.

Hayat, M. A. (1978). *Introduction to biological scanning electron microscopy.* University Park Press. Baltimore. London. Tokyo.

Hayat, M. A. (1989). *Principles and techniques of electron microscopy. Biological applications.* 3rd edition. Macmillan Press Ltd. London.

Hedner, P. and Rorsman, G. (1968). Structures essential for the effect of cholecystokinin on the Guinea pig small intestine in vitro. *Acta Physiol.Scand.* 74, 58-68.

Hedner,P.; Persson, H. and Rorsman, G. (1967). Effect of cholecystokinin on small intestine. *Acta Physiol.Scand.* 70, 250-254.

Helander, H.F. (1981). The cells of the gastric mucosa. *Int.Rev.Cytol.* 70, 217-289.

Hemmings, C.C. (1966). Factors influencing the visibility of objects underwater. In *Light as an Ecological Factor* (R. Bainbridge; G.W. Evans and O. Rackham, eds.), pp. 359-374. Oxford:Blackwell.

Hess, G.P. (1971). Chymotrypsin-chemical properties and catalysis. In *The Enzynes*, Vol.3, 3rd. ed. (Boyer, P.D. ed.). Acad. Press. New York.

Hester, F.J. (1968). Visual contrasts thresholds in goldfish. *Vision Res.* 8, 1315-1336.

Higgins, P.J. and Talbot, C. (1985). Growth and feeding in juvenile Atlantic salmon (*Salmo salar* L.). In *Nutrition and Feeding in Fish* (C.B. Cowey; A.M. Mackie and J.G. Bell, eds.). Academic Press. New York.

Higgins, P.J. (1985). Metabolic differences between Atlantic salmon (*Salmo salar*) parr and smolts. *Aquaculture*, 45, 33-53.

Hill, R.L. (1965). Hydrolysis of proteins. *Advan. Protein Chem.* 20, 37-107.

Hirschowitz, B.I. (1984). Pepsinogen. *Postgraduate Medical Journal*, 60, 743-750.

Hjelmeland, K. and Raa, J. (1982). Characteristics of two trypsin type izozymes isolated from the Arctic fish capelin (*Mallotus villosus*). *Comp.Biochem.Physiol.* 71B, 557-562.

Hoar, W.S. (1942). Diurnal variations in feeding activity of young salmon and trout. *J.Fish.Res.Bd.Can.* 6(1), 90-101.

Hoar, W.S. (1976). Smolt transformation: evolution, behaviour and physiology. *J.Fish.Res.Bd Can.* 33, 1233-1252.

Hochberg, Y. and Tamhane, A.C. (1987). *Multiple Comparison Procedures.* Wiley. New York. Chichester.

Hofer, R.; Ladurner, H.; Gattringer, A. and Wieser, W. (1975). Relationship between the temperature preferenda of fishes, amphibians and reptiles and the substrate affinities of their trypsins. *J. Comp. Physiol.* 99, 345-355.

Hofer, R. and Schiemer, F. (1981). Proteolytic activity in the dgestive tract of several species of fish with different feeding habits. *Oecologia (Berl)*, 48, 342-345.

Holm, J.-C. (1992). Salmonids in duoculture: Growth rate gain and behavioural changes.

In *The Importance of Feeding Behaviour for the Efficient Culture of Salmonid Fishes* (J.E. Thorpe and F.A. Huntingford, eds.). *World Aquaculture Workshops*, Number 2. The World Aquaculture Society. Louisiana State University. Pp: 49-52.

Holmgren, S.; Jönsson, A.C. and Holstein, B. (1986). Gastro-intestinal peptides in fish. In *Fish Pysiology: Recent Advances* (eds. S. Nilsson and S. Holmgren), Groom Helm, London, pp. 119-139.

Holmgren, S.; Vaillant, C. and Dimaline, R. (1982). VIP-, substance P-, gastrin/CCK-, bombesin-, somatostatin-and glucagon-like immunoreactivities in the gut of the rainbow trout, *Salmo gairdneri. Cell & Tissue Res.* 223, 141-153.

Holmgren, S.; Grove, D.J. and Nilsson, S. (1985). Substance P acts by releasing 5-hydroxytryptamine from enteric neurons in the stomach of the rainbow trout, *Salmo gairdneri. Neuroscience*, 14, 683-693.

Holmquist, A.L.; Dockray, G.J.; Rosenquist, G.L. and Walsh, J.H. (1979). Immunochemical characterization of cholecystokinin-like peptides in lamprey gut and brain. *Gen.Comp.Endocrinol.* 37, 474-481.

Holstein, B. (1983). Effect of vasoactive intestinal polypeptide on gastric acid secretion and mucosal blood flow in the Atlantic cod, *Gadus morhua. General and Comparative Endocrinology*, 52, 471-473.

Holstein, B. (1982). Inhibition of gastric acid secretion in the Atlantic cod, *Gadus morhua*, by sulphated and desulphated gastrin, caerulein, and CCK-octapeptide. *Acta Physiol.Scand.* 114, 453-459.

Holstein, B. (1979a). Gastric acid secretion and water balance in the marine teleost *Gadus morhua. Acta Physiol.Scand.* 105, 93-107.

Holstein, B. (1979b). Gastric acid secretion and drinking in the Atlantic cod (*Gadus morhua*) during acidic or hyperosmotic perfusion of the intestine. *Acta Physiol.Scand.* 106,

257-265.

Holstein, B. (1977). Effect of atropine and SC-15396 on stimulated gastric acid secretion in the Atlantic cod, *Gadus morhua*. *Acta Physiol.Scand.* 101, 185-193.

Holstein, B. (1976). Effect of the H_2-receptor antagonist metiamide on carbachol-and histamine-induced gastric acid secretion in the Atlantic cod, *Gadus morhua*. *Acta Physiol.Scand.* 97, 189-195.

Holstein, B. (1975). Gastric acid secretion in the teleostean fish: A method for the continuous collection of gastric effluence from a swimming fish and its response to histamine and pentagastrin. *Acta Physiol. Scand.* 95, 417-423.

Holstein, B. and Cederberg, C. (1980). Effect of vagotomy and glucose administration on gastric acid secretion in the Atlantic cod, *Gadus morhua*. *Acta Physiol.Scand.* 109, 37-44.

Holstein, B. and Cederberg, C. (1984). Effect of 5-HT on basal and stimulated secretions of acid and pepsin and on gastric volume outflow in the *in vivo* gastrically and intestinally perfused cod, *Gadus morhua*. *Agents and Actions*, 15(3/4), 290-305.

Holstein, B. and Cederberg, C. (1986). Effects of tachykinins on gastric acid and pepsin secretion and on gastric outflow in the Atlantic cod, *Gadus morhua*. *Am.J.Physiol.* 250, G309-G315.

Holstein, B. and Humphrey, C.S. (1980). Stimulation of gastric acid secretion and suppression of VIP-like immunoreactivity by bombesin in the Atlantic codfish, *Gadus morhua*. *Acta Physiol.Scand.* 109, 217-223.

Hong, S.S. (1960). Mechanism of gallbladder evacuation. *New Med.J. (Korea)*, 3, 49-62.

Hong, S.S. and Magee, D.F. (1970). Pharmacological studies on the regulation of pancreatic secretion in pigs. *Ann.Surg.* 172, 41-48.

Hong, S.S.; Magee, D.F. and Crewdson, F. (1956). The physiological regulation of gallbladder evacuation. *Gastroenterology* 30, 625-630.

Honkanen, R.E.; Crim, J.W. and Patton, J.S. (1988). Effects of cholecystokinin peptides on digestive enzymes in killifish *in vivo*. *Comp.Biochem.Physiol.* 89A(4), 655-660.

Hsu, Y.-L. and Wu, J.-L. (1979). The relationship between feeding habits and digestive proteases of some freshwater fishes. *Bull.Inst.Zool., Academia sinica* 18(1), 45-53.

Huntingford, F.A.; Metcalfe, N.B.; and Thorpe, J.E. (1988). Choice of feeding station in Atlantic salmon, *Salmo salar*, parr: effects of predation risk, season and life history strategy. *J.Fish Biol.* 33, 917-924.

Iro, S. (1967). Anatomic structure of the gastric mucosa. In *Handbook of Physiology*, (C. F. Code, ed.). Sect. 6, Vol. 2, pp. 705-741. Am. Physiol. Soc., Washington, D. C.

Ivlev, V.S. (1961). Experimental ecology of the feeding of fishes. Yale Univ.Press, New Haven.

Iwai, K.; Fukuoha, S-I.; Fushiki, T.; Tsujikawa, M.; Hirose, M.; Tsunasawa, S. and Sakiyama, F. (1987). Purification and sequencing of trypsin-sensitive cholecystokinin-releasing peptide from rat pancreatic juice. *J.Biol.Chem.* 262, 8956-8959.

Jaffe, B.M.; Akande, B.; Modlin, I.M. Reilly, P. and Albert, D. (1982). Cholinergic modulation of substance P release. *Dig.Dis.Sci.* 27, 28-32.

Jamieson, J.D. and Palade, G.E. (1971). Synthesis, intracellular transport, and discharge of secretory proteins in stimulated pancreatic exocrine cells. *J. Cell. Biol.* 50, 135-158.

Jany, K.D. (1976). Studies on the digestive enzymes of the stomachless bonefish *Carassius auratus gibelio* (Bloch): endopeptidases. *Comp. Biochem. Physiol.* 53 B, 31-38.

Jensen, R.T.; Lemp, G.F. and Gardner, J.D. (1980). Interaction of cholecystokinin with

specific membrane receptors on pancreatic acinar cells. *Proc.Natl Acad.Sci.USA*, 77, 2079-2083.

Johnson, L.R. and Guthrie, P.D. (1976). Stimulation of DNA synthesis by big and little gastrin (G34 and G17). *Gastroenterology*, 71, 599-602.

Johnson, W.C. and Lindsay, A.J. (1939). An improved universal buffer. *Analyst, Lond.* 64, 490-492.

Jónás, E.; Rágyanszki, M.; Oláh, J. and Boross, L. (1983). Proteolytic digestive enzymes of carnivorous (*Silurus glanis* L.), herbivorous (*Hypophthalmichthys molitrix*Val.) and omnivorous (*Cyprinus carpio* L.) fishes. *Aquaculture*, 30, 145-154.

Jonsson, B. and Ruud-Hansen, J. (1985). Water temperature as the primary influence on timing of seaward migration of Atlantic salmon (*Salmo salar*) smolts. *Can.J.Fish.Aquat.Sci.* 42(3), 593-595.

Jönsson, A-C; Holmgren, S. and Holstein, B. (1987). Gastrin/CCK-like immunoreactivity in endocrine cells and nerves in the gastrointestinal tract of the cod, *Gadus morhua*, and the effect of peptides of the gastrin/CCK family on cod gastrointestinal smooth muscle. *Gen. Comp. Endocrinol.* 66, 190-202.

Jönsson, A-C. and Holmgren, S. (1989). Gut secretion. In *The Comparative Physiology of Regulatory Peptides.* (S. Holmgren, ed.). Chapman and Hall. London. New York.

Jönsson, A-C. (1991). Regulatory peptides in the pancreas of two species of elasmobranchs and in the Brockmann bodies of four teleost species. *Cell & Tissue Res.* 266, 163-172.

Jorpes, J.E. and Mutt, V. (1962). *Exocrine Pancreas, Ciba Foundation Symposium.* Curchill. London. p. 150.

Jorpes, J.E. and Mutt, V. (1973). Secretin and cholecystokinin (CCK). In *Secretin,*

Cholecystokinin, Pancreozymin and Gastrin (J.E. Jorpes and V. Mutt, eds.), pp. 1-179. Spriner-Verlag New York Inc. New York.

Kadri, S.; Metcalfe, N.B.; Huntingford, F.A. and Thorpe, J.E. (1991). Daily feeding rhythms in Atlantic salmon in sea cages. *Aquaculture*, 92, 219-224.

Kalac, J. (1978a). Studies on herring (*Clupea harengus* L.) and capelin (*Mallotus villosus* L.) pyloric caeca proteases I: Partial purification and separation and identification of proteases. *Biológia* (Bratislava), 33(6), 485-495.

Kalac, J. (1978b). Studies on herring (*Clupea harengus* L.) and capelin (*Mallotus villosus* L.) pyloric caeca proteases III: Characterization of the anionic fractions of chymotrypsins. *Biológia* (Bratislava), 33(12), 939-950.

Kalleberg, H. (1958). Observations in a stream tank of territoriality and competition in juvenile salmon and trout (*Salmo salar* L. and *S. trutta*). *Report of the Institute of Freshwater Research, Drottningholm*, 39, 55-98.

Kapoor, B.G.; Smit, H. and Verighina, I.A. (1975). The alimentary canal and digestion in teleosts. *Adv. mar. Biol.* 13, 109-239.

Kaspar, P.; Moeller, G.; Wahlefeld, A.W. and Staehler, F. (1982). A new photometric method for the determination of chymotrypsin in stool. *Frezenius Z.Anal.Chem.* 311, 391-392.

Kaspar, P.; Möller, G. and Wahlefeld, A. (1984). New photometric assay for chymotrypsin in stool. *Clin. Chem.* 30(11),1753-1757.

Kawai, S. and Ikeda, S. (1973). Studies on digestive enzymes of fishes-III. Development of digestive enzymes of rainbow trout after hatching and the effect of dietary change on the activities of digestive enzymes in the juvenile stage. *Bull.Jap.Soc.Sci.Fish.* 39(7), 819-823.

Keenleyside, M.H.A. and Yamamoto, F.T. (1962). Territorial behaviour of juvenile Atlantic salmon (*Salmo salar* L.). *Behaviour*, 19(1), 139-169.

Kennedy, G.J.A. (1984). Evaluation of techniques for classifying habitats for juvenile Atlantic salmon (*Salmo salar* L.). *Atlantic Salmon Trust Workshop on Stock Enhancement*, University of Surrey.

Kitamikado, M. and Tachino, S. (1960). Studies on digestive enzymes of rainbow trout-II. Proteases. *Bull.Jap.Soc.Sci.Fish.* 26(7), 685-690.

Kitamikado, M.; Morishita, T. and Tachino, S. (1965). Digestion of dietary protein in rainbow trout. II. Effect of starch and oil contents in diets and the size of fish on digestibility. *Chem. Abstracts.* 62, 15129.

Knutsson, S. and Grav, T. (1976). Seawater adaptation in Atlantic salmon (*Salmo salar*) at different experimental temperatures and photoperiods. *Aquaculture*, 8, 169-187.

Komourdjian, M.P.; Saunders, R.L. and Fenwick, J.C. (1976). Evidence for the role of growth hormone as a part of a ' light-pituitary axis' in growth and smoltification of Atlantic salmon (*Salmo salar*). *Can.J.Zool.* 54, 544-551.

Kraut, J. (1977). Serine proteases: Structure and mechanism of catalysis. *Ann. Rev. Biochem.* 46, 331-358.

Krogdahl, Å. and Holm, H. (1983). Pancreatic proteinases from man, trout, rat, pig, cow, chicken, mink and fox. Enzyme activities and inhibition by soybean and lima bean proteinase inhibitors. *Comp. Biochem. Physiol.* 74 B(3), 403-409.

Kunitz, M. (1947). Crystalline soybean trypsin inhibitor. *J. Gen. Physiol.* 30, 291-310.

Langer, M.; Van Noorden, S.; Polak, J.M. and Pearse, A.G.E. (1979). Peptide hormone-like immunoreactivity in the gastrointestinal tract and endocrine pancreas of eleven teleost species. *Cell Tissue Res.* 199, 493-508.

Larsson, L-I. and Rehfeld, J.F. (1977). Evidence for a common evolutionary origin of gastrin and cholecystokinin. *Nature*, 269, 335-338.

Larsson, L.-I. and Rehfeld, J.F. (1978). Evolution of CCK-like hormones. In *Gut Hormones* (S.R. Bloom, ed.). Churchill Livingston. Edinburgh. London. New York. pp. 68-73.

Lawrence, J.M. (1958). Estimated sizes of various forage fishes largemouth bass can swallow. *Proc.11th Ann.Conf.S.E.Assoc.of Game and Fish Commissioners.* 220-225.

Leániz, C.G. de (1988). Site fidelity and homing of Atlantic salmon parr in a small Scottish stream. In *Proceedings of the Second International Symposium on Salmon and Trout Migratory Behaviour*, Trondheim, June 1987 (eds. E. Brannon and B. Jonsson).

Lehninger, A.L. (1975). *Biochemistry.* Worth Publishers, Inc. New York.

Lindstrom, T. (1956). On the relation fish size-food size. *Rep.Inst.Fw.Res.Drottningholm* 36, 133-147.

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* 56, 658-666.

Logson, C.D. and Williams, J.A. (1986). Pancreatic acinar cell in monolayer culture: direct trophic effects of caerulein in vitro. *Am.J.Physiol.* 250, G440-G447.

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

Luzanne, L. (1970). La sèlection des proies chez *Alestes Baremoze. Cah. O.R.S.T.O.M.Sèr.Hydrobiol.* 4, 71-76.

MacArthur, R.H. and Pianka, E.R. (1966). On optimal use of a patchy environment.

Am.Nat. 100, 603-609.

MacCrimmon, H.R. and Gots, B.L. (1979). World distribution of Atlantic salmon, *Salmo salar. J.Fish.Res.Bd Can.*, 33, 2616-2621.

Maraux, S.; Rovery, M. and Desnuelle, P. (1966). Hydrolysis of bovine trypsin of some aromatic bonds in high molecular weight peptides. *Biochim. Biophys. Acta* 122, 145-147.

Martensson, H.G.; Akande, B.; Yeo, C. and Jaffe, B.M. (1984). The role of substance P in the control of gastric acid secretion. *Surgery*, 95, 567-571.

Martin, N.V. (1952). A study of the lake trout, *Salvelinus namaycush*, in two Algonquin Park, Ontario, lakes. *Trans.Am.Fish.Soc.* 81, 111-137.

Martin, B.F.; Levin, R.J. and Kugler, J.H. (1969). A light and electron microscopic study of the exocrine pancreas following administration of 5-fluorouracil. *J.Anat.* 104, 93-107.

Martínez, A. and Olsen, R.L. (1989). Characterization of pepsins from cod. *United States Biochemical Corp.* 16(1), 22-23, 20.

Martínez, A. and Serra, J.L. (1989). Proteolytic activities in the digestive tract of anchovy (*Engraulis encrasicholus*). *Comp. Biochem. Physiol.* 93 B(1), 61-66.

Martínez, A.; Olsen, R.L. and Serra, J.L. (1988). Purification and characterization of two trypsin-like enzymes from the digestive tract of the Anchovy *Engraulis encrasicholus. Comp. Biochem. Physiol.* 91 B(4), 667-684.

Martínez, A. and Olsen, R.L. (1986). Studies on pepsins from cod stomach. Abstact presented at the *22nd Annual Meeting of the Norwegian Biochemical Society.*

Mason, J.C. (1976). Some features of coho salmon *Oncorhynchus kisutch* fry emerging from simulated redds, and concurrent changes in photobehaviour. *Fish.Bull.Fish Wildl.Serv.U.S.* 74, 165-175.

McLeese, J.M. and Stevens, E.D. (1982). The effect of acclimation temperature, assay temperature, and ration on the specific activity of trypsin and chymotrypsin from rainbow trout (*Oncorhynchus mykiss*). *Comp.Biochem.Physiol.* 73B(3), 631-634.

Melchiorri, P. (1978). Bombesin and bombesin-like peptides of amphibian skin. In *Gut Hormones* (S.R. Bloom, ed.). Churchill livingstone. Edinburgh. London. New York.

Metcalfe, N.B. (1990). Competitive ability influences seaward migration age in Atlantic salmon. *Can.J.Zool.* 69, 815-817.

Metcalfe, N.B.; Huntingford, F.A. and Thorpe, J.E. (1986). Seasonal changes in feeding motivation of juvenile Atlantic salmon (*Salmo salar*). *Can.J.Zool.* 64, 2439-2446.

Metcalfe, N.B.; Huntingford, F.A. and Thorpe, J.E. (1992). Social effects on appetite and development in Atlantic salmon. In *The Importance of Feeding Behaviour for the Efficient Culture of Salmonid Fishes* (J.E. Thorpe and F.A. Huntingford, eds.). *World Aquaculture Workshops*, Number 2. The World Aquaculture Society. Louisiana State University. Pp: 29-40.

Metcalfe, N.B.; Huntingford, F.A. and Thorpe, J.E. (1988). Feeding intensity, growth rates, and the establishment of life-history patterns in juvenile Atlantic salmon *Salmo salar*. *J.Anim.Ecol.* 67, 463-474.

Metcalfe, N.B.; Huntingford, F.A.; Graham, W.D. and Thorpe, J.E. (1989). Early social status and the development of life history strategies in Atlantic salmon. *Proceedings of the Royal Society of London* B236: 7-19.

Metz, J.P. (1974). Die Invertebratendrift an der Oberfläche eines Voralpenflusses und ihre selektive Ausnutzung durch die Regenbogenforellen (*Salmo gairdneri*). *Oecologia* 14, 247-268.

Mihalyi, E. (1978). Proteolytic enzymes. In *Application of proteolytic enzymes to protein*

structure studies, 2nd edn, Vol. I, 43-127. CRC Press. West Palm Beach.

Mills, D.H. (1965). Observations on the effects of hydro-electric developments on salmon migration in a river system. *International Council for the Exploration of the Sea*, C.M.1965/M:32.

Mills, D.H. (1964). The ecology of the young stages of the Atlantic salmon in the River Bran, Ross-shire. *Freshwat.Salm.Fish.Res., Scotland*, 32, 58 pp.

Mills, D.H. (1969a). The survival of juvenile Atlantic salmon and brown trout in some scottish streams. In *Symposium on Salmon and Trout in Streams* (ed. T.G. Northcote), H.R. MacMillan Lectures in Fisheries, 1968, University of British Columbia, Vancouver, pp. 217-228.

Mills, D.H. (1969b). The survival of hatchery-reared salmon fry in some scottish streams. *Freshwat.Salm.Fish.Res., Scotland*, 39, 10 pp.

Mills, D. (1989). *Ecology and Management of Atlantic Salmon.* Chapman and Hall. London New York.

Miner, R.W. ed. (1955). The regulation of hunger and appetite. *Ann.N.Y.Acad.Sci.* 63, 1-144.

Mironova, N.N. (1974). Selected problems of fish bioenergetics. *Proc.World Symp.Finfish.Nutr.Fishfeed.Technol.Hamb.* 1, 17-44.

Moore, J.W. and Moore, I.A. (1974). Food and growth of anadromous arctic char, *Salvelinus alpinus* L., in the Cumberland Sound area of Baffin Island. *J.Fish.Biol.* 6,79-92.

Moore, J.W. and Moore, I.A. (1976). The basis of food selection in flounders, *Platichthys flesus* L., in the Severn estuary. *J.Fish.Biol.* 9, 139-156.

Moriarty, D.J.W. and Moriarty, C.M. (1973). The assimilation of carbon from

phytoplankton by two herbivorous fishes: *Tilapia nilotica* and *Haplochromis nigripinnis*. *J.Zool.Lond.* 171, 41-55.

Műller, K. (1954). Investigations on the organic drift in north Swedish streams. *Rep.Inst.Fw.Res.Drottningholm.* 35, 133-148.

Munro, A. L. S.; Ellis, A. E.; McVicar, A. H.; McLay, H. A. and Needham, E. A. (1984). An exocrine pancreas disease of farmed Atlantic salmon in Scotland. *Helgoländer Meeresuntersuchungen*, 37, 571-586.

Mutt, V. (1976). Further investigations on intestinal hormonal polypeptides. *Clin.Endocrinol.* 5 suppl. 183-197.

Mutt, V. and Jorpes, J.E. (1971). Hormonal polypeptides of the upper intestine. *Biochem.J.* 125, 57P-58P.

Mutt, V. and Jorpes, J.E. (1968). Structure of porcine cholecystokinin-pancreozymin. *Eur.J.Biochem.* 6, 156-162.

Mutt, V. and Jorpes, J.E. (1967). Isolation of aspartyl-phenylalanine amide from cholecystokinin-pancreozymin. *Biochem.Biophys.Res.Comm.* 26, 392-397.

Nakamura, E.L. (1968). Visual acuity of two tunas, *Katsuwonus pelamis* and *Euthynnus affinis*. *Copeia* 41-49.

Nevalainen, T.J. (1970). Effects of pilocarpine stimulation on rat pancreatic acinar cells. *Acta Pathol.Microbiol.Scand.* Section A. Supplement no. 210, 10-43.

Niemela E.; McComas, R.L. and Niemela, M. (1985). Salmon (*Salmo salar*) parr densities in the Teno river (Finland). *International Council for the Exploration of the Sea*, C.M.1985/M:23.

Nilsson, A. and Fänge, R. (1969). Digestive processes in the holocephalon fish *Chimaera*

monstrosa (L.). *Comp. Biochem. Physiol.* 31, 147-165.

Nilsson, A. and Fänge, R. (1970). Digestive processes in the cyclostome *Myxine glutinosa* (L.). *Comp.Biochem.Physiol.* 32, 237-250.

Nilsson, S. and Holmgren, S. (1989). Novel neurotransmitters in the autonomic nervous systems of nonmammalian vertebrates. *Pharmac.Ther.* 41, 257-287.

Nilsson, N.-A. (1965). Food segregation between salmonid species in northern Sweden. *Rep.Inst.Fw.Rest.Drottningholm.* 46, 58-78.

Nilsson, N.-A. (1958). On the food competition between two species of Coregonus in a north Swedish lake. *Rep.Inst.Fw.Res.Drottningholm.* 36, 146-161.

Nilsson, N.-A. (1957). On the feeding habits of trout in a stream in northern Sweden. *Rep.Inst.Fw.Res.Drottningholm.* 38, 154-166.

Noaillac-Depeyre, J. and Gas, N. (1978). Ultrastructural and cytochemical study of the gastric epithelium in a freshwater teleosteian fish (*Perca fluviatilis*). *Tissue & Cell.* 10 (1), 23-27.

Noillac-Depeyre, J. and Gas, N. (1974). Fat absorption by the enterocytes of the carp (*Cyprinus carpio* L.). *Cell Tiss. Res.* 155, 353-365.

Noaillac-Depeyre, J. and Hollande, E. (1981). Evidence for somatostatin, gastrin and pancreatic polypeptide-like substances in the mucosa cells of the gut in fishes with and without stomach. *Cell Tissue Res.* 216, 193-203.

Noda, M. and Murakami, K. (1981). Studies on proteinases from the digestive organs of sardine. II. Purification and characterization of two acid proteinases from the stomach. *Biocimica et Biophysica Acta* 658, 27-34.

Norden, C.R. (1961). Comparative osteology of representative salmonid fishes with

particular reference to thr grayling (*Thymallus thymallus*) and its phylogeny. *J.Fish.Res.Bd.Can.* 18, 679-791.

Norris, J.S.; Norris, D.O. and Windell, J.T. (1973). Effect of simulated meal size on gastric acid and pepsin secretory rates in bluegill (*Lepomis macrochirus*). *J.Fish.Res.Bd.Can.* 30(2), 201-204.

Norris, E.R. and Elam, D.W. (1940). Preperation and properties of chrystalline salmon pepsin. *J. Biol. Chem.* 134, 443-454.

Norris, E. R. and Mathies, J. C. (1953). Preparation, properties and crystallization of tuna pepsin. *J. Biol. Chem.* 204, 673-680.

Northcote, T. (1954). Observations on the comparative ecology of two species of fish, *Cottus asper* and *C. rhotheus*, in British Columbia. *Copeia*, 25-28.

Okada, S. and Taniguchi, A. (1971). Size relationships between salmon juveniles in shore waters and their prey animals. *Bull.Fac.Fish.Hokkaido Univ.* 22, 30-36.

Ondetti, M.A.; Rubin, B.; Engel, S.L.; Pluscec, J. and Sheehan, J.T. (1970). Cholecystokinin-pancreozymin: recent developments. *Amer. J. Dig. Dis.* 15, 149-156.

Ong, E.B.; Shaw, E. and Schoellmann, G. (1965).The identification of the histidine residue at the active center of chymotrypsin. *J. Biol. Chem.* 240(2), 694-698.

Onishi, T.; Murayama, S. and Takeuchi, M. (1973). Sequence of digestive enzyme levels in carp after feeding-II. *Bull.Tokai Reg.Fish.Res.Lab.* 75, 33-38.

Onishi, J.; Murayama, S. and Takiuchi, M. (1976). Changes in digestive enzyme levels in carp after feeding-III. *Bull.Jap.Soc.Scient.Fish.* 42, 921-929.

Osborn, R.H.; Simpson, T.H. and Youngson, A.F. (1978). Seasonal and diurnal rythms of thyroidal status in the rainbow trout, *Salmo gairdneri* Richardson. *J.Fish.Biol.* 12, 531-

540.

Osborn, R.H. and Simpson, T.H. (1973). A possible relationship between thyroidal status and the ease of capture of fish. *ICES C.M.* 1973. *Gear and Behaviour Cttee*, Doc. No. B: 23.

Overnell, J. (1973). Digestive enzymes of the pyloric caeca and their associated mesenteries in the cod (*Gadus morhua*). *Comp.Biochem.Physiol.* 46B, 519-531.

Owen, T.G. and Wiggs, A.J. (1971). Thermal compensation in the stomach of brook trout (*Salvelinus fontinalis* Mitchill). *Comp.Biochem.Physiol.* 40B, 465-473.

Owyang, C.; Louie, D.S. and Tatum, D. (1986). Feedback regulation of pancreatic enzyme secretion: suppression of cholecystokinin release by trypsin. *J.Clin.Invest.* 77, 2042-2047.

Palmer, T. (1981). *Understanding Enzymes*. Ellis Horwood Ltd. England.

Pedersen, B.H. (1993). Protein digestion in herring (*Clupea harengus*) larvae: Trypsinogen secretion, and effects of transitory food restriction on mortality, growth and digestive enzyme content. In *Physiological and Biochemical Aspects of Fish Development* (B.T. Walther and H.J. Fyhn, eds.). University of Bergen. Bergen. Norway. Pp. 220-225.

Pernow, B. (1983). Substance P. *Pharmacol.Rev.* 35, 85-141.

Peterson, R.H. (1978). Physical characteristics of Atlantic salmon spawning gravel in some new Brunswick streams. *Fisheries and Environment, Canada, Fisheries and Marine Service Technical Report*, 785, 28 pp.

Pilpott, C. W. and Copeland, D. E. (1963). Fine structure of chloride cells from three species of *Fundulus*. *J. Cell. Biol.* 18, 389-404.

Pilz, J. and Plantikow, H. (1992). Sterile isolation and long-term culture of exocrine cells from the pancreas of pike (*Esox lucius* L.): Influence of culture media and hormone

CCK/Pz. Pp. 1-19 in: Braunbeck, Th.; W. Hanke and H. Segner (eds.). *Fish in Exotoxicology and Ecophysiology*. VCH Verlag Chemie.

Polyak, S. (1957). *The Vertebrate Visual System*. Chicago Univ.Press.

Poort, C. and Geuze, J.J. (1969). The effect of temperature elevation and feeding on the pancreas of *Rana esculenta* in late winter. A bichemical and ultrastructural study. *Zeitschrift für Zellforchung und Mikroscopische Anatomie*, 98, 1-8.

Pope, J.A.; Mills, D.H. and Shearer, W.M. (1961). The fecundity of Atlantic salmon (*Salmo salar* L.). *Freshwat. Salm. Fish. Res. Scotland*, 26, 12 pp.

Pringle, G.M.; Houlihan, D.F.; Callanan, K.R.; Mitchell, A.I.; Raynard, R.S. and Houghton, G.H. (1992). Digestive enzyme levels and histopathology of pancreas disease in farmed Atlantic salmon (*Salmo salar*). *Comp.Biochem.Physiol.* 102A(4), 759-768.

Protozov, V.R. (1968). *Vision and Near Orientation in Fish*. Israel Program Sci.Trans., Jerusalem 1970.

Pulliam, H.R. (1974). On the theory of optimal diets. *Am.Nat.* 108, 59-75.

Raae, A.J. and Walther, B.T. (1989). Purification and characterization of chymotrypsin, trypsin and elastase like proteinases from cod (*Gadus Morhua* L.). *Comp. Biochem. Physiol.* 93 B(2), 317-324.

Rabinovitch, M.; Valeri, V.; Rotschild, H.A.;Camara, S.;Sesso, A. and Junqueira, L.C.U. (1952). Nucleic acid phosphorus of mouse pancreas after pilocarpine administration. *J.Biol.Chem.* 198, 815-820.

Rajjo, I.M.; Vigna, S.R. and Crim, J.W. (1988a). Actions of cholecystokinin related peptides on the gallbladder of bony fishes *in vitro*. *Comp. Biochem. Physiol.* 90 C (1), 267-273.

Rajjo, I.M.; Vigna, S.R. and Crim, J.W. (1988b). Cholecystokinin immunoreactivity in the digestive tract of the bowfin (*Amia calva*), bluegill (*Lepomis macrochirus*), and bullfrog (*Rana catesbeiana*). *Gen.Comp.Endocrinol.* 70, 133-144.

Ranirez, M. and Farrar, J.T. (1970). The effect of secretin and cholecystokinin-pancreozymin on the intraluminal pressure of the jejunum in the unanesthetized dog. *Amer.J.Dig.Dis.N.S.* 15, 539-544.

Reddi, P.K.; Constantinides, S.M. and Dymsza, H.A. (1972). Catheptic activity of fish muscle. *J. Food. Sci.* 37, 643-648.

Reggio, H.; Cailla-Deckmyn, H.; and Marchis-Moureen, G. (1971). Effect of pancreozymin on rat pancreatic enzyme biosynthesis. *J.Cell.Biol.* 50, 333-343.

Rehfeld, J.F. (1978). Multiple molecular forms of cholecystokinin. In *Gut Hormones* (S.R. Bloom, ed.). Churchill Livingstone. Edinburgh, London, New York.

Reifel, C.W.; Marin-Sorensen, M. and Samloff, I.M. (1985). Cellular localization of pepsinogens by immunofluorescence in the gastriontestinal tracts from four species of fish. *Canad.J.Zool.* 63, 1692-1694.

Reith, A.; and Mayhew, T.M., eds. (1988). *Sterology and Morphometry in Electron Microscopy. Problems and Solutions.* Hemisphere Publishing Corporation. New York. London.

Ricker, W.E. (1932). Studies on speckled trout in Ontario. *Publ.Ont.Fish.Res.Lab.* 44, 69-110.

Rimmer, D.M.; Paim, U. and Saunders, R.L. (1984). Changes in the selection of microhabitat by juvenile Atlantic salmon (*Salmo salar*) at the summer-autumn transition in a small river. *Can.J.Fish.Aquat.Sci.* 41, 469-475.

Rodriguez, J. and Deinhardt, F. (1960). Preperation of semi-permanent mounting medium

for fluorescent antibody studies. *Virology* 12, 316-317.

Rothman, S.S. (1972). The release of cholecystokinin-pancreozymin: The effects of duodenal perfusion of various amino acids. In *Nobel SymposioumxVI. Frontiers in Gastrointestinal Hormone Research, Stockholm, July 20-21* (S. Andersson ed.). Almqvist and Wiksell. Upsala.

Rungruangsak, K. and Utne, F. (1981). Effect of different acidified wet feed on protease activities in the digestive tract and on growth rate of Rainbow trout (*Salmo gairdneri* Richardson). *Aquaculture* 22, 67-79.

Sainte-Marie, G. (1962). A paraffin embedding technique for studies employing immunofluorescence. *J. Histochem. Cytochem.* 10, 250-256.

Sakai-Suzuki, J.; Sakaguchi, Y.; Hoshino, S. and Matsumoto, J.J. (1983). Separation of chatepsin D-like proteinase of squid mantle muscle. *Comp. Biochem. Physiol.* 75 B, 409-414.

Sànches-Chiang, L.; Cisternas, E. and Ponce, O. (1987). Partial purification of pepsins from adult and juvenile salmon fish *Oncorhynchus keta*. Effect of NaCl on proteolytic activities. *Comp. Biochem. Physiol.* 87 B(4), 793-797.

Sankaran, H.; Goldfine, I.D.; Deveney, C.W.; Wong, K-Y. and Williams, J.A. (1980). Binding of cholecystokinin to high affinity receptors on isolated rat pancreatic acini. *J.Biol.Chem.* 255, 1849-1853.

Sankaran, H.; Goldfine, I.D.; Bailey, A.; Licko, V. and Williams, J.A. (1982). Relationship of cholecystokinin receptor binding to regulation of biological functions in pancreatic acini. *Am.J.Physiol.* 242, G250-G257.

Saunders, R.L. and Sreedharan, A. (1977). The incidence and genetic implications of sexual maturity in male Atlantic salmon parr. *International Council for the Exploration of the Sea, C.M.*1977/M:21.

Saunders, R.L.; Henderson, E.B. and Glebe, B.D. (1982). Precocious sexual maturation and smoltification in male Atlantic salmon (*Salmo salar*). *Aquaculture*, 28, 211-229.

Saunders, R.L. and Henderson, E.B. (1970). Influence of photoperiod on smolt development and growth of Atlantic salmon (*Salmo salar*). *J.Fish.Res.Board Can.* 27, 1295-1311.

Scapin, S. (1982). Seasonal fluctations of pancreatic activities and cyclic nucleotide levels in the frog *Rana esculenta*. *Comp.Biochem.Physiol.* 71A, 205-209.

Scheele, G.A. and Palade, G.E. (1975). Studies on the Guinea pig pancreas. Parallel discharge of exocrine enzyme activities. *J.Biol.Chem.* 250(7), 2660-2670.

Schick, J.; Kern, H. and Scheele, G. (1984). Hormonal stimulation in the exocrine pancreas results in coordinate and anticoordinate regulation of protein synthesis. *J.Cell.Biol.* 99, 1569-1574.

Schoellmann, G. and Shaw, E. (1963). Direct evidence for the presence of histidine in the active center of chymotrypsin. *Biochemistry* 2(2), 252-255.

Schoener, T.W. (1969). Optimal size and specialisation in constant and fluctating environments: an energy time approach. *Brookhaven Symp.Biol.* 22, 103-114.

Sedar, A.W. (1962). The fine structure of the oxyntic cell in relation to functional activity of the stomach. *Ann.N.Y.Acad.Sci.* 99, 9-29.

Shaw, E.; Mares-Guia, M. and Cohen, W. (1965). Evidence for an active center histidine in trypsin through use of special reagent, 1-chloro-3-tosylamido-7-amino-2-heptanone, the chlorometyl ketone derived from N-tosyl-L-lysine. *Biochemistry* 4(10), 2219-2224.

Shaw, E. and Springhorn, S. (1967). Identification of histidine residue at the active center of trypsin labelled by TLCK. *Biochem. Biophys. Res. Commun.* 27(3), 391-397.

Shiratori, K.; Watanabe, S.; Chey, W.Y.; Lee, K.Y. and Chang, T.-M. (1986). Endogenous cholecystokinin drives gallbladder emptying in dogs. *Am.J.Physiol.* 251(4), G553-G558.

Sigler, P.B.; Blow, D.M.; Matthews, B.W. and Henderson, R. (1968). Structure of crystalline α-chymotrypsin II. A preliminary report including a hypothesis for the activation mechanism. *J. Mol. Biol.* 35, 143-164.

Simpson, B.K. and Haard, N.F. (1984). Purification and characterization of trypsin from the Greenland cod (*Gadus ogac*). 1. Kinetic and thermodynamic charactersitcs. *Can. J. Biochem. Cell. Biol.* 62, 894-900.

Simpson, T.H. and Thorpe, J.E. (1976). Growth bimodality in the Atlantic salmon. *International Council for the Exploration of the Sea.* CM 1976/M22. 7pp.

Sircus, W. (1958). Studies on the mechanism in the duodenum which inhibits gastric secretion. *Quart.J.Exp.Physiol.* 43, 114-133.

Smillie, L.B. and Hartley, B.S. (1966). Histidine sequences in the active centres of some 'serine' proteases. *Biochem. J.* 101, 232-241.

Smit, H. (1967). Influence of temperature on the rate of gastric juice secretion in the brown bullhead, *Ictalurus nebulosus. Comp.Biochem.Physiol.* 21, 125-132.

Smit, H. (1968). Gastric secretion in the lower vertebrates and birds. In *Handbook of Physiology.* Alimentary Canal, Sect. 6, Vol. 5, pp. 2791-2805. Am. Physiol. Soc. Washington, D. C.

Smith, G.P.; Jerome, C.; Cushing, B.J.; Eterno, R. and Simansky, K.J. (1981). Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. *Science,* 213, 1036-1037.

Solomon, D.J. (1978). Some observations on salmon smolt migration in a chalk stream. *J.Fish Biol.* 12, 571-574.

Somero, G.N. (1977). Temperature as a selective factor in protein evolution: the adaptational strategy of "compromise". *J. Exp. Zool.* 194, 175-188.

Sømme, S. (1941). On the high age of smolts at migration in Northern Norway. A preliminary report. Norske Vidensk. Akademie, Oslo. I. Mat.-Naturvklasse No. 16, 5 pp.

Sosiak, A.J.; Randall, R.G. and McKenzie, J.A. (1979). Feeding by hatchery-reared and wild Atlantic salmon (*Salmo salar*) parr in streams. *J.Fish.Res.Bd.Can.* 36, 1408-1412.

Squires, E.J.; Haard, N.F. and Feltham, L.A.W. (1986). Gastric proteases of the Greenland cod, Gadus ogac. II. Structural properties. *Biochem. Cell. Biol.* 64, 215-222.

Steedman, H.F. (1957). Polyester wax. A new ribboning embedding medium for histology. *Nature*, 4574, 1345.

Stevens, E.D. and McLeese, J.M. (1988). Kinetics of trypsin from rainbow trout are not influenced by the level of feeding. *Comp. Biochem. Physiol.* 90 B (2), 375-377.

Stewart, J.A.; Lee, H.S. and Dobson, J.E. (1963). Evidence for a functional carboxyl group in trypsin and chymotrypsin. *J. Am. Chem.Soc.* 85, 1537-1538.

Stradmeyer, L. (1992). Appearance and taste of pellets influence feeding behaviour of Atlantic salmon. In *The Importance of Feeding Behaviour for the Efficient Culture of Salmonid Fishes* (J.E. Thorpe and F.A. Huntingford, eds.). *World Aquaculture Workshops*, Number 2. The World Aquaculture Society. Louisiana State University. Pp: 21-28.

Stroud, R.M. (1974). A family of protein-cutting proteins. *Sci. Amer.* 231(1), 74-88.

Stryer, L. (1981). *Biochemistry*. W.H. Freeman and Co. New York. San Fransisco.

Susini, C.; Estival, A.; Scanini, J.L.; Ruellan, C.; Vayasse, N.; Clemente, F.; Esteve, J.P.; Fourmy, D. and Ribet, A. (1986). Studies on human pancreatic acini: action of

secretagogues on amylase release and cellular cyclic AMP accumulation. *Pancreas*, 1, 124-129.

Talbot, C. and Higgins, P.J. (1982). Observations on the gall bladder of juvenile Atlantic salmon *Salmo salar* L., in relation to feeding. *J.Fish.Biol.* 21, 663-669.

Talbot, C. and Higgins, P.J. (1983). A radiographic method for feeding studies on fish using metallic iron powder as a marker. *J.Fish Biol.* 23, 211-220.

Talbot, C.; Higgins, P.J. and Shanks, A.M. (1984). Effects of pre-and post-prandial starvation on meal size and evacuation rate of juvenile Atlantic salmon, *Salmo salar* L. *J.Fish Biol.* 25, 551-560.

Tamura, T. (1957). A study of visual perception in fish, especially on resolving power and accommodation. *Bull.Jap.Soc.Sci.Fish.* 22, 536-557.

Tang, J. (1976). Pepsin and pepsinogen: models for carboxyl (acid) proteases and their zymogens. *Trends in Biochem. Sci.* 1, 205-208.

Tardini, A.; Amersa, P.; Bordi, C.; Bertaccini, G. and Impecciatore, M. (1971). Ultrastructural and biochemical changes after marked caerulein stimulation of the exocrine pancreas of the dog. *Am.J.Pathol.* 62, 35-56.

Tartakoff, A.M.; Jamieson, J.D.; Scheele, G.A. and Palade, G.A. (1975). Studies on the pancreas of the Guinea pig. Parallel processing and discharge of exocrine proteins. *J.Biol.Chem.* 250(7), 2671-2677.

Theo, L.H.; Chen, T.W. and Tan, L.L. (1990). The proteases of the common Malayan toad, *Bufo melanostictus* Schneider. *Comp. Biochem. Physiol.* 96 B(4), 715-720.

Thomas, J.D. (1962). The food of brown trout (Salmo trutta) and its feeding relationship with the salmon parr (Salmo salar) and the eel (Anguilla anguilla) in the River Teify, West Wales. *J.Anim.Ecol.* 31, 175-205.

Thorpe, J.E. (1977). Bimodal distribution of length of juvenile Atlantic salmon (*Salmo salar* L.) under artificial rearing conditions. *J.Fish Biol.* 11, 175-184.

Thorpe, J.E. (1981). Rearing salmonids in freshwater. In *Aquarium Systems* (A.D. Hawkins, ed.). Academic Press. London, pp. 325-344.

Thorpe, J.E.; Adams, C.E.; Miles, M.S. and Keay, D.S. (1989). Some influences of photoperiod and temperature on opportunity for growth in juvenile Atlantic salmon, *Salmo salar* L. *Aquaculture*, 82, 119-126.

Thorpe, J.E.; Miles, M.S. and Keay, D.S. (1984). Developmental rate, fecundity and egg size in Atlantic salmon, *Salmo salar* L. *Aquaculture*, 43, 289-305.

Thorpe, J.E. and Morgan, R.I.G. (1978). Parental influence on growth rate, smolting rate and survival in hatchery reared juvenile Atlantic salmon, *Salmo salar* L. *J.Fish Biol.* 13, 549-556.

Thorpe, J.E.; Morgan, R.I.G.; Ottaway, E.M. and Miles, M.S. (1980). Time of divergence of growth groups between potential 1+ and 2+ smolts among sibling Atlantic salmon. *J.Fish Biol.* 17, 13-21.

Thorpe, J.E.; Metcalfe, N.B. and Huntingford, F.A. (1991). Behavioral influences on life-history variation in juvenile Atlantic salmon, *Salmo salar. Environmental Biology of Fishes.* Kluwer Academic publishers. Netherlands.

Tomarelli, R.M.; Charney, J. and Harding, M.L. (1949). The use of azoalbumin as a substrate in the colorimetric determination of peptic and tryptic activity. *J. Lab. Clin. Med.* 34, 428-433.

Torrissen, K.R. and Torrissen, O.J. (1985). Protease activities and carotenoid levels during the sexual maturation of Atlantic salmon (*Salmo salar*). *Aquaculture*, 50, 113-122.

Torrissen, K.R. and Torrissen, O.J. (1984). Digestive proteases of Atlantic salmon (*Salmo*

salar) from different river strains: Development after hatching, rearing temperature effect and effect of sexand maturation. *Comp.Biochem.Physiol.* 77B(1), 15-20.

Torrissen, K.R. (1991). Genetic variation in growth rate of Atlantic salmon with different trypsin-like isozyme patterns. *Aquaculture*, 93, 299-312.

Torrissen, K.R. and Shearer, K.D. (1992). Protein digestion, growth and food conversion in Atlantic salmon and Arctic charr with different trypsin-like isozyme patterns. *J.Fish.Biol.* 41, 409-415.

Tracy, H.J. and Gregory, A.A. (1964). Physiological properties of a series of synthetic peptides structurally related to gastrin I. *Nature (London)* 204, 935-938.

Trier, J. S. and Rubin, C. E. (1965). Electron microscopy of the small intestine: a review. *Gastroenterology* 49, 574-603.

Twining, S.S.; Alexander, P.A.; Huibregtse, K. and Glick, D.M. (1983). A pepsinogen from rainbow trout. *Comp. Biochem. Physiol.* 75 B, 109-112.

Tyler, J.E. and Preisendorfer, R.W. (1962). Transmission of energy within the sea: 8, light. In *The Sea* (M.N. Hill, ed.), pp.397-451. John Wiley. London.

Uchida, N.; Tsukayama, K. and Nishide, E. (1984). Properties of two main anionic trypsins from the pyloric caeca of the Chum salmon. *Bull.Jap.Soc.Sci.Fish.* 50(2), 313-321.

Usher, M.L.; Talbot, C. and Eddy, F.B. (1988). Drinking in Atlantic salmon smolts transferred to seawater and the relationship between drinking and feeding. *Aquaculture*, 73, 237-246.

Usher, M.L.; Talbot, C. and Eddy, F.B. (1990). Effects of transfer to seawater on digestion and gut function in Atlantic salmon smolts (*Salmo salar* L.). *Aquaculture*, 90, 85-96.

Ushiyama, H. (1968). Studies on the proteolytic enzymes of the salmon pyloric caeca II. Purification and some properties of trypsin-like enzymes. *Bull.Fac.Fish.Hokkaido Univ.* 19(2), 147-155.

Van Venrooij, W.J. and Poort, C. (1971). Rate of protein synthesis and polyribosome formation in the frog pancreas after fasting and feeding. *Biochim.Biophys.Acta*, 247, 468-470.

Vigna, S.R. and Gorbman, A. (1977). Effects of cholecystokinin, gastrin, and related peptides on coho salmon gallbladder contraction in vitro. *Am.J.Physiol.* 232, E485-E491.

Vigna, S.R.; Fischer, B.L.; Morgan, J.L.M. and Rosenquist, G.L. (1985). Distribution and molecular heterogeneity of cholecystokinin-like immunoreactive peptides in the brain and gut of the rainbow trout, *Oncorhynchus mykiss*. *Comp.Biochem.Physiol.* 82C(1), 143-146.

Vigna, S.R. (1983). Evolution of endocrine regulation of gastrointestinal function in lower vertebrates. *Amer.Zool.* 23, 729-738.

Vigna, S.R. (1979). Distinction between cholecystokinin-like and gastrin-like biological activities extracted from gastrointestinal tissues of some lower vetebrates. *Gen. Comp. Endocrinol.* 39, 512-520.

Vonk, H.J. (1937). The specificity and collaboration of digestive enzymes in Metazoa. *Biol. Rev.* 12, 245-284.

Vonk, H.J. and Western, J.R.H. (1984). *Comparative Biochemistry and Physiology of Enzyme Digestion*. Academic Press. New York.

Walsh, J.H. (1978). Gastrointestinal hormones. In *Physiology of the Gastrointestinal Tract*, 2nd edn. (L.R. Johnson, ed.), pp. 181-254. Raven Press. New York.

Walsh, J.H.; Maxwell, V.; Ferrari, J. and Varner, A.A. (1981). Bombesin stimulates gastrin function by gastrin-dependent and independent mechanisms. *Peptides*, 2, 193-198.

Wang, C.C. and Grossman, M.I. (1951). Physiological determination of release of secretin and pancreozymin from intestine of dogs with transplanted pancreas. *Am.J.Physiol.* 164, 527-544.

Wankowski, J.W.J. (1977). *The role of prey size in the feeding behaviour and growth of juvenile Atlantic salmon* (*Salmo salar* L.). PhD. Thesis, University of Stirling, Scotland.

Wankowski, J.W.J. (1981). Behavioural aspects of predation by juvenile Atlantic salmon (*Salmo salar* L.) on particulate, drifting prey. *Anim.Behav.* 29, 557-571.

Wankowski, J.W.J. (1979). Morphological limitations, prey size selectivity, and growth response of juvenile atlantic salmon, *Salmo salar*. *J.Fish.Biol.* 14, 89-100.

Wankowski, J.W.J. and Thorpe, J.E. (1979a). Spatial distributionin and feeding the Atlantic salmon, *Salmo salar* L. juveniles. *J.Fish.Biol.* 14, 351-370.

Wankowski, J.W.J. and Thorpe, J.E. (1979b). The role of food particle size in the growth of juvenile Atlantic salmon (*Salmo salar* L.). *J.Fish.Biol.* 14, 351-370.

Ware, D.M. (1973). Risk of epibenthic prey to predation by rainbow trout (*Salmo gairdneri*). *J.Fish.Res.Bd.Can.* 30, 787-797.

Ware, D.M. (1971). Predation by rainbow trout (*Salmo gairdneri*): the effect of experience. *J.Fish.Res.Bd.Can.* 28, 1847-1852.

Weinreb, E.L. and Bilstad, N.M. (1955). Histology of the digestive tract and adjacent structures of the rainbow trout, *Salmo gairdneri irideus*. *Copeia*, 3, 194-204.

Werner, E.E. (1974). The fish size, prey size, handling time relation in several sunfishes and some implications. *J.Fish.Res.Bd.Can.* 31, 1531-1536.

Western, J. R. H. and Jennings, J. B. (1970). Histochemical demonstration of hydrochloric

acid in the gastric tubules of teleosts using an *in vivo* prussian blue technique. *Comp.Biochem.Physiol.* 35, 879-884.

Whitehead, P.J.P.; Bauchot, M.-L.; Hureau, J.-C.; Nielsen, J. and Tortonese, E., eds., (1986). *Fishes of the North-eastern Atlantic and the Mediterranean.* United Nations Educational, Scientific and Cultural Organization. Paris.

Wicker, C.; Puigserver, A. and Scheele, G. (1984). Dietary regulation of levels of active mRNA coding for amylase and serine protease zymogens in the rat pancreas. *Eur.J.Biochem.* 139, 381-387.

Wicker, C.; Puigserver, A.; Rausch, U.; Scheele, G. and Kern, H. (1985). Multiple-level caerulein control of the gene expression of secretory proteins in the rat pancreas. *Eur.J.Biochem.* 151, 461-466.

Wolf, K. (1963). Physiological salines for freshwater teleosts. *Progre. Fish Cult.* 25, 135-140.

Wong, B. and Ward, F.J. (1972). Size selection of *Daphnia pulicaria* by yellow perch fry in West Blue Lake, Manitoba. *J.Fish.Res.Bd.Can.* 29, 1761-1764.

Yasuda, F. (1960). The feeding mechanisms of some carnivorous fishes. *Record of Oceanographic works in Japan* 5, 153-160.

Yasugi, S. (1988). Presence of pepsinogens immunoreactive to anti-embryonic chicken pepsinogen antiserum in fish stomachs: Possible ancestor molecules of chymosin of higher vertebrates. *Comp.Biochem.Physiol.* 91A(3), 565-569.

Yasugi, S. (1987). Pepsinogen-like immunoreactivity among vertebrates: Occurrence of common antigenicity to an anti-chicken pepsinogen antiserum in stomach gland cells of vertebrates. *Comp. Biochem. Physiol.* 86 B(4), 675-680.

Yasugi, S.; Matsunaga, T. and Mizuno, T. (1988). Presence of pepsinogens

immunoreactive to anti-embryonic chicken pepsinogen antiserum in fish stomachs: Possible ancestor molecules of chymosin of higher vertebrates. *Comp.Biochem.Physiol.* 91A(3), 565-569.

Yasugi, S.; Hayashi, K.; Takiguchi, K.; Mizuno, T.; Mochii, M.; Kodama, R.; Agata, K. and Eguchi, G. (1987). Immunological relationships among embryonic and adult chicken pepsinogens: A study with monoclonal and polyclonal antibodies. *Develop. Growth and Differ.* 29(1), 85-91.

Yasutake, W.T. and Wales, J.H. (1983). *Microscopic Anatomy of Salmonids: An Atlas.* United States Department of the Interior Fish and Wildlife Service. Washington, D.C.

Yoshinaka, R.; Tanaka, H.; Sato, M. and Ikeda, S. (1982). Purification and some properties of elastase from the pancreas of catfish. *Bull. Jap. Soc. Sci. Fish.* 48(4), 573-579.

Yoshinaka, R.; Sato, M. and Ikeda, S. (1981a). Distribution of trypsin and chymotrypsin and their zymogens in digestive system of catfish. *Bull.Jap.Soc.Sci.Fish.* 47(12), 1615-1618.

Yoshinaka, R.; Sato, M. and Ikeda, S. (1981b). *In vitro* activation of trypsinogen and chymotrypsinogen in the pancreas of catfish. *Bull. Jap.Soc. Sci. Fish.* 47(11), 1473-1478.

Yoshinaka, R.; Sato, M.; Sato, T. and Ikeda, S. (1984). Distribution of trypsin and chymotrypsin, and their zymogens in digestive organs of eel (*Anguilla japonica*). *Comp.Biochem.Physiol.* 78B(3), 569-573.

Yoshinaka, R.; Sato, M. and Ikeda, S. (1978). Participation of collagenase in the digestion of collagen by rainbow trout. *Bull.Jap.Soc.Sci.Fish.* 44, 639-641.

Zar, J.H. (1984). *Biostatistical Analysis.* Second edition. Simon & Schuster Co. New Jersey.

Zendzian, E.N. and Barnard, E.A. (1967). Distributions of pancreatic ribonuclease, chymotrypsin and trypsin in vertebrates. *Arch. Biochem. Biopys.* 122, 699-713.

Zordidi, Z.K. (1970). Seasonal and ontogenic changes in the feeding of coho salmon juveniles in the Plotnikova and Avacha rivers. *Izv.T.I.N.R.O.* 78, 129-150. *Fish.Res.Bd.Can.Transl.Ser.* 2440, 33pp.

X.Appendices.

Chapter III.

Kinetics of pepsin activity (See III. 3.1.1.):

The regression eqution is

$1/V_o = 0.00503 + 0.00107 * 1/S_o$

Table III.1:

Predictor	Coefficient	Standard dev.	t-ratio	p-value
Constant	0.0050255	0.0001481	33.94	< 0.0005
1/So	0.00107499	0.00004284	25.09	< 0.0005

Table III.2:

Standard error	Correlation coefficient= R-squared	Correlelation coefficient= R-squared (adj.)
0.0003621	0.980	0.978

Table III.3: Analysis of variance:

Source	DF	SS	MS	F	p-value
Regression	1	0.000082564	0.000082564	629.54	< 0.0005
Error	13	0.000001705	0.000000131		
Total	14	0.000084269			

Table III.4: Pure error test:

F	DF (pure error)	p-value
1.68	10	0.2335

Kinetics of trypsin activity (See III. 3.2.1.):

The regression eqution is

$1/V_o = 0.0117 + 0.00191 * 1/S_o$

Table III.5:

Predictor	Coefficient	Standard dev.	t-ratio	p-value
Constant	0.0116717	0.0007433	15.70	< 0.0005
1/So	0.00190934	0.00006619	28.85	< 0.0005

Table III.6:

Standard error	Correlation coefficient= R-squared	Correlelation coefficient= R-squared (adj.)
0.003159	0.972	0.971

Table III.7: Analysis of variance:

Source	DF	SS	MS	F	p-value
Regression	1	0.0083062	0.0083062	832.17	< 0.0005
Error	24	0.0002396	0.0000100		
Total	25	0.0085458			

Table III.8: Pure error test:

F	DF (pure error)	p-value
0.67	13	0.7424

Kinetics of chymotrypsin activity (See III. 3.3.1.):

The regression eqution is

$1/V_o = 0.00746 + 0.000218 * 1/S_o$

Table III.9:

Predictor	Coefficient	Standard dev.	t-ratio	p-value
Constant	0.0074640	0.0001353	55.16	< 0.0005
1/So	0.00021825	0.00001045	20.89	< 0.0005

Table III.10:

Standard error	Correlation coefficient= R-squared	Correlelation coefficient= R-squared (adj.)
0.0005776	0.948	0.946

Table III.11: Analysis of variance:

Source	DF	SS	MS	F	p-value
Regression	1	0.00014560	0.00014560	436.37	< 0.0005
Error	24	0.00000801	0.00000033		
Total	25	0.00015361			

Table III.12: Pure error test:

F	DF (pure error)	p-value
0.64	13	0.7645

The effect of trypsin on the chymotrypsin assay (See III. 3.5.):

Tukey's tests:

Table III.13: The effect of TLCK on α-chymotrypsin:

Source	DF	SS	MS	F	p-value
Factor	1	0.12	0.12	0.02	> 0.50
Error	10	61.39	6.14		

Total	11	61.51			

Table III.14: The effect of TLCK on chymotrypsin activity in salmon intestine:

Source	DF	SS	MS	F	p-value
Factor	1	35.16	35.16	13.78	0.005 > p > 0.001
Error	10	25.52	2.55		
Total	11	60.67			

Chapter IV.

The *in vivo* effect of cholecystokinin at 4.6 °C for fish acclimatised in the temperature range 1.3-8.8 °C (See IV.3.1.):

Table IV.1: Trypsin activity in the pancreatic tissue and the pyloric caeca; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	4	11816	2954	2.87	0.044
Error	25	25753	1030		
Total	29	37569			

Table IV.2: Trypsin activity in the pancreatic tissue and the pyloric caeca; Tukey's tests:

CCK doses			
(C.u./g)	q	p	Null hypothesis: H_o
0.000 vs 0.001	0.19	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	1.12	3	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.100	2.40	4	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.500	2.56	5	$p > 0.05 \Rightarrow$ Accept H_o

Standard error = 18.52925. Degrees of freedom = 25. k = 5.

Table IV.3: Trypsin activity in the intestinal digesta; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	4	4023	1006	4.47	0.007
Error	25	5630	225		
Total	29	9654			

Table IV.4: Trypsin activity in the intestinal digsta; Tukey's tests:

CCK doses			
(C.u./g)	q	p	**Null hypothesis: H_o**
0.000 vs 0.001	3.36	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	4.19	3	$p < 0.05 \Rightarrow$ Reject H_o
0.000 vs 0.100	4.25	4	$p < 0.05 \Rightarrow$ Reject H_o
0.000 vs 0.500	4.93	5	$p < 0.05 \Rightarrow$ Reject H_o

Standard error = 8.660254. Degrees of freedom = 25. k = 5.

Table IV.5: Chymotrypsin activity in the pancreatic tissue and the pyloric caeca; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	4	2288759	572190	1,37	0.273
Error	25	10456166	418247		
Total	29	12744925			

Table IV.6: Chymotrypsin activity in the pancreatic tissue and the pyloric caeca; Tukey's tests:

CCK doses			
(C.u./g)	q	p	**Null hypothesis: H_o**
0.000 vs 0.001	0.51	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	1.19	3	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.100	2.02	4	$p > 0.05 \Rightarrow$ Accept H_o

0.000 vs 0.500	1.67	5	$p > 0.05 \Rightarrow$ Accept H_o

Standard error = 373.3827. Degrees of freedom = 25. k = 5.

Table IV.7: Chymotrypsin activity in the intestinal digesta; one way analysis of variance:

Source	**DF**	**SS**	**MS**	**F**	**p-value**
Factor	4	898176	224544	3.54	0.020
Error	25	1584517	63381		
Total	29	2482693			

Table IV.8: Chymotrypsin activity in the intestinal digsta; Tukey's tests:

CCK doses			
(C.u./g)	**q**	**p**	**Null hypothesis: H_o**
0.000 vs 0.001	3.01	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	3.45	3	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.100	4.21	4	$p < 0.05 \Rightarrow$ Reject H_o
0.000 vs 0.500	4.37	5	$p < 0.05 \Rightarrow$ Reject H_o

Standard error = 145.3570. Degrees of freedom = 25. k = 5.

Table IV.9: Discharge of the gallbladder; Kruskal-Wallis's test:

CCK (C.u./g):	**0.000**	**0.001**	**0.010**	**0.100**	**0.500**
Gallbladder	No	No	No	Yes	Yes
discharge	No	No	No	Yes	Yes
	No	No	No	Yes	Yes
	No	No	Yes	Yes	Yes
	No	No	Yes	Yes	Yes
	No	No	Yes	Yes	Yes

n:	6	6	6	6	6

Table IV.10:

Rank:	1	2	3	4	5
Gallbladder	8	8	8	23	23
discharge	8	8	8	23	23
	8	8	8	23	23
	8	8	23	23	23
	8	8	23	23	23
	8	8	23	23	23
Rank sum:	48	48	93	138	138
$(\text{Rank sum})^2$:	2304	2304	8649	19044	19044
$(\text{Rank sum})^2/n$:	384	384	1441.5	3174	3174
Mean ranks:	8.00	8.00	15.50	23.00	23.00

Number of groups of tied ranks = 2. C = 0.750834. k = 5. N = 30.

Sum of rank sums = 465. Sum of $(\text{Rank sum})^2/n$ = 8557.5.

H_o: No significant difference for the gallbladde discharge for the various CCK doses.

H = 17.41935. Sum of T = 6720.

H(C) = 23.2. v = k-1 = 4.x(0.001,4) = 18.467. H(C) >x(0.001,4) $\Rightarrow$ Reject H_o ($p < 0.001$).

Table IV.11: Multiple comparisons of a control with ties:

Ranks	Diff.in means	Stand.err.	Q	k
R1 vs R2	0.0	4.40	0.000	2
R1 vs R3	7.5	4.40	1.703	3
R1 vs R4	15.0	4.40	3.406	4
R1 vs R5	15.0	4.40	3.406	5

Table IV.12:

Null hypothesis: H_o	Conclusion
0.000 C.u./g = 0.001 C.u./g	H_o cannot be rejected
0.000 C.u./g = 0.010 C.u./g	H_o rejected: $0.1 > p > 0.05$

0.000 C.u./g = 0.100 C.u./g	H_o rejected: 0.001 > p > 0.0005
0.000 C.u./g = 0.500 C.u./g	H_o rejected: 0.0025 > p > 0.001

The *in vivo* effect of cholecystokinin at 8.0 °C for fish acclimatised in the temperature range 8.0-10.0 °C (See IV.3.1.):

Table IV.13: Trypsin activity in the pancreatic tissue and the pyloric caeca; one way analysis of variance:

Source	**DF**	**SS**	**MS**	**F**	**p-value**
Factor	4	8652	2163	9.37	< 0.0005
Error	25	5771	231		
Total	29	14424			

Table IV.14: Trypsin activity in the pancreatic tissue and pyloric caeca; Tukey's tests:

CCK doses			
(C.u./g)	**q**	**p**	**Null hypothesis: H_o**
0.000 vs 0.001	0.63	2	p > 0.05 ⇒ Accept H_o
0.000 vs 0.010	0.47	3	p > 0.05 ⇒ Accept H_o
0.000 vs 0.100	5.35	4	p < 0.01 ⇒ Reject H_o
0.000 vs 0.500	5.23	5	p < 0.01 ⇒ Reject H_o

Standard error = 8.774964. Degrees of freedom = 25. k = 5.

Table IV.15: Trypsin activity in the intestinal digesta; one way analysis of variance:

Source	**DF**	**SS**	**MS**	**F**	**p-value**
Factor	4	478	120	1.18	0.344
Error	25	2536	101		
Total	29	3014			

Table IV.16: Trypsin activity in the intestinal digesta; Tukey's tests:

CCK doses			
(C.u./g)	q	p	Null hypothesis: H_o
0.000 vs 0.001	0.57	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	1.30	3	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.100	0.51	4	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.500	0.46	5	$p > 0.05 \Rightarrow$ Accept H_o

Standard error = 5.802298. Degrees of freedom = 25. k = 5.

Table IV.17: Chymotrypsin activity in the pancreatic tissue and the pyloric caeca; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	4	3635005	908751	14.16	< 0.0005
Error	25	1604051	64162		
Total	29	5239056			

Table IV.18: Chymotrypsin activity in the pancreatic tissue and pyloric caeca; Tukey's tests:

CCK doses			
(C.u./g)	q	p	Null hypothesis: H_o
0.000 vs 0.001	1.71	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	2.01	3	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.100	6.50	4	$p = 0.001 \Rightarrow$ Reject H_o
0.000 vs 0.500	5.58	5	$p < 0.005 \Rightarrow$ Reject H_o

Standard error = 146.2418. Degrees of freedom = 25. k = 5.

Table IV.19: Chymotrypsin activity in the intestinal digesta; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	4	143861	35965	1.33	0.288
Error	25	677772	27111		

Total	29	821633			

Table IV.20: Chymotrypsin activity in the intestinal digesta; Tukey's tests:

CCK doses			
(C.u./g)	**q**	**p**	**Null hypothesis: H_o**
0.000 vs 0.001	0.06	2	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.010	1.73	3	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.100	1.51	4	$p > 0.05 \Rightarrow$ Accept H_o
0.000 vs 0.500	0.48	5	$p > 0.05 \Rightarrow$ Accept H_o

Standard error = 95.06839. Degrees of freedom = 25. k = 5.

Table IV.21: Discharge of the gallbladder; Kruskal-Wallis's test:

CCK (C.u./g):	**0.000**	**0.001**	**0.010**	**0.100**	**0.500**
Gallbladder	No	No	No	No	Yes
discharge	No	No	No	No	Yes
	No	No	No	Yes	Yes
	No	No	No	Yes	Yes
	No	No	No	Yes	Yes
	No	No	Yes	Yes	Yes

n:	6	6	6	6	6

Table IV.22:

Rank:	**1**	**2**	**3**	**4**	**5**
Gallbladder	10	10	10	10	25
discharge	10	10	10	10	25
	10	10	10	25	25
	10	10	10	25	25
	10	10	10	25	25

	10	10	25	25	25
Rank sum:	60	60	75	120	150
(Rank sum)^2:	3600	3600	5625	14400	22500
(Rank sum)^2/n:	600	600	937.5	2400	3750
Mean ranks:	10.00	10.00	12.50	20.00	25.00

Number of groups of tied ranks = 2. C = 0.697441. k = 5. N = 30.

Sum of rank sums = 465. Sum of (Rank sum)^2/n = 8287.5.

H_o: No significant difference for the gallbladde discharge for the various CCK doses.

H = 13.93548. Sum of T = 8160.

H(C) = 19.98086. v = k-1 = 4.x(0.001,4) = 18.467. H(C) >x(0.001,4) $\Rightarrow$ Reject H_o ($p < 0.001$).

Table IV.23: Multiple comparisons of a control with ties:

Ranks	**Diff.in means**	**Stand.err.**	**Q**	**k**
R1 vs R2	0.0	4.24	0.000	2
R1 vs R3	2.5	4.24	0.589	3
R1 vs R4	10.0	4.24	2.356	4
R1 vs R5	15.0	4.24	3.534	5

Table IV.24:

Null hypothesis: H_o	**Conclusion**
0.000 C.u./g = 0.001 C.u./g	H_o cannot be rejected
0.000 C.u./g = 0.010 C.u./g	H_o cannot be rejected
0.000 C.u./g = 0.100 C.u./g	H_o rejected: 0.05 > p > 0.025
0.000 C.u./g = 0.500 C.u./g	H_o rejected: 0.001 > p > 0.0005

The *in vitro* effect of cholecystokinin (See IV.3.2.):

Table IV.25: *t*-test for pairwise data, n = 6, tested against 0:

Enzyme	Stand.err.of mean	*t*-value	*p*-value
Trypsin	0.71	29.44	< 0.00005
Chymotrypsin	34.09	9.72	0.0002

Chapter V.

Pepsin activity in the stomach (See V.3.1.2.):

Table V.1: Pepsin activity in the stomach wall; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	3	770.46	256.82	27.26	< 0.0005
Error	16	150.73	9.42		
Total	19	921.19			

Table V.2: Pepsin activity in the stomach wall; Bonferroni; one tailed:

Stomach area	DF	*t*-value	*p*-value
OP vs CA	4	5.09	0.0035
CA vs CP	4	0.78	0.23
CP vs PA	4	8.32	0.0006

Mobilisation of pancreatic granules by CCK (See V.3.2.1.):

Table V.3: Three factor analysis of variance of transformed granule to acinar tissue point counts:

Source	DF	SS	MS	F-value	*p*-value
Fish	1	0.01	0.01	0.00	0.977
Sample	1	18.94	18.94	1.32	0.253

Treatment	1	5514.16	5514.16	384.07	< 0.0005
Fish * Sample	1	29.88	29.88	2.08	0.151
Fish * Treatment	1	4.45	4.45	0.31	0.578
Sample * Treatment	1	4.28	4.28	0.30	0.586
Fish * Sample * Treatment	1	23.62	23.62	1.65	0.202
Error	152	2182.28	14.36		
Total	159	7777.63			

Trypsin and chymotrypsin in pancreas, pyloric caeca and intestine (See V.3.2.2.):

Table V.4: Trypsin and chymotrypsin activities in the pancreas, pyloric caecal wall and intestinal wall; Bonferroni; one tailed:

CCK vs control	**DF**	***t*-value**	***p*-value**
Trypsin: pancreas	7	6.35	0.0002
Chymotrypsin: pancreas	8	6.02	0.0001
Trypsin: pyloric caecal wall	13	0.58	0.29
Chymotrypsin: pyloric caecal wall	13	0.16	0.44
Trypsin: Intestinal wall	12	0.72	0.76
Chymotrypsin: intestinal wall	7	2.10	0.96

Chapter VI.

The effect of starvation on pepsin, trypsin and chymotrypsin, and the gallbladder (See VI.3.1.):

Table VI.1: Stored pepsin during starvation; two way ANOVA:

Source	**DF**	**SS**	**MS**	**F**	**p-values**
Feed/Starv	1	81085	81085	15.96	< 0.0005

Days	8	100832	12604	2.48	0.018
Interaction	8	58867	7358	1.45	0.188
Error	90	457335	5082		
Total	107	698120			

Table VI.2: Stored pepsin during starvation; Bonferroni; one tailed test:

Pepsin	**DF**	***t*-value**	***p*-value**
Day 2	8	1.8	0.054

Table VI.3: Stored trypsin during starvation; two way ANOVA:

Source	**DF**	**SS**	**MS**	**F**	**p-values**
Feed/Starv	1	1259947	1259947	89.61	< 0.0005
Days	8	886439	110805	7.88	< 0.0005
Interaction	8	543667	67958	4.83	< 0.0005
Error	90	1265445	14060		
Total	107	3955499			

Table VI.4: Stored trypsin during starvation; Bonferroni; one tailed test:

Trypsin	**DF**	***t*-value**	***p*-value**
Day 2	6	3.36	0.0076
Day 16	9	5.60	0.0002
Day 20	9	0.95	0.18

Table VI.5: Stored chymotrypsin during starvation; two way ANOVA:

Source	**DF**	**SS**	**MS**	**F**	**p-values**
Feed/Starv	1	364493856	364493856	98.64	< 0.0005
Days	8	270947136	33868392	9.17	< 0.0005
Interaction	8	157694016	19711752	5.33	< 0.0005

Error	90	332572864	3695254		
Total	107	1.126E+09			

Table VI.6: Stored chymotrypsin during starvation; Bonferroni; one tailed test:

Chymotrypsin	**DF**	***t*-value**	***p*-value**
Day 2	6	3.96	0.0037
Day 16	8	10.94	< 0.00005

The effect of food on pepsin (See VI.3.2.):

Table VI.7: Stomach digesta; two way ANOVA:

Source	**DF**	**SS**	**MS**	**F**	**p-values**
Feed/Starv	1	14.8160	14.8160	174.60	< 0.0005
Hours	6	9.4656	1.5776	18.59	< 0.0005
Interaction	6	9.5700	1.5950	18.80	< 0.0005
Error	70	5.9401	0.0849		
Total	83	39.7917			

Table VI.8: Stomach digesta; Bonferroni; one tailed test:

Digesta	**DF**	***t*-value**	***p*-value**
1 hours	5	4.50	0.0032

Table VI.9: Pepsin in mucosa; two way ANOVA:

Source	**DF**	**SS**	**MS**	**F**	**p-values**
Feed/Starv	1	20632	20632	5.66	0.020
Hours	6	94970	15828	4.34	0.001
Interaction	6	78927	13155	3.61	0.004
Error	70	255231	3646		

Total	83	449760			

Table VI.10: Pepsin in mucosa; Bonferroni; one tailed test:

Pepsin	DF	*t*-value	*p*-value
1 hours	9	2.77	0.011
7 hours	6	2.82	0.015
25 hours	9	3.33	0.0044

Table VI.11: Pepsin in digesta; two way ANOVA:

Source	DF	SS	MS	F	p-values
Feed/Starv	1	81074	81074	79.76	< 0.0005
Hours	6	80720	13453	13.24	< 0.0005
Interaction	6	83664	13944	13.72	< 0.0005
Error	70	71151	1016		
Total	83	316609			

Table VI.12: Pepsin in digesta; Bonferroni; one tailed test:

Pepsin	DF	*t*-value	*p*-value
1 hours	9	6.90	< 0.00005
4 hours	8	5.13	0.0005
7 hours	9	2.19	0.028

Table VI.13: Total pepsin; two way ANOVA:

Source	DF	SS	MS	F	p-values
Feed/Starv	1	19908	19908	4.09	0.047
Hours	6	270307	45051	9.25	< 0.0005
Interaction	6	262557	43760	8.98	< 0.0005
Error	70	340982	4871		

Total	83	893754			

Table VI.14: Total pepsin; Bonferroni; one tailed test:

Pepsin	DF	*t*-value	*p*-value
1 hours	9	6.15	0.0001
2 hours	9	4.52	0.0007
4 hours	7	0.26	0.40
7 hours	6	2.07	0.042
25 hours	9	2.67	0.013

The effect of food on trypsin and chymotrypsin, and on the gallbladder (See VI.3.3.):

Digesta (See 3.3.1.):

Table VI.15: Digesta in pyloric caecal area; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Feed/Starv	1	0.27743	0.20959	0.20959	17.68	< 0.0005
Hours	5	0.65617	0.47137	0.09427	7.95	< 0.0005
Interaction	5	0.23785	0.23785	0.04757	4.01	0.002
Error	158	1.87255	1.87255	0.01185		
Total	169	3.04401				

Table VI.16: Digesta in the pyloric caecal area; Bonferroni; one tailed test:

Digesta	DF	*t*-value	*p*-value
3.73 hours	18	2.65	0.0082
13.65 hours	25	5.70	< 0.00005

Table VI.17: Total intestinal digesta; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values

Feed/Starv	1	0.72616	0.55087	0.55087	25.82	< 0.0005
Hours	5	1.49185	1.05050	0.21010	9.85	< 0.0005
Interaction	5	0.71746	0.71746	0.14349	6.72	< 0.0005
Error	158	3.37151	3.37151	0.02134		
Total	169	6.30699				

Table VI.18: Total intestinal digesta; Bonferroni; one tailed test:

Digesta	**DF**	***t*-value**	***p*-value**
3.73 hours	22	0.15	0.44
13.65 hours	26	5.21	< 0.00005
25.32 hours	23	2.06	0.025

Discharge of the gallbladder and secretion of trypsin and chymotrypsin (See 3.3.2.):

Table VI.19: The gallbladder index; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	805.16	592.21	592.21	22.70	< 0.0005
Hours	5	776.47	667.87	133.57	5.12	< 0.0005
Interaction	5	461.80	461.80	92.36	3.54	0.005
Error	158	4121.64	4121.64	26.09		
Total	169	6165.06				

Table VI.20: The gallbladder index; Bonferroni; one tailed test:

GB ind.	**DF**	***t*-value**	***p*-value**
3.73 hours	14	1.77	0.049
13.65 hours	10	4.52	0.0006

Table VI.21: Trypsin in the pancreatic tissue; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**

Feed/Starv	1	33872	27802	27802	5.18	0.024
Hours	5	28874	55957	11191	2.09	0.070
Interaction	5	145690	145690	29138	5.43	< 0.0005
Error	158	847895	847895	5366		
Total	169	1056332				

Table VI.22: Trypsin in the pancreatic tissue; Bonferroni; one tailed test:

Trypsin	**DF**	***t*-value**	***p*-value**
3.73 hours	21	0.12	0.45
13.65 hours	18	4.93	0.0001

Table VI.23: Chymotrypsin in the pancreatic tissue; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	4343940	3789648	3789648	3.03	0.083
Hours	5	18350452	11841078	2368215	1.90	0.098
Interaction	5	14436394	14436394	2887279	2.31	0.046
Error	158	197355520	197355520	1249086		
Total	169	234486320				

Table VI.24: Chymotrypsin in the pancreatic tissue; Bonferroni; one tailed test:

Chymotrypsin	**DF**	***t*-value**	***p*-value**
13.65 hours	20	4.17	0.0002

Trypsin and chymotrypsin activities in the intestinal digesta (See 3.3.3.):

Table VI.25: Trypsin in the pyloric caecal region; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	6026.4	5107.5	5107.5	7.05	0.009
Hours	5	43298.9	28985.7	5797.1	8.00	< 0.0005

Interaction	5	8023.8	8023.8	1604.8	2.21	0.055
Error	158	114491.8	114491.8	724.6		
Total	169	171840.9				

Table VI.26: Trypsin in the pyloric caecal region; Bonferroni; one tailed test:

Trypsin	**DF**	***t*-value**	***p*-value**
3.75 hours	27	0.75	0.23
13.65 hours	27	2.52	0.0090

Table VI.27: Trypsin in the mid intestinal region; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	3184.04	2614.76	2614.76	44.17	< 0.0005
Hours	5	3837.30	2074.80	414.96	7.01	< 0.0005
Interaction	5	2276.40	2276.40	455.28	7.69	< 0.0005
Error	158	9353.50	9353.50	59.20		
Total	169	18651.24				

Table VI.28: Trypsin in the mid intestinal region; Bonferroni; one tailed test:

Trypsin	**DF**	***t*-value**	***p*-value**
3.75 hours	27	2.42	0.011
13.65 hours	19	5.57	< 0.00005

Table VI.29: Trypsin in the rectum; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	3491.1	3021.0	3021.0	27.59	< 0.0005
Hours	5	5218.2	2689.1	537.8	4.91	< 0.0005
Interaction	5	3019.1	3019.1	603.8	5.51	< 0.0005
Error	158	17299.4	17299.4	109.5		

Total	169	29027.8				

Table VI.30: Trypsin in the rectum; Bonferroni; one tailed test:

Trypsin	**DF**	***t*-value**	***p*-value**
3.75 hours	23	3.13	0.0024
13.65 hours	19	5.25	< 0.00005

Table VI.31: Chymotrypsin in the pyloric caecal region; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	828284	721249	721249	2.99	0.086
Hours	5	11878161	8028553	1605710	6.65	< 0.0005
Interaction	5	1815281	1815281	363056	1.50	0.192
Error	158	38162300	38162300	241534		
Total	169	52684024				

Table VI.32: Chymotrypsin in the pyoric caecal region; Bonferroni; one tailed test:

Chymotrypsin	**DF**	***t*-value**	***p*-value**
3.75 hours	26	0.16	0.44
13.65 hours	27	2.20	0.018

Table VI.33: Chymotrypsin in the mid intestinal region; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Feed/Starv	1	984015	803078	803078	47.11	< 0.0005
Hours	5	1477995	840810	168162	9.87	< 0.0005
Interaction	5	694928	694928	138986	8.15	< 0.0005
Error	158	2693194	2693194	17046		
Total	169	5850132				

Table VI.34: Chymotrypsin in the mid intestinal region; Bonferroni; one tailed test:

Chymotrypsin	DF	*t*-value	*p*-value
3.75 hours	23	2.76	0.0056
13.65 hours	19	5.76	< 0.00005

Table VI.35: Chymotrypsin in the rectum; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Feed/Starv	1	2474738	2017726	2017726	45.56	< 0.0005
Hours	5	4172851	2298920	459784	10.38	< 0.0005
Interaction	5	2102748	2102748	420550	9.50	< 0.0005
Error	158	6997907	6997907	44291		
Total	169	15748244				

Table VI.36: Chymotrypsin in the rectum; Bonferroni; one tailed test:

Chymotrypsin	DF	*t*-value	*p*-value
3.75 hours	20	3.88	0.005
13.65 hours	19	7.47	< 0.00005

Chapter VII.

Lower and upper modal salmon in their first year (See VII.3.1.):

Changes with time in body weight and gut digesta (See VII.3.1.1.)

Table VII.1: Bodymass from December to July; Bonferroni; one tailed test:

Bodymass	DF	*t*-value	*p*-value
LMG: March vs May	24	5.97	< 0.00005
LMG: May vs July	20	15.38	< 0.00005
UMG: March vs May	27	14.63	< 0.00005

UMG: May vs July	23	13.77	< 0.00005

Table VII.2: Stomach digesta for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	65.959	13.192	40.97	< 0.0005
Error	114	36.710	0.322		
Total	119	102.669			

Table VII.3: Stomach digesta for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	8.635	1.727	16.68	< 0.0005
Error	114	11.801	0.104		
Total	119	20.437			

Table VII.4: Stomach digesta for the LMG and the UMG fish; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	0.0222	0.0222	0.0222	0.10	0.747
Hours	5	57.5506	57.5506	11.5101	54.10	< 0.0005
Interaction	5	17.0438	17.0438	3.4088	16.02	< 0.0005
Error	228	48.5115	48.5115	0.2128		
Total	239	123.1281				

Table VII.5: Intestinal digesta for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	9.412	1.883	5.05	< 0.0005
Error	114	42.542	0.372		
Total	119	51.864			

Table VII.6: Intestinal digesta for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	0.9623	0.1925	4.83	< 0.0005
Error	114	4.5447	0.0399		
Total	119	5.5071			

Table VII.7: Intestinal digesta for the LMG and the UMG fish; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	7.3430	7.3430	7.3430	35.62	< 0.0005
Hours	5	6.4548	6.4548	1.2910	6.26	< 0.0005
Interaction	5	3.9195	3.9195	0.7839	3.80	0.003
Error	228	46.9964	46.9964	0.2061		
Total	239	64.7137				

Table VII.8: Pepsin in the stomach mucosa for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	7485219	1497044	37.71	< 0.0005
Error	114	4525160	39694		
Total	119	12010380			

Table VII.9: Pepsin in the stomach mucosa for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	421427	84285	20.67	< 0.0005
Error	114	464757	4077		
Total	119	886184			

Table VII.10: Pepsin in the stomach mucosa for the LMG and the UMG fish; two way

ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	18721256	18721256	18721256	855.41	< 0.0005
Hours	5	3603341	3603341	720668	32.93	< 0.0005
Interaction	5	4303305	4303305	860661	39.33	< 0.0005
Error	228	4989917	4989917	21886		
Total	239	31617820				

Table VII.11: Pepsin in the stomach digesta for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	959108	1911822	15.90	< 0.0005
Error	114	1375332	12064		
Total	119	2334440			

Table VII.12: Pepsin in the stomach digesta for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	268201	53640	21.85	< 0.0005
Error	114	279823	2455		
Total	119	548025			

Table VII.13: Pepsin in the stomach digesta for the LMG and the UMG fish; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	1656217	1656217	1656217	228.15	< 0.0005
Hours	5	761418	761418	152284	20.98	< 0.0005
Interaction	5	465891	465891	93178	12.84	< 0.0005
Error	228	1655155	1655155	7259		

Total	239	4538681				

Table VII.14: Trypsin in the pancreas and pyloric caeca for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	476526	95305	4.19	0.002
Error	114	2593315	22748		
Total	119	3069841			

Table VII.15: Trypsin in the pancreas and pyloric caeca for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	1630965	326193	20.00	< 0.0005
Error	114	1858942	16307		
Total	119	3489907			

Table VII.16: Trypsin in the pancreas and pyloric caeca for the LMG and the UMG fish; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	76920	76920	76920	3.94	0.048
Hours	5	1060269	1060269	212054	10.86	< 0.0005
Interaction	5	1047222	1047222	209444	10.73	< 0.0005
Error	228	4452257	4452257	19527		
Total	239	6636668				

Table VII.17: Trypsin in the intestinal digesta for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	10531987	2106397	39.39	< 0.0005

Error	114	6095927	53473		
Total	119	16627914			

Table VII.18: Trypsin in the intestinal digesta for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	3021448	604290	14.42	< 0.0005
Error	114	4776252	41897		
Total	119	7797700			

Table VII.19: Trypsin in the intestinal digesta for the LMG and the UMG fish; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	36897	36897	36897	0.77	0.38
Hours	5	11900109	11900109	2380022	49.91	< 0.0005
Interaction	5	1653327	1653327	330665	6.93	< 0.0005
Error	228	10872179	10872179	47685		
Total	239	24462512				

Table VII.20: Chymotrypsin in the pancreas and pyloric caeca for the LMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value
Factor	5	3.332E+08	6.664E+07	4.31	0.001
Error	114	1.762E+09	1.545E+07		
Total	119	2.095E+09			

Table VII.21: Chymotrypsin in the pancreas and pyloric caeca for the UMG fish; one way analysis of variance:

Source	DF	SS	MS	F	p-value

Factor	5	6.537E+08	1.307E+08	17.99	< 0.0005
Error	114	8.286E+08	7.269E+06		
Total	119	1.482E+09			

Table VII.22: Chymotrypsin in the pancreas and pyloric caeca for the LMG and the UMG fish; two way ANOVA:

Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**p-values**
Mode	1	2.448E+06	2.448E+06	2.448E+06	0.22	0.643
Hours	5	5.258E+08	5.258E+08	1.052E+08	9.26	< 0.0005
Interaction	5	4.611E+08	4.611E+08	9.222E+07	8.12	< 0.0005
Error	228	2.590E+09	2.590E+09	1.136E+07		
Total	239	3.580E+09				

Table VII.23: Chymotrypsin in the intestinal digesta for the LMG fish; one way analysis of variance:

Source	**DF**	**SS**	**MS**	**F**	**p-value**
Factor	5	5.269E+09	1.054E+09	25.10	< 0.0005
Error	114	4.787E+09	4.199E+07		
Total	119	1.006E+10			

Table VII.24: Chymotrypsin in the intestinal digesta for the UMG fish; one way analysis of variance:

Source	**DF**	**SS**	**MS**	**F**	**p-value**
Factor	5	5.038E+09	1.008E+09	32.19	< 0.0005
Error	114	3.569E+09	3.131E+07		
Total	119	8.607E+09			

Table VII.25: Chymotrypsin in the intestinal digesta for the LMG and the UMG fish; two way ANOVA:

Source	DF	Seq SS	Adj SS	Adj MS	F	p-values
Mode	1	5.627E+07	5.627E+07	5.627E+07	1.54	0.217
Hours	5	6.075E+09	6.075E+09	1.215E+09	33.15	< 0.0005
Interaction	5	4.233E+09	4.233E+09	8.466E+08	23.10	< 0.0005
Error	228	8.356E+09	8.356E+09	3.665E+07		
Total	239	1.872E+10				

The effect of starvation, cholecystokinin and feeding (See VII.3.2.):

Table VII.26: Pepsin; Bonferroni; two tailed test:

Pepsin	DF	*t*-value	*p*-value
LMG: Starving vs feeding	11	2.40	0.035
UMG: Starving vs feeding	19	6.53	< 0.00005

Table VII.27: Trypsin in the pancreas and pyloric caeca; Bonferroni; two tailed test:

Trypsin	DF	*t*-value	*p*-value
LMG: Starving vs CCK	11	6.41	< 0.00005
LMG: CCK vs feeding	23	8.82	< 0.00005
LMG: Feeding vs starving	15	0.55	0.59
UMG: Starving vs CCK	17	6.38	< 0.00005
UMG: CCK vs feeding	22	4.61	0.0001
UMG: Feeding vs starving	12	10.63	< 0.00005

Table VII.28: Chymotrypsin in the pancreas and pyloric caeca; Bonferroni; two tailed test:

Chymotrypsin	DF	*t*-value	*p*-value
LMG: Starving vs CCK	13	5.99	< 0.00005
LMG: CCK vs feeding	24	10.09	< 0.00005

LMG: Feeding vs starving	18	1.88	0.076
UMG: Starving vs CCK	17	5.49	< 0.00005
UMG: CCK vs feeding	24	2.85	0.0089
UMG: Feeding vs starving	14	8.31	< 0.00005

Table VII.29: Trypsin in the intestinal digesta; Bonferroni; two tailed test:

Trypsin	**DF**	***t*-value**	***p*-value**
LMG: Starving vs CCK	17	2.65	0.017
LMG: CCK vs feeding	18	7.20	< 0.00005
LMG: Feeding vs starving	14	8.78	< 0.00005
UMG: Starving vs CCK	15	4.34	0.0006
UMG: CCK vs feeding	26	1.98	0.058
UMG: Feeding vs starving	16	6.26	< 0.00005

Table VII.30: Chymotrypsin in the intestinal digesta; Bonferroni; two tailed test:

Chymotrypsin	**DF**	***t*-value**	***p*-value**
LMG: Starving vs CCK	18	2.24	0.038
LMG: CCK vs feeding	19	11.96	< 0.00005
LMG: Feeding vs starving	15	13.94	< 0.00005
UMG: Starving vs CCK	21	3.39	0.0028
UMG: CCK vs feeding	17	6.71	< 0.00005
UMG: Feeding vs starving	15	8.22	< 0.00005